This series of books addresses a wide range of topics in nutritional science. The books are aimed at advanced undergraduate and graduate students, researchers, university teachers, policy makers and nutrition and health professionals. They offer original syntheses of knowledge, providing a fresh perspective on key topics in nutritional science. Each title is written by a single author or by groups of authors who are acknowledged experts in their field. Titles include aspects of molecular, cellular and whole body nutrition and cover humans and wild, captive and domesticated animals. Basic nutritional science, clinical nutrition and public health nutrition are each addressed by titles in the series.

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Contents

Contributors vii

Preface ix

Part 1: The Immune System

1. The Immune System: an Overview
   G. Devereux 1

2. Evaluation of the Effects of Nutrients on Immune Function
   S. Cunningham-Rundles 21

Part 2: Individual Nutrients, Infection and Immune Function

3. Effect of Post-natal Protein Malnutrition and Intrauterine
   Growth Retardation on Immunity and Risk of Infection
   R.K. Chandra 41

4. Fatty Acids, Inflammation and Immunity
   P.C. Calder and C.J. Field 57

5. Arginine and Immune Function
   M.D. Duff and J.M. Daly 93

6. Glutamine and the Immune System
   P.C. Calder and P. Newsholme 109

7. Sulphur Amino Acids, Glutathione and Immune Function
   R.F. Grimble 133
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>8. Vitamin A, Infection and Immune Function</td>
<td>151</td>
</tr>
<tr>
<td>R.D. Semba</td>
<td></td>
</tr>
<tr>
<td>9. Antioxidant Vitamins and Immune Function</td>
<td>171</td>
</tr>
<tr>
<td>D.A. Hughes</td>
<td></td>
</tr>
<tr>
<td>10. Zinc, Infection and Immune Function</td>
<td>193</td>
</tr>
<tr>
<td>A.S. Prasad</td>
<td></td>
</tr>
<tr>
<td>11. Role of Iron in Immunity and Infection</td>
<td>209</td>
</tr>
<tr>
<td>S. Kuvibiidila and B.S. Baliga</td>
<td></td>
</tr>
<tr>
<td>12. Selenium and the Immune System</td>
<td>229</td>
</tr>
<tr>
<td>13. Probiotics and Immune Function</td>
<td>251</td>
</tr>
<tr>
<td>H.S. Gill and M.L. Cross</td>
<td></td>
</tr>
<tr>
<td>Part 3: Nutrition and Immunity through the Life Cycle</td>
<td></td>
</tr>
<tr>
<td>14. Role of Local Immunity and Breast-feeding in Mucosal Homoeostasis and Defence against Infections</td>
<td>273</td>
</tr>
<tr>
<td>P. Brandtzaeg</td>
<td></td>
</tr>
<tr>
<td>15. Food Allergy</td>
<td>321</td>
</tr>
<tr>
<td>E. Opara</td>
<td></td>
</tr>
<tr>
<td>16. Exercise and Immune Function – Effect of Nutrition</td>
<td>347</td>
</tr>
<tr>
<td>E.W. Petersen and B.K. Pedersen</td>
<td></td>
</tr>
<tr>
<td>17. Nutrition and Ageing of the Immune System</td>
<td>357</td>
</tr>
<tr>
<td>B. Lesourd, A. Raynaud-Simon and L. Mazari</td>
<td></td>
</tr>
<tr>
<td>A. Tomkins</td>
<td></td>
</tr>
<tr>
<td>Index</td>
<td>413</td>
</tr>
</tbody>
</table>
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Preface

‘This fortress built by Nature for herself
Against infection and hand of war’
(The Tragedy of King Richard II, Act II, Scene I, lines 43 and 44, William Shakespeare)

It has been recognized for many years that states of nutrient deficiency are associated with an impaired immune response and with increased susceptibility to infectious disease. In turn, infection can affect the status of several nutrients, thus setting up a vicious circle of under nutrition, compromised immune function and infection. Thus, the focus of much of the research into nutrition, infection and immunity has been related to identifying the effects of nutrient deficiencies upon components of the immune response (often using animal models) and, importantly, upon attempts to reduce the occurrence and severity of infectious diseases (often in human settings). Although it is often considered that the problems of under nutrition relate mainly to the developing world, they exist in developed countries, especially among the elderly, individuals with eating disorders, alcoholics, patients with certain diseases and premature and small-for-gestational-age babies. Thus, immunological problems in these groups probably relate, at least in part, to nutrient status. In addition, many diseases that exist among the apparently well nourished have a strong immunological component and it is now recognized that at least some of these diseases relate to diet and that their course may be modified by specific changes in nutrient supply. Examples of these diseases include rheumatoid arthritis, Crohn's disease and atopic diseases. Furthermore, it is now recognized that atherosclerosis, a disease strongly influenced by diet, has an immunological component. Thus, understanding the interaction between nutrition and immune function is fundamental to understanding the development of a multitude of communicable and non-communicable diseases and will offer preventive and therapeutic opportunities to control the incidence and severity of those diseases. It is also now recognized that immune dysfunction plays a role in
the events that follow trauma, burns or major surgery, and which, in some patients, can lead to organ failure and death. Thus, understanding the interaction between nutrition and immune function is fundamental in designing therapies to control the severity of these aberrant responses and to improve patient outcome.

The aim of this book is to provide a state of the art description of the interaction between nutrition and immunity, with an emphasis on the mechanism(s) of action of the nutrients concerned and the impact on human health. The book is divided into three parts.

Part 1 contains two chapters. The first is an overview of the immune system, its components and the way in which it functions and regulates its activities. The second is a description, using examples from the recent literature, of the methodological approaches that can be used to investigate the impact of altered nutrient supply on immune outcomes.

Part 2 contains 11 chapters. The first of these is devoted to the immunological effects of protein-energy malnutrition and of intrauterine growth retardation. Each of a further nine chapters is devoted to a specific nutrient or a family of nutrients: fatty acids, arginine, glutamine, sulphur amino acids, vitamin A, antioxidant vitamins (vitamins C and E and β-carotene), zinc, iron and selenium are all featured. The final chapter in this section deals with probiotics, an emerging area of great interest.

Part 3 contains five chapters. Rather than taking a nutrient-led approach these deal with changes in immune competence through the life cycle and with how nutrition affects these. The development of immunity in early life and the role of breast-feeding are covered in one chapter. A later chapter describes the current understanding of the impact of ageing on immune competence and how nutrient status plays a role in accelerating or delaying this ageing process. In between these two chapters are chapters on food allergy and on the influence of exercise on immune function. The final chapter tackles the public health implications of our increased understanding of the interaction between nutrition and immune function and poses important questions about how we can harness our knowledge for greater benefit.

Each chapter of this book includes an extensive reference list, which will guide the reader who wishes to seek more detailed information.

The true remedy for all diseases is Nature’s remedy. Nature and Science are at one … Nature has provided, in the white corpuscles as you call them – in the phagocytes as we call them – a natural means of devouring and destroying all disease germs. There is at bottom only one genuinely scientific treatment for all diseases, and that is to stimulate the phagocytes. Stimulate the phagocytes... The phagocytes are stimulated; they devour the disease; and the patient recovers.

The Doctor’s Dilemma, Bernard Shaw

P.C. Calder, C.J. Field and H.S. Gill
Editors
December 2001
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P.C. Calder, C.J. Field and H.S. Gill
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1 The Immune System: an Overview
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Aberdeen Royal Infirmary, Foresterhill, Aberdeen AB25 2ZD, UK

Introduction

To parasitic microorganisms, the human body represents an extremely attractive environment and source of nutrients. Consequently, we live under the constant threat of overwhelming attack by viruses, bacteria and parasites. Microorganisms evolve more rapidly than humans, so that the nature of the microbiological threat to humans is changing as exposure to new or variant organisms occurs. To combat this potentially devastating threat, evolution has provided humans with a highly sophisticated, flexible and potent immune system, which is able to protect humans against rapidly evolving microorganisms. The critical protective function of the immune system becomes apparent when it fails. The inherited and acquired immunodeficiency states are characterized by increased susceptibility to all infections, including those organisms not normally considered to be pathogenic.

The immune system is a two-edged sword: the extremely potent and toxic biological effector mechanisms of the immune system can destroy not only threatening microorganisms but also body tissues. Usually the tissue destruction and inflammation associated with the eradication of a microbiological threat are acceptable and functionally insignificant. However, in several human diseases, the immunologically associated tissue destruction and inflammation are harmful, e.g. tuberculosis, fulminant hepatitis and meningitis, and, although this may be advantageous to the species as a whole, the effect on the individual may be devastating. It is because of their potential to destroy tissues that the effector mechanisms of the immune system are very tightly regulated. Failure of these regulatory mechanisms results in the full might of the immune system being inappropriately directed against body tissues and the development of autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus (SLE), myasthenia gravis and multiple sclerosis. If immune responses are directed against innocuous targets, such as allergens or transplanted...
organs, the resulting immunologically mediated tissue damage and inflammation are the basis of allergy and transplant rejection. The immune response to microorganisms is divided into two general systems: innate (natural) immunity and adaptive (specific, acquired) immunity.

**Innate Immunity** (Medzhitov and Janeway, 1997)

Innate immunity comprises physical barriers, soluble factors and phagocytic cells, which can be considered to provide an immediate first line of defence against invading microorganisms. Innate immunity is encoded in the germline, it is very similar among normal individuals and there is no memory effect, with re-exposure to the same pathogen eliciting the same response. Innate immunity is directed against molecular structures of microorganisms that are essential for microbial survival, present in many types of microorganisms and unique to pathogenic microorganisms, e.g. bacterial lipopolysaccharides and teichoic acids. The major cells of innate immunity are phagocytic macrophages and neutrophils, which possess surface receptors specific for common bacterial surface molecules. Engagement of these receptors triggers phagocytosis and destruction of the microorganism. Although pathogenic microorganisms have evolved mechanisms to evade the innate immune response, e.g. bacterial capsules, they are usually eliminated by the adaptive immune response, which is able to mount an appropriate neutralizing response directed specifically against the invading microorganism. Although innate immunity is inflexible, it provides a very rapid first line of defence until the more powerful and flexible adaptive immune response takes effect. The innate and adaptive immune systems are not independent; the innate immune response probably influences the character of the adaptive response and the effector arm of the adaptive response harnesses innate effector mechanisms, such as phagocytes (Fearon and Locksley, 1996).

**Adaptive Immunity** (Huston, 1997)

**Cells and tissues involved**

It is the functional properties of B lymphocytes (B-cells) and T lymphocytes (T-cells) that enable the adaptive immune response to be extremely powerful and yet, at the same time, regulated and flexible. Lymphocytes originate in the bone marrow from a common lymphoid stem cell. Further development and maturation of B- and T-cells occur in the bone marrow and thymus, respectively. Mature T- and B-cells enter the bloodstream; specific receptors enable adherence to capillary endothelial cells and migration into peripheral lymphoid organs. These comprise the lymph nodes, spleen, bronchial-associated lymphoid tissue, mucosa-associated lymphoid tissue and gut-associated lymphoid tissues (tonsils, adenoids, appendix and the Peyer’s patches of the small intestine). Peripheral lymphoid organs are highly anatomically and functionally organized to facilitate interactions between migrating lymphocytes and antigens.
transported actively (by antigen-presenting cells) or passively (in lymph) to the peripheral lymphoid organs from the tissues. Lymphocytes that do not encounter antigen re-enter the bloodstream by way of efferent lymphatics and the thoracic duct. The functional consequence of this T- and B-cell circulation is that all of the body tissues are under continuous immunological surveillance for invading pathogens.

**Clonal expansion of lymphocytes**

Each T- and B-cell bears surface receptors with a single antigenic specificity, but the specificity of each individual lymphocyte is different. The population of T- and B-cells in a human is able to recognize an estimated $10^{11}$ different antigens. This huge receptor repertoire is generated during lymphocyte development by the random rearrangement of a limited number of receptor genes (Fanning *et al.*, 1996). Although the immune system is able to recognize a huge number of antigens, any single antigen is recognized by relatively few lymphocytes, typically 1 in 1,000,000; consequently, there are not enough lymphocytes to immediately eliminate an invading microorganism. When a lymphocyte antigen receptor engages its complementary antigen, the lymphocyte ceases migration, enlarges and rapidly proliferates so that, within 3–5 days, there are a large number of effector cells, each specific for the initiating antigen. This antigen-driven clonal expansion accounts for the characteristic delay of several days before adaptive immune responses become effective. Some of the effector cells generated by clonal expansion are very long-living and are the basis of the immunological memory that is characteristic of adaptive immunity. Functionally, immunological memory enables a more rapid and effective immune response upon re-exposure to microorganisms. In contrast to innate immunity, the antigen specificities of adaptive immunity reflect the individual’s lifetime exposure to infectious agents and will consequently differ between individuals.

**B-cells, immunoglobulins and humoral immunity**

Protection against certain infections can be transferred by serum. This is called humoral immunity and is mediated by circulating antibodies, also known as immunoglobulins (Ig). The cell surface of B-cells incorporates the membrane-bound form of immunoglobulin, which functions as an antigen-specific receptor. Engagement of surface Ig by complementary antigen initiates B-cell proliferation, with the majority of the resulting cells transforming into plasma cells secreting large amounts of antibody with the same specificity as the progenitor B-cell surface Ig receptor.

*Structure of immunoglobulins* (Huston, 1997)

The general structural features of antibodies can be demonstrated by immunoglobulin G (IgG) (molecular weight 150 kDa), which comprises two
identical heavy chains (50 kDa each) and two identical light chains (25 kDa each). Each of the two heavy chains is linked to the other and to a light chain by disulphide bonds, giving a roughly Y-shaped molecule (Fig. 1.1). Each immunoglobulin molecule possesses two antigen-binding (Fab) sites, each with the same specificity situated at the amino ends of the light and heavy chains. The Fab segments are divided into a variable (V) and a constant (C) region and the structural diversity of the V regions produces the diversity of antibody specificity. There are five main types of heavy chain, μ, δ, γ, α and ε, which confer differing functional properties between the five major classes (isotypes) of immunoglobulin, namely IgM, IgD, IgG, IgA and IgE, respectively. The functional activity of antibodies resides at the carboxyl-terminal (Fc) region of the heavy chains.

**Immunoglobulin isotypes**

The antigen specificity of antibodies is mediated by the two antigen-binding sites, while the differing Fc regions of the various immunoglobulin isotypes engage differing effector mechanisms. Monomeric IgM and IgD act as B-cell surface antigen-specific receptors. The affinity of each IgM antigen-binding site tends to be low; however, IgM in serum usually polymerizes into a pentamer with ten antigen-binding sites, which give the antibody high binding strength.

IgM dominates the initial humoral immune response; however, IgG and IgA predominate later, although IgE is prominent during an allergic response. This process is known as isotype switching and is the consequence of DNA

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**Fig. 1.1.** Schematic representation of an IgG molecule. The two domains of each of the two light chains are termed V<sub>L</sub> and C<sub>L</sub>. The four domains of each of the two heavy chains are termed V<sub>H</sub>, C<sub>H1</sub>, C<sub>H2</sub> and C<sub>H3</sub>. The amino terminal (dark) domain of each chain is the variable region and it is the tips of these regions that form the two antigen-binding sites of the molecule.
rearrangements in the genes encoding for the C (but not the V) regions of the heavy chains (Stavnezer, 1996). Isotype switching results in differing classes of antibodies with differing functional properties, although antigen specificity remains constant. Isotype switching is dependent on T-cells and their secretion of cytokines, with interleukin-4 (IL-4) inducing B-cell switching to IgE; this is antagonized by interferon-γ (IFN-γ) (Pene et al., 1988). Switching to IgA is promoted by transforming growth factor-β (TGF-β), in combination with IL-10 (Defrance et al., 1992). In addition to isotype switching, as the humoral immune response matures, point mutations in the immunoglobulin V-region genes occur. A T-cell-dependent process, known as affinity maturation, selects those B-cells with point mutations producing antibodies with an increased affinity for the stimulating antigen. Consequently, as the humoral immune response progresses, the affinity and specificity of the antibodies increase and the resulting memory cells provide highly effective protection against reinfection by the same microorganism (Neuberger and Milstein, 1995).

IgG antibodies are monomeric and are further subdivided into IgG1, IgG2, IgG3 and IgG4, with IgG1 being found in the greatest quantities in serum. IgG1 and IgG3 are transferred across the placenta to the fetus. IgA circulates in the bloodstream but, of more functional importance, IgA is secreted across mucous membranes and is found in intestinal and bronchial secretions, tears and breast milk. Circulating IgA is monomeric, while secreted IgA polymerizes into dimers; polymerization is required for transport across epithelia. IgA is subdivided into IgA1 and IgA2. IgE is the principal antibody isotype involved in the immune response to parasites and in allergic reactions. The ε heavy chains possess an extra constant heavy-chain (C_{H}) domain and the Fc component binds with high affinity to the FceR1 receptor found on the surface membranes of mast cells, basophils and activated eosinophils.

**Effector functions of immunoglobulins**

The humoral arm of the adaptive immune responses is particularly effective against extracellular microorganisms and their toxins. Antibodies bind to functionally critical antigenic sites on soluble toxins and to the surface antigens of extracellular microorganisms. Such binding effectively neutralizes toxins and microorganisms by preventing binding to host-cell surface molecules. Antibodies bound to bacteria are also able to activate a series of plasma proteins, known as complement, to produce molecules that are chemotactic for phagocytes, promote phagocytosis and can also directly destroy bacteria (Lambris et al., 1999).

Antibodies bind to bacteria by the amino-terminal antigen-binding sites, leaving the Fc component of the antibody exposed. Engagement of these exposed Fc fragments by surface Fc receptors on phagocytic cells induces phagocytosis and destruction of the coated bacterium; this process is known as opsonization. Macrophages and neutrophils possess IgM- and IgG-specific Fc receptors, while eosinophils possess IgE-specific Fc receptors. Phagocytes form part of the innate immune system and possess very limited antigen-specific receptors. Opsonizing antibodies enable phagocytes to recognize a wide range
of antigens by effectively converting an antigen to an Fc segment that is easily recognized by phagocytes that are otherwise unable to engage and destroy the bacteria.

Antibodies are mainly directed against extracellular pathogens; however, they can be effective against virally infected cells that express viral antigens on their surfaces. These exposed viral antigens are bound by antigen-specific antibodies and the infected cell is destroyed by natural killer (NK) cells. NK cells are large granular lymphocytes, defined by the absence of surface immunoglobulin or T-cell receptors and the presence of Fc receptors. NK cells do not undergo clonal expansion; instead, they provide innate cytotoxic immune responses directed against virally infected cells, although they can interact with the adaptive immune response as outlined above (Fearon and Locksley, 1996).

T-cells and cell-mediated immunity

Antibodies are highly effective against extracellular pathogens, but they have very limited potency against intracellular pathogens, such as viruses and certain bacteria. T-cells, however, are particularly effective against intracellular pathogens, because of their ability to identify infected cells and then mount and coordinate an effective cell mediated immune response.

The T-cell receptor

Each T-cell possesses approximately 30,000 antigen-specific T-cell receptor (TCR) molecules on its surface, each with the same antigen specificity. Unlike B-cell immunoglobulin molecules, TCR is always surface-bound, is not secreted and does not undergo any form of isotype switching or somatic hypermutation. The TCR (Fig. 1.2) comprises two transmembrane glycoprotein chains, linked by a disulphide bond. A single α and a single β chain associate to form the majority (90%) of TCRs. However, 10% of T-cell TCRs are composed of a single γ chain and a single δ chain. The true functional significance of αβ and γδ T-cells is unknown. Each TCR traverses the T-cell membrane, and the external part of each TCR chain consists of a V and a C domain, with the V region being highly polymorphic, and the single antigen-binding site is formed by the apposition of the two amino-terminal V regions. TCR antigen-specificity diversity is generated during T-cell maturation by random rearrangement of gene segments encoding the TCR Vα and Vβ regions. Rearrangement of the genes encoding the αβ TCR produces an estimated $10^{15}$ variants, each with a different antigen specificity; γδ chain diversity is even greater, with an estimated $10^{18}$ specificities. In contrast to B-cells, T-cells are only able to recognize antigens displayed on cell surfaces. Infection of a cell by an intracellular pathogen is signalled by the surface expression of pathogen-derived peptide fragments, expressed in conjunction with glycoproteins encoded by the major histocompatibility complex (MHC). It is the combination of pathogen peptide fragment bound to MHC molecule that is recognized by T-cells (Fremont et al., 1996).
The **MHC** (Germain, 1994; Huston, 1997)

The MHC is a large complex of genes that encode the major histocompatibility glycoproteins. These large cell-surface glycoproteins are present in some form on every nucleated cell and there are two structural variants (MHC class I and MHC class II). The MHC was originally identified and characterized by its profound influence on the rejection or acceptance of transplanted organs. The MHC is the molecular basis by which T-cells recognize intracellular pathogens in order to initiate or effect an immune response.

An MHC class I molecule (Fig. 1.3) consists of a highly polymorphic 44 kDa α chain that is non-covalently associated with a smaller non-polymorphic 12 kDa β₂-microglobulin chain. The α chain spans the cell membrane and forms a cleft into which the pathogen-derived peptide fragment is inserted during assembly of the MHC molecule. An MHC class II molecule comprises a 34 kDa α chain and a 29 kDa β chain; both span the cell membrane (Fig. 1.4). Each chain is divided into two domains, with association of the α₁ and β₁ domains forming an open-ended peptide-binding cleft into which a processed antigen peptide fragment is incorporated. MHC class I molecules bind peptides of eight to ten amino acids that originate from pathogen proteins synthesized within the cell cytosol, typically from viruses and certain bacteria. MHC class II molecules bind peptides derived from pathogens that have been phagocytosed by macrophages or endocytosed by antigen-presenting cells' such as macrophages, B-cells and professional antigen-presenting cells. MHC–pathogen–peptide complexes are very stable and are expressed on the cell surface, ready for recognition by a T-cell with TCRs specific for the peptide–MHC complex; this is known as MHC restriction.
T-cells expressing the CD8 antigen recognize peptides complexed with MHC class I molecules, which are expressed by all nucleated cells. The CD8 antigen is a surface molecule that acts as a co-receptor by simultaneously binding to the TCR and the MHC class I \( \alpha_3 \) domain. MHC class II–peptide complexes are recognized by T-cells expressing the CD4 antigen, which acts as a co-receptor (like CD8) by binding to the \( \beta_2 \) domain of the MHC class II molecules already bound by TCR. In humans, approximately one-third of peripheral blood T-cells are CD8, two-thirds are CD4 and approximately 5–10% are CD4– CD8–, the functions of which are unclear.

Fig. 1.3. Schematic representation of an MHC class I molecule. A single \( \alpha \) chain is composed of three domains, \( \alpha_1, \alpha_2 \) and \( \alpha_3 \), and the apposition of the \( \alpha_1 \) and \( \alpha_2 \) domains forms the peptide-binding cleft. The \( \alpha \) chain is non-covalently associated with a smaller non-polymorphic protein \( \beta_2 \)-microglobulin.

Fig. 1.4. Schematic representation of an MHC class II molecule. Each of the constituent \( \alpha \) and \( \beta \) chains comprises two domains. Apposition of the \( \alpha_1 \) and \( \beta_1 \) domains forms the peptide-binding cleft.
The structure of the peptide-binding cleft determines the peptide-binding specificity of an MHC molecule, such that it binds to peptides with a broadly similar structure. There are several genetic organizational features of the MHC that result in nucleated cells expressing a highly polymorphic set of MHC molecules, each with differing peptide-binding specificities. The polymorphic nature of the MHC is the consequence of the MHC being formed by three major class I genes designated human leucocyte antigen (HLA)-A, HLA-B and HLA-C, and three main class II genes, HLA-DP, HLA-DQ and HLA-DR; each of these loci is highly polymorphic. Furthermore, most individuals are heterozygous for MHC genes and there is co-dominant expression of the antigens coded by the maternally and paternally derived loci. Consequently, nearly all individuals express six class I and ten class II molecules, each with differing specificities. During an infection, it is highly likely that the proteins of a pathogen include peptide sequences that are recognized and presented to T-cells by at least one MHC molecule. The general explanation for MHC polymorphism is that it is an evolutionary response to pathogenic diversity, enabling the immune systems of individuals to respond to a wide range of existing and evolving pathogens. MHC polymorphism results in individuals with differing immunological capabilities to combat an individual pathogen, but on a population scale it is highly unlikely that any individual pathogen will be able to evade the immune system of every individual.

The generation of effector T-cells (Janeway and Bottomly, 1994)

Activation of a T-cell is a complex, tightly regulated process. This is necessary in order to ensure that T-cell activation is directed only against pathogens and not against body tissues. Furthermore, increased complexity decreases the likelihood that a microorganism can evolve mechanisms to subvert T-cell activation. T-cell activation takes place in the peripheral lymphoid organs. However, before this can occur, antigen is processed and presented in association with MHC molecules, and the antigen is then transported from the site of infection to the peripheral lymphoid organs and presented to T-cells. The processing, transportation and presentation of antigen are performed by antigen-presenting cells, the most important and efficient of which are dendritic cells. Dendritic cells are mandatory for the initiation of a primary immune response against a new pathogen, although both dendritic cells and non-professional antigen-presenting cells, such as macrophages and B-cells, are able to initiate secondary (memory) responses against reinfecting organisms.

Dendritic cells (Banchereau and Steinman, 1998)

These are generated in the bone marrow but are subsequently widely distributed throughout the tissues, typically in association with epithelial surfaces. When viewed by phase-contrast microscopy, dendritic cells extend long, delicate, motile processes in all directions. In peripheral tissues, so-called 'immature' dendritic cells have poor T-cell stimulatory activity. Instead, they act as
sentinels, constantly sampling the surrounding tissues for pathogens. Immature dendritic cells accumulate foreign antigens in their surroundings by macropinocytosis of soluble antigens and phagocytosis of particulate antigens and microorganisms. These processes are so efficient that dendritic cells can initiate immune responses with pico- and nanomolar concentrations of antigens, compared with the micromolar concentrations required by non-professional antigen-presenting cells, such as B-cells and macrophages.

After a dendritic cell captures a pathogen-associated antigen, its sampling function declines and, instead, it starts to process pathogenic antigens and present them in association with MHC molecules on its cell surface. Endocytosed antigens are presented in association with MHC class II molecules, while endogenously produced antigen, e.g. from a virus infecting the dendritic cell, is presented in association with MHC class I molecules. Dendritic cells are able to process and present, in a class I-restricted manner, antigens that do not enter the cytosolic compartment, e.g. viruses unable to infect dendritic cells. However, the mechanism for this is unclear. As antigens are processed and expressed, dendritic cells up-regulate surface expression of T-cell co-stimulatory molecules, such as CD40 and B7. Dendritic-cell maturation is also associated with secretion of cytokines and chemotactic cytokines (chemokines), which recruit macrophages, granulocytes, NK cells and more dendritic cells to counter the invading pathogen.

After processing and presenting antigen, dendritic cells bearing processed antigen migrate from the site of infection to the T-cell areas of local lymph nodes. There migration stops and they interact with T- and B-cells to initiate an immune response. Mature dendritic cells are extremely potent activators of T-cells, with a single dendritic cell being able to activate 100–3000 T-cells. This is because of the high density of MHC, co-stimulatory and adhesion molecules expressed by dendritic cells and the secretion of cytokines that profoundly influence T-cells, e.g. IL-12.

**Dendritic–T-cell interactions**

As T-cells circulate around the body, they pass through the peripheral lymphoid organs, where they transiently adhere to antigen-presenting cells. Contact is made with many thousands of dendritic cells every day. This enables T-cells to ‘sample’ the many MHC–peptide complexes on the surface of the antigen-presenting cells. Rarely, a circulating T-cell will possess TCRs that conform to the peptide–MHC complex. Binding of the TCR and peptide–MHC complex induces conformational changes in adhesion molecules that increase the interaction between the antigen-presenting cell and the T-cell and keep the T-cell and its progeny in close proximity to the source of their stimulation. T-cell activation is not induced solely by ligation of a TCR, CD4 or CD8 co-receptor with a specific MHC–peptide complex. T-cell proliferation requires a further stimulus from the antigen-presenting cell and this is provided by the antigen-presenting cell surface glycoproteins B7.1 (CD80) and B7.2 (CD86) binding to their receptor (CD28) present on the T-cell. Typically, a TCR binding to an MHC–peptide complex in the absence of co-stimulation leads to T-cell anergy (unresponsiveness) or apoptosis.
Clonal expansion and differentiation of T-cells into effector cells

Antigen-specific and co-stimulatory interaction between T-cell and antigen-presenting cell triggers T-cell proliferation. After a few days, thousands of T-cell progeny emerge from the peripheral lymphoid organs and localize to the areas of infection or inflammation. Each of these effector T-cells possesses the same antigen specificity as the parent T-cell and they are now available to counteract the stimulating pathogen. These effector T-cells differ from the parent T-cell, because they do not require the co-stimulation provided by antigen-presenting cells; therefore, further encounters by effector T-cells with their specific antigen results in immunological attack. The nature of immunological attack depends on the effector T-cell CD4/CD8 status.

Effector CD8 T-cells

Effector CD8 T-cells (also known as cytotoxic T-cells) play a vital role in countering viral infections (Fig. 1.5), which are intracellular and almost completely hidden from the humoral immune response. Effector CD8 T-cells are

Fig. 1.5. Schematic representation of virally infected cell by destruction CD8+ effector T-cell.
induced by antigen-presenting cell presentation of MHC class I–peptide complexes to CD8 T-cells. The anti-viral activity of CD8 cytotoxic T-cells depends on the ability of virally infected cells to signal their corrupted state by the cell-surface expression of viral peptide sequences in association with MHC class I molecules. It is these MHC–peptide complexes that are recognized by CD8 TCRs and trigger immunological attack by the CD8 T-cell. It is the interaction between CD8 T-cell and infected cell that enables precise destruction of infected cells and preservation of uninfected cells. After migrating to a site of viral infection, CD8 cytotoxic T-cells sample cell surfaces. If the CD8 T-cell adheres to and identifies an infected cell, the corrupted cell is destroyed by directed localized secretion of cytotoxic enzymes (perforin and granzymes) by the CD8 cell. This effectively neutralizes the viruses infecting the cell. Other anti-viral properties of CD8 cytotoxic T-cells include the secretion of the anti-viral cytokine IFN-γ and expression of Fas ligand (CD95L), which induces apoptosis in target cells bearing the Fas (CD95) receptor protein. Clearly, if control of this extremely destructive but precise process is lost and CD8 T-cells start destroying ‘self’ cells, the consequences are potentially catastrophic. Such a breakdown in control is probably the basis of the immunological destruction of insulin-secreting β cells of the pancreatic islets, resulting in type I (insulin-dependent) diabetes mellitus.

**Effector CD4 T-cells**

Although CD8 effector T-cells are of major importance in the defence against viruses, they are ineffective in eliminating certain intracellular bacteria, fungi and parasites that are not neutralized by destruction of their host cell. These microorganisms are also resistant to the humoral immune response. These particularly resistant organisms are neutralized by effector CD4 T-cells, which are generated by MHC class II-restricted presentation of peptide by antigen-presenting cells. Effector CD4 T-cells are more commonly known as T-helper (Th) cells.

*Th-cells and macrophages* (Stout and Bottomly, 1989)

Macrophages usually destroy phagocytosed microorganisms. However, certain pathogens (e.g. *Mycobacteria, Leishmania* and *Pneumocystis*) have evolved mechanisms that resist macrophage destruction. After directed migration of Th-cells to the site of infection, Th-cells sample the peptide–MHC class II surface-molecular repertoire of adjacent cells. Macrophage activation occurs if the surface-expressed peptide–MHC class II is recognized by a Th-cell possessing the complementary TCR. This macrophage–Th-cell interaction alone is insufficient to activate the macrophage, and two further signals are required (Fig. 1.6). The first is IFN-γ; this is usually secreted by the engaged Th-cell, but other sources of IFN-γ are also important, e.g. CD8 cytotoxic T-cells. The second signal sensitizes the macrophage to IFN-γ and this second signal can also be provided by Th-cells, which express surface CD40 ligand molecules; these interact with macrophage surface CD40 molecules.
Clearly, Th-cells are extremely potent antigen-specific macrophage activators, because they provide both the IFN-γ and the CD40 signals required for macrophage activation. Th-cell-induced activation greatly enhances macrophage antimicrobial and antigen-presenting capacity. The increased antimicrobial capacity of activated macrophages in part derives from the following:

1. Increased efficiency of lysosome fusion with microbe-containing phagosomes.
2. Increased synthesis of antimicrobial proteases and peptides, such as defensins.

Fig. 1.6. Schematic representation of Th-cell activation of macrophage infected with resistant microorganism.
3. Induction of the respiratory burst produces extremely microbiocidal products, such as the superoxide anion ($O_2^-$), singlet oxygen ($^{1}O_2$), the hydroxyl radical (OH$^-$), and hydrogen peroxide ($H_2O_2$).

4. Production of the reactive nitrogen metabolite nitric oxide (NO) is increased by activation of the enzyme inducible NO synthase (iNOS).

Macrophage activation is associated with the release of anti-microbial mediators that are not only toxic to microorganisms but also extremely toxic to host cells, resulting in host tissue damage. If macrophages constitutively remained in this activated state, massive tissue damage would occur. Therefore, macrophage activation is tightly regulated and extremely pathogen-specific. The control and antigen specificity of macrophage activation is provided by antigen-specific Th-cells. Thus the price paid by the host, in terms of tissue damage, in order to destroy these difficult invading intracellular organisms is minimized.

**Th-cells and B-cells**

Certain bacterial-associated antigens can elicit a T-cell-independent B-cell response (Mond et al., 1995). These thymus-independent (TI) antigens tend to have highly repetitive epitopes, which enable extensive cross-linking of surface immunoglobulin molecules, resulting in B-cell activation. Typical bacterial TI antigens are capsular polysaccharides, lipopolysaccharides and polymeric proteins. T-cell-independent B-cell responses provide a rapid specific response directed against bacteria possessing anti-phagocytic polysaccharide capsules, e.g. *Streptococcus pneumoniae*.

In general, B-cell activation requires signals from two sources; the first arises from the binding of B-cell surface-bound IgM/D to the complementary microorganism surface epitope and the second is Th-cell-derived (Fig. 1.7). This Th-cell facilitation of B-cell activation is essential for full expression of the humoral immune response, particularly isotype switching, affinity maturation and the efficient development of memory B-cells. To enable Th-cell facilitation of B-cell activation, B-cells are able to internalize antigen–immunoglobulin complexes and then express the resulting pathogen peptide sequences in an MHC class II-restricted fashion on the B-cell surface. It is these peptide–MHC class II complexes that are recognized by the Th-cell. It is essential that the peptide sequences recognized by the Th-cell originate from the antigen recognized by the B-cell. This process of linked recognition means that the B-cell and the Th-cell respond to different epitopes; however, the epitopes originate from the same antigen. Typically, the B-cell recognizes a surface epitope and the Th-cell possibly an internal peptide sequence.

The second signal provided by the Th-cell to enable B-cell activation takes the form of secreted and cell-bound signals. Effector Th-cells express surface CD40 ligand and this binds to B-cell surface CD40. Th-cell cytokine secretion is also critical in B-cell activation and maturation. Once activated, B-cells undergo clonal expansion and differentiation into immunoglobulin-secreting plasma cells, each secreting immunoglobulin isotypes with the same antigen specificity.
as the parent B-cell. Although plasma cells tend to localize to lymph nodes and bone marrow, their anti-microbial actions are widespread because of the extensive distribution of their secreted immunoglobulins.

Although macrophages and B-cells are critically dependent on Th-cells, clinical and experimental observations suggest that there is selective utilization of these cells during immune responses. Some CD4 Th-cell-mediated responses are predominantly antibody-based, while others are macrophage-dependent. For example, healing tuberculoid leprosy is associated with strong macrophage-mediated immunity with low antibody levels, whereas non-healing lepromatous leprosy is associated with high (but ineffective) antibody levels, weak macrophage-based effector responses and uncontrolled proliferation of microorganisms. The discovery that Th-cells are functionally diverse has helped in the understanding of these observations.
**Th-cell functional diversity** (Abbas et al., 1996; Mosmann and Sad, 1996)

The ability of CD4 Th-cells to initiate immune responses with differing effector mechanisms was clarified by a demonstration by Mosmann and Coffman (1989) that murine CD4 T-cell clones could be categorized into two broad functional groups, Th1 and Th2, depending on their secreted cytokines. Th1 cells secrete IFN-γ, IL-2 and tumour necrosis factor β (TNF-β), while Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Human Th1 and Th2 secretory cytokine patterns are similar to the murine model, although the synthesis of IL-2, IL-6, IL-10 and IL-13 is not so tightly restricted to a single subset. Additionally, however, individual human Th-cells can secrete both Th1 and Th2 cytokines and these are commonly known as Th0 cells. Human Th-cells appear to form a continuum, with some extremely polarized cells secreting either typically Th1 or Th2 cytokines but the majority are Th0 cells, secreting a mixture of Th1 and Th2 cytokines. The subdivision of Th-cells is complicated further by the recognition that some Th2 cells secrete the suppressive regulatory cytokine TGF-β, with some authorities terming these cells Th3. In recent years, it has become apparent that the Th1/Th2 subdivision is overly simple, but the concept of the functional dichotomy of Th1/Th2 is extremely useful in aiding the understanding of immune responses.

Th1 and Th2 cytokines have important effector and Th-cell regulatory functions (Fig. 1.8). Th1 and Th2 cytokines augment Th-cell differentiation in favour of the secreting subset, i.e. Th1 cytokines promote differentiation towards the Th1 phenotype and Th2 cytokines towards the Th2 phenotype. In addition, Th cytokines inhibit Th-cell differentiation towards the reciprocal phenotype, i.e. Th1 cytokines inhibit differentiation towards the Th2 phenotype and Th2 cytokines antagonize development of Th1 cells. The consequence of this self-amplification and mutual antagonism of the reciprocal phenotype is that, once a Th-cell-mediated immune response deviates towards either the Th1 or Th2 phenotype, the Th-cell response becomes increasingly polarized towards that phenotype.

**Factors affecting Th1/Th2 differentiation**

CD8 T-cells are predestined to mature into cytotoxic T-cells. However, Th1 and Th2 cells develop from a common CD4 T-cell precursor. Differentiation of precursor Th-cells is determined by genetic and environmental factors influential at the time of T-cell antigen recognition. Several factors influencing Th1/Th2 polarization have been proposed and demonstrated, but the most potent factor is the local cytokine milieu present at the time of T-cell activation.

The most potent cytokine promoting development of the Th1 phenotype is IL-12 in the absence of IL-4 (Trinchieri and Gerosa, 1996). Macrophages and professional antigen-presenting cells, such as dendritic cells, secrete IL-12 in response to bacteria, bacterial products and intracellular parasites. IL-12 is extremely potent in promoting Th1-biased differentiation by direct influences on the Th-cells. The most potent Th2-promoting stimulus is IL-4 in the absence of IFN-γ, but the initial source of polarizing IL-4 is not established (Ricci et al.,
Environmental and/or genetic factors may induce IL-4 secretion during activation of CD4 cells; a small specialized subset of CD4 T-cells known as CD4 NK1.1 secrete IL-4 on stimulation, and antigen presentation by B-cells can stimulate Th2 differentiation (Mason, 1996).

Although the cytokine microenvironment is the most potent determinant of Th1/Th2 polarization, Th1/Th2 differentiation is also influenced by complex interactions between antigen dose, TCR and MHC antigen affinities. Influential antigenic properties include the nature of the antigen, with viruses and bacteria favouring Th1 differentiation and helminths Th2. Th2 differentiation appears to be promoted by the small, highly soluble proteins characteristic of allergens. Some important allergens (house dust-mite allergen Der p1, subtilisin and papain) are proteases, and it is suggested that this favours Th2 differentiation, because helminths secrete proteases to aid tissue penetration. It is apparent that many factors influence Th1/Th2 differentiation, but it is highly unlikely that any single criterion is the sole determinant of Th-cell differentiation, because this would be quickly perverted by rapidly evolving pathogens. The complex matrix
of factors that eventually determine Th0, Th1 or Th2 polarization is probably an immunological evolutionary adaptation to reduce the scope for pathogen interference.

**Effector mechanisms of Th1-mediated immunity**

Th1 cells appear to be critical in effecting an antigen-specific phagocytic-mediated defence against microorganisms, principally bacteria, fungi and some parasites. If, however, Th1-biased immunity is directed against self-antigens, extensive tissue destruction and autoimmune disease may ensue. Common autoimmune diseases resulting from inappropriate Th1 responses include autoimmune haemolytic anaemia, autoimmune thrombocytopenic purpura, Goodpasture’s syndrome, type I insulin-dependent diabetes mellitus, rheumatoid arthritis and multiple sclerosis. Disease may also ensue if a Th1-biased immune response is inappropriately directed against innocuous antigens, such as occurs in coeliac disease.

The effector mechanisms of Th1-biased immune responses include activation of macrophages that have phagocytosed microorganisms normally resistant to lysosomal destruction. Th1 cytokines direct isotype switching of B-cells towards IgG production. In mice, Th1 cytokines promote secretion of the opsonizing antibodies IgG2a and IgG3; in humans, the equivalent IgG subtypes are probably IgG1 and IgG3. These opsonizing antibodies bind to microorganisms and promote their phagocytosis by macrophages and neutrophils, because of their affinity for phagocytes possessing Fcγ receptors and their ability to activate components of complement. Th1 cytokines also mobilize and localize appropriate phagocytic cells to sites of infection. IL-3 and granulocyte–macrophage colony-stimulating factor (GM-CSF) promote bone-marrow stem-cell proliferation and differentiation and the generation of large numbers of phagocytes. Localization of these phagocytes to sites of infection is achieved by Th1-cell secretion of TNF-α and TNF-β and chemokines that alter the adhesive properties of local endothelial cells and act as chemotactic agents. Therefore, in an elegantly efficient, controlled and microorganism-specific manner, Th1-biased Th-cells secrete cytokines that not only promote Th1 differentiation and inhibit Th2 development but also induce a complex package of biological responses directed towards the phagocytic destruction of invading microorganisms.

**Effector mechanisms of Th2-mediated immunity**

Th2-biased immune responses are believed to be important in the immune responses against helminth infections. If, however, Th2-biased immune responses are inappropriately directed against innocuous antigens, such as allergens, tissue damage and inflammation may ensue. These inappropriate Th2 responses underlie asthma, eczema, hay fever and some food allergies.

Th2 cytokines induce the isotype switching of B-cells to the synthesis of IgE. They also promote the growth, differentiation and release of mast cells and eosinophils from the bone marrow. Eosinophils are directed towards sites of
helminth infection and allergy by chemokines, such as eotaxin, which are released by Th-cells. Th2 cytokines also activate eosinophils. In a situation analogous to Th1-biased responses, Th2-biased Th-cells induce a package of biological responses that are characteristic of allergy and helminth infection, namely, high levels of circulating IgE, mastocytosis and tissue eosinophilia.

**Summary**

The immune system has evolved to combat the constant threat of tissue invasion by microorganisms. If, however, the immune system is directed against innocuous antigens or tissue antigens, the same immune responses that are vital for defence against microorganisms can result in autoimmune disease and allergy. The adaptive immune response is reliant on the properties of B- and T-cells that enable the response to be powerful, flexible and antigen-specific and exhibit immunological memory. B-cells secrete antibodies that are effective against extracellular bacteria and their toxins, whereas CD8 T-cells are adept at neutralizing virally infected cells. CD4 T-cells, also known as Th-cells, do not directly neutralize invading pathogens; instead, they interact with other cells (e.g. macrophages and B-cells) to direct a coordinated, antigen-specific immune response against microorganisms. CD4 Th-cell differentiation can be usefully considered to be either Th1- or Th2-biased. Th1-biased immune responses are characterized by IgG production and macrophage activation; such a response is vital for defence against extra/intracellular bacteria, fungi and some parasites. Conversely, inappropriate Th1-biased immune responses underlie autoimmune diseases. Th2-biased immune responses are characterized by IgE secretion, mastocytosis and eosinophilia, and, although useful in eliminating helminth infestations, such responses underlie the allergic diseases of asthma, eczema and hay fever.

This chapter is an attempt to provide an outline of the immune system; inevitably space constraints have necessitated oversimplification and the omission of some aspects. The major aspects of the immune system have been covered but if readers require further detail, they should consult one of the many readily available large immunological textbooks.

**References**


Evaluation of the Effects of Nutrients on Immune Function

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Introduction and Overview

Nutrients are primary factors in the regulation of the human immune response. Both macronutrients and micronutrients derived from the diet affect immune-system function through actions at several levels in the gastrointestinal tract, thymus, spleen, regional lymph nodes and immune cells of the circulating blood (Chandra, 1997; Cunningham-Rundles and Lin, 1998; Wallace et al., 2000; Cunningham-Rundles, 2001). Effects at one level may be opposed or modified at another level. Thus, the development of an experimental approach capable of revealing critical interactions requires study of more than one aspect of immune function (Cunningham-Rundles, 1993; Muga and Grider, 1999; Beisel, 2000). The effect of any single nutrient is dependent upon concentration, interactions with other key nutrients, host genetic expression and internal environmental conditions. In situations of nutrient imbalance, duration of the altered condition and age of the host are also often critical factors (Cunningham-Rundles and Cervia, 1996; Hirve and Ganatra, 1997; Miles et al., 2001).

Nutrients affect specific immune–cell types differently through influencing intrinsic cell function and by influencing cell–cell interactions. Much of the critical action appears to occur in the local microenvironment during the response to antigen. Classically, the immune system has been considered as an operational duality divided into an innate system, mediating immune reactions that do not functionally change with re-exposure to signal, and an adaptive immune system, which is capable of developing the response to antigen encounter and evolving with re-exposure. Adaptive immunity has been further characterized according to cell type, as the response of bone-marrow-derived B-cells of the humoral immune system and thymus-derived T-cells of the cellular immune system. This rather static picture of compartmentalized function is changing. Now, it is increasingly clear that significant T-
cell differentiation does occur independently of the thymus – for example, in the gastrointestinal tract. Current studies also show that the innate immune system, mediated by such cells as natural killer (NK) and NK T-cells, monocytes and dendritic cells, influences the nature of cytokine production by the adaptive immune system. This occurs through secretion of cytokines by innate immune cells into the microenvironment (Doherty et al., 1999; Garcia et al., 1999; see also Devereux, Chapter 1, this volume). The effect of the microenvironment is to drive the immune response towards either a T-helper type 1 (Th1) or a T-helper type 2 (Th2) response (see Devereux, Chapter 1, this volume). Micronutrients, such as trace elements and vitamins, are present in the local environment and have important regulatory effects on adaptive immune-cell function. For example, the trace element zinc supports a Th1 response, whereas vitamin A appears to produce a Th2 response (Frankenburg et al., 1998; Shankar and Prasad, 1998). Thus the new immunology provides a more fluid representation of a potentially evolving process that presents as a defined pattern according to an environmental dynamic rather than a static programme that is derived from fixed cellular characteristics. The basic elements are shown in Fig. 2.1.

**Fig. 2.1. Microenvironment of immune response.** APC, antigen-presenting cell; IFN-γ, interferon-γ; IL, interleukin; MHC, major histocompatibility complex.
Age of the host or developmental stage is often a critical variable. Antigen-specific humoral and cellular immunity are central to the adaptive immune response generated in the adult host. In contrast, neonates and infants rely primarily on innate immunity, specifically complement, maternal antibody, circulating mediators of the inflammatory response and phagocytes (see Brandtzaeg, Chapter 14, this volume). However, many of the components of innate immunity are not as functional in young children as in adults (Insoft et al., 1996; see also Chapter 14). Encounters with potential pathogens, such as parasitic infections or viruses, may easily compromise these resources. Study of this permits a glimpse of how the naive immune system copes with the sudden influx of signals, new antigens and potential pathogens. When malnutrition is present, the overall development and expression of the immune response are significantly impaired (Cunningham-Rundles et al., 2000, 2002; see Chandra, Chapter 3, this volume). Similarly, the ageing process affects nutrient needs and the immune response in an interactive fashion. The effect of ageing on the response to immunization and the enhancing effects of micronutrients are well known (Lesourd, 1997; Pallast et al., 1999; see Lesourd et al., Chapter 17, this volume). In addition, there are fundamental age-related changes, which may reflect inflammatory processes (see Chapter 17), such as the report that plasma levels of certain adhesion molecules increase with age and appear to influence the impact of dietary fish oil supplementation (Miles et al., 2001).

Assessment of how nutrients may interact in human immune function is a complex undertaking, more difficult than the assay of the response to a specific antigen of interest – for example, the serological antibody response to a virus. In the latter case, it is usually possible to know what level of response correlates with protection. Because of the great specificity and sensitivity of this information, some of the best data regarding nutrient interaction with the human immune system have been based on the use of response to specific pathogens as the point of reference. However, extrapolation from specific settings may be hazardous. It is seldom clear that immune deficiencies in vitro will predict immune deficiency in vivo. Therefore, investigators often seek to strengthen inferences by inclusion of in vivo tests, such as delayed-type hypersensitivity measured by skin testing, and by assessment of the humoral immune response through assay of specific antibodies arising in response to primary or secondary (booster) immunization. Consistency of an altered immune response in the absence of acute clinical presentation continues to serve as the benchmark indicator of a putative intrinsic immune defect. By analogy, repeated studies in the absence of the acute clinical process are crucial for the study of immune changes secondary to chronic malnutrition.

General assessment of the anatomy of the immune system in humans includes measurement of serum immunoglobulins and complement and the evaluation of lymphocyte subsets by immunophenotyping. Analytical studies require selection among a wide range of tests that measure immune function in vitro or ex vivo as a reflection of the immune response in vivo (Kramer and Burri, 1997; Jaye et al., 1998; Cunningham-Rundles, 1999; Bergquist et al., 2000). A basic panel of tests is also required to reveal how the overall balance
of the immune system has been affected. Immune studies are often based on
limited studies of immune-cell subsets, serum or plasma concentrations of
cytokines or the functional response of mononuclear cells cultured in highly
standardized systems, using a chosen stimulus and often a single end-point.
Newer methods have made it possible to assess differentiation in antigen
expression on peripheral-blood mononuclear cells in response to activation, to
study early events in the activation pathway and to analyse gene activation.

The development of cytokine biology has provided a critical means of clar-
ifying the fundamental impact of nutrients on immune response. In general,
nutrients appear to affect the immune system most profoundly through regulatory mechanisms affecting the expression and production of cytokines (e.g.
Savendahl and Underwood, 1997; Rink and Kirchner, 2000). Since the type of
cytokine pattern produced is crucial for the response to infectious pathogens,
serious nutrient imbalance will ultimately compromise the development of the
future immune response. However, while malnutrition promotes susceptibility
to pathogens, even subclinical infections directly affect nutrient intake and
metabolism. Severe, acute infection will have a very strong impact. The fact
that cytokine production during the acute-phase response to generalized sepsis
can lead to loss of lean tissue and body fat is well known (Lin et al., 1998).
Interestingly, this cascade of events can be altered by nutritional intervention
(Jeevanandam et al., 1999). Immune deficiency and susceptibility to infection
are often directly linked with malnutrition, which was the leading cause of acquired immune deficiency before the appearance of the human immunodeficiency virus (HIV). Malnutrition is also a major factor contributing to the progression of HIV infection, especially in less developed countries. Since malnutrition and HIV affect the host in similar ways, the combination is particularly devastating. Many of the infections observed in human protein–energy malnutrition (PEM), such as tuberculosis, herpes, Pneumocystis carinii pneumonia and measles, are caused by intracellular pathogens, indicating that the cellular immune system is particularly affected (Keusch, 1993; see Chandra,
Chapter 3, this volume).

While the effects of infection and malnutrition on the immune response are
interactive, the effects of each upon immune response are also independent. A
recent examination by Mishra et al. (1998) of graded PEM in children in rela-
tionship to tuberculosis infection and response to a skin-test anergy panel,
including purified protein derivative of Mycobacterium tuberculosis (PPD), has shown that impaired cellular immunity was observable in all grades of malnutrition, except
for response to PPD in grade I, and that infection did not affect this.

Differentiation of lymphocyte subpopulations is also directly affected by
malnutrition. Studies show that T-cells from children with severe PEM are
immature, compared with those from well-nourished children, and that the
degree of immaturity is directly associated with thymic involution, as measured by echo radiography (Parent et al., 1994). While nutritional repletion
affected anthropometric measures within 1 month, regrowth of the thymus
took longer (Chevalier et al., 1996, 1998). The long-term consequences of
slow thymic regrowth are unknown. These studies underscore the importance
of longitudinal studies.
Response to certain pathogens may actually be enhanced in some states of malnutrition. Genton et al. (1998) assessed the incidence of malaria in children in Papua New Guinea, and found that increased height-for-weight at baseline (an indicator of a better nutritional state) predicted susceptibility to malaria during the year of study and that the lymphocyte response to malarial antigens was lower among the less wasted children. Furthermore, cytokine production towards malarial antigens was greater among malnourished children, suggesting that a favourable cytokine regulatory shift might be the basis of improved response among stunted, but not wasted, children. Stunting has often been considered as an adaptive and partially protective host response to prolonged nutrient deprivation. Rikimaru et al. (1998) evaluated lymphocyte subpopulations and immunoglobulins among healthy children and children with kwashiorkor, marasmus and marasmic kwashiorkor in Ghana. Interestingly, immunoglobulin A (IgA) and C4 were higher, whereas C3 and relative B-cell percentage were lower, in the severely malnourished groups. These studies demonstrate the advantages of using linked measurements to develop a full immunological profile.

In summary, the study of nutrient immune interaction requires consideration of the setting and a design that includes evaluation of possible complementary effects at more than one level. Longitudinal studies are often useful and permit assessment of the evolution of the immune response and characterization of downstream effects, which may modulate outcome.

Evaluation of Human Immune Response

Until recently, methods for evaluating the human immune system were derived largely from experimental approaches designed to analyse deficits in host defence in specific clinical settings. With the advent of molecular approaches, immune function has been studied more directly and has led to clarification of specific pathways. As a result, the molecular basis of primary and acquired immune deficiency syndromes is better understood. In addition, the development of vaccines and the study of the natural response to infectious exposure have expanded exponentially in the wake of the HIV crisis, leading to the development of increasingly targeted methods of measuring the immune response. While assessment of the humoral immune response at the level of specific antibody is now well standardized and often routine, evaluation of the complex interactions that are needed to produce specific antibody and the idiootypic interactions that govern this remains a specialized research endeavour. The study of the cellular immune response as a whole continues to remain largely a research activity, although this is beginning to change. This discussion will focus on methods that have been applied to the study of nutrients, and will include approaches that have led to new discoveries in other areas.

The most widely applied methods of evaluating T lymphocyte activation have used peripheral-blood mononuclear cells, isolated by density-gradient centrifugation and cultured with plant lectins (mitogens), or bacterial or viral activators, or antigens, which elicit a secondary response that depends upon prior priming or natural exposure in vitro (Paxton et al., 2001). The typical
mononuclear-cell culture contains a mixture of T-cells, B-cells and monocytes. After several days in culture, the cells are pulse-labelled with a radioactive precursor (usually thymidine), and incorporation is measured by assessing incorporation into DNA. The amount of incorporated tracer is closely related to the amount of DNA synthesis and ensuing cell division. The use of whole blood diluted and cultured in the presence of activators also provides an index of mononuclear-cell response but is fundamentally different, since the concentration of cells is not standardized, as it is when mononuclear cells are isolated from whole blood. However, the advantage of this kind of ex vivo test is that plasma proteins and soluble factors present in blood are not removed (Sottong et al., 2000). Further, the interrelationships among cell types are preserved.

The development of monoclonal antibodies directed against cell-surface determinants has evolved from the detection of lymphocyte-subset differentiation antigens defining T-cells, B-cells and NK cells to the elucidation of critical receptors, such as cytokine and growth-factor receptors, as well as many molecules involved in the activation, differentiation and dissemination of immune response. These methods are applicable to a wide range of studies (Cunningham-Rundles, 1998). Examples include monoclonal antibodies recognizing intracellular cytokines, adhesion molecules and early surface markers produced in response to antigen. Flow cytometry provides a means of studying lymphocyte-subset activation without resort to the use of radioactive tracers. In the following section, examples from current work will be discussed.

Overall design

Nutrition research offers a very interesting and potentially novel way to study the human immune system, and provides an important counterpart to the study of the immune response in primary or secondary immune deficiency where infection, autoimmunity or malignancy are manifest at clinical presentation. While it is clear that there is substantial variation in the normal immune response, the basis of this difference, whether genetic or environmental, remains to be determined. Fundamental studies are needed to determine how nutrient status may influence the development and expression of host genes involved in the immune response. Bendich (1995) has proposed that tests of immune function should be considered in determining the recommended daily allowance (RDA) of certain nutrients, since the levels of several micronutrients needed to support optimal immune function are often higher than those levels needed to qualify as clinical nutrient deficiency, which are usually defined in association with secondary clinical presentation. While there is good evidence that reduced immune function as measured in vitro or ex vivo is linked to risk of infection or to the development of tumours in vivo, tests of immune function are not specific for specific nutrients. A valid test of the effect of nutrient deficiency on immune function would probably require that repletion be proved to correct the defect induced by depletion. This has been achieved for zinc by Prasad (2000), who has demonstrated that experimental human zinc depletion by dietary means leads to reduced levels of Th1 cytokines.
Evidence that nutrients have direct effects on human host defence has come mainly from clinical observations and field studies in settings of severe or chronic nutrient deficiency. These investigations are often complicated by host environmental factors or by exposure to toxins, carcinogens, pathogens or endemic infection (Blot et al., 1993; Zhang et al., 1995; Giuliano et al., 1997; Dai and Walker, 1999). While many studies have described interesting and potentially critical associations, few have identified causal relationships. No single investigational design is necessarily capable of revealing the causal links that govern these intricate relationships.

The choice of study population is fundamental and this directly affects the kinds of controls that are needed. Laboratory controls are highly informative for internal technical quality if run in parallel with subject studies. In some cases, this can be achieved by using aliquots of frozen cells from the same donor, but this has the disadvantage of not providing information concerning the normal range. Parallel controls should include fresh samples from subjects matched for age, sex and clinical status. Longitudinal studies may be crucial and, in some cases, may enable the use of each subject as his/her own control.

When the study design is observational and a nutrient or immune abnormality is known or suspected, study of other potentially related immune-function variables becomes critical. For example, both Th1 and Th2 cytokines should be measured when a Th1 deficiency is suspected. In the context of intervention studies, reliable data can be obtained using different designs, such as placebo-controlled, double-blind and crossover. Inferences may also be drawn from some single-arm studies with unambiguous and quantifiable endpoints. In some cases, it has been possible to use experimental depletion and repletion of the same study group. In other cases, lingering effects have blurred distinctions. For greater stringency, it may be necessary to include several repletion arms at graded doses and to follow changes for a length of time, since the immune system often shows a transient rebound effect that is not seen at later time points. It is also essential to measure other nutrient levels that are positively or negatively regulated by the nutrient under study.

**Experimental approach**

**Immune activators**

Immune activation requires a signal when circulating blood is used as the cell source, since the peripheral-blood lymphocyte is a resting cell. This signal is often a plant lectin, or another signal, such as certain divalent cations, calcium ionophores or surface-reactive molecules, including monoclonal antibodies to CD3, which provide a non-antigenic stimulus that activates T lymphocytes independently of antigenic history. Impaired response to mitogens in human settings of PEM may or may not be accompanied by loss of response to pathogens. Examples include the study of response to PPD in malnourished children at risk of tuberculosis and the effect of stunting on the response to malarial antigens (discussed above). It is well known that infections with even
relatively non-pathogenic viruses, such as measles, are often fatal in children with PEM, because measles-virus infection causes a serious but usually transient suppression of the cellular immune response (Schlender et al., 1996; Ito et al., 1997), which, in the malnourished host, may continue to prevent immune clearance. Longitudinal studies are often essential to demonstrate long-term effects, such as the lingering effect of vitamin A deficiency, which increases mortality from infections (West et al., 1999). Current studies suggest that the specificity of the response, defined as a Th1 or Th2 cytokine-pattern, to a specific microbe is critically associated with host defence. Study designs that incorporate antigens that are actually being encountered at the time of study or that focus on the type of cytokine production may therefore provide important and unique information.

Selection of methodology

Good study design is based on the formulation of a clear question that addresses a critical issue. Table 2.1 illustrates how the integration of the study design with a well-chosen methodology can lead to informative results in different areas of research. A balance of human and experimental animal-model studies is presented, since development of this field has depended upon both.

The study of whole foods, fats and certain micronutrients and how these could influence immune function is currently under development. Fundamental observation of human PEM has shown that generalized malnutrition leads to impaired immune response and susceptibility to infection (see Chandra, Chapter 3, this volume). However, direct examination of how dietary intake of any particular nutrient affects the immune response is a complex undertaking. Table 2.1 includes four studies on dietary intake. Labeta et al. (2000) addressed the fundamental question of how human milk might activate the neonatal immune system by molecular mimicry through the isolation and sequencing of a relevant polypeptide. Fawzi et al. (2000) focused on how a whole food, specifically tomatoes, may protect against morbidity and mortality, an idea that has come from studies implicating antioxidants as improving immune function (see Hughes, Chapter 9, this volume). The relationship held true even with correction for total vitamin A level (Fawzi et al., 2000). The strength of this study is derived from the large scale – more than 28,000 infants studied – and the careful surveys conducted, combined with excellent statistical analysis.

When stress is added to the equation, the nutrient requirements for immune response are further altered, and development of hypotheses often requires more than one approach. For example, there are extensive observations showing that total parenteral nutrition suppresses immune response in the surgical patient and related studies showing that glutamine becomes a conditional essential amino acid during metabolic stress (Calder and Yaqoob, 1999; O'Flaherty and Bouchier-Hayes, 1999; see also Calder and Newsholme, Chapter 6, this volume). These observations have led to the discovery that nutrients provide an essential stimulus for the induction, differentiation and maintenance of the mucosal immune system. Lack of enteral dietary intake
Table 2.1. Experimental approach to nutrient–immune function interaction.

<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Methods</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect of glutamine on mucosal immunity</td>
<td>Randomized study; groups of rats given food, total parenteral nutrition (TPN) or isocaloric/isonitrogenous TPN with 2% glutamine</td>
<td>Respiratory tract and intestinal washings obtained for IgA and Th2 cytokines measured</td>
<td>TPN decreased IgA, IL-4 and IL-10; supplementation with 2% glutamine enhanced levels of IgA and Th2 cytokines</td>
<td>Kudsk et al. (2000)</td>
</tr>
<tr>
<td>Effect of gene for haemochromatosis (HFE) on iron metabolism and immunity</td>
<td>Deletion of HFE α1 and α2 putative ligand-binding domains in vivo</td>
<td>HFE-deficient animals were analysed for a comprehensive set of metabolic and immune parameters</td>
<td>Plasma iron, transferrin saturation and hepatic iron were increased – traceable to augmented duodenal iron absorption; no obvious effect on immune system</td>
<td>Bahram et al. (1999)</td>
</tr>
<tr>
<td>Dietary fats and immune response</td>
<td>Mice fed high-fat (saturated, n-6 or n-3) or low-fat diet</td>
<td>Fatty-acid composition, spleen lymphocyte proliferation, Th1 and Th2 cytokine profile of spleen cells measured</td>
<td>Polyunsaturated but not saturated fatty acids decreased Th1 cytokine production; little effect on Th2 cytokines; effect shown at level of mRNA</td>
<td>Wallace et al. (2001)</td>
</tr>
<tr>
<td>Tomato intake and morbidity/mortality</td>
<td>Large-scale longitudinal study of infants in Sudan</td>
<td>Morbidity/mortality from diarrhoeal/respiratory disease and intake of tomatoes during previous 2–3 days</td>
<td>Intake linked to reduced mortality after adjustment for total vitamin A intake, inversely correlated with diarrhoeal incidence, respiratory infection</td>
<td>Fawzi et al. (2000)</td>
</tr>
<tr>
<td>Risk of mortality in selenium-deficient HIV+ children</td>
<td>Perinatally exposed children enrolled over a 3-month period studied for 5 years</td>
<td>CD4 cell count and nutritional status were studied; regression models were used to test relationship to survival</td>
<td>Low plasma selenium and CD4+ T-cell number below 200 linked independently with mortality</td>
<td>Campa et al. (1999)</td>
</tr>
</tbody>
</table>

IL4, interleukin; IgA, immunoglobulin A.
impairs mucosal IgA and secretory-component production, the number of IgA-containing cells and the level of IgG and promotes mucosal growth (Heel et al., 1998; Kudsk et al., 2000). Even foods such as indigestible saccharides can have a stimulating effect upon the immune system (Kudoh et al., 1998). The study of Kudsk et al. (2000), included in Table 2.1, has added significantly to this field, showing specific differences among animals fed on laboratory food, by total parenteral nutrition and by parenteral nutrition supplemented with glutamine on the pattern of cytokine and IgA production. Loss of nutrient stimulation led to loss of total lymphocyte number in Peyer’s patches, in the intraepithelial layer and in the lamina propria, a reduced CD4+ T-cell to CD8+ T-cell ratio and a reduced intestinal level of IgA (Kudsk et al., 2000). Furthermore, lack of enteral nutrition may signal increased neutrophil recruitment through up-regulation of the intercellular adhesion molecule 1 (ICAM-1), causing increased leucocyte binding in the intestine (Fukatsu et al., 1999). These studies indicate how the immune response during stress may be modulated experimentally by specific amino acids in the diet.

The study of lipids provides great challenges for study design, since incorporation into membranes, as well as direct effects on metabolic pathways, must be considered. There is increasing evidence that increase in fat intake may impair immune function, as well as leading to obesity (Nieman et al., 1996). A relationship between fat intake and cancer risk has been indicated (Risch et al., 1994), but the mechanisms remain unclear. Recent data demonstrate that the fatty-acid composition of cellular membranes can cause immune perturbation. Mechanisms of action include modulation of adhesion-molecule expression (Miles et al., 2000) and are apparently related to specific fatty-acid composition. The activation state of the cell is a determining factor in how fatty acids affect the immune response (Wallace et al., 2000). This topic has been addressed by Wallace et al. (2001) in a thorough study in which mice were fed low-fat diets or high-fat diets, containing either saturated or unsaturated fats. Both n-3 and n-6 polyunsaturated fatty acids were used, permitting distinction of their effects on cytokine production. Data showed that n-3 fatty acids were strongly suppressive of Th1 cytokines (see also Calder and Field, Chapter 4, this volume). This classic feeding study included measurement of fatty-acid incorporation, cytokine secretion and cytokine mRNA production.

Other work has shown how emerging information about the human genome may be used to study basic mechanisms. For example, the discovery of the gene HFE has revealed that the molecular basis of hereditary haemochromatosis, which involves increased iron uptake from the gastrointestinal tract, can be attributed to homozygous inheritance of one mutation (Feder et al., 1998; Gross et al., 1998). HFE regulates the metabolism and distribution of iron by affecting the binding of iron to transferrin, is a major histocompatibility complex (MHC) class I protein and is also non-covalently associated with β₂-microglobulin (β₂m). The significance of this physical association is unclear. Excess iron has been observed in association with loss of CD8+ T lymphocytes in the β₂m-knockout mouse. CD8+ T-cells are also reduced in a subgroup of haemochromatosis patients who show an increased rate of iron loading (Porto et al., 1997). The low-CD8 phenotype is also
observed in a subset of patients with transfusion-related iron overload (Cunningham-Rundles et al., 2000). Interestingly, studies in compound mutant mice lacking both HFE and $\beta_{2m}$ have shown that more iron was deposited in various tissues than was observed in mice with either mutation alone (Levy et al., 2000). However, studies in genetic-deletion models (e.g. the work of Bahram et al., 1999) indicate that the basis of a putative link between immune function and iron handling remains unresolved.

Good study design is critically important for studies in complex settings, such as HIV infection, where nutrient imbalance is fundamentally linked to infection but hard to study in a clear-cut manner. Weight loss is a common occurrence in general chronic viral illness and, in the case of HIV infection, can evolve into a wasting condition, which may become intractable with failure of antiretroviral therapy. Infection-induced malnutrition, as discussed above, is primarily cytokine-mediated and is associated with the acute-phase response. This is accompanied by multiple effects on metabolism, such as altered fluxes of iron and zinc and loss of nitrogen, potassium, magnesium, phosphate, zinc and vitamins. This process is accompanied by retention of salt and water. Malnutrition may also present during the asymptomatic phase of HIV infection (Niyongabo et al., 1997; Peters et al., 1998). Many studies have shown that micronutrient status is profoundly affected in HIV infection, but the aetiological significance of these changes has been difficult to demonstrate (Cunningham-Rundles, 2000). Therefore, the work of Campa et al. (1999), included in Table 2.1, has provided an important advance. Using careful longitudinal studies and good statistical design, this group was able to establish that selenium deficiency in children with acquired immune deficiency syndrome (AIDS) was independently associated with mortality.

**Immune assessment**

New assay methods have enabled the design of experiments addressing different stages involved in immune-cell activation and the study of effects on signalling pathways, which may then lead to the characterization of causal relationships. Table 2.2 outlines some of the types of methods currently in use. Most investigations begin with a general assessment of how a nutrient or altered nutritional state affects the general parameters of the immune system, immune-cell subsets and function. Measurement of changes in frequency and number of circulating lymphocyte subpopulations in the course of observation or dietary intervention is now accepted as a useful and widely comparable procedure, but attention must be given to the issue of controls. This analysis should include standardized performance of immunophenotyping, using correction for purity of the gating region, quantitative recovery of the cell type and positive identification of cellular subsets. For human studies, a complete blood count and differential are needed to quantify effects on absolute numbers of cells. Although there is frequently a limitation on blood to be drawn for nutritional studies, it is essential that the baseline evaluation includes parallel studies providing a complete blood count, haematological analysis of haemoglobin, haematocrit, etc., on an aliquot of the same specimen of blood.
Table 2.2. Assessment of functional immune response.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Function</th>
<th>Determinant of specificity</th>
<th>Principle</th>
<th>Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early activation event</td>
<td>Response to signal</td>
<td>Signal, identity of responder cell</td>
<td>Biochemical or monoclonal antibody assay or gene activation of responder cell</td>
<td>ATP production Flow-cytometric assay of CD69 up-regulation mRNA</td>
</tr>
<tr>
<td>Proliferation: magnitude of cellular response to signal</td>
<td>Cell division</td>
<td>Signal, cell population, culture conditions</td>
<td>Radioisotopic tracer incorporation measures DNA synthesis after cell culture with activator; DNA-binding dyes</td>
<td>Microtitre culture Whole blood Mononuclear cells isolated by density gradient Purified cell populations</td>
</tr>
<tr>
<td>Cytokine response Cytokine pattern</td>
<td>Specific cytokine Th1/Th2 response to signal</td>
<td>Reagent specificity, identity of producer cell</td>
<td>ELISA and ELISPOT use antibody/antigen; intracellular cytokine use monoclonal antibodies</td>
<td>ELISA: cytokine level ELISPOT can identify secreting-cell frequency Intracellular detection can identify producer cell</td>
</tr>
<tr>
<td>Immune-cell subsets</td>
<td>Subpopulation analysis</td>
<td>Monoclonal antibody and accuracy of the gating strategy</td>
<td>Monoclonal antibodies coupled to fluorochromes</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>Antigen-specific cells</td>
<td>Functional response</td>
<td>Specificity depends on signal and detection system</td>
<td>Detection of interferon secretion as enzyme-labelled antibody reaction, specific activation</td>
<td>ELISPOT Flow-cytometric detection of activated cell ATP production by specific cells</td>
</tr>
<tr>
<td>Antibody secretion</td>
<td>Antibody-secreting cell</td>
<td>Antigen/antibody – may require antigenic stimulation</td>
<td>Recombinant antigens, monoclonal antibody, limiting-dilution methods</td>
<td>ELISA RIA ELISPOT Chromium release ELISPOT Flow cytometry</td>
</tr>
<tr>
<td>Cytotoxicity</td>
<td>Specific and non-specific target-cell killing</td>
<td>Depends upon target and effector cell</td>
<td>Specificity of target-cell killing, relative strength/restriction measured</td>
<td></td>
</tr>
</tbody>
</table>

ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme linked immune spot; RIA, radioimmune assay.
In addition to assessment of relative percentages of T-cells, B-cells and NK cells, immunophenotyping for activation-antigen expression (e.g. CD69), coexpression of critical molecules involved in cell–cell interaction (e.g. CD28), T-cell receptor (TCR) changes and percentages of naive and memory cells may be informative. Functional studies should be carried out on fresh anticoagulated blood whenever possible (or blood stored at room temperature in the dark for under 24 h) before mononuclear cells are isolated. When blood is being sent by air or transported to a distant laboratory, it is extremely important to include a control specimen drawn in parallel to serve as an internal standard for the shipping process. In addition, the type of tube chosen to draw the blood is important. Lithium heparin- or ethylenediamine tetra-acetic acid (EDTA)-containing tubes cannot be used for functional studies. Sodium heparin (preservative-free) or acid citrate dextrose (ACD) tubes should be used and consistency of tube type is important. There may be differences between venous and arterial blood. The question of when the blood should be drawn is important. In general, most data have been obtained with blood drawn in the morning and there are circadian effects on hormones and immune-cell phenotypes that may influence results. When this cannot be done, it is helpful to continue to maintain a uniformity of drawing time for an individual subject or group. Concurrent control blood must be drawn to ensure that technical performance standards are met. It is important that positive and negative (normal range and abnormal range) controls be included. Double-baseline studies – as a minimum, before and after intervention is undertaken – are recommended.

Studies of immune function usually start with a general assessment of mononuclear response in vitro to a mitogen, to another non-specific activator or to antigen, as discussed above. These methods are generally based on assay of cell division at the peak of response following microtitre plate culture for several days. Culture methods profoundly affect results, and conditions need to be optimized according to the kinetics of the response. Responses measured under most conditions favour T-cell proliferation, as the T-cell is the most prevalent lymphocyte in peripheral blood. The elicited composite response is highly quantitative when radioactive tracers – usually thymidine – are used. Recently, whole-blood methodology has been introduced as an alternative ex vivo method that can reflect potential response in vivo (Sottong et al., 2000); this method correlates with the level of DNA synthesis found when isolated mononuclear cells are cultured under optimal standard conditions. Comparative studies have also shown that there is a significant correlation between the whole-blood method and isolated mononuclear cells for cytokine production (Yaqoob et al., 1999). Some laboratories have replaced thymidine incorporation assays with a combination of cell-surface marker-induction assays and a measurement of the percentage of cells in various phases of the cell cycle following activation. Dyes have been developed that stably integrate into the membranes of live lymphocytes, such that, with each successive division, the amount of dye per cell is decreased. Fluorescence can be used to measure the number of cell divisions. Other assays based on whole blood measure early responses of cells selected through adherence to magnetic beads to which monoclonal antibodies recognizing cells of particular interest are attached. Assessment is achieved by an assay of adenosine
riphosphate (ATP) production by the luciferin/luciferase reaction (Sottong et al., 2000). Assays such as this may provide accurate assessment of in vivo response in vitro. This method may be combined with a quantitative measure of specific lymphocyte subsets by flow cytometry for examination of response per cell.

Other approaches use measurement of cytokine response, receptor up-regulation or activation antigen to assess initial immune response, rather than the secondary response of cells recruited in the amplified reaction. Also, in vivo regulation of the immune response can be assessed through evaluation of unstimulated levels of secreted products when the producer-cell source of these products and normal levels are known. Methods measuring early events in T lymphocyte activation may or may not correlate with cell division, since cell division is only one aspect of the immune response. One of the earliest events that occurs following T-cell activation is the rapid increase in intracellular free calcium. This is followed by a change in pH and changes in the membrane potential. All of these effects can be measured by flow cytometry, using functional probes. Following T-cell activation via CD3/TCR or via CD2 (the alternate T-cell activation pathway), the first measurable surface marker induced is CD69. This marker is a disulphide-linked homodimer that is present on 20–30% of normal thymocytes, but which is not expressed on resting peripheral-blood lymphocytes. CD69 reaches peak levels within 18–24 h and declines if the stimulus is removed. Using flow cytometry, it is possible to measure increase in CD69 expression on specific lymphocyte subsets. It is apparent that CD69 induction is not part of the pathway leading to cell division, as induction of CD69 can occur without subsequent cell proliferation. A good way to measure CD69 expression is to consider the relative expression of this marker on the subpopulation of interest, as this removes the confounding effect of subpopulation size. Other cell-surface markers appear on activated T-cells at variable times following activation, including CD25 (the α chain of the interleukin-2 (IL-2) receptor) and the transferrin receptor CD71 (both within 24–48 h) and human leucocyte antigen (HLA)-DR (after 48 h).

Finally, statistical evaluation is crucial to all of the studies described here. This includes evaluation of both the internal and the study-group controls. Studies of certain types may be suitable for the collection and banking of specimens prior to assay, such as cytokine supernatants. This may be helpful in giving a homogeneous data set with a low coefficient of variation, as long as controls and experimental specimens are run simultaneously. Good design is often based on internal cross-checks, which can be developed from the working hypothesis and which allow for different elements in the same pathway to be considered.

In summary, the emerging field of nutritional immunology has benefited from the evolution of cellular and molecular immunology. New approaches have provided a strong foundation for experimental design and offer a choice of analytical methods for approaching hypothesis testing. The key to any specific investigation is the identification of clear questions and the choice of relevant and practical methods. These methods then need to be tested in a pilot study, before launching the investigation. The use of an integrated design, including biostatistical considerations and complementary assays, is important in the development of meaningful data and of critical knowledge.
References


Effect of Post-natal Protein Malnutrition and Intrauterine Growth Retardation on Immunity and Risk of Infection

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Introduction

In spite of projections and plans announced by both politicians and professionals in the last 25 years, protein–energy malnutrition (PEM) continues to be widely prevalent, particularly in Asia and Africa. This is associated with considerable morbidity due to infectious illness. Work during the last 30 years has demonstrated the important pathogenetic role of impaired immune responses in the two-way interaction between malnutrition and infection. Similarly, intrauterine growth retardation (IUGR) resulting from a variety of maternal and fetal factors is associated with impaired immune responses and enhanced susceptibility to infection. Unlike the reversibility of reduced immunity in post-natal PEM, decreased immunity in small-for-gestational-age (SGA) infants is prolonged and may last for months, even years.

Protein–Energy Malnutrition

The clinical spectrum of PEM is somewhat varied, depending upon the age of occurrence, the concurrent presence of infection and the area of the world where it occurs. The same applies, to some extent, to the immunological effects of PEM.

From a historical perspective, it is useful to cite the clinical stimulus that led to the first comprehensive examination of the immune system in PEM (Chandra, 1972). Interest in nutrition–immune interactions was kindled by the story, with an unhappy ending, of a child. Eighteen-month-old Kamala was thin, her skin pale as wax and her lungs screaming for air. She wore a spectral...
white death-mask in a frame of black hair. Her shrivelled body and swollen legs were typical of marasmic kwashiorkor, and she had an obvious fulminating infection. Lung aspirate revealed the opportunistic organism *Pneumocystis carinii*. Despite our best efforts, we lost the child. We speculated that malnutrition had robbed Kamala of her defences against infection and led to her premature demise. Against this background in 1969, I applied the available techniques to study the immunocompetence of undernourished children. To convey a sense of time, the discipline of immunology did not even involve the general use of terms such as cell-mediated immunity, lymphocyte subsets, immunoregulation and so on. In malnourished patients, we found a number of impaired immune responses, including delayed cutaneous hypersensitivity, lymphocyte-proliferation responses to mitogens, complement activity and secondary antibody responses to some antigens. These findings were soon confirmed by several investigators (Anon., 1987).

Any discussion of the effects of nutritional deficiencies on immune responses must be prefaced by emphasizing the complexities and heterogeneity of both clinical malnutrition and immune responses. The critical role of nutrition in modulation of immune responses is based on physiological considerations. The severity and extent of dysfunction caused by malnutrition in various organ systems depend on several factors, including the rate of cell proliferation, the amount and rate of protein synthesis and the role of individual nutrients in metabolic pathways. Lymphoid tissues are very vulnerable to marked involutions as a result of nutritional deficiency. Many cells of the immune system are known to depend for their function on metabolic pathways that employ various nutrients as critical cofactors. Numerous enzymes require micronutrients.

The consistent impairment of immunity in PEM and the recognized increase in infections in patients with primary immunodeficiencies are compatible with the hypothesis that a depressed immune system in malnutrition enhances the risk and severity of infection. The work on children has now been extended to other age-groups and to other parts of the world, including the malnourished groups seen in hospitals and in underprivileged communities in industrialized affluent countries. For example, the cellular immune changes seen in young children with PEM in developing countries are replicated to a large extent in subjects with primary or secondary PEM in industrialized countries, such as those with anorexia nervosa (for a review, see Marcos et al., 2001). It should be pointed out that malnutrition is a complex syndrome where several deficiencies exist simultaneously. Even in laboratory animals deprived of a single nutrient, the functional effects may be the consequence of changes in the absorption or body stores of other substances. Thus, what is observed in an undernourished individual is the sum of the contributions and responses of many components of the immune system that have been altered by one or more nutrient deficiencies.

The interaction between malnutrition and infection is bidirectional: one aggravates the other. Scrimshaw *et al.* (1968) proposed the interesting concept of synergism and antagonism between the host's nutritional status and the microbe's ability to produce disease; the direction of interaction is more often synergistic, namely, PEM increases the incidence, duration and severity of infectious illness.
Longitudinal prospective studies of infants have shown that reduction in various parameters of immunocompetence preceded clinical infection and growth faltering. Findings such as these suggest, first that altered immune responses are early functional indices of growth failure secondary to latent nutritional deficiency and, second, that episodes of infection worsen the child’s nutritional state.

PEM has been documented to increase morbidity and mortality caused by diarrhoea and respiratory illness (James, 1972; Tomkins, 1981; Chandra, 1983a). The incidence of infectious diarrhoea is increased and there is a more profound and consistent effect on the duration of each episode. Victora et al. (1990) studied the synergism between nutritional status and hospital admissions due to diarrhoea and pneumonia in a cohort of 5914 live births in southern Brazil and found that malnutrition was a more important risk factor for pneumonia than for diarrhoea, whereas diarrhoea was a stronger predictor of malnutrition than was pneumonia, the association being strongest in the first 2 years of life. In rural India, there was a significant correlation between weight-for-height as an index of protein–energy status and risk of death from infectious disease (Chandra, 1983b).

The term ‘nutritional thymectomy’ has been used to dramatize the extensive reduction in the size and weight of the thymus that occurs with malnutrition (see Chandra and Newberne, 1977). Histologically, there is a loss of corticomedullary differentiation, there are fewer lymphoid cells and the Hassal bodies are enlarged, degenerated and, occasionally, calcified. These changes are easily differentiated from findings in primary immune deficiency, such as DiGeorge’s syndrome. In the spleen, there is a loss of lymphoid cells around small blood vessels. In the lymph node, the thymus-dependent paracortical areas show depletion of lymphocytes.

The consistent adverse effects of PEM on cell-mediated immunity, production of cytokines, phagocyte function, the complement system and mucosal immunoglobulin A (IgA) antibody responses have been demonstrated in several studies in many countries. These observations have been reviewed several times (see Chandra and Newberne, 1977; Keusch et al., 1983; Gershwin et al., 1984; Watson, 1984; Chandra, 1992, 1996, 1999; Woodward, 2001).

In PEM, most of the host defence mechanisms are breached (Fig. 3.1). Delayed cutaneous hypersensitivity responses to both recall and new antigens are markedly depressed. It is not uncommon to have complete anergy to a battery of different antigens (Chandra, 1972). These changes are observed in moderate nutrient deficiencies as well (Kielmann et al., 1976; McMurray et al., 1981). Findings in patients with kwashiorkor are more striking compared with those in marasmus. There is a significant correlation between the cumulative diameter of induration response to five common antigens and the serum concentration of albumin, an index of visceral protein synthesis. Similarly, there is a significant correlation between the size of the delayed-hypersensitivity skin-test response and lean body mass (Fig. 3.2). The skin reactions are restored after appropriate nutritional therapy for several weeks or months.

The cellular and molecular reason for impaired skin responses lies in changes in the number and function of T lymphocyte subsets and macrophages.
Fig. 3.1. In PEM, most of the host defence mechanisms are breached, allowing microbes to invade and produce clinical infections that are more severe and prolonged (copyright ARTS Biomedical Publishers 1981).

Fig. 3.2. Correlation between the diameter of maximum skin induration, in response to delayed hypersensitivity challenge, and lean body mass. Those with a negative response, defined as induration of less than 5 mm (shaded box), had a lean body mass of 80% of standard for age or less.
and the production of various cytokines. There is a significant reduction in the number of mature, fully differentiated T lymphocytes, which can be recognized by the classical technique of rosette formation or by the newer method of fluorescent labelling with monoclonal antibodies. The reduction in serum thymic factor activity observed in primary PEM, including in adolescents with anorexia nervosa (Wade et al., 1985), may underlie the impaired maturation of T lymphocytes. There is an increase in deoxynucleotidyl transferase activity in leukocytes (Chandra, 1983a), a feature of immaturity. The proportion of helper/inducer T lymphocytes, recognized by the presence of CD4+ antigen on the cell surface, is markedly decreased in PEM (Fig. 3.3; Chandra, 1983c). There is a moderate reduction in the number of suppressor/cytotoxic CD8+ cells. Thus the ratio CD4+/CD8+ is significantly decreased compared with that in well-nourished controls.

The proliferative response to mitogens and microbial antigens is decreased. The synthesis of DNA is reduced, especially when autologous patient’s plasma is used in cell cultures. This may be the result of the presence of inhibitory factors, as well as deficiency of essential nutrients in the patient’s plasma (Beatty and Dowdle, 1978). Another aspect of lymphocyte function that changes in PEM is the traffic and ‘homing’ pattern (Chandra, 1991a). For example, lymphocytes derived from mesenteric lymph nodes of immunized rodents normally revert back to the intestine in large numbers, whereas in malnutrition this homing is reduced.

Co-culture experiments have shown a reduction in the number of antibody-producing cells in malnutrition (Fig. 3.4) and in the amount of immunoglobulin secreted (Chandra, 1983c). These observations may reflect the amount of ‘help’ provided by T-cells, since the impairment is reversed when T-cells are derived from well-nourished controls.

![Fig. 3.3. The proportion of T lymphocyte subsets in children with PEM and well-nourished controls matched for age and gender. The CD4/CD8 ratio is decreased.](image-url)
Serum antibody responses are generally intact in PEM, particularly when antigens are administered with an adjuvant or in the case of those materials that do not evoke a T-cell response. Rarely, the antibody response to organisms such as *Salmonella typhi* and influenza virus (Fig. 3.5) may be decreased. However, before an impaired antibody response can be attributed to nutritional deficiency, infection as a confounding factor must be ruled out. Antibody affinity is decreased in patients who are malnourished (Fig. 3.5; Chandra *et al.*, 1984). This may provide an explanation for a higher frequency of antigen–antibody complexes found in such patients. As opposed to serum antibody responses, secretory IgA antibody levels after deliberate immunization with viral vaccines are decreased (Chandra, 1975a); there is a selective reduction in secretory IgA levels. This may have several clinical implications, including the increased frequency of septicaemia commonly observed in undernourished children.

The production of several cytokines, particularly interleukin-2 and interferon-γ, is decreased in PEM (Chandra, 1992). Moreover, PEM alters the ability of T lymphocytes to respond appropriately to cytokines (Hoffman-Goetz *et al.*, 1984).

Phagocytic function is deranged in PEM. Chemotactic migration of phagocytes is slower and less efficient (Chandra *et al.*, 1976). In the presence of control serum that provides the normal concentrations and activity of various opsonins, phagocytes are able to ingest particles such as bacteria (Seth and Chandra, 1972). However, the next steps of metabolic activation – discharge of digestive enzymes into phagolysosomes, and microbial killing – are reduced (Seth and Chandra, 1972).

Many components of the complement system are reduced in concentration and activity in PEM (Chandra, 1975b; Haller *et al.*, 1978). The most affected are complement C3, C5 and factor B. Total haemolytic activity is decreased. These changes affect the opsonic activity that facilitates phagocytosis.
There is very little work on the effect of malnutrition on the integrity of physical barriers, quality of mucus or several other innate immune defences. However, lysozyme levels are decreased, largely as a result of reduced production by monocytes and neutrophils, but also due to increased excretion in the urine (Chandra et al., 1977a). Adherence of bacteria to epithelial cells is a first step before invasion and infection can occur. The number of bacteria adhering to respiratory epithelial cells is increased in PEM (Fig. 3.6; Chandra and Gupta, 1991). Work in laboratory-animal models of PEM has demonstrated a reduction in ciliary movement, particularly in the presence of mucosal infection (Fig. 3.7).

**Intrauterine Growth Retardation**

There is much clinical evidence that neonates have suboptimal immune responses and are susceptible to infection. When growth retardation and nutritional deficiency complicate the picture, as in low-birth-weight (LBW) infants, impairment of immunocompetence and risk of infection are more marked and longer-lasting (Chandra, 1991b). This results in higher morbidity (Ashworth, 2001; Table 3.1), enhanced occurrence of admission to hospital and increased mortality (Ashworth, 2001; Table 3.2).

The worldwide incidence of LBW, defined as a weight less than 2500 g, varies considerably from one population group to another, from 8% in some industrialized countries to 41% in some developing countries of Africa. In the former, the majority are preterm appropriate for gestational age (AGA), whereas, in the latter, the majority are SGA. The aetiology of fetal growth retardation includes maternal malnutrition.
Fig. 3.6. Correlation between the number of *Klebsiella* adhering to tracheal epithelial cells and nutritional status, assessed by weight-for-height.

![Graph showing correlation between adherence and weight-for-height](image)

**Adherence (bacteria per cell)**

**Weight-for-height (% standard)**

$r = 0.74$

$P < 0.001$

Fig. 3.7. Movement of tracheal-cell cilia in dogs with PEM and well-nourished controls. The experiment was run with phosphate-buffered saline (PBS) and after infection with *Bordetella* sp.

![Graph showing ciliary movement](image)

- **Well-nourished**
- **Malnourished**

<table>
<thead>
<tr>
<th>Incubation period (min)</th>
<th>PBS</th>
<th>Bordetella</th>
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<td>12.5</td>
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<tr>
<td>60</td>
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*Fig. 3.6.* Correlation between the number of *Klebsiella* adhering to tracheal epithelial cells and nutritional status, assessed by weight-for-height.

*Fig. 3.7.* Movement of tracheal-cell cilia in dogs with PEM and well-nourished controls. The experiment was run with phosphate-buffered saline (PBS) and after infection with *Bordetella* sp.
Table 3.1. Low birth weight and risk of mortality.

<table>
<thead>
<tr>
<th>Country</th>
<th>Design</th>
<th>Gestation</th>
<th>Age (months)</th>
<th>Sample size (deaths)</th>
<th>Birth weight (g)</th>
<th>Risk ratio (95% CI)</th>
<th>Outcome</th>
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<tr>
<td>Brazil</td>
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<td>Term</td>
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<td>6.6a (1.4–31.2)</td>
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<td>2500</td>
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<td>2500</td>
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<td>2500</td>
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<td>1.9a (1.1–3.6)b</td>
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*aAdjusted for confounders.
*b90% confidence intervals.
CI, confidence interval; ARI, acute respiratory infections.
See Ashworth (2001) for references.
### Table 3.2. Low birth weight and risk of morbidity.

<table>
<thead>
<tr>
<th>Country</th>
<th>Design</th>
<th>Gestation</th>
<th>Sample size</th>
<th>Birth weight (g)</th>
<th>Risk ratio (95% CI)</th>
<th>Outcome</th>
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<td>&lt; 2500</td>
<td>1.5 (1.1–2.1)</td>
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<td>&lt; 2500</td>
<td>1.2</td>
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<td>&lt; 2000</td>
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^aAdjusted for confounders.

ARI, acute respiratory-tract infection; CI, confidence interval; ALRI, acute lower respiratory-tract infection. See Ashworth (2001) for references.
LBW is associated with higher mortality. Whereas the total proportion of infants who die or are handicapped is similar in AGA and SGA groups, the former are at a higher risk of death in the immediate post-natal period, whereas the latter are at a higher risk of morbidity in the first year of life (Chandra, 1984). Infection is one of the recognized causes of increased illness in SGA infants. Upper and lower respiratory-tract infections are three times more frequent in SGA infants compared with AGA infants (Chandra, 1984). It appears that the morbidity pattern in the former group shows a bimodal distribution; about two-thirds exhibit a near-normal rate of illness, comparable to that of healthy full-term infants, whereas one-third have an increased illness rate – almost three times that of the full-term infants (Chandra, 1984). The SGA group is also at risk of developing infection with opportunistic microorganisms, such as *P. carinii*, as observed in post-natal malnutrition (Chandra, 1984).

SGA infants show atrophy of the thymus and prolonged impairment of cell-mediated immunity (Chandra, 1975c; Moscatelli *et al.*, 1976). Delayed cutaneous hypersensitivity to a variety of microbial recall antigens, as well as to the strong chemical sensitizer 2,4-dinitrochlorobenzene, is impaired. Serum thymic-factor activity is lower in SGA infants tested at 1 month of age or later. In contrast to AGA LBW infants, who recover immunologically by about 2–3 months of age, SGA infants continue to exhibit impaired cell-mediated immune responses for several months or even years (Chandra *et al.*, 1977b; Chandra, 1980). This is particularly true of those infants whose weight-for-height is less than 80% of standard. The prolonged immunosuppression in some SGA infants correlates with clinical experience of infectious illness (Chandra, 1991b) and thus may have considerable biological significance. In animal models of intrauterine nutritional deficiency, PEM results in reduced immune responses in the offspring (Chandra, 1975d).

Phagocyte function is deranged in LBW infants (Chandra, 1975c). There is a slight reduction in ingestion of particulate matter and a significant reduction in both metabolic activity and bactericidal capacity.

IgG from the mother, acquired through placental transfer, is the principal immunoglobulin in cord blood. The half-life of IgG is 21 days and thus all infants show physiological hypoimmunoglobulinaemia between 3 and 5 months of age. This is pronounced and prolonged in LBW infants (Chandra, 1975c), since their level of IgG at birth is significantly lower compared with that of full-term infants. There is a progressive rise in IgG concentration with gestational age and birth weight, especially in infants below 2500 g. All four subclasses of IgG are detected in fetal sera as early as 16 weeks of gestation, the bulk being IgG1 (Chandra, 1988). In SGA LBW infants, the cord-blood level of IgG1 is reduced much more than that of other subclasses (Chandra, 1988). Thus the infant:maternal ratio is significantly low for IgG1 but not for IgG2. The number of immunoglobulin-producing cells and the amount of immunoglobulin secreted are decreased in SGA infants who are symptomatic, i.e. those who have recurrent infection (Chandra, 1986). In the second year of life, SGA infants show a marked reduction in IgG2 levels and often show infections with organisms that have a polysaccharide capsule.
In preterm infants with a birth weight between 1800 and 2200 g, moderate oral zinc supplementation accelerates immunological recovery (Chandra, 1991a). In SGA infants, zinc supplements given from birth to 6 months of age improve immune responses and reduce mortality from diarrhoea and respiratory illness. A micronutrient supplement is more beneficial (Chandra, 2001).

Clinical Application and Intervention Strategies

The interactions between nutrition, the immune system and infection have much clinical and public health significance (Chandra, 1992). The fact that changes in immune responses occur early in the course of nutritional deficiency has led to the suggestion that immunocompetence can be used as a sensitive functional indicator of nutritional status. In patients with obvious primary or secondary malnutrition, the number of T lymphocytes is a useful measure of response to supplementation therapy. Anergy and other immunological changes correlate with poor outcome, in both medical and surgical patients, if impaired immunity is considered in association with hypoalbuminaemia (Chandra, 1983a,b,c). Opportunist infections occur more frequently among those patients with cancer who are also malnourished. The incidence of complicating infections can be reduced if appropriate preventive and therapeutic nutritional management is carried out in patients with leukaemia. It has been postulated that nutritional deficiency may influence the biological gradient and natural history of human immunodeficiency virus (HIV) infection (Jain and Chandra, 1984). Recent surveys indicate that attention to the nutritional needs of the HIV-infected individual is an important part of the overall management of this life-threatening infection (Subcommittee on Nutrition, 2001). Response to immunization is modulated by the nutritional status of the host, and the protective efficacy of vaccines may be suboptimal in the undernourished individual (Chandra, 1972). Finally, immune responses can be used to define safe upper and lower limits of nutrient intake.

We are now able to outline intervention strategies that will reduce the incidence and adverse health impact of both PEM and infection (Chandra, 1992). We have much of the knowledge needed to improve health; this needs to be supported by political commitment and effective management (see also Tomkins, Chapter 18, this volume). The preventive and intervention measures discussed below are well within the combined resources of the world. Even within the health sector, there are glaring anomalies. We must assign priorities and implement methods to prevent and control contributors to morbidity and mortality.

The major intervention strategies and their relative importance in tackling the twin problems of malnutrition and infection are shown in Fig. 3.8. Improvement in socio-economic status and education and ensuring the availability of sufficient food to every individual will most certainly eliminate much of malnutrition and infection, the two diseases of poverty. Health and self-limitation of family size will usually follow these measures. There is a negative correlation between rates of adult female literacy and infant mortality.
Promotion of breast-feeding should be continued. The anti-infective properties of human milk are well known and depend in part upon various cellular and soluble factors, as well as its buffering capacity and several antigen-non-specific protective factors (see also Brandtzaeg, Chapter 14, this volume). Secretory IgA antibodies against a variety of common pathogens have been found in human milk and correlate negatively with morbidity due to specific diseases, such as cholera (Chandra, 1992). The protective effect is particularly dramatic in underprivileged communities with poor sanitation, inadequate housing and contaminated food and water. Furthermore, breast-feeding contributes to birth spacing, an important factor in both maternal and child health.

More effective immunization programmes against the common communicable diseases are required for the majority of the susceptible population. There are still a large number of children in developing countries who die from or are disabled by preventable infectious diseases. Immunization programmes should include universal coverage of all the population at risk. Moreover, there is a need to develop new vaccines, such as those for malaria, *Shigella* and *Pneumococcus*, and to improve the quality of those against typhoid, cholera and tuberculosis. In addition, new methods of vaccine preparation, such as genetic recombination, subunit antigens, synthetic-peptide antigens, anti-idiotypes and host-cell receptor-specific vaccines, show great promise. It would be ideal to have a single, stable, efficacious, inexpensive vaccine containing immunizing antigens for several infections, which can be given at birth, be easy to administer and have no serious adverse effects.
Other useful preventive measures include the availability of plentiful clean water and improved sanitation and housing. The early and adequate management of diarrhoea and respiratory infections using oral rehydration solution and antibiotics, respectively, has already proved useful and found applicability worldwide. The early detection of growth faltering, using simple weight and height charts, together with subsequent dietary advice, will reduce the prevalence and severity of malnutrition and its adverse consequences. Lastly, targeted subsidies during times of acute need, such as in famines and wars, and massive campaigns to eliminate specific nutrient deficiencies, such as those of vitamin A, iron and iodine, are justified.

References


Introduction

In Western countries, an adult eats, on average, 75–150 g of fat each day and fat contributes 30–45% of dietary energy. By far the most important component of dietary fat in quantitative terms is triacylglycerol, which, in most diets, constitutes > 95% of dietary fat. Each triacylglycerol molecule is composed of three fatty acids esterified to a glycerol backbone. Thus, fatty acids are major constituents of dietary fat. In recent years, it has become clear that fatty acids, especially polyunsaturated long-chain fatty acids (PUFAs), are important regulators of numerous cellular functions, including those related to inflammation and immunity. Interest in the effects of fatty acids upon inflammation and immunity has intensified during the past two decades. The influence of various fatty acids on the functional responses of cells of the immune system has been examined in numerous in vitro studies and in animal feeding models and human intervention studies. The effects of linoleic acid and the n-3 PUFAs found in fish oil have been most extensively investigated. There is now convincing evidence that the type of fat in the diet has a major impact on inflammation and other aspects of immune function, and this has formed the basis for interventions with fish oil in diseases characterized by immune dysfunction. This chapter will describe the nature of the fatty acids available in the human diet, the influence of different types of fatty acids on inflammation and immune function, the mechanisms by which fatty acids might exert their effects and the potential applications of those effects. However, it is not possible in this chapter to review the breadth of information available. The reader is referred to recent detailed reviews of the many aspects of fatty acids, inflammation and immunity in health and disease (Kinsella et al., 1990; Kelley and Daudu, 1993; Blok et al., 1996; Calder, 1996, 1997, 1998a, b, c, 2001a, b, c; Alexander, 1998; Fernandes et al., 1998; Grimble, 1998; Harbige, 1998; Hughes, 1998; Miles and Calder, 1998; Sperling, 1998; Wu and Meydani, 1998; Yaqoob, 1998a, b; de Pablo and Alvarezda Cienfuegos, 2000; James et al., 2000; Field et al., 2001; Calder et al., 2002).
Nomenclature, Synthesis and Dietary Sources of Fatty Acids

Because of the wide range of foods consumed, the human diet contains a great variety of fatty acids. The most abundant fatty acids have straight chains of an even number of carbon atoms. The chain lengths vary from four (e.g. in milk) to 30 (e.g. in some fish oils) and may contain double bonds (unsaturated fatty acids) (Fig. 4.1). It is the nature of the constituent fatty acids (their chain length and degree of unsaturation) that gives a fat its physical properties. Fatty acids are often referred to by their common names, but are more correctly identified by a systematic nomenclature (Table 4.1). This nomenclature indicates the number of carbon atoms and the number and position of double (unsaturated) bonds in the chain (see Fig. 4.1). It is the position of the first double bond in the hydrocarbon chain that is indicated by the n-7, n-9, n-6 or n-3 part of the shorthand notation for a fatty acid. Note that n-6 and n-3 are sometimes referred to as omega-6 and omega-3.

Mammalian cells are able to synthesize (from non-fat precursors) saturated fatty acids and unsaturated fatty acids of the n-9 and n-7 series but lack the delta-12 and delta-15 desaturase enzymes (found in most plants) for insertion of a double bond at the n-6 or n-3 position (Figs 4.1 and 4.2). Thus, mammalian cells cannot synthesize n-6 or n-3 PUFAs de novo. The n-6 and n-3 fatty acids are essential substrates for many of the major regulatory lipids in the body and, as they cannot be synthesized in the body, the body must obtain them from the diet. The commonly consumed PUFAs are linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3). Once consumed, these fatty acids can be converted to the longer-chain, more unsaturated derivatives (Fig. 4.2). Thus linoleic acid is converted via γ-linolenic (18:3n-6) and dihomo-γ-linolenic

![Fig. 4.1. Structure of some fatty acids.](image-url)
<table>
<thead>
<tr>
<th>Systematic name</th>
<th>Trivial name</th>
<th>Shorthand notation</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decanoic</td>
<td>Capric</td>
<td>10:0</td>
<td>De novo synthesis; coconut oil</td>
</tr>
<tr>
<td>Dodecanoic</td>
<td>Lauric</td>
<td>12:0</td>
<td>De novo synthesis; coconut oil</td>
</tr>
<tr>
<td>Tetradecanoic</td>
<td>Myristic</td>
<td>14:0</td>
<td>De novo synthesis; milk</td>
</tr>
<tr>
<td>Hexadecanoic</td>
<td>Palmitic</td>
<td>16:0</td>
<td>De novo synthesis; milk; eggs; animal fats; meat; cocoa butter; palm oil (other vegetable oils contain lesser amounts); fish oils</td>
</tr>
<tr>
<td>Octadecanoic</td>
<td>Stearic</td>
<td>18:0</td>
<td>De novo synthesis; milk; eggs; animal fats; meat; cocoa butter</td>
</tr>
<tr>
<td>9-Hexadecenoic</td>
<td>Palmitoleic</td>
<td>18:1n-7</td>
<td>Desaturation of palmitic acid; fish oils</td>
</tr>
<tr>
<td>9-Octadecenoic</td>
<td>Oleic</td>
<td>18:1n-9</td>
<td>Desaturation of stearic acid; milk; eggs; animal fats; meat; cocoa butter; most vegetable oils, especially olive oil</td>
</tr>
<tr>
<td>9,12-Octadecadienoic</td>
<td>Linoleic</td>
<td>18:2n-6</td>
<td>Cannot be synthesized in mammals; some milks; eggs; animal fats; meat; most vegetable oils, especially maize, sunflower, safflower and soybean oils; green leaves</td>
</tr>
<tr>
<td>9,12,15-Octadecatrienoic</td>
<td>α-Linolenic</td>
<td>18:3n-3</td>
<td>Cannot be synthesized in mammals; green leaves; some vegetable oils, especially rapeseed, soybean and linseed oils</td>
</tr>
<tr>
<td>6, 9,12-Octadecatrienoic</td>
<td>γ-Linolenic</td>
<td>18:3n-6</td>
<td>Synthesized from linoleic acid; borage and evening primrose oils</td>
</tr>
<tr>
<td>11,14,17-Eicosatrienoic</td>
<td>Mead</td>
<td>20:3n-9</td>
<td>Synthesized from oleic acid; indicator of essential fatty acid deficiency</td>
</tr>
<tr>
<td>8,11,14-Eicosatrienoic</td>
<td>Dihomo-γ-linolenic</td>
<td>20:3n-6</td>
<td>Synthesized from γ-linolenic acid</td>
</tr>
<tr>
<td>5, 8,11,14-Eicosatetraenoic</td>
<td>Arachidonic</td>
<td>20:4n-6</td>
<td>Synthesized from linoleic acid via γ-linolenic and dihomo-γ-linolenic acids; meat</td>
</tr>
<tr>
<td>5, 8,11,14,17-Eicosapentaenoic</td>
<td>Eicosapentaenoic</td>
<td>20:5n-3</td>
<td>Synthesized from α-linolenic acid; fish oils</td>
</tr>
<tr>
<td>7,10,13,16,19-Docosapentaenoic</td>
<td>Docosapentaenoic</td>
<td>22:5n-3</td>
<td>Synthesized from α-linolenic acid via eicosapentaenoic acid</td>
</tr>
<tr>
<td>4,7,10,13,16,19-Docosahexaenoic</td>
<td>Docosahexaenoic</td>
<td>22:6n-3</td>
<td>Synthesized from α-linolenic acid via eicosapentaenoic acid; fish oils</td>
</tr>
</tbody>
</table>
(20:3n-6) acids to arachidonic acid (20:4n-6) (Fig. 4.2). Likewise, \(\alpha\)-linolenic acid is converted to eicosapentaenoic acid (EPA) (20:5n-3) (Fig. 4.2). There is some controversy about the extent to which docosahexaenoic acid (DHA) (22:6n-3) can be synthesized from EPA in humans.

In the past 40 years, the absolute consumption of saturated fatty acids in Western diets has declined. For example, in the UK saturated fatty acid intake has declined by 40% since 1970, while the consumption of monounsaturated fatty acids has declined by 30% (Department of Health, 1994). The consumption of PUFAs increased by 25% over this period of time (Department of Health, 1994). This was largely the result of increased consumption of linoleic acid, which became generally available in margarines and cooking oils. This has also resulted in an alteration in the amounts of n-6 and n-3 PUFAs consumed, with the n-6 to n-3 PUFA ratio of the diet increasing. According to the UK Adult Survey conducted in 1986, the daily diet of the average adult male in the UK contains 42 g saturated fatty acids, 31 g monounsaturated fatty acids (mainly oleic acid) and 15.8 g PUFAs (Department of Health, 1994). The main PUFA in the diet is linoleic acid (intake is approximately 14 g day\(^{-1}\) for adult males), with \(\alpha\)-linolenic acid contributing approximately 2 g day\(^{-1}\) (British
Nutrition Foundation, 1999). Adult females show a similar pattern of fatty-acid consumption to that of males, but the absolute amounts of each type of fatty acid consumed are about 70% of those consumed by males. Fat intakes are similar in North America to those in the UK, with the exception that the intake of n-3 fatty acids may be even lower (Kennedy et al., 1999; Cavadini et al., 2000). Longer-chain PUFAs are consumed in lower amounts than are linoleic and α-linolenic acids. Estimates of the intake of arachidonic acid intakes in Western populations vary between 50 and 300 mg day$^{-1}$ for adults (Sinclair and O’Dea, 1993; Jonnalagadda et al., 1995; Mann et al., 1995). EPA and DHA are found in high quantities in many marine (e.g. herring, mackerel, fresh (i.e. not tinned) tuna, sardines) oils and in the oils extracted from the livers of fish that live in warmer waters (e.g. cod). EPA and DHA comprise 20–30% of the fatty acids in a typical preparation of fish oil, which means that a 1 g fish oil capsule can provide 200–300 mg of EPA plus DHA. In the absence of oily fish or fish oil consumption, α-linolenic acid is the main dietary n-3 PUFA. Average intake of the long-chain n-3 PUFAs in the UK is estimated at 250 mg day$^{-1}$ (British Nutrition Foundation, 1999).

### Fatty Acids and the Innate Immune System

#### Amount of fat in the diet and innate immune function

Several studies have compared the effects of feeding laboratory animals low- and high-fat diets on innate immune responses, such as natural killer cell activity. Most studies have found that high-fat diets result in diminished innate immune responses (for references, see Calder, 1998a), but the precise effect depends upon the exact level of fat used in the high-fat diet and its source. Human natural killer cell activity was significantly increased by a reduction in fat intake to less than 30% of energy (Barone et al., 1989; Hebert et al., 1990).

#### Linoleic and α-linolenic acids and innate immune function

Feeding rats or mice diets deficient in n-6 or n-3 fatty acids decreased neutrophil chemotaxis and macrophage-mediated phagocytic and cytotoxic activity, as compared with animals fed diets containing adequate amounts of these fatty acids (for references, see Kelley and Daudu, 1993). Thus, the immunological effects of essential fatty-acid deficiencies on innate immune responses are similar to the effects of other essential nutrient deficiencies. However, again as seen with other essential nutrients, an excess of essential fatty acids can impair aspects of the innate immune response. Animal studies have reported lower natural killer cell activity following the feeding of high fat including oils rich in linoleic acid (maize, sunflower or safflower oil) or in α-linolenic acid (e.g. linseed (flaxseed) oil), when compared with feeding high-saturated-fat diets (for references, see Kelley and Daudu, 1993; Calder, 1998a, b). These data suggest that a very high intake of linoleic or α-linolenic acid, compared with saturated
fatty acids, has the potential to suppress natural killer cell activity. However, in humans, increasing linoleic acid intake by 6 g day\(^{-1}\) did not affect natural killer cell activity or the production of cytokines (interleukin (IL)-1\(\beta\), tumour necrosis factor (TNF-\(\alpha\)) by monocytes (Yaqoob et al., 2000). Furthermore, increasing \(\alpha\)-linolenic acid intake by 2 or 4 g day\(^{-1}\) (less than usually fed in animal studies) did not affect natural killer cell activity or the production of TNF-\(\alpha\), IL-1\(\beta\) or IL-6 by monocytes (Thies et al., 2001a, b), neutrophil respiratory burst (Healy et al., 2000; Thies et al., 2001b) or monocyte respiratory burst (Thies et al., 2001b). This suggests that the amount of essential fatty acids that humans could potentially consume in the habitual diet is not sufficient to negatively influence innate immunity. However, feeding a high dose of \(\alpha\)-linolenic acid (approx. 15 g day\(^{-1}\)) was reported to decrease IL-1 and TNF production by human monocytes (Caughey et al., 1996).

**EPA and DHA and innate immune function**

**Animal studies**

There are many published animal studies investigating the effects of fish oil on aspects of inflammation and innate immunity. Most of these studies indicate that feeding high amounts of fish oil decreases a wide range of responses. However, not all studies agree with this generalization. Animal studies are often designed to demonstrate effects and to identify potential mechanisms and so result in the use of diets that differ markedly from human diets in both the level and the type of fat. Additional reasons for apparent contradictions in this literature might relate to the species of animal studied, the comparison being made (e.g. to a low-fat diet or to another high-fat diet; to saturated fat or to a diet high in n-6 PUFAs), the amount of vitamin E in the diets and the conditions used for ex vivo cell-culture experiments.

Feeding fish oil to laboratory animals has been reported to decrease macrophage functions, including generation of reactive oxygen species and production of TNF-\(\alpha\), IL-1 and IL-6 (e.g. Billiar et al., 1988; Hubbard et al., 1991; Renier et al., 1993). Fish oil, compared with other fat sources, resulted in lower concentrations of TNF-\(\alpha\), IL-1\(\beta\) and IL-6 in the bloodstream after endotoxin injection or burns (e.g. Hayashi et al., 1998; Sadeghi et al., 1999). Thus, these studies support the idea that fish oil has anti-inflammatory effects. There are, however, opposing studies. For example, Somers et al. (1989) reported that cultured peritoneal macrophages from mice fed fish-oil-supplemented diets exhibited higher TNF-\(\alpha\) activity after endotoxin stimulation than did macrophages from mice fed a diet high in linoleic acid.

Animal feeding studies indicate that feeding high levels of fish oil decreases natural killer cell activity (e.g. Meydani et al., 1988; Yaqoob et al., 1994a), while lower levels (e.g. EPA plus DHA fed at less than 5% w/w of the diet) are reported to enhance this activity (Brouard and Pascaud, 1993; Robinson and Field, 1998). This effect may also be fatty-acid-specific, as a recent study reported that relatively low levels (4.4% of fatty acids; 1.7% of energy) of dietary EPA (but not DHA) inhibit rat natural killer cell activity (Peterson et al., 1998).
Human studies

Human studies have generally fed proportionately less fish oil than the amount provided in most animal studies. Nevertheless, a number of studies in healthy humans reveal significant immunomodulatory effects of long-chain n-3 PUFAs. Providing more than 2.3 g EPA plus DHA day\(^{-1}\) (and in some studies up to 14.5 g day\(^{-1}\)) has been reported to decrease chemotaxis and superoxide production by neutrophils (Lee et al., 1985; Schmidt et al., 1989, 1992; Luostarinen et al., 1992; Sperling et al., 1993) and by monocytes (Endres et al., 1989; Schmidt et al., 1989, 1992; Fisher et al., 1990). Daily consumption of more than 2.4 g EPA plus DHA day\(^{-1}\) has been shown in some studies to decrease production of TNF, IL-1 and IL-6 by mononuclear cells (Endres et al., 1989; Meydani et al., 1991; Gallai et al., 1993; Caughey et al., 1996). Similarly, adding oily fish (providing 1.2 g EPA plus DHA day\(^{-1}\)) to a low-fat diet resulted in decreased production of TNF, IL-1 and IL-6 (Meydani et al., 1993). Parenteral nutrition supplemented with fish oil decreased serum TNF-\(\alpha\) and IL-6 concentrations in patients following major abdominal surgery, compared with n-6 fatty-acid-rich parenteral nutrition (Wachtler et al., 1997). In contrast to these observations, a number of studies that provided from 0.55 g to 3.4 g EPA plus DHA day\(^{-1}\) failed to demonstrate an effect on neutrophil chemotaxis, neutrophil or monocyte respiratory burst or the production of TNF, IL-1 and IL-6 (Molvig et al., 1991; Cooper et al., 1993; Schmidt et al., 1996; Blok et al., 1997; Healy et al., 2000; Yaqoob et al., 2000; Thies et al., 2001b).

Thus, studies in animals and humans have demonstrated that high levels of fish oil or its component n-3 PUFAs in the diet exert potent anti-inflammatory effects, particularly decreasing neutrophil and monocyte chemotaxis, superoxide production and production of pro-inflammatory cytokines. Reduced production of pro-inflammatory mediators may be beneficial in diseases characterized by excess production of these mediators (see later sections). On the other hand, these effects may compromise immune function in healthy and immune-compromised individuals. The effects of these lipids are probably dose-dependent (and disease-specific), as studies providing more modest amounts of long-chain n-3 PUFAs have not consistently demonstrated these effects on the innate immune system.

Fatty Acids and the Acquired Immune System

Amount of fat in the diet and acquired immune function

A number of studies have compared the effects of feeding laboratory animals low- and high-fat diets (usually high in saturated fat) upon lymphocytes. These studies have concluded that high-fat diets are associated with suppressed T-cell proliferation (for references, see Calder, 1998a). This conclusion is supported by studies in humans that showed significantly enhanced lymphocyte proliferation in response to mitogens if healthy subjects were fed a diet where fat contributed 25% of energy (Kelley et al., 1989, 1992).
Linoleic and α-linolenic acids and acquired immune function

Essential fatty-acid deficiency is reported to decrease thymus and spleen weight and suppress cell-mediated immune responses and antibody production (for references, see Kelley and Daudu, 1993; Calder 1998a). However, a large number of studies in rats, mice, rabbits, chickens and monkeys have reported lower mitogen-stimulated lymphocyte proliferation and antibody production following the feeding of diets rich in linoleic acid (maize, sunflower or safflower oils), compared with feeding high-fat diets rich in saturated fatty acids (for references, see Kelley and Daudu, 1993; Calder, 1998a, b). These data suggest that linoleic acid has the potential to suppress acquired immune function. However, no difference in blood lymphocyte proliferation, circulating immunoglobulins or the delayed-type hypersensitivity response was seen in volunteers consuming low-fat diets (25% energy as fat) that were rich (12.9% of energy) or poor (3.5% of energy) in linoleic acid (Kelley et al., 1989, 1992). Furthermore, increasing linoleic acid intake by 6 g day$^{-1}$ did not affect blood lymphocyte proliferation or the production of a range of cytokines by lymphocytes (Yaqoob et al., 2000). Again, the reason for the apparent discrepancy between animal and human studies most probably relates to the amount of linoleic acid in the diets studied.

Similarly to linoleic acid, feeding rodents diets containing very high levels of α-linolenic acid (linseed oil) is reported to decrease lymphocyte proliferation (e.g. Marshall and Johnston, 1985; Jeffery et al., 1996). The precise effect of α-linolenic acid on lymphocyte function appears to depend on both the level of the fatty acid and the total PUFA content of the diet (Jeffery et al., 1997). Feeding linseed oil (providing about 15 g α-linolenic acid day$^{-1}$) as part of a low-fat diet (total fat provided 29% energy) for 6 weeks resulted in significant decreases in human blood lymphocyte proliferation and in the delayed-type hypersensitivity response (Kelley et al., 1991). Thus, as was the case for linoleic acid, it appears that both a deficiency and an excess of α-linolenic acid can lead to suppressed immune function. However, increasing α-linolenic acid consumption by 2 g day$^{-1}$ in healthy humans did not affect lymphocyte proliferation or the production of a range of cytokines by lymphocytes (Thies et al., 2001c), suggesting a limited immunological impact of a more moderate increase in α-linolenic acid intake.

EPA and DHA and acquired immune function

Animal studies

Studies in rabbits, chickens, rats and mice have clearly demonstrated that long-chain n-3 PUFAs can inhibit lymphocyte proliferation, IL-2 and interferon (IFN)-γ production, delayed-type hypersensitivity and antigen presentation, as compared with diets rich in lard, or hydrogenated coconut, safflower, maize or linseed oils (e.g. Fujikawa et al., 1992; Yaqoob et al., 1994b; Sanderson et al., 1995, 1997; Byleveld et al., 1999; Wallace et al., 2001).
The addition of either EPA or DHA to a diet was demonstrated to suppress T-cell proliferation in rats (Peterson et al., 1998) and mice (Jolly et al., 1997). Although mechanistically important, the physiological importance of many of the animal studies in this area is not clear, as the diets used to identify the effects of long-chain n-3 PUFAs often contain very high amounts of these fatty acids and very low amounts of linoleic acid. Indeed, in contrast to many other studies, adding EPA and DHA at 5% by weight in both a high- and low-PUFA diet improved rat lymphocyte responses, measured as activation-marker expression and cytokine production (Robinson and Field, 1998; Robinson et al., 2001).

Human studies

Human studies have generally provided less fish oil (as a proportion of fat or energy) in the diet than the amount fed in most studies in animals. Despite this, data from studies investigating the influence of fish oil on human lymphocyte functions are also conflicting. Supplementation of the diet of healthy human volunteers with fish oil providing 2.4 g EPA plus DHA day$^{-1}$ resulted in decreased proliferation of lymphocytes from older women (aged 51–68 years) but not young women (aged 21–33 years) and decreased IL-2 production (Meydani et al., 1991). Molvig et al. (1991) reported decreased lymphocyte proliferation after providing 1.7 or 3.4 g EPA plus DHA day$^{-1}$ to men, while Gallai et al. (1993) reported that 5.2 g EPA plus DHA day$^{-1}$ decreased IL-2 and IFN-$\gamma$ production. Providing 1.2 g EPA plus DHA to healthy subjects aged 55–75 years resulted in decreased lymphocyte proliferation (Thies et al., 2001c), but did not affect IL-2 or IFN-$\gamma$ production (Thies et al., 2001c). Inclusion of oily fish providing 1.2 g EPA plus DHA day$^{-1}$ in the diet of volunteers consuming a low-fat, low-cholesterol diet decreased lymphocyte proliferation, IL-2 production and the delayed-type hypersensitivity response to seven recall antigens (Meydani et al., 1993). In contrast to these observations, there are reports of no effect of 3.2 g EPA plus DHA day$^{-1}$ on lymphocyte proliferation and IL-2 and IFN-$\gamma$ production (Yaqoob et al., 2000) by peripheral-blood lymphocytes and of no effect of 4.6 g EPA plus DHA day$^{-1}$ on lymphocyte proliferation and IL-2 production (Endres et al., 1993). Thus, feeding moderate amounts of long-chain n-3 PUFAs is not clearly immunosuppressive, although feeding high amounts might be.

Mechanisms of the Effect of Dietary Fatty Acids on Immune Function

While it is widely recognized that dietary fatty acids can potentially alter immune and inflammatory responses, current understanding of how they act is incomplete. Several candidate mechanisms have been proposed, including alterations in membrane structure and composition, changes in membrane-mediated functions and signals (i.e. proteins, eicosanoids), changes in gene expression and effects on the development of the immune system (Fig. 4.3).
Immune-cell activation results in both \textit{de novo} synthesis and an increased turnover of membrane phospholipids (e.g. Resch \textit{et al.}, 1972; Ferber \textit{et al.}, 1975). Therefore, essential fatty acids would be required for the synthesis of new membranes during immune-cell responses, especially those involving increased membrane synthesis and turnover (e.g. cell proliferation, phagocytosis).

The fluidity of the plasma membrane or of regions of the plasma membrane is important in the functioning of cells (see Stubbs and Smith, 1984). The fluidity of a membrane is determined by its lipid components and their fatty-acid composition (Stubbs and Smith, 1984). Membrane fluidity is an important regulator of phagocytosis (Calder \textit{et al.}, 1990). The function of the immune system depends on interactions between different cell types and, through effects on membrane composition, dietary fatty acids have the potential to influence these interactions. For example, the interaction of cytotoxic T-cells with target cell membranes, a necessary interaction to induce effector function, is affected by the fluidity of the plasma membrane of the T-cells (Bialick \textit{et al.}, 1984). Cell culture experiments have demonstrated that changes in fatty-acid composition of immune cells alter membrane fluidity (e.g. Calder \textit{et al.}, 1994), but this has been less easy to demonstrate after dietary manipulations (e.g. Yaqoob \textit{et al.}, 1995), probably because the fatty acid composition changes induced by diet are less extreme than those seen in culture and because, in the intact animal, mechanisms to counter the fluidizing effect of increasing the PUFA content of membranes (e.g. insertion of cholesterol) can be achieved more readily than in culture.
Since there has been significant focus on the effects of n-6 and n-3 PUFAs in inflammation and immunity, the proportions of those classes of fatty acids in immune cells are of interest. The exact proportion of arachidonic acid in human immune cells varies according to cell type and the lipid fraction examined (Gibney and Hunter, 1993; Sperling et al., 1993). The phospholipids of human mononuclear cells (an approximately 70 : 20 : 10 mixture of T lymphocytes, B lymphocytes and monocytes purified from human blood) contain 6–10% linoleic acid, 1-2% dihomo-γ-linolenic acid (DGLA) and 15–25% arachidonic acid (Gibney and Hunter, 1993; Yaqoob et al., 2000; see Table 4.2). In contrast, the proportions of n-3 fatty acids are low: α-linolenic acid is generally found only in trace amounts and EPA and DHA comprise only 0.1–0.8% and 2–4%, respectively (Gibney and Hunter, 1993; Yaqoob et al., 2000; see Table 4.2).

Animal studies show that decreasing the availability of linoleic acid in the diet, especially by replacing it with n-3 fatty acids (either α-linolenic acid or long-chain n-3 fatty acids), results in decreased proportions of all n-6 fatty acids, including arachidonic acid, in immune-cell phospholipids (Marshall and Johnston, 1985; Lokesh et al., 1986; Brouard and Pascaud, 1990; Yaqoob et al., 1995; Jeffery et al., 1996; Peterson et al., 1998; Robinson and Field, 1998; Wallace et al., 2000, 2001; Robinson et al., 2001). When α-linolenic acid is added to the human diet in significant quantities, it appears in immune cells and there is also an increase in the proportion of EPA, although the proportion of DHA may not be elevated (Caughey et al., 1996). More moderate increases in the amount of α-linolenic acid in the human diet appear to have a limited impact on immune-cell fatty-acid composition (Healy et al., 2000; Thies et al., 2001c). When fish oil is provided in the human diet, the proportions of EPA and DHA in immune cells are significantly elevated and the n-6/n-3 PUFA ratio is decreased (Lee et al., 1985; Endres et al., 1989; Fisher et al., 1990; Molvig et al., 1991; Gibney and Hunter, 1993; Sperling et al., 1993; Caughey et al.,

### Table 4.2. Fatty-acid composition of human mononuclear cells before and after supplementation of the diet with evening primrose oil or fish oil.

Healthy volunteers supplemented their diet with 9 g evening primrose oil (providing 1 g γ-linolenic acid) day⁻¹ or with 9 g fish oil (providing 3.2 g EPA plus DHA) day⁻¹ for 8 weeks. Mononuclear cells were isolated by standard techniques and the fatty-acid composition determined. (Data are mean ± standard error of mean (SEM) for six subjects per group and are taken from Yaqoob et al., 2000.)

<table>
<thead>
<tr>
<th>Fatty acid (g 100 g⁻¹ of total fatty acids)</th>
<th>Evening primrose oil</th>
<th>Fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Dihomo-γ-linolenic acid</td>
<td>1.2 ± 0.4</td>
<td>2.2 ± 0.4ᵃ</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>20.6 ± 2.1</td>
<td>21.2 ± 0.5</td>
</tr>
<tr>
<td>EPA</td>
<td>1.0 ± 0.3</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>DHA</td>
<td>2.7 ± 0.4</td>
<td>2.6 ± 0.4</td>
</tr>
</tbody>
</table>

ᵃIndicates significantly different from before supplementation.
1996; Healy et al., 2000; Yaqoob et al., 2000; Thies et al., 2001c; see Table 4.2) in a dose-dependent manner. Similar effects occur in neutrophils, monocytes and T and B lymphocytes (Gibney and Hunter, 1993). In many studies, the degree of enrichment of EPA is greater than that of DHA (e.g. 300% vs. 95%; Yaqoob et al., 2000), although this probably depends upon the relative amounts of EPA and DHA in the fish oil preparation. The incorporation of the long-chain n-3 fatty acids is at least partly at the expense of arachidonic acid (see Table 4.2) and is considered to be near maximal 4 weeks after a dietary change (e.g. Healy et al., 2000; Yaqoob et al., 2000; Thies et al., 2001c). Since n-3 PUFAs oxidize more readily than n-6 PUFAs, they may increase susceptibility of cellular membranes to lipid peroxidation. Increased free radical production has been demonstrated in animals fed diets rich in n-3 PUFAs. The risk of oxidation associated with increased intake of n-3 PUFA has been shown to be minimized by intake of extra antioxidants, such as vitamin E.

Alterations in membrane-mediated functions and signals

Alterations in the function of membrane proteins

Changes in plasma membrane structural characteristics can change the activity of proteins that serve as ion channels, adhesion molecules, transporters, receptors, signal transducers or enzymes (Stubbs and Smith, 1984; Clandinin et al., 1991). Many membrane-associated proteins in immune cells have been shown to be modulated by membrane lipid changes. For example, feeding 5% w/w long-chain n-3 PUFAs to rats resulted in a higher proportion of T- and B-cells and macrophages expressing the transferrin receptor (CD71) after stimulation with mitogen (Robinson and Field, 1998), although feeding a higher amount of fish oil did not induce this effect (Yaqoob et al., 1994b). The binding of cytokines to their receptor has been reported to be altered with changes in membrane composition (Grimble and Tappia, 1995). Additionally, the expression of several cell surface molecules was reported to be altered after fish oil feeding (Sanderson et al., 1995; Hughes et al., 1996; Robinson and Field, 1998; Sanderson and Calder, 1998a; Field et al., 2000; Hughes and Pinder, 2000; Robinson et al., 2001). Many of these molecules are involved in the co-stimulation processes necessary for lymphocyte activation, and some of the effects are suggestive of improved cell-mediated immune function.

Changes in membrane-mediated signals (signal transduction)

Lipids, derived from either endogenous or exogenous sources, affect many cell signalling pathways via a variety of mechanisms. Many of the established cell signalling molecules are generated directly from membrane phospholipids (e.g. inositol-1,4,5-trisphosphate, diacylglycerol, phosphatidic acid, choline, ceramide, platelet-activating factor, arachidonic acid). These have important roles in regulating the activity of proteins involved in immune-cell responses.
The concentration and/or composition of lipid-derived signalling molecules have been shown to be sensitive to n-3 PUFA availability either in cell culture (Jenski et al., 1995) or through the diet (Huang et al., 1992; Fowler et al., 1993; Sperling et al., 1993; Marignani and Sebaldt, 1995; Jolly et al., 1997; Sanderson and Calder, 1998b). This may be due to either altered activity of the enzymes that generate the signals or to altered composition of the substrate molecules. There is evidence to support each of these possibilities (see Miles and Calder, 1998). For example, lymphocyte phospholipase Cγ activity is reduced after feeding a diet rich in fish oil, which might account for the decreased generation of signalling molecules observed (Sanderson and Calder, 1998b). There is also evidence that arachidonic acid released from the plasma membrane has a direct role in regulating some immune-cell functions, such as natural killer cell granule release and cell-mediated toxicity (Cifone et al., 1993). In addition, arachidonic acid is an intracellular activator of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme in neutrophils (Sakata et al., 1987), and enrichment of arachidonic acid in neutrophil membranes is reported to increase the oxidative burst of neutrophils (Badwey et al., 1981, 1984; Hardy et al., 1991). Dietary lipids have been demonstrated to influence the pattern of fatty acids released from lymphocytes (Sanderson et al., 2000).

Changes in eicosanoid synthesis

A key link between fatty acids, inflammation and immune function is a group of bioactive mediators termed eicosanoids (prostaglandins, leukotrienes, thromboxanes), which are synthesized from 20-carbon PUFAs (Fig. 4.4). The two

![Fig. 4.4. Outline of synthesis of eicosanoids from 20-carbon n-6 and n-3 polyunsaturated fatty acids. COX, cyclo-oxygenase; DGLA, dihomo-γ-linolenic acid; EPA, eicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leucotriene; PG, prostaglandin; TX, thromboxane.](image-url)
major pathways for eicosanoid synthesis are via the enzymes cyclo-oxygenase (COX) and lipoxygenase (LOX). These enzymes initiate pathways that result in the production of prostaglandins/thromboxanes and leucotrienes/hydroxyeicosatetraenoic acids/lipoxins, respectively. Membrane arachidonic acid is the main precursor of these mediators, giving rise to dienoic prostaglandins (e.g. PGE₂) and thromboxanes (TXA₂) and tetraenoic leucotrienes (e.g. LTB₄). Arachidonic acid in cell membranes is mobilized by various phospholipase enzymes, most notably phospholipase A₂, and the released arachidonic acid is the substrate for COX or one of the three LOX enzymes (Fig. 4.5). There are at least 16 different 2-series PG and these are formed in a cell-specific manner. For example, monocytes and macrophages produce large amounts of PGE₂ and PGF₂α, neutrophils produce moderate amounts of PGE₂ and mast cells produce PGD₂. The LOX enzymes have different tissue distributions, with 5-LOX being found mainly in mast cells, monocytes, macrophages and granulocytes and 12- and 15-LOX being found primarily in epithelial cells. Metabolism of arachidonic acid by the 5-LOX pathway gives rise to hydroxy and hydroperoxy derivatives (5-hydroxyeicosatetraenoic acid) (5-HETE) and 5-hydroperoxyeicosatetraenoic acid (5-HPETE), respectively and the 4-series LT (Fig. 4.5).

Eicosanoids (particularly PGE₂ and 4-series LT) are involved in modulating the intensity and duration of inflammatory and immune responses (for reviews, see Kinsella et al., 1990; Lewis et al., 1990; Tilley et al., 2001). The pro-inflammatory effects of PGE₂ include inducing fever, increasing vascular permeability

![Fig. 4.5. Synthesis of eicosanoids from arachidonic acid. COX, cyclo-oxygenase; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leucotriene; PG, prostaglandin; TX, thromboxane.](image-url)
and vasodilatation and enhancing pain and oedema caused by other agents, such as histamine. Additionally, PGE₂ suppresses lymphocyte proliferation and natural killer cell activity and inhibits production of TNF-α, IL-1, IL-6, IL-2 and IFN-γ (Fig. 4.6); thus, in these respects, PGE₂ is immunosuppressive and anti-inflammatory. PGE₂ does not affect the production of the T-helper 2 (Th2)-type cytokines IL-4 and IL-10, but promotes immunoglobulin E (IgE) production by B lymphocytes (Fig. 4.6). LTB₄ increases vascular permeability, enhances local blood flow, is a potent chemotactic agent for leucocytes, induces release of lysosomal enzymes, enhances generation of reactive oxygen species, inhibits lymphocyte proliferation and promotes natural killer cell activity (Fig. 4.7). In addition, 4-series LT regulate the production of pro-inflammatory cytokines; for example, LTB₄ enhances production of TNF-α, IL-1, IL-6, IL-2 and IFN-γ (Fig. 4.7). Whereas 15-HETE inhibits lymphocyte proliferation, 5-HETE enhances it. Thus, arachidonic acid gives rise to a range of mediators that have opposing effects to one another, so the overall physiological effect will be the result of the balance of these mediators, the timing of their production and the sensitivities of target cells to their effects.

Dietary fatty acids can influence eicosanoid synthesis by affecting the supply of substrates. Feeding animals or humans increased amounts of fish oil results in a decrease in the amount of arachidonic acid in the membranes of most cells in the body, including those involved in inflammation and immunity.

![Fig. 4.6. Immunoregulatory roles of PGE₂. IFN-γ, interferon-γ; IgE, immunoglobulin E; IL, interleukin; PG, prostaglandin; TNF, tumour necrosis factor.](image-url)
such as monocytes, macrophages, neutrophils and lymphocytes (see earlier section). This means that there is less arachidonic acid available for the synthesis of eicosanoids. Consequently, dietary fish oil decreases the production of arachidonic acid-derived eicosanoids from animal (Lokesh et al., 1986; Brouard and Pascaud, 1990; Yaqoob and Calder, 1995; Peterson et al., 1998; Fig. 4.8) and human (Lee et al., 1985; Endres et al., 1989; Meydani et al., 1991; Sperling et al., 1993; Caughey et al., 1996) immune cells. EPA is also a substrate for the COX and LOX enzymes, resulting in the synthesis of the trienoic prostanoids (e.g. PGE₃) and pentaenoic leucotrienes (e.g. LTB₅) (Fig. 4.4). The eicosanoids produced from EPA are often less biologically potent than the analogues synthesized from arachidonic acid. For example, LTB₅ is only about 10% as potent as LTB₄ as a chemotactic agent and in promoting lysosomal enzyme release (see Kinsella et al., 1990). Since dietary fish oil leads to decreased PGE₂ production, it is often stated that feeding n-3 lipids should result in a reversal of the effects of PGE₂. Thus, fish oil is expected to
result in less inflammation, enhanced cytokine production by monocytes/macrophages and Th1 lymphocytes and enhanced lymphocyte proliferation (Fig. 4.9). The reduction in the generation of arachidonic acid-derived mediators that accompanies fish oil consumption has led to the idea that fish oil is anti-inflammatory and might enhance immune function (Fig. 4.9). However, the in vivo situation is likely to be more complex than this, because PGE$_2$ is not the sole mediator produced from arachidonic acid and the range of mediators produced have varying, sometimes opposite, actions (see above). Furthermore, EPA will give rise to mediators with varying actions, some of which may actually be the same as those of the analogues produced from arachidonic acid. Thus, the overall effect of fish oil feeding cannot be predicted solely on the basis of an abrogation of PGE$_2$-mediated effects. Furthermore, a number of the effects of n-3 PUFA have been shown to occur independent of changes in eicosanoid production (Santoli et al., 1990; Calder et al., 1992; Soyland et al., 1993).

Changes in gene expression

Fatty acids, especially PUFA, are known to modulate the expression of a variety of genes coding for key regulatory proteins in numerous metabolic pathways in hepatocytes and adipocytes (Clarke and Jump, 1994). These effects are mediated by both indirect mechanisms (e.g. by eicosanoids, hor-
mones) and direct effects on gene expression. There is now emerging evidence that PUFAs regulate the expression of genes for cytokines, adhesion molecules, COX, inducible nitric oxide synthase and other inflammatory proteins (Renier et al., 1993; de Caterina and Libby, 1996; Khair-el-Din et al., 1996; Robinson et al., 1996; Curtis et al., 2000; Miles et al., 2000; Wallace et al., 2001). Since the expression of many of these genes is regulated by the transcription factor nuclear factor kappa B (NFκB), these observations suggest that PUFAs might somehow affect the activity of this transcription factor. This might be through effects on cell signalling leading to NFκB activation. There is recent evidence that dietary fish oil affects NFκB activity (Lo et al., 1999; Xi et al., 2001), in a manner that is consistent with its ability to down-regulate the production of inflammatory mediators.

A second group of transcription factors currently undergoing scrutiny for their role in inflammation are the peroxisome proliferator-activated receptors (PPARs). The main members of this family are PPARα and PPARγ. PPARα and γ play important roles in liver and adipose tissue, respectively (Schoonjans et al., 1996). However, they are also found in inflammatory cells (Chinetti et al., 1998; Ricote et al., 1998). PPARs can bind, and appear to be regulated by, PUFAs and eicosanoids (Kleiwer et al., 1995; Devchand et al., 1996). PPARα-deficient mice have a prolonged response to inflammatory stimuli (Devchand et al., 1996), suggesting that PPARα activation might be anti-inflammatory. More recently, activators of both PPARα and PPARγ have been shown to inhibit the activation of a number of inflammatory genes (Jiang et al., 1998; Poynter and Daynes, 1998; Ricote et al., 1998; Jackson et al., 1999; Marx et al., 1999; Takano et al., 2000; Wang et al., 2001; Xu et al., 2001). Two mechanisms for the anti-inflammatory actions of PPARs have been proposed (for reviews, see Chinetti et al., 2000; Delerive et al., 2001). The first is that PPARs might stimulate the breakdown of inflammatory eicosanoids through the induction of peroxisomal β-oxidation. The second is that PPARs might interfere with/antagonize the activation of other transcription factors, including NFκB. Although the effect of fish oil on PPAR expression in inflammatory cells has not been reported, studies in other tissues (e.g. Berthou et al., 1995) suggest that n-3 PUFAs might act by increasing the level of these anti-inflammatory transcription factors in such cells.

Fig. 4.9. Theoretical basis for the immunoregulatory effects of eicosapentaenoic acid (EPA).
Effects on the development of the immune system

Despite the amount of work done in healthy adults, human diseases and animal models of disease, little work has been done on the effect of dietary PUFAs on T-cell development in the infant or young animal. However, a recent study examined the effect of altered long-chain PUFA availability on the functional indices of immune development during the first 42 days of human life (Field et al., 2000). A group of clinically stable preterm infants were fed human milk, standard preterm infant formula or a preterm infant formula containing DHA (0.4%) and arachidonic acid (0.6%) for the first 42 days of life. Using blood samples obtained at 14 and 42 days of age, the effect of diet on some parameters of immune development was studied. Compared with standard formula, feeding long-chain PUFAs significantly increased the proportion of antigen mature (CD45RO+) CD4+ cells (by approximately 25%), compared with non-supplemented formula-fed infants and lowered the proportion of immature (CD45RA+) CD4+ cells to levels not different from human milk-fed infants. These changes in the sialylation of the CD45 region (RA vs. RO) are believed to reflect the maturation of the immune system (Bofill et al., 1994). These data suggest that adding DHA and arachidonic acid to preterm formula may have assisted in the maturation of peripheral CD4+ cells. Between 14 and 42 days of age, the ability of peripheral mononuclear cells from unsupplemented formula-fed infants to produce IL-10 was lower than that of human milk-fed infants (Field et al., 2000). IL-10 production by cells from infants fed the formula containing DHA plus arachidonic acid did not differ from that of the human milk-fed infants. Feeding the formula containing DHA plus arachidonic acid resulted in a significant decrease in the amount of secretory IL-2 receptor (SIL-2R) produced by stimulated peripheral mononuclear cells at 42 days of age, as compared with 14 days (Field et al., 2000). This work supports an effect of dietary lipids, particularly long-chain PUFAs, on immune development.

Fatty Acids and Diseases Involving the Immune System

Chronic inflammatory (autoimmune) diseases

Chronic inflammatory or autoimmune diseases are often characterized by a dysregulated Th1-type response and by an inappropriate production of pro-inflammatory cytokines (e.g. TNF-α) and arachidonic acid-derived eicosanoids (e.g. PGE2 and LTB4). The effects of fish oil outlined above suggest that n-3 PUFAs might have a role in the prevention and therapy of chronic inflammatory diseases. In support of this idea, dietary fish oil has been shown to have beneficial clinical, immunological and biochemical effects in various animal models of human diseases. These effects include: increased survival and decreased proteinuria and anti-DNA antibodies in mice with autoimmune glomerulonephritis (Prickett et al., 1983; Kelley et al., 1985; Robinson et al., 1985, 1986), decreased incidence and severity of joint inflammation in mice with collagen-induced arthritis (Leslie et al., 1985) or rats with streptococcal cell
wall-induced arthritis (Volker et al., 2000) and reduced inflammation in rats with inflammatory bowel disease (Vilaseca et al., 1990). These improvements are associated with abolition of pro-inflammatory cytokine production and induction of anti-inflammatory cytokines in some models (Chandrasekar and Fernandes, 1994; Fernandes et al., 1994; Kleemann et al., 1998; Venkatraman and Chu, 1999).

There have been a number of clinical trials assessing dietary supplementation with fish oils in several chronic inflammatory diseases in humans. N-3 PUFAs in the diet could affect the course of these diseases by altering the immune or inflammatory responses, thus modifying clinical symptoms. In some of the human trials of fish oil in inflammatory disease, anti-inflammatory effects of fish oil, including decreased production of circulating concentrations of pro-inflammatory mediators (LTB$_4$, TNF-α, IL-1 and C-reactive protein), were observed. Many of the short-term placebo-controlled, double-blind trials of fish oil in chronic inflammatory diseases (cyclosporin-induced nephrotoxicity and hypertension, lupus, nephritis, IgA nephropathy, Crohn’s disease, ulcerative colitis) reveal significant benefit, including decreased disease activity and a lowered use of anti-inflammatory drugs (Table 4.3). However, the evidence for a beneficial effect of fish oil is strongest in rheumatoid arthritis (see Table 4.3). Further research is needed to identify which components of fish oil might be most effective, which doses should be used, what qualitative alterations of dietary fatty acids and background diet might be important, what the optimal duration of therapy should be, which disease or subsets of patients might respond and what interaction between other anti-inflammatory therapy and n-3 PUFAs might occur.

**Asthma and related diseases**

Eicosanoids synthesized from arachidonic acid have a role in allergic diseases: PGD$_2$, LTC$_4$, LTD$_4$ and LTE$_4$ are produced by the cells that mediate pulmonary inflammation in asthma, such as mast cells, and are believed to be the major mediators of asthmatic bronchoconstriction (Fig. 4.10). Although its action as a precursor to leucotrienes has highlighted the significance of arachidonic acid in the aetiology of allergic disease, a second link with this fatty acid has been made. This is because PGE$_2$ regulates the activities of lymphocytes. Of particular relevance in the context of allergic disease is the ability of PGE$_2$ to inhibit the production of the Th1-type cytokines IL-2 and IFN-γ without affecting the production of the Th2-type cytokines IL-4 and IL-5, and to stimulate B-cells to produce IgE (Fig. 4.10). These observations suggest that PGE$_2$ promotes the development of IgE (Fig. 4.10). These observations suggest that PGE$_2$ promotes the development of allergic disease. Since n-3 fatty acids potentially antagonize the effects of arachidonic acid, there may be a role for fish oil in treating, or in protecting against the development of, allergic diseases, including asthma. Hence, a number of trials of fish oil in asthma have been performed. Although some of these trials show fish oil-induced changes in production of some inflammatory mediators (e.g. LTB$_4$), a number report no effects on clinical outcomes (e.g. Arm et al., 1988, 1989; see Calder and Miles, 2000).
<table>
<thead>
<tr>
<th>Disease</th>
<th>Number of double-blind, placebo-controlled studies</th>
<th>Doses of EPA + DHA used (g day(^{-1}))</th>
<th>Duration (weeks)</th>
<th>Key findings</th>
<th>Reviews</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rheumatoid arthritis</td>
<td>13</td>
<td>1–6.4</td>
<td>12–52</td>
<td>All studies reported improvements, including reduced duration of morning stiffness, reduced number of tender or swollen joints, reduced joint pain, reduced time to fatigue and increased grip strength. Twelve studies reported improvement in at least two clinical measures, and four studies reported improvement in at least four clinical measures. Ten studies reported decreased joint tenderness. Three studies reported significant decrease in the use of non-steroidal anti-inflammatory drugs.</td>
<td>Volker and Garg (1996); James and Cleland (1997); Geusens (1998); Calder (2001d); Calder and Zurier (2001)</td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td>3</td>
<td>2.7–5.1</td>
<td>12–52</td>
<td>Two studies reported no benefit. One study reported a significant decrease in relapses. One other study, which used oily fish (100–250 g day(^{-1}) for 2 years), reported a significant decrease in relapses.</td>
<td>Belluzzi and Miglio (1998)</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>4</td>
<td>1.8–5.4</td>
<td>12–52</td>
<td>One study reported no benefit (this study used the lowest dose of EPA plus DHA). One study reported a non-significant decrease in disease activity and a significant decrease in use of corticosteroids. Two studies reported benefit, including improved histological appearance of the colon, decreased disease activity, weight gain and decreased use of prednisolone. Two other ‘open’ studies reported improved symptoms, improved histological appearance of the rectal mucosa and decreased use of prednisolone.</td>
<td>Rodgers (1998)</td>
</tr>
<tr>
<td>Psoriasis</td>
<td>2</td>
<td>1.8</td>
<td>8–12</td>
<td>One study reported significant improvement in itching, scaling and erythema. One study reported no benefit. Three open studies (providing 10–18 g EPA + DHA day(^{-1}) for 6–8 weeks) reported mild to moderate (two studies) or moderate to excellent (one study) improvements in scaling, itching, lesion thickness and erythema in the majority of patients. One open study that combined fish oil with a low-fat diet reported improvements.</td>
<td>Ziboh (1998)</td>
</tr>
</tbody>
</table>
In contrast, some studies have shown significant clinical improvements in patients (e.g. Dry and Vincent, 1991) and there are suggestions that this type of approach may be useful in conjunction with other drug- and diet-based therapies (see Calder and Miles, 2000). Broughton et al. (1997) compared the effects of low-dose and high-dose fish oil in adult atopic asthmatics; the actual amount of fish oil each patient consumed was calculated according to their regular n-6 PUFA intake, such that the ratio of n-6 to n-3 fatty acid in the diet was 10:1 (low fish oil) or 2:1 (high fish oil). At baseline and after each treatment period (i.e. with low and then high fish oil), lung function was measured in response to increasing doses of methacholine. With low n-3 fatty acid ingestion, methacholine-induced respiratory distress increased. In contrast, high n-3 fatty

Fig. 4.10. Putative role of arachidonic acid in atopic disease. PGE₂ inhibits production of the Th1-type cytokine, IFN-γ, so allowing the production of Th2-type cytokines (e.g. IL-4) to proceed without inhibition. IL-4 promotes Ig class switching in B-cells to produce IgE; PGE₂ also directly promotes IgE production by B-cells. Thus, PGE₂ acts to promote the Th2-type response and IgE production. The 4-series LT (and some 2-series PG, such as PGD₂) are the direct mediators of allergic inflammation. HETE, hydroxyeicosatetraenoic acid; IFN, interferon; Ig, immunoglobulin; IL, interleukin; LT, leucotriene; PG, prostaglandin; Th, T-helper.
acid ingestion resulted in improved lung function in more than 40% of subjects; all measures of respiratory function were improved in this group of patients, who also showed a markedly elevated appearance of the EPA-derived 5-series LT in their urine. However, some patients did not respond to the high n-3 polyunsaturated fatty acid intake, which in some cases worsened respiratory function. This study suggests that there may be asthmatic subjects who respond positively to fish oil intervention but others whose response may be worsened by such a dietary intervention. Thus, this therapy should be approached cautiously until more is known about the factors that determine sensitivity to n-3 PUFAs.

Endotoxaemia, sepsis and trauma

The importance of a hyperinflammatory response, characterized by overproduction of TNF-α, IL-1β, IL-6 and IL-8, in the progression of trauma patients towards sepsis is now recognized. Enhanced production of arachidonic acid-derived eicosanoids, such as PGE₂, is also associated with trauma and burns. The inflammatory effects of infection can be mimicked by administration of endotoxin (bacterial lipopolysaccharide). Essential fatty acid deficiency in rats increased mortality after endotoxin challenge (Cook et al., 1981). Arachidonic acid administration increased mortality following endotoxin (Cook et al., 1981), while feeding a high linoleic acid diet increased mortality in guinea pigs recovering from burns injury (Alexander et al., 1986). Fish oil feeding or infusions enhanced the survival of guinea pigs following endotoxin challenge (Mascioli et al., 1988, 1989) and decreased the accompanying metabolic perturbations in guinea pigs and rats (for references, see Calder, 1997). Mice fed fish oil and then injected with endotoxin had lower plasma TNF-α, IL-β and IL-6 concentrations than mice fed safflower oil (Sadeghi et al., 1999), while fish oil-containing parenteral nutrition decreased serum TNF-α, IL-6 and IL-8 concentrations in burned rats (Hayashi et al., 1998; Tashiro et al., 1998). Total parenteral nutrition using fish oil as the lipid source was found to prevent the endotoxin-induced reduction in blood flow to the gut and to reduce the number of viable bacteria in mesenteric lymph nodes and liver following exposure to live bacteria (Pscheidl et al., 2000). Fish oil did not, however, decrease bacterial translocation across the gut and the authors concluded that fish oil must have improved bacterial killing. Fish oil administration prior to exposure to live pathogens decreased the mortality of rats compared with vegetable oil (Barton et al., 1991; Rayon et al., 1997). These studies did not measure inflammatory cytokine levels, but they showed that PGE₂ levels were decreased by fish oil (Barton et al., 1991; Rayon et al., 1997). More recently, fish oil infusion after induction of sepsis by caecal ligation and puncture in rats was shown to decrease mortality (and PGE₂ production) compared with vegetable oil (Lanza-Jacoby et al., 2001).

An understanding of the inflammatory changes occurring during sepsis and of the anti-inflammatory effects of fish oil, combined with the outcome of these animal experiments, has prompted clinical studies investigating the influence of
fish oil administered either parenterally or enterally. Patients receiving parenteral fish oil following major abdominal surgery had lower serum concentrations of TNF-α and IL-6 than those receiving a control mix (Wachtler et al., 1997). This study did not report clinical outcome. A large number of clinical trials (at least 20) have been performed in intensive care or surgical patients using enteral formulae containing n-3 PUFAs. The majority of these trials have used the commercially available product IMPACT®, which contains arginine, yeast RNA and n-3 PUFAs. Many of these trials report beneficial outcomes, including decreased numbers of infections and infectious or wound complications, decreased severity of infection, decreased need for mechanical ventilation, decreased progression to systemic inflammatory response syndrome and decreased length of intensive-care unit and/or total hospital stay. A comprehensive meta-analysis of 15 randomized, controlled studies using IMPACT® or Immun-Aid® (also rich in arginine, RNA and n-3 PUFAs) has been performed (Beale et al., 1999). This analysis confirmed significant reductions in infection rate, number of ventilator days and length of hospital stay, but not in overall mortality. Few of the studies reviewed measured immune outcomes. However, some other studies of IMPACT®, not included in the meta-analysis, did so, focusing especially on inflammatory cytokines. Several of these studies show decreased circulating TNF-α and/or IL-6 concentrations in patients given IMPACT® or similar formulae before (Braga et al., 1999; Gianotti et al., 1999; Tepaske et al., 2001) or after (Braga et al., 1996; Wu et al., 2001) major surgery. Although these observations fit with the predicted effects of n-3 PUFAs and could be used as evidence of their efficacy in the trauma and post-surgery settings, the complex nature of the formulae prevents such a clear interpretation. The effects could be due to any one of the specified nutrients (i.e. arginine, RNA, n-3 PUFAs) or to a combination of these nutrients. Indeed, the positive outcomes from the use of IMPACT® and similar formulae have often been used as evidence for the benefit of arginine in these settings (see Duff and Daly, Chapter 5, this volume).

Cancer

The immune system obviously plays an important role in anti-cancer defence. There is a progressive decrease in many immune surveillance defences in animal models of cancer (Shewchuk et al., 1996) and in humans with cancer (Keissling et al., 1999). A major focus of current research in immunology and oncology is the development of methods to augment host antitumour immune defences. Feeding fish oil to experimental animals protects against the development of carcinogen-induced mammary tumours, reduces the growth of mammary tumours and prevents the development of cachexia and metastatic diseases (see Cave, 1991). Although dietary fat can modulate anti-cancer defences, such as natural killer cell cytotoxicity and humoral and T-cell responses, the application of studies in healthy humans and animals to the cancer state may not be as straightforward. The influence of dietary n-3 PUFAs on the immune response may be different between healthy animals and those with
suppressed immune systems (Robinson et al., 2001, 2002). Tumour-bearing rats fed long-chain n-3 PUFAs as part of a low-PUFA diet had significantly increased natural killer cell cytotoxicity, a higher proportion of CD8+ and CD28+ cells that were activated (i.e. expressing CD25) and increased nitric oxide and IL-2 production after mitogen stimulation, whereas these immune enhancements were not found when n-3 PUFAs were supplemented in a high-PUFA diet.

**Conclusions**

Several fatty acids can potentially exert effects on inflammation and immunity. Arachidonic acid gives rise to inflammatory mediators (eicosanoids) and through these regulates the activities of inflammatory cells, the Th1 versus Th2 balance and B-cell function. It is generally considered that n-3 PUFAs act as arachidonic acid antagonists. As such, among the fatty acids, it is the n-3 PUFAs that are believed to possess the most potent immunomodulatory activities, and, among the n-3 fatty acids, those from fish oil (EPA and DHA) are more biologically potent than α-linolenic acid. Components of both natural and acquired immunity, including the production of key inflammatory mediators, can be affected by n-3 PUFAs. Although some of the effects of n-3 fatty acids may be brought about by modulation of the amount and types of eicosanoids made, it is possible that these fatty acids might exert some of their effects by eicosanoid-independent mechanisms, including actions upon intracellular signalling pathways and transcription-factor activity. There is some evidence that n-3 fatty-acid-induced effects may be of use as a therapy for acute and chronic inflammation and for disorders that involve an inappropriately activated immune response. However, more needs to be understood about the effects of individual fatty acids against different backgrounds (e.g. levels and types of fat in the diet), about the dose-response effects of n-3 PUFAs, about interactions between PUFAs and other dietary components, especially antioxidant vitamins, and about the differences in immune effects of fatty acids between health and disease.

**References**


acid composition and prostaglandin E₂ production but have different effects on lymphocyte functions and cell-mediated immunity. *Lipids* 33, 171–180.


Sanderson, P. and Calder, P.C. (1998b) Dietary fish oil appears to inhibit the activation of phospholipase C-γ in lymphocytes. *Biochimica et Biophysica Acta* 1392, 300–308.


Introduction

Arginine is a semi-essential amino acid in mammals. While dietary arginine is not an absolute requirement under normal conditions, it can become essential at times of growth and metabolic stress, such as following trauma, sepsis or burn injuries. This dibasic amino acid is found in all mammalian cells and is an intermediate in many metabolic pathways from protein synthesis to energy storage to the clearance of nitrogenous waste. The importance of arginine for the normal functioning of the immune system has become progressively apparent over the last 15 years and has led to the development of arginine-supplemented enteral feeding regimes and the concept of immunonutrition (for a review, see Evoy et al., 1998).

The Biochemistry of Arginine

The structure of the arginine molecule is shown in Fig. 5.1. It is the most basic of the amino acids, with a $pK_a$ of 12.5, and, as such, contributes to the positive charge of proteins of which it is a component. The metabolic pathways involving arginine are complex and are outlined in Fig. 5.2. Adding to the complexity of these pathways is the varied location of the enzymes involved, both at the intracellular level (mitochondrial versus cytosolic enzymes) and between tissues. With the exception of enterocytes in neonates, no single cell type contains all the necessary enzymes for arginine synthesis (Wu and Morris, 1998).

Uptake and synthesis of arginine

Arginine is actively absorbed from the gut via a sodium-dependent transport mechanism. High arginase activity within enterocytes converts 40% of dietary
arginine to citrulline, which is released into the circulation (Castillo et al., 1993). Of the arginine reaching the portal venous blood, 15% is cleared by the liver; the remainder enters the systemic circulation (O'Sullivan et al., 1998). Dietary glutamine and glutamate can also be used by enterocytes to generate citrulline.

Arginine is synthesized from citrulline, primarily in the kidney, and is then released into the circulation to be taken up and used by other tissues. While arginine is also generated in large quantities by the liver, this arginine is recycled by the urea cycle within the hepatocyte, with little net production (Rabier et al., 1991).

**Nitric oxide pathway of arginine metabolism**

No one molecule has attracted more attention in the last decade than nitric oxide (NO). This small molecule plays a pivotal role in a diverse range of functions, including vasodilatation, memory, peristalsis, penile erection, cytotoxicity and the control of various endocrine and exocrine secretions in the cardiovascular, reproductive, central nervous and immune systems (Nathan and Xie, 1994; MacMicking et al., 1997). NO is synthesized from arginine by nitric oxide synthase (NOS), with the formation of citrulline. There are three known forms of this enzyme: neuronal (nNOS) and endothelial cell (ecNOS) NO synthases, which are both constitutively expressed and calcium-activated, and an inducible form (iNOS), which is controlled at the transcriptional level and is of most interest in the setting of the immune system.

iNOS expression, and hence NO production, is induced in macrophages in response to a variety of stimuli, particularly the T-helper-1 cytokines interferon (IFN)-γ and tumour necrosis factor (TNF)-α and the Gram-negative bacterial wall component endotoxin (lipopolysaccharide (LPS)). Inhibition of
NO production increases the host susceptibility to viral, bacterial, fungal, protozoal and helminthic infections (MacMicking et al., 1997). In addition, the anti-tumour activity of stimulated mouse macrophages is absent in iNOS knockout mice (Stuehr and Nathan, 1989). The mechanisms of NO cytotoxicity are complex, involving inhibition of DNA synthesis, mitochondrial inactivation, cell membrane lysis, cell cycle arrest, DNA strand break formation and induction of apoptosis (Burney et al., 1997). In addition, NO can react with superoxide to form peroxynitrite, a powerful oxidizing agent capable of
inducing cell injury and death (Samar et al., 1997). Apart from its cytotoxic effects, NO is involved in regulating the expression of major histocompatibility complex (MHC) II expression in antigen-presenting cells, in modulating T-cell mitogenic responses and in the induction and suppression of many cytokines (Niedbala et al., 1999; Akaike and Maeda, 2000). However, the full extent of NO involvement in the functioning of the immune system has yet to be established.

Arginase/ornithine pathways of arginine metabolism

Two forms of the arginase enzyme exist. While both forms catalyse the conversion of arginine to ornithine, they are encoded by separate gene sequences, are located in different subcellular compartments and are expressed to varying degrees in separate tissues. Type I arginase or hepatic arginase is constitutively expressed in hepatocytes. It is a cytosolic enzyme and is a central component of the urea cycle. While not constitutively present, its expression can be induced in macrophages by stimulation with T-helper-2 cytokines (especially by interleukins (IL)-4, -10 and -13) (Munder et al., 1999; Chang et al., 2000). Arginase II, on the other hand, is localized to the mitochondria and is found in high concentrations in kidney, brain and small intestine. Arginase II can also be induced in macrophages but by different stimuli, namely, LPS and dexamethasone (Corraliza et al., 1995). The expression of both forms of arginase in macrophages is reduced by T-helper-1 cytokines, such as IFN-γ (Munder et al., 1999).

Following conversion to ornithine, a number of pathways may be followed for further metabolism. What directs a cell to choose one pathway over another is not yet understood.

Urea cycle

The reactions involved in the urea cycle are shown in Fig. 5.3 and occur primarily in the liver. The function of the urea cycle is to clear nitrogenous waste, by converting ammonia to urea for excretion by the kidneys. Nitrogen can enter the cycle either through conversion to carbamoyl phosphate and subsequent passage of the carbamoyl molecule to ornithine, forming citrulline, or via glutamate to aspartate, which enters the cycle by reacting with citrulline to form arginosuccinate. Arginosuccinate lyase converts arginosuccinate to arginine, and fumarate, and arginase catalyses the degradation of arginine to ornithine with the loss of one molecule of urea. The reactions involving carbamoyl phosphate and glutamate occur in the mitochondrion, whereas the remaining reactions take place in the cytosol.

Proline

Ornithine aminotransferase catalyses the transfer of one amine residue from α-keto-glutarate to ornithine, with the formation of pyrroline-5-carboxylate,
which can then be reduced to proline or pass, via glutamate semialdehyde, to glutamate. Proline and its derivative hydroxyproline (formed in situ by the action of ascorbic acid) constitute 25% of the collagen molecule and therefore play a vital role in wound healing and tissue repair. Glutamate can be used by the cell for energy production, by complete oxidation to CO₂ through the citric acid cycle, or can be used for protein or amino acid synthesis.

**Polyamines**

The polyamines – putrescine, spermidine, spermine – are ubiquitous molecules found in all eukaryotic cells. They are synthesized from arginine via ornithine and ornithine decarboxylase, as shown in Fig. 5.2. The precise physiological role of polyamines has yet to be fully elucidated. It is known that they are required at low concentrations for cell viability and that levels increase during cell growth, differentiation and proliferation. They have been demonstrated to act through altering the three dimensional structure of tRNA thereby stimulating protein synthesis, through the phosphorylation of protein kinases, thereby accelerating intracellular signalling pathways, through modulation of transcription and mRNA turnover and through DNA editing. Inhibition of polyamine synthesis, using DL-alpha-difluoromethylornithine (DMFO) (a competitive inhibitor of ornithine decarboxylase), leads to a reduction in cell viability, cell-cycle arrest in S-phase and inhibition of cell differentiation.
Other pathways of arginine metabolism

Apart from incorporation into proteins or degradation by arginase or NOS, as described above, arginine can follow other pathways catalysed by the enzymes arginine decarboxylase and arginoglycine amidinotransferase. The latter leads to the formation of a recently discovered molecule, agmatine, which is thought to be involved in intracellular signalling. The former leads to the synthesis of phosphocreatine, an important molecule for energy storage in skeletal muscle.

Endocrine effects of arginine

Elevated plasma levels of arginine have been found to correlate with increased secretion of various hormones, including prolactin and growth hormone from the pituitary, insulin, glucagon, insulin-like growth factor 1 (IGF-1) and adrenal catecholamines (see Barbul, 1996). These hormones, in turn, can affect the functioning of the immune system. While the powerful secretagogue action of arginine is largely unexplained, a direct cholinergic effect, membrane depolarization by this highly cationic molecule and subsequent calcium influx, and the use of NO as an intermediary in cell signalling have all been demonstrated.

Prolactin

Prolactin can play a role in various stages of dendritic-cell maturation and T-helper-1 development. Prolactin induces maturation of dendritic cells from monocytes, by increasing their expression of the antigen-presenting MHC class II molecules and co-stimulatory molecules. The expression of CD40 is also up-regulated by prolactin, the final effect being increased T-cell activation. Prolactin can also stimulate the release of T-helper-1 cytokines by T-cells in the absence of dendritic cells (Matera et al., 2000).

Growth hormone

The growth hormone receptor is a member of the haematopoietin/cytokine receptor family and induces tyrosine phosphorylation through the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways. While growth hormone is not an absolute requirement for normal lymphoid and myeloid development, under situations of stress it can potentiate the cytokine responses of human T-cells, improve the antigen-presenting capability of dendritic cells, increase the numbers of haematopoietic progenitor cells in the bone marrow and induce thymic hyperplasia (Murphy and Longo, 2000).

IGF-1

IGF-1 plays an important role in the maturation of lymphocytes in the bone marrow and in their function in the periphery. In rodents, IGF-1 can restore age-related thymic involution and increase lymphocyte number and activity (Clark et al., 1993; Hinton et al., 1995). In addition, the thymotropic effects of growth hormone appear to be mediated through IGF-1.
Arginine and Immune Function – Animal Studies

Stress to the body, such as trauma with fracture or haemorrhage, burn injury or major elective surgical procedures, leads to alterations in immune functions and an attenuated immune response (Faist et al., 1986). This predisposes to the development of infectious complications in the days or weeks following injury. It is estimated that, among patients who die more than 24 h after hospitalization following trauma, 75% die as a result of complications of infection and the inflammatory response (Miller et al., 1982). It is in limiting this immune dysfunction that supplemental dietary arginine appears to hold most promise (see Evoy et al., 1998).

Barbul and co-workers demonstrated that supplemental dietary arginine reduced trauma-induced thymic involution, lessened weight loss, improved wound healing and prolonged survival in injured rats (see Barbul, 1977). The same group also demonstrated that a dietary supplement of 1% arginine by weight increased thymic weight and the number of thymic lymphocytes in normal healthy mice and rats (see Barbul, 1986, 1996). In a burn model in guinea pigs, Saito et al. (1987) showed an increase in delayed hypersensitivity and in survival in arginine-supplemented animals. Madden et al. (1988) demonstrated a similar survival advantage in animals subjected to lethal bacterial peritonitis. This finding was replicated by Gianotti et al. (1993), who, in addition to improved survival, found a reduction in bacterial translocation and increased bactericidal activity in the arginine-supplemented animals.

The anti-tumour activity of arginine has been studied in a number of models, with variable results. In the setting of protein–calorie malnutrition and tumour inoculation, supplemental arginine (1% by weight) reduced the growth rate of the immunogenic neuroblastoma C1300 and improved host survival compared with glycine-supplemented controls (Fig. 5.4), while a similar effect was not seen in mice bearing a poorly immunogenic neuroblastoma TBJ (Reynolds et al., 1990). Similar differences have been seen in different models of breast and colon cancer. The impression is of a dual effect of arginine: improving host resistance while simultaneously improving tumour-cell survival, the end result depending on the balance between these two opposing actions. The potential benefit of arginine supplementation in cancer therapy was further highlighted in a study by Lieberman et al. (1992), in which mice bearing the C1300 neuroblastoma were treated with a combination of arginine and IL-2: combined therapy led to a reduction in tumour growth and prolonged host survival, compared with the control group or either treatment alone.

Mechanism(s) of Arginine Action on Immune Function

Arginine is required for the normal growth and proliferation of lymphocytes in vitro. While the diminished mitogenic response seen in arginine-free conditions was initially attributed to the reduction in protein and polyamine synthesis, the demonstration that normal function and DNA synthesis can be returned by the addition of NO donors (sodium nitroprusside, S-nitrosoacetyl penicillamine) has forced a reconsideration of this premise (Efron et al., 1991).
Early mechanistic studies of the in vivo effect of supplemental arginine focused on T-cell activity. Increased delayed-type hypersensitivity responses, measured by foot-pad or ear-lobe thickness following inoculation of foreign material in previously sensitized hosts has been demonstrated in normal mice given supplemental arginine and in the settings of tumour, burn and sepsis (Saito et al., 1987; Reynolds et al., 1988b, 1990; Lewis and Langkamp-Henken, 2000). This has been found to correlate with increased T-cell mitogenesis in response to stimulation by concanavalin A, phytohaemagglutinin or tumour antigens, in addition to increases in specific T-cell cytotoxicity (Reynolds et al., 1988b, 1990). Improvements in mononuclear-cell response to stimulation with concanavalin A have also been demonstrated, using cells from human subjects given arginine (Daly et al., 1988). The mechanisms behind these effects remain unclear. While early reports suggested the involvement of a thymic hormone, similar effects have been produced in athymic nude mice (see Barbul, 1986). Up-regulation of IL-2 receptor expression and, in tumour-bearing mice, IL-2 production have been demonstrated and suggested as a mechanism behind increased T-cell activity (Reynolds et al., 1988b). Gianotti et al. (1993) found that the survival advantage conferred on mice by arginine in the setting of abdominal sepsis was eliminated by the concomitant administration of the NOS inhibitor N-omega-nitro-L-arginine (NNA) and proposed the arginine–NO pathway as the key factor. Meanwhile, the importance of the hypothalamic–pituitary axis in arginine-mediated immune modulation was demonstrated by Barbul et al. (1983), who found that the thymotropic effects of supplemental arginine following injury were not reproduced in hypophysectomized rats.

**Fig. 5.4.** The effect of supplemental arginine on the growth of a C1300 neuroblastoma in mice with protein–calorie malnutrition (adapted from Reynolds et al., 1988b).
In addition to the above alterations seen in T-cell functions, supplemental arginine also benefits the innate immune response, with increases in macrophage and natural killer cell cytotoxicity (Reynolds et al., 1988a). The link between arginine metabolism and the tumoricidal activity of macrophages was highlighted by Mills et al. (1992) in a study looking at macrophage function following intraperitoneal implantation of P815 mastocytoma in naive and pre-immunized mice. Tumour rejection was associated with elevated levels of NO production and iNOS expression in peritoneal macrophages and with a reduction in arginase activity. In contrast, during times of exponential tumour growth, arginase activity was increased, with a corresponding elevation in urea and ornithine production, while NO and citrulline production were reduced. The balance between iNOS and arginase activity in macrophages has been demonstrated in many different models and is considered to be central to the shift in phenotype from wound to cytotoxic macrophage.

Both human and animal models have demonstrated the beneficial effect of arginine in wound healing. Arginine supplementation of the diet of injured rats resulted in accelerated wound healing, increased wound tensile strength and increased collagen deposition (see Barbul et al., 1983). Wound healing was assessed by fresh wound strip breaking strength, fixed breaking strength and the amount of reparative collagen deposition indexed by the hydroxyproline content of implanted sponges. These findings can be explained, in part, by the increased requirement for arginine to synthesize reparative connective tissue. However, as with T-cell mitogenesis, the improvement in wound healing was not reproducible in hypophysectomized animals, suggesting a more complex mechanism.

In human studies, Kirk et al. (1993) examined the effect of arginine supplementation (17 g day\(^{-1}\)) on wound healing in an otherwise healthy elderly population. While epithelialization of a partial-thickness wound was not improved, collagen synthesis (as determined by hydroxyproline and protein deposition in subcutaneous polytetrafluoroethylene implants) was significantly increased in those subjects given arginine.

Clinical Studies with Arginine in Patients at Risk of Sepsis and Septic Complications

The first clinical study to demonstrate a benefit from supplemental dietary arginine in surgical patients was performed in 30 patients undergoing major operations for gastrointestinal malignancy (Daly et al., 1988). Patients were commenced on enteral feeding post-operatively and were randomized to receive either arginine (25 g day\(^{-1}\)) or isonitrogenous glycine (43 g day\(^{-1}\)) for 7 days. Arginine supplementation resulted in elevated plasma arginine and ornithine levels and was associated with an enhanced response of peripheral-blood lymphocytes to mitogens by day 7 and with an increased number of circulating CD4+ cells. Only the arginine-supplemented group achieved a positive nitrogen balance, which was attained by day 6 (Fig. 5.5). However, there was no difference in clinical outcome between the two groups.
Fig. 5.5. Some immunological and metabolic effects of supplemental arginine in patients undergoing surgery for upper gastrointestinal malignancy (adapted from Reynolds et al., 1990). Con A, concanavalin A.
At this time, two other dietary factors were emerging as playing an important role in modulating host defence – namely nucleotides and n-3 fatty acids. In mice fed nucleotide-free diets, supplemental RNA or uracil is required to restore cellular immunity, anti-fungal resistance, anti-bacterial resistance and, the bactericidal activity of macrophages (Van Buren et al., 1994). n-3 fatty acid supplementation, in clinical and laboratory studies, has been associated with improved survival after burn injury, reduced post-injury infectious complications and diminished immunosuppression secondary to transfusion (Daly et al., 1992). The proposed mechanisms of omega-3 fatty acid-induced improvements in immune function are related to an alteration of prostaglandin synthesis pathways from 2-series to 3-series prostaglandins (see Calder and Field, Chapter 4, this volume).

A study was therefore performed to examine the effect of enteral nutrition with supplemental arginine, RNA and n-3 fatty acids on immunological, metabolic and clinical outcome in patients after surgery (Daly et al., 1992). Eighty-five patients requiring operations for upper gastrointestinal malignancies were randomized to receive a supplemental diet (IMPACT®, Sandoz Nutrition, Minneapolis, Minnesota, USA) or a standard enteral diet. Patients receiving the supplemental diet had a significantly greater nitrogen balance over the course of the study. Lymphocyte mitogenesis was reduced in both groups in the immediate post-operative period but returned to normal levels only in the supplemented group, by post-operative day 7. Infectious and wound complications occurred less commonly in the supplemented than in the control group (11% vs. 37%; \( P = 0.02 \)) and mean length of hospital stay was significantly shorter in the supplemented group. A subsequent study (Daly et al., 1995) used the same feeding protocol in 60 patients requiring surgery with or without adjuvant radiation/chemotherapy for upper gastrointestinal malignancy. Patients receiving the supplemental diet had fewer wound and infectious complications (10% vs. 43%; \( P < 0.05 \)), and shorter length of hospital stay (16 vs. 22 days; \( P < 0.05 \)) than patients receiving the standard enteral diet.

Since then, an increasing number of studies have been performed comparing immunonutrition (using one of the two formulas outlined in Table 5.1) to standard enteral feeding regimes in critically ill patients. In one of the largest such studies, a multicentre, prospective randomized trial compared early enteral nutrition by IMPACT® with standard enteral nutrition in 296 patients in intensive care units (Bower et al., 1995). While mortality rates and infectious complications were the same in the treatment groups, subgroup analysis showed two interesting results. First, the mean length of hospital stay of septic patients was significantly reduced (\( P < 0.05 \)). Second, in the 100 patients who were able to complete the total planned intake of the enteral diet within the first 7 days, there was a significant reduction in infectious complications (0.54 ± 0.78 vs. 0.94 ± 0.87) between the supplemented and control groups.

Comparing the same feeding formula with standard enteral nutrition, Braga et al. (1998) looked at 60 patients undergoing surgery for malignancy. While there was no demonstrable difference in the incidence of infectious complications between the two groups, the infectious complications occurring in the patients receiving the supplemental diet were assessed as being significantly less severe.

Kudsk et al. (1996) prospectively randomized 35 severely injured trauma patients to an enteral diet containing glutamine, arginine, n-3 fatty acids and
nucleotides (Immun-Aid®, McGaw, Irvine, California, USA) or to an isonitrogenous, isocaloric diet to investigate the effect on septic outcome. Significantly fewer major infectious complications developed in patients who received the supplemental diet than in the control group (6% vs. 41%; \( P = 0.02 \)). Hospital stay was also significantly shorter.

One study to date has shown improvement in the mortality rate in patients receiving immunonutrition (Galban et al., 2000). This was a prospective, randomized, multicentre study of 176 septic patients in intensive care units: 89 patients received IMPACT®, while 87 patients received a standard high-protein enteral feed. The mortality rate was reduced in the treatment group compared with the control group (17 of 89 vs. 28 of 87; \( P < 0.05 \)), and this was most marked in moderately ill patients with APACHE (Acute Physiological and Chronic Health Evaluation) II scores between 10 and 15 (1 of 26 vs. 8 of 29; \( P = 0.02 \)). There was also a significant difference in the incidence of bacteraemia between the two groups (7 of 89 vs. 19 of 87; \( P = 0.01 \)) and in the number of patients developing more than one nosocomial infection (5 of 89 vs. 17 of 87; \( P = 0.02 \)).

In a prospective, randomized, double-blind trial Senkal et al. (1999) examined the effects of IMPACT® enteral nutrition compared with the standard enteral diet, when commenced 5 days pre-operatively in patients with upper gastrointestinal-tract malignancy. These authors used as end-points the incidence of postoperative infectious complications, the cost of treating these complications, and the overall cost-effectiveness of immunonutrition. One hundred and fifty-four patients were eligible for analysis. The number of patients developing infectious complications after day 3 was significantly reduced in the immunonutrition group (7 of 78 vs. 16 of 76; \( P = 0.04 \)), as was the total number of complication events (14 vs. 27; \( P = 0.05 \)). While the total number of patients developing complications (10 of 78 vs. 18 of 76) and the mean length of stay (22.2 ± 4.1 days vs. 25.8 ± 3.8 days) in the treated group were decreased, these values did not reach statistical significance (\( P = 0.08 \) and \( P = 0.09 \), respectively). Despite higher product costs, the savings accrued by a substantially lower complication rate led to better cost-effectiveness in the group receiving immunonutrition.

### Table 5.1. Compositions of two enteral feeding formulae.

<table>
<thead>
<tr>
<th>Component</th>
<th>IMPACT®</th>
<th>Immun-Aid®</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (g)</td>
<td>56</td>
<td>37</td>
</tr>
<tr>
<td>Free arginine (g)</td>
<td>12.5</td>
<td>14</td>
</tr>
<tr>
<td>Free glutamine (g)</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Other free amino acids (g)</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Nucleic acids (g)</td>
<td>1.2</td>
<td>1</td>
</tr>
<tr>
<td>Total fat (g)</td>
<td>27.8</td>
<td>22</td>
</tr>
<tr>
<td>n-3 fatty acids (g)</td>
<td>2.8</td>
<td>1</td>
</tr>
<tr>
<td>Vitamins and minerals</td>
<td>Selectively enriched above 100% US RDA</td>
<td></td>
</tr>
</tbody>
</table>

RDA, recommended daily allowance.
Atkinson et al. (1998) performed a prospective, randomized, double-blind, controlled clinical trial in a heterogeneous group of critically ill patients in an intensive care unit. Infectious complications were not reported in this study, but, in the subgroup of patients \((n = 101)\) who successfully achieved early enteral nutrition \((> 2.5 \text{ l in the first 72 h})\), there was a significant reduction in the requirement for mechanical ventilation and in the length of hospital stay in the immunonutrition group.

In a meta-analysis of 12 studies containing 1482 critically ill patients, Beale et al. (1999) sought to address the clinical benefits derived from immunonutrition \((\text{IMPACT}^\circledast \text{ or Immun-Aid}^\circledast)\) over standard enteral feeds. This detailed analysis found that there was no overall effect on mortality. However, there were significant reductions in infection rate \((P = 0.006)\), ventilator days \((P = 0.04)\) and length of hospital stay \((P = 0.0002)\) in the immunonutrition group. These benefits were found to be most impressive among surgical patients.

**Conclusion**

The favourable modulatory effects of arginine in the immune system have been well documented in animal models and some have been reproduced in humans. The most prominent effect of supplemental arginine is in abrogating trauma-induced immunosuppression – in particular, the reductions seen in T-cell mitogenesis, delayed-type hypersensitivity response, macrophage and natural killer cell cytotoxicity and the improvement of wound healing. In the clinical setting, immunonutrition, comprising supplemental arginine, nucleic acids and n-3 fatty acids, has been demonstrated to reduce infectious complications (e.g. see Table 5.2) and length of hospital stay in critically ill patients.

### Table 5.2. The incidence of septic complications in prospective randomized trials comparing arginine-supplemented patients with non-supplemented controls.

<table>
<thead>
<tr>
<th>Patient cohort</th>
<th>Author</th>
<th>N</th>
<th>Experimental diet</th>
<th>Infectious complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer surgery</td>
<td>Daly et al. (1992)</td>
<td>85</td>
<td>IMPACT®</td>
<td>11\textsuperscript{a}</td>
</tr>
<tr>
<td>Cancer surgery</td>
<td>Daly et al. (1995)</td>
<td>60</td>
<td>IMPACT®</td>
<td>10\textsuperscript{a}</td>
</tr>
<tr>
<td>Cancer surgery</td>
<td>Braga et al. (1998)</td>
<td>60</td>
<td>IMPACT®</td>
<td>10</td>
</tr>
<tr>
<td>Cancer surgery</td>
<td>Senkal et al. (1999)</td>
<td>164</td>
<td>IMPACT®</td>
<td>18\textsuperscript{a}</td>
</tr>
<tr>
<td>Trauma</td>
<td>Brown et al. (1994)</td>
<td>37</td>
<td>IMPACT®</td>
<td>16\textsuperscript{a}</td>
</tr>
<tr>
<td>Trauma</td>
<td>Kudsk et al. (1996)</td>
<td>33</td>
<td>Immun-Aid®</td>
<td>6\textsuperscript{a}</td>
</tr>
<tr>
<td>Trauma</td>
<td>Moore et al. (1994)</td>
<td>98</td>
<td>Immun-Aid®</td>
<td>0\textsuperscript{b}</td>
</tr>
<tr>
<td>Mixed ICU</td>
<td>Bower et al. (1995)</td>
<td>296</td>
<td>IMPACT®</td>
<td>6\textsuperscript{b}</td>
</tr>
<tr>
<td>Mixed ICU</td>
<td>Galban et al. (2000)</td>
<td>181</td>
<td>IMPACT®</td>
<td>8\textsuperscript{a,b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}P < 0.05 vs. control group.

\textsuperscript{b}Bacteraemia.

ICU, intensive care unit.
References


Glutamine and the Immune System

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Glutamine Synthesis and Interorgan Transport

Glutamine (Fig. 6.1) is the most abundant free amino acid in the bloodstream and in the body. It contributes about 50% of the free α-amino acid pool within the human body and is quantitatively the most important amino acid involved in inter-organ nitrogen transport (Lund and Williamson, 1985). Classically, glutamine is a non-essential amino acid. Indeed, it can be synthesized in many cells and tissues of the body. The immediate precursor of glutamine is glutamate and the enzyme responsible for glutamine synthesis is glutamine synthetase (Fig. 6.2). In turn, glutamate can be formed from 2-oxoglutarate by transamination. Thus, the transamination reaction serves to transfer amino groups from amino acids to glutamine via glutamate (Fig. 6.2). Although any amino acid can potentially participate in the transamination reaction with 2-oxoglutarate, it is considered that the branched-chain amino acids play an important role in amino-group donation. The ammonia for the glutamine synthetase reaction could be generated from any deamination reaction; however, it is likely that in muscle the glutamate dehydrogenase and AMP deaminase reactions play important roles here.

Although many tissues can synthesize glutamine, only certain tissues are able to release significant amounts of it into the bloodstream. These include the lung, brain, skeletal muscle and perhaps adipose tissue. Because of its large mass, skeletal muscle is considered to be the most important glutamine producer in the body (see Elia and Lunn, 1997). In skeletal muscle, glutamine contributes approximately 60% of the total free amino acid pool and it has a concentration of approximately 20 mM (Bergstrom et al., 1974; Lund, 1981). It is estimated that skeletal muscle releases up to 9 g of glutamine day⁻¹ (Elia and Lunn, 1997). This is a greater amount of glutamine than that typically provided by the diet (approximately 5 g day⁻¹). It is estimated that about 60% of glutamine released by human skeletal muscle in healthy individuals comes from de novo synthesis, with the remaining 40% coming from protein breakdown (Hankard et al., 1995).
Once released from skeletal muscle, glutamine acts as an interorgan nitrogen transporter (Lund and Williamson, 1985; Newsholme et al., 1989; see Fig. 6.3). The plasma glutamine concentration in healthy adult humans is typically in the range 0.5–0.8 mM, with a mean concentration of approximately 0.65 mM. Important users of glutamine include the kidney (see Tizianello et al., 1982), liver (see Haussinger, 1989), small intestine (see Windmueller and Spaeth, 1974; Souba, 1991) and cells of the immune system (for reviews, see Calder, 1994, 1995a; Wilmore and Shabert, 1998; Calder and Yaqoob, 1999; Newsholme et al., 1999; Newsholme, 2001). Glutamine has a number of metabolic roles in these user organs (Table 6.1).
In the liver, the carbon skeleton of glutamine is an important precursor for glucose synthesis, while glutamine itself can be used for the synthesis of other amino acids and proteins, with excess nitrogen disposed of via ureagenesis. Glutamine can also be used as the precursor for the glutamate portion of glutathione, which is synthesized primarily in the liver. In the kidney, glutamine participates in acid–base balance, donating its amido and amino nitrogens to join with protons to form ammonium ions, which are excreted in the urine. The remaining carbon skeleton can be used to generate energy or as a precursor for glucose synthesis (gluconeogenesis). Glutamine is the major energy source in the small intestine and is an important energy source for immune cells. Glutamine is a nitrogen donor for the synthesis of purines and pyrimidines.

**Table 6.1. Metabolic roles of glutamine.**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Metabolic role of glutamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Glucose synthesis (C skeleton)</td>
</tr>
<tr>
<td></td>
<td>Amino acid synthesis</td>
</tr>
<tr>
<td></td>
<td>Urea synthesis</td>
</tr>
<tr>
<td></td>
<td>Glutathione synthesis (via glutamate)</td>
</tr>
<tr>
<td>Kidney</td>
<td>Glucose synthesis (C skeleton)</td>
</tr>
<tr>
<td></td>
<td>Energy (C skeleton)</td>
</tr>
<tr>
<td></td>
<td>Acid–base balance</td>
</tr>
<tr>
<td>Small intestine</td>
<td>Energy (C skeleton)</td>
</tr>
<tr>
<td>Immune system</td>
<td>Energy (C skeleton)</td>
</tr>
<tr>
<td>All tissues</td>
<td>Protein synthesis</td>
</tr>
<tr>
<td></td>
<td>Purine synthesis (RNA, DNA)</td>
</tr>
<tr>
<td></td>
<td>Pyrimidine synthesis (RNA, DNA)</td>
</tr>
</tbody>
</table>

In the liver, the carbon skeleton of glutamine is an important precursor for glucose synthesis, while glutamine itself can be used for the synthesis of other amino acids and proteins, with excess nitrogen disposed of via ureagenesis. Glutamine can also be used as the precursor for the glutamate portion of glutathione, which is synthesized primarily in the liver. In the kidney, glutamine participates in acid–base balance, donating its amido and amino nitrogens to join with protons to form ammonium ions, which are excreted in the urine. The remaining carbon skeleton can be used to generate energy or as a precursor for glucose synthesis (gluconeogenesis). Glutamine is the major energy source in the small intestine and is an important energy source for immune cells. Glutamine is a nitrogen donor for the synthesis of purines and pyrimidines.
Since these are the building blocks of RNA and DNA, this role of glutamine is likely to be a particularly important one in cells that have high rates of division and/or of protein secretion. These include cells of the immune system and cells of the small intestine, such as enterocytes.

The importance of glutamine to cell survival and proliferation in vitro was first reported by Ehrensvand et al. (1949) but was more fully described by Eagle et al. (1956). Glutamine needed to be present at ten- to 100-fold in excess of any other amino acid in cell culture and could not be replaced by glutamate or glucose. This work led to the development of the first tissue-culture medium, which contained essential growth factors, glucose, 19 essential and non-essential amino acids at approximately physiological concentrations and a high concentration of glutamine (2 mM).

**Glutamine Metabolism by Cells of the Immune System**

The possible fates of glutamine carbon are shown in Fig. 6.4. One possible rate-determining step in the pathway of glutamine utilization is that catalysed by the enzyme phosphate-dependent glutaminase (hereafter referred to as glutaminase), which is found within mitochondria. The activity of glutaminase is high in all lymphoid organs examined, including lymph nodes, spleen, thymus, Peyer's patches and bone marrow (Ardawi and Newsholme, 1985), and in lymphocytes (Ardawi, 1988a; Keast and Newsholme, 1990), macrophages (Newsholme et al., 1986), and

---

**Fig. 6.4.** The pathway of glutamine utilization. Enzymes are indicated as: 1, glutaminase; 2, transaminase; 3, enzymes of part of the citric acid cycle; 4, malate dehydrogenase; 5, malic enzyme; 6, phosphoenolpyruvate carboxykinase; 7, pyruvate kinase; 8, lactate dehydrogenase. PEP phosphoenolpyruvate.
Glutamine and the Immune System

neutrophils (Curi et al., 1997). Glutaminase activity increases in the popliteal lymph node in response to an immunological challenge (Ardawi and Newsholme, 1982). Consistent with the high activity of glutaminase, glutamine is utilized at a high rate by cultured lymphocytes (Ardawi and Newsholme, 1983; Brand, 1985; Ardawi, 1988a; Brand et al., 1989; O'Rourke and Rider, 1989), macrophages (Newsholme et al., 1987; Newsholme and Newsholme, 1989; Spolarics et al., 1991) and neutrophils (Curi et al., 1997; see Table 6.2). Mitogenic stimulation of lymphocytes increases both glutaminase activity (Brand, 1985) and the rate of glutamine utilization (Ardawi and Newsholme, 1983; Brand, 1985; Ardawi, 1988a; Brand et al., 1989; O'Rourke and Rider, 1989). Glutamine utilization by macrophages was increased by bacillus Calmette–Guérin (BCG) activation in vivo or by bacterial lipopolysaccharide (LPS) stimulation in vitro (Murphy and Newsholme, 1998).

The major products of glutamine utilization by cultured lymphocytes and macrophages are glutamate, aspartate, lactate and ammonia, although alanine and pyruvate are also produced and some glutamine (approx. 25%) is completely oxidized (Ardawi and Newsholme, 1983; Brand, 1985; Newsholme et al., 1987; Ardawi, 1988a; Brand et al., 1989; Newsholme and Newsholme, 1989; O'Rourke and Rider, 1989). Macrophages are known to have a large oxidative capacity and their rate of O₂ consumption (515 nmol h⁻¹ mg⁻¹ protein) is similar to those of sheep heart (696 nmol h⁻¹ mg⁻¹ protein) and rat liver (520 nmol h⁻¹ mg⁻¹ protein) in vitro (Newsholme, 1987). Newsholme (1987) calculated ATP generation rates for isolated, incubated macrophages, taking into account oxygen utilized by the NADPH oxidase of these cells. The ATP generation rate in the presence of both glucose and glutamine was 930 nmol h⁻¹ mg⁻¹ protein, based on known pathways of metabolism. Glucose contributed 62% and glutamine 38% of the energy requirement of the cell. Since the ATP concentration of the macrophage is approximately 7 nmol mg⁻¹ protein (Newsholme et al., 1987), the total ATP concentration of the cell must have been turned over at least twice per minute. It has been calculated that glutamine can contribute up to 35% of the energy requirement of other immune cells in culture (Spolarics et al., 1991).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Addition</th>
<th>Rate of utilization (nmol h⁻¹ mg⁻¹ cell protein)</th>
<th>Rate of production (nmol h⁻¹ mg⁻¹ cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage</td>
<td>Glucose</td>
<td>355</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Glutamine</td>
<td>-</td>
<td>186</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>Glucose</td>
<td>42</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Glutamine</td>
<td>-</td>
<td>223</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>Glucose</td>
<td>460</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Glutamine</td>
<td>-</td>
<td>770</td>
</tr>
</tbody>
</table>
Glutamine and Immune-cell Functions

Introduction

The high rate of glutamine utilization by neutrophils, macrophages and lymphocytes and its increase when these cells are challenged suggests that provision of glutamine might be important to the function of these cells and so to the ability to mount an efficient immune response. Over 30 years ago, it was reported that the addition of asparaginase or glutaminase to cultures of lymphocytes prevented the cells from proliferating (Hirsch, 1970; Simberkoff and Thomas, 1970). Furthermore, asparaginase treatment of animals leads to immunosuppression (Brambilla et al., 1970; Chakrabaty and Friedman, 1970; Ashworth and MacLennan, 1974; Kafkewitz and Bendich, 1983). The immunosuppressive effect of asparaginase was shown to be due to its ability to hydrolyse glutamine and so decrease its availability to the immune system (Ashworth and MacLennan, 1974; Durden and Distasio, 1981; Kafkewitz and Bendich, 1983). These observations suggest that a supply of glutamine is required for the immune system to function optimally. A number of specific immunomodulatory actions of glutamine have now been reported.

Influence of glutamine on T lymphocyte proliferation in vitro

The proliferative response of rat (Ardawi and Newsholme, 1983; Szondy and Newsholme, 1989), mouse (Griffiths and Keast, 1990; Yaqoob and Calder, 1997) and human (Chuang et al., 1990; Parry-Billings et al., 1990a; Chang et al., 1999a, b) lymphocytes to T-cell mitogens is dependent upon the availability of glutamine: in the absence of glutamine, these cells do not proliferate, but, as the glutamine concentration in the culture medium increases, lymphocyte proliferation increases (Fig. 6.5). Lymphocyte proliferation increases greatly over the glutamine concentration range between 0.01 and 1 mM and appears to be maximal at normal physiological concentrations. Other amino acids, including glutamate, aspartate and arginine, cannot substitute for glutamine to support lymphocyte proliferation (Ardawi and Newsholme, 1983; Calder, 1995b). However, hydrolysable dipeptides that contain glutamine (e.g. alanyl-glutamine or glycyl-glutamine) can act as a replacement for glutamine to support in vitro T lymphocyte proliferation (Brand et al., 1989; Kweon et al., 1991; Kohler et al., 2000).

Influence of glutamine on B lymphocyte differentiation in vitro

The differentiation of B lymphocytes into antibody-synthesizing cells in vitro is glutamine-dependent and increases greatly over the physiological range of glutamine concentrations (Crawford and Cohen, 1985). This effect of glutamine cannot be mimicked by glutamate or asparagine.
Influence of glutamine on macrophage functions in vitro

In contrast to lymphocytes, which are rapidly dividing cells, macrophages are terminally differentiated cells that have lost their ability to divide. However, they remain very active cells, characterized by high rates of phagocytosis, protein secretion and membrane recycling. The level of cell surface expression of various molecules involved in phagocytosis and in antigen presentation (major histocompatibility complex (MHC) II) on human blood monocytes is influenced by the concentration of glutamine in which the cells are cultured (Spittler et al., 1995, 1997). This is associated with increased function (i.e. increased phagocytosis of immunoglobulin (Ig) G or complement opsonized particles and increased antigen presentation) with increasing glutamine availability (Spittler et al., 1995, 1997). Glutamine availability influenced the phagocytic uptake of unopsonized yeast-cell walls (Parry-Billings et al., 1990a) and of opsonized sheep red blood cells (Wallace and Keast, 1992) by incubated murine macrophages. The dipeptide alanyl-glutamine can replace glutamine to support in vitro phagocytosis by rat macrophages (Kweon et al., 1991).

Influence of glutamine on neutrophil functions in vitro

Addition of glutamine to cultures of blood neutrophils taken from patients with burns or post-surgery improved the defective anti-microbial activities (e.g. decreased reactive oxygen species production, phagocytosis and bactericidal activity) of those cells (Ogle et al., 1994; Furuwaka et al., 1997, 2000a, b). A study by Garcia et al. (1999) suggests a mechanism by which glutamine may
promote increased antimicrobial activity by neutrophils in stress states: 2 mM extracellular glutamine was able to attenuate the adrenaline-induced inhibition of superoxide production in these cells, suggesting that glutamine may protect cells from the suppressive effects of stress hormones.

**Influence of glutamine on cytokine production in vitro**

Increased availability of glutamine enhanced interleukin (IL)-2 production by concanavalin A (Con A)-stimulated rat (Calder and Newsholme, 1992), mouse (Yaqoob and Calder, 1997) and human (Rohde et al., 1996a; Yaqoob and Calder, 1998; Chang et al., 1999b) lymphocytes and also increased expression of the IL-2 receptor on stimulated rat lymphocytes (Yaqoob and Calder, 1997). The latter study also reported that the proportion of CD4+ lymphocytes increased with increasing concentration of glutamine in the culture medium (Yaqoob and Calder, 1997). Interferon (IFN)-γ production by human blood lymphocytes was enhanced with increasing availability of glutamine (Heberer et al., 1996; Rohde et al., 1996a; Yaqoob and Calder, 1998; Chang et al., 1999b), with maximum production occurring at a concentration below 0.5 mM.

Wallace and Keast (1992) demonstrated that murine macrophages stimulated with bacterial LPS secreted increasing amounts of IL-1 as the supply of glutamine increased, while more recently Murphy and Newsholme (1999) reported similar enhancement of tumour necrosis factor (TNF)-α release by rat macrophages with increasing glutamine availability. Glutamine addition to cultured rat macrophages stimulated with LPS increased IL-1β and IL-6 mRNA and secreted protein levels (Yassad et al., 2000). In contrast to the observations with rodent macrophages, production of TNF-α, IL-1β and IL-6 by human blood monocytes (Rohde et al., 1996a; Yaqoob and Calder, 1998) and lymphocytes (Heberer et al., 1996) appears to be little affected by glutamine availability, although one study suggests otherwise for IL-6 production (Peltonen et al., 1997). IL-8 production by LPS-stimulated human blood monocytes was markedly increased with increasing glutamine concentration (Murphy and Newsholme, 1999).

**Glutamine feeding studies in healthy animals**

A recent study compared the effects of feeding mice for 2 weeks on a diet that included 200 g casein kg⁻¹, providing 19.6 g glutamine kg⁻¹, or a glutamine-enriched diet, which provided 54.8 g glutamine kg⁻¹, partly at the expense of casein. Spleen lymphocytes from mice fed on the glutamine-enriched diet proliferated better in response to Con A than those from mice fed on the control diet (Kew et al., 1999). The glutamine-enriched diet also increased the proportion of CD4+ lymphocytes in the spleen and increased the proportion of stimulated lymphocytes bearing the IL-2 receptor. IL-2, but not IFN-γ, production was significantly greater for Con A-stimulated spleen lymphocytes from mice.
fed the glutamine-enriched diet (Kew et al., 1999), while the production of all three cytokines investigated (TNF-α, IL-1β and IL-6) was greater for LPS-stimulated macrophages from mice fed the glutamine-enriched diet (Wells et al., 1999). These observations suggest that increasing the amount of glutamine in the murine diet enhances the ability of both macrophages and T lymphocytes to respond to stimulation, at least in terms of cytokine production. Feeding rats a glutamine-free diet for 7 days resulted in decreased mucosal wet weight and a decreased number of intraepithelial lymphocytes (Horvath et al., 1996). This study suggests that glutamine is required for maintenance of the gut-associated immune system.

### Plasma and Muscle Glutamine Levels in Catabolic Stress

One of the early responses to stress that occurs in skeletal muscle is the increased rate of export of glutamine from the intracellular free amino acid pool. This lowers the intracellular glutamine concentration, leading to protein breakdown and *de novo* synthesis of glutamine from other amino acids. Glutamine synthetase in skeletal muscle is up-regulated by glucocorticoids (Max et al., 1988) and by TNF-α (Chakrabarti, 1998), and glucocorticoids increase glutamine efflux from skeletal muscle (Muhlbacher et al., 1984; Parry-Billings et al., 1990b). Thus, there appears to be an attempt in stress states to increase the supply of glutamine from muscle to the rest of the body. Nevertheless, glucocorticoid treatment decreases skeletal muscle and plasma glutamine concentrations (Muhlbacher et al., 1984; Parry-Billings et al., 1990b), suggesting that the demand for glutamine exceeds the supply.

Animal studies indicate that intramuscular and plasma glutamine concentrations are decreased in catabolic-stress situations, such as in sepsis and cancer cachexia and following burn injury and surgery (Table 6.3). In humans, plasma glutamine levels are lowered (by up to 50%) by sepsis, major injury and burns and following surgery (see Table 6.4). A recent study reported that low plasma glutamine concentration (< 0.42 mM) at admission to intensive care was associated with higher severity of illness and higher mortality (Oudemans-van Straaten et al., 2001). In humans, the skeletal-muscle glutamine concentration is lowered by more than 50% in catabolic stress (see Table 6.4). These observations indicate that a significant depletion of the skeletal-muscle glutamine pool is characteristic of catabolic stress. The lowered plasma glutamine concentrations that occur are most probably the result of demand for glutamine (by the liver, kidney, gut and immune system) exceeding the supply, and it is proposed that glutamine be considered a conditionally essential amino acid during catabolic stress (Lacey and Wilmore, 1990; Wilmore and Shabert, 1998). It has been suggested that the lowered plasma glutamine availability contributes, at least in part, to the immunosuppression that accompanies such situations (Newsholme and Calder, 1997). Because of the apparent immunostimulatory actions of glutamine described above, it seems sensible to provide glutamine for patients following surgery, radiation treatment or bone-marrow transplantation or suffering from injury, sepsis or burns.
There are also reports of decreased plasma glutamine concentrations after endurance exercise (Parry-Billings et al., 1992b; Rohde et al., 1996b; Castell et al., 1997) and athletic training (Keast et al., 1995; Hack et al., 1997) and in the overtrained athlete (Parry-Billings et al., 1992b).

Table 6.3. Effect of catabolic stress on plasma and muscle glutamine concentrations in animals. Values separated by → indicate the concentrations observed in control and stressed animals, respectively.

<table>
<thead>
<tr>
<th>Model</th>
<th>Plasma glutamine (mM)</th>
<th>Skeletal-muscle glutamine (mM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat injury</td>
<td>ND</td>
<td>9.9 → 5.9</td>
<td>Albina et al. (1987)</td>
</tr>
<tr>
<td>Rat sepsis</td>
<td>1.1 → 0.8</td>
<td>3.8 → 1.5</td>
<td>Parry-Billings et al. (1989)</td>
</tr>
<tr>
<td>Rat cancer cachexia</td>
<td>1.0 → 0.8</td>
<td>5.1 → 2.3</td>
<td>Parry-Billings et al. (1991)</td>
</tr>
<tr>
<td>Rat burn injury</td>
<td>0.7 → 0.5</td>
<td>4.1 → 2.7</td>
<td>Ardawi (1988b)</td>
</tr>
<tr>
<td>Dog burn injury</td>
<td>0.7 → 0.5</td>
<td>7.6 → 6.0</td>
<td>Stinnett et al. (1982)</td>
</tr>
<tr>
<td>Pig post-surgery</td>
<td>0.3 → 0.2</td>
<td>ND</td>
<td>Deutz et al. (1992)</td>
</tr>
</tbody>
</table>

Table 6.4. Effect of stress on plasma and muscle glutamine concentrations in humans. Values separated by → indicate the concentrations observed in healthy controls and in patients with the indicated catabolic stress, respectively.

<table>
<thead>
<tr>
<th>Catabolic stress</th>
<th>Plasma glutamine (mM)</th>
<th>Skeletal-muscle glutamine (mM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trauma/burns</td>
<td>0.60 → 0.70</td>
<td>20.0 → 10.0</td>
<td>Furst et al. (1979)</td>
</tr>
<tr>
<td>Injury</td>
<td>0.78 → 0.51</td>
<td>20.5 → 9.1</td>
<td>Askanazi et al. (1980)</td>
</tr>
<tr>
<td>Sepsis</td>
<td>0.53 → 0.37</td>
<td>19.3 → 6.7</td>
<td>Roth et al. (1982)</td>
</tr>
<tr>
<td>Sepsis</td>
<td>0.78 → 0.62</td>
<td>20.5 → 9.5</td>
<td>Askanazi et al. (1980)</td>
</tr>
<tr>
<td>Sepsis</td>
<td>0.38 → 0.30</td>
<td>22.0 → 4.0</td>
<td>Milewski et al. (1982)</td>
</tr>
<tr>
<td>Burns</td>
<td>0.62 → 0.30</td>
<td>ND</td>
<td>Parry-Billings et al. (1990a)</td>
</tr>
<tr>
<td>Burns</td>
<td>0.83 → 0.50</td>
<td>ND</td>
<td>Stinnett et al. (1982)</td>
</tr>
<tr>
<td>Surgery</td>
<td>0.65 → 0.48</td>
<td>ND</td>
<td>Parry-Billings et al. (1992a)</td>
</tr>
<tr>
<td>Surgery</td>
<td>0.46 → 0.36</td>
<td>ND</td>
<td>Lund et al. (1986)</td>
</tr>
<tr>
<td>Surgery</td>
<td>0.69 → 0.59</td>
<td>18.8 → 9.5</td>
<td>Askanazi et al. (1978)</td>
</tr>
<tr>
<td>Surgery</td>
<td>0.60 → 0.70</td>
<td>20.0 → 10.0</td>
<td>Askanazi et al. (1980)</td>
</tr>
<tr>
<td>Surgery</td>
<td>0.62 → 0.48</td>
<td>ND</td>
<td>Powell et al. (1994)</td>
</tr>
</tbody>
</table>

There are also reports of decreased plasma glutamine concentrations after endurance exercise (Parry-Billings et al., 1992b; Rohde et al., 1996b; Castell et al., 1997) and athletic training (Keast et al., 1995; Hack et al., 1997) and in the overtrained athlete (Parry-Billings et al., 1992b).

Effect of Exogenous Glutamine on Immune Function and Survival in Animal Models of Infection and Trauma

A number of animal studies have been performed to investigate the effect of glutamine on the ability to withstand challenges with various pathogens or tumour bearing. Glutamine-supplemented parenteral nutrition improved survival (75% vs. 45% in the control group receiving standard parenteral nutri-
tion) in rats following caecal ligation and puncture (Ardawi, 1991). Likewise, intravenous glutamine improved survival (92% vs. 55% in the control group) following an intraperitoneal injection of live *Escherichia coli* into rats (Inoue et al., 1993). Parenteral administration of alanyl-glutamine into rats improved survival (86% vs. 44% in the control group) in response to intraperitoneally infused *E. coli* (Naka et al., 1996). Suzuki et al. (1993) fed mice for 10 days on diets containing casein or casein supplemented with 20 g or 40 g glutamine kg⁻¹ and then inoculated them intravenously with live *Staphylococcus aureus*. Over the following 20 days, during which the mice were maintained on the same diets they had been fed prior to infection, 80% of the control animals died, while mortality was 60% in the 20 g glutamine kg⁻¹ group and 30% in the 40 g glutamine kg⁻¹ group. Another study showed that inclusion of glutamine in parenteral nutrition decreased mortality to intratracheally inoculated *Pseudomonas* (23% and 30% mortality at 24 and 48 h in the glutamine group vs. 55% and 75% mortality at 24 and 48 h in the control group) (DeWitt et al., 1999). In addition to enhanced survival, these studies showed that glutamine improved nitrogen balance diminished the sepsis-induced decrease in muscle glutamine concentration and decreased muscle protein breakdown (Ardawi, 1991), increased plasma glutamine concentration (Inoue et al., 1993), increased intestinal function and/or integrity (Inoue et al., 1993; Naka et al., 1996), and enhanced muscle protein synthesis (Ardawi, 1991; Naka et al., 1996). These studies did not measure indices of immune function. However, Yoo et al. (1997) reported that proliferation of blood lymphocytes from *E. coli*-infected piglets was significantly higher if the piglets consumed a diet containing 40 g glutamine kg⁻¹ compared with a diet that did not contain glutamine. Shewchuk et al. (1997) reported that Con A-stimulated proliferation of spleen lymphocytes taken from tumour-bearing rats fed a diet containing an increased amount of glutamine was greater than that of those taken from rats fed a standard casein-containing diet. Furthermore, infusion of alanyl-glutamine into tumour-bearing rats increased the *in vitro* phagocytic capacity of alveolar macrophages (Kweon et al., 1991), while infusion into septic rats increased *in vitro* proliferation of mitogen-stimulated blood lymphocytes (Yoshida et al., 1992). These studies indicate that provision of glutamine either parenterally or enterally increases the function of various immune cells and that this might account for the enhanced resistance to infection observed in other studies.

A series of studies has examined the influence of glutamine on the gut-associated and respiratory lymphoid systems in mice undergoing various challenges. Parenteral glutamine or alanyl-glutamine maintained the lymphocyte yield from Peyer’s patches and intestinal integrity in mice given an intranasal inoculation of influenza virus (Li et al., 1997, 1998). More recently, enteral glutamine was found to increase total cellularity of Peyer’s patches (and spleen) in LPS-treated mice (Manhart et al., 2000); this effect was mainly due to an increase in T-cell number. In another recent study, inclusion of glutamine in parenteral nutrition improved the concentration of secretory immunoglobulin A in the intestinal lumen and improved intestine IL-4 and IL-10 concentrations (Kudsk et al., 2000).
In an animal model of haemorrhagic shock, standard parenteral nutrition decreased the ex vivo release of TNF-α and IL-6 by LPS-stimulated gut mononuclear cells and spleen macrophages and was associated with injury to the gut mucosa and bacterial translocation into the mesenteric lymph nodes (Schroder et al., 1998). Inclusion of alanyl-glutamine and glycyl-glutamine in the parenteral regimen improved mucosal structure and prevented the fall in ex vivo IL-6, but not TNF-α, release (Schroder et al., 1998).

Provision of Glutamine in Catabolic-stress States in Humans

The provision of glutamine or glutamine ‘precursors’ (glutamine-containing dipeptides, N-acetylglutamine, 2-oxoglutarate, branched-chain amino acids), usually by the parenteral route, has been used in various catabolic situations in humans. In most cases, the intention was not to support the immune system but rather to maintain nitrogen balance, muscle mass and/or gut integrity (for a review, see Furst et al., 1997). Nevertheless, the maintenance of plasma glutamine concentrations in such a group of patients very much at risk of immunosuppression might have the added benefit of maintaining immune function.

The provision of glutamine intravenously for patients following bone-marrow transplantation resulted in a lower level of infection (12% of patients with clinical infections vs. 42% in the control group) and a shorter stay in hospital (29 ± 1 days vs. 36 ± 2 days) than for patients receiving glutamine-free parenteral nutrition (Ziegler et al., 1992). A later report by this group (Ziegler et al., 1998) showed that glutamine treatment resulted in greater numbers of total lymphocytes, T lymphocytes and CD4+ lymphocytes (but not B lymphocytes or natural killer cells) in the bloodstream after the patients were discharged. The authors suggested that glutamine specifically enhances T lymphocyte number and that this might be responsible for the diminished infection rate observed.

Very low-birth-weight babies who received a glutamine-enriched premature feeding formula (providing 0.3 g glutamine kg⁻¹ body weight day⁻¹) had a much lower rate of sepsis (11% vs. 31%) than babies who received a standard formula (Neu et al., 1997). In a study of patients in intensive care, glutamine provision decreased mortality compared with standard parenteral nutrition (43% vs. 67%) and changed the pattern of mortality (Griffiths et al., 1997). Neither of these studies reported immunological outcomes of the treatments. However, another study of patients in intensive care reported that enteral glutamine increased the blood CD4:CD8 ratio (Jensen et al., 1996). In a more recent study, in which patients received enteral glutamine vs. standard enteral feed from within 48 h of the trauma, there was a significant reduction in the 15-day incidence of pneumonia (17% vs. 45% in the control group), bacteraemia (7% vs. 42%) and severe sepsis (4% vs. 26%) in the glutamine group, although this was not associated with reduced mortality (Houdijk et al., 1998). Parenteral administration of glutamine into patients postcolorectal surgery increased mitogen-stimulated proliferation of blood lymphocytes (O’Riordain et al., 1994), suggesting that glutamine does improve T lymphocyte function in patients at risk of sepsis; glutamine did not affect ex vivo TNF or IL-6 production. In
another study, post-operative patients who received alanyl-glutamine parenterally had increased blood lymphocyte numbers, increased ex vivo production of cysteinyl leucotrienes by blood neutrophils and a shorter stay in hospital (Morlion et al., 1998). Most recently, infusion of a parenteral mixture containing glycyl-glutamine for 48 h after major abdominal surgery resulted in better maintenance of the human leucocyte antigen (HLA)-DR expression on circulating monocytes than in control patients who received a standard parenteral mixture (Spittler et al., 2001). There was no effect of the glutamine dipeptide on production of TNF-α or IL-6 by LPS-stimulated whole blood (Spittler et al., 2001). Patients with oesophageal cancer being treated with radiochemotherapy had higher blood lymphocyte counts and better lymphocyte proliferative responses if they consumed glutamine (30 g day$^{-1}$) for 28 days (Yoshida et al., 1998). These studies indicate that glutamine is able to maintain lymphocyte numbers and (some) immune-cell responses in patients normally at risk of immunosuppression and infection.

In addition to a direct immunological effect, glutamine, even provided parenterally, improves the gut barrier function in patients at risk of infection (van der Hulst et al., 1993). This would have the benefit of decreasing the translocation of bacteria from the gut and so eliminating a key source of infection. Animal studies indicate that providing glutamine does decrease bacterial translocation (Burke et al., 1989).

**Role of Glutamine in the Pathogenesis of Type 1 Diabetes**

Since glutamine appears to act to promote lymphocyte activity, it has been proposed that increased availability of glutamine could play a role in the pathogenesis of some autoimmune conditions, such as type 1 diabetes (Wu et al., 1991). Indeed, the administration of the anti-glutamine-utilization drug acivicin delayed or stopped the progression of the disease in diabetes-prone rats (Misra et al., 1996). Addition of the glutaminase inhibitor 6-diazo-5-oxo-norleucine to macrophages before exposure to rat pancreatic β cells in vitro virtually abolished the lytic capacity of the macrophage towards the target β cells (Murphy and Newsholme, 1999). The glutamine concentration in the plasma of moderately ketoacidotic diabetics at diagnosis is significantly elevated compared with that of age- and sex-matched normal control individuals (P. Newsholme, unpublished observations), adding further weight to the argument that this amino acid is important to the pathogenic process.

**Mechanism of Glutamine Action**

There has been much speculation about the mechanism by which glutamine acts to preserve, or even improve, immune function. Similar metabolic characteristics apply to various cells of the immune system, despite the fact that their cell biology is different. Hence any hypothesis must explain high rates of glutamine utilization in cells with widely different cell-biological characteristics. As
indicated earlier, glutamine makes a significant contribution to energy generation in cells of the immune system. However, oxidation of glutamine is only partial and immune cells can, and do, generate energy from other substrates (see Calder, 1995a). These observations suggest that the importance of glutamine to immune function is not simply through its action as an energy-yielding substrate. Another suggestion is that glutamine metabolism can generate intermediates for the synthesis of purines and pyrimidines and so provides the building blocks for mRNA and DNA. However, the rate of synthesis of nucleotides in lymphocytes is reported to be much less than the rate of glutamine utilization (Szondy and Newsholme, 1989). On the basis of ‘metabolic control logic’, it was suggested that the importance of a high rate of glutamine utilization in immune cells relates to maintenance of a high flux through the pathway of glutaminolysis (i.e. the pathway of partial glutamine oxidation (Fig. 6.4)), which would allow high sensitivity to regulatory molecules controlling biosynthetic pathways (Newsholme et al., 1989). This hypothesis has proved difficult to test. While the capacity for rapid cell division is retained by isolated lymphocytes, this does not apply to isolated neutrophils or macrophages, which are terminally differentiated cells with little capacity for cell division. However, neutrophils and macrophages have a large phagocytic capacity (requiring a high rate of lipid turnover and synthesis) and a high secretory activity. The mechanism by which glutamine can act to allow high rates of secretory-product formation and release and sustain cell proliferation must account for the diverse nature of these secretory products and the requirements for cell division and should include at least one common metabolic product.

NADPH is required by the enzymes responsible for the formation of the reactive species nitric oxide and superoxide, inducible nitric oxide synthase (iNOS) and NADPH oxidase, respectively. NADPH is also required for the formation of reduced glutathione (see below) and for de novo synthesis of DNA, RNA and fatty acids. Glutamine, via metabolism involving NADP+-dependent malate dehydrogenase (malic enzyme (enzyme 5 in Fig. 6.4)), can generate considerable NADPH for cell requirements. The NADP+-dependent malate dehydrogenase step will result in the formation of pyruvate, which can either be converted to lactate (ending the pathway of glutaminolysis) or be converted to acetyl-coenzyme A (CoA) and on to CO₂. Thus, depending upon the energy demands placed on the cell, glutamine may be partially oxidized in the pathway of glutaminolysis or may be fully oxidized, but the outcome of metabolism in either case is NADPH production. Glucose may also, via metabolism through the pentose-phosphate pathway, generate NADPH. However, during periods of active pinocytosis and phagocytosis, glucose carbon may be diverted towards lipid synthesis and therefore the pentose-phosphate pathway may be compromised (Newsholme et al., 1996). Additionally, glutamine carbon may be used for new amino acid synthesis in periods of active synthesis and secretion. It is possible that NADPH is the ‘common factor’ that links the diverse effects that glutamine has in cells of the immune system (Newsholme, 2001). Evidence in support of this hypothesis is provided by the enhancing effect of glutamine on superoxide generation in neutrophils and monocytes (Garcia et al., 1999; Saito et al., 1999; Furukawa et al., 2000a, b) and recent in vitro data that cell prolif-
Superoxide generation in cells requires the electron-donating ability of NADPH if generated via the enzyme NADPH oxidase, which directly reduces molecular oxygen. The latter enzyme is quantitatively the most significant source of superoxide in immune cells.

It is also possible that the importance of glutamine relates to its many roles as a biosynthetic precursor. Of particular importance may be its role as the precursor of glutamate for the synthesis of glutathione. Glutathione is a tripeptide antioxidant composed of glutamate, cysteine and glycine (see also Grimble, Chapter 7, this volume). Glutathione concentrations in the liver, lung, small intestine and immune cells fall in response to infection, inflammatory stimuli and trauma. The fall in hepatic glutathione concentration and in the export of glutathione from the liver can be prevented by provision of oral glutamine for rats (Hong et al., 1992; Welbourne et al., 1993). Glutamine-enriched parenteral nutrition elevated plasma glutathione concentration in rats (Denno et al., 1996; Cao et al., 1998) and promoted the release of glutathione from the rat gut into the bloodstream (Cao et al., 1998).

Culture of human lymphocytes in the presence of glutathione enhances cytotoxic T-cell activity (Droge et al., 1994) and depletion of intracellular glutathione diminishes lymphocyte proliferation (Chang et al., 1999a) and the generation of cytotoxic T lymphocytes (Droge et al., 1994). Depletion of glutathione through an exercise regimen decreased the number of CD4+ cells by 30% in a subset of individuals (Kinscherf et al., 1994). Treatment with N-acetylcysteine (400 mg day⁻¹ for 4 weeks) prevented the exercise-induced fall in intracellular glutathione concentrations and increased the number of CD4+ cells by 25%. Glutathione depletion is associated with diminished IFN-γ, but not IL-2 or IL-4, production by antigen-stimulated murine lymph-node cells (Peterson et al., 1998); this effect was mediated by antigen-presenting cells and the authors suggest that glutathione acts via inducing IL-12 production by these cells to alter the T-helper (Th)1/Th2 balance in favour of a Th1 response. Thus, glutathione appears to promote a range of cell-mediated immune responses. Although glutamine is able to preserve glutathione concentrations in the liver, gut, kidney and bloodstream (see above and also Welbourne and Dass, 1982; Harward et al., 1994), it is not clear whether it also preserves glutathione concentrations within immune cells. However, it was recently reported that incubation of human blood mononuclear cells with increasing concentrations of glutamine resulted in higher intracellular glutathione concentrations in both CD4+ and CD8+ cells (Chang et al., 1999a). Thus, one means by which glutamine might exert its immunological effects is through maintenance of glutathione status. However, this hypothesis requires further investigation.

**Conclusion**

Glutamine depletion in vivo results in immunosuppression, and catabolic-stress situations in humans are associated with lowered plasma (and muscle) glutamine levels. Glutamine is used at a high rate by cells of the immune system and
there is much evidence that key functions of these cells, tested in vitro, are dependent upon the provision of glutamine. Evidence is now emerging that glutamine supplied orally or intravenously improves immune function in vivo and in cells cultured ex vivo, while additionally protecting against infectious challenges. Thus, administration of glutamine or its precursors should prove beneficial as a therapy for individuals whose immune system is compromised by catabolic stress. Nevertheless, more information is required about the mechanism by which glutamine provides beneficial effects for cells of the immune system in vivo and in vitro, whether this mechanism is altered in disease states and the importance of the route of glutamine administration.

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Sulphur Amino Acids, Glutathione and Immune Function

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The Biochemistry of Sulphur Amino Acids

Sulphur amino acid metabolism

The sulphur amino acids are methionine and cysteine. Their metabolism is interlinked. As a result of this metabolism, the sulphur moiety is incorporated into a number of end-products, three of which, glutathione, taurine and proteins, have important roles in immune function. Methionine is a nutritionally essential amino acid, due to the inability of mammals to synthesize its carbon skeleton. Cysteine is considered to be semi-essential, in that it is synthesized from methionine provided that the dietary supply of the latter is sufficient. The methyl group of methionine can be removed from and reattached to the carbon skeleton of the amino acid by a cyclical process referred to as the transmethylation pathway (Fig. 7.1). The formation of homocysteine, part way along the transmethylation pathway, is an important branch point in the metabolism of methionine. Homocysteine can be remethylated to form methionine or can be metabolized by the transulphuration pathway to form cysteine (Fig. 7.1). Both the remethylation of homocysteine and the formation of cysteine utilize serine. This latter amino acid forms the carbon skeleton of cysteine and acts as a methyl-group donor to tetrahydrofolic acid, once the methyl version of the latter compound has donated its methyl group to homocysteine during the formation of methionine.

Methionine is intimately involved in the synthesis of the polyamines spermine and spermidine, in which the carbon chain of methionine is donated to a third polyamine, putrescine, which is derived from ornithine (Fig. 7.2). The polyamines are present in high concentrations in rapidly dividing cells, such as those of an activated immune system. Their role is poorly defined but appears to be important. Polyamines have been likened to ‘molecular grease’, in that they are permissive metabolites, ensuring the fidelity of DNA transcription and RNA translation (Grimble and Grimble, 1998). In in vitro studies, cells depleted...
of polyamines exhibit increased error rates in both processes. The first enzyme
in the step from ornithine to putrescine is highly induced in rapidly dividing cells.

Methionine also acts as a methyl donor in the synthesis of creatine (Fig.
7.3), which is essential for muscle energy generation through its phosphoryla-
tion to creatine phosphate. Creatine phosphate can transfer its phosphate to
ADP to restore cellular ATP supplies during periods of high metabolic activity.

In addition to incorporation into proteins, cysteine can be incorporated
into the key antioxidant glutathione (GSH), or converted to taurine and inor-
ganic sulphate. The possession of an SH group by cysteine and GSH allows
the formation of an S–S bridge between two molecules of cysteine or of GSH
to form cystine and oxidized glutathione (GSSG), respectively. Taurine has
many roles, including formation of the bile salt taurocholic acid, and is a puta-

Fig. 7.1. Outline of sulphur amino acid metabolism. Enzymes: 1, methionine adenosyl
transferase; 2, methyl transferase; 3, adenosyl homocysteinase; 4, betaine methyltransferase;
5, S-methyltetrahydrofolate methyl transferase; 6, cystathionine β-synthase; 7, cystathionine
γ-lyase; 8, L-cysteiny-l-tRNA synthetase; 9, γ-glutamyl cysteine synthase; 10, cysteine
dioxygenase. S-AH, S-adenosyl homocysteine; S-AM, S-adenosyl methionine; THFA,
tetrahydrofolic acid.

Fig. 7.2. Polyamine biosynthesis.
tive antioxidant and cell membrane stabilizer. Taurine is the predominant nitrogenous compound in immune cells.

The synthesis of glutathione from its three constituent amino acids is mainly limited to the liver. Two consecutive steps are required to synthesize glutathione, each step consuming one ATP molecule (Fig. 7.4). The rate-limiting enzyme in the pathway is \( \gamma \)-glutamyl cysteine synthetase (step 1 of Fig. 7.4). Under normal physiological conditions, there is feedback on the activity of this enzyme by GSH. Thus, conversion of cysteine to GSH is strongly influenced by the rate of utilization/transport of GSH within and between the cells of the body. In other words, synthesis is a 'demand-led' process, provided that cysteine is available.

Glutathione is transferred to the blood and transported around the body in both plasma and cells mainly in its reduced form (GSH).

Thus, apart from protein synthesis, sulphur amino acids are involved as direct and indirect participants in pathways involved in cell replication and stabilization, antioxidant defence, assimilation of lipids and energy metabolism. As the immune response involves major changes in cell replication, oxidant stress and lipid and energy metabolism, it is not surprising that the availability of sulphur amino acids has a major impact on immune function.

![Fig. 7.3. Creatine biosynthesis.](image)

![Fig. 7.4. Formation of glutathione and its role in the \( \gamma \)-glutamyl cycle. Enzymes: 1, \( \gamma \)-glutamyl cysteine synthetase; 2, glutathione synthase.](image)
Control of sulphur amino acid metabolism

The $K_m$ values for the homocysteine transferase enzymes (steps 4 and 5 of Fig. 7.1) (which lead to the recycling of methionine) are two orders of magnitude lower than those for cystathionine synthase (step 6 of Fig. 7.1) and cystathionine $\gamma$-lyase (step 7 of Fig. 7.1) (which process methionine towards catabolism via the transsulphuration pathway). Thus, at low intracellular concentrations of methionine, remethylation will be favoured over transsulphuration and methionine will be conserved. When these two pathways were examined in vivo in rats fed diets containing 3, 15 and 30 g L-methionine kg$^{-1}$ of diet, the percentage of methionine metabolized by the two competing pathways changed (Finkelstein and Martin, 1984, 1986). With increasing dietary methionine intake, substrate flux through the transmethylating pathway fell and flux through the transsulphuration pathway increased.

Examination of the $K_m$ values for rate-limiting enzymes processing the major cysteine metabolites provides a further insight into how sulphur amino acid metabolism is influenced by alteration in the supply of cysteine. The $K_m$ for L-cysteinyl-tRNA synthetase (step 8 of Fig. 7.1) (essential for incorporation of cysteine into protein) is less than one-tenth of that for $\gamma$-glutamyl cysteine synthetase (step 9 of Fig. 7.1) (the rate-limiting enzyme for GSH synthesis) or cysteine dioxygenase (step 10 of Fig. 7.1) (forming cysteine sulphinate, the precursor for sulphate and taurine). Thus, under conditions of low cysteine availability, protein synthesis will be maintained and synthesis of sulphate, taurine and GSH curtailed.

From the kinetics of the key enzymes in sulphur amino acid metabolism reported above, it can be seen that, when the diet is low in sulphur amino acids, cellular methionine is highly conserved. Flux down the transsulphuration pathway, which ultimately leads to methionine catabolism, increases only as dietary methionine intake increases. It can also be seen that, at low flux rates of substrate down the transsulphuration pathway, conversion of cysteine into its main metabolites will be affected, so that protein synthesis will be relatively maintained while sulphate and GSH synthesis rates will fall. Synthesis of GSH and sulphate will increase in concert as increasing levels of substrate flow through the pathway. In a study in rats, seven molecules of cysteine were incorporated into GSH for every ten incorporated into protein in liver at adequate sulphur amino acid intake (Grimble and Grimble, 1998). At inadequate sulphur amino acid intake, the ratio fell to $< 3:10$. This response to a low intake of sulphur amino acids will not necessarily be advantageous since GSH is an important component of antioxidant defence. Thus, at low sulphur amino acid intakes, antioxidant defences will become weakened. The immune response makes large demands on these defences and sulphur amino acid metabolism in particular.

Sulphur Amino Acid and Glutathione Metabolism Following Infection and Injury

The immune system has a great capacity for immobilizing invading microbes, creating a hostile environment for them and bringing about their destruction (Fig. 7.5). The immune system can also become activated, in a similar way to
the response to microbial invasion, by a wide range of stimuli and conditions; these include burns, penetrating and blunt injury, the presence of tumour cells, environmental pollutants, radiation, exposure to allergens and the presence of chronic inflammatory diseases. The strength of the response to this disparate range of stimuli will vary, but it will contain many of the hallmarks of the response to invading pathogens. The immune response has a high metabolic cost, and inappropriate prolongation of the response will exert a deleterious effect upon the nutritional status of the host.

The pro-inflammatory cytokines interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF)-α have widespread metabolic effects upon the body and stimulate the process of inflammation. Many of the signs and symptoms experienced after infection and injury, such as fever, loss of appetite, weight loss, negative nitrogen, sulphur and mineral balance and lethargy are caused directly or indirectly by pro-inflammatory cytokines (Fig. 7.5). The indirect effects of cytokines are mediated by actions upon the adrenal glands and endocrine pancreas, resulting in increased secretion of the catabolic hormones adrenaline, noradrenaline, glucocorticoids and glucagon. Insulin insensitivity occurs, in addition to this ‘catabolic state’. The biochemistry of an infected individual is thus fundamentally changed in a way that will ensure that the immune system receives nutrients from within the body. Muscle protein is catabolized to provide amino acids for synthesizing new cells, GSH and proteins for the immune response.
Furthermore, amino acids are converted to glucose (a preferred fuel, together with glutamine, for the immune system). An increase in urinary nitrogen and sulphur excretion occurs as a result of this catabolic process. The extent of this process is highlighted by the significant increase in urinary nitrogen excretion, from 9 g day\(^{-1}\) in mild infection to 20–30 g day\(^{-1}\) following major burn or severe traumatic injury (Wilmore, 1983). The loss of nitrogen from the body of an adult during a bacterial infection may be equivalent to 60 g of tissue protein and, in a period of persistent malarial infection, equivalent to over 500 g of protein. However, during the response to infection and injury, the urinary excretion of sulphur increases to a lesser extent than that of nitrogen (Cuthbertson, 1931), suggesting that sulphur amino acids are preferentially retained and so 'spared' from catabolism. Infection with human immunodeficiency virus (HIV) has been shown to cause substantial excretion of sulphate in the urine during the asymptomatic phase of the disease (Breitkreutz et al., 2000). The losses reported were equivalent to 10 g of cysteine day\(^{-1}\), in contrast to losses of approximately 3 g day\(^{-1}\) for healthy individuals on a ‘Westernized diet’. As cysteine is the precursor for both sulphate and GSH this finding may be linked with the decline in tissue glutathione pools that has been observed in HIV infection (De Rosa et al., 2000). Clearly, such a depletion of antioxidant defences will not be sustainable over a long period.

Large decreases in plasma glycine, serine and taurine concentrations occur following infection and injury. These changes may be due to enhanced utilization of a closely related group of amino acids, namely, glycine, serine, methionine and cysteine. Many substances produced in enhanced amounts in response to pro-inflammatory cytokines are particularly rich in these amino acids. These substances include GSH, which comprises glycine, glutamic acid and cysteine, metallothionein (the major zinc-transport protein), which contains glycine, serine, cysteine and methionine to a composite percentage of 56%, and a range of acute-phase proteins, which contain up to 25% of these amino acids in their structure. If an increased demand for sulphur and related amino acids is created by the inflammatory response, then provision of additional supplies of these amino acids may assist the response.

Many of the components of antioxidant defence interact to maintain antioxidant status (see also Hughes, Chapter 9, Prasad, Chapter 10, and McKenzie et al., Chapter 12, this volume). Glutathione and the enzymes that maintain it in its reduced form are central to effective antioxidant status. For example, when oxidants interact with cell membranes, the oxidized form of vitamin E that results is restored to its reduced form by ascorbic acid. The dehydroascorbic acid formed in this process is reconverted to ascorbic acid by interaction with the reduced form of glutathione. Subsequently, oxidized glutathione formed in the reaction is reconverted to the reduced form of glutathione by glutathione reductase (Fig. 7.6). Vitamins E and C and glutathione are thus intimately linked in antioxidant defence. The interdependence of the various nutritional components of antioxidant defence is illustrated in a study in which healthy subjects were given 500 mg ascorbic acid day\(^{-1}\) for 6 weeks (Johnston et al., 1993). A 47% increase in the glutathione content of red blood cells occurred. Vitamin B\(_6\) and riboflavin, which have no antioxidant properties
per se, also contribute to antioxidant defences indirectly. Vitamin B₆ is the cofactor in the metabolic pathway for the biosynthesis of cysteine (Fig. 7.1). Cellular cysteine concentration is rate limiting for glutathione synthesis. Riboflavin is a cofactor for glutathione reductase, which maintains the major part of cellular glutathione in the reduced form (Fig. 7.6).

**Antioxidant Defences Following Infection and Injury**

Although pro-inflammatory cytokines are essential for the normal operation of the immune system, they play a major damaging role in many inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, asthma, psoriasis and multiple sclerosis, and in cancer (Tracey and Cerami, 1993; Grimble, 1996). They are also thought to be important in the development of atheromatous plaques in cardiovascular disease (Ross, 1993). In conditions such as cerebral malaria, meningitis and sepsis, they are produced in excessive amounts and are an important factor in increased mortality (Tracey and Cerami, 1993). Clearly, in these diseases, the cytokines are being produced in the wrong biological context. In malaria, tuberculosis, sepsis, cancer, HIV infection and rheumatoid arthritis, inflammatory cytokines bring about a loss of lean tissue, which is associated with depleted tissue GSH content and an increased output of nitrogenous and sulphur-containing excretion products in the urine (see above).

Although the body strives to maintain them, observations in experimental animals and patients indicate that antioxidant defences become depleted during infection and after injury. For example, in mice infected with influenza virus, there were 27%, 42% and 45% decreases in the vitamin C, vitamin E and glutathione contents of blood, respectively (Hennett et al., 1992). In asymptomatic HIV infection, substantial decreases in glutathione concentrations in blood and lung epithelial-lining fluid have been noted (Staal et al., 1992). In patients undergoing elective abdominal operations, the glutathione content of blood and skeletal muscle fell by over 10% and 42%, respectively, within 24 h of the operation (Luo et al.,...
While values in blood slowly returned to pre-operative values, concentrations in muscle were still depressed 48 h post-operatively. Furthermore, reduced tissue glutathione concentration has been noted in hepatitis C, ulcerative colitis and cirrhosis. In patients with malignant melanoma, metastatic hypernephroma and metastatic colon cancer, plasma ascorbic acid concentrations fell from normal to almost undetectable levels within 5 days of commencement of treatment with IL-2 (Grimble, 1999). In patients with inflammatory bowel disease, substantial reductions in ascorbic acid concentrations occurred in inflamed gut mucosa (Buffinton and Doe, 1995). As a general consequence of the weakening of antioxidant defences during disease that is attested to by these observations, oxidative damage is apparent in a wide range of clinical conditions in which cytokines are produced. Lipid peroxides and increased thiobarbituric acid reactive substances are present in the blood of patients with septic shock, asymptomatic HIV infection, chronic hepatitis C, breast cancer, cystic fibrosis, diabetes mellitus and alcoholic liver disease. Peroxides also increase following cancer chemotherapy, open heart surgery, bone marrow transplantation and haemodialysis. When glutathione status was reduced in rats by injection of diethyl maleate, which binds irreversibly to GSH, rendering it inactive, a sublethal dose of TNF became lethal (Zimmerman et al., 1989), thus illustrating the importance of GSH in protection from the adverse effects of pro-inflammatory cytokines. The onset of sepsis in patients leads to a transient decrease in the total antioxidant capacity of blood plasma (a functional measure of the total antioxidant content) (Cowley et al., 1996). The capacity returns to normal values over the following 5 days. However, this was not the case for patients who subsequently died, in whom values remained well below the normal range.

As well as increasing the risk of direct oxidant damage, a reduction in the strength of antioxidant defences also indirectly increases the risk of damage to the host via transcription-factor activation, leading to up-regulation of pro-inflammatory cytokine production (see below).

Glutathione and the Immune System

Direct effects of glutathione

One of the first indications that glutathione influences aspects of immune function that are related to T lymphocytes came from a study in which the GSH content of lymphocytes was measured in a group of healthy volunteers (Kinscherf et al., 1994). The numbers of helper (CD4+) and cytotoxic (CD8+) T-cells increased in parallel with intracellular glutathione concentrations up to 30 nmol mg\(^{-1}\) protein. However, the relationship between cellular glutathione concentrations and cell numbers was complex, with numbers of both subsets declining at intracellular glutathione concentrations between 30 and 50 nmol mg\(^{-1}\) protein. The study also revealed that cell numbers were responsive to long-term changes in GSH content. When the subjects engaged in a programme of intensive physical exercise daily for 4 weeks, a fall in glutathione concentrations occurred. Individuals with glutathione concentrations in the optimal range before exercise who experi-
enced a fall in concentration after exercise showed a 30% fall in CD4+ T-cell numbers. The decline in T-cell number was prevented by administration of N-acetyl-cysteine (NAC) (this is metabolized to cysteine (see later)). This study suggests that immune cell function may be sensitive to a range of intracellular sulphhydril compounds, including glutathione and cysteine. In HIV+ individuals and patients with acquired immune deficiency syndrome (AIDS), a reduction in cellular and plasma glutathione has been noted (Staal et al., 1992). It is unclear at present whether the depletion in lymphocyte population that occurs in these subjects is related to this phenomenon. However, in a large, randomized, double-blind, placebo-controlled trial, administration of 600 mg day\(^{-1}\) of NAC for 7 months resulted in both anti-inflammatory and immunoenhancing effects (Breitkreutz et al., 2000). A decrease in plasma IL-6 concentration occurred, together with an increase in lymphocyte count and in the stimulation index of T lymphocytes in response to tetanus toxin. The precise mechanism underlying the complex effects of changes in cellular glutathione content are not clear, and whether they are related to GSH function as an antioxidant or to some other property is not apparent. However, a recent study suggests that glutathione promotes IL-12 production by antigen-presenting cells, so driving T-helper (Th) cells along the Th1 pathway of differentiation (Peterson et al., 1998).

**Effects of other nutrients that might have an impact on glutathione status**

**Vitamin B\textsubscript{6}**

Vitamin B\textsubscript{6} although having no antioxidant properties, plays an important part in antioxidant defences, because of its action in the metabolic pathway for the formation of cysteine, which, as indicated earlier, is the rate-limiting precursor in glutathione synthesis. Vitamin B\textsubscript{6} status has widespread effects upon immune function (Rall and Meydani, 1993). Vitamin B\textsubscript{6} deficiency causes thymic atrophy and lymphocyte depletion in lymph nodes and spleen. Antigen processing is unaffected. However, the ability to make antibodies to sheep red blood cells is depressed. In human studies, the ability to make antibodies to tetanus and typhoid antigens is not seriously affected. Various aspects of cell-mediated immunity are also influenced by vitamin B\textsubscript{6} deficiency. Skin grafts in rats and mice survive longer during deficiency, and guinea pigs exhibit decreased delayed-hypersensitivity reactions to bacillus Calmette–Guérin (BCG) administration. Deficiency of vitamin B\textsubscript{6} is rare in humans but can be precipitated with the anti-tuberculosis (anti-TB) drug isoniazid. However, experimental deficiency in elderly subjects has been shown to reduce total blood lymphocyte numbers and decrease the proliferative response of lymphocytes to mitogens (Meydani et al., 1991). Likewise, IL-2 production is reduced by deficiency of the vitamin. Restoration of vitamin B\textsubscript{6} intake to normal by dietary supplements restores immune function. However, intakes that are higher than current recommended values are required to normalize all immune functions, suggesting that this vitamin can only restore immune function in this way. It is unclear, at present, whether a similar situation occurs in younger subjects.
One mechanism for the effect of vitamin $B_6$ on immune function may be due to the importance of the vitamin in cysteine synthesis, as outlined earlier. Deficiency of the vitamin may limit the availability of cysteine for glutathione synthesis. In rats, vitamin $B_6$ deficiency resulted in decreases of 12 and 21% in glutathione concentrations in plasma and spleen, respectively (Takeuchi et al., 1991). In healthy young women, large doses of vitamin $B_6$ (27 mg day$^{-1}$ for 2 weeks) resulted in a 50% increase in plasma cysteine content (Kang-Yoon and Kirksey, 1992), presumably by increased flux through the transulphuration pathway. As cysteine is a rate-limiting substrate for glutathione synthesis, these findings may have implications for the response to pathogens, because of the importance of glutathione in lymphocyte proliferation and antioxidant defence. However, while vitamin $B_6$ has cellular effects on the immune system, evidence is lacking of any effect upon the inflammatory response.

**Ascorbic acid**

High concentrations of vitamin C are found in phagocytic cells. While the role of vitamin C as a key component of antioxidant defence is well established (Fig. 7.6), most studies have shown only minor effects upon a range of immune functions (see Hughes, Chapter 9, this volume), except in cases where the vitamin may be acting by interacting with GSH metabolism. Unlike deficiencies in vitamins $B_6$, and E and riboflavin, deficiency of vitamin C does not cause atrophy of lymphoid tissue. In a study of ultramarathon runners, dietary supplementation with 600 mg day$^{-1}$ of ascorbic acid reduced the incidence of upper respiratory-tract infections after a race by 50% (Peters et al., 1993). It is interesting to note that strenuous exercise has been shown to deplete tissue glutathione content. The interrelationship between glutathione and ascorbic acid may therefore play a role in the effect of exercise on immune function.

When immunological parameters and antioxidant status were measured in adult males fed 250 mg day$^{-1}$ of vitamin C for 4 days, followed by 5 mg day$^{-1}$ for 32 days, plasma ascorbic acid and glutathione decreased and impairment of antioxidant status became evident from a doubling in semen 8-hydroxydeoxyguanosine concentration (a measure of oxidative damage to nucleic acids) during the second dietary period (Jacob et al., 1991). A fall in vitamin content in peripheral-blood mononuclear cells was noted and the delayed-type hypersensitivity reaction to seven recall antigens was significantly reduced in intensity.

**Mechanism of the Effect of Oxidants and Antioxidants on Inflammation and Immune Function**

There is a growing body of evidence that antioxidants suppress inflammatory components of the response to infection and trauma and enhance components related to cell-mediated immunity (see Hughes, Chapter 9, Prasad, Chapter 10, and McKenzie et al., Chapter 12, this volume). The reverse situation applies when antioxidant defences become depleted.
The oxidant molecules produced by the immune system to kill invading organisms may activate at least two important families of proteins that are sensitive to changes in cellular redox state. The families are nuclear transcription factor kappa B (NFκB) and activator protein 1 (AP1). These transcription factors act as ‘control switches’ for biological processes, not all of which are of advantage to the individual. NFκB is present in the cytosol in an inactive form, by virtue of being bound to IκB. Phosphorylation and dissociation of IκB renders the remaining NFκB dimer active. Activation of NFκB can be brought about by a wide range of stimuli, including pro-inflammatory cytokines, hydrogen peroxide, mitogens, bacteria and viruses and their related products, and UV and ionizing radiations. The dissociated IκB is degraded and the active NFκB is translocated to the nucleus, where it binds to response elements in the promoter regions of genes. A similar translocation of AP1, a transcription factor composed of the proto-oncogenes c-fos and c-jun, from cytosol to nucleus also occurs in the presence of oxidant stress. Binding of the transcription factors is implicated in the activation of a wide range of genes associated with inflammation and the immune response, including those encoding cytokines, cytokine receptors, cell-adhesion molecules, acute-phase proteins and growth factors (Schreck et al., 1991).

Unfortunately, NFκB also activates transcription of the genes of some viruses, such as HIV. This sequence of events in the case of HIV accounts for the ability of minor infections to speed the progression of individuals who are infected with HIV towards AIDS, since, if antioxidant defences are poor, each encounter with general infections results in cytokine and oxidant production, NFκB activation and an increase in viral replication. It is thus unfortunate that reduced cellular concentrations of GSH are a common feature of asymptomatic HIV infection (Staal et al., 1992).

Oxidant damage to cells will indirectly create a pro-inflammatory effect by the production of lipid peroxides. This situation may lead to up-regulation of NFκB activity, since the transcription factor has been shown to be activated in endothelial cells cultured with linoleic acid, the main dietary n-6 polyunsaturated fatty acid, an effect inhibited by vitamin E and NAC (Hennig et al., 1996). The interaction between oxidant stress and an impaired ability to synthesize glutathione, which results in enhanced inflammation, is clearly seen in cirrhosis, a disease that results in high levels of oxidative stress and an impaired ability to synthesize GSH (Pena et al., 1999). In this study, an inverse relationship between glutathione concentration and the ability of monocytes to produce IL-1, IL-8 and TNF-α was observed. Furthermore, treatment of the patients with the GSH pro-drug oxothiazalidin-4-carboxylate (procysteine) (Fig. 7.7) increased monocyte GSH content and reduced IL-1, IL-8 and TNF-α production. Thus, antioxidants might act to prevent NFκB activation by quenching oxidants. However, not all transcription factors respond to changes in cell redox state in the same way. When rats were subjected to depletion of effective tissue GSH pools by administration of diethyl maleate, there was a significant reduction in lymphocyte proliferation in spleen and mesenteric lymph nodes (Robinson et al., 1993). In an in vitro study using HeLa cells and cells from human embryonic kidney, both TNF and hydrogen peroxide resulted in
activation of NF\(_k\)B and AP1 (Wesselborg et al., 1997). Addition of the antioxidant sorbitol to the medium suppressed NF\(_k\)B activation (as expected) but (unexpectedly) activated AP1. Thus, the antioxidant environment of the cell might exert opposite effects upon transcription factors closely associated with inflammation (e.g. NF\(_k\)B) and cellular proliferation (e.g. AP1). Evidence for this biphasic effect was seen when glutathione was incubated with immune cells from young adults (Wu et al., 1994). A rise in cellular glutathione content was accompanied by an increase in IL-2 production and lymphocyte proliferation and a decrease in production of the inflammatory mediators prostaglandin E\(_2\) (PGE\(_2\)) and leucotriene B\(_4\) (LTB\(_4\)). Modification of the glutathione content of liver, lung, spleen and thymus in young rats, by feeding diets containing a range of casein (a protein with a low sulphur amino acid content) concentrations, changed immune cell numbers in the lung (Hunter and Grimble, 1994). It was found that, in unstressed animals, the number of lung neutrophils decreased as dietary protein intake and tissue glutathione content fell. However, in animals given an inflammatory challenge (endotoxin), liver and lung GSH concentrations increased directly in relation to dietary protein intake. Lung neutrophils, however, became related inversely with tissue glutathione content. Addition of methionine to the protein-deficient diets normalized tissue glutathione content and restored lung neutrophil numbers to those seen in unstressed animals fed a diet of adequate protein content.

Thus it can be hypothesized that antioxidants exert an immunoenhancing effect, by activating transcription factors that are strongly associated with cell proliferation (e.g. AP1), and an anti-inflammatory effect, by preventing activation of NF\(_k\)B by oxidants produced during the inflammatory response.

### Strategies for Modulating Tissue GSH Content and Improving Immune Function

A number of strategies have evolved to raise levels in depleted individuals. As shown in Fig. 7.7, there are three potential ways of enhancing cellular GSH content: administration of the three amino acids (cysteine, glutamic acid and
glycine) that comprise the tripeptide, either singly or in various combinations; administration of cofactors for the metabolic pathways leading to GSH production, i.e. vitamin B₆, riboflavin and folic acid; and administration of synthetic compounds that become converted to precursors of GSH.

While cysteine supplies are the primary determinant of the ability to synthesize GSH, in some circumstances an insufficiency in the other two amino acids from which it is made might limit synthesis. Glutamine (a precursor of glutamate), for example, has been shown to maintain hepatic GSH in animals poisoned with acetaminophen, to enhance gut GSH synthesis in rats when given by gavage and to enhance hepatic GSH synthesis when given intravenously to rats (Cao et al., 1998). In human studies a similar effect on gut GSH concentrations was noted (O’Riordain et al., 1996). Glycine supplements have been shown to raise hepatic GSH in rats exposed to haemorrhagic shock (Spittler et al., 1999). In this condition, however, the metabolic demand for glycine is increased, since glycine is the sole nitrogen donor for haem synthesis and would therefore become rate-limiting for GSH synthesis. There are many studies that illustrate the ability of sulphur amino acid availability to influence tissue GSH concentrations (e.g. Stipanuk et al., 1992).

Studies using animal models of inflammation have shown that a low-protein diet will suppress glutathione synthesis, a situation that is reversed by the provision of cysteine or methionine (Hunter and Grimble, 1994, 1997). Beneficial effects on immune function, morbidity and mortality were observed in burned children when additional protein in the form of whey protein (the milk protein richest in sulphur amino acids) was fed (Alexander et al., 1980).

Because cysteine is unstable in its reduced form, toxic in high doses and mostly degraded in the extracellular compartment, several compounds have been used to deliver cysteine directly to cells. These include L-2-oxothiazalidine-4-carboxylate (OTZ) and NAC. OTZ is an analogue of 5-oxoproline, in which the 4-methylene moiety has been replaced with sulphur. It provides an excellent substrate for 5-oxoprolinase (an intracellular enzyme). The enzyme converts OTZ to S-carboxy-L-cysteine, which is rapidly hydrolysed to L-cysteine. NAC rapidly enters the cell and is speedily deacylated to yield L-cysteine. Recent animal and clinical trials with NAC and OTZ have demonstrated the ability of the compounds to enhance GSH status (Bernard et al., 1997; Deneke, 2000; De Rosa et al., 2000). In studies on patients with sepsis, NAC infusion was shown to increase blood GSH, decrease plasma concentrations of IL-8 and soluble TNF receptors (an index of TNF production), improve respiratory function and reduce the number of days needed in intensive care (Bernard et al., 1997; Spapen et al., 1998). While not affecting mortality rates, NAC shortened hospital length of stay by > 60%. OTZ increased whole-blood GSH in peritoneal-dialysis patients, normalized tissue GSH in rats fed a sulphur amino acid-deficient diet and decreased the extent of inflammation in a rat peritonitis model (Bernard et al., 1997). In a randomized, double-blind, controlled study on asymptomatic HIV-infected patients, oral OTZ treatment increased GSH concentrations in whole blood (Breitzkreutz et al., 2000). Other randomized studies on asymptomatic HIV+ patients in the presence and absence of anti-retroviral therapy (ART) have shown that NAC can raise blood
GSH, increase natural killer cell activity and enhance stimulation indices of T-cells incubated with mitogen or tetanus toxin (Simon et al., 1994; Breitzkreutz et al., 2000). Interestingly, the rise in T-cell function was accompanied by a fall in plasma IL-6 in subjects receiving ART as well as the drug. Furthermore, studies have shown that survival time was improved in HIV+ patients who maintained high concentrations of GSH in CD4+ T lymphocytes (Herzenberg et al., 1997). It could therefore be surmised that improved T-cell function and reduced inflammation are modulated by improvement in antioxidant status in these patients. Alpha-lipoic acid provides a further means of enhancing tissue GSH content (Deneke, 2000). The compound is reduced to dihydrolipoic acid, which converts cystine to cysteine. This change has functional significance for glutathione status in lymphocytes, since the xc transport system, which is needed to take up cysteine into the cells, is weakly expressed and is inhibited by glutamate, while the neutral amino acid transport system, which takes up cysteine, is functional. Upon gaining entry to the immune cells, cysteine is rapidly converted to GSH. Flow-cytometric analysis of freshly prepared human peripheral-blood lymphocytes shows that lipoic acid is able to normalize a subpopulation of cells with severely compromised thiol status, rather than increasing the level in all cells above normal values (Sen et al., 1997). Hence lipoic acid may also prove to be a useful clinical agent for restoring cellular GSH concentration in immunocompromised subjects.

Taurine and Immune Function

Taurine, along with sulphate, can be regarded as a biochemical end-product of cysteine metabolism. However, it is apparent that taurine also plays a role in immune function. It is the most abundant free nitrogenous compound (often incorrectly classified as an amino acid) in cells. It is a membrane stabilizer and regulates calcium flux, thereby controlling cell stability. It has been shown to possess antioxidant properties and to regulate the release of pro-inflammatory cytokines in hamsters, rats and humans (Grimble, 1994; Huxtable, 1996; Kontny et al., 2000).

The possibility that taurine might have immunomodulatory properties was indicated in studies in obligate carnivores, such as cats, in which taurine is an essential nutrient, due to an inability to synthesize the compound. Premature infants have similar metabolic difficulties. In cats deprived of taurine, substantial impairment of immune function occurs (Grimble, 1994). A large decline in lymphocytes, an increase in mononuclear cells and a decrease in the ability of these cells to produce a ‘respiratory burst’ and to phagocytose bacteria occur. There was a rise in gamma globulin concentrations in deficient animals. Spleen and lymph nodes showed regression of follicular centres and depletion of mature and immature B lymphocyte numbers. The changes were reversed by inclusion of taurine in the diets. Studies in other species have also reported effects of supplementation on immune system and function. In mice, administration of taurine prevented the decline in T-cell number that occurs with ageing and enhanced the proliferative responses of T-cells in both young and old mice.
The effect was more marked in cells from old than from young animals. Taurine has been shown to ameliorate inflammation in trinitrobenzene sulphonic acid-induced colitis.

Taurine interacts with hypochlorous acid, produced during the ‘oxidant burst’ of stimulated macrophages, to produce taurine chloramine (TauCl). This compound may have important immunomodulatory properties and may be responsible for properties that have been ascribed earlier to taurine. TauCl has been shown to inhibit nitric oxide, PGE₂, TNF-α and IL-6 production from stimulated macrophages in culture and to inhibit the ability of antigen-presenting cells to process and present ovalbumin (Grimble, 1999). In in vitro studies with murine dendritic cells, the compound altered the balance of Th1 to Th2 cytokines, suggesting that it might play a role in maintaining the balance between the inflammatory response and the acquired immune response.

Taurolidine, which is a derivative of taurine, has been used as a bactericidal and anti-lipopolysaccharide agent. However, it may also have an immunomodulatory influence, since it is hydrolysed to taurine in vivo. Indeed, in a murine model of sepsis, the former compound was shown to decrease mortality (Grimble, 1999).

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Vitamin A, Infection and Immune Function

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Introduction

Vitamin A deficiency is a leading cause of morbidity and mortality worldwide, especially among infants, children and women in developing countries. An estimated 253 million children are at risk of immunodeficiency due to Vitamin A deficiency (World Health Organization, 1995), and millions of pregnant and lactating women are also at high risk in developing countries. Among the micronutrients, the role of vitamin A in immune function has probably been the most extensively characterized, and studies have shown a multifaceted role of vitamin A in many aspects of immunity. Vitamin A plays a role in the maintenance of mucosal surfaces, in the generation of antibody responses, in haematopoiesis and in the function of T and B lymphocytes, natural killer (NK) cells and neutrophils (for reviews, see Semba, 1994, 1998). The influence of vitamin A on different aspects of immune function is attributed to the action of vitamin A and related metabolites as modulators of gene transcription. The purpose of this chapter is to summarize the role that vitamin A plays in immune function and resistance to infectious diseases. In addition to compromising the immune system, vitamin A deficiency causes night-blindness, xerophthalmia, retardation of growth, impaired reproductive capacity and anaemia. Recently, this array of adverse health problems was described in a comprehensive manner and has been aptly termed the ‘vitamin A deficiency disorders’ (McLaren and Frigg, 2001).

Biochemistry and Metabolism of Vitamin A

Vitamin A is available in dietary sources as either preformed vitamin A or pro-vitamin A carotenoids. Rich dietary sources of preformed vitamin A include egg yolk, liver, butter, cheese, whole milk and cod-liver oil. In many developing countries, the consumption of foods containing preformed vitamin A is limited, and
pro-vitamin A carotenoids often comprise the major dietary source of vitamin A. The major pro-vitamin A carotenoids consist of α-carotene and β-carotene, found in such foods as dark green leafy vegetables, carrots, sweet potatoes, mangos, and papayas and β-cryptoxanthin, found in oranges and tangerines.

Digested foods that contain preformed vitamin A are emulsified with bile salts and lipids in the small intestine. Retinol is esterified in the intestinal mucosa, packaged into chylomicrons and carried to the bloodstream via the lymphatic circulation. Pro-vitamin A carotenoids, such as β-carotene, may be converted to retinaldehyde through cleavage by carotenoid-15,15'-dioxygenase or by an asymmetrical cleavage pathway. The bioavailability of pro-vitamin A carotenoids is less than that of preformed vitamin A, due to a variety of factors, including differences in efficacy of absorption and biochemical conversion (De Pee et al., 1995; West, 2000). About 90% of the vitamin A in the body is stored in the liver as retinyl esters, and the liver has the capacity to store enough vitamin A to last for several months, with a larger storage capacity among adults than among children. Retinol is released from the liver in combination with plasma retinol-binding protein (RBP) and transthyretin (TTR). Retinol is poorly soluble in water and is carried in the blood sequestered inside the carrier proteins, RBP and TTR. Retinol seems to enter cells via specific receptors, although it is unclear whether all cells contain these receptors.

Vitamin A exerts its effects via retinoic acid and retinoid receptors, which are found in the nucleus of the cell. Retinol is converted to all-trans-retinoic acid and 9-cis-retinoic acid in the cytoplasm. Retinoic acid influences gene activation through specific receptors, which belong to the superfamily of thyroid and steroid receptors (Chambon, 1996). Retinoic acid receptors (RARs) act as transcriptional activators for many specific target genes. The RAR is expressed as several isoforms (RAR α, β and γ), and the retinoid-X receptor (RXR) is also expressed as several isoforms (RXR α, β and γ) (Kliewer et al., 1992). All-trans-retinoic acid is a ligand for RARs whereas 9-cis-retinoic acid is a ligand for both RARs and RXRs. The DNA sequences that interact with RAR and RXR are known as retinoic acid response elements. RARs and RXRs form heterodimers, which bind to DNA and control gene expression. In addition, RXRs can also form heterodimers with the thyroid hormone receptor, vitamin D3 receptor, peroxisome proliferator activator receptors and a number of newly described ‘orphan receptors’. Most retinoic acid response elements occur in the regulatory region of genes.

Vitamin A and Immune Function

Historical overview

In the years soon after its discovery, vitamin A became suspected of being a factor essential for the development of the lymphoid system and for the maintenance of mucosal surfaces of the gastrointestinal, respiratory and genitourinary tracts (Clausen, 1934; Robertson, 1934) and the high childhood morbidity and mortality in Europe and the USA in the early 20th century – comparable to those found in many developing countries today – were ascribed to the
deficiency of vitamin A (Bloch, 1924). Milk, cream and butter were advocated to reduce infections in children (Bloch, 1924). Subsequently, vitamin A was evaluated in at least 30 therapeutic trials in various infections (e.g. Green and Mellanby, 1928; Ellison, 1932). It is now recognized that vitamin A modulates many different aspects of immune function, including components of both non-specific immunity (e.g. phagocytosis, maintenance of mucosal surfaces) and specific immunity (e.g. generation of antibody responses). Much of our knowledge of vitamin A and immune function is derived from experimental animal studies involving mice, rats and chickens. The effects of vitamin A deficiency on aspects of immune function are summarized in Table 8.1.

**Mucosal immunity**

Vitamin A deficiency impairs mucosal function through several mechanisms: (i) loss of cilia in the respiratory tract; (ii) loss of microvilli in the gastrointestinal tract; (iii) loss of mucin and goblet cells in the respiratory, gastrointestinal and genitourinary tracts; (iv) squamous metaplasia with abnormal keratinization in the respiratory and genitourinary tracts; (v) alterations in antigen-specific secretory immunoglobulin A (IgA) concentrations; (vi) impairment of mucosal-associated immune-cell function; and (vii) decreased integrity of the gut. The first four were among the most striking findings described in early studies of vitamin A-deficient animals and humans (Wolbach and Howe, 1925; Blackfan and Wolbach, 1933; Sweet and K’ang, 1935). The ocular surface has also been intensively studied during vitamin A deficiency, and loss of mucin and goblet cells and squamous metaplasia of the conjunctiva and cornea are well known (McLaren, 1963; Sommer, 1982). There is a close relationship between vitamin A status and the expression of mucins (Koo et al., 1999; Tei et al., 2000) and keratins (Gijbels et al., 1992; Darwiche et al., 1993). Mucins are large glycoconjugates that are found on cell surfaces and secreted into the lumens of the gastrointestinal, respiratory and genitourinary tracts. Mucins are also secreted on the bulbar and palpebral conjunctivae of the eye. The loss of mucin that occurs in vitamin A deficiency constitutes a serious impairment of mucosal immunity.

**Table 8.1. Effects of vitamin A deficiency on host defence.**

<table>
<thead>
<tr>
<th>Effect</th>
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<tr>
<td>Abnormal expression of keratins in the respiratory tract, genitourinary tract and ocular surface</td>
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<tr>
<td>Loss of cilia from respiratory epithelium</td>
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<tr>
<td>Loss of microvilli from small intestine</td>
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<tr>
<td>Decrease in goblet cells and mucin production in mucosal epithelia</td>
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<td>Impaired neutrophil function</td>
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<td>Impaired natural killer (NK) cell function and decreased number of NK cells</td>
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<td>Impaired aspects of haematopoiesis</td>
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<td>Shift towards T-helper type 1-like immune responses</td>
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<tr>
<td>Decrease in number and function of B lymphocytes</td>
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<tr>
<td>Impaired antibody responses to T-cell-dependent and independent antigens</td>
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</table>
In vitamin A-deficient chickens, the concentrations of total IgA were lower in the gut than in control animals (Rombout et al., 1992). Vitamin A-deficient BALB/c mice that were challenged with influenza A virus had a lower influenza-specific IgA response than control mice (Gangopadhyay et al., 1996). Vitamin A-deficient mice had significantly lower serum antibody responses against epizootic diarrhoea of infant mice (EDIM) rotavirus infection compared with control mice (Ahmed et al., 1991). An impaired ability to respond with IgA antibodies to oral cholera vaccine was demonstrated in vitamin A-deficient rats (Wiedermann et al., 1993a). Using the urinary lactulose/mannitol excretion test, increased gut permeability was found in vitamin A-deficient infants, and the gut integrity improved following vitamin A supplementation (Thurnham et al., 2000).

NK cells

Vitamin A deficiency reduces the number of circulating NK cells and impairs NK cell cytolytic activity. NK cells play a role in anti-viral and anti-tumour immunity that is not major histocompatibility complex (MHC)-restricted, and they are involved in the regulation of immune responses. In experimental animal models, vitamin A deficiency reduced the number of NK cells in the spleen (Nauss and Newberne, 1985; Bowman et al., 1990) and peripheral blood (Zhao et al., 1994). The cytolytic activity of NK cells was reduced by vitamin A deficiency (Zhao et al., 1994). In ageing Lewis rats, marginal vitamin A status reduced the number of NK cells in peripheral blood and the cytolytic activity of NK cells (Dawson et al., 1999). There have been few studies of vitamin A status and NK cells in humans. Children with acquired immune deficiency syndrome (AIDS) who received two doses of oral vitamin A (60 mg retinol equivalents (RE)) had large increases in the number of circulating NK cells, compared with children who received placebo (Hussey et al., 1996).

Neutrophils

The function of neutrophils appears to be impaired during vitamin A deficiency. Neutrophils play an important role in non-specific immunity, because they phagocytose and kill bacteria, parasites, virus-infected cells and tumour cells. Retinoic acid plays an important role in the normal maturation of neutrophils (Lawson and Berliner, 1999). Vitamin A-deficient rats had widespread defects in neutrophil function, including impaired chemotaxis, adhesion, phagocytosis and ability to generate active oxidant molecules, compared with neutrophils from controls (Twining et al., 1996). In rats challenged with Staphylococcus aureus, impaired phagocytosis and decreased complement lysis activity were found in vitamin A-deficient rats compared with control rats (Wiedermann et al., 1996). Vitamin A treatment was shown to increase superoxide production by neutrophils from Holstein calves (Higuchi and Nagahata, 2000).
Haematopoiesis

Vitamin A deficiency appears to impair haematopoiesis of some lineages, such as CD4+ lymphocytes, NK cells and erythrocytes. In humans, vitamin A deficiency has been characterized by lower total lymphocyte counts and decreased CD4+ lymphocytes in peripheral blood; furthermore, CD4+ lymphocyte counts and/or percentage increased after vitamin A supplementation of deficient individuals (Semba et al., 1993a, b; Hussey et al., 1996). In the vitamin A-deficient rat, lower NK-cell, B-cell and CD4+ lymphocyte counts were found in peripheral blood, and these counts responded to retinoic acid supplementation (Zhao and Ross, 1995). Retinoids have been implicated in the maturation of pluripotent stem cells to cell lineages that produce different haematopoietic cell lines, such as lymphocytes, granulocytes and megakaryocytes. Retinoids also appear to play a role in the maturation of differentiation of pluripotent stem cells into multipotent colony-forming unit granulocyte–erythroid–macrophage mixed (CFU-GEMM) cells, and differentiation and commitment of CFU-GEMM into erythroid burst-forming units (BFU-E) and then into erythroid colony-forming units (CFU-E) (Perrin et al., 1997; Zermati et al., 2000).

T lymphocytes

Vitamin A appears to modulate the balance between T-helper type 1- and T-helper type 2-like responses. *Trichinella spiralis* infection in mice usually stimulates strong T-helper type 2-like responses, characterized by parasite-specific IgG responses and a cytokine profile dominated by interleukin (IL)-4, IL-5 and IL-10 production. However, in vitamin A-deficient mice, infection by *T. spiralis* results in low production of parasite-specific IgG and a cytokine profile dominated by interferon (IFN)-γ and IL-12 production, more characteristic of a T-helper-1 profile (Carman et al., 1992; Cantorna et al., 1994, 1996). Lymphocyte responsiveness to stimulation by concanavalin A or β-lactoglobulin was higher and production of IL-2 and IFN-γ was higher in lymphocyte supernatants from vitamin A-deficient rats, compared with control rats, further supporting the idea that vitamin A deficiency modulates a shift towards T-helper type 1-like responses (Wiedermann et al., 1993b). Vitamin A appears to inhibit production of IFN-γ, IL-2 and granulocyte–macrophage colony-stimulating factor (GM-CSF) by type 1 lymphocytes in vitro (Frankenburg et al., 1998). The effect of high-level dietary vitamin A on the shift to T-helper type 2-like responses in BALB/c mice has been used to explain the apparent lack of benefit of vitamin A supplementation for acute lower respiratory infections in humans (Cui et al., 2000).

Monocytes/macrophages

Retinoids appear to play a role in the differentiation and activity of cells of the monocyte/macrophage lineage. The effect of vitamin A deficiency on macrophage function is less clear, as most studies have addressed the effects of all-trans-retinoic acid on the function of murine macrophages (Dillehay et al., 1988) or myeloid cell lines.
B lymphocytes

Vitamin A deficiency impairs the growth, activation and function of B lymphocytes. B lymphocytes have been shown to utilize a metabolite of retinol, 14-hydroxy-4,14-retro-retinol, instead of retinoic acid, as a mediator for growth (Buck et al., 1991). The effects of retinol and all-trans-retinoic acid on immunoglobulin synthesis by B lymphocytes have been examined in human cord-blood and adult peripheral-blood mononuclear cells (Israel et al., 1991; Wang and Ballow 1993; Wang et al., 1993; Ballow et al., 1996). A T-cell-dependent antigen was used to induce differentiation of sensitized human B lymphocytes into immunoglobulin-secreting cells, and all-trans-retinoic acid increased the synthesis of IgM and IgG by these cells. Highly purified T lymphocytes incubated with retinoic acid enhanced IgM synthesis by cord-blood B lymphocytes, suggesting that retinoic acid modulates T-cell help through cytokine production (Ballow et al., 1996).

Antibody responses

The hallmark of vitamin A deficiency is an impaired capacity to generate an antibody response to T-cell-dependent antigens (Smith and Hayes, 1987; Semba et al., 1992, 1994; Wiedermann et al., 1993a, b) and T-cell-independent type 2 antigens, such as pneumococcal polysaccharide (Pasatiempo et al., 1989). Antibody responses are involved in protective immunity to many types of infections and are the main basis for immunological protection for most types of vaccines. Depressed antibody responses to tetanus toxoid have been observed in vitamin A-deficient children (Semba et al., 1992) and animals (Lavasa et al., 1988; Pasatiempo et al., 1990). Vitamin A deficiency appears to impair the generation of primary antibody responses to tetanus toxoid, but, if animals are replete with vitamin A prior to a second immunization, the secondary antibody responses to tetanus toxoid are comparable to those of control animals (Kinoshita et al., 1991). These findings suggest that formation of immunological memory and class switching are intact during vitamin A deficiency, despite an impaired IgM and IgG response to primary immunization. Human peripheral-blood lymphocytes from subjects previously immunized against tetanus toxoid were used to reconstitute control and vitamin A-deficient mice with severe combined immunodeficiency (SCID). After challenge with tetanus toxoid, vitamin A-deficient SCID mice had a 2.9-fold increase in human anti-tetanus toxoid antibody, compared with a 74-fold increase in control SCID mice (Molrine et al., 1995). In healthy children without vitamin A deficiency, vitamin A supplementation did not enhance antibody responses to tetanus toxoid (Kutukculer et al., 2000). These findings suggest that vitamin A supplementation is unlikely to enhance antibody responses in subjects who are not vitamin A-deficient.

Role of Vitamin A in Resistance to Infectious Disease

Vitamin A deficiency increases susceptibility to some types of infections, and there is currently an extensive literature regarding vitamin A deficiency and
infection in experimental animal models (Clausen, 1934; Robertson, 1934; Scrimshaw et al., 1968; Beisel, 1982; Nauss, 1986; Semba, 1994). After an extensive global survey of vitamin A deficiency, Oomen et al. (1964) recognized that there was a vicious circle of vitamin A deficiency and infection: ‘Not only may deficiency of vitamin A itself play an important role in lowering the resistance to infection … but infectious diseases themselves predispose to and actually precipitate xerophthalmia.’ There have been over 100 clinical trials of vitamin A conducted in humans, and these studies show that vitamin A supplementation can reduce morbidity and mortality due to measles and diarrhoeal disease, the morbidity of *Plasmodium falciparum* malaria and maternal morbidity and mortality related to pregnancy (see below). Vitamin A supplementation does not appear to reduce morbidity and mortality from acute lower respiratory infections or reduce mother-to-child transmission of human immunodeficiency virus (HIV) type 1 (see below).

## Measles

Vitamin A supplementation reduces the morbidity and mortality from acute measles in infants and children in developing countries. Children with low circulating vitamin A concentrations had higher mortality from measles in a study from Kinshasa, Zaire (Markowitz et al., 1989). An early clinical trial from London showed that vitamin A supplementation could reduce mortality in children with acute measles (Ellison, 1932). Clinical trials showed that high-dose vitamin A reduces morbidity and mortality in children with acute measles infection (Barclay et al., 1987; Hussey and Klein, 1990; Coutsoudis et al., 1991; Ogaro et al., 1993). In acute, complicated measles, high-dose vitamin A supplementation (60 mg RE upon admission and the following day) was shown to reduce mortality by up to 80% in Cape Town, South Africa (Hussey and Klein, 1990). Vitamin A supplementation seems to reduce the infectious complications associated with measles immune suppression, such as pneumonia and diarrhoeal disease.

Vitamin A supplementation appears to modulate antibody responses to measles and increases total lymphocyte counts. Children with acute measles infection who received high-dose vitamin A supplementation (60 mg RE upon admission and the following day) had significantly higher IgG responses to measles virus and higher circulating lymphocyte counts during follow-up, compared with children who received placebo (Coutsoudis et al., 1992). When vitamin A supplementation is given simultaneously with live measles vaccine, there appears to be an effect upon antibody titres to measles if maternal antibodies are present. In 6-month-old infants in Indonesia, administration of vitamin A (30 mg RE) at the time of immunization with standard-titre Schwarz measles vaccine interfered with seroconversion to measles in infants who had maternal antibody present, and significantly reduced the incidence of measles vaccine-associated rash (Semba et al., 1995). A separate clinical trial also showed that vitamin A (30 mg RE) reduced antibody responses to measles virus in 9-month-old infants who had maternal antibody present, but did not interfere with overall seroconversion rates to measles (Semba et al., 1997).
In Guinea-Bissau, vitamin A supplementation (30 mg RE) enhanced geometric-mean titres to measles when given simultaneously with standard-titre Schwarz measles vaccine in 9-month-old infants (Stabell Benn et al., 1997). In a two-dose measles immunization schedule at ages 6 and 9 months, simultaneous vitamin A supplementation did not interfere with seroconversion to measles when measured at 18 months of age (Stabell Benn et al., 1997). It was not possible to determine whether vitamin A supplementation interfered with seroconversion rates after measles vaccine in 6-month-old infants in the study in Guinea-Bissau, as with the study in Indonesia, as antibody titres were not measured until after two vaccinations. Although the results of the studies involving 6-month-old infants in Indonesia and Guinea-Bissau have been viewed as contradictory (Ross and Cutts, 1997; Stabell Benn et al., 1997), the differences in the design of the measles-vaccine studies lend little validity to making direct comparisons between these two studies, and the findings may be complementary.

Diarrhoeal diseases

In developing countries, diarrhoeal diseases among children are caused by a wide variety of pathogens, including rotavirus, *Escherichia coli*, *Shigella*, *Vibrio cholerae*, *Salmonella* and *Entamoeba histolytica*. The epidemiology, clinical features, immunology and pathogenesis of diarrhoea may differ according to characteristics of the pathogen, such as production of toxins, tissue invasion, fluid and electrolyte loss and location of infection. Vitamin A supplementation or fortification has been shown to reduce the morbidity and mortality of diarrhoeal diseases among preschool children in developing countries. The reduction in diarrhoeal disease mortality appears to account for most of the reduction in overall mortality when vitamin A is given through fortification or supplementation on a community level. Clinical vitamin A deficiency is associated with diarrhoeal disease in children (Sommer et al., 1984; Brilliant et al., 1985; DeSole et al., 1987; Gujral et al., 1993; Schaumberg et al., 1996). Large community-based clinical trials of vitamin A supplementation in Tamil Nadu, Nepal and Ghana show that vitamin A has a major impact upon the overall mortality of diarrhoeal disease but not on pneumonia in preschool children (Beaton et al., 1993; Vitamin A and Pneumonia Working Group, 1995). The severity of diarrhoeal disease was reduced by vitamin A supplementation in a clinical trial in Brazil (Barreto et al., 1994). Urinary losses of vitamin A during *Shigella* infection may be substantial in some children (Mitra et al., 1998), and vitamin A supplementation (60 mg RE) has been shown to reduce morbidity in children with acute shigellosis (Hossain et al., 1998). Although improvement of vitamin A status has been shown to protect against diarrhoeal diseases, it is not clear whether this is a general effect against all diarrhoeal pathogens or only against certain types of pathogens.

Acute lower respiratory infections

Acute lower respiratory infections (ALRI) are a major cause of death among children in developing countries, and major causes of ALRI include respiratory
syncytial virus (RSV) infection, parainfluenza, *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Bordetella pertussis*. Secondary bacterial infection with high case fatality may follow a primary viral infection in the lungs. Community-based trials failed to demonstrate any effect of vitamin A supplementation upon morbidity and mortality of ALRI (Vitamin A and Pneumonia Working Group, 1995). Hospital-based studies have also shown that high-dose vitamin A supplementation has no therapeutic effect upon the morbidity of ALRI in children (Kjolhede et al., 1995; Nacul et al., 1997; Fawzi et al., 1998). In Chile and the USA, hospital-based trials showed that vitamin A supplementation had little impact upon RSV infection among infants and young children (Bresee et al., 1996; Dowell et al., 1996; Quinlan and Hayani, 1996).

High-dose vitamin A supplementation may have adverse consequences for some children who are not malnourished (Sempertegui et al., 1999; Fawzi et al., 2000a). In a study conducted in Dar Es Salaam, Tanzania, children hospitalized with pneumonia received high-dose vitamin A supplementation and, after discharge, they were monitored for diarrhoeal and respiratory disease. Vitamin A supplementation was associated with a higher rate of diarrhoeal disease among children who were better nourished, whereas a reduction in diarrhoeal morbidity was noted among wasted children. This apparent bidirectional effect has been termed ‘the vitamin A paradox’ (Griffiths, 2000). A recent controlled clinical trial conducted in Quito, Ecuador, also suggested that weekly vitamin A supplementation to children, aged 6–36 months, significantly reduced the incidence of ALRI in underweight (weight-for-age Z score $< -2$) children, but significantly increased the incidence of ALRI in normal-weight children (weight-for-age Z score $> -1$), compared with placebo (Sempertegui et al., 1999).

Although vitamin A status has been shown to be related to the severity of acute respiratory infection in children (Dudley et al., 1997), it is unclear why vitamin A therapy has no apparent effect in some trials upon the morbidity of acute respiratory infections among preschool children. Young age might be one contributing factor to the lack of an effect, as large community-based studies suggest that vitamin A supplementation has little effect on morbidity and mortality of infants (West et al., 1995; WHO/CHD Immunisation-Linked Vitamin A Supplementation Study Group, 1998). Studies have also been conducted in populations where vitamin A deficiency is not considered a public-health problem. In the recent clinical trials involving RSV infection, the apparent lack of impact of vitamin A supplementation on RSV infection might be due to the young age of the subjects and the lack of vitamin A deficiency in the population. It would be erroneous to consider vitamin A as ineffective in increasing immunity to ALRI completely, as vitamin A supplementation has been shown to reduce the life-threatening complication of pneumonia after acute measles infection (Barclay et al., 1987; Hussey and Klein, 1990).

**Malaria**

There is new evidence that vitamin A supplementation may help reduce the morbidity of *P. falciparum* malaria – an important observation since *P.*
Plasmodium falciparum causes an estimated 1–2 million deaths worldwide each year. There appears to be an association between poor vitamin A status and malaria (Stürchler et al., 1987; Galan et al., 1990; Friis et al., 1997). A randomized, placebo-controlled clinical trial was conducted in Papua New Guinea to examine the effects of vitamin A supplementation (60 mg RE every 3 months) on malarial morbidity in preschool children aged 6–60 months (Shankar et al., 1999). Weekly morbidity surveillance and clinic-based surveillance were established for monitoring acute malaria, and children were followed for 1 year. Vitamin A significantly reduced the incidence of malaria attacks by about 20–50% for all except extremely high levels of parasitaemia. Similarly, vitamin A supplementation reduced clinic-based malaria relapses, which consisted of self-solicited visits to the clinic by mothers who thought that their children should be seen because of fever. Vitamin A supplementation had little impact in children under age 12 months and the greatest effect was from 13 to 36 months of age.

**HIV infection**

Vitamin A supplementation may have some benefit for HIV-infected children and pregnant women in developing countries. Low plasma or serum concentrations of vitamin A or intake of vitamin A have been associated with increased disease progression and mortality and higher mother-to-child transmission of HIV (Kennedy et al., 2000). Periodic high-dose vitamin A supplementation seems to reduce morbidity among children born to HIV-infected mothers (Coutsoudis et al., 1995) and diarrhoeal-disease morbidity in HIV-infected children after discharge from the hospital for ALRI (Fawzi et al., 1999). Vitamin A supplementation did not reduce mother-to-child transmission of HIV (Coutsoudis et al., 1999; Fawzi et al., 2000b). However, a trial in Durban, South Africa, showed that vitamin A supplementation reduced preterm birth (Coutsoudis et al., 1999). Vitamin A supplementation does not appear to influence HIV load in the blood (Semba et al., 1998). A study from Cape Town, South Africa, suggests that vitamin A supplementation modulates lymphopoiesis in children with AIDS (Hussey et al., 1996).

**Tuberculosis**

Although malnutrition and vitamin A deficiency seem to be major risk factors for the progression of tuberculosis, clinical management usually involves chemoprophylaxis and chemotherapy alone, rather than any special concern for host nutritional status. Cod-liver oil, a rich source of vitamins A and D, was used as treatment strategy for tuberculosis for over 100 years (Williams and Williams, 1871). The role of nutrition and tuberculosis remains a major area of neglect, despite the promise that micronutrients have shown as therapy for other types of infections and the long record of the use of vitamins A and D for treatment of pulmonary and miliary tuberculosis in both Europe and the USA.
A recent clinical trial suggests that high-dose vitamin A supplementation does influence the morbidity of tuberculosis in children (Hanekom et al., 1997). Studies have not been conducted to address the use of multivitamins and minerals or vitamins A plus D as adjunct therapy for tuberculosis.

Infections in pregnant and lactating women

Recent data from Nepal suggest that pregnant women with clinical vitamin A deficiency (i.e. night-blindness) are at higher risk of infectious-disease morbidity (Christian et al., 1998) and mortality (Christian et al., 2000b). Weekly vitamin A or β-carotene supplementation appeared to reduce the risk of infectious disease morbidity and mortality among these women, suggesting that vitamin A status may be important in pregnancy-related morbidity and mortality (West et al., 1999; Christian et al., 2000a). Vitamin A or β-carotene reduced all-cause mortality, and further work is needed both to replicate these findings and to determine the types of infections that might be reduced through improving vitamin A status during pregnancy. The recent trial in Nepal appears to corroborate earlier trials from England, which showed that vitamin A supplementation reduced the morbidity of puerperal sepsis (Cameron, 1931; Green et al., 1931).

Conclusions and Future Directions

Vitamin A has been used as both disease-targeted and prophylactic therapy to reduce morbidity and mortality from infectious diseases for hundreds of years. Vitamin A plays an important role in haematopoiesis, the maintenance of mucosal surfaces, the function of T and B lymphocytes, NK cells and neutrophils, and the generation of antibody responses to T-cell-dependent and independent antigens. As an immune modulator, vitamin A reduces the severity but not the incidence of certain types of infections: measles, diarrhoeal diseases, malaria and, possibly, infections related to pregnancy. Vitamin A does not appear to reduce the morbidity and mortality from ALRI. As a general rule, there appears to be little value in vitamin A supplementation in populations that are already relatively well-nourished and thus clinical investigation of immune modulation by vitamin A should be focused on populations at high risk of vitamin A deficiency. Despite the tremendous advances that have been made in our understanding of the role that vitamin A plays in immune function, many gaps in knowledge remain:

- The relationship between vitamin A status in humans and the function of immune effector cells, such as neutrophils, macrophages, NK cells and cytotoxic T-cells.
- The relationship between vitamin A status in humans and the balance between T-helper type 1-like and T-helper type 2-like immune responses.
- The relationship between vitamin A status and gut integrity in humans.
- The role of vitamin A in resistance to *P. falciparum* malaria.
• The role of vitamin A in resistance to tuberculosis in humans.
• The more precise biological mechanism(s) by which vitamin A reduces measles severity.
• The more precise biological mechanism(s) by which vitamin A reduces diarrhoeal-disease severity.
• The role of vitamin A in immune senescence.
• The role of vitamin A in apoptosis.
• The relationship between vitamin A and other micronutrients (e.g. zinc) in immune modulation.
• The relationship between vitamin A status and expression of pro-inflammatory cytokines.
• The relationship between vitamin A status and specific infections during pregnancy.
• The uses of synthetic retinoids in immune modulation.

These are promising areas for future investigation, which should be addressed in order to gain further insight into the biological functions of this important vitamin.

Acknowledgements

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Antioxidant Vitamins and Immune Function

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Introduction

Oxidative stress, resulting from cumulative damage caused by reactive oxygen species (ROS), is present throughout life and is thought to be a major contributor to the ageing process. The immune system is particularly vulnerable to oxidative damage, since many immune cells produce these reactive compounds as part of the body’s defence mechanisms. Higher organisms have evolved a variety of antioxidant defence systems either to prevent the generation of ROS or to intercept any that are generated. These defence systems exist in both the aqueous and membrane compartments of cells and can be enzymic or non-enzymic in nature. The enzymes contain metal ions at their active sites – these must be obtained from the diet – while the diet is the source of many non-enzymic components of the body’s antioxidant defence system (e.g. antioxidant vitamins).

Reactive Oxygen Species and Antioxidant Defences

Reactive oxygen species

Free radicals are highly reactive molecules containing one or more unpaired electrons. Examples of free radicals are the superoxide anion (O\textsuperscript{2−}) and the hydroxyl radical (OH·). The term ‘reactive oxygen species’ is a collective one that includes not only oxygen-centred radicals but also some non-radical derivatives of oxygen, such as hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), singlet oxygen and hypochlorous acid (HOCI). Hydrogen peroxide can very easily break down, particularly in the presence of transition-metal ions (e.g. ferrous (Fe\textsuperscript{2+}) iron), to produce the hydroxyl radical, the most reactive and damaging of the oxygen free radicals:

\[
\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{OH}^{-} + \text{OH}^{-} + \text{Fe}^{3+}
\]
Exogenous sources of free radicals include ozone, UV radiation and cigarette smoke. Free radicals are also generated endogenously, mainly from two sources. The first is by leakage from the mitochondrial electron-transfer chain, as part of normal cellular metabolism. The second is as part of the respiratory-burst activity of leucocytes, which is involved in microbial killing.

ROS can cause damage to all of the major classes of macromolecules. They cause strand breaks in DNA (Halliwell and Aruoma, 1991), which can potentially lead to subsequent misrepair, mutation and tumour-cell formation. An example of free-radical-mediated damage to proteins is the formation of cataracts, resulting from the damage to the crystallins in the lens of the eye. However, lipids are probably most susceptible to free radical attack, particularly long chain polyunsaturated fatty acids (PUFA) that contain several double bonds. The oxidative destruction of PUFA, known as lipid peroxidation, can be extremely damaging, since it proceeds as a self-perpetuating chain reaction.

Generation of ROS in excess of the amounts that can be dealt with by the body’s antioxidant protective mechanisms is thought to be a major contributor to several degenerative disorders, such as cancer and cardiovascular diseases (Table 9.1), and to the ageing process. Strong associations between diets rich in antioxidant nutrients and a reduced incidence of cancer have been observed in several epidemiological studies (Block et al., 1992; Giovannucci, 1999), and it has been suggested that a boost to the body’s immune system by antioxidants might, at least in part, account for this (Bendich and Olson, 1989). Indeed, it is probably crucial to attempt to balance the production of ROS and the antioxidant defence system, ideally by dietary means rather than by taking supplements, from as early an age as possible, in order to delay the onset of, if not prevent, many age-related disorders.

The immune system appears to be particularly sensitive to oxidative stress. Immune cells rely heavily on cell–cell communication, particularly via membrane-bound receptors, to work effectively. Cell membranes are rich in PUFA, which, if peroxidized, can lead to a loss of membrane integrity, altered membrane fluidity (Baker and Meydani, 1994) and alterations in intracellular signalling and cell function. It has been shown that exposure to ROS can lead to a reduction in cell-membrane-receptor expression (Gruner et al., 1986). In addition, the production of ROS by phagocytic immune cells can damage the cells themselves if they are not sufficiently protected by antioxidants.

<table>
<thead>
<tr>
<th>Table 9.1. Degenerative disorders associated with oxidative damage.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
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<tr>
<td>Cardiovascular disease</td>
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<tr>
<td>Stroke</td>
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<tr>
<td>Cataract</td>
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<tr>
<td>Degeneration of the macula region of the retina</td>
</tr>
<tr>
<td>Immunosenescence</td>
</tr>
<tr>
<td>Ageing</td>
</tr>
</tbody>
</table>
Antioxidant defences

The enzyme superoxide dismutase decomposes superoxide radicals by converting them to hydrogen peroxide plus oxygen. Catalase and glutathione (GSH) peroxidase are enzymes that decompose peroxides, particularly hydrogen peroxide.

\[
\text{Superoxide dismutase:} \quad 2O_2^-+ 2H^+ \rightarrow H_2O_2 + O_2
\]

\[
\text{Catalase:} \quad 2H_2O_2 \rightarrow 2H_2O + O_2
\]

\[
\text{Glutathione peroxidase:} \quad 2GSH + H_2O_2 \rightarrow GSSG + 2H_2O
\]

(oxidized glutathione)

There are two forms of superoxide dismutase: a mitochondrial enzyme, which contains manganese, and a cytosolic enzyme, which contains copper and zinc. Catalase contains iron, while glutathione peroxidase contains selenium. These metal ions must come from the diet. At least some of the effects that selenium, copper, zinc, iron and glutathione have on immune function relate to their roles in antioxidant defence (see Grimble, Chapter 7, Prasad, Chapter 10, Kuvibidila and Baliga, Chapter 11, and McKenzie et al., Chapter 12, this volume). The diet also provides many other, non-enzymic, components of the body’s antioxidant defence system. These include the antioxidants vitamins C and E and the carotenoids.

Dietary sources of antioxidant vitamins

The most important antioxidant in cell membranes is \( \alpha \)-tocopherol, the major member of the vitamin E family. This molecule acts as a ‘chain-breaking antioxidant’, intercepting lipid peroxyl radicals and so terminating lipid-peroxidation chain reactions. Vitamin E is found in many dietary fats and oils, especially those containing PUFA (Table 9.2). Thus, the dietary intake of vitamin E is related to the intake of PUFA. Intakes among adults in the UK vary between 3.5 and 19.5 (median 9.3) mg \( \alpha \)-tocopherol equivalents day\(^{-1}\) for men and

Table 9.2. Dietary sources of antioxidant vitamins.

<table>
<thead>
<tr>
<th>Vitamin C</th>
<th>Citrus fruits, blackcurrants, kiwi fruit, strawberries</th>
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</thead>
<tbody>
<tr>
<td>Vitamin E</td>
<td>Whole grains, vegetable oils, wheat germ, eggs</td>
</tr>
<tr>
<td>Carotenoids</td>
<td></td>
</tr>
<tr>
<td>( \beta )-carotene</td>
<td>Carrots, broccoli, watercress, spinach, apricots</td>
</tr>
<tr>
<td>Lycopene</td>
<td>Tomatoes and processed tomato products (sauce and paste)</td>
</tr>
<tr>
<td>Lutein</td>
<td>Peas, spinach, broccoli and dark green leafy vegetables</td>
</tr>
<tr>
<td>( \beta )-cryptoxanthin</td>
<td>Mandarins, satsumas, apricots, orange peppers</td>
</tr>
</tbody>
</table>
between 2.5 and 15.2 (median 6.7) mg day\(^{-1}\) \(\alpha\)-tocopherol equivalents for women (Department of Health, 1991). Another group of lipid-soluble compounds that can act as antioxidants are the carotenoids, such as \(\beta\)-carotene, lycopene and lutein, found in highly pigmented fruits and vegetables (Mangels et al., 1993). The major water-soluble free radical scavenger is ascorbic acid (vitamin C), which also plays a role in 'sparing' vitamin E, by regenerating \(\alpha\)-tocopherol from the oxidized tocopheroxyl radical (Bendich et al., 1986). The estimated average requirement for vitamin C in adults in the UK is 25 mg day\(^{-1}\) (Department of Health, 1991). More recently, attention has also focused on the antioxidant properties of plant polyphenols, found in tea and red wines (Rice-Evans, 1995), but considerably more information on the absorption, metabolism and excretion of these compounds in humans is required before their relative contribution to preventing oxidative damage can be assessed.

A balanced diet, containing at least five or six varied portions of fruits and vegetables per day, should provide an adequate supply of antioxidants for healthy individuals. Concerns regarding the taking of supplements centre around the possibilities that certain compounds might have a toxic effect if taken in doses significantly higher than can be obtained from a healthy diet and that a reliance on supplements will lead to a reduced consumption of fresh fruit and vegetables, which probably contain a multitude of compounds whose health benefits we have yet to appreciate. However, in elderly individuals, whose diet might be restricted (e.g. by loss of appetite, dental conditions) and where absorption of nutrients is impaired, there might be a case for supplementation with certain nutrients. The case is probably strongest for vitamin E, because it is impossible to obtain high intakes of this nutrient without consuming a high-fat diet. Table 9.2 identifies dietary sources of antioxidant vitamins.

Antioxidant Vitamins and Immune Function

Vitamin C and immune function

Vitamin C is found in high concentrations in white blood cells and is rapidly utilized during infection; reduced plasma concentrations are often associated with reduced immune function (see Siegel, 1993). Animal and human studies have suggested that the dietary requirements for vitamin C are increased in cancer, surgical trauma and infectious diseases (see Siegel, 1993). The belief that high intakes of vitamin C will prevent the onset of the common cold has not been substantiated scientifically, although the associated symptoms following infection appear to be reduced by a moderate intake (Coulehan et al., 1974). Pauling's claims regarding the effects of vitamin C on the common cold (Pauling, 1970) inspired a great deal of research into its effect on immune function in the 1970s and early 1980s (reviewed by Thomas and Holt, 1978; Siegel, 1993). Vitamin C deficiency in the guinea pig impairs lymphocyte proliferation, the delayed-type hypersensitivity (DTI) response to tuberculin, the ability of neutrophils to kill bacteria and the
activity of cytotoxic T-cells and delays the rejection of skin allografts, but has little effect on antibody responses (for references, see Siegel, 1993). Providing vitamin C to mice increased spleen lymphocyte proliferation in response to mitogens but did not affect natural killer (NK)-cell activity or the antibody response to sheep red blood cells or lipopolysaccharide (LPS) (see Siegel, 1993). Vitamin C decreased or slowed tumour development in some animal models, but not others (see Siegel, 1993). Vitamin C deficiency in humans did not impair lymphocyte proliferation or alter the number of CD4+ or CD8+ cells in the circulation (Kay et al., 1982).

However, Vitamin C (1–5 g daily for 3 days to several weeks) increased human T lymphocyte proliferation (Yonemoto et al., 1976; Anderson et al., 1980; Panush et al., 1982) and neutrophil motility towards LPS-activated autologous serum (Anderson et al., 1980). Some studies indicate that vitamin C increases circulating immunoglobulin (Ig) levels in humans (Prinz et al., 1977; Vallance, 1977; Ziemlanski et al., 1986), but other studies fail to show this (Anderson et al., 1980; Panush et al., 1982; Kennes et al., 1983). Jacob et al. (1991) studied the effect of vitamin C at different levels in the diet on immune function in a group of young, healthy non-smokers. The subjects first consumed a vitamin C-deficient diet and then gradually increased their vitamin C intake (from 5 to 250 mg day\(^{-1}\)). The vitamin C-deficient diet decreased plasma and white-cell vitamin C concentrations by 50% and decreased the DTH response to seven recall antigens, but did not alter lymphocyte proliferation. Sixty or 250 mg vitamin C day\(^{-1}\) led to recovery of the DTH response, but did not affect lymphocyte proliferation. The authors suggest that the inconsistency regarding the influence of vitamin C on these two outcomes, both indicators of cell-mediated immunity, may result from the higher sensitivity of the DTH test, involvement of cells other than those isolated for in vitro cultures in the in vivo DTH response or other unknown factors. The lack of an effect on lymphocyte proliferation at an intake of 250 mg day\(^{-1}\) suggests that, at least in young individuals, only levels of vitamin C that approach pharmacological doses can produce a quantifiable effect on this parameter of immune function. It has been suggested that vitamin C intakes of 600 mg day\(^{-1}\) may be beneficial in reducing infections in individuals who undertake a large amount of physical activity: for instance, studies of marathon runners have found a significantly lower incidence of post-race upper respiratory infections in runners taking a daily supplement of 600 mg vitamin C (Peters, 1997).

One of the major problems in assessing the beneficial effects of dietary components on the immune system is the lack of a reliable marker of immune function that is known to be indicative of a long-term beneficial effect in terms of reducing the incidence of degenerative disorders in later life. Although not an immunological one, one recent study does provide an excellent example of the potential need to maintain adequate intakes of antioxidant nutrients in the middle years of life to prevent the accumulative damage caused by ROS being made manifest in later years. Jacques et al. (1997) examined the cross-sectional relationship between age-related lens opacities and vitamin C supplement use over a 10–12-year period in women without diagnosed cataract or diabetes. Use of vitamin C supplements for 10 years or more was associated with a 77% lower prevalence of early lens opacities and an 83% lower prevalence of moderate lens opacities, compared with women who did not use sup-
plements. Women who consumed vitamin C supplements for less than 10 years showed no evidence of a reduced prevalence of early opacities, suggesting that long-term consumption of vitamin C supplements may substantially reduce the development of age-related lens opacities. While the use of supplements might be required to obtain sufficient intakes of vitamin C to prevent this form of oxidative damage, it is hoped that the intake required to maintain optimal immune function can be obtained from a healthy diet containing fruit and vegetables rich in antioxidants. This should be the case, since epidemiological studies of populations having a lower incidence of cancer suggest that the benefits are associated with the intake of increased amounts of these foodstuffs rather than the taking of supplements (Block et al., 1992).

Vitamin C has been used to treat some clinical phagocytic cell dysfunctions. In Chediak–Higashi syndrome, which is characterized in part by defective neutrophil functions, vitamin C supplementation has been shown to increase neutrophil chemotaxis, improve bactericidal activity and reduce the length of clinical illness (Boxer et al., 1976). Vitamin C also appears to be beneficial in the treatment of chronic granulomatous disease (Anderson, 1982).

Vitamin C provides important antioxidant protection for plasma lipids and lipid membranes and can also neutralize phagocyte-derived oxidants released extracellularly, thereby preventing oxidant-mediated tissue damage, particularly at sites of inflammatory activity. Other mechanisms that have been proposed for the immunostimulatory effects of vitamin C include modulation of intracellular cyclic-nucleotide levels, modulation of prostaglandin synthesis, enhancement of cytokine production, antagonism of the immunosuppressive interaction between histamines and white blood cells and the protection of 5-lipoxygenase (Anderson et al., 1990). There is a need for further research, not only into the mechanisms by which vitamin C can enhance immune-cell function, but also to define the level of intake required to maintain an optimal immune responsiveness throughout life and to reduce the incidence of degenerative disorders in later life.

**Vitamin E and immune function**

Since vitamin E is the most effective chain-breaking, lipid-soluble antioxidant present in cell membranes, it is considered likely that it plays a major role in maintaining cell membrane integrity by limiting lipid peroxidation by ROS.

Studies conducted in humans and animals, using either states of deficiency or supra-dietary levels, suggest strongly that vitamin E is involved in maintaining immune cell function (for a review, see Meydani and Beharka, 1998). For example, Canadian 3-year-olds with the lowest serum vitamin E levels had the lowest lymphocyte proliferative responses and serum IgM concentrations (Vobecky et al., 1984). In addition, there was a positive association between plasma vitamin E levels and DTH responses and a negative association between plasma vitamin E levels and incidence of infections in healthy adults aged over 60 (see Chavance et al., 1989). Administration of vitamin E to premature infants enhanced neutrophil phagocytosis (Baehner et al., 1977; Chirico et al., 1983) but decreased the ability of neutrophils to kill bacteria
(Baehner et al., 1977); this latter effect is most probably due to a vitamin E-induced decrease in production of free radicals and related reactive species. In laboratory animals, vitamin E deficiency decreased spleen lymphocyte proliferation in response to mitogens, NK-cell activity, specific antibody production following vaccination and phagocytosis by neutrophils (for a review, see Meydani and Beharka, 1998). Vitamin E deficiency also increases susceptibility of animals to infectious pathogens (for references, see Meydani and Beharka, 1998; Han and Meydani, 1999). Vitamin E supplementation of the diet of laboratory animals enhances antibody production, lymphocyte proliferation, NK-cell activity, and macrophage phagocytosis (for references, see Meydani and Beharka, 1998). Dietary vitamin E promoted resistance to pathogens in chickens, turkeys, mice, pigs, sheep and cattle (for references, see Meydani and Beharka, 1998; Han and Meydani, 1999); some of these studies report improved immune-cell functions in the animals receiving additional vitamin E (see Han and Meydani, 1999). For example, vitamin E prevented the retrovirus-induced decrease in production of interleukin-2 (IL-2) and interferon-γ (IFN-γ) by spleen lymphocytes and in NK-cell activity in mice (Wang et al., 1994).

One application of the effects of vitamin E on immune function is in the elderly. This has been investigated in both murine models and human trials. Adding vitamin E to the diet of aged mice increased lymphocyte proliferation, IL-2 production and the DTH response (Meydani et al., 1986). A high level of vitamin E in the diet (500 mg kg⁻¹ food) also increased NK-cell activity of spleen cells from old (but not young) mice (Meydani et al., 1988). In another study, young and old mice were fed diets containing adequate (30 mg kg⁻¹ diet) or high (500 mg kg⁻¹ diet) levels of vitamin E for 6 weeks and infected with influenza A virus: young mice and old mice fed the high level of vitamin E had lower lung titres of virus than old mice fed the adequate vitamin E diet (Hayek et al., 1997). The high level of vitamin E caused increased production of IL-2 and IFN-γ by spleen lymphocytes from influenza-infected old mice (Han et al., 1998; Han and Meydani, 2000). Supplementation of the diet of elderly human subjects with 800 mg vitamin E day⁻¹ for 4 weeks increased lymphocyte proliferation stimulated by concanavalin A, IL-2 production and the DTH response, but did not affect IL-1 production, the number of CD4 cells or circulating Ig concentrations (Meydani et al., 1990). In a more recent study, 60, 200 and 800 mg vitamin E day⁻¹ increased DTH response in elderly subjects, with 200 mg day⁻¹ having the maximal effect (Meydani et al., 1997). The two higher vitamin E doses improved antibody responses to hepatitis B, but only the 200 mg day⁻¹ dose increased the antibody response to tetanus toxoid (Meydani et al., 1997). The authors conclude that 200 mg vitamin E day⁻¹ represents the optimal level for the immune response. In another study, young and elderly individuals were supplemented with 800 mg vitamin E day⁻¹ for 48 days before being asked to run down an inclined treadmill for 45 min. Vitamin E supplementation was found to eliminate the age-associated difference in exercise-induced neutropenia, to prevent the exercise-induced increase in IL-1 production and to inhibit IL-6 production (Cannon et al., 1991). Since these cytokines are involved in the inflammatory process and in exercise-induced muscle damage, their inhibition by vitamin E during exercise might have important implications. However, on a
cautious note, studies have reported that prolonged high intakes of vitamin E (> 1000 mg day\(^{-1}\)) can lead to inhibition of neutrophil phagocytosis (Boxer, 1986). Further research is needed to assess the optimal intake of this nutrient required to provide benefit for different groups of individuals.

Cigarette smoke contains millions of free radicals per puff, and other compounds present can stimulate the formation of other highly reactive molecules (Pryor and Stone, 1993). Serum levels of vitamin E (as well as of vitamin C and \(\beta\)-carotene) and lung vitamin E concentrations are significantly lower in smokers compared with non-smokers and even supplementation with 2400 mg \(\alpha\)-tocopherol equivalents day\(^{-1}\) for 3 weeks failed to restore the lung vitamin E level to that found in non-smokers (Pacht et al., 1986). Circulating phagocytes from smokers produce high levels of free radicals, which probably in part accounts for the depressed immune function observed in smokers (Johnson et al., 1990), and there is some evidence that vitamin E supplementation can reduce the overproduction of ROS by phagocytic cells from smokers (Richards et al., 1990).

Reduced vitamin E status has also been reported in human immunodeficiency virus (HIV)-infected individuals. Passi et al. (1993) found that plasma vitamin E concentrations were significantly lower in a group of 200 HIV-positive individuals compared with controls, but whether this is related to an inadequate intake of this vitamin is unclear. Dietary diaries from a group of 100 HIV-infected asymptomatic men did not indicate an inadequate intake of vitamin E, but plasma levels were low or marginally low in 74% of the men (Beach et al., 1992). In a study of patients who had developed acquired immune deficiency syndrome (AIDS), an inverse relationship was observed between serum vitamin E levels and severity of disease (Favier et al., 1994). A recent study of 49 HIV-infected subjects provided with vitamin E and vitamin C observed a significant reduction in oxidative stress and a trend towards a reduction in viral load after 3 months (Allard et al., 1998). These studies suggest that larger trials of these and other antioxidant nutrients in the treatment of HIV-infected persons should be encouraged, since there is a need to find alternative, cheaper, treatments than the combination therapies currently employed.

In terms of mechanisms of action, in addition to its role as a protective antioxidant, vitamin E, at higher intakes, is associated with a reduced production of prostaglandin E\(_2\) (PGE\(_2\)) (e.g. Meydani et al., 1986, 1988, 1990). Since PGE\(_2\) inhibits lymphocyte proliferation and NK-cell activity, it is possible that this may be one immunomodulatory mechanism of vitamin E action. It is also possible that vitamin E and, indeed, other antioxidant nutrients can influence a variety of inflammatory processes by inhibiting the activity of a transcription factor called nuclear factor kappa B (NF\(\kappa\)B). Transcription factors are intracellular regulators of gene expression. Once activated, the transcription factor binds to the promoter region of a specific gene within the DNA in the nucleus, resulting in that gene being ‘turned on’. NF\(\kappa\)B is required for maximal transcription of many proteins that are involved in inflammatory responses, including several cytokines, such as IL-1\(\beta\), IL-2 and tumour necrosis factor (TNF)-\(\alpha\). NF\(\kappa\)B is a redox-sensitive transcription factor and it is thought that the generation of ROS is a vital link in mediating NF\(\kappa\)B activation by a variety of stimuli (Lavrovsky et al., 2000).
Carotenoids and immune function

The carotenoids are a group of over 600 naturally occurring coloured pigments that are widespread in plants, of which only about 20 commonly occur in human foodstuffs. In nature, they serve two essential functions: as accessory pigments in photosynthesis, and in photoprotection. These two functions are achieved through the chemical structure of carotenoids (Fig. 9.1), which allows the molecules to absorb light and to quench singlet oxygen and free radicals.

Many epidemiological studies have shown an association between diets rich in carotenoids and a reduced incidence of many forms of cancer, and it has been suggested that the antioxidant properties of these compounds are a causative factor (Block et al., 1992). Since the publication of an article by Peto et al. (1981), a great deal of attention has focused on the potential role of one particular carotenoid, β-carotene, in preventing cancer. Numerous publications have described epidemiological studies, in vitro experiments, animal studies and clinical trials that suggest that this carotenoid can protect against not only cancer, but also other oxidative damage-associated disorders, listed in Table 9.1 (reviewed by Mayne, 1996). Because the immune system plays a major role in the prevention of cancer, it has been suggested that β-carotene may enhance immune cell function (Bendich and Olson, 1989). In animals, adding carotenoids to the diet prevented stress-related thymic involution, increased the number of circulating lymphocytes, enhanced lymphocyte proliferation and cytotoxic T-cell activity and increased resistance to infective pathogens (for references, see Roe and Fuller, 1993).

Several studies have examined the effect of β-carotene on human immune function. Various doses of β-carotene have been employed in these studies, ranging from 15 mg day$^{-1}$, which could be achieved through the diet, up to pharmacological doses of 180 mg day$^{-1}$, provided over periods of 14–365 days. These studies

![Fig. 9.1. Chemical structure of some carotenoids found in the diet.](image-url)
have reported increases in the numbers of CD4+ cells or in the ratio of CD4+ to CD8+ cells in the circulation, in the percentages of lymphocytes expressing the IL-2 and transferrin receptors, and in NK-cell activity (Alexander et al., 1985; Watson et al., 1991; Murata et al., 1994), particularly in elderly subjects. The potential for increasing the numbers of CD4+ cells led to the suggestion that β-carotene might be useful as an immunoenhancing agent in the treatment of HIV infection. Preliminary studies have shown a slight but insignificant increase in CD4+ numbers in response to β-carotene (60 mg day$^{-1}$ for 4 weeks) in patients with AIDS (Fryburg et al., 1995), but long-term effectiveness has not been reported.

Other investigators have been unable to confirm the increase in T-cell-mediated immunity in healthy individuals following β-carotene supplementation. Santos et al. (1996, 1997) reported the results of two studies in the elderly: a short-term, high-dose study (90 mg day$^{-1}$ for 21 days) in women and a longer-term, lower-dose trial (50 mg alternate day$^{-1}$ for 10–12 years) in men. Both studies concluded that there was no significant difference in T-cell function as assessed by DTH response, lymphocyte proliferation, IL-2 and PGE$_2$ production and composition of lymphocyte subsets (Santos et al., 1997). However, these workers also examined the effect of β-carotene supplementation on NK-cell activity in the longer-term trial with male volunteers and observed that supplementation of the diet of older males (> 65 years) with β-carotene resulted in significantly greater NK-cell activity compared with subjects of a similar age given placebo treatment (Santos et al., 1996). Since patients with Chediak–Higashi syndrome, a disorder associated with defective NK-cell function, show a higher susceptibility to tumour formation (Roder et al., 1980), and homozygous mice genetically deficient in NK cell activity grow tumours and develop leukaemia more rapidly than do heterozygous littermates with normal NK-cell function (Lotzova, 1993), the enhancing effect of β-carotene on NK-cell activity has been postulated to be a link between raised intakes of this nutrient and cancer prevention. As shown in Fig. 9.2, the study by Santos et al. (1996) highlighted both the reduction in NK-cell activity that is observed with age and the fact that the increase in NK-cell activity observed in older males (65–86 years) following β-carotene supplementation restored it to the level seen in a group of younger males (51–64 years). The mechanism for this is unclear, but it was not due to an increase in the percentage of NK cells or to an increase in IL-2 production. The authors suggest that β-carotene may be acting directly on one or more of the lytic stages of NK-cell cytotoxicity or on NK-cell activity-enhancing cytokines other than IL-2, such as IL-12.

Individuals who are repeatedly exposed to UV light show suppression of immune function (Rivers et al., 1989). Because carotenoids can provide photoprotection, several studies have assessed the ability of β-carotene to protect the immune system from UV-induced free radical damage. In one study, a group of young males were placed on a low-carotenoid diet (< 1.0 mg day$^{-1}$ total carotenoids) and given either placebo or 30 mg β-carotene day$^{-1}$ for 28 days prior to periodic exposure to UV light. DTH responses were significantly suppressed in the placebo group after UV treatments and the suppression was inversely proportional to plasma β-carotene concentrations in this group (Fuller et al., 1992). In contrast, no significant suppression of DTH responses was seen in the β-carotene-treated group (Fuller et al., 1992). The ability of β-carotene
to protect against the harmful effects of natural UV sunlight has also been demonstrated by exposing healthy female students to time- and intensity-controlled sunlight exposure: a Berlin-based study involved taking volunteers to the Red Sea and exposing areas of their skin to the sunlight by lifting discretely placed flaps in their specially designed swimsuits (Gollnick et al., 1996).
Since antigen-presenting cells initiate cell-mediated immune responses, one aspect of the immune-enhancing effect of β-carotene might be improved antigen-presenting cell function. A prerequisite for antigen presentation is the expression of major histocompatibility complex (MHC) class II molecules (human leucocyte antigen (HLA)-DR, HLA-DP and HLA-DQ) (Bach, 1985), which are present on the majority of human monocytes. The antigenic peptide is presented to the T-helper lymphocyte within a groove of the MHC class II molecule (Fig. 9.3). Since the degree of immune responsiveness of an individual has been shown to be proportional to both the percentage of MHC class II-positive monocytes and the density of these molecules on the cell surface (Janeway et al., 1984), it is possible that one mechanism by which β-carotene may enhance cell-mediated immune responses is by enhancing the cell surface expression of these molecules. In addition, cell-to-cell adhesion is critical for the initiation of a primary immune response, and it has been shown that the intercellular adhesion molecule 1 (ICAM-1)–leucocyte function-associated antigen-1 (LFA-1) ligand–receptor pair is also capable of co-stimulating an immune response (Springer, 1990), enhancing T-cell proliferation and cytokine production.

![Fig. 9.3.](image) Cell surface molecules involved in the initiation of cell-mediated immune responses. LFA, leucocyte function-associated antigen; ICAM-1, intercellular adhesion molecule 1; HLA, human leucocyte-associated antigen; MHC, major histocompatibility complex.
The effect of β-carotene supplementation (15 mg day\(^{-1}\) for 26 days; equivalent to 150 g carrots day\(^{-1}\)) on expression of MHC class II and adhesion molecules on the monocyte surface has been investigated. Following dietary supplementation, there were significant increases in plasma levels of β-carotene and in the percentages of monocytes expressing the MHC class II molecule HLA-DR and the adhesion molecules ICAM-1 and LFA-3 (Hughes et al., 1997). These results suggest that moderate increases in the dietary intake of β-carotene can enhance cell-mediated immune responses within a relatively short period of time, providing a potential mechanism for the anti-carcinogenic properties attributed to this compound. The increase in surface molecule expression may also, in part, account for the ability of β-carotene to prevent the reduction in DTH response following exposure to UV radiation, since the latter can inhibit both HLA-DR and ICAM-1 expression. This finding could certainly be relevant to the preventive action of β-carotene towards skin cancer (Mathews-Roth, 1989), since immunosuppressed individuals, such as renal-transplant patients, have an increased risk of skin cancer.

As well as preventing oxidative damage, it has been suggested that β-carotene, like vitamin E, can influence immune cell function by modulating the production of PGE\(_2\). This eicosanoid is the major prostaglandin (PG) synthesized by monocytes and macrophages and is known to possess a number of immunosuppressive properties (see Calder and Field, Chapter 4, this volume). It has been suggested that β-carotene might enhance immune responses by altering the activation of the arachidonic acid cascade (from which PGE\(_2\) is derived), since it has been shown to be capable of suppressing the generation of arachidonic acid products \textit{in vitro} from non-lymphoid tissues (Halevy and Sklan, 1987). This possibility requires further investigation.

There have been very few studies examining the influence of carotenoids other than β-carotene on human immune function, even though there is strong epidemiological evidence to suggest that lycopene (found in tomatoes) and lutein (found in peas, watercress and other vegetables) can protect against the development of prostate and lung cancer, respectively (Le Marchand \textit{et al.}, 1993; Gann \textit{et al.}, 1999). In addition, tomato intake has been found to be inversely associated with the risk of diarrhoeal and respiratory infections in young children in Sudan (Fawzi \textit{et al.}, 2000). In terms of mechanisms of action, the effect of dietary supplementation with lycopene and lutein on the expression of monocyte surface molecules involved in antigen presentation has been investigated. It was found that these carotenoids appear to be less influential than β-carotene, when given at the same level in the diet (Hughes \textit{et al.}, 2000). In addition, enriching the diet with lycopene (by drinking 330 ml of tomato juice daily) for 8 weeks did not modify cell-mediated immune responses in the elderly (Watzl \textit{et al.}, 2000). In another study, performed in a group of older volunteers (over 65 years) living in Ireland, the effects of placebo, β-carotene (8.2 mg day\(^{-1}\)) and lycopene (13.3 mg day\(^{-1}\)) for 12 weeks on various parameters of cell-mediated immunity were examined. There were no significant changes in circulating T-cell subsets, mitogen-stimulated lymphocyte proliferation or surface molecule expression following any of these interventions, in spite of significant increases in the plasma levels of the carotenoids (Corridan \textit{et al.}, 2001). The
authors concluded that in well-nourished, free-living, healthy individuals, supplementation with relatively low levels of β-carotene or lycopene is not associated with either beneficial or detrimental effects on cell-mediated immunity.

Other investigators have shown an opposing effect of β-carotene and lutein upon human lymphocyte proliferation (Watzl et al., 1999), emphasizing further the fact that different carotenoids might affect immune function in different ways. Therefore, in fruits and vegetables, the influence of the combination of carotenoids they contain on immune function may represent the sum total of these different effects and, indeed, the potential for synergistic effects remains to be investigated.

One possible factor to explain the different effects seen with different carotenoids might be the preferred location of these compounds within the cell and within the body. Carotenoids are lipid-soluble and thus it is thought that most will be concentrated in the lipid-rich membranes of the cell. However, their exact location may influence their effectiveness in modulating specific cellular events. Within the body, lycopene appears to be selectively taken up within the prostate, a finding that may help explain the association between higher intakes of lycopene and a reduced incidence of prostate cancer (Giovannucci, 1999). Thus, it is possible that tests on peripheral blood cells to determine immune function will not detect any localized effects, suggesting that there might be ‘hidden’ benefits associated with certain dietary components that we have yet to discover.

The strongest epidemiological evidence supporting a beneficial effect of carotenoids in preventing cancer is the protective effect of β-carotene intake in reducing the incidence of cancer of the lung. Carotenoid intake has been associated with a reduced lung-cancer risk in eight of eight prospective studies and in 18 of 20 retrospective studies (for a review, see Zeigler et al., 1996). As a result, three major intervention trials were initiated, examining the efficacy of β-carotene in the prevention of lung cancer (Alpha-tocopherol Beta-carotene Cancer Prevention Study Group, 1994; Hennekens et al., 1996; Omenn et al., 1996). The failure of these trials to show a protective effect, with two of the studies showing an increase in lung cancer in smokers receiving β-carotene supplementation, has been widely publicized. The mechanism for the increased lung-cancer risk associated with the supplementation is unclear, but several suggestions have been made. Since the participants in these studies could be classified as ‘high-risk’ for developing lung cancer (long-term smokers or previously exposed to asbestos), it is possible that many of them had undetected tumours prior to the commencement of supplementation. The stage (or stages) of carcinogenesis that β-carotene might be effective against is unclear, but, if the effect is mediated via the immune system, it is likely to occur during the promotional stages preceding the formation of a malignant tumour. A recent analysis of the Cancer Prevention Study II (CPS-II), a prospective mortality study of more than 1 million US adults, investigated the effects of supplementation with multivitamins and/or vitamins A, C and/or E on mortality during a 7-year follow-up period. The use of a multivitamin plus vitamins A, C and/or E significantly reduced the risk of cancer in former smokers and in never-smokers, but increased the risk of lung cancer in male smokers who had used a
multivitamin plus vitamins A, C and/or E, compared with men who had reported no vitamin supplement use. Interestingly, in this study, no association with smoking was seen in women (Watkins et al., 2000).

One of the major unresolved dilemmas of research into β-carotene is what intake is required for optimal immune function and other health-related properties. Most studies of this compound have been undertaken at levels that are not achievable within a normal healthy diet. It is still unclear whether different intakes are associated with different outcomes or, in mechanistic studies, with different effects on various aspects of immune function. In addition, there remains the possibility that, at supra-dietary levels, β-carotene may exhibit pro-oxidant activity, particularly in the presence of high oxygen tensions, as occur in the lungs (reviewed by Palozza, 1998). Of course, the probability remains that the apparent protection of consuming a diet rich in fruits and vegetables is the result of a multifactorial effect of a number of components of these foods. In support of this, two of the prospective studies mentioned above found that higher plasma β-carotene concentrations upon entry into the trials, resulting from dietary consumption, as opposed to taking supplements, were associated with a lower risk of lung cancer (McDermott, 2000). Greater emphasis should be placed on studying the effects of enriching the diet with antioxidants via real foodstuffs rather than by supplementation.

Conclusions

Because the immune system is critically dependent on accurate cell–cell communication in order to mount a response, immune cell integrity is essential. Antioxidant nutrients help to maintain this integrity, reducing the damage caused by reactive oxygen species to cell membranes and their associated receptors, as well as modulating immune cell function by influencing the activity of redox-sensitive transcription factors and the production of cytokines and PGs. The effects of antioxidants appear to be particularly beneficial during periods of oxidative stress, whether the periods are acute, such as during infections, or chronic, such as in the elderly. However, the results of the prospective studies with β-carotene in smokers show that caution must still be taken in making recommendations regarding the taking of supplements that provide a greater intake than can be achieved by eating a diet rich in fruits and vegetables. In this regard, it is important to remember that the strongest evidence supporting a beneficial effect of antioxidant nutrients in reducing the risk of developing chronic oxidative stress-related disorders has come from epidemiological studies of populations consuming whole foods and not from supplementation studies. Further research needs to be undertaken to examine the interaction between different antioxidant nutrients and to establish the levels of intake required to optimize immune responsiveness in different sectors of the population (e.g. the elderly, cigarette smokers). In addition, greater emphasis should be placed on studying the effects of enriching the diet with antioxidants via real foodstuffs rather than by supplementation, since these foods undoubtedly contain beneficial compounds that we have still to discover.
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References


10 Zinc, Infection and Immune Function

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Introduction

Animal studies in the 1930s first documented the essential requirement of zinc (Zn) for the growth and survival of animals (Todd et al., 1934). It was not until the 1960s that the importance of Zn deficiency for human populations was appreciated (see Prasad, 1991). Later, it became clear that Zn was also crucial for patients maintained on parenteral nutrition (Kay and Tasman-Jones, 1975). A central clinical feature of Zn deficiency in humans was the increased susceptibility to infectious diseases. Studies in the Middle East revealed that most Zn-deficient dwarfs succumbed to infections before they reached 25 years of age, leading researchers to speculate that Zn must be important for host immunity. The last two decades have witnessed a rapid growth in knowledge of the underlying mechanisms whereby Zn exerts its ubiquitous effects on immune function and disease resistance. The effects of Zn deficiency and of Zn supplementation on immune cells underscore the essential role of Zn in the normal development and function of many key tissues, cells and effectors of immunity. In vitro studies have elucidated the role of Zn at the cellular level and recent advances in molecular and cell biology have begun to clarify the role of Zn in gene expression, mitosis and apoptosis of lymphoid cells. It is clear that even mild Zn deficiency can impair multiple mediators of host immunity, ranging from the physical barrier of the skin to acquired cellular and humoral immunity (see Frost et al., 1977; Oleske et al., 1979; Good, 1981; Walsh et al., 1994).

Zinc Deficiency

Most studies indicate that, among healthy adults consuming Western-style diets, Zn intake is in the range 8–12 mg day\(^{-1}\); mean Zn intakes among adults in the UK are 11.4 mg day\(^{-1}\) for men and 8.4 mg day\(^{-1}\) for women (Department of
Health, 1991). The estimated average requirement for adults in the UK is about 7 (men) and 5.5 (women) mg day\(^{-1}\) (Department of Health, 1991). In persons suffering from marginal Zn deficiency (intake less than 5 mg day\(^{-1}\)), clinical signs consist of depressed immunity, impaired taste and smell, onset of night-blindness, impairment of memory and decreased spermatogenesis in males (Prasad et al., 1961; Sandstead et al., 2000). Severe Zn deficiency is characterized by severely depressed immune function, frequent infections, bullous pustular dermatitis, diarrhoea, alopecia and mental disturbances (Barnes and Moynahan, 1973). Similar effects of mild and severe Zn deficiency arise in Zn-deficient laboratory animals (see Shankar and Prasad, 1998). A rare genetic disorder, known as acrodermatitis enteropathica (AE), occurs in cattle and humans, resulting in decreased Zn absorption, accompanied by characteristic hyperpigmented skin lesions, poor growth and low plasma Zn levels (Walsh et al., 1994). It is estimated that nutritional Zn deficiency may affect approximately 2000 million people in the developing world.

The Cell Biology of Zinc with Relevance to the Immune System

Zinc and the cell cycle

Treatment of lymphocytes with mitogens results in a fairly rapid increase in cellular Zn (see Zalewski, 1996; Shankar and Prasad, 1998; Prasad, 2000a). These findings are consistent with studies indicating a requirement for Zn during the mid to late G1 phase of the cell cycle in promotion of thymidine kinase expression (Chesters et al., 1993) and in another less well-defined step involved in cell transition to S phase. Activated lymphocytes take up Zn via multiple mechanisms, including receptors for Zn-transferrin, metallothionein, albumin and \(\alpha_2\)-macroglobulin (see Walsh et al., 1994; Shankar and Prasad, 1998; Prasad, 2000a) and also by other less well-characterized mechanisms, such as anionic channels or transporters. The Zn-dependent activity of DNA polymerase may account, in part, for the influence of Zn during the S phase of the cell cycle. Zn may also play a role in transition to the G2 and M phases. A greater proportion of S- compared with G2-phase cells was observed among mitogen-stimulated lymphocytes from mildly Zn-deficient patients suffering from sickle-cell anaemia (Prasad, 2000a). The ratio returned to normal following a period of Zn supplementation. The M phase of the cell cycle may also be affected by Zn deficiency, since defective tubulin polymerization is seen in tissues from Zn-deficient animals (see Shankar and Prasad, 1998), and Zn is known to bind the N-terminal of tubulin, thereby stabilizing microtubule formation.

Zinc and cell replication

Zn influences the activity of multiple enzymes, which act at the very basic levels of replication and transcription. These include DNA polymerase, thymidine kinase, DNA-dependent RNA polymerase, terminal deoxynucleotidyl trans-
Zinc and immune function

Zinc and aminoacyl tRNA synthetase (Walsh et al., 1994; Zalewski, 1996; Shankar and Prasad, 1998), and the family of transcriptional regulators known as Zn-finger DNA-binding proteins. In addition, Zn forms the active enzymatic sites of many metalloproteases. The activity of the major enzyme regulating DNA replication, DNA polymerase, is Zn-dependent. It is inhibited by Zn deficiency and Zn chelators and is enhanced by addition of low concentrations of Zn in vitro. Thymidine kinase, crucial for the synthesis of phosphorylated pyrimidines, is also very sensitive to dietary Zn depletion. Zn is, in fact, required for expression of multiple genes regulating mitosis, including thymidine kinase, ornithine decarboxylase and c-myc. Several transcription factors, such as nuclear factor kappa B (NFκB), metallothionein transcription factor 1 (MTF-1) and really interesting new gene (RING), contain Zn-finger-like domains, which may be influenced by changes in intracellular pools of Zn. In addition, Zn deprivation affects the activity of RNA polymerase, needed for transcription.

Zinc and lymphocyte activation

Zn plays a role in multiple aspects of T lymphocyte activation and signal transduction. Zn has been implicated in the non-covalent interaction of the cytoplasmic tails of CD4 and CD8 with the tyrosine kinase p56ck, an essential protein in the early steps of T-cell activation (Turner et al., 1990). Through this and possibly other pathways, Zn stimulates autophosphorylation of tyrosine residues by p56ck and subsequent phosphorylation of the T-cell-receptor complex involving CD45. Zn is also involved in the activity of phospholipase C to give rise to inositol trisphosphate and diacylglycerol (see Zalewski, 1996). In addition, Zn affects the phosphorylation of proteins mediated by protein kinase C. Subsequent changes through protein phosphorylation regulate activation and cell proliferation.

Zinc and apoptosis

The major mechanism of cell death in the body and in cell culture is apoptosis, a form of cell suicide characterized by a decrease in cell volume, dramatic condensation of the chromatin and cytoplasm and fragmentation of nuclear DNA. Apoptosis is a normal physiological process, enabling a variety of important processes, from epithelial turnover to T- and B-cell development. The dysregulation of such a basic process would, therefore, have important health consequences.

Zn-deficient animals exhibit enhanced spontaneous and toxin-induced apoptosis in multiple cell types (see Zalewski and Forbes, 1993; Shankar and Prasad, 1998). Thymic atrophy is a central feature of Zn deficiency (see later). It is now known that this atrophy is accompanied by apoptotic cell death of thymocytes. Several studies have demonstrated that Zn is a regulator of lymphocyte apoptosis in vivo and in vitro (see Zalewski and Forbes, 1993; Shankar and Prasad, 1998; Prasad, 2000a). Zn supplementation decreased mycotoxin-induced apoptosis of macrophages and T-cells in mice. In addition, Zn administration to mice 48 h prior to intraperitoneal injection of endotoxin (lipopolysaccharide (LPS)) greatly
abrogated subsequent apoptotic DNA cleavage in thymocytes and loss in thymic weight. In vitro, greater numbers of lymphocytes and thymocytes undergo apoptosis when cultured with Zn-free medium or with Zn chelators. Conversely, apoptosis of T lymphocytes induced by in vitro exposure to toxins and other agents is prevented by the addition of high concentrations of Zn salts. Cells could also be rescued from apoptosis with physiological levels (5–25 μM) of Zn salts if uptake was facilitated by the Zn ionophore pyrithione. It has been suggested that Zn is a major intracellular regulator of apoptosis, since lymphocytes maintain intracellular Zn at levels slightly above those needed to suppress apoptosis. In addition, a dose–response relationship exists between intracellular Zn levels and the degree of susceptibility to apoptosis.

The mechanisms whereby Zn affects apoptosis are not well understood, but it is likely that Zn acts at multiple levels. There is a good correlation between inhibition of Ca²⁺/Mg²⁺ DNA endonuclease activity and inhibition of apoptotic DNA fragmentation (see Shankar and Prasad, 1998). Although in vivo data are lacking, the Ca/Zn balance may regulate endonuclease activity. Nucleoside phosphorylase, another Zn-dependent enzyme, may inhibit apoptosis by preventing the accumulation of toxic nucleotides (Prasad, 1993). Likewise, poly (ADP-ribose) polymerase, the Zn-dependent nuclear enzyme, interacts with and inhibits the Ca²⁺/Mg²⁺ DNA endonuclease. In lymphocytes undergoing apoptosis, there is a large increase in cytoplasmic Zn, possibly originating from release of Zn from nuclear proteins.

**The Role of Zinc in Antioxidant Defence**

Zn plays a role in antioxidant defence, protecting cells from the damaging effects of oxygen radicals, which are generated during immune activation. Zn is a component of the cytosolic superoxide dismutase enzyme. Zn also regulates the expression of metallothionein and metallothionein-like proteins in lymphocytes, which have antioxidant activity (Prasad, 1993). Membrane Zn levels are strongly influenced by dietary Zn levels, and Zn concentrations in cell membranes appear to be important in preserving their integrity through poorly defined mechanisms involving binding to thiolate groups. Zn release from thiolate bonds can prevent lipid peroxidation. In addition, nitric oxide induces Zn release from metallothionein, the primary Zn-binding and transport protein in the body, which may limit free-radical membrane damage during inflammation. Indeed, Zn supplementation could prevent pulmonary pathology due to hyperoxia in rats (Taylor and Bray, 1991).

**Thymulin**

Thymulin is a nine-peptide hormone (Glu–Ala–Lys–Ser–Gln–Gly–Gly–Ser–Asn) secreted by thymic epithelial cells. Zn is bound to thymulin in a 1:1 stoichiometry via the side-chain of asparagine and the hydroxyl groups of the two serines. The binding of Zn results in a conformational change, which produces
the active form of thymulin. Thymulin binds to high-affinity receptors on T-cells and promotes T-cell maturation, cytotoxicity and interleukin (IL)-2 production (see Shankar and Prasad, 1998). Thymulin activity in vitro and in vivo in both animals and humans is dependent on plasma Zn levels, such that marginal changes in Zn intake or availability affect thymulin activity (Prasad et al., 1988; Shankar and Prasad, 1998). Thymulin is readily detectable in the serum of Zn-deficient patients, but is not active. The overall role of thymulin in the immunological lesions caused by Zn deficiency has not been well studied. The use of thymulin as an indicator of Zn deficiency has been suggested, although thymulin concentration is also modulated by Zn-independent factors. Assays regarding Zn status and thymulin Zn saturation may prove more useful, much as the index of transferrin saturation provides useful information regarding iron status.

Zinc and Immune Function

Effects of fetal zinc deficiency on immunological development

Gestational Zn deficiency in mice and non-human primates has short- and long-term deleterious effects on the offspring. Substantial reductions are seen in lymphoid organ size and circulating immunoglobulin (Ig) levels in pups born to marginally Zn-deficient mice (Beach et al., 1982). Additional murine studies showed that many of the immunodeficiencies seen at birth persisted into adulthood, despite the pups having been raised on a normal Zn diet after weaning (Beach et al., 1983). Indirect evidence for such effects in humans is also available. Intrauterine growth retardation, which has been linked to maternal Zn deficiency (Dutz et al., 1976), results in depressed cell-mediated immunity, which can persist for years (Dutz et al., 1976; Ferguson, 1978).

Effects of zinc deficiency on barrier function

Zn deficiency damages epidermal cells, resulting in the characteristic skin lesions of AE or severe Zn deficiency (see Shankar and Prasad, 1998). Damage to the linings of the gastrointestinal and pulmonary tracts is also observed during Zn deficiency (see Shankar and Prasad, 1998).

Effects of zinc on immune-cell numbers

Lymphopenia is common in Zn-deficient humans and animals and occurs in both the central and peripheral lymphoid tissues (Walsh et al., 1994). B-cell development in the bone marrow is adversely affected by Zn-deficiency (see Walsh et al., 1994; Shankar and Prasad, 1998). When mice were fed a marginally Zn deficient diet for 30 days, the number of nucleated bone-marrow cells was reduced by one-third, with a preferential reduction in small non-granular cells. The numbers of B-cells and their precursors were reduced by nearly 75%.
Losses were predominantly in pre-B and immature B-cells, which declined by about 50% and 25%, respectively. Thus, Zn deficiency blocks development of B-cells in the marrow, resulting in fewer B-cells in the spleen.

Studies of Zn deficiency in bovine, porcine, rat and murine models and in severely Zn-deficient children describe substantial reductions in the size of the thymus (see Shankar and Prasad, 1998; Prasad, 2000a). Mice maintained on a Zn-deficient diet for as little as 2 weeks showed moderate thymic involution. After 4 weeks, the thymus retained only 25% of its original size and, at 6 weeks, only a few thymocytes remained in the thymic capsule. Thymic atrophy exceeded that of other organs and overall weight loss, which declined only 20% by 6 weeks. The reduction in thymic size and cellularity was seen mostly in the thymic cortex, where immature thymocytes develop. Such changes were not observed in control animals, confirming that Zn was responsible for the effect. Following only 1 week of normal Zn intake, thymic size increased and cellular repopulation of the cortex was seen.

Adult mice maintained for 2 weeks on a Zn-deficient diet had reduced numbers of T and B lymphocytes in peripheral blood, lymph nodes and spleen; numbers of peripheral-blood lymphocytes (and macrophages) were eventually reduced by more than 50% (see Good, 1981; Shankar and Prasad, 1998). Even marginal Zn deficiency substantially suppresses the numbers of peripheral blood immune cells in mice and in humans (Moulder and Steward, 1989; Zalewski, 1996). Zn-deficient children with AE have reduced numbers of lymphocytes, particularly T-cells, in the blood and peripheral lymphoid tissues. Decreased CD4+/ CD8+ cell ratios are also seen. Recent studies in an experimental human model show that the percentage of CD8+CD73+ T lymphocytes (these are precursors to cytotoxic T lymphocytes (CTL)) is decreased in Zn deficiency (see Prasad, 2000a). This and the other effects described are reversed with Zn supplementation.

**Effects of zinc deficiency and repletion on immune-cell functions**

**Neutrophil functions**

Neutrophil chemotaxis and function are impaired in Zn-deficient animals and patients with AE and other types of Zn deficiency (see Walsh et al., 1994; Shankar and Prasad, 1998). These impairments are reversible by in vitro addition of Zn to the cells. In addition, in vitro addition of Zn improved the neutrophil response against *Staphylococcus*. One study observed that exercise-induced potentiation of superoxide formation by neutrophils was attenuated by Zn supplementation (Singh et al., 1994); this might be due to the role of Zn in superoxide dismutase.

**Monocyte/macrophage functions**

Effects on monocyte/macrophage function are also seen during Zn deficiency. In humans, the chemotactic response of monocytes from AE patients is suppressed and can be restored following addition of Zn to cells in vitro (see Walsh
et al., 1994; Shankar and Prasad, 1998). Monocytes from Zn-deficient mice have impaired killing of intracellular parasites, which is rapidly corrected in vitro by addition of Zn. Reduced macrophage phagocytosis of Candida has also been observed in deficient animals. In other studies, however, the ability of macrophages from Zn-deficient rodents to phagocytose particles either was enhanced and accompanied by greater numbers of Fc- and C3b-bearing cells or remained unchanged. High concentrations of Zn in vitro inhibited macrophage activation, mobility, phagocytosis and oxygen consumption. When marasmic children were rehabilitated with a Zn-containing regimen, monocyte phagocytic and fungicidal activity was suppressed (Schlesinger et al., 1993). Since elevated Zn levels can inhibit complement activation (Montgomery et al., 1979), complement-mediated phagocytosis may be adversely affected by high Zn levels. Additional studies are clearly needed to understand more fully the conditions under which Zn affects monocyte/macrophage phagocytosis.

Natural killer cell function

Human and animal studies showed decreased natural killer (NK)-cell activity in Zn deficiency (see Walsh et al., 1994; Shankar and Prasad, 1998; Prasad, 2000a). NK-cell function was depressed following treatment of cells with 1,10-phenanthroline, a Zn chelator, and was reversed by adddition of Zn but not Ca or Mg. Exogenous Zn also stimulated production of interferon (IFN)-γ by human peripheral-blood NK cells (Salas and Kirchner, 1987). However, exposure of NK cells to high levels of Zn in vitro inhibited cytotoxicity by rendering target cells more resistant to damage. This and other reports of Zn-mediated inhibition of NK activity may be partially explained by the demonstration that the NK-cell-inhibitory receptor requires Zn (Rajagopalan et al., 1995).

T- and B-cell functions

T-cell responses, such as proliferation in response to mitogens, cytotoxicity and delayed-type hypersensitivity (DTH), are suppressed during Zn deficiency and reversed by Zn supplementation (Good et al., 1976; Cunningham-Rundles et al., 1981; Moulder and Steward, 1989; see also Shankar and Prasad, 1998; Prasad, 2000a). Suppressed DTH responses in malnourished children are also restored following Zn supplementation (e.g. Golden et al., 1978). Patients receiving total parenteral nutrition devoid of Zn had reduced T-cell responses to mitogens, which returned to normal after 20 days of Zn supplementation (Allen et al., 1981). Mitogen-induced lymphocyte responses were greater after feeding rats for 14 days on a diet containing 0.1% by weight Zn than after feeding 0.004% Zn (see Chvapil et al., 1976; Shankar and Prasad, 1998). In an experimental human model of Zn deficiency, the production of IL-2 and IFN-γ was decreased, whereas the production of IL-4, IL-6 and IL-10 was not affected (Beck et al., 1997; Prasad et al., 1997, 1999; Prasad, 2000b). IL-2 production in patients with sickle-cell disease and Zn deficiency is decreased and Zn
supplementation results in increased production of IL-2 (Prasad et al., 1999). Thus, Zn deficiency in humans appears to be accompanied by an imbalance of T-helper-1 and T-helper-2 function.

Since NFκB binds to the promoter enhancer area of the IL-2 and interleukin 2 receptor alpha (IL-2Rα) genes, we investigated the effect of Zn deficiency on activation of NFκB and its binding to DNA in HUT-78, a Th0 malignant human lymphoblastoid cell line (Prasad et al., 2001). We showed for the first time that in Zn-deficient HUT-78 cells, phosphorylation of the NFκB-inhibitory subunit (IκB) and of IκB kinase, ubiquitination of IκB and binding of NFκB to DNA were significantly decreased in comparison with Zn-sufficient cells. Zn increased the translocation of NFκB from cytosol to nucleus. We concluded that Zn plays an important role in the activation of NFκB in HUT-78 cells, which may regulate IL-2 gene expression.

B-cell proliferative and antibody responses are inhibited by Zn deficiency (see Moulder and Steward, 1989; Walsh et al., 1994; Shankar and Prasad, 1998). Interestingly, T-dependent antibody responses are more affected by Zn deficiency than T-independent ones: the plaque-forming colony responses to a T-dependent antigen (sheep red blood cells) and a T-independent antigen (dextran) were reduced 90% and 50%, respectively, in Zn-deficient mice (Fraker et al., 1977, 1978, 1984, 1986). Mice fed a high-Zn diet had increased numbers of splenic plaque-forming colonies in response to T-dependent antigens (Salvin et al., 1987).

### Effects of high-dose zinc on immune cell functions

One study reported that 11 men receiving 300 mg Zn daily (20 times the US recommended intake) for 6 weeks experienced decreased proliferative responses of lymphocytes to mitogens and reductions in chemotaxis and phagocytosis of neutrophils (Chandra, 1984). Very high Zn intakes in adults and children can result in copper deficiency and this could be the cause of the immunosuppression (Porter et al., 1977; Prasad et al., 1978; Fosmire, 1990). Importantly, other larger and longer-term controlled trials of high-dose Zn supplementation in adults did not document deleterious effects on cellular immunity (Bogden et al., 1988, 1990). In one study, 103 apparently healthy elderly subjects age 60 to 89 years were randomly assigned to one of three treatments: placebo, 15 mg Zn day⁻¹, or 100 mg Zn day⁻¹ for 3 months (Bogden et al., 1988). None of the treatments significantly altered the DTH response to a panel of seven recall antigens or in vitro lymphocyte proliferative responses to mitogens and antigens. Bogden et al. (1990) administered 100 mg Zn daily to elderly subjects for 12 months and found no deleterious immunological effects. Moreover, deleterious immunological effects were not observed in trials where clinically healthy, and otherwise normal, children received daily Zn supplementation up to twice the US recommended intake (see Shankar and Prasad, 1998). Therefore, intake of Zn twofold above the recommended intake is considered well within the safety range for preschool children and adults. As for any nutritional supplement, caution must be exercised in taking excessive doses for prolonged periods of time.
Zinc and Glucocorticoids

The release of glucocorticoid hormones from the adrenal glands can cause thymic atrophy. Since Zn deficiency raises blood glucocorticoid levels, thymic atrophy may be mediated, in part, by glucocorticoids (DePasquale-Jardieu and Fraker, 1980; Concordet and Ferry, 1993). Indeed, when adrenalectomized mice were maintained up to 6 weeks on a Zn-deficient diet, changes in thymic weight were small or absent (DePasquale-Jardieu and Fraker, 1980). In addition, when adult mice were given a slow-release corticosteroid implant, thymus size was reduced more than 80%. Steroid-implanted mice also showed large reductions in pre-B and immature B-cells in the bone marrow, suggesting that the effects of Zn deficiency on early B-cell development may also involve glucocorticoids. The contribution of glucocorticoids to the effects of Zn deficiency must, however, be interpreted with caution. Zn deficiency has profound effects on human thymocytes, which are relatively resistant to glucocorticoids (DePasquale-Jardieu and Fraker, 1980). In addition, although the thymus of Zn-deficient adrenalectomized mice remained normal in size, Zn-dependent decreases in the ratio of the areas of the cortex to the medulla still occurred. Likewise, in adrenalectomized mice, Zn deficiency reduced IgM and IgG responses to sheep red blood cells, with 50% of the loss in T-cell helper function occurring before detectable increases in plasma corticosterone. Lastly, in marginally Zn-deficient mice, loss of lymphocytes in the spleen, depressed immunity and decreased IL-2 production were observed despite the absence of thymic shrinkage or increased glucocorticoid levels.

The Influence of Zinc on Conditions Involving Immunosuppression

Individuals suffering from sickle-cell disease have depressed peripheral T-cell numbers, decreased CD4+/CD8+ T-cell ratios, loss of DTH, decreased NK-cell activity, decreased production of IL-2 and suppressed activity of thymulin (for references, see Prasad, 2000a). Zn supplementation restores these immunological parameters to near-normal levels. Likewise, in patients with Down's syndrome, Zn supplements restore immediate hypersensitivity, lymphocyte functions and neutrophil chemotaxis, and increase resistance to infection (Bjorksten et al., 1980). Zn supplements also restored DTH in alcoholics and stimulated cell-mediated and humoral immunity in humans and mice with hypo-gammaglobulinaemia (for references, see Shankar and Prasad, 1998).

In elderly human beings and animals, impairments in wound healing and resistance to infection were corrected by Zn supplementation (see Prasad, 2000a), suggesting that immunodeficiency in the elderly is due in part to Zn deficiency. Low plasma Zn levels in elderly patients were associated with peripheral blood lymphopenia, although not with depressed serum IgA levels or DTH responses. Zn supplementation restored normal lymphocyte and neutrophil Zn levels, increased numbers of circulating T-cells, and improved DTH and IgG antibody responses to tetanus toxoid (for references, see Prasad, 2000a). As might be expected from these effects, increased resistance to infection was also observed.
Zinc and Infectious Diseases

Numerous animal studies indicate that Zn deficiency decreases resistance to a range of bacterial, viral, fungal and parasitic pathogens (for a review, see Shankar and Prasad, 1998), probably because of the immune impairments induced by the deficiency. Thus, the enhancing effect upon the immune response of providing Zn should translate into improved host defence and increased resistance to pathogens. However, most microorganisms require Zn for basic cellular processes (e.g. replication) and, during the acute-phase response, Zn is redistributed from the plasma to the liver and to lymphocytes (see Shankar and Prasad, 1998). It has been suggested that this is an adaptive response intended to deprive invading pathogens of Zn, facilitate immune function and prevent free-radical damage to cells. However, plasma Zn levels resulting from the acute-phase response remain generally well above the levels at which the growth of most pathogens is affected. The balance between Zn availability for host immunity and for the invading pathogen is affected by multiple factors. It appears, however, that in most cases any benefit to the pathogen of Zn availability is well compensated by the concomitant improvement in host immune function.

A number of studies have demonstrated the benefits of Zn supplementation in regard to infectious diseases in human populations (for a review, see Shankar and Prasad, 1998). Controlled trials of Zn supplementation demonstrated a reduction in the incidence and duration of acute and chronic diarrhoea by 25–30%, and in the incidence of pneumonia by up to 50% (Castillo-Duran et al., 1987; Sazawal et al., 1995, 1996, 1998; Ninh et al., 1996; Rosado et al., 1997; Roy et al., 1997, 1999; Ruel et al., 1997). Some studies implied that Zn may reduce clinical disease caused by Plasmodium falciparum (Gibson et al., 1991; Bates et al., 1993), and it has been demonstrated in a controlled trial carried out in Papua New Guinea that Zn supplements could reduce health-centre attendance attributable to malaria by over 35% (Shankar et al., 1998). Decreased Schistosoma mansoni egg counts were observed among children given Zn supplements versus those given a placebo (Friis et al., 1997). Humans suffering from AE also suffer fewer infections when given supranormal levels of Zn, and plasma Zn levels are substantially lower in patients with diffuse lepromatous leprosy compared with those with the more limited bacillary form (for references, see Shankar and Prasad, 1998). Zn deficiency is frequently seen in patients with human immunodeficiency virus (HIV), and disease progression is accompanied by decreased serum Zn levels and depressed lymphocyte mitogenic responses (Pifer et al., 1987; Falutz et al., 1988; Wang et al., 1994). These changes are partially reversible by Zn supplementation.

The role of Zn in preventing and treating the common cold has been discussed for many years. It appears that Zn is effective in reducing the severity of cold symptoms. For example, in a recent placebo-controlled study, 12.8 mg of Zn was administered in lozenges to a group of subjects every 2 to 3 h while awake within 24 h of developing symptoms of the common cold (Prasad et al., 2000). In the Zn-treated group, the duration and severity of cold symptoms were decreased approximately 50% in comparison with the placebo group.
Conclusion

Zn has a number of key roles relating to cell signalling, cell activation, gene expression, protein synthesis and apoptosis. Zn is crucial for the normal development of immune cells. Zn plays an important role in maintaining the activity of a range of immune cells, including neutrophils, monocytes, NK cells, B-cells and T-cells, and Zn-deficient individuals have increased susceptibility to a variety of pathogens. Providing Zn for deficient individuals improves immune function and host defence. Studies in at-risk groups indicate significant decreases in the incidence and severity of infectious disease when Zn is provided.

References


Role of Iron in Immunity and Infection

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Introduction

Iron is the fourth most common element on earth and is one of the most studied nutrients in human health (see Yip and Dallman, 1996). Iron exists in two main forms: ferric (Fe3+) and ferrous (Fe2+). The ease of oxidation and reduction of iron makes it a unique trace element for many cellular redox reactions. Iron is required by virtually all living cells for many biochemical reactions, especially for aerobic and anaerobic energy metabolism and cell proliferation (Cazzalo et al., 1990). In spite of our knowledge on the role of iron in human health related to haematology since the 18th century, the importance of iron in immunity was first recognized only in the late 1960s and early 1970s. This field evolved from clinical observations of an association between iron deficiency and infection (see Strauss, 1978; Humbert and Moore, 1983; Kuvibidila et al., 1989). It was later shown that some immune responses were altered by iron deficiency (see Dallman, 1987; Kuvibidila et al., 1989). This chapter will summarize the current knowledge of the effects of iron deficiency and iron overload on immunity and the implications for infection.

Iron Status

Between two-thirds and three-quarters of body iron circulates in blood in the form of haemoglobin. Iron status is evaluated by three methods: clinical evaluation, haematological and biochemical laboratory tests and therapeutic iron trials. The four blood indexes that distinguish iron deficiency, normal iron status and iron overload are serum ferritin concentration, blood haemoglobin concent-
Iron deficiency differs from normal iron status by reduced blood levels of haemoglobin, transferrin saturation below the levels defined for age and gender, serum ferritin concentration less than 12 μg l\(^{-1}\), and soluble transferrin receptor concentration above 9 mg l\(^{-1}\). Groups at risk of iron deficiency include infants, young children and women of childbearing age. Iron overload differs from normal iron status by increased serum ferritin concentration (> 200 μg l\(^{-1}\)) and transferrin saturation (> 50%). Groups at risk of iron overload include individuals who receive frequent blood transfusions, such as those with beta-thalassaemia or sickle-cell disease, patients with renal disease who receive medicinal iron due to impaired erythropoiesis, people with idiopathic haemochromatosis and the Bantu of southern Africa, who frequently consume traditional beer, which is very rich in iron.

Iron Absorption and Transport

Iron is predominantly absorbed in the duodenum. In its free form, iron is a potent pro-oxidant known to induce peroxidation of lipids, proteins and nucleic acids. Extracellular iron circulates in blood bound to transferrin. One transferrin molecule has two iron-binding sites. Under physiological conditions, cells take up iron from plasma by endocytosis, whereby one transferrin molecule binds to one transferrin receptor molecule, and the complex is transferred to the cytoplasm by invagination. As a result of low pH in the endosome, iron is released to the cytoplasm, where it is either used for various cellular functions or incorporated into ferritin. Upon loss of iron, the apotransferrin–transferrin-receptor complex is transported back to the cell membrane, where apotransferrin is released into the bloodstream, and the transferrin receptor is available for a new round of transferrin binding. The mechanism by which the apotransferrin–transferrin-receptor complex is transported to the cell membrane remains unclear. However, Sainte-Marie et al. (1997) suggest that changes in free cytoplasmic calcium concentrations might be involved in the recycling of transferrin receptor.

Effects of Iron Deficiency on Immunity

Iron metabolism by T-cells and the effects of iron deficiency on T-cell functions

Resting T-cells do not express the transferrin receptor on their cell surface, and therefore either do not take up iron from their environment or take up very little (Tormey et al., 1972). Upon T-cell activation, T-cells express surface transferrin receptors in the G0/G1 phase of the cell cycle before the initiation of DNA synthesis, but after induction of interleukin (IL)-2 secretion (Neckers and Cossman, 1983). The increase in transferrin receptor concentrations is believed to be to ensure sufficient iron uptake to support the activity of ribonucleotide reductase for the biosynthesis of deoxyribonucleotides.
There are many cell-mediated immune responses that have been investigated in iron-deficient subjects and laboratory animals (Table 11.1). In children and adults, iron deficiency resulting from dietary restriction reduces the proportion of T lymphocytes in blood, although the absolute number of T-cells can be either reduced or unchanged (Chandra and Saraya, 1975; Srikantia et al., 1976; Bagchi et al., 1980; Prema et al., 1982; Swarup-Mitra and Sinha, 1984; Kemahli et al., 1988; Vydyborets, 2000). However, based on the report of Santos and Falcao (1990), it appears that iron deficiency resulting from blood loss does not reduce the proportion of T-cells in blood, but it does decrease total T-cell numbers. The discrepancy between these types of iron deficiency is probably related to the long period of time required for the development of iron deficiency by dietary restriction as compared with blood loss. The absolute numbers and proportions of CD4+ and CD8+ T-cells are either decreased or normal in iron deficiency.

In mice, iron deficiency reduces the proportion of total T-cells, helper T-cells, and cytotoxic/suppressor T-cells in the spleen (Kuvibidila et al., 1990;

### Table 11.1. Iron and T-cell functions.

<table>
<thead>
<tr>
<th></th>
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<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Thymus weight</td>
<td>↓ or ↔</td>
<td>↑</td>
<td>Not determined</td>
</tr>
<tr>
<td>Spleen weight</td>
<td>↑</td>
<td>↓</td>
<td>Not determined</td>
</tr>
<tr>
<td>Total lymphocytes</td>
<td>↓ or ↔</td>
<td>↑</td>
<td>↔</td>
</tr>
<tr>
<td>Total T-cell number</td>
<td>↓ or ↔</td>
<td>↑</td>
<td>↔</td>
</tr>
<tr>
<td>Proportion of T-cells (% CD3+)</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Proportion of CD4 cells</td>
<td>↓ or ↔</td>
<td>↔</td>
<td>↓</td>
</tr>
<tr>
<td>Total CD4 cells</td>
<td>↓ or ↔</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Proportion of CD8 cells</td>
<td>↓ or ↔</td>
<td>↔</td>
<td>↑ or ↔ (↓ iron chelation)</td>
</tr>
<tr>
<td>Total CD8 cells</td>
<td>↓ or ↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>↓ or ↔</td>
<td>↔</td>
<td>↓</td>
</tr>
<tr>
<td>Delayed-type hypersensitivity</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Antibody-dependent cytotoxicity</td>
<td>↓</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>Splenic T-cell cytotoxicity (mice)</td>
<td>↓</td>
<td>Not determined</td>
<td>Not determined</td>
</tr>
<tr>
<td>Lymphocyte proliferation (in most studies)</td>
<td>↓</td>
<td>↑</td>
<td>↓, ↑ or ↔</td>
</tr>
<tr>
<td>Interleukin-2 secretion</td>
<td>↓ or ↔</td>
<td>↓ or ↔</td>
<td>↓</td>
</tr>
<tr>
<td>Interleukin-4 secretion</td>
<td>Not determined</td>
<td>Not determined</td>
<td>↑</td>
</tr>
<tr>
<td>Interleukin-10 secretion</td>
<td>Not determined</td>
<td>Not determined</td>
<td>↑</td>
</tr>
<tr>
<td>Interferon-γ secretion</td>
<td>↓</td>
<td>Not determined</td>
<td>↓</td>
</tr>
<tr>
<td>Hydrolysis of PIP₂</td>
<td>↓</td>
<td>↑</td>
<td>Not determined</td>
</tr>
<tr>
<td>Protein kinase C activity</td>
<td>↓</td>
<td>↑</td>
<td>Not determined</td>
</tr>
<tr>
<td>Protein kinase C</td>
<td>↓</td>
<td>↑</td>
<td>Not determined</td>
</tr>
<tr>
<td>translocation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein kinase C mRNA</td>
<td>↓</td>
<td>Not determined</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

PIP₂, cell-membrane phosphatidylinositol-4,5-bisphosphate; ↑, increase; ↓, decrease; ↔, no significant change from normal.
However, it does not alter the ratio of helper to cytotoxic T-cells (Table 11.2), which is also sometimes the case in humans. Iron deficiency induces thymus atrophy in mice, but does not affect the proportion of total T-cells, helper T-cells and cytotoxic/suppressor T-cells or the ratio of helper to cytotoxic T-cells in the thymus (Kuvibidila et al., 1990). The mechanisms of thymus atrophy are unclear but are probably multifactorial. Recent data suggest that iron deficiency decreases thymocyte proliferation in vivo but does not increase apoptosis (Kuvibidila et al., 2001). A defect in endocrine function of the thymus is not likely to be responsible for thymus atrophy, since plasma thymulin concentration is normal in iron-deficient mice (Kuvibidila et al., 1990).

Iron deficiency in humans and laboratory animals consistently induces anergy (Joynson et al., 1972; Bhaskaram and Reddy, 1975; Chandra and Saraya, 1975; MacDougall et al., 1975; Kuvibidila et al., 1981; Swarup-Mitra and Sinha, 1984; Kemahli et al., 1988). In most though not all studies, iron deficiency has been shown to decrease secretion of IL-2 (Galan et al., 1992; Kuvibidila et al., 1992; Latunde-Dada and Young, 1992; Thibault et al., 1993; Omara and Blakley, 1994), and interferon (IFN)-γ (Omara and Blaker, 1994), the lymphocyte proliferative responses to mitogens (Fig. 11.1) and antigens (Joynson et al., 1972; Bhaskaram and Reddy, 1975; Chandra and Saraya, 1975; MacDougall et al., 1975; Kuvibidila et al., 1983b, 1998, 1999; Swarup-Mitra and Sinha, 1984; Omara and Blakley, 1994) and antibody-dependent cytotoxicity (Bagchi et al., 1980). Most affected immune responses in humans are corrected within 1–3 months by iron therapy.

### Iron metabolism by B-cells and the effects of iron deficiency on humoral immunity

In contrast to T-cells, resting B-cells express low levels of the transferrin receptor, which implies that they continuously take up small quantities of iron (Neckers et al., 1984). Upon activation with a mitogen, up to 80% of B-cells express surface transferrin receptor, and hence exhibit increased iron uptake.

### Table 11.2. Distribution of B- and T-cell subsets in the spleen of iron-deficient and control mice (adapted, with permission from the American Society for Clinical Nutrition, from Kuvibidila et al., 1990).

<table>
<thead>
<tr>
<th>Subset</th>
<th>Control (%)</th>
<th>Iron-deficient (%)</th>
<th>Pair-fed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-cells</td>
<td>53.6 ± 4.1</td>
<td>28.5 ± 9.1a</td>
<td>50.8 ± 5.5</td>
</tr>
<tr>
<td>T-cells</td>
<td>29.3 ± 3.6</td>
<td>11.4 ± 6.9a</td>
<td>26.3 ± 6.2</td>
</tr>
<tr>
<td>Helper T-cells</td>
<td>17.4 ± 2.6</td>
<td>6.5 ± 4.1a</td>
<td>16.7 ± 4.0</td>
</tr>
<tr>
<td>Suppressor T-cells</td>
<td>11.1 ± 1.6</td>
<td>4.1 ± 2.7a</td>
<td>9.9 ± 2.7</td>
</tr>
<tr>
<td>Helper T-cells/suppressor T-cells</td>
<td>1.6 ± 0.2</td>
<td>1.6 ± 0.3</td>
<td>1.7 ± 1.2</td>
</tr>
</tbody>
</table>

aP < 0.001 versus control and pair-fed mice.

Values are mean ± standard error of mean. Sample sizes are 14 control, 16 iron-deficient, 16 pair-fed mice.
This suggests that iron deprivation may also affect certain B-cell functions. Indeed, murine splenic B-cell proliferation in response to bacterial lipopolysaccharide is significantly reduced by iron deficiency (Kuvibidila et al., 1983a; Fig. 11.2). However, when parameters of humoral immunity are compared in iron-deficient and control individuals, it is noticed that, in general, humoral immunity is quite well preserved. The percentage and total number of B-cells and the concentration of immunoglobulins (Ig) are either unchanged or slightly increased, and antibody production in response to tetanus toxoid immunization is also normal (Table 11.3; Chandra and Saraya, 1975; Srikantia et al., 1976; Bagchi et al., 1980; Krantman et al., 1982; Prema et al., 1982). In contrast to humans, iron deficiency in laboratory animals decreases the percentage of B-cells in the spleen (Table 11.2; Kuvibidila et al., 1990; Helyar and Sherman, 1992), antibody production against tetanus toxoid (Nalder et al., 1972), secondary antibody response to influenza vaccine (Dhur et al., 1990), the number of plaque-forming cells (Kuvibidila et al., 1982) and Ig levels (Kochanowski and Sherman, 1985; Table 11.3). In addition, the number of intestinal cells containing IgM and secretory IgA (sIgA) in rats is reduced by iron deficiency, an alteration that may affect intestinal mucosal immunity (Perkkio et al., 1987). The discrepancy between humans and laboratory animals could be due to species differences.
Fig. 11.2. Proliferation in response to lipopolysaccharide of spleen cells and enriched B-cell fractions as a function of iron status. Values are mean ± standard error of the mean (SEM) thymidine incorporation. C, control; PF, pair-fed; ID, iron-deficient; R, iron-replete; CPM, counts per minute. Bars with different letters are significantly different from each other (P < 0.05). (Adapted, with permission from the American Society for Clinical Nutrition, from Kuvibidila et al., 1983b.)

Table 11.3. Iron and humoral immunity.

<table>
<thead>
<tr>
<th>Immune function</th>
<th>Iron deficiency: before treatment</th>
<th>Iron deficiency: after treatment</th>
<th>Iron overload</th>
</tr>
</thead>
<tbody>
<tr>
<td>% B-cells</td>
<td>↓, ↑ or ↔</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Total B-cell number</td>
<td>↓, ↑ or ↔</td>
<td>↔</td>
<td>↑ or ↔</td>
</tr>
<tr>
<td>Immunoglobulin levels</td>
<td>↑ or ↔</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>SIgA, IgM, IgG</td>
<td>↓ (rats)</td>
<td>Not determined</td>
<td>↑*</td>
</tr>
<tr>
<td>Antibody production (influenza vaccine)</td>
<td>↓ (rats)</td>
<td>Not determined</td>
<td>Not determined</td>
</tr>
<tr>
<td>Antibody production (tetanus toxoid)</td>
<td>↓ in animals</td>
<td>Not determined</td>
<td>↔</td>
</tr>
<tr>
<td>Plasma IgE after Candida infection in mice</td>
<td>↔ in humans</td>
<td>Not determined</td>
<td>↑</td>
</tr>
<tr>
<td>B-cell proliferation</td>
<td>↓</td>
<td>↑</td>
<td>↓ or ↔</td>
</tr>
</tbody>
</table>

*Activated cells from patients with hereditary haemochromatosis. ↓, decreased; ↑, increased; ↔, no significant change from normal.
Iron metabolism by monocytes and macrophages and the effects of iron deficiency on their functions

Although monocytes do not express transferrin receptor, macrophages do (Testa et al., 1991). Macrophages differ from lymphocytes or other cell types because they up-regulate the expression of surface transferrin receptor when cultured in an iron-rich medium. This makes sense because macrophages are involved in iron storage and require iron for cytotoxic activity (Jiang and Baldwin, 1993).

Although the production of macrophage migration inhibitory factor is reduced in iron-deficient adults (Joyson et al., 1972; Swarup-Mitra and Sinha, 1984), production of IL-1 is not, and macrophage cytotoxicity is only slightly reduced (Table 11.4; Bhaskaram et al., 1989). In vitro iron chelation by desferrioxamine led to reduced secretion of tumour necrosis factor (TNF)-α by alveolar macrophages from both healthy non-smokers and smokers, which implies that iron deficiency may reduce the production of this pro-inflammatory

Table 11.4. Iron and non-specific immunity.

<table>
<thead>
<tr>
<th>Immune function</th>
<th>Iron deficiency: before treatment</th>
<th>Iron deficiency: after treatment</th>
<th>Iron overload</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage/monocyte phagocytosis</td>
<td>↓</td>
<td>Not determined</td>
<td>↓ or ↔</td>
</tr>
<tr>
<td>Macrophage/monocyte killing capacity</td>
<td>↓</td>
<td>Not determined</td>
<td>↓ or ↔</td>
</tr>
<tr>
<td>Peritoneal macrophage tumoricidal activity</td>
<td>↓</td>
<td>Not determined</td>
<td>Not determined</td>
</tr>
<tr>
<td>Neutrophil phagocytosis</td>
<td>↔</td>
<td>↔</td>
<td>↓</td>
</tr>
<tr>
<td>Neutrophil bactericidal activity</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Myeloperoxidase activity</td>
<td>↓</td>
<td>↑</td>
<td>Not determined</td>
</tr>
<tr>
<td>Neutrophil migration to inflammation site</td>
<td>↓</td>
<td>Not determined</td>
<td>Not determined</td>
</tr>
<tr>
<td>Natural killer cell activity</td>
<td>↓</td>
<td>Not determined</td>
<td>↓</td>
</tr>
<tr>
<td>In vivo macrophage clearance of particles</td>
<td>↓ (mice)</td>
<td>↑</td>
<td>Not determined</td>
</tr>
<tr>
<td>Interleukin-1 secretion</td>
<td>↓ in rats</td>
<td>Not determined</td>
<td>↓</td>
</tr>
<tr>
<td>Interleukin-12 secretion</td>
<td>↔ in humans</td>
<td>Not determined</td>
<td>↓ for neutrophils ↔ for macrophages</td>
</tr>
<tr>
<td>Interferon-α secretion</td>
<td>↓</td>
<td>Not determined</td>
<td>Not determined</td>
</tr>
<tr>
<td>Tumour necrosis factor-α secretion</td>
<td>↓</td>
<td>Not determined</td>
<td>↔</td>
</tr>
<tr>
<td>Nitroblue reduction</td>
<td>↓ or ↔</td>
<td>Not determined</td>
<td>↓</td>
</tr>
<tr>
<td>Zymosan opsonization</td>
<td>Not determined</td>
<td>Not determined</td>
<td>↓</td>
</tr>
<tr>
<td>Nitric oxide production</td>
<td>Not determined</td>
<td>Not determined</td>
<td>↓ for neutrophils ↔ for macrophages</td>
</tr>
<tr>
<td>Complement C3</td>
<td>↓</td>
<td>↑</td>
<td>Not determined</td>
</tr>
<tr>
<td>Haemolytic activity CH50</td>
<td>↓ or ↔</td>
<td>↑</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

↓, decreased; ↑, increased; ↔, no significant change from normal.
cytokine (O’Brien-Ladner et al., 1998). Such an effect would be beneficial under circumstances associated with lung injury. In contrast to humans, macrophages from iron-deficient laboratory animals show reduced in vitro secretion of IL-1 (Helyar and Sherman, 1987) and IFN-γ (Omara and Blakley, 1994), decreased in vivo clearance of polyvinyl-pyrrolidone (Kuvibidila and Wade, 1987) and decreased tumoricidal activity (Kuvibidila et al., 1983b).

**Iron metabolism by neutrophils and the effects of iron deficiency on their functions**

Iron concentrations in neutrophils are affected by the iron status of the host: they can be low in iron deficiency and elevated in iron overload. Neutrophils can take up iron from iron-saturated transferrin (Brieland and Fantone, 1991), although transferrin receptors have never been demonstrated on the neutrophil surface (Parmley et al., 1983).

Several neutrophil responses have been assessed in both iron-deficient humans and laboratory animals (Table 11.4). Although neutrophil phagocytosis remains normal in iron deficiency, intracellular killing of bacteria is significantly impaired in both humans and laboratory animals (Yetgin et al., 1979; Walter et al., 1986; Murakawa et al., 1987; Chwang et al., 1988). In parallel with reduced bactericidal killing, the activity of myeloperoxidase, an iron-dependent enzyme involved in neutrophil killing of bacteria, is impaired. The impaired functions return to normal after a few weeks of iron repletion (Walter et al., 1986; Murakawa et al., 1987).

**Iron deficiency and natural killer (NK)-cell activity**

Similarly to T lymphocytes, resting NK cells do not express surface transferrin receptor, and they probably take up very little iron from the environment (Kemp, 1993). However, upon activation, they express the transferrin receptor on their surface. There is no information on the effects of iron deficiency on NK cell activity in human subjects. In rats, moderate, as well as severe, iron deficiency markedly reduces NK cytotoxicity against the YAC-1 target cell line (Spear and Sherman, 1992).

**Mechanisms of impaired immunity in iron deficiency**

The mechanisms by which iron deficiency impairs cell-mediated and non-specific immunity are not fully understood, but they are multifactorial (Table 11.5). They include, though are not limited to, reduced activity of iron-dependent enzymes (specifically ribonucleotide reductase), reduced cytokine secretion, a reduced number of immunocompetent T-cells and, very probably, altered signal transduction. Specific steps of signal-transduction pathways that are potentially regulated by iron remain to be identified. However, protein kinase C activity and its translo-
cation to the plasma membrane in murine spleen lymphocytes and human T-cell lines are impaired by iron deficiency (Alcantara et al., 1991, 1994; Kuvibidila et al., 1991, 1999; Fig. 11.3). Furthermore, iron chelation reduces production of mRNA for protein kinase C (Alcantara et al., 1991, 1994). One early event in T-cell activation pathways that is also reduced by iron deprivation is the hydrolysis of cell-membrane phosphatidylinositol-4,5-bisphosphate by phospholipase C (a zinc-dependent enzyme) (Kuvibidila et al., 1998). The end-products of this enzymatic reaction, inositol-1,3,5-trisphosphate and diacylglycerol, regulate protein kinase C activity. Both protein kinase C activation and the hydrolysis of cell-membrane phospholipids are crucial for signal transduction that leads to T-cell proliferation and many functions. The altered protein kinase C activation and hydrolysis of cell-membrane phospholipids may lead to impaired immune responses in iron-deficient humans and laboratory animals. However, a defect in the activity of other protein kinases involved in the regulation of the cell-cycle progression cannot be ruled out (Lucas et al., 1995).

Effects of Iron Overload on Immunity

In contrast to the numerous studies conducted in humans and laboratory animals on the effects of iron deficiency on immune responses, less work has been conducted on the effects of iron overload on immune functions. Part of the reason for this is the fact that primary iron overload is not as common as iron deficiency. However, iron overload due to repeated blood transfusion in patients with haemoglobinopathies or renal disease is not rare.

Iron overload and T-cell functions

Patients with iron overload due to multiple transfusion (beta-thalassaemia, sickle-cell disease) generally have reduced proportions of T lymphocytes and
CD4+ T-cells and a reduced ratio of CD4+/CD8+ T-cells (Gugliemo et al., 1984; Kaplan et al., 1984; Dwyer et al., 1987; Table 11.1). Lymphocyte proliferative responses to mitogens and delayed-type hypersensitivity skin responses to antigens are also reduced in these patients (Hernandez et al., 1980; Munn et al., 1981; Escalona et al., 1987). However, it is not always certain whether the reduction in the proportion of T-cells is due to iron overload alone or to a combination with other factors, such as alloantigen sensitization or the coexistence of other nutrient deficiencies (protein–energy malnutrition, zinc, vitamin A, vitamin E), which are also known to impair cell-mediated and non-specific immunity (Kuvibidila et al., 1993). However, it is not always certain whether the reduction in the proportion of T-cells is due to iron overload alone or to a combination with other factors, such as alloantigen sensitization or the coexistence of other nutrient deficiencies (protein–energy malnutrition, zinc, vitamin A, vitamin E), which are also known to impair cell-mediated and non-specific immunity (Kuvibidila et al., 1993). However, it is not always certain whether the reduction in the proportion of T-cells is due to iron overload alone or to a combination with other factors, such as alloantigen sensitization or the coexistence of other nutrient deficiencies (protein–energy malnutrition, zinc, vitamin A, vitamin E), which are also known to impair cell-mediated and non-specific immunity (Kuvibidila et al., 1993).
percentages of CD3+ and CD4+ T-cells returned to normal levels, while those of CD8+ decreased below normal levels.

Data on T-cell functions in laboratory animals with iron overload are inconsistent. While lymphocyte proliferation is impaired in mice (Omara and Blakley, 1994), it is increased in iron-overloaded rats (Wu et al., 1990). The secretion of IL-2 (Omara and Blakley, 1994) and IFN-γ (Mencacci et al., 1997) by mitogen-activated murine spleen cells is suppressed by iron overload. In contrast to this down-regulation of T-helper-1-type response, iron overload up-regulates the T-helper-2-type response: the secretion of IL-4 and IL-10 in response to Candida albicans is significantly increased when compared with spleen cells from mice with normal iron status (Mencacci et al., 1997).

**Iron overload and B-cell functions**

As is the case for T-cells, very little information is available on B-cell functions in non-transfusion patients with iron overload. However, it appears that the humoral immunity is not impaired by iron overload (Table 11.3). In fact, IgG,

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**Fig. 11.4.** Hydrolysis of cell-membrane phosphatidyl inositol-4,5-bisphosphate in murine lymphocytes as a function of iron status. Data are mean + standard error of the mean IP₃ generation in the presence of Con A/IP₃ generation in the absence of Con A, expressed as a percentage of that ratio at zero time. C, control; PF, pair-fed; ID, iron-deficient; Con A, concanavalin A; IP₃, inositol-1,3,5-trisphosphate; a, P < 0.01 vs. control and pair-fed mice. (Adapted, with permission from the American Society for Nutritional Sciences, from Kuvibidila et al., 1998.)
IgA, IgM and total Ig secretion by non-activated and pokeweed mitogen-stimulated peripheral blood mononuclear cells obtained from patients with hereditary haemochromatosis is higher than that of cells from control individuals (Bryan et al., 1991). In patients with β-thalassaemia and those with sickle-cell disease, B-cell numbers and Ig concentrations are elevated (Glassman et al., 1980; Escalona et al., 1987). Serum concentrations of IgE specific for *C. albicans* are increased several-fold in iron-overloaded mice compared with mice with normal iron status (Mencacci et al., 1997).

Iron overload and macrophage functions

While iron overload has no effects on the secretion of TNF-α by bacterial lipopolysaccharide-activated alveolar macrophages, it reduces the secretion of IL-1β (O’Brien-Ladner et al., 1998; Table 11.4). Further support for a negative effect of iron overload on IL-1 secretion is provided by increased levels following iron chelation by desferrioxamine. Macrophage phagocytosis and the secretion of nitric oxide and IL-12 are not affected by iron overload (Mencacci et al., 1997).

The role of iron in microbial killing by monocytes/macrophages is complex. Iron is required for the generation of hydroxyl radicals, which are more potent anti-microbial agents than hydrogen peroxide and superoxide anion, which are produced during the oxidative burst. The limited existing data suggest that macrophage killing capacity is either normal or slightly decreased in iron overload (Brock, 1992). However, when iron is added to the culture medium, macrophage killing capacity of certain microorganisms, such as *Brucella abortus*, *Staphylococcus aureus* and *Mycobacterium tuberculosis*, is increased (Jiang and Baldwin, 1993; Byrd, 1997).

Iron overload and neutrophil functions

Iron overload also has deleterious effects on neutrophil functions. Secondary iron overload due to multiple blood transfusions in patients with beta-thalassaemia (Cantinieaux et al., 1987) and those with renal disease (Waterlot et al., 1985; Flamant et al., 1986) is associated with reduced phagocytosis of various microorganisms (yeast, *S. aureus*, *Escherichia coli*) (Table 11.3). Nitroblue tetrazolium reduction, zymozan opsonization, myeloperoxidase activity and bactericidal capacity are also impaired in patients who are iron-overloaded due to transfusion (Waterlot et al., 1985; Cantinieaux et al., 1987). Although there are other factors that can contribute to impaired neutrophil functions in transfused patients, the improved neutrophil phagocytosis and bactericidal capacity following decreasing body iron stores by iron chelation or increased erythropoiesis provide good evidence for the negative effects of iron overload on neutrophil functions (Boelaert et al., 1990; Cantinieaux et al., 1999). The secretion of nitric oxide and IL-12 by neutrophils obtained from uninfected and *C. albicans*-infected mice is abolished.
by iron overload when compared with cells obtained from mice with normal iron status (Mencacci et al., 1997).

Iron overload and NK cell function

Although NK activity is not depressed in patients with haemochromatosis (Chapman et al., 1988), it is severely reduced in transfused patients with beta-thalassaemia or sickle-cell disease (Kaplan et al., 1984). However, while in vitro incubation with desferrioxamine improved NK activity, in vivo administration of the same chelator to patients with beta-thalassaemia failed to correct it. This is most probably due to insufficient reduction of iron levels in NK cells or to the presence of some confounding variables that also impair NK activity. There is no information on NK activity in laboratory animals with iron overload.

Iron Status and Infection

The role of iron in cell division and cellular functions is well established. Almost all living cells, including bacteria, fungi, protozoa, mammalian cells from various tissues (including those of the immune system) require iron for DNA synthesis and many other cellular functions (Cazzalo et al., 1990). In addition to its role for ribonucleotide reductase activity, iron is also a cofactor of enzymes involved in cell respiration, antioxidant defence (catalase) and neutrophil bacterial killing (myeloperoxidase). Microorganisms need iron in the concentration range of 22–220 μg l⁻¹ (Payne and Finkelstein, 1978). Although an insufficient supply of iron will diminish the growth of microorganisms (Patruta and Horl, 1999), the iron in human body fluids and tissues is potentially more than sufficient to sustain optimal growth of microorganisms (Fairbank, 1988; Cook and Skikne, 1989; Cazzalo et al., 1990). However, this iron is tightly bound to various proteins (haemoglobin, myoglobin, ferritin, lactoferrin, transferrin and various enzymes) and therefore is, in general, unavailable to microorganisms. On the host’s side, too little iron may impair immune responses, especially those that require cell proliferation and bactericidal activity. Too much iron is toxic to cells, as it induces peroxidation of intracellular and cell-membrane macromolecules, and it may also impair immune functions. Thus, there must be a suitable amount of iron available to support immune function, while not promoting the growth of infectious agents above that with which the host defence can cope. The importance of this subtle balance probably explains the variety of observations that have been made with regard to the influence of iron status on susceptibility to infection. Some observations demonstrate that iron promotes infection, suggesting that iron deficiency is ‘beneficial’, as it may protect from infectious illnesses. Other observations show that iron protects from infection, which implies that iron deficiency is deleterious and may promote infection. Yet other observations indicate that iron status alone is probably insufficient to determine susceptibility.
Evidence that iron may promote infection

Several studies have been published that suggest that iron may promote infections. For example, administration of iron, especially to neonates and school-age children, increased various types of infection (Barry and Reeve, 1977; Murray et al., 1978a, b; Smith et al., 1989) and, in some studies, mortality was significantly increased (see McFarlane et al., 1970; Brock, 1993). In support of the idea that iron promotes infection is the observation that, during infection, the host responds by decreasing serum iron concentration and shifting it to storage in the reticuloendothelial cells; it may be that this is an attempt by the host to deprive the invading microorganisms of iron.

The situation regarding iron status and malaria is complicated by the fact that it is the red blood cell that is parasitized. The malaria parasite is totally dependent upon red blood cells of the host to complete its life cycle. This might explain the observations that malaria is more common in iron-replete than in iron-deficient individuals (Oppenheimer et al., 1986) and that the levels of malaria infection and the severity of disease were increased by iron supplementation (Murray et al., 1978a, b).

Evidence that iron status alone may not determine susceptibility to infections

Several studies suggest that iron status does not affect susceptibility to infections (e.g. Snow et al., 1991; Heresi et al., 1995; Menendez et al., 1997). In a study, conducted in Tanzania, of more than 800 infants, iron supplementation for 24 weeks starting at 8 weeks of age did not significantly affect the rate of malarial infection (Menendez et al., 1997). Unfortunately, the rate of other types of infection was not reported. Iron fortification during the first year of life in Chilean children did not alter the rate of diarrhoeal diseases and respiratory infections (Heresi et al., 1995). In fact, the rate of infection was higher in children with iron deficiency compared with children with adequate iron status, regardless of iron treatment. However, it was unclear from the paper what came first, iron deficiency or infection.
Conclusions

In spite of the numerous human and laboratory animal studies showing that many cell-mediated and non-specific immune responses are impaired in iron deficiency, the relationship between iron deficiency and infection is far less clear. Unfortunately, the issue of susceptibility to infection is very complex and depends not only on iron status, but also on many host, parasite and environmental factors (Keush, 1990). Some of these factors include exposure to microorganisms, the presence of other nutritional deficiencies, the type of population (neonates, young children, women, men, elderly), the severity and duration of iron deficiency, the type, dose and duration of iron therapy and pre-existing conditions (primary and secondary immunodeficiencies). There is no doubt that these confounding factors affect susceptibility to and severity of infection, regardless of iron status. However, based on published data, the two extremes of iron nutritional status – iron deficiency and iron overload – both have detrimental effects on cell-mediated and non-specific immunity. Iron deficiency and iron overload will therefore affect susceptibility to certain types of infections, and the severity and duration of infection will vary according to host and parasite factors (extracellular versus intracellular microorganisms). In summary, oral and intramuscular iron administration of therapeutic doses to immunocompromised (malnourished) individuals is associated with increased risk of morbidity due to malaria and other infectious diseases and should therefore be avoided. In contrast, since there is no evidence of deleterious effect of oral iron supplementation to immunocompetent individuals, prevention of iron deficiency either by iron supplementation or food fortification, should remain among the priorities of public health.

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Selenium and the Immune System

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Introduction

Selenium (Se) modulates immunity: Se deficiency impairs immunity, Se intakes above those habitually consumed in many Western countries boost immunity and high Se intakes lead to toxic effects and suppression of immunity. This chapter will first review the metabolism of Se and its incorporation into Se-containing proteins (selenoproteins), which mediate many of the immune effects of Se. The function of individual selenoproteins will be reviewed briefly and this will be followed by a description of the effects of Se on immunity and the protective role of Se in inflammation and viral infection.

Historical Background

Se can be highly toxic, with signs and symptoms starting to occur in humans if the daily intake exceeds approximately 0.8–1 mg. Studies in the 1940s suggested that Se may be a potential carcinogen, but, paradoxically, it is now recognized that Se has powerful anti-cancer properties. Although initially it was the toxic properties of Se that generated scientific interest, in 1957 Schwartz and Foltz (Schwartz and Foltz, 1957) found that Se could prevent hepatic...
necrosis in vitamin E-deficient rats. This observation led to the acceptance that Se was an essential trace element. The discovery in 1973 that Se was a constitutive part of cytoplasmic glutathione peroxidase (cyGPX) provided a mechanism by which the trace element could exert its biological actions. It was thus hypothesized that Se exerted its effects through modifying the expression of this important antioxidant enzyme (Vernie, 1984). However, it soon became apparent that Se must have other means for exerting some of its biological actions, since, for example, changes in the expression of drug-metabolizing enzymes observed in Se-deficient mice could be reversed using doses of Se that had no effects on GPX expression. Labelling experiments on cells and animal tissue with $^{75}$Se-selenite demonstrated that more than 35 selenoproteins, with diverse roles, are expressed by tissues. It is now widely recognized that, in addition to exerting important antioxidant activity, Se can act as a growth factor and an anti-cancer agent and is required to ensure thyroid hormone homeostasis and optimal fertility and immune function. Although many of the biological effects of Se operate through modifying the expression of selenoenzymes, there is good evidence to suggest that some actions of Se (such as its anti-cancer properties) may operate independently of these selenoproteins (Reilly, 1993; Arthur and Beckett, 1994; Foster and Sumar, 1997; Allan et al., 1999).

**Selenoproteins**

**Synthesis**

Mammalian selenoproteins contain selenocysteine residues, usually at their active sites. The importance of the selenocysteine residue lies in the fact that, at physiological pH, the residue is fully ionized, allowing it to participate effectively in redox-type reactions. In contrast, cysteine residues, which may also participate in redox reactions, are only approximately 10% ionized at physiological pH. Selenocysteine residues are incorporated at specific sites in the selenoproteins through a co-translational event directed by the UGA codon. Although the UGA codon can be recognized by the cell as a termination codon, in selenoprotein synthesis the UGA codon also signals the insertion of a selenocysteine residue. The recognition of the UGA codon to signal the insertion of a selenocysteine residue requires selenocysteine insertion sequence (SECIS) elements. In eukaryotes, the SECIS elements are located in the 3' untranslated region of the mRNA and comprise a small number of conserved nucleotides, which form a stem–loop structure. These SECIS elements are different for each selenoprotein but form a similar-shaped stem loop and are functionally interchangeable (Berry, 1991).

The synthesis of selenocysteine and its insertion into specific selenoproteins in prokaryotes involves the products of four genes ($selA$, $selB$, $selC$ and $selD$). The products are: a selenocysteine-specific tRNA species (tRNA$^{Sec}$) ($selC$), which carries the anticodon for UGA, the enzymes selenocysteine synthase ($selA$) and selenophosphate synthetase ($selD$), which are essential for the formation of selenocysteine-tRNA$^{Sec}$ from seryl-tRNA$^{Sec}$, and the elongation fac-
tor, which specifically recognizes the selenocysteine-tRNA (selB) (Bermano et al., 1995; Allan et al., 1999).

The lack of a series of mutants has delayed the description of the mechanism of selenocysteine incorporation and selenoprotein synthesis in eukaryotes. However, two forms of the tRNA_{Sec} have been isolated in eukaryotes and both contain the UGA anticodon, which is functional in *Escherichia coli* (Kollmus et al., 1996; Low and Berry, 1996). Like bacterial tRNA_{Sec}, in eukaryotes tRNA_{Sec} is esterified with serine and is subsequently converted to seryl-tRNA{Sec}. The major steps of this co-translational event in eukaryotes are illustrated in Fig. 12.1, highlighting the role of SECIS-binding protein 2 (SBP2) and multiple selenophosphate synthetases (Mansell and Berry, 2001).

**Selenoproteins that have been characterized**

Although 30–40 selenoproteins have been demonstrated using isotopic labelling, only approximately 20 have been further characterized. The biological actions of some of these selenoproteins are known and are summarized in Table 12.1 (see also Holmgren, 1985, 1989; Beckett and Arthur, 1994; Burk, 1995/1998).

![Fig. 12.1. The synthesis of specific selenoproteins. A tRNA that recognizes the stop codon UGA on mRNA forms a complex with the selenocysteine insertion sequence (SECIS) loop in the 3' untranslated region of the mRNA. Selenophosphate converts a serine bound to the tRNA to selenocysteine; this is then incorporated into the backbone of the protein. The part of the protein complex that carries out this reaction on the ribosome is SBP2; SEL C is the tRNA and SEL D is one of the selenophosphate synthetases.](image-url)
Table 12.1. Properties of selenoproteins.

<table>
<thead>
<tr>
<th>Selenoprotein family</th>
<th>Member</th>
<th>Where found</th>
<th>Structural features</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione peroxidase (GPX) (cyGPX)</td>
<td>Cytoplasmic GPX</td>
<td>All cells</td>
<td>Tetramer: four identical subunits (molecular weight 19–25 kDa). Each subunit has a glutathione-binding site and a single selenocysteine residue at the active site.</td>
<td>Catalyses reduction of a variety of hydroperoxides, including ( \text{H}_2\text{O}_2 ) and fatty-acid hydroperoxides.</td>
</tr>
<tr>
<td>Phospholipid hydroperoxide GPX (PHGPX)</td>
<td>Cytosol and membranes of many cells. High activity in testis.</td>
<td>Monomer (molecular weight 19 kDa)</td>
<td>Catalyses reduction of a variety of hydroperoxides, especially fatty-acid and cholesterol hydroperoxides.</td>
<td></td>
</tr>
<tr>
<td>Extracellular GPX</td>
<td>Plasma. Synthesized mainly in proximal tubules of kidney; also by thyrocytes</td>
<td>Tetramer</td>
<td>? Accounts for all hydroperoxide-reducing activity of plasma.</td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal GPX</td>
<td>Closely related to cyGPX</td>
<td></td>
<td>? Protection of gastrointestinal tract against ingested hydroperoxides.</td>
<td></td>
</tr>
<tr>
<td>Iodothyronine deiodinase (ID)</td>
<td>ID I</td>
<td>Liver and kidney provide 80% of plasma T3</td>
<td></td>
<td>Thyroid hormone metabolism: catalyses 5- and 5'-monodeiodination of iodothyronines (including thyroid hormones). 5'-Deiodination promotes conversion of T4 to its active form T3; 5'-deiodination promotes conversion of T4 to the inactive reverse T3.</td>
</tr>
<tr>
<td>Protein Name</td>
<td>Location</td>
<td>Function</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------------------</td>
<td>---------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iodothyronine deiodinase (ID) ID II</td>
<td>Brain, CNS, Placenta, pituitary</td>
<td>Catalyses 5'-monodeiodination of iodothyronines (including thyroid hormones)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iodothyronine deiodinase (ID) ID III</td>
<td>Brain, CNS, Placenta, pituitary</td>
<td>Catalyses 5-monodeiodination of iodothyronines (including thyroid hormones)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selenoprotein P</td>
<td>Extracellular (accounts for 40% of plasma Se)</td>
<td>Function not known</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thioredoxin reductase</td>
<td>At least three isoforms exist</td>
<td>All tissues: FAD-containing homodimer with a single selenocysteine residue near the carboxyl terminus of each subunit chain. Along with thioredoxin (substrate) and NADPH (cofactor) forms a powerful dithiol-disulphide oxidoreductase system. Can catalyse the reduction of a variety of chain substrates. Thioredoxin involved as hydrogen donor for ribonucleotide reductase (key step of DNA synthesis). Involved in many cell functions (cell growth, apoptosis inhibition, maintenance of cellular redox state).</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selenoprotein W</td>
<td>Four forms identified (in the rat)</td>
<td>Intracellular. High amounts in brain, muscle, testis and spleen; low amount in liver</td>
<td>? Antioxidant</td>
<td></td>
</tr>
</tbody>
</table>

CNS, central nervous system; NADPH, nicotinamide adenine dinucleotide phosphate; FAD, flavin adenine dinucleotide.
Hierarchy of Selenium Supply in Selenium Deficiency

When Se intake is limited there is a clear hierarchy of Se supply, both to different tissues and to different selenoenzymes within a tissue. Thus, it appears that regulatory mechanisms exist, which ensure that, in Se deficiency, Se levels are maintained in certain priority organs and selenoproteins. Se is well retained by brain, endocrine and reproductive organs, indicating the relative importance of the trace element for the biological functions of these organs. In contrast, Se is lost rapidly from liver and muscle. Within a tissue, iodothyronine deiodinase I and phospholipid hydroperoxide GPX (PHGPX) take priority for expression over cyGPX in Se deficiency (Behne et al., 1988; Bermano et al., 1995).

The mechanisms that regulate the supply of Se to selenoproteins have probably evolved, at least in part, to cope with differing amounts of the micronutrient in the diet. The processes whereby dietary Se finds its way into selenocysteine within specific selenoproteins are complicated, allowing for regulation at many levels. The major portion of Se in the diet is probably in the form of the amino acid selenomethionine. This amino acid is the seleno analogue of methionine and can take part in many of the metabolic pathways for the sulphur-containing amino acid. Se is also found in the diet as selenocysteine and as various inorganic salts, such as selenite and selenate. There is also a large variety of selenosulphur compounds, but quantitatively these may only represent a very small portion of the dietary total Se. Once selenomethionine is absorbed into an animal, it can be metabolized in a similar fashion to methionine and can be incorporated non-specifically into proteins (Fig. 12.2).

The rate of incorporation of selenomethionine is very dependent on the adequacy of methionine levels. Thus, when methionine is limiting, larger portions of selenomethionine are incorporated, due to a mass-action effect. Conversely, when higher levels of methionine are consumed, less selenomethionine is incorporated. Similarly, it is possible that any selenocysteine absorbed into the animal is also incorporated non-specifically into protein. This non-specific incorporation of Se into protein has no known physiological role, although these amino acids may provide a source of Se for production of specific selenoproteins during Se deficiency.

Incorporation of Se into specific selenoproteins requires the complex mechanism described previously. The selenophosphate synthetases operating in this mechanism produce selenophosphate from ATP and a form of Se, which is chemically similar to selenide. Many of the inorganic Se compounds that are absorbed from the diet would be reduced by molecules such as glutathione to selenide-like compounds (Fig. 12.2). However, Se from selenomethionine has to go through a transamination pathway and then reduction. Thus, in mammalian tissue, there are non-specifically incorporated seleno amino acids and selenocysteine incorporated at the active site of the selenoproteins.
Se metabolism is thus a complex process and this is perhaps not surprising, given the potential chemical reactivity of inorganic Se compounds. The Se as selenocysteine at the active site of enzymes is a very efficient biological catalyst and large amounts of non-specific incorporation of the amino acid might lead to inappropriate biochemical reactions within the body. The predominance of selenomethionine as a dietary source of the micronutrient provides a relatively inert source, which through well-regulated metabolic pathways can be specifically incorporated into the active site of the specific selenoproteins (for a review, see Daniels, 1996).

**Effects of Selenium on the Immune System**

Studies over the past 30 years have demonstrated that an adequate Se intake is essential for both cell-mediated and humoral (antibody-mediated) immunity (for reviews, see Spallholz et al., 1990; Kiremidjian-Schumacher and Roy, 1998; McKenzie et al., 1998; Kiremidjian-Schumacher et al., 2000; Rayman, 2000). The immunomodulatory effects of Se occur through three principal mechanisms: (i) anti-inflammatory effects of selenoproteins or selenocompounds; (ii) selenoenzymes or Se compounds altering the redox state of the cell by acting as antioxidants; and (iii) through the generation of cytostatic and anticancer compounds as products of Se metabolism.

![Diagram of selenium (Se) metabolism](image)

**Fig. 12.2.** Some pathways of selenium (Se) metabolism. The different forms of Se in the diet are absorbed and the major pool of selenomethionine passes either non-specifically into proteins instead of methionine or passes through an inorganic intermediate similar to selenide, which is then specifically incorporated into selenoproteins. Not shown are the pathways for methylation of excess Se, prior to excretion. GSH, glutathione.
Effects of selenium on respiratory burst and microbe killing

The respiratory burst is a microbicidal reaction that takes place in neutrophils and monocyte/macrophages. Using a partial reduction of oxygen, it produces superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and other reactive oxygen species (ROS) that kill bacteria. As a defence mechanism, it is extremely effective, but the host must be able to remove the peroxides that are generated in the process; otherwise, host cell damage will result. Superoxide is converted by superoxide dismutase to H$_2$O$_2$, which can, if not removed, decompose to the extremely reactive hydroxyl radical (OH$^\cdot$) (see Trenam et al., 1992).

In the 1970s, it was realized that Se deficiency led to decreased GPX activity and an inability to produce a respiratory-burst reaction that was effective at killing microbes (Spallholz et al., 1990). The reason for this is that the production of O$_2^-$ is sensitive to H$_2$O$_2$, which damages the O$_2^-$-generating enzyme. This loss of respiratory-burst reaction impairs effective killing of bacteria and results in granuloma formation (a mass of activated, but ineffective, leucocytes) and an inability of the host to eliminate microbes (references in Spallholz et al., 1990). Furthermore, nitric oxide (NO) is used by a variety of host cells to destroy bacteria and viruses. Reaction of NO with O$_2^-$ leads to the formation of peroxynitrite (ONOO$^-$), which causes oxidative damage to lipids, proteins and DNA (references in McKenzie, 2000). In Se deficiency, there is an impaired capability to detoxify organic and inorganic peroxides, generated by oxidative stress or through general metabolism. As a consequence, damage to macromolecules and cell membranes can occur.

Effects of selenium on eicosanoid metabolism

Potent lipid modulators of inflammation are synthesized from arachidonic acid cleaved from membrane phospholipids by the action of phospholipases A$_2$ and C, followed by the action of cyclo-oxygenase (see Fig. 12.3; see also Calder and Field, Chapter 4, this volume). This family of metabolites of arachidonic acid, known collectively as eicosanoids (Gerritsen, 1996), has both pro-inflammatory and immunosuppressive properties (see Calder and Field, Chapter 4, this volume). Furthermore, the excessive generation of hydroperoxides formed by the lipoxygenase and cyclo-oxygenase enzymes in circulating leucocytes can lead to oxidative damage to endothelial cells.

The leucotrienes (LTs), such as LTB$_4$, are pro-inflammatory compounds (see Calder and Field, Chapter 4, this volume). Some, like LTB$_4$, are important chemoattractants for neutrophils, bringing them into the inflamed tissue. The LTB$_4$ synthase enzyme requires reduction of 12-hydroperoxyeicosatetraenoic acid (12-HPETE) by the PHGPX or other GPX enzymes (reviewed in Parnham and Graf, 1987; Spallholz et al., 1990). Se deficiency results in decreased LTB$_4$ synthesis and impaired neutrophil chemotaxis. Diminished peroxidase capacity in Se deficiency also leads to a decrease in the synthesis of prostacyclins (Cao et al., 2000). These mediators prevent arterial thrombosis and platelet aggregation. Instead, Se deficiency promotes the synthesis of thromboxanes, which
cause platelet aggregation. Platelet degranulation results in the release of pro-inflammatory mediators, including vasoactive amines, eicosanoids and pro-inflammatory cytokines. Thromboxane synthesis and platelet aggregation and activation are decreased by Se (Zbikowska et al., 1999).

**Fig. 12.3.** The effects of selenium (Se) on the production of eicosanoids. The enzymes catalysing each step are indicated in italic. GPX indicates either a GPX or the phospholipid hydroperoxide GPX. Reactions stimulated or inhibited by Se are indicated. Cyclo-oxygenase is a key enzyme in eicosanoid synthesis. High levels of peroxides inactivate cyclo-oxygenase; these can be broken down by GPX – thus GPX is an activator of cyclo-oxygenase. PGG₂ and PGH₂ are unstable endoperoxides, which are converted to thromboxanes (TX), prostacyclins (PGI₂) or prostaglandins (PG). 5-HETE, 5-hydroxyeicosatetraenoic acid. LT, laktotriene.
Se supplementation stimulates several activities of lymphocytes, natural killer (NK) cells and lymphokine-activated killer cells (summarized in Figs 12.4 and 12.5). Preservation of protein structure is partly Se-dependent, because of the role of thioredoxin reductases in maintaining proteins in their correctly folded configuration. For cell-mediated immunity to function, interaction between many immunologically active proteins and their receptors needs to occur. Interleukin (IL)-2 is a vitally important paracrine growth and activation cytokine for immune cells (see Devereux, Chapter 1, this volume). A major immunostimulatory effect of Se is by Se-induced up-regulation of expression of the α and β subunits of the IL-2 receptor, which are expressed on many immune cells and notably on T and B lymphocytes. This increases the ability of these cells to respond to IL-2. Stimulation with IL-2 from activated CD4+ T-helper cells then potentiates the cytotoxicity of killer cells, increases numbers of lymphocytes, promotes antibody production by B lymphocytes and improves the responsiveness of immature bone-marrow cells to other cytokines in order to produce immune-cell precursors. The combination of IL-2 and interferon gamma binding to monocytes and macrophages boosts resistance to and killing of microbes (Kiremidjian-Schumacher et al., 1996).

Se also causes increases in the cytotoxicity of CD8+ cells, increases CD4+ cell numbers and responses to mitogens and greater survival of CD4+ cells in human immunodeficiency virus (HIV)-infected patients (for references, see Spallholz et al., 1990; Kiremidjian-Schumacher et al., 1996, 2000; Kiremidjian-Schumacher and Roy, 1998; McKenzie et al., 2002). The diminished proliferative response of lymphocytes to mitogenic stimuli in aged mice can be reversed by dietary Se supplementation, acting by up-regulation of the IL-2 receptor (Roy et al., 1995). Se supplementation also appears to reverse the age-related decline in NK-cell function in elderly humans (Ravaglia et al., 2000). The loss of NK-cell activity is one means by which cancer cells may evade immunemediated destruction.

The thioredoxin reductases and the GPXs protect host cells from their own respiratory-burst reaction. The thioredoxin reductases also have the ability to inactivate NK lysin, a protein produced by cytotoxic cells to kill bacteria and tumour cells. In Se deficiency, it is possible that host cells are vulnerable to damage from both types of ‘friendly fire’. Proof of the importance of this mechanism is that tumour cells often have elevated levels of thioredoxin reductase, which protects them from NK lysin. Thus, Se deficiency weakens the immune response at several key points.

In 1973, it was discovered that mice fed Se-enriched diets had increased titres for immunoglobulin G (IgG) and IgM antibodies and had higher levels of complement proteins. Most studies since then in various animals and in humans have confirmed that Se alone or in combination with vitamin E raises B-cell numbers and antibody production in response to vaccination. Studies showing that Se supplementation increases antibody production, complement responses and cell-mediated killing are catalogued in Spallholz et al. (1990) and McKenzie et al. (1998). Studies in farm animals have shown that Se
**Fig. 12.4.** Effects of selenium (Se) deficiency (left-hand column) or Se supplementation (right-hand column) on cells and molecules mediating innate immunity. ↑ signifies an increase in activity or numbers and ↓ denotes a decline in activity or numbers. Ros, reactive oxygen species. IL-X, various interleukins.
Fig. 12.5. Effects of selenium (Se) deficiency (left-hand column) or Se supplementation (right-hand column) on cells and molecules mediating acquired immunity. ↑ signifies an increase in activity or numbers and ↓ denotes a decline in activity or numbers. HIV, human immunodeficiency virus; IL-2, interleukin-2; DTH, delayed-type hypersensitivity; Ig, immunoglobulin.
deficiency increases the severity of and mortality from parasitic, viral, fungal and bacterial infections. It is now common farm practice to supplement animal feed with Se to boost growth and disease resistance. However, excessive amounts of Se (equivalent to > 400 µg day\(^{-1}\) in humans) were found to lead to toxic effects and immune suppression (Spallholz et al., 1990).

Anti-inflammatory effects of selenium

**Effects on cell signalling and gene transcription**

Se compounds have anti-inflammatory properties, probably resulting from their ability to influence the redox state of the cell and to remove ROS (Parnham and Graf, 1987). Recently, it has been realized that ROS serve as secondary messengers within cells, conveying signals of oxidative damage from the plasma membrane through the cellular signalling cascades by influencing kinase activity and by regulating the induction of transcription factors and their DNA-binding ability in the nucleus (Lander, 1997; McKenzie et al., 2002). Many protein–protein and protein–DNA interactions require thiol groups or cysteine residues on the proteins to be in a reduced state. This can be regulated by the thioredoxin reductase/thioredoxin system. The complexities of these interactions are still being unravelled. However, two key anti-inflammatory targets of Se are the transcription factors, activated protein 1 (AP-1) and nuclear factor kappa B (NF\(\text{\kappa}\)B). These factors and the genes encoding them are activated by changes in the redox state of the cell and oxidative stress, such as may be caused by chemicals or radiation. For example, ROS activate a protease, which cleaves the inhibitory subunit from the inactive NF\(\text{\kappa}\)B in the cytoplasm, allowing the activated NF\(\text{\kappa}\)B to move to the nucleus, where it activates gene transcription. Many pro-inflammatory cytokine genes have binding sites for these factors in their promoter regions. Se prevents gene induction by metabolizing ROS to prevent transcription-factor activation and possibly by inhibiting binding of the transcription factor to its response element on the DNA (reviewed in McKenzie et al., 2002).

**Effects of selenium on cytokines and adhesion molecules**

Se compounds block the constitutive expression of IL-1, IL-6, tumour necrosis factor alpha (TNF-\(\alpha\)) and IL-8 in skin cells (see Figs 12.4 and 12.6). Induction of these pro-inflammatory cytokines leads to up-regulation of adhesion molecules on endothelial cells and binding and recruitment of leucocytes to damaged tissue (Fig. 12.4). These cytokines also activate leucocytes, increasing their release of inflammatory mediators. IL-8 acts as a potent chemotaxant for neutrophils and T-cells. Endothelial cells grown under Se-deficient conditions and stimulated with TNF-\(\alpha\) bind more neutrophils than endothelial cells from Se-replete controls and have higher levels of E-selectin, P-selectin and intercellular adhesion molecule-1 expression (see Maddox et al., 1999; McKenzie et al., 2002).
Conversely, Beck and Levander have shown that Se deficiency down-regulates the production of certain cytokines in the hearts of mice infected with a cardiotoxic virus. Mice fed Se-deficient diets did not differ in the production of IL-1 from the Se-replete controls, but mRNA for IL-6, a co-stimulant for IL-2 production, was decreased in Se deficiency. A decrease in the production of mRNA for TNF-α was also observed and the authors suggest that the decrease in the availability of these cytokines could radically disrupt anti-viral immunity (Beck and Levander, 1998).

Cytokines can also be induced by non-specific damage to DNA. When skin cells (keratinocytes) are irradiated with ultraviolet B radiation (UVB), thymidine dimer formation (cross-linking of adjacent pyrimidines), single-strand breaks in DNA and oxidative damage to DNA occur. Two cytokines induced by DNA damage are TNF-α and IL-10. In the skin, TNF-α causes emigration of the Langerhans cells, the principal dendritic antigen-presenting cells in the skin. Without these antigen-presenting cells, local immune suppression occurs and neoplastic cells cannot be readily detected. Release of IL-10 potentiates this situation by inhibiting the release of pro-inflammatory cytokines and by inhibiting antigen presentation and cell-mediated immune responses. Se, as selenite or

**Fig. 12.6.** Inhibition of cytokine release from normal human keratinocytes in culture by selenium. Primary keratinocytes were supplemented with either sodium selenite or selenomethionine for 24 h prior to the media being collected and the protein levels measured by ELISA. Control cells had no selenium added. Results are expressed as the mean ± standard error of the mean, n = 3. Significant difference from the control cells, * P < 0.05. IL-8, interleukin-8.
selenomethionine, protects keratinocytes from oxidative DNA damage and the UVB-induced release of IL-10 and TNF-α proteins, as well as inhibiting UVB induction of the pro-inflammatory cytokines, IL-6 and IL-8. Peroxynitrite can cause DNA single-strand breaks; strand breakage is prevented by GPX and thioredoxin reductase. Overall, Se can prevent cytokine release in response to DNA damage, preventing inflammation and helping to prevent immune suppression following UVB irradiation (reviewed in McKenzie, 2000).

Cytokines can also influence the expression of selenoproteins. The immunosuppressive cytokine transforming growth factor β inhibits GPX gene transcription (Mostert et al., 1999). Also, activation of a plasmid construct consisting of the selenoprotein P promoter linked to a reporter gene was suppressed by treatment of cells harbouring the construct with IL-1β, interferon-γ or TNF-α (Dreher et al., 1997).

**Effects of selenium on the skin immune system**

The skin is the frontier of the immune system, the interface of the body with the external environment and the site of exposure of the immune system to mutagenic and oxidative damage from UV radiation (reviewed in Duthie et al., 1999). Oxidizing agents are also produced by commensal microorganisms that reside on the skin. Se has been shown to have a vital role in protecting the skin from carcinogenesis and from oxidative damage (reviewed in McKenzie, 2000). In mice, dietary Se supplements or even topical application of Se significantly decrease the incidence of skin tumours, tumour size and mortality. Mice on Se-deficient diets also have significantly lower numbers of Langerhans cells in the skin, which may result from increased secretion of TNF-α, which triggers emigration of these dendritic cells. In humans, low Se intake has been correlated with increased incidence of skin cancer, but it is not clear yet whether Se supplements protect humans from skin damage and malignancy (reviewed in McKenzie, 2000).

In vitro, selenite and selenomethionine protect keratinocytes from UVB-induced cell death by necrosis and apoptosis. The mechanisms are thought to involve protection from lipid peroxidative damage to membranes, a decrease in oxidative DNA damage and inhibition of caspase-3, one of the proteases involved in triggering apoptosis (reviewed in McKenzie, 2000). For maximum protection, at least 12 h preincubation of the cells with the Se compounds is necessary, suggesting that the protection is mediated by selenoproteins. The GPX family seem to have an important role in this protection; for example, the molluscum contagiosum virus, which causes skin papules, carries an open reading frame encoding a GPX-like transcript. Transfection of this cDNA into keratinocytes protects them from UVB-induced cell death (Shisler et al., 1998). Furthermore, unlike catalase, which also breaks down H₂O₂, GPX is not inactivated by UVB. Tanning of the skin protects against UVB, and thioredoxin reductase has been proposed to regulate the production of the melanin tanning pigment (reviewed in McKenzie, 2000). Thus, Se plays an important role in protecting the skin immune system from oxidative damage and carcinogenesis.
Effects of selenium on viral infections

Oxidative stress is produced during viral infections and contributes to the pathology of the disease. Nutritional deficiencies can exacerbate the problem (reviewed in Beck and Levander, 1998). The Coxsackie B3 virus, which is implicated in the pathology of Keshan disease, mutates to a more cardiotoxic form when passaged through Se-deficient mice (see Beck, 1999). This seems to be a common theme for RNA viruses and it has been proposed that the oxidizing environment of an Se-deficient host may contribute to faster mutation of the viral genome (Beck et al., 1995). These viruses lack proofreading capability and this contributes to their high mutation rates. Beck and Levander (1998) have noted that the severe forms of the influenza virus, demonstrating a large degree of antigenic drift, have evolved in Se-deficient parts of China and that HIV is thought to have evolved in areas of Central Africa where Se intake is very low. The importance of selenoenzymes for anti-viral protection was demonstrated in experiments where mice were fed gold thioglucose or gold sodium thiomalate, which inhibit selenocysteine residues. In these animals, injection with normally non-lethal Semliki Forest virus or Sindbis viruses was fatal (Beck and Levander, 1998). Furthermore, injection of non-virulent Coxsackie B3 virus into GPX knockout mice led to mutation to a more virulent strain (Beck, 1999).

The long terminal repeat of HIV controls replication and is activated by binding of NFκB, which is regulated by the cell redox state and oxidative stress. TNF-α stimulates NFκB activation in T-cells. As we have described, Se compounds can inhibit TNF-α release. An inverse correlation between plasma Se concentration, red-cell GPX activity and the progression of acquired immune deficiency syndrome (AIDS) has been shown (reviewed in Chen et al., 1997; McKenzie et al., 2002). In culture, Se supplementation of HIV-infected monocytes and CD4+ T-cells inhibits TNF-α-induced viral replication (Hori et al., 1997). Thus, it seems that Se may be useful in the treatment of AIDS (Chen et al., 1997). Dietary Se supplements have been used for the treatment of hepatitis-B-induced liver cancer in China. The incidence of hepatitis-B-virus-induced liver cancer in humans decreased in a previously Se-deficient, hepatitis-B+ population given Se supplements of 200 μg day⁻¹.

Viruses illustrate the importance of the GPX system in protection from ROS. Several different viral genomes (HIV, Ebola, molluscum and hepatitis C) encode GPX-like molecules (Zhang et al., 1999). This presumably protects them against the ROS produced by host phagocytes.

The Relationship between Selenium Intake and Other Diseases

Overview

The intakes of Se recommended by health authorities in different countries vary between 50 and 70 μg day⁻¹. These values are based on the observation that such intakes should produce maximal expression of extracellular GPX
(eGPX) in plasma or cyGPX activity in red cells. Thus, the Se requirements are based on biochemical parameters and, as yet, there is no consensus as to other biochemical or physiological effects of Se status that may be used to determine optimal intake. The recognition that the anti-cancer effects of Se in humans can occur at intakes of 200 μg day\(^{-1}\) or more suggests that a reappraisal of optimal Se intake may be needed.

Daily Se intakes of humans can vary across the globe from less than 5 μg day\(^{-1}\) up to 3000 μg day\(^{-1}\). However, these intakes represent extremes and, in most cases, Se intakes are between 30 and 200 μg day\(^{-1}\). With regard to human health issues, most debate revolves around whether intakes of 30–75 μg day\(^{-1}\) are associated with an increased incidence of a range of diseases, including cardiovascular disease and cancers. Low dietary Se intake has been implicated in the development of numerous health disorders in humans. These include Kashin–Beck disease, cancer, cardiovascular disease (including Keshan disease), muscular dystrophy, malaria, alopecia areata, pregnancy hypertension syndrome, altered immune function, male infertility and even AIDS (reviewed in Rayman, 2000). Patients on long-term parenteral nutrition without Se supplementation in their formulation run the risk of Se deficiency, which is manifested in myopathy and cardiomyopathy. Low Se status (low Se and erythrocyte GPX activity) in the elderly was correlated with lower tri-iodothyronine (T3)-to-thyroxine (T4) ratios, due to raised T4 concentrations, and was seen with advancing age (Olivieri et al., 1996). Se supplementation decreased the serum T4 concentration in these patients. The age-related decline in T3 content was ascribed to the requirement for iodothyronine-5'-deiodinase, which is a selenoprotein, to catalyse the conversion of T4 to T3. A deficiency in T4-to-T3 conversion will impair general metabolism, including immunity.

**Cancer**

In 1965, Shamberger and Rudolph demonstrated a significant reduction of skin-cancer incidence in carcinogen-treated mice given a topical application of sodium selenite. This initiated a great number of subsequent studies, using animal models, which consistently demonstrated the anti-carcinogenic nature of Se. The anti-cancer effects seem to be of two types: one is the ability to protect against DNA damage and to bolster immune effectiveness and the second is the ability of selenocompounds to cause growth inhibition and apoptosis of tumour cells, while leaving normal cells unaffected (reviewed in Combs and Clark, 1999; Ganther, 1999). Se compounds inhibit the cellular oncogene AP-1, which is required for cell growth. Other selenocompounds, such as selenodiglutathione, do not kill normal cells, but cause apoptosis of tumour cells by inducing the tumour suppressor protein, p53, and apoptosis (Lanfear et al., 1994). For p53 to bind to DNA, thiol groups on nine critical cysteine residues must be in a reduced state, this is mediated by thioredoxin. Inactivation of thioredoxin reductase prevents activation of a functional p53 (references in McKenzie et al., 2002). Se compounds also prevent metastasis and angiogenesis of tumours, possibly by inhibition of release of cytokines, such as IL-8.
In humans, some, but not all, epidemiological studies have suggested an inverse correlation between Se intake and the prevalence of malignancy. The most convincing evidence for Se having an anti-cancer effect in humans comes from a recent randomized, double-blind, placebo-controlled, supplementation study (Combs and Clark, 1999). In this study, 1300 subjects from the USA received 200 μg of Se daily (given as Se-rich yeast) or a placebo for approximately 5 years. Total cancer incidence was 42% lower in the Se-supplemented group compared with the placebo group, with significant decreases in the incidence of prostate, gastric and colorectal cancers. Similarly, the total death rate from malignancy was 52% lower in the subjects who received Se supplementation when compared with the placebo group. The multicentre PRECISE trial has recently been started in the USA, Finland, Denmark, Sweden and the UK with a view to extending these observations to European populations (Rayman, 2000).

Cardiovascular disease

The risk of developing atherosclerosis and heart disease may be higher in people who have a low dietary Se intake. Endothelial dysfunction is a primary factor in the pathogenesis of atherosclerosis. Laboratory-based research has provided considerable evidence to suggest that Se may be beneficial to the endothelium and thus help to prevent atherosclerotic disease. Se supplementation of cell cultures protects the endothelium from oxidative damage and can alter platelet function, cytokine signalling and transcription of pro-atherogenic adhesion molecules (Fig. 12.4).

The contribution of Se deficiency to the pathogenesis of cardiovascular disease was originally suggested from epidemiological studies that correlated low Se content of forage crops, drinking water and blood with regional mortality rates from cardiovascular disease (Schamberger et al., 1979). Other studies have failed to confirm this link. Huttunen (1997) postulated that the conflicting data from these studies can be explained by a threshold effect of Se intake on the risk of cardiovascular disease; that is, in populations with low Se status, a correlation between serum Se and cardiovascular risk is observed, while populations with a high Se intake (serum Se levels > 45 μg l⁻¹) would show no such correlation (Korpela, 1993). There are data that support this 'threshold hypothesis' (Kardinaal et al., 1997): from the ten centres across nine European countries, only one (Germany, with the lowest measured Se concentrations) demonstrated a statistically significant inverse association between toenail Se levels and risk of myocardial infarction.

Conclusion

In conclusion, Se deficiency seriously impairs cell-mediated and humoral immunity and appears to play a part in the pathogenesis and exacerbation of some chronic inflammatory and viral diseases. Dietary Se supplements may be
a useful additional therapy in the treatment of some of these conditions. However, excessive Se intakes also impair immune function.

References


13 Probiotics and Immune Function

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Introduction: Microbes and the Intestinal Environment

Microorganisms represent an essential, functioning component of the mammalian intestinal lumen. While the stomach is sparsely populated by acid-tolerant microbes, post-gastric sites support an increasing microbial population density, which in humans can reach concentrations of up to $10^{11}$ bacteria g$^{-1}$ of lumen contents in the large intestine (Salminen et al., 1998). Indeed, the human body contains approximately tenfold as many bacterial cells as somatic cells. Colonization of the human intestinal tract by microorganisms begins perinatally, when a newborn baby first encounters maternal and environmental microbes during and immediately following delivery. As neonatal development continues, there is a succession of colonization of the infant’s developing intestinal tract by major groups of bacteria, which, under normal circumstances, begins to stabilize during weaning (Mitsuoka and Hayakawa, 1973). A stable intestinal microflora is typically attained post-weaning and during early childhood, and forms an essential component of the functioning human body. Perturbations of this resident microflora (for example, by external stressors, dramatic alterations of the diet or antibiotic treatment) can lead to a deterioration of physiological function and decline in health, including poor digestion and nutrient assimilation, immune dysfunctions and susceptibility to infection by diarrhoea-causing pathogens.

The intestinal microflora constitutes a metabolically active microbial environment, dominated by a relatively low diversity of genera, which, in the gut of healthy individuals, exist as part of a stable community (Fuller, 1992). Under normal circumstances, these resident gut bacteria cause neither pathogenesis nor inflammation in the host, but instead contribute to health maintenance, by forming a barrier layer against colonization by pathogens and by aiding in nutrient digestion and assimilation (Salminen et al., 1998). In addition, the resident intestinal microflora plays other important physiological roles in health.
maintenance: deconjugating potentially damaging oxidative metabolites and
toxins in the gut; degrading potentially allergenic food proteins; regulating cho-
lesterol and triglyceride uptake; increasing vitamin biosynthesis; and providing
immunosurveillance signals to limit intestinal-tract inflammation. Thus, a stable,
properly functioning and active intestinal-tract microflora is essential to the con-
tinuance of human health.

Probiotic Supplementation of the Intestinal Microflora

Among the most predominant microbes in the human intestinal tract are the
Gram-positive lactic acid-producing genera *Lactobacillus* and *Bifidobacterium*.
*Lactobacilli* and bifidobacteria are also common fermentative microbes in
yoghurt, cheese and soured vegetable foods (such as sauerkraut and suguki).
The majority of fermentative microorganisms present in such foodstuffs are sus-
ceptible to low stomach pH and bile-salt secretions and cannot survive gastric
processing. However, following oral delivery, a few strains are able to survive
gastric transit and can persist in the intestinal lumen. These strains are thus able
to transiently colonize the gut by integrating into the existing microflora, and
are termed ‘probiotics’.

Probiotics can be defined as dietary supplements containing living
microbes that are able to persist in (or transiently colonize) the human intestinal
tract and impart a beneficial influence on host physiology, such that this effect
is able to improve health. This process is particularly important at times when
the normal indigenous microflora has been perturbed: at this point, exoge-
nously supplied probiotics of a defined species/strain are able to temporarily
colonize the intestinal tract and stabilize the microfloral composition, thus
restoring the vital physiological functioning of the microbial community. Thus,
the use of probiotics in health improvement relies on the principle that exoge-
nous microbes (from food sources) augment the beneficial physiological effects
of the normal (indigenous) gut microflora.

Among the many purported physiological influences of probiotic microor-
ganisms, a large proportion of research attention over the last decade has
focused on the interaction of probiotics with the immune system (Salminen et
al., 1998). It is evident that several probiotic strains of lactic acid bacteria (LAB)
are able to influence the immune system and, in many cases, this effect has
been linked to a measurable improvement in health. The immune system com-
prises innate and adaptive components, and these play vital interacting roles in
health maintenance, in both regulating and stimulating the body’s responses.

Probiotics and the Immune System: Regulation and Stimulation

With regard to the role that the immune system plays in health maintenance
and improvement, the traditional viewpoint has been one of immunity as a
defence system against intrinsic (neoplasms and tumours) and extrinsic disease-
causing agents (pathogens). However, this definition forms only part of the pic-
ture. Through control and orchestration of immune responses, the immune system is also able to regulate inflammatory events and control or limit the development of pathologies. This occurs mainly via the production of modulatory hormones (cytokines) that are able to shape and modify the character of a developing immune or inflammatory reaction (see Devereux, Chapter 1, this volume). In this context, it should be realized that gut-dwelling microbes are far from passive inhabitants of the intestinal-tract mucosa in an inert immunological sense. Paradoxically, it is the very signals generated by gastrointestinal (GI)-tract microbial interactions with the immune system that probably constitute the beneficial impact of probiotics on health. Clinical case studies have indicated that children raised in environments rich in early-life bacterial exposure (including lactobacilli-containing foods) develop fewer immune dysfunctional diseases than those experiencing more sterile environments (Alm et al., 1999). In this case, it has been suggested that early stimulation by ‘appropriate’ bacterial signals may regulate the development of the immune system, such that immunopathologies (e.g. atopic reactions and mucosal allergies) are limited (Matricardi et al., 1999). Indeed, a recent study has shown that supplementing the diets of newborn babies with the probiotic *Lactobacillus rhamnosus* (strain GG) can effectively reduce the incidence of atopic eczema during infancy and early childhood (Kalliomaki et al., 2001), suggesting that augmentation of the neonatal intestinal microflora with exogenous bacteria can provide the bacterial signals necessary to combat allergic sensitization.

There is also more direct evidence that orally delivered probiotic organisms can interact with the immune system to limit pathologies. Further studies on *L. rhamnosus* GG have indicated that this probiotic can alleviate immune-mediated atopy following oral delivery to infants or to nursing mothers (Majamaa and Isolauri, 1997; Isolauri et al., 2000), can partially control immune-mediated inflammatory responses in adults (via regulation of leucocyte inflammatory receptor expression) (Pelto et al., 1998) and can reduce the incidence and severity of infant diarrhoea concomitant with an increase in circulating antibody responses (Kaila et al., 1992; Majamaa et al., 1995; Table 13.1). Clearly, there is scope to exploit the beneficial effects of probiotics on the immune system, with a view to the development of safe, dietary adjuncts/food-borne alternatives to pharmaceutical intervention for the control of a wide range of human pathologies (Elmer et al., 1996).

**Immune Signalling by Orally Delivered Probiotics**

The first point of contact for orally delivered probiotics with intestinal tissues occurs as the microorganisms form lectin-like attachments to epithelial cells of the intestinal tract, as they begin to colonize the mucosa (Fuller, 1992). Recent research has shown that human intestinal epithelial cells are immunocompetent and can transcribe cytokine messenger RNA in response to contact with probiotic bacteria (Delneste et al., 1998). This response is heightened in cells that have been subjected to cytokine activation (e.g. during an inflammatory reaction) and is accompanied by an up-regulation of cell surface receptors.
<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Immunological effect</th>
<th>Subjects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus acidophilus</em></td>
<td>↑ Phagocytic activity of blood mononuclear and polymorphonuclear cells</td>
<td>Healthy adult and elderly volunteers</td>
<td>Schiffrin <em>et al.</em> (1995, 1997); Donnet-Hughes <em>et al.</em> (1999); Arunachalam <em>et al.</em> (2000); Chiang <em>et al.</em> (2000); Gill and Rutherfurd (2001); Gill <em>et al.</em> (2001a, b); Sheih <em>et al.</em> (2001)</td>
</tr>
<tr>
<td>La1/Lactobacillus johnsonii; <em>Lactobacillus rhamnosus</em> HN001; <em>Bifidobacterium bifidum</em> Bb12; <em>Bifidobacterium lactis</em> HN019</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>Lactobacillus casei</em> Shirotā; <em>Bifidobacterium lactis</em> HN019</td>
<td>↑ Tumoricidal activity of blood mononuclear cells</td>
<td>Healthy adult and elderly volunteers; patients with colorectal cancer</td>
<td>Sawamura <em>et al.</em> (1994); Chiang <em>et al.</em> (2000); Gill <em>et al.</em> (2001a,b,c); Sheih <em>et al.</em> (2001)</td>
</tr>
<tr>
<td><em>Lactobacillus brevis</em> Labre; <em>Bifidobacterium lactis</em> HN019</td>
<td>↑ Production of interferons (cytokines) by peripheral blood mononuclear cells <em>in vitro</em> and pro-interferon enzymes in circulation</td>
<td>Healthy adult and elderly volunteers</td>
<td>de Simone <em>et al.</em> (1989, 1993); Kishi <em>et al.</em> (1996); Aattouri and Lemonnier (1997); Arunachalam <em>et al.</em> (2000)</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em> GG; <em>Bifidobacterium breve</em> YIT4064</td>
<td>↑ Anti-rotavirus antibody responses during infection</td>
<td>Children with rotavirus diarrhoea</td>
<td>Kaila <em>et al.</em> (1992); Majamaa <em>et al.</em> (1995); Yasui <em>et al.</em> (1999b)</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em> GG</td>
<td>↑ Specific antibody responses following vaccination</td>
<td>Volunteer adult vaccinees</td>
<td>Link-Amster <em>et al.</em> (1994); Isolauri <em>et al.</em> (1995); Fangac <em>et al.</em> (2000)</td>
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</table>
During the regulation of potential inflammatory events, it now seems that bacterial signalling from the gut microflora plays an important role in the communication between gut epithelial cells and associated intraepithelial lymphocytes. *In vitro* studies with the CaCo-2 human intestinal epithelial cell line have shown that fermentative (*Lactobacillus sakei*) and probiotic (*Lactobacillus johnsonii*) species can induce the expression of the anti-inflammatory mediator transforming growth factor (TGF)-β, but not pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α or interleukin (IL)-1β. Addition of leucocytes to CaCo-2 *Lactobacillus* co-cultures promotes the production of pro-inflammatory molecules by the epithelial cells, but also induces secretion of the leucocyte-derived anti-inflammatory mediator IL-10 (Haller *et al.*, 2000). Thus, the picture that emerges is that gut microflora and/or probiotic microbes play an active role in the maintenance of gut homoeostasis by inducing the release of anti-inflammatory mediators and that, under pro-inflammatory conditions, the cross-talk between epithelial cells and leucocytes augments this regulatory role via additional cytokine mediation. In this context, contact between gut-dwelling bacteria and intestinal cells may be considered part of the routine microbial signalling processes of a healthy gut microflora, forming a homoeostatic mechanism for the regulation of intestinal inflammation. Indeed, removal of these routine signals at the gut epithelial surface can lead to a breakdown in these regulatory immune mechanisms and consequently promote aggressive and uncontrolled inflammatory responses (Kuhn *et al.*, 1993; Kulkarni and Karlsson, 1993).

While it has been suggested that routine signalling between resident/probiotic microbes and gut epithelial cells plays a maintenance role for gut homoeostasis, it is arguably direct immunostimulation by an interaction of the microbes with lymphoid foci that has received most research attention. In this situation, the interactions are quite different: microbes traverse the epithelial boundary and contact leucocytes directly (e.g. in the organized capsular foci of Peyer’s patches), enabling direct immunoactivation. Evidence for this direct interaction has been obtained experimentally in animal models (de Simone *et al.*, 1987; Yasui *et al.*, 1989; Herias *et al.*, 1999; Perdigon *et al.*, 1999), and has an important consequence: unlike immunoinflammatory events that take place solely in the common mucosal immune system, immunostimulation via lymphoid foci facilitates ready access of messenger cells to the systemic circulation, via drainage to the mesenteric lymph node and thoracic duct. Thus, the consequence of an interaction between probiotic bacteria and lymphoid foci in the GI tract could include effects on systemic immune responses involving circulating leucocytes (Perdigon *et al.*, 1988, 1995). Several Gram-positive bacterial cell-wall components (including lipoteichoic acid, peptidoglycan and muramyl dipeptide) have been shown to bind leucocyte pattern-recognition receptors, including the endotoxin receptor (CD14), Toll 2 and type I macrophage scavenger receptor (Dunne *et al.*, 1994; Cleveland *et al.*, 1996; Cauwels *et al.*, 1997; Dziarski *et al.*, 1998), and this could represent the mechanism by which probiotics are able to stimulate the immune system directly.
Immunoregulation and Stimulation by Probiotics: Laboratory and Clinical Studies

Although the primary site of immunological signalling is at the gut mucosal interface, there is evidence that the immunomodulatory effects of probiotics can be expressed systemically. Typically, this is manifested by changes in leucocyte or humoral function, which can be assessed by ex vivo assays. To date, several compartments of the immune system have been identified as affected by probiotic delivery, including lymphocyte function (proliferation, cytokine secretion and cellular cytotoxicity); innate cell defences (e.g. phagocytosis, oxidative radical production, lysosomal enzyme secretion); natural cytoidal function of macrophages and natural killer (NK) cells, and antibody responses (both in terms of total immunoglobulin (Ig) levels and antigen-specific responses) (Table 13.1). In addition, there is evidence that oral delivery of probiotics can influence cellular phenotype expression, both at the mucosal interface and systemically, to reflect a state of activation.

Probiotic effects on lymphocytes

The majority of research to characterize probiotic effects on lymphocyte function has utilized animal models for study. Oral delivery of different strains of Lactobacillus has been shown to confer an increased capacity for splenic lymphocytes to proliferate in response to T-cell and B-cell mitogenic stimulation (Vesely et al., 1985; de Simone et al., 1987; Kirjavainen et al., 1999; Gill et al., 2000) and, in at least one case, this general enhancement of lymphocyte function has also been demonstrated at the local level in lymphoid foci of the intestinal tract (i.e. Peyer’s patches) (Perdigon et al., 1991). What is not clear at the moment is whether this enhanced capacity for lymphocytes to undergo activation/mitosis is due to increases in population levels (i.e. proportionally more lymphocytes) and/or increases in responsiveness to stimuli (i.e. lymphocytes at a heightened state of preactivation). However, a study by Perdigon et al. (1999) has shown that T-helper (CD4+) lymphocyte numbers are increased in the gut-associated lymphoid tissue (GALT) following oral delivery of Lactobacillus casei, providing evidence that probiotic stimulation can increase the size of lymphocyte populations.

Oral delivery of probiotics has also been shown to increase the capacity of systemic lymphocytes to secrete T-cell cytokines in response to appropriate in vitro stimulation. Some strains of Lactobacillus and Bifidobacterium have been demonstrated to increase the capacity of murine splenic lymphocytes (Pereyra et al., 1991; Gill, 1998; Matsuzaki and Chin, 2000) and human peripheral-blood lymphocytes (Solis-Pereyra and Lemonnier, 1991) to secrete the cytokine interferon-γ (IFN-γ), following mitogen stimulation in vitro. Clinical studies have confirmed that certain probiotic LAB can induce increased expression of both type I and type II interferons among peripheral blood mononuclear cells (Kishi et al., 1996; Aattouri and Lemonnier 1997; Solis-Pereyra et al., 1997; Arunachalam et al., 2000; Table 13.2).
### Table 13.2. Health benefits of probiotic microorganisms that interact with the immune system.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Immunological effect</th>
<th>Health benefit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus acidophilus</em></td>
<td>↑ Production of anti-allergy cytokine (IFN-γ)</td>
<td>↓ Eosinophil count in asthmatic subjects; ↓ IgE levels in elderly subjects with nasal allergies</td>
<td>Trapp <em>et al.</em> (1993); Wheeler <em>et al.</em> (1997)</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus GG</em></td>
<td>↓ Expression of inflammatory receptor molecules in milk-hypersensitive subjects; ↓ expression of pro-allergy cytokine (IL-4) in milk-hypersensitive subjects</td>
<td>↓ Atopic responses in milk-hypersensitive infants and adults</td>
<td>Sutas <em>et al.</em> (1996b); Majamaa &amp; Isolauri (1997); Pelto <em>et al.</em> (1998); Kalliomaki <em>et al.</em> (2001)</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus GG</em></td>
<td>↑ Anti-pathogen antibody responses</td>
<td>Promotes recovery from acute rotavirus diarrhoea in children; reduces viral shedding</td>
<td>Kaila <em>et al.</em> (1992); Majamaa <em>et al.</em> (1995)</td>
</tr>
<tr>
<td><em>Lactobacillus casei Shirota</em></td>
<td>↑ Cellular immune responses</td>
<td>↓ Tumour recurrence in adult bladder cancer patients following resection</td>
<td>Sawamura <em>et al.</em> (1994); Aso <em>et al.</em> (1995)</td>
</tr>
</tbody>
</table>

IFN-γ, interferon-γ; IL-4, interleukin-4; IgE, immunoglobulin E.
Probiotic effects on innate cell defences

A large body of work concerned with definition of probiotic effects on the immune system has focused on innate cell responses. Early studies had shown that oral delivery of *L. casei* probiotic strains to mice could activate mononuclear phagocytes for increased phagocytic activity and lysosomal enzyme production and that this enhancement could be detected in cells derived from peritoneal exudates (Perdigon et al., 1986, 1988). Subsequent studies have confirmed that certain strains of probiotic LAB can prime peritoneal macrophage populations for enhanced phagocytosis, lysosomal enzyme production and free radical oxidant production (Perdigon et al., 1988; Gill, 1998; Matsuzaki and Chin, 2000). Further studies in murine models have reported that probiotic feeding can also enhance the activity of blood-derived phagocytes and that both mononuclear (monocyte) and polymorphonuclear (neutrophil) populations are stimulated by probiotics (Gill et al., 2000). Human studies have confirmed this effect in circulating phagocytes of adult subjects (Schiffrin et al., 1995; Donnet-Hughes et al., 1999; Yoon et al., 1999; Chiang et al., 2000; O’Mahony et al., 2000; Sheih et al., 2001) including the elderly (Arunachalam et al., 2000; Gill et al., 2001a, b, c; Table 13.1).

In common with studies on the effects on lymphocyte proliferation, it is at present unclear whether oral probiotic delivery enhances phagocytic cell function as a reflection of increased cell numbers and/or increased cellular avidity to phagocytose. It is likely that bacterial signalling will activate a general release of phagocytically active cells into circulation (Herich et al., 1999), and this is possibly achieved by microbial stimulation of phagocytic precursor cells. It is important to note that mononuclear phagocytes, in particular, are also capable of secreting immunomodulatory cytokines and that stimulation of these cells by oral probiotics has been shown to increase production of key cytokines, which modify and shape the character of the immune response (Tejada-Simon et al., 1999b). Thus, it is possible that phagocyte activation is the first and key event in immune stimulation by probiotics and that enhanced phagocytic capacity is a reliable index of this activation, prior to the initiation of downstream events, such as cytokine-mediated enhancement of leucocyte cytotoxicity and lymphocyte activation.

Studies in murine models have shown that the cytocidal activity of splenic leucocytes can also be increased following delivery of certain strains of probiotics. Systemic priming of mice with viable *L. casei* (Shirota strain) can enhance ex vivo tumoricidal activity of splenic NK cells and macrophages (Kato et al., 1983, 1984) and can also increase cytocidal activity against cytomegalovirus-infected target T-cells (Ohashi et al., 1988). Oral delivery of *L. rhamnosus* HN001 or *L. casei* Shirota to mice has also been shown to increase ex vivo NK-cell tumoricidal activity (Gill et al., 2000; Matsuzaki and Chin, 2000). In human studies, feeding of *L. rhamnosus* (strain HN001) or *Bifidobacterium lactis* (strain HN019) has been demonstrated to up-regulate peripheral blood NK-cell-mediated cytotoxicity against tumour cells (Chiang et al., 2000; Gill et al., 2001a, b, c; Sheih et al., 2001; Table 13.1).
Probiotic effects on antibody responses

Several studies have investigated the ability of probiotics to regulate antibody production. Initial animal studies showed that probiotics were able to potentiate systemic antibody responses to parenterally delivered foreign antigens in mice (Portier et al., 1993) and that serum levels of IgG and IgM isotypes were elevated (Perdigon et al., 1991, 1999). Subsequent studies have indicated that probiotic strains such as *L. rhamnosus* HN001 or *B. lactis* HN019 can potentiate antibody responses to both systemically and orally administered T-dependent antigens in mice and that increases in specific antibody titre can be measured in both the serum and intestinal-tract secretions, the latter involving a rise in IgA levels (Yasui et al., 1989; Yasui and Ohwaki, 1991; Herias et al., 1999; Tejada-Simon et al., 1999a; Gill et al., 2000). Since the major GI antibody secretion is derived from plasma cells of the lamina propria, these results suggest that probiotics are able to stimulate the mucosal immune system, possibly via direct interaction with immunocompetent T-cells of the GI tract. Indeed, recent studies in mice have indicated that probiotic LAB are able to increase the mucosal density of IgM- and IgA-secreting plasma cells in both gut epithelial and broncho-alveolar lymphoid tissues (Bibas Bonet et al., 1999; Perdigon et al., 1999).

Under disease conditions, animal studies have also indicated that probiotic delivery can increase GI tract and systemic antibody responses to bacterial pathogens, including *Escherichia coli* (Perdigon et al., 1990, 1991), *Shigella sonnei* (Nader de Marcias et al., 1992) and *Salmonella typhimurium* (Paubert-Braquet et al., 1995; Shu et al., 2000). Clinical studies have demonstrated that the orally delivered probiotic *L. rhamnosus* GG can also increase the frequency of pathogen-specific and total antibody-secreting cells in children during convalescence from rotavirus diarrhoea (Kaila et al., 1992; Majamaa et al., 1995). However, in the case of non-infectious diseases, such as atopy, it appears that certain probiotic bacteria are able to exert a regulatory, rather than enhancing, effect on antibody production. Several studies have shown that IgE responses in allergen-primed mice can be attenuated by the oral or systemic delivery of probiotic LAB (Matsuzaki et al., 1998; Shida et al., 1998; Yasui et al., 1999a; Matsuzaki and Chin, 2000), suggesting that an ability to regulate immune responses may play an important role. Indeed, *in vitro* studies by Murosaki et al. (1998) have shown that adding *L. casei* (Shirota strain) to cultures of allergen-reactive murine splenocytes can directly suppress IgE production.

Health Benefits of Probiotic-mediated Immunomodulation

As described previously, probiotics are capable of modulating the immune system via both immunostimulation and immunoregulation, and thus have the potential to have an impact on health status and disease conditions that have an inherent immune component. In the case of immunostimulation, probiotics may provide a boosting of the immune system in key aspects of effector mechanisms that are tailored towards combating infectious diseases or intrinsic pathologies, such as neoplasm development. In addition, the ability of probi-
otics to stimulate cytokine secretion may provide an important immunoregulatory function for the control of immune dysfunctional conditions, such as chronic inflammation and allergies. Research that has sought to investigate these potential outlets for probiotics in health has drawn on both animal studies and human clinical trials for supportive evidence.

Probiotics and infectious diseases

There is clear evidence that certain probiotic LAB strains are able to potentiate pathogen-specific antibody responses, both in animal models and in humans. Yasui et al. (1999b) have demonstrated that mice immunized with influenza vaccine and fed Bifidobacterium breve (strain YIT4064) as a probiotic developed enhanced virus-specific antibody responses and showed greater protection against respiratory challenge than non-probiotic-fed mice. In addition, some studies have confirmed an increase in innate and lymphoid cell-mediated events in pathogen-infected mice, which may contribute to enhanced disease resistance. Shu et al. (2000) have recently shown that the probiotic B. lactis HN019 could enhance pathogen-specific antibody responses in S. typhimurium-infected mice, as well as promoting increased peritoneal cell phagocytosis and splenic lymphoproliferative potential; correlation analyses indicated that elevated immune function in probiotic-fed mice corresponded with reduced pathogen translocation in these mice and promoted enhanced survival. Other strains of bifidobacteria (such as B. breve) have been shown to increase murine antibody titres in nursing dams and to provide increased protection to weanling mice against rotavirus (Yasui et al., 1995; Fukushima et al., 1999). Recent studies have confirmed this phenomenon in weanling piglets that have been fed B. lactis HN019, which exhibit enhanced cellular and humoral immunity and increased protection against naturally acquired weanling diarrhoea (Shu et al., 2001).

In human studies, the probiotic L. rhamnosus GG has been shown to promote recovery from both rotavirus and non-bloody diarrhoea in children and infants (Raza et al., 1995; Saxelin, 1997), by reducing virus shedding as well as the duration and intensity of diarrhoeal disease (Table 13.2). Two studies have demonstrated a concomitant rise in the frequency of antibody-secreting plasma cells in the circulation of probiotic-fed children, strongly suggesting that enhanced humoral immunity plays a role in reducing convalescence time by aiding viral elimination (Kaila et al., 1992; Majamaa et al., 1995). Studies using B. breve have shown that oral administration of this probiotic to hospitalized children can also support a reduction in both the incidence of diarrhoea and of viral shedding, concomitant with elevated titres of anti-rotavirus IgA antibody in the stools (Araki et al., 1999).

Probiotics and tumour growth

Several studies in animal models have investigated the effects of probiotic administration on immune responses and tumour regression. Initial studies had
indicated that systemically delivered LAB cells could potentiate ex vivo leucocyte tumoricidal and lymphoproliferative responses and could limit the growth of both primary and secondary tumours at several tissue sites in vivo (Kato et al., 1981, 1983). More recent studies have focused on the use of orally delivered probiotics and anti-tumour immunity. *L. casei* Shirota has received a great deal of research attention. Orally delivered *L. casei* Shirota was shown to reduce the establishment and growth of inoculated syngeneic sarcoma cells in BALB/c mice, concomitant with an increased lymphoproliferative response and capacity to secrete the cytokine IL-2 by splenic T-cells in these animals (Yokokura, 1994). Furthermore, growth of secondary tumours was inhibited in probiotic-fed mice following tumour resection, again linked to enhanced lymphocyte responsiveness (Kato et al., 1994). *Lactobacillus plantarum* (strain L-137) has also been shown to retard the growth of implanted P3881D tumour cells in syngeneic DBA/2 mice, and in this case the mechanism was suggested to be a systemic elevation of the pro-cellular-immunity cytokine IL-12, favouring anti-tumour cellular immune responses (Murosaki et al., 2000).

Additional studies on *L. casei* Shirota have indicated that this strain may also have anti-carcinogenic effects related to enhanced immune activity. Takagi et al. (1999) have recently demonstrated that mice fed the probiotic developed fewer systemic tumours following injection of the hydrocarbon carcinogen 3-methylcholanthrene and that lymphoproliferative responses and the IL-2-secreting activity of splenic T-cells were retained, while the comparative immune responses in non-probiotic-fed mice declined markedly during tumour development. In similar studies, probiotic-containing yoghurt has been shown to limit intestinal-tract tumour development in mice injected with the carcinogen 1,2-dimethylhydrazine (Perdigon et al., 1998), and this reduction was associated with enhanced infiltrations of CD4+ T lymphocytes into the intestinal tissues in these mice. Other strains of *Lactobacillus* have also been shown to limit the incidence and mean developmental size of colonic adenocarcinomas in Sprague–Dawley rats fed 1,2-dimethylhydrazine (Balansky et al., 1999; McIntosh et al. 1999), although associated immune responses were not investigated in these studies. A further anti-cancer mechanism of probiotics involves the deconjugation of potentially mutagenic enzymes in the gut lumen, although this mechanism is not thought to have an immune component.

No longitudinal clinical trials have yet been undertaken to determine the potentially protective effects of immunoactive probiotics in the reduction of tumour incidence/development. However, a few studies have investigated the ability of probiotic LAB strains to retard tumour growth in cancer patients. *L. casei* Shirota was shown to reduce the recurrence of superficial bladder cancer in adult patients following resection (Aso et al., 1995) and also to delay the onset of tumour recurrence (Aso et al., 1992; Table 13.2). Although associated cellular immune parameters were not reported in these studies, work in adult colon-cancer patients has shown that oral *L. casei* Shirota delivery can enhance circulating NK-cell activity (Sawamura et al., 1994), suggesting that tumour limitation may be the result of enhanced immunoactivity imparted by the probiotic. In contrast, however, a recent study has reported that *L. casei* Shirota does not enhance NK-cell tumoricidal activity in healthy adult subjects (Spanhaak et al., 1998).
Probiotics and the control of immune dysfunctions

The immune system plays an essential role in the regulation of inflammatory-type diseases, and consequently a dysfunction of the immune system can lead to exacerbation of disease. Due to their potential for immune regulation, it has been suggested that probiotics offer potential for the alleviation of several immuno-inflammatory diseases. Perhaps most attention has been given to the ability of probiotics to regulate allergic/atopic responses. In animal studies, *L. casei* Shirota has been shown to reduce cutaneous anaphylaxis in allergen-sensitized mice following dermal challenge (Yasui *et al.*, 1999). Both *L. casei* Shirota and *L. plantarum* L-137 have been shown to exhibit anti-allergy properties in mice, reportedly due to their ability to induce high-level systemic expression of IL-12 (Murosaki *et al.*, 1998; Kato *et al.*, 1999), which can down-regulate allergic responses. Indeed, some strains of lactobacilli have been shown to elevate systemic levels of IL-12 following oral delivery (Murosaki *et al.*, 1999; Tejada-Simon *et al.*, 1999), suggesting that this is a major mechanism by which probiotics effect anti-allergy-type immunoregulation.

In human studies of allergic disease, there is longitudinal evidence that consumption of probiotic-supplemented yoghurt over a period of 1 year can lower the circulating levels of IgE and reduce nasal allergies in elderly subjects (Halpern *et al.*, 1991; Trapp *et al.*, 1993; Table 13.2). Wheeler *et al.* (1997) have shown that shorter-term consumption of probiotics (i.e. 1 month) by adult allergy sufferers can generate a trend towards reduced peripheral blood eosinophil counts and increased IFN-γ-secreting activity of lymphocytes, suggesting that probiotic-induced anti-allergy immune regulation may be effective in humans also. A report by Pelto *et al.* (1998) demonstrated an alternative mechanism for the ability of *L. rhamnosus* GG to limit hypersensitivity responses in subjects with cows'-milk allergy, namely, that the probiotic can prevent the up-regulation of pro-inflammatory receptors on leucocytes (a response that normally precedes GI tract inflammation in milk-sensitive subjects). Other potential mechanisms by which probiotics might limit food-hypersensitivity responses include their ability to stabilize the gut intestinal barrier against macromolecular sensitization (Majamaa and Isolauri, 1997) and/or the enzymatic hydrolysis of potentially allergenic macromolecules (Rokka *et al.*, 1997). In the latter case, an additional mechanism may be the generation of immunoregulatory peptides from milk substrates by the enzymatic action of probiotics, since Sutas *et al.* (1996a, b) have shown that milk or casein hydrolysed with *L. rhamnosus* GG invokes lower levels of pro-allergy immune responses in antigen-stimulated peripheral blood lymphocytes from milk-sensitive subjects than do intact macromolecules.

In addition to anti-allergy immunoregulation, several studies have suggested that probiotics could be used to combat inflammatory-type diseases. There is some evidence that dietary consumption of immunoregulating LAB might assist in combating autoimmune diseases, including juvenile chronic arthritis (Malin *et al.*, 1996), although the potential mechanism for this is uncertain. A recent report has shown that a diet rich in lactobacilli could decrease subjective symptoms of arthritis among rheumatoid patients, although whether
this effect was a result of anti-inflammatory immune regulation is uncertain (Nenonen et al., 1998). The potential use of probiotics to augment the routine immune signalling events of the gut microflora, as a means of restoring vital anti-inflammatory immunoregulatory control mechanisms, has recently gained a great deal of attention as a promising means of combating inflammatory bowel disease (Gionchetti et al., 2000). However, definitive proof for the effectiveness of this mechanism remains to be obtained.

Overview and Conclusions

It is clear, from the foregoing discussions, that there is significant evidence, both experimental and clinical, to indicate that certain strains of probiotic organisms can modulate the immune system of the host. The two major impacts that have been demonstrated so far include immunostimulation and immunoregulation. Immunostimulation involves an elevation of immune function(s) to a heightened state of responsiveness, and may provide an important role in conditions where an elevation of immune function is not achievable by conventional means or in boosting responses among individuals with sub-optimal immunity. Experimentally, several strains of *Lactobacillus* and *Bifidobacterium* have been shown to boost humoral antibody responses to experimentally administered T-cell-dependent antigens (Portier et al., 1993; Perdigon et al., 1995; Gill et al., 2000). In human studies, *Lactobacillus* GG has been shown to enhance the humoral immune response to orally administered rotavirus and *Salmonella typhi* vaccines (Isolauri et al., 1995; Fangac et al., 2000), while *B. breve* enhances IgA antibody responses to poliomyelitis vaccine (Fukushima et al., 1998), thus providing evidence of the potential use of probiotics as oral adjuvants to boost immune responses at the gut mucosal surface. Future uses of probiotics may be expanded to their use as oral adjuvants to promote immune responses against vaccines that currently can only be administered parenterally – for example, to boost circulating antibody responses to orally administered influenza vaccine (Maassen et al., 2000).

A further role for immune-stimulating probiotics is their use in boosting immune function in individuals with suboptimally functioning immunity. *Lactobacillus* GG has already been mentioned as an oral immunostimulator to enhance antibody responses in children combating rotavirus infection, and probiotics may prove very useful in this context of boosting immunity among malnourished children or infants with poorly developed sensitization. At the other end of the age spectrum, probiotics may prove useful in boosting immunity among elderly subjects. Studies have shown that senescence of the immune system can predispose the elderly to infectious and non-infectious diseases and that a decline in immune function with age can contribute to decreased life expectancy (Roberts-Thomson et al., 1974; Goodwin, 1995). Immunosenescence is characterized by a suboptimal functioning of the cellular immune system in particular, mainly involving T-cell-mediated responses but also some NK-cell and phagocyte functions (Lesourd and Meaume, 1994; Butcher et al., 2000; Solana and Mariani, 2000). In this respect, it has been demonstrated that
L. rhamnosus (strain HN001) and B. lactis (strain HN019) are both effective at boosting cellular immune function among healthy middle-aged and elderly subjects (Arunachalam et al., 2000; Chiang et al., 2000; Gill and Rutherfurd, 2001; Gill et al., 2001a, b, c; Sheih et al., 2001). Thus, certain probiotic strains may offer benefit to elderly consumers by stimulating the very compartments of the immune system that are adversely affected by ageing.

The immunoregulatory role of probiotics has probably received the greatest degree of attention in experimental research. A large proportion of this work has thus far focused on probiotic LAB, which induce the anti-allergy cytokines IL-12 and IFN-γ, for their potential use in preventing atopic responses and combating allergies. Yet there is still only limited clinical evidence that orally delivered probiotics are effective at combating allergic symptoms among at-risk groups (Trapp et al., 1993; Wheeler et al., 1997). In contrast, there is gathering clinical evidence that certain probiotic strains can be used effectively in neonatal and paediatric care to provide the necessary bacterial signals which, in early life, enable the immune system to develop appropriately and to avoid allergic sensitization (Isolauri et al., 2000; Kalliomaki et al., 2001).

Other potential uses of immunoregulatory probiotics (e.g. in controlling inflammatory diseases at the gut surface) have only recently begun to attract research attention (Venturi et al., 1999), partly because the microbial: gut mucosal signalling mechanisms are only beginning to be understood by microbiological researchers (Haller et al., 2000). A recent pilot study (Gupta et al., 2000) showed promising preliminary results for the use of L. rhamnosus GG as a dietary supplement to reduce clinical indices of GI-tract inflammation in children with Crohn's disease. As research starts to define the interactions of the gut microflora and the immune system in the maintenance of health, so it is likely that new avenues for dietary intervention will become the focus of research efforts.

The Future for Probiotics in Immune Health

For both immunostimulatory and immunoregulatory roles, contemporary research has already identified a few promising strains of immunoactive probiotics (predominantly LAB) and these strains either are currently being commercialized or are near commercialization. An on-going need for research in this area is for continued safety monitoring, particularly among individuals with pre-existing health conditions. For example, among patients with active autoimmune conditions, probiotic strains that stimulate cellular immune function must receive particular and thorough attention to avoid the potential for disease exacerbation; moreover, the safety of probiotics in subjects with deficient immune systems (e.g. acquired immune deficiency syndrome (AIDS) patients) should be considered. That aside, the essential requirement in all of these cases is not only that the probiotic under consideration is effective at influencing immunity but that it influences the immune system in the appropriate manner and, moreover, that this action contributes in a meaningful way to health improvement. In the former case, this suggests that well-designed and appro-
appropriate clinical trials are conducted to determine the impact of any probiotic on
the immune system and that this research is conducted on the target population
(with regard to demographics, etc.) (Meydani and Ha, 2000). In the latter case,
where an effect of probiotics on the immune system has already been demon-
strated, there remains the need to correlate immunoactivity with health
improvement. In many cases (e.g. boosting of anti-tumour immunity) such evi-
dence will only come from longitudinal/cross-sectional studies of significant
duration (Macfarlane and Cummings, 1999). Nevertheless, major progress in
the use of defined probiotics for health improvement is likely to become appar-
ent in the coming decade.

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Introduction

The mammalian host defence has successfully handled environmental confrontations for millions of years. To this end, numerous genes involved in innate and acquired (adaptive) immune protection have been subjected to evolutionary modifications, thus being shaped according to the microbial pressure and environmental (including dietary) impact. In humans, this modulation has been influenced by various ways of living, such as hunting, fishing, gathering, agriculture and animal husbandry.

In the process of evolution, the mucosal immune system has generated two arms of adaptive defence: (i) antigen exclusion, performed by secretory antibodies of the immunoglobulin (Ig)A and IgM classes, to modulate or inhibit surface colonization of microorganisms and dampen penetration of potentially dangerous soluble agents; and (ii) suppressive mechanisms to avoid local and peripheral overreaction (hypersensitivity) against innocuous substances bombarding the mucosal surfaces (Fig. 14.1). The latter arm is referred to as ‘oral tolerance’ when induced via the gut against dietary antigens (Brandtzaeg, 1996a); it probably explains why overt and persistent hypersensitivity to food proteins is relatively rare (Bischoff et al., 2000). Similar down-regulatory mechanisms apparently operate against antigens from the commensal microbial flora (Duchmann et al., 1997; Karlsson et al., 1999; Helgeland and Brandtzaeg, 2000).

Oral tolerance generally seems to be a rather robust adaptive immune function, in view of the fact that more than a ton of food may pass through the
gut of an adult every year, resulting in substantial uptake of intact antigens even in the healthy state. Nevertheless, the neonatal period is particularly critical in terms of mucosal defence, in regard to both infections and priming for allergic disease (Holt and Jones, 2000). This is so because the mucosal barrier function and the immunoregulatory network are poorly developed for a variable period after birth (Brandtzaeg et al., 1991; Holt, 1995). Notably, the post-natal development of mucosal immune homoeostasis appears to depend on the establishment of a normal commensal microbial flora, as well as on the adequate timing and dose of dietary antigens when first introduced (Brandtzaeg, 1996b, 1998; Helgeland and Brandtzaeg, 2000).
Antibody-mediated Defence in the Neonate

Striking species differences

The enterocytes of mammals play a vital role in defence of the neonate, not only by forming a mechanical barrier but also by transferring breast-milk-derived maternal antibodies from the gut lumen, thus providing passive systemic immunity in the newborn period. This enterocytic Ig transmission differs remarkably among species. In the ungulate (horse, cattle, sheep, pig), the whole length of the intestine is involved in a non-selective protein uptake, including all Ig isotypes, in a poorly defined pinocytic process. Because colostrum of these animals is particularly rich in IgG, this antibody class will preferentially reach the circulation of the neonate via its gut epithelium during the two first postnatal days, after which so-called ‘gut closure’ takes place (Mackenzie, 1990). Rodents, on the other hand, express an Fc receptor specific for IgG apically on neonatal enterocytes in the proximal small intestine. This receptor (FcRn), which disappears at weaning, has been particularly well characterized on enterocytes of the neonatal rat; it is a major histocompatibility complex (MHC) class I-related molecule, associated with β₂-microglobulin (Simister and Mostov, 1989). Complexes of FcRn and IgG are internalized in clathrin-coated pits at the base of the microvilli; binding of the ligand takes place in the acidic luminal environment, and IgG release occurs at physiological pH on the basolateral face of the enterocyte, after which the receptor is recycled.

In contrast to the animal species mentioned above, the human fetus acquires maternal IgG via the placenta (Mackenzie, 1990) and perhaps, to some extent, from swallowed amniotic fluid via FcRn expressed by fetal enterocytes (Israel et al., 1993). Indeed, a bidirectional transport mechanism for IgG was recently demonstrated in a human intestinal epithelial cell line (Dickinson et al., 1999), but the functional significance of FcRn on enterocytes in the human newborn remains unknown. Intestinal uptake of secretory IgA (sIgA) antibodies after breast-feeding appears of little or no importance in the support of systemic immunity (Ogra et al., 1977; Klemola et al., 1986), except perhaps in the preterm infant (Weaver et al., 1991). Although gut closure in humans normally seems to occur mainly before birth, a patent mucosal barrier function may not be established until after 2 years of age; the different variables involved in this process are poorly defined (van Elburg et al., 1992). Interestingly, the post-natal colonization of commensal bacteria is important both to establish (Hooper et al., 2001) and to regulate (Neish et al., 2000) an appropriate epithelial barrier.

Immediately after birth, the mucosae are bombarded by a large variety of microorganisms, as well as by protein antigens from the environment, the latter particularly in formula-fed infants. The mucosal surface to be protected is enormous, probably more than 100 times that of the skin. In fact, the various mucosae are favoured as portals of entry by the majority of infectious agents, allergens and carcinogens. In most mucosal tissues, the epithelial barrier is monolayered and therefore quite vulnerable, so the defence of this large surface area is a formidable task. Nevertheless, most babies growing up under
privileged conditions show remarkably good resistance to infections if their innate non-specific mucosal defence mechanisms are normally developed. This can be explained by the fact that immune protection of their mucosae is additionally provided by maternal IgG antibodies, which are distributed in interstitial tissue fluid at a concentration 50–60% of the intravascular level. In the first postnatal period, only occasional traces of SlgA and SlgM normally occur in the intestinal juice, whereas some IgG is more often present. This might be a result of external FcRn-mediated transmission or, perhaps more probably, it reflects passive epithelial ‘leakage’ from the highly vascularized lamina propria, which, particularly after 34 weeks of gestation, contains readily detectable maternal IgG (Brandtzaeg et al., 1991). However, an optimal mucosal barrier function in the neonatal period unquestionably depends on an adequate supply of breast milk, as highlighted in relation to mucosal infections, especially in developing countries (Anon., 1994). In the Westernized part of the world, the anti-infectious protective value of breast-feeding is clinically most apparent in preterm infants (Hylander et al., 1998).

Critical role of breast-feeding

When much of the transferred maternal IgG has been catabolized around 2 months of age, the infant becomes still more dependent on antibodies from breast milk for specific humoral immunity. At least 90% of the pathogens attacking humans use the mucosae as portals of entry; mucosal infections are in fact a major killer of children below the age of 5 years, being responsible for more than 14 million deaths of children annually in developing countries. Diarrhoeal disease alone claims a toll of 5 million children per year, or about 500 deaths every hour. These sad figures document the need for mucosal vaccines against common infectious agents, in addition to the importance of advocating breast-feeding. Convincing epidemiological documentation suggests that the risk of dying from diarrhoea is reduced 14–24 times in nursed children (Hanson et al., 1993; Anon., 1994). Indeed, exclusively breast-fed infants are better protected against a variety of infections (Pisacane et al., 1994; Wold and Hanson, 1994; Newman, 1995; Wright et al., 1998) and apparently also against allergy, asthma (Saarinen and Kajosaari, 1995; Oddy et al., 1999; Kull et al., 2001) and coeliac disease (Brandtzaeg, 1997a). Interestingly, experiments in neonatal rabbits strongly suggest that SlgA is a crucial anti-microbial component of breast milk (Dickinson et al., 1998). The role of secretory antibodies for mucosal homoeostasis is furthermore supported by the fact that knockout mice lacking SlgA and SlgM show increased mucosal leakiness (Johansen et al., 1999).

After the peak of passive immunity mediated by maternal IgG and antibodies from breast milk, the survival of the infant will, to an increasing extent, depend on its own adaptive immune responses. At mucosal surfaces, such responses are largely expressed by local antibody production (Brandtzaeg et al., 1999a). The cellular basis for this first-line humoral defence is the fact that exocrine glands and secretory mucosae contain most of the body’s activated B-
Local Immunity and Breast-feeding

cells, terminally differentiated to Ig-producing blasts and plasma cells (collectively called immunocytes). These cells produce mainly dimers and some larger polymers of IgA (collectively called pIgA), which, along with pentameric IgM, can be actively transported through the serous type of secretory epithelia (Brandtzaeg, 1973, 1974a, b, 1975; Brandtzaeg et al., 1968), including lactating mammary glands (Brandtzaeg, 1983), to act in a first-line mucosal defence (Fig. 14.2). As discussed later, this function depends on the epithelial polymeric Ig receptor (pIgR), which consists of a transmembrane glycoprotein, also known as membrane secretory component (SC).

**Fig. 14.2.** Model for local generation of secretory immunoglobulin (Ig)A and secretory IgM. J-chain-containing dimeric IgA (IgA + J) and pentameric IgM (IgM + J) are produced by local plasma cells (left). Polymeric Ig receptor (pIgR) or membrane secretory component (SC) is synthesized by secretory epithelial cell in the rough endoplasmic reticulum (ER) and matures in the Golgi complex by terminal glycosylation (●). In the trans-Golgi network (TGN), pIgR is sorted for delivery to the basolateral plasma membrane. The receptor becomes phosphorylated (○) on a serine residue in its cytoplasmic tail. After endocytosis, ligand-complexed and unoccupied pIgR is delivered to basolateral endosomes and sorted for transcytosis to apical endosomes. Some recycling from basolateral endosomes to the basolateral surface may occur for unoccupied pIgR (not shown). Receptor recycling also takes place at the apical cell surface as indicated, although most pIgR is cleaved to allow extrusion of SlgA, SlgM and free SC to the lumen. During epithelial translocation, covalent stabilization of SlgA regularly occurs (disulphide bond between bound SC and one IgA subunit indicated), whereas free SC in secretions stabilizes the non-covalently bound SC in SlgM (dynamic equilibrium indicated). (Modified from Brandtzaeg *et al.*, 1999a.)
IgA-producing immunocytes are normally undetectable in human intestinal mucosa before 10 days of age but thereafter a rapid increase takes place, although IgM immunocytes usually remain predominant up to 1 month (Brandtzaeg et al., 1991; Brandtzaeg, 1996b, 1998). Adult salivary IgA levels are reached quite late in childhood, but only a small increase of IgA-producing cells has been reported to take place in the intestinal mucosa after 1 year. These observations have been made in industrialized countries; a faster development of the IgA immune system is usually seen in children from developing countries, reflecting the adaptability of mucosal immunity according to the environmental antigenic load, as discussed below. This should not detract from the fact that breast-feeding is highly desirable for both its immunological and its nutritional value (Børresen, 1995).

Immune Induction in Mucosa-associated Lymphoid Tissue (MALT)

Integration and regionalization

Lymphoid cells are located in three distinct compartments in the gut: organized gut-associated lymphoid tissue (GALT), the lamina propria and the surface epithelium. GALT comprises the Peyer’s patches, the appendix and numerous solitary lymphoid follicles, especially in the large bowel (O’Leary and Sweeney, 1986). All these lymphoid structures are believed to represent inductive sites for intestinal immune responses (Brandtzaeg et al., 1999a). The lamina propria and epithelial compartment constitute effector sites but are nevertheless important in terms of cellular expansion and differentiation within the mucosal immune system. GALT and other MALT structures (see below) are covered by a characteristic follicle-associated epithelium (FAE), which contains membrane (M) cells (Figs 14.1 and 14.3). These specialized thin epithelial cells are particularly effective in the uptake of live and dead antigens from the gut lumen, especially when they are of a particulate nature (Hathaway and Kraehenbuhl, 2000). Many enteropathogenic infectious bacterial and viral agents use the M cells as portals of entry.

GALT structures resemble lymph nodes with B-cell follicles, intervening T-cell areas and a variety of antigen-presenting cell (APC) subsets, but there are no afferent lymphatics supplying antigens for immunological stimulation. Therefore, the exogenous stimuli must come directly from the gut lumen, probably in the main via the M cells. Among the T-cells, the CD4+ helper subset predominates, the ratio between CD4 and CD8 cells being similar to that of other peripheral T-cell populations (Brandtzaeg et al., 1999a). In addition, B-cells aggregate together with T-cells in the M cell pockets, which thus represent the first contact site between immune cells and luminal antigens (Brandtzaeg, 2001; Yamanaka et al., 2001). The B-cells may perform important antigen-presenting functions in this compartment, perhaps promoting antibody diversification and immunological memory or contributing to tolerance induction (Brandtzaeg et al., 1999b). Other types of professional APCs, macrophages and dendritic cells (DCs), are located below the FAE and between the follicles.
Pioneer studies performed in animals almost 30 years ago demonstrated that immune cells primed in GALT are functionally linked to mucosal effector sites by an integrated migration or ‘homing’ pathway (Brandtzaeg, 1996a). T-cells activated by microbial and other antigens in GALT preferentially differentiate to CD4+ helper cells, which, aided by DCs and the secretion of cytokines,
such as transforming growth factor (TGF)-β and interleukin (IL)-10, induce the
differentiation of antigen-specific B-cells to predominantly IgA-committed plasma blasts. These blasts proliferate and differentiate further on their route through mesenteric lymph nodes and the thoracic duct into the bloodstream (Fig. 14.3). Thereafter, they home preferentially to the gut mucosa, where they complete their terminal differentiation to IgA-producing plasma cells (see below). As reviewed elsewhere (Brandtzaeg et al., 1999a, c), this migration of lymphoid cells is facilitated by ‘homing receptors’ interacting with ligands on the microvascular endothelium at the effector site (‘addressins’), with an additional fine-tuned navigation mechanism conducted by chemoattractant cytokines (chemokines). Under normal conditions, therefore, the local microvasculature exerts a ‘gatekeeper’ function to allow selective extravasation of primed lymphoid cells belonging to the mucosal immune system (Fig. 14.3).

Although GALT constitutes the major part of MALT, induction of mucosal immune responses can also take place in the palatine tonsils and other lymphoepithelial structures of Waldeyer’s pharyngeal ring, including nasal-associated lymphoid tissue (NALT), such as the adenoids in humans (Brandtzaeg, 1999; Brandtzaeg et al., 1999b, c), and probably also bronchus-associated lymphoid tissue (BALT). Because BALT is lacking in normal lungs of newborns and adults (Pabst and Gehrke, 1990; Tschernig et al., 1995), Waldeyer’s ring may represent a significant component of human MALT. Accumulating evidence suggests that a certain regionalization exists in the mucosal immune system, especially a dichotomy between the gut and the upper aerodigestive tract with regard to homing properties and terminal differentiation of B-cells (Brandtzaeg et al., 1999a, b, c). This disparity may be explained by microenvironmental differences in the antigenic repertoire as well as the adhesion molecules and chemokines involved in preferential local leucocyte extravasation. It appears that primed immune cells selectively home to effector sites corresponding to the inductive sites where they were initially triggered by antigens. Such regionalization within the ‘common’ or integrated mucosal immune system has to be taken into account in the development of local vaccines.

B-cell homing to mammary glands

Lactating mammary glands are part of the integrated mucosal immune system, and milk antibodies reflect antigenic stimulation of MALT in the gut as well as in the airways. This fact has been documented by showing that SIgA from breast milk exhibits antibody specificities for an array of common intestinal and respiratory pathogens (Goldman, 1993). The secretory antibodies are thus highly targeted against infectious agents in the mother’s environment, which are those likely to be encountered by the infant during its first weeks of life. Therefore, breast-feeding represents an ingenious immunological integration of mother and child (Fig. 14.4). Although the protection provided by this humoral defence mechanism is most readily demonstrable in populations living in poor sanitary conditions (Hanson et al., 1993; Anon., 1994), a beneficial clinical effect is also apparent in the industrialized world (Wold and Hanson, 1994),
even in relation to relatively common diseases, such as otitis media and acute lower respiratory tract infections (Pisacane et al., 1994; Newman, 1995; Golding et al., 1997).

Antibodies to various dietary antigens, such as cow's milk proteins (Savilahti et al., 1991) and gluten (Juto and Holm, 1992), are also present in breast milk. However, little is known about the preferential site where soluble luminal antigens exert immune priming. Thus, dietary proteins may be taken up mainly through the extensive epithelial surfaces covering the diffuse immunological effector tissue of the intestinal mucosa rather than by M cells, and may therefore be largely transported to the mesenteric lymph nodes. As discussed below, their fate and possible immune-inductive or tolerogenic effects will depend on how they are handled locally and whether they reach lymph or portal blood (Brandtzæg et al., 1987; Sanderson and Walker, 1993; Brandtzæg, 1996a).
Post-natal Development of Mucosal Immunity

Activation of the local B-cell system

Peyer’s patches are the best studied MALT structures and start to develop in fetal life (Cornes, 1965; Husband and Gleeson, 1990), with discrete T- and B-cell areas being apparent as early as 19 weeks of gestation (Spencer and MacDonald, 1990). The primary lymphoid follicles seem to be generated around follicular dendritic cells (FDCs). However, lymphoid hyperplasia, with secondary follicles containing germinal centres (signifying B-cell activation), does not occur until shortly after birth (Bridges et al., 1959; Spencer et al., 1986a; Gebbers and Laissue, 1990); this reflects the dependency of MALT on exogenous environmental stimulation. Furthermore, animal studies have shown an absence of secondary follicles in Peyer’s patches of germ-free mice (Parrott, 1976). The germinal-centre B-cells express small amounts of membrane IgA, along with less IgM or IgG (Butcher et al., 1982). Such isotype skewing reflects differentiation to precursors for IgA-producing cells. The drive for isotype switching towards IgA, together with J-chain expression, in B-cells is much more evident in Peyer’s patches than in other MALT structures, but the reasons for this are unclear (Brandtzaeg et al., 1999a, b). The combination of IgA and J-chain production is a prerequisite for the generation of SIgA antibodies (Fig. 14.2).

The retarded post-natal immune activation of MALT parallels the functionally decreased systemic immunocompetence in the newborn period (MacDonald and Spencer, 1993; Holt, 1995; Holt and Jones, 2000). Thus, peripheral CD4+ T-cells of infants show a reduced capacity for the production of interferon (IFN)-γ (Taylor and Bryson, 1985; Holt et al., 1992) and IL-4 (Lewis et al., 1991), as well as for B-cell help (Splawski and Lipsky, 1991; MacDonald and Spencer, 1993). One reason might be that there are relatively few circulating memory (CD45RO+) T-cells in infancy. Interestingly, the responsiveness of neonatal naïve (CD45RA+) T-cells does not differ significantly from that of virgin counterparts in adults, and more recent animal studies suggest that the chief explanation for the apparent immunological immaturity is to be found in a deficient APC function (Ridge et al., 1996). Thus, in the neonate macrophages, DCs and B-cells are all unable to deliver adequate co-stimulatory signals to naïve T-cells (Lu et al., 1979; Taylor and Bryson, 1985; Morris et al., 1992; Ridge et al., 1996).

Homing of primed lymphoid cells to mucosal effector sites

After antigen-induced activation, proliferation and partial differentiation in MALT, lymphoid memory and effector cells migrate rapidly via regional lymph nodes and the peripheral blood circulation to various secretory effector sites (Fig. 14.3). The presence of SIgA antibodies to bovine β-lactoglobulin in neonatal breast milk (’witch milk’) from infants fed cow’s milk formula (Roberton et al., 1986) documents such early postnatal homing of primed
intestinal B-cells to mammary glands. However, very few B-cells with IgA-producing capacity are actually present in the blood of newborns (< 8 per million mononuclear cells), although this number is remarkably increased (~ 600 per million mononuclear cells) after 1 month, reflecting the progressive exogenous stimulation of GALT (Nahmias et al., 1991). An initial early elevation of Ig-producing cells can be seen in preterm infants, especially in those with intrauterine infections, although the IgM class not unexpectedly dominates in such cases (Stoll et al., 1993). Altogether, these observations support the notion that mucosal immune cells are competent even before birth, at least during the final trimester, but that APCs need to undergo an activation process initiated by exogenous ‘danger signals’ enabling them to provide sufficient co-stimulatory signals to naive T-helper (Th) cells (Medzhitov and Janeway, 1997). This is further supported by the finding that fetal lamina propria T-cells (mainly CD4+) can be activated by mitogens or bacterial super-antigens in vitro (MacDonald and Spencer, 1993).

In this context, it is also interesting to note that intraepithelial lymphocytes (IELs) are present in human intestinal epithelium as early as 11 weeks of gestation (Orlic and Lev, 1977). As in adults, fetal IELs occur mainly in the villi of the small intestine and are dominated by CD3+ CD8+ T-cells (Brandtzaeg et al., 1998). Their numbers increase throughout the gestational period (Spencer et al., 1986b), which suggests that the migration of IELs into the epithelium is, to some extent, antigen-independent. However, stimulation by luminal factors clearly determines the numbers of IELs, as shown by their rapid post-natal increase, up to tenfold by the age of 1–2 years (Cerf-Bensussan and Guy-Grand, 1991; Machado et al., 1994); this probably reflects the development of GALT, from which the intestinal IEL precursors may largely be derived (Guy-Grand et al., 1978; Dunkley and Husband, 1987; Cuff et al., 1993). In a similar manner, germ-free animals have few IELs. Moreover, conventionalization of germ-free mice and rats has demonstrated a marked stimulatory effect of the commensal intestinal microflora and also its apparent impact on the T-cell receptor (TCR) repertoire of IELs (Helgeland and Brandtzaeg, 2000).

Effects of antigen exposure and nutrition on secretory immunity

The degree of antigenic and mitogenic exposure is decisive not only for the post-natal development of IELs, but also for the secretory immune system. Antigenic constituents of food clearly exert a stimulatory effect on the intestinal B-cell system, as suggested by the occurrence of fewer lamina propria IgA immunocytes both in mice fed on hydrolysed milk proteins (Sagie et al., 1974) and in parenterally fed babies (Knox, 1986). Likewise, mice given total parenteral (intravenous) nutrition have reduced numbers of B- and T-cells in the gut, as well as decreased SlgA levels (Li et al., 1995a, b; Janu et al., 1997), and they show impaired SlgA-dependent influenza-specific immunity (Renegar et al., 2001). The effect of food in the gut lumen could be direct immune stimulation or mediated via release of gastrointestinal neuropeptides. The indigenous microbial flora is also extremely important for secretory immunity, as shown by
the fact that the intestinal IgA system of germ-free or specific pathogen-free mice is normalized after about 4 weeks of conventionalization (Crabbé et al., 1970; Horsfall et al., 1978). Bacteroides and Escherichia coli strains seem to be particularly stimulatory for the development of intestinal IgA immunocytes (Lodinová et al., 1973; Moreau et al., 1978). The large dietary and bacterial antigen load in the gut lumen therefore explains why the greatest density of IgA immunocytes is seen in the intestinal lamina propria, amounting to some 10¹⁰ cells m⁻¹ of adult gut (Brandtzaeg et al., 1999a).

In human lactating mammary glands the immunocyte density is much less, one gland showing an IgA-producing capacity similar to that of only 1 m of intestine (Brandtzaeg, 1983). Thus, the daily output of IgA kg⁻¹ wet weight of tissue (minus fat) is no more for lactating mammary glands than for salivary glands. In fact, it remains an enigma how any terminal plasma cell differentiation at all is accomplished in these secretory effector organs, which are at considerable distances from antigen-exposed mucosal surfaces (Brandtzaeg et al., 1999a). Anyhow, the large capacity for storage of pIgA/SIgA in the mammary-gland epithelium and duct system, rather than a high immunocyte density, explains the remarkable output of SIgA during breast-feeding (Brandtzaeg, 1983).

In keeping with an important stimulatory effect of antigens on local B-cell differentiation, defunctioning colostomies in children showed a 50% numerical reduction of mucosal IgA and IgM immunocytes after 2–11 months (Wijesinha and Steer, 1982). Prolonged studies of defunctioned ileal segments in lambs revealed an even more striking scarcity of mucosal immunocytes. This was caused by decreased local proliferation and differentiation of B-cell blasts and perhaps reduced homing from GALT (Reynolds and Morris, 1984). Accordingly, the post-natal establishment of the mucosal IgA system is usually much faster in developing countries than in the industrialized part of the world, a difference that seems to hold true even in undernourished children (Nagao et al., 1993). However, severe vitamin A deficiency has been reported to have an adverse effect on mucosal IgA antibody responses in rodents (Wiedermann et al., 1993), but with no consistent down-regulation of epithelial IgA transport (Stephensen et al., 1996).

It has been reported that undernourished children respond to bacterial overgrowth in the gut with enhanced synthesis, as well as up-regulated external transport, of IgA (Beatty et al., 1983). It is of great clinical importance that detrimental effects of severe malnutrition exerted on the SlgA system can apparently be reversed with nutritional rehabilitation (Watson et al., 1985). In a recent study based on whole gut lavage obtained from healthy adult volunteers in Dhaka, Bangladesh, the intestinal concentration of IgA was found to be almost 50% higher than that of comparable samples collected in Edinburgh, UK; the intestinal IgA antibody titre against lipopolysaccharide (LPS) core types of E. coli was almost seven times higher in the former group of subjects, in contrast to the lower levels of ovalbumin antibodies (Hoque et al., 2000).

In view of the above information, the possibility exists that sub-optimal stimulatory reinforcement of the SlgA-dependent mucosal barrier function might contribute to the increased frequency of certain diseases in industrialized
countries, particularly allergies and other inflammatory mucosal disorders. This ‘hygiene hypothesis’ has been tested in several experimental and clinical studies by evaluating the beneficial effect of probiotic bacterial preparations. In particular, viable strains of the commensal intestinal microflora, such as lactobacilli and bifidobacteria, have been reported to enhance IgA responses, in both humans and experimental animals, apparently in a T-cell-dependent manner (Kaila et al., 1992, 1995; Isolauri et al., 1995; Yasui et al., 1995; Malin et al., 1996; Prokesová et al., 1999). Interestingly, early colonization of infants with a non-enteropathogenic strain of *E. coli* has been reported to have a long-term beneficial effect by reducing both infections and allergies (Lodinová-Zádniková and Cukrowská, 1999). Likewise, a recent double-blind study of infants with a family history of atopic (IgE-mediated) allergy reported the prevalence of atopic eczema to be reduced by 50% at the age of 2 years in those receiving the probiotic *Lactobacillus* GG strain daily for 6 months, compared with those receiving placebo (Kalliomäki et al., 2001a). It remains to be shown whether this striking beneficial effect was mediated via SIgA enhancement or by promotion of oral tolerance, as discussed later.

**Individual variations**

Post-natal mucosal B-cell development shows large individual variations, even within the same population (Brandtzaeg et al., 1991). This disparity could partly reflect a genetically determined effect on the establishment of the mucosal barrier function. Thus, it has been proposed, on the basis of serum IgA levels, that a hereditary risk of atopy is related to a retarded post-natal development of the IgA system (Taylor et al., 1973; Soothill, 1976). This notion was later supported by a report showing significantly reduced IgA immunocyte numbers (with no compensatory IgM enhancement) in the jejunal mucosa of atopic children (Sloper et al., 1981). Also, an inverse relationship was found between the serum IgE level and the jejunal IgA cell! population in children with food-induced atopic eczema (Perkkiö, 1980). It was subsequently reported that infants born to atopic parents showed a significantly higher prevalence of salivary IgA deficiency than age-matched control infants (van Asperen et al., 1985). Interestingly, Kilian et al. (1995) found that the throats of 18-month-old infants with presumably IgE-mediated allergic problems contained significantly higher proportions of IgA₁ protease-producing bacteria than age-matched healthy controls, thus supporting a previous report showing much less intact IgA in nasopharyngeal secretions from children with a history of atopic allergy than from controls with episodes of acute otitis (Sørensen and Kilian, 1984). In this context, it is important to note that it takes up to 3 months after birth before the IgA₂-to-IgA₁ immunocyte ratio in salivary glands has increased to the adult value, with approximately 33% IgA₂-producing cells (Thrane et al., 1991).

Altogether, a poorly developed or enzymatically reduced SIgA-dependent mucosal barrier function, combined with a hereditary and/or cytokine-driven hyper-IgE responsiveness (see below), could contribute to the pathogenesis of
allergy. This notion accords with the increased frequency not only of infections, but also of atopic allergy and coeliac disease seen in subjects with permanent selective IgA deficiency (Burrows and Cooper, 1997), although compensatory over-production of SIgM may apparently counteract the adverse consequences of their absent mucosal IgA responses, particularly in the gut (Brandtzaeg et al., 1991; Brandtzaeg and Nilssen, 1995).

**Mucosal Induction of Tolerance**

**Suggestive evidence of oral tolerance in humans**

The concept of oral tolerance is mainly based on feeding experiments in rodents and has a long history (Brandtzaeg, 1996a). The understanding of this mucosally-induced down-regulatory or suppressive phenomenon has been hampered by an overwhelming mechanistic complexity. Identifiable experimental variables include genetics, age, dose and timing of post-natal feeding, antigenic structure and composition of fed protein, epithelial barrier integrity and the degree of concurrent local immune activation, as reflected by microenvironmental cytokine profiles and the expression of co-stimulatory molecules on mucosal APCs (Brandtzaeg, 1996a; Nagler-Anderson, 2000; Mayer et al., 2001). Also, rodent studies suggest that the commensal microflora is important both for induction of oral tolerance and for reconstitution of this mechanism after its experimental abrogation (Helgeland and Brandtzaeg, 2000). This effect is probably mediated mainly through immune stimulation of GALT, as discussed above.

Although there is little direct evidence that oral tolerance operates in humans, it seems justified to believe that this is the case. Circumstantial evidence is provided by the fact that, in the normal state, the vulnerable gut mucosa, which is separated only by a monolayered epithelium from the enormous intestinal load of live and dead antigenic material, exhibits no substantial IgG response (Brandtzaeg et al., 1987, 1999a) and contains very few T-cells with markers of hyperactivation, such as CD25 (the IL-2 receptor) (Brandtzaeg et al., 1998). Moreover, the systemic IgG response to dietary antigens tends to decrease in humans with increasing age (Rothberg and Farr, 1965; Scott et al., 1985), and direct evidence for a hyporesponsive state in regard to bovine serum albumin has been obtained by intradermal testing with this antigen in adults (Korenblat et al., 1968).

Interestingly, experimental feeding in healthy adults with a protein to which humans are not normally exposed, keyhole limpet haemocyanin (KLH), did result in down-regulation of the peripheral T-cell response, although stimulation of local as well as systemic humoral immunity was observed (Husby et al., 1994). Conversely, intranasal application of KLH tended to suppress both cell-mediated and humoral peripheral immunity to this antigen (Waldo et al., 1994). The mechanisms remain unclear, however, and sequestration of specific immune cells into the antigen-exposed mucosae or regional lymph nodes is an alternative possibility, which may be difficult to refute because local immunity
was enhanced in both studies. Such a mechanism has been suggested in untreated coeliac-disease patients whose circulating T-cells show a decreased response to gluten compared with treated patients on a gluten-free diet (Scott et al., 1983). Nevertheless, feeding humans with KLH was recently repeated with parallel systemically immunized controls, and mucosally-induced T-cell tolerance was indeed confirmed in peripheral blood (Mayer et al., 2001, and their unpublished observations). Also notably, feeding low doses of myelin basic protein to patients with multiple sclerosis resulted in a higher frequency of circulating T-cells with a potency for production of the down-regulatory cytokine TGF-β, compared with T-cells from placebo-fed patients (Fukaura et al., 1996).

Putative involvement of lymphoepithelial interactions

A central role of the gut epithelium in oral tolerance is suggested by the observation that its experimental induction depends on the preserved integrity of the mucosal barrier (Nicklin and Miller, 1983; Strobel et al., 1983). Suppressive effects resulting from interactions between the dominating TCRα/β CD8+ IEL subset and a normal epithelium represent one intriguing possibility, and there is some supporting evidence to this effect (Sachdev et al., 1993). It is possible that luminal antigenic peptides are presented by resting enterocytes, with inadequate co-stimulation to IELs or subepithelial CD4+ T-cells (Hoyne et al., 1993). Experiments in CD8 knockout mice have suggested that CD8+ T-cells are crucial for the down-regulation of enterically-elicited mucosal immunity but not for mucosally-induced suppression of systemic antibody responses (Grdic et al., 1998). Moreover, the chief effect obtained when enterocytes have been used as unconventional APCs in various test systems has been stimulation of CD8+ T-cells with a suppressor function (Hershberg and Mayer, 2000; Mayer et al., 2001). Human enterocytes express a ligand (gp180) that, by interaction with the α chain of CD8, may rapidly activate the tyrosine kinase p56Lck and thereby trigger preferentially CD8+ T-cells (Li et al., 1995). Antigen presentation by MHC or CD1d molecules on enterocytes in this context could theoretically leave cognate IELs and even CD4+ lamina propria Th cells in an unresponsive state or induce an active down-regulatory potential by a deviated cytokine profile (Fig. 14.5). Moreover, basolateral exosomes with MHC class II-dependent antigen-presenting capacity may be released from the gut epithelium and act as ‘tolerosomes’ (Karlsson et al., 2001), either locally or at distant sites, such as mesenteric lymph nodes or the liver (Fig. 14.5).

The additional involvement of TCRγδ+ IELs in oral tolerance is also an intriguing possibility (Fig. 14.5), in view of the suggestion that this subset in the mouse may act as ‘contrasuppressor cells’, thereby being able to release intestinal IgA responses from T-cell-mediated suppression (Fujihashi et al., 1992). Subsequent studies have shown that this effect can probably be ascribed to IL-10 secreted by CD4+ T-cells, which are controlled by γδ T-cells operating through this down-regulatory cytokine in low-dose tolerance (Fujihashi et al., 1999). If this mechanism also operates in humans, the preferential expansion of intraepithelial γδ T-cells in the coeliac lesion might contribute to the striking
increase of Ig-producing immunocytes and activated lamina propria CD4+ T-cells seen in untreated patients (Scott et al., 1997). However, the increase of TCRγ/δ+ IELs in coeliac disease could instead reflect that they are cytotoxic cells involved in the clearance of microorganisms or damaged epithelium to preserve the surface barrier (Brandtzaeg, 1996a; Groh et al., 1998; Hershberg and Mayer, 2000).

**Role of co-stimulation by antigen-presenting cells**

Productive T-cell activation with appropriate proliferation and cytokine secretion requires two signalling events, one through the TCR and another through a receptor for some co-stimulatory molecule (Fig. 14.6). Without the latter signal, the T-cells mount only a partial response and, more importantly, may be sub-
jected to active tolerance induction (Nagler-Anderson, 2000) or anergy, with no capacity for production of their own growth-factor IL-2 upon restimulation (Janeway and Bottomly, 1994). The required co-stimulation for productive immunity is provided by soluble mediators, such as IL-1, and through cellular interactions, especially ligation of B7 (CD80/CD86) on professional APCs with CD28 on the T-cells (Robey and Allison, 1995). There is particularly great interest in the role of DCs in shaping the phenotypes of naive T-cells during such initial priming. Also, because DCs have migratory properties, they largely determine the tissue site in which primary immune responses will take place (Holt and Stumbles, 2000; Lanzavecchia and Sallusto, 2001).

Immature DC subsets are found both in the circulation and in most peripheral tissues, from which, after endocytosis of antigen, they generally migrate via draining lymphatics into regional lymph nodes to perform antigen presentation (Sallusto and Lanzavecchia, 1999). The actual expression level of various co-stimulatory molecules on the matured and activated DCs during the priming process influences the differentiation of naive T-cells in terms of cytokine

**Fig. 14.6.** Schematic representation of polarized patterns of cytokines produced by activated T-helper (Th) cells. When naive CD4+ Th cells are primed by a professional antigen-presenting cell (APC) providing adequate co-stimulatory signals, they differentiate into Th1 or Th2 cells. Such skewing of the immune response depends on the presence of microenvironmental factors, such as lipoproteins (LPs), lipopolysaccharide (LPS) and unmethylated CpG nucleotide motifs. Their interaction with APC receptors determine the expression level of various co-stimulatory signals. For simplicity, only the LPS receptor CD14 and Toll-like receptors (TLRs) are indicated, together with the co-stimulatory molecules B7.1 and B7.2. Th1 cells produce predominantly interferon (IFN)-γ, interleukin (IL)-2 and tumour necrosis factor (TNF)-α, while Th2 cells are mainly capable of IL-4, IL-5, IL-10 and IL-13 secretion. Distinct Th1 and Th2 profiles are further promoted by inhibitory feedback loops, as indicated. Ag, antigen; MHC II, major histocompatibility complex class II molecules; TCR, T-cell receptor.
production – that is, a Th1 (IFN-γ, IL-2 and tumour necrosis factor (TNF)-α) versus a Th2 (IL-4, IL-5, IL-10 and IL-13) profile. Interaction of the T-cell CD28 receptor with B7.1 (CD80) appears to favour the former and with B7.2 (CD86) the latter cytokine profile (Kuchroo et al., 1995). This Th1/Th2 paradigm is important in relation to atopic allergy, because IgE production as a basis for type I hypersensitivity is highly dependent on IL-4 and IL-13 (Corry and Kheradmand, 1999). Also, homoeostatic cross-regulation should ideally take place between the Th1 and Th2 responses (Romagnani, 2000).

Considerable information exists about putative aberrant immunoregulatory functions of non-professional APCs, such as keratinocytes, because they lack the appropriate co-stimulatory molecules necessary for productive immunity (Nickoloff and Turka, 1994). As alluded to above, this also applies to enterocytes (Fig. 14.5). Thus, both B7 and intercellular adhesion molecule 1 (CD54) are virtually absent on normal human enterocytes (Bloom et al., 1995). Low levels of B7 might actually engage the high-affinity co-stimulatory molecule cytotoxic T lymphocyte antigen (CTLA)-4 on Th cells (Chambers and Allison, 1999), which could result in a down-regulatory response contributing to oral tolerance (Read et al., 2000).

In the normal state, even the subepithelial professional APCs in human gut mucosa, which have both macrophage and DC properties, show an extremely low level of B7 expression (Rugtveit et al., 1997; Brandtzaeg, 2001) and might therefore ligate CTLA-4 rather than CD28 on T-cells. Also, only B7.2 (CD86) is normally detectable, and this molecule has been shown in animal experiments to be important for low-dose oral tolerance (Liu et al., 1999). Functional characteristics of normal human lamina propria CD4+ T-cells do suggest that they are tightly controlled by suppression. First, they are remarkably unresponsive to signalling via the classical TCR/CD3 pathway alone, whereas anti-CD2 (particularly together with engagement of CD28) induces proliferation and cytokine secretion (Boirivant et al., 1996; Fuss et al., 1996). Second, they appear to be particularly susceptible to Fas (CD95)-mediated apoptosis, which might contribute to the limitation of clonal proliferation in the normal gut (De Maria et al., 1996). Third, they may be kept in check by prostaglandin E2 released by the gut epithelium or lamina propria macrophages (Newberry et al., 1999).

The fact that resident APCs from normal human gut mucosa are quite inert in terms of immune-productive stimulatory properties (Qiao et al., 1996) supports the notion that they play a central role in the induction of oral tolerance. One possibility is that, in the normal state (i.e. when subjected to only low-grade activation), they carry penetrating dietary and innocuous microbial antigens away from the mucosa, thereby avoiding local hyperactivation of immune cells (Fig. 14.5). Indeed, normal human intestinal mucosa shows only very low expression levels of mRNA for IFN-γ, the key cytokine of activated Th1 cells (Nilsen et al., 1998). The same is true for Th2 cytokines, such as IL-4 and IL-5. Moreover, animal experiments have demonstrated that intestinal APCs can be triggered by pro-inflammatory factors to become mobilized (MacPherson et al., 1995) and even constitutively migrate rapidly with acquired epithelial elements and antigens away from the intestinal mucosa (Gütgemann et al., 1998; Huang et al., 2000). Such successful ‘silent’ antigen clearance probably depends on
relatively low doses of absorbed antigen and may result in systemic T-cell-dependent tolerance induction (Fig. 14.5). Interestingly, in vivo expansion of the intestinal APC population enhanced the induction of oral tolerance in mice (Viney et al., 1998), whereas concurrent APC activation by immunization with cholera toxin or treatment of the animals with IL-1 resulted in productive immunity against the fed antigen (Williamson et al., 1999).

Animal studies have suggested differential effects of antigen dose and feeding frequency on the mechanisms of tolerance induction (Brandtzaeg, 1998). At very high doses, both Th1 and Th2 cells were shown to be deleted following initial activation, an event apparently depending on apoptosis in Peyer’s patches (Chen et al., 1995). Anergy and clonal deletion would be antigen-specific events, in contrast to active suppression resulting from deviation of cytokine profiles induced by T-cell stimulation locally or in regional lymph nodes or the liver (Knolle et al., 1999; Limmer et al., 2000) after distant transport of antigen in APCs or epithelial exosomes (Fig. 14.5). Experiments performed to induce therapeutic tolerance via the gut in various autoimmune disease models have relied on a bystander effect of stimulated T-cells, which, through immune deviation, have preferentially secreted down-regulatory cytokines, particularly TGF-β (Weiner et al., 1994). It has been suggested that the gut harbours T-cells with a propensity for secretion of TGF-β (so-called Th3 cells), which appear to be particularly resistant to apoptosis (Chen et al., 1995), but this subset has not been clearly identified in humans. Another regulatory T-cell subset (Tr1), with a remarkable propensity for IL-10 production, has been identified in both the murine and the human gut (Groux et al., 1997; Khoo et al., 1997). This subset probably belongs to the activated (CD25+) and CTLA-4-expressing suppressive CD4+ T-cells induced after antigen feeding in mice (Zhang et al., 2001).

Altogether, a complex scenario may be proposed for oral tolerance, depending on apoptosis, when intestinal antigen exposure is excessive, and on anergy, due to lack of co-stimulatory APC molecules, antigen clearance from the mucosa and induction of immune deviation (skewing of T-cell cytokine profile) at lower antigen doses (Fig. 14.5). This scenario is further complicated by the fact that several cytokines contributing to the local profile are produced not only by T-cells, but also by APCs and epithelial cells – for instance, the down-regulatory cytokines TGF-β and IL-10. Furthermore, it remains unclear whether the most important immunoregulatory events for oral tolerance against dietary antigens take place in Peyer’s patches, in the lamina propria, in systemic lymphoid organs or in the liver (Chen et al., 2000; Alpan et al., 2001; Fujihashi et al., 2001).

**Importance of homoeostatic immune regulation**

It may seem paradoxical that mucosal disorders, such as inflammatory bowel disease (IBD) and coeliac disease, appear to depend, at least initially, on putative Th1-cell-driven pathogenic mechanisms (Scott et al., 1997; Brandtzaeg et al., 1999d), while atopic (IgE-mediated) allergy originates from Th2-cell responses (Brandtzaeg, 1997b; Corry and Kheradmand, 1999), which generate the essential cytokines IL-4 and IL-13 (early phase) as well as IL-3, IL-5 and
granulocyte–macrophage colony-stimulating factor (GM-CSF) (late phase). According to the ‘hygiene hypothesis’, the increasing incidence of allergy in Westernized societies may to some extent be explained by a reduced microbial load early in infancy, resulting in too little Th1-cell activity and therefore an insufficient level of IFN-\(\gamma\) to cross-regulate Th2-cell responses optimally (Rook and Stanford, 1998; Erb, 1999; Kirjavainen and Gibson, 1999). In this context, an appropriate composition of the commensal bacterial flora (Isolauri et al., 2000) and exposure to food-borne and orofaecal microbes (Herz et al., 2000; Matricardi et al., 2000) most probably exert an important homeostatic impact, both by enhancing the SlgA-mediated barrier function (see above) and by promoting oral tolerance through a shift from a predominant Th2-cell activity in the newborn period (Prescott et al., 1998) to a more balanced cytokine profile later on (Fig. 14.5). Thus, the intestinal microflora of young children in Sweden was found to contain a relatively large number of Clostridium spp., whereas high levels of Lactobacillus spp. and Eubacterium spp. were detected in an age-matched population from Estonia (Sepp et al., 1997). Perhaps this difference could explain the lower incidence of allergy in the Baltic countries compared with Scandinavia. Interestingly, the intestinal microflora of children in Estonia was deemed to be somewhat similar to that of Swedish children in the 1960s. Also, the intestinal microflora of Estonian children with allergy appeared to differ from that of their healthy counterparts, particularly by containing fewer lactobacilli (Björkstén et al., 1999). A recent Finnish study likewise reported that atopic infants had more clostridia and tended to have fewer bifidobacteria in their stools than non-atopic controls (Kalliomäki et al., 2001b).

Such observations make a good case for studying the potential clinical benefits of prebiotics and probiotic bacterial strains from the indigenous gut flora (Collins and Gibson, 1999; Kirjavainen and Gibson, 1999; Isolauri et al., 2001). Similarly, there is some hope that immunization with mycobacterial antigens might skew the cytokine profile towards Th1 and thereby, through cross-regulation, dampen Th2-dependent allergic (atopic) symptoms (von Reyn et al., 1997; Hopkin et al., 1998). Newborns are in fact able to mount a Th1-type immune response when appropriately stimulated (Marchant et al., 1999). Also notably, the bacterial endotoxin or LPS receptor CD14, together with the Toll-like receptor (TLR) 4 on APCs, as well as other TLRs that recognize microbial products (e.g. lipoproteins and peptidoglycans) as danger signals or pathogen-associated molecular patterns (PAMPs), are in this respect an important link between innate and specific immunity (Fig. 14.6). This link operates via the nuclear factor kappa B (NF\(\kappa\)B) activation pathway to release pro-inflammatory cytokines (Modlin, 2000; Kaisho and Akira, 2001), including the Th1-inducing IL-12 and IL-18 (McInnes et al., 2000; Manigold et al., 2000). Even certain CpG motifs of bacterial DNA have been shown to promote Th1-cell activity through interaction with TLR9 (Klinman et al., 1996; Kadowaki et al., 2001; Peng et al., 2001). Subepithelial intestinal APCs most probably express TLRs, although this has not yet been studied properly in the human gut (MacDonald and Pettersson, 2000). However, low levels of CD14 are normally present on these cells, and its expression is enhanced, together with that of B7.1 and B7.2, by pro-inflammatory factors (Rugtveit et al., 1997; Brandtzaeg, 2001).
Altogether, it appears that the human intestinal immune system preferentially responds with a dominating Th1 profile (Nilsen et al., 1998), even against various food antigens in the seemingly normal state (Nagata et al., 2000). This appears to be true for T-cells also in the duodenal mucosa of children with cow’s milk hypersensitivity (Hauer et al., 1997) and might, to some extent, reflect a high expression level of the Th1-promoting cytokine IL-12 observed for putative APCs situated below the FAE of Peyer’s patches in children (MacDonald and Monteleone, 2001). The strong bias towards Th1-cell responses in the human gut could thus contribute to the fact that the majority of food-allergic children outgrow their problems (Bischoff et al., 2000). This is in contrast to respiratory atopic allergy, which tends to persist and even increase in severity (Hattevig et al., 1993; Brandtzaeg et al., 1996). Most probably, danger signals from an established intestinal bacterial flora, as well as the environmental microbial exposure, exert an important drive towards an adequate Th1 skewing in the gut, thus counterbalancing excessive Th2 responses (Fig. 14.6). Nevertheless, allergen-specific mucosal Th2 cells have been detected in patients with (presumably) cow’s-milk-induced gastroenteritis (Beyer et al., 2001).

Although the immune system in the airways also responds to antigen stimulation in the presence of danger signals (infection or inflammation) with a Th1 profile (Holt and Stumbles, 2000), an increasingly prominent Th2 profile generally develops as the basis for IgE-mediated (atopic) respiratory allergy (Hattevig et al., 1993; Holt et al., 1999) in individuals with a hereditary predisposition (Anderson and Cookson, 1999; Barnes, 2000). This skewing towards Th2-cell responses may be influenced by the so-called ‘lymphoid’ DC type, recently named plasmacytoid DCs (P-DCs), which can be identified by their high level of IL-3 receptor (CD123) in allergic nasal mucosa (Jahnsen et al., 2000). In vitro, P-DCs have been shown to drive naïve T-cells towards a Th2 response, with IL-4 and IL-5 production (Rissoan et al., 1999). Interestingly, we have been unable to detect P-DCs in the intestinal lamina propria, even in IBD and coeliac disease (Jahnsen et al., 2000). Therefore, the apparent inability of this DC subset to home to intestinal effector sites might contribute to the Th1 dominance of immune responses in the human gut as a result of little cross-regulation from local Th2 responses. The paucity of human intestinal Th2 responsiveness (MacDonald and Monteleone, 2001) is emphasized by the fact that there is usually no detectable IgE production at this mucosal effector site, even in adult food-allergic individuals with overt atopy (Bengtsson et al., 1991). Hence, there may be several mechanisms other than a local mucosal Th2 response to explain gastrointestinal allergy against dietary antigens (Bruijnzeel-Koomen et al., 1995; Bischoff et al., 2000), including recruitment of mast cells armed with IgE from mesenteric lymph nodes, type III (immune complex)-mediated reactions and type IV (delayed type) hypersensitivity (Brandtzaeg, 1997b).

The feeding and treatment regimen (e.g. antibiotics) to which the newborn is subjected and its nutritional state have a significant impact on the composition of its indigenous microbiota, as well as on its gut integrity, and may hence disturb the balance of its developing mucosal immune system (Zeiger, 2000;
Hoppu et al., 2001; Isolauri et al., 2001). The role of commensal bacteria for mucosal tolerance induction in humans was highlighted in a recent clinical trial with postnatal colonization (for 6 months) of a probiotic lactobacillus strain (Kalliomäki et al., 2001a); after 2 years, a 50% reduction of atopic eczema was observed in these children, compared with placebo controls. Intestinal colonization of lactobacilli and bifidobacteria is promoted by breast milk, because of its large amounts of oligosaccharides, which have prebiotic properties (Hoppu et al., 2001); these microorganisms may directly enhance the Th1 profile in the gut by inducing IL-12, IL-18 and IFN-γ (Miettinen et al., 1998; Hessle et al., 1999). Also notably, E. coli is a strong inducer of IL-10 secretion, apparently derived from APCs (Hessle et al., 2000a, b). This has been suggested to be an important suppressive cytokine in the gut (Steidler et al., 2000). Thus, the indigenous microbiota may have an impact on mucosal homoeostasis beyond that of enhancing the SIgA system or promoting a Th1-cytokine profile that counterbalances Th2-cell responsiveness (Holt, 2000).

Immune Exclusion and IgA-mediated Mucosal Homoeostasis

The secretory antibody system and its function

The remarkable magnitude of GALT as an inductive site for B-cells is documented by the fact that more than 80% of all Ig-producing blasts and plasma cells in an adult are located in the intestinal lamina propria (Brandtzaeg et al., 1999a). As mentioned above, most such terminally differentiated mucosal B-cells (immunocytes) produce J-chain-containing dimers and some larger polymers of IgA, collectively called pIgA. These polymers (as well as pentameric IgM with J chain) are efficiently transported externally as SIgA (and SIgM) antibodies by the pIgR (Norderhaug et al., 1999; Johansen et al., 2000), which is constituted by the membrane SC expressed basolaterally on the intestinal crypt cells and other secretory epithelia (Fig. 14.2).

The main purpose of the secretory antibody system is, in cooperation with innate mucosal defence mechanisms, to perform immune exclusion (Fig. 14.7). Most importantly, SIgA inhibits colonization and invasion of pathogens, and pIgR-transported pIgA and pentameric IgM antibodies may even inactivate viruses (e.g. rotavirus and influenza virus) inside secretory epithelial cells and carry the pathogens and their products back to the lumen (Fig. 14.7), thus avoiding cytolytic damage to the epithelium (Norderhaug et al., 1999). Both the agglutinating and the virus-neutralizing antibody effects of pIgA are superior to those of monomeric antibodies (Brandtzaeg et al., 1987), and SIgA antibodies may block microbial invasion quite efficiently. This has been particularly well documented in relation to the human immunodeficiency virus (Mazzoli et al., 1997), and specific SIgA antibodies isolated from human colostrum have been shown to be more efficient in this respect than comparable IgG antibodies (Hocini and Bomsel, 1999).

Induction of SIgA responses has likewise been shown to interfere significantly with mucosal uptake of soluble macromolecules in experimental animals
Collectively, therefore, the function of locally produced pIgA, including antibodies in breast milk, would be to inhibit or modulate the epithelial colonization of microorganisms and to dampen the penetration of soluble antigens; this effect is most probably enhanced by the relatively high levels of polyreactive SIgA antibodies (Quan et al., 1997). In the gut, interaction of SlgA with the endogenous protein Fv (Fv fragment binding protein) may, moreover, build an immune fortress by forming large complexes of intact or degraded antibodies with different specificities, thereby reinforcing immune exclusion (Bouvet and Fischetti, 1999). It has also been claimed that SlgA can enhance the sticking of certain bacteria to mucus, interfere with growth factors (e.g. iron) and enzymes necessary for pathogenic bacteria and parasites (Brandtzaeg et al., 1999a) and exert positive influences on the inductive phase of mucosal immunity, by promoting antigen uptake in GALT via

**Fig. 14.7.** Schematic representation of three levels at which dimeric immunoglobulin (Ig)A or secretory IgA (SlgA) may provide immune protection after being produced with J chain by plasma cells in the mucosal lamina propria. Left: Dimeric IgA is transported by the polymeric Ig receptor (pIgR) across epithelial cells and released into the lumen as SlgA antibodies, which perform immune exclusion by interaction with luminal antigens (black bars). Middle: Dimeric IgA antibodies interact with viral antigens within epithelial cells during pIgR-mediated transport, thereby performing intracellular virus neutralization and removal of viral products. Right: Dimeric IgA antibodies interact with penetrating antigens in the lamina propria and shuttle them back to the lumen by pIgR-mediated transport.
putative IgA receptors on the M cells of FAE (Frey and Neutra, 1997). The latter possibility adds to the importance of breast-feeding in providing a supply of relevant IgA antibodies for the infant’s gut.

Interestingly, free SC released to the lumen (Fig. 14.2) may on its own be able to block epithelial adhesion of *E. coli* (Giugliano et al., 1995) and can bind the potent toxin of *Clostridium difficile* (Dallas and Rolfe, 1998). Also, a pneumococcal surface protein (SpsA) has been shown to interact with both free and bound SC (Hammerschmidt et al., 1997). Such observations suggest that SC has phylogenetically originated from the innate defence system before being exploited by the adaptive secretory immune system to function as pIgR.

**Handling of absorbed food antigens**

Intact antigens have in several studies been shown to cross the normal gut barrier and enter the bloodstream even in adults, particularly after food intake (Brandtzaeg et al., 1987), although the actual amount reaching the intestinal lamina propria remains uncertain. Work performed in experimental animals with mucosal application of 125I-labelled albumin has been difficult to interpret, due to marker instability; both degradation of the carrier molecule and release of the label can result in considerable overestimation of protein penetrability as determined by scintillation counting, compared with data based on immunological quantification (Brandtzaeg and Tolo, 1977). Intact dietary antigens appear in the circulation of healthy adults 2–5 h after a meal, being partly present in immune complexes. Thus, intake of 1.2 l of bovine milk resulted in some 3 ng ml\(^{-1}\) of β-lactoglobulin in peripheral blood (Paganelli and Levinsky, 1980). Ovalbumin up to 10 ng ml\(^{-1}\) has likewise been found, corresponding to approximately 10\(^{-5}\) of the amount consumed (Husby et al., 1985). Furthermore, both β-lactoglobulin and ovalbumin have been detected in the breast milk of lactating women, but with unexplained large intra- and inter-individual variations, the levels ranging from 0.9 to 150 μg l\(^{-1}\) (Kilshaw and Cant, 1984; Høst et al., 1990).

Several routes may be visualized for the penetration of intact soluble antigens through the normal intestinal epithelium: paracellular diffusion bypassing the tight junctions; via epithelial discontinuities, such as the cell extrusion zones of the villus tips; translocation through enterocytes by endocytosis and subsequent exocytosis; or transport by M cells in GALT. As discussed elsewhere (Brandtzaeg et al., 1987; Brandtzaeg, 1996a), the relative importance of these mechanisms remains unknown, and the consequences in terms of sensitization or induction of oral tolerance probably depend on the route of uptake, as well as on the nature of the antigen – that is, soluble, lectin-like or particulate (Fig. 14.8). There is likewise no definite knowledge about the effects transmission of food antigens to breast milk might have on the suckling’s immune system (Zeiger, 2000; Hoppu et al., 2001), although animal experiments have suggested the possibility of tolerance induction (Johansen et al., 2001).

External transport of pIgA-containing immune complexes by the pIgR has been suggested as an efficient, non-inflammatory antigen clearance mechanism.
from the gut lamina propria (Fig. 14.7). This notion has recently been supported by experiments performed in vivo (Robinson et al., 2001). Pentameric IgM (in contrast to hexameric IgM without J chain) also appears to have little or no complement-activating properties and therefore could probably support the non-inflammatory functions of pIgA in competition with corresponding pro-inflammatory IgG antibodies (Brandtzaeg et al., 1999a, b). Interestingly, monomeric IgA or IgG antibodies, when cross-linked via antigen to pIgA of the same specificity, might contribute to such pIgR-mediated epithelial transcytosis of foreign material (Mazanec et al., 1993). Conversely, complement-activating IgG antibodies against infectious agents and dietary proteins could on its own adversely affect mucosal penetrability for a variety of exogenous proteins while contributing to local protection. This possibility has been suggested by experiments ex vivo (Brandtzaeg and Tolo, 1977) and in vivo (Lim and Rowley, 1982); IgG antibodies against one antigen were shown to enhance mucosal penetration of bystander molecules. Mucosal integrity was apparently damaged by lysosomal enzymes released from polymorphonuclear granulocytes, which

**Fig. 14.8.** Various theoretical routes of antigen uptake in the gut and putative immunological consequences. Pathogenic microorganisms and dead particulate antigens (1), as well as proteins with special lectin-like properties (2), are rapidly transported through M cells (M) of follicle-associated epithelium covering gut-associated lymphoid tissue (to the left). Breaching of the gut epithelium (3) also allows rapid antigen uptake. Soluble proteins may be taken up by the paracellular route through the villus epithelium and then endocytosed by subepithelial antigen-presenting cells (APCs), or they are transported and presented by enterocytes to intraepithelial (CD8) or subepithelial T-cells (T). The transcellular route through the enterocyte is presumably speeded up by the lectin-like properties of the antigen (2). If the antigen is aggregated, luminal endogenous or bacterial enzymes may degrade it extensively to become non-immunogenic (to the right).
are attracted when immune complexes form locally. Perhaps antigen interaction with maternal IgG antibodies could explain why abrupt introduction of native cow’s milk proteins in infants often causes gastrointestinal bleeding (Ziegler et al., 1990).

The pro-inflammatory potential of maternally-derived or locally-produced IgG antibodies is probably less important in the gut of infants who are breast-fed, because milk SlgA antibodies will exert a non-inflammatory blocking effect. Moreover, breast milk contains large amounts of the soluble complement inhibitor protectin, CD59 (Bjørge et al., 1993). Also, this factor and other complement regulatory proteins are expressed by the gastrointestinal epithelium (Berstad and Brandtzaeg, 1998), and these probably counteract immune complex-mediated (type III hypersensitivity) damage of the epithelial barrier.

There is further experimental evidence to suggest that IgA may influence mucosal homeostasis in various ways through its binding to the Fcα receptor (CD89) when present on lamina propria leucocytes, although in the normal state CD89 expression is extremely low on human intestinal macrophages (Smith et al., 2001). Interestingly, IgA can down-regulate the secretion of the pro-inflammatory cytokine TNF-α from activated monocytes and inhibit activation-dependent generation of reactive oxygen intermediates in neutrophils and monocytes (Wolf et al., 1994a, b). On the other hand, plgA or aggregated monomeric IgA can trigger monocytes to show increased activity, such as TNF-α secretion (Devière et al., 1991), and also up-regulate B7 on APCs (Geissmann et al., 2001) and induce eosinophil degranulation (Abu-Ghazaleh et al., 1989). This pro-inflammatory potential of IgA probably reflects the need for reinforcement of mucosal antigen elimination mechanisms when immune exclusion fails, such as in intestinal parasitic infestations.

Protective and Immunoregulatory Effects of Breast Milk

Secretory antibodies and free SC

The initial breast milk, or colostrum, is much richer in antibodies than other secretions, because of its remarkably high concentration of SlgA (~12 g l⁻¹) and SlgM (~0.6 g l⁻¹). The individual variations are large, however, and the level decreases by a factor of approximately four after 2 weeks and then remains fairly stable throughout lactation (Goldman, 1993; Hanson et al., 1993). Antibodies of these two classes are produced locally by plasma cells as plgA and pentameric IgM in the lactating breast (Brandtzaeg, 1983). Unoccupied plgR molecules are also cleaved and released to the lumen as free SC (Fig. 14.2), which is present at a relatively high level in colostrum (~2 g l⁻¹). Free SC exerts a stabilizing effect on SlgM (Brandtzaeg, 1975) and may by itself contribute to intestinal defence through inhibition of E. coli colonization and C. difficile toxin blocking, as mentioned above. When present in a bound form, SC may activate eosinophils (Lamkhioued et al., 1995), but it may counteract such pro-inflammatory stimulation in its free, soluble form (Motegi and Kita, 1998).
Because the lactating mammary glands are part of the integrated mucosal immune system (Fig. 14.4), milk antibodies will reflect antigenic stimulation of MALT in the gut as well as in the airways, as mentioned earlier. The secretory antibodies are thus highly targeted against infectious agents in the mother’s environment, which are those likely to be encountered by the baby during its first weeks of life. As mentioned previously, antibodies against various dietary antigens, such as gluten and cow’s milk proteins, as well as against other potential allergens (Casas et al., 2000), are also often present in breast milk. The possible role of these IgA antibodies for the clinical presentation of immune-mediated adverse reactions to food in the infant will be discussed below.

**Non-specific anti-microbial factors**

Numerous constituents of breast milk are thought to exert innate defence functions, and the possible role of free SC was alluded to above. Other factors include lysozyme, lactoferrin, peroxidase, complex oligosaccharides (receptor analogues), fatty acids and mucins (Goldman, 1993; Hoppu et al., 2001). A further discussion of their potential protective effects is beyond the scope of this chapter.

**Immune cells and macrophages**

A variety of leucocytes occur in colostrum (~ $4 \times 10^6$ ml$^{-1}$) and later in milk (~ $10^5$ ml$^{-1}$). Macrophages (55–60%) and polymorphonuclear granulocytes (30–40%) dominate over lymphocytes (5–10%), the latter being mainly (75–80%) T lymphocytes (Goldman, 1993; Wold and Hanson, 1994). Oral administration of macrophages in newborn mice showed survival of these cells for several hours in the gut and even some mucosal uptake (Hughes et al., 1988). Experiments with milk leucocytes in newborn rats, calves and lambs likewise demonstrated transepithelial migration, lymphocytes apparently being the predominant cell type (Slade and Schwartz, 1987). Also, the distribution of labelled human colostral leucocytes after enteral administration in premature baboons suggested epithelial adherence in the gut, as well as mucosal uptake and persistence for more than 60 h, along with some peripheral migration (Jain et al., 1989). The contribution of milk lymphocytes to the infant’s developing intestinal immune system therefore remains an intriguing possibility.

Milk leucocytes generally express markers of previous priming and respond readily to restimulation. The macrophages contain engulfed SlgA that they may release on contact with bacteria in the gut (Slade and Schwartz, 1987). They may also secrete an array of immunologically important cytokines (Wold and Hanson, 1994). It is furthermore of interest that, compared with peripheral blood, breast milk is relatively enriched in T lymphocytes with the alternative TCRγ/δ; this subset mainly employs the V81/J81-encoded receptor, as do intraepithelial γ/δ T-cells in the gut (Bertotto et al., 1991). T lymphocytes of this
phenotype with specificity for *Mycobacterium tuberculosis* appear in colostrum of tuberculin-positive nursing mothers (Bertotto *et al.*, 1993). This observation is interesting in view of the direct antimicrobial activity mediated by various T-cell phenotypes upon interaction with microbial targets (Levitz *et al.*, 1995). In this respect, mucosal defence may especially engage γ/δ T-cells. Their supply from breast milk could thus be immunologically important before the baby’s IEL population has developed numerically and functionally.

**Putative immunoregulatory factors**

Glycoproteins from human colostrum have an enhancing effect on T-cell proliferation at low concentrations but an inhibitory effect at high concentrations (Mincheva-Nilsson *et al.*, 1990). The biological significance of this *in vitro* phenomenon is obscure as long as the active factors remain unidentified. One inhibitory mechanism of breast milk was suggested to be down-regulation of IL-2 production (Hooton *et al.*, 1991) and multimeric colostral α-lactalbumin was shown to induce apoptosis in lymphocytes (Håkansson *et al.*, 1995). Conversely, the stimulatory effect of milk on T-cells was tentatively ascribed to lactoferrin (Mincheva-Nilsson *et al.*, 1990), but great confusion exists about the possible immunoregulatory properties of this protein (Brock, 1995).

Several studies have reported that unfractionated supernatants of breast milk cell cultures preferentially stimulate IgA production by peripheral blood lymphocytes (Slade and Schwartz, 1987). An explanation for this effect may be the various cytokines that are secreted by stimulated milk macrophages (Wold and Hanson, 1994). The same soluble cytokines are found in breast milk (Goldman, 1993), and the presence of TGF-β, IL-6 and IL-10 is of particular interest for the development and differentiation of IgA-producing cells (Brandtzaeg *et al.*, 1999a). Evidence to this end has been provided for IL-6 (Saito *et al.*, 1991), as well as for IL-10 and TGF-β (Böttcher *et al.*, 2001), by relating the levels of cytokines in breast milk to salivary IgA concentrations in breast-fed children. Even if these cytokines are unable to survive the passage through the gastrointestinal tract, they may be released locally from milk macrophages in the neonatal gut and promote the development of a balanced mucosal immune system, thus contributing to a subsequent responder phenotype compatible with health. In this context, the balance between the immunosuppressive IL-10 and the Th2-promoting IL-4 in breast milk (Böttcher *et al.*, 2000) might be of particular significance. Also, the high levels of soluble Fas (CD95) could be important, because this protein might protect the intestinal epithelial barrier against apoptosis and favour tolerance induction (Srivastava and Srivastava, 1999).

**Effect on productive mucosal immunity development**

In addition to the remarkable reinforcement of mucosal defence provided by maternal SlgA (and SlgM) antibodies as a natural immunological 'substitution
therapy’, it is important to emphasize the positive nutritional effect of breast-feeding on immune development (Brandtzaeg, 1996b). Also, as mentioned above, breast milk contains a number of immune cells, cytokines and growth factors that may exert a significant biological effect in the suckling’s gut, apparently enhancing in an indirect way even the long-term health of the individual (Wold and Hanson, 1994; Newman, 1995).

Numerous studies of the effect of breast-feeding on secretory immunity have been performed with salivary IgA measurements as a read-out system. Discrepant observations have been made, probably to some extent reflecting different cytokine levels in the milk as discussed above. The influence of contaminating the saliva sample with milk SIgA, shielding of the suckling’s mucosal immune system by maternal SIgA antibodies, and altered growth and composition of the infant’s gut flora have been discussed as additional uncontrollable variables (Brandtzaeg et al., 1991). However, the balance of accumulated data suggests that breast-feeding promotes the post-natal development of secretory immunity (Wold and Hanson, 1994; Brandtzaeg, 1998), apparently even in the urinary tract (Newman, 1995); and there are reports of enhanced secretory, as well as systemic, immune responses to oral and parenteral vaccines in breast-fed babies (Hahn-Zoric et al., 1990; Pabst and Spady, 1990).

Nevertheless, several prospective studies have reported that the early physiological increase of salivary IgA (and IgM) is more prominent in formula-fed than in solely breast-fed infants (Gleeson et al., 1986; Stephens, 1986; Brandtzaeg et al., 1991), although this difference seems to disappear after weaning (Tappuni and Challacombe, 1994). It likewise seems that breast-feeding, in comparison with formula-feeding, reduces the salivary IgA antibody titres to cow’s milk proteins; this decrease was seen after a nursing period of only 3 weeks and appeared also in infants receiving mixed feeding (Gleeson et al., 1986; Renz et al., 1991). Altogether, therefore, although breast-feeding initially appears to reduce induction of salivary IgA, it will later on in infancy (up to 8 months) boost this response (Avanzini et al., 1992; Fitzsimmons et al., 1994). In a similar manner, experiments in mice have demonstrated that SIgA antibodies from breast milk affect the stimulatory properties of the gut flora in the suckling by retarding bacterial contact with the developing GALT (Cebra et al., 1999). When the host’s mucosal immune response subsequently is successfully elicited, GALT will be further shielded by the SIgA antibodies produced in the gut; local immunostimulation is thereby attenuated despite the continued presence of microorganisms (Shroff et al., 1995). This could contribute to the hypo-responsiveness or tolerance that normally exists towards members of the autologous commensal gut bacteria, in both rodents and humans (Helgeland and Brandtzaeg, 2000).

**Effect on oral tolerance development**

Through avoidance of too early local immune activation – for instance, limiting the intestinal up-regulation of the co-stimulatory B7 molecules (Brandtzaeg, 1998; Chen et al., 2000), the shielding effect exerted by SIgA from breast milk
on the suckling’s GALT (see above) may contribute to the establishment of oral tolerance not only against the indigenous microflora, but also against dietary antigens, such as gluten. Antibodies to gluten peptides are present in breast milk (Juto and Holm, 1992) and breast-feeding has in fact been shown to protect significantly against the development of coeliac disease in children, an effect that is unrelated to the time of solid food introduction (Brandtzaeg, 1997a). Early exposure to cow’s milk has been suggested to be associated with predisposition to type 1 (insulin-dependent) diabetes, and investigations have particularly focused on immune stimulation by bovine serum albumin (Karjalainen et al., 1992), β-lactoglobulin (Dahlquist et al., 1992) and insulin (Vaarala et al., 1999). In a recent study, short-term breast-feeding and early introduction of cow’s milk were found to be associated with progressive signs of type 1 diabetes-related autoimmunity (Kimpimäki et al., 2001).

On the basis of such observations, it may be tentatively concluded that mixed feeding, rather than abrupt weaning, appears to promote tolerance to food proteins and thereby also avoidance of potentially harmful cross-reactive autoantibodies. This notion is further supported by reports suggesting that cow’s milk allergy is more likely to develop in infants whose mothers have relatively low levels of milk IgA antibodies to bovine proteins (Savilahti et al., 1991; Järvinen et al., 2000). It is also noteworthy in this context that allergic mothers appear to have decreased levels of ovalbumin-specific IgA (Casas et al., 2000) and elevated levels of IL-4 (Böttcher et al., 2000) in their breast milk.

The presence of TGF-β and IL-10 in breast milk might contribute to its tolerogenic properties, because these cytokines exert pronounced immunosuppressive effects in the gut (Ishizaka et al., 1994; Steidler et al., 2000) and TGF-β enhances the epithelial barrier function (Planchon et al., 1994). Although still a somewhat controversial issue (Zeiger, 2000), the balance of epidemiological studies supports the view that breast-feeding protects against atopic allergy and asthma (Saarinen and Kajosaari, 1995; Oddy et al., 1999; Kull et al., 2001). Interestingly, TGF-β has been reported to be present at a higher level in maternal colostrum provided for infants that did not develop atopic eczema during exclusive breast-feeding, compared with those with early-onset symptoms (Kalliomäki et al., 1999). As discussed above, food antigens do appear in breast milk, but dietary restriction during pregnancy and breast-feeding has shown no conclusive effect on the development of atopic diseases in the child (Zeiger, 2000; Hoppu et al., 2001). It remains an open question whether early exposure to small amounts of food antigens may actually have a positive effect on tolerance induction, especially when occurring in its natural context in the gut lumen of a suckling (Johansen et al., 2001).

**Conclusions**

Several more or less well-defined variables influence the development of productive mucosal immunity and oral tolerance, therefore constituting a complex and rather enigmatic mechanistic basis for adaptive immune defence and adverse immunological reactions to food. An inadequate epithelial barrier
against luminal antigens is an important primary or secondary event in the pathogenesis of several mucosal diseases – being influenced by the individual’s age (e.g. preterm versus term infant), activation of the epithelium and subepithelial elements, such as APCs and mast cells (e.g. by infection, cytokines or neuropeptides), and the shielding effect of SIgA provided by breast milk or produced by adaptive B-cell responses in the infant’s gut. The consequences will depend on how quickly mucosal homoeostasis can be attained or re-established after abrogation.

SIgA is the best-defined effector component of the mucosal immune system, and this first-line specific defence against infectious agents and other harmful substances is of considerable clinical interest. The large capacity for storage of plgA in the mammary-gland epithelium and duct system explains the remarkable output of SIgA during feeding. Breast milk also contains an array of important immunoregulatory factors and promotes colonization of lactic acid-producing bacteria. These members of the indigenous microbiota are powerful in combating pathogenic intruders that may break oral tolerance (Collins and Gibson, 1999; Isolauri et al., 2001), and they also appear to exert a beneficial effect on the cytokine balance of the host and thereby on the developing immunological responder phenotype (Fig. 14.9). Animal experiments have indeed documented that the commensal bacterial flora is crucial both for the induction of oral tolerance and for its re-establishment after abrogation (Helgeland and Brandtzaeg, 2000). Altogether, this effect might not only be mediated through immune modulation, but could also be explained by the

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**Fig. 14.9.** Multiple direct or indirect effects of breast-feeding may be imprinted in the immune-responder phenotype of the infant. Details are discussed in the text. (Modified from Hoppu et al., 2001.)
enzymatic activity of the indigenous flora that degrades food proteins to tolerated peptides (Barone et al., 2000).

Convincing evidence exists for an important role of breast-feeding in the defence against mucosal infections. Its role in oral tolerance and protection against food allergy has been much more difficult to establish conclusively. This is not surprising in view of the complex and poorly understood interface between these two enigmatic biological phenomena, with multiple potential interactions influenced by the numerous bioactive components of breast milk (Fig. 14.9). For ethical reasons, it will not be possible to assign infants to breast- or formula-feeding for long-term follow-up studies. Therefore, perhaps we shall just have to take it on trust that exclusive breast-feeding for several months, followed by mixed feeding, is the natural way to begin life for all mammals, including humans.

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15 Food Allergy

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Introduction

Over the past few years, food allergy has become a topical issue, and a question that is commonly asked is why do some foods elicit immune responses in certain individuals? To answer this question requires knowledge of:

- The immune system.
- The reactions that result in the symptoms associated with food allergy.
- How and why such reactions are generated.
- The characteristics and behaviour of allergenic foods and their constituents.

Research in these areas has allowed scientists to provide a gradually expanding picture of food-induced allergic reactions, which, although rare, are increasing in incidence and are, in some instances, life-threatening.

Food Allergy

Definition

In 1995, the European Academy of Allergy and Clinical Immunology (EAACI) prepared a position paper on adverse reactions to food (Bruijnzeel-Koomen et al., 1995). In this paper, the EAACI presented a new classification, which divides adverse reactions to food into toxic and non-toxic reactions and further clearly distinguishes food allergy from food intolerance in the non-toxic category (Fig. 15.1). Based on the classification of the EAACI, food intolerance is defined as a non-immune-mediated adverse reaction to food, while food allergy is defined as an immune-mediated adverse reaction to food.
Food intolerance can be further divided into:

1. Enzymatic food intolerance, e.g. lactose intolerance.
2. Pharmacological food intolerance: this may be caused by biogenic amines such as histamine and tyramine, which are found in large amounts in cheese, red wine and tinned food, and which may play a role in migraine. Food additives and histamine-releasing factors present in foods may also be responsible for pharmacological food intolerance.
3. Undefined: adverse reactions to certain food additives for which the mechanisms remain largely unknown.

For further information on food intolerance, see European Commission (1997), Committee on Toxicity (2000) and British Nutrition Foundation (2001).

Food allergy can be further divided into immunoglobulin E (IgE)-mediated and non-IgE-mediated reactions to food. The IgE-mediated reaction to food, also known as the immediate or type I response, is the most common and frequently reported and its role in food allergy is well established. Thus, unless otherwise stated, food allergy refers to the IgE-mediated type I response. There are three non-IgE-mediated reactions, which will also be discussed, albeit briefly.

**Foods that cause allergic reactions**

A range of foods are known to trigger type I allergic responses (Table 15.1). These foods contain specific proteins that, in certain individuals, initiate immunological reactions to food. These proteins, called allergens, possess common immunological characteristics that allow them to elicit an allergic, specifi-
cally IgE-mediated type I, response. In addition, these proteins (with some exceptions) possess certain common physical and chemical properties that may influence their allergenic potency (see Taylor and Lehrer, 1996; Lehrer et al., 1996; see later section on ‘Allergenic Foods and Food Allergens’).

**Symptoms of food allergy**

A range of symptoms result from allergic reactions to food (Table 15.2). These symptoms, which affect different organs, are sometimes mild but some, such as anaphylaxis, can be life-threatening. Symptoms generated by an IgE-mediated response can occur very quickly (within minutes). However, such responses may also give rise to delayed symptoms/reactions. An example of this is food-induced eosinophilic gastroenteritis. Symptoms of this condition include post-prandial nausea, vomiting and diarrhoea and it occurs mainly in infants, children and young adults. This condition appears to involve cellular activity suggestive of the late phase of an IgE-mediated type I allergic reaction (Sampson, 1997).

Other, non-IgE, immune-mediated responses may be responsible for certain food-induced disorders, such as coeliac disease (seen in infants, children and adults) and food protein-induced enterocolitis syndromes, which occur primarily in infants and young children. However, the specific mechanisms that cause these disorders are unknown (see section on ‘Other Immunological Reactions to Food’).

**IgE-mediated (Type I) Allergic Response**

There are four allergic reactions, of which the IgE-mediated (type 1) is the most common. The components of the immune system and the ways in which they interact are described by Devereux (Chapter 1, this volume). The IgE-mediated response is a prime example of the interaction and interdependence of innate

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**Table 15.1.** A list of known allergenic foods.

<table>
<thead>
<tr>
<th>Commonly allergenic foods</th>
<th>Less commonly allergenic foods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow’s milk</td>
<td>Molluscs:</td>
</tr>
<tr>
<td>Egg</td>
<td>• Mussels</td>
</tr>
<tr>
<td>Fish:</td>
<td>• Clams</td>
</tr>
<tr>
<td>• Cod</td>
<td>• Oysters</td>
</tr>
<tr>
<td>Crustacea:</td>
<td>Lupin</td>
</tr>
<tr>
<td>• Shrimp</td>
<td>Peas</td>
</tr>
<tr>
<td>Peanuts</td>
<td>Rice</td>
</tr>
<tr>
<td>Soybeans</td>
<td>Apples</td>
</tr>
<tr>
<td>Tree nuts:</td>
<td>Celery</td>
</tr>
<tr>
<td>• Brazil nuts</td>
<td>Peach</td>
</tr>
<tr>
<td>• Hazelnuts</td>
<td>Tomato</td>
</tr>
<tr>
<td>• Pistachio</td>
<td>Melons</td>
</tr>
<tr>
<td>Wheat</td>
<td>Cabbage</td>
</tr>
</tbody>
</table>
and adaptive immunity. However, in this case, the antigen is not a harmful pathogen but a food protein, which contains an IgE epitope recognized by specific T- and B-cells (clonal selection). These cells are thus activated and proliferation (clonal expansion) and antibody (IgE) production occur. These antibodies then interact with cells of innate immunity (mast cells and basophils) and trigger a reaction that results in the symptoms listed in Table 15.2. The IgE-mediated response is divided into two stages: sensitization (which includes the production of IgE antibodies) and the allergic reaction itself.

### Table 15.2. Symptoms of allergic reactions to foods.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Symptoms</th>
<th>Type of Food Allergy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral allergy syndrome (OAS)</td>
<td>Itching and swelling of the lip, tongue and larynx, other symptoms, such as urticaria, rhinitis, asthma, laryngeal oedema and anaphylaxis may also occur (see below)</td>
<td>Type I IgE-mediated</td>
</tr>
<tr>
<td>Cutaneous reactions</td>
<td></td>
<td>All type I IgE-mediated</td>
</tr>
<tr>
<td>• Urticaria</td>
<td>Wheals</td>
<td></td>
</tr>
<tr>
<td>• Angio-oedema</td>
<td>Swelling of subcutaneous tissue</td>
<td></td>
</tr>
<tr>
<td>• Atopic dermatitis (eczema)</td>
<td>Itching (pruritus), dry and lined skin</td>
<td></td>
</tr>
<tr>
<td>Respiratory reactions</td>
<td></td>
<td>All type I IgE-mediated</td>
</tr>
<tr>
<td>• Rhinitis</td>
<td>Inflammation of the nasal passages causes runny nose</td>
<td></td>
</tr>
<tr>
<td>• Asthma</td>
<td>Narrowing of the airways caused by inflammation Wheezing, tightness and breathlessness</td>
<td></td>
</tr>
<tr>
<td>• Laryngeal oedema</td>
<td>Constriction of the throat</td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal reactions or gastrointestinal anaphylaxis</td>
<td>Abdominal pain, nausea, vomiting, diarrhoea</td>
<td>Type I IgE-mediated</td>
</tr>
<tr>
<td>Allergic eosinophilic gastroenteritis</td>
<td>Nausea, vomiting and diarrhoea caused by intestinal immune</td>
<td>Type I IgE-mediated</td>
</tr>
<tr>
<td>Gluten-sensitive enteropathy (coeliac disease)</td>
<td>response to gluten (a protein complex in wheat); immune response causes damage to wall of small intestine. This condition is characterized by chronic diarrhoea, weight loss, vomiting and anorexia</td>
<td>Non-IgE-mediated; possibly type III or type IV (see section on ‘Other Immunological Reactions to Food’)</td>
</tr>
<tr>
<td>Systemic reaction</td>
<td></td>
<td>Type I IgE-mediated</td>
</tr>
<tr>
<td>• Anaphylaxis</td>
<td>A severe, generally rapid, reaction, which provokes itching and swelling of the oral cavity, cutaneous, respiratory, gastrointestinal and cardiovascular (chest pain, feeling faint and hypotension) symptoms</td>
<td></td>
</tr>
</tbody>
</table>

Ig, immunoglobulin.
Sensitization

In allergic individuals, initial exposure to a food allergen (the antigen) causes production of IgE antibodies. The events leading to the production of IgE are:

1. The allergen is processed by antigen-presenting cells and class II major histocompatibility (MHC) proteins present a fragment of the allergen to allergen-specific T-helper (Th) cells.
2. Following binding to the allergen fragment, allergen-specific Th cells proliferate. Sensitization to a food allergen favours a T-helper (Th) 2 rather than a Th1-type response, and leads to the production of interleukin (IL)-4, IL-5, IL-10 and IL-13.
3. Th2-type cytokines promote the differentiation and proliferation of allergen-specific B-cells.
4. B-cells produce allergen-specific IgE antibodies (Fig. 15.2).
5. These allergen-specific IgE antibodies then bind to high-affinity IgE receptors present on mast cells and basophils.
6. Mast cells and basophils are now sensitized (Fig. 15.3).

The allergic reaction

With repeated exposure/ingestion, the food allergen comes into contact with a mast cell/basophil-bound IgE antibody. Since this IgE antibody is specific to the allergen (via the allergen’s epitope – an IgE epitope), binding of the allergen to the IgE antibody occurs. This binding causes one IgE antibody to become cross-linked with another IgE antibody. Cross-linking triggers degranulation of the mast cells and basophils, leading to the release of pre-formed mediators, such as histamine, and the synthesis and release of tumour necrosis factor alpha (TNF-α), IL-4, prostaglandins and leucotrienes (Fig. 15.4). Release of these mediators causes an inflammatory reaction, which results in one or more of the symptoms listed in Table 15.2.

Allergenic Foods and Food Allergens

Allergenic foods

The list of foods that have been shown to cause allergic reactions in sensitive individuals is extensive. However, there are a number of foods that are consumed on a regular basis and which account for the majority of IgE-mediated reactions to food. An Expert Consultation of the Food and Agriculture Organization proposed a list of the most common allergenic foods (see Bousquet et al., 1998). This list includes:

- Barley, oats, wheat and products of these (gluten and starch included).
- Crustaceans and other shellfish and products of these.
- Eggs and egg products.
Antigen is presented by antigen presenting cells to receptors on the surface of a T-cell. Antibody on the surface of a B-cell also recognizes the antigen and, after contact with the T-cell, B-cell and its daughter cells are primed to produce IgE. Interleukin-2 (IL-2), secreted by T-cells, promotes proliferation of B-cells. Interleukin-4 (IL-4), also secreted by T-cells, promotes IgE production.

Fig. 15.2. Production of immunoglobulin (Ig)E antibodies. N.B. T-helper cells do not recognize free antigen but antigen processed and presented by antigen-presenting cells and class II major histocompatibility complex protein, respectively (see text). (Reprinted with permission from the International Life Sciences Institute from the ILSI Europe Concise Monograph on Food Allergy.)
**Fig. 15.3.** Sensitization of mast cells (reprinted with permission from the International Life Sciences Institute from the ILSI Europe Concise Monograph on Food Allergy). IgE, immunoglobulin E.

**Fig. 15.4.** Allergic reaction initiated by mast-cell degranulation (reprinted with permission from the International Life Sciences Institute from the ILSI Europe Concise Monograph on Food Allergy).
Major and minor allergens

Table 15.3 lists the systematic and original names of the allergens present in some allergenic foods. The first three letters of the systematic name come from the genus of the food in which the allergen is found, then follows the first letter of the species, then a number; the number assigned is based on the order of identification. For example, the first allergen described in peanut, *Arachis hypogea*, is designated *Ara h* 1.

Researchers have divided food allergens into major and minor allergens. Major allergens are generally defined as proteins for which 50% or more of the allergic individuals studied have specific IgE. Major allergens are normally abundant in a food. However, there are exceptions, such as *Gad c 1*, which is not abundant in cod but is a major allergen. Minor allergens, although they may have structures similar to major allergens, are unable to cause degranulation of mast cells and basophils because they do not possess the appropriate conformation (see section on ‘Biochemical and immunological properties’). However, it has been suggested that minor allergens could be parts of larger major allergens and may therefore have the potential to cause allergic reactions in certain individuals (see Bush and Hefle, 1996).

Why do certain food proteins elicit an allergic response?

Food allergens possess biochemical and immunological characteristics that allow them to elicit an IgE-mediated immune response. In addition, they possess physical and chemical factors that may influence and/or indicate allergenicity (Table 15.4).

Biochemical and immunological properties

For a molecule to ‘select’/bind to a T-cell or a B-cell and its antibodies and thus activate an immune response, it must possess an epitope. Thus, an allergen (whether a food, inhalant or venom allergen) must possess an epitope, specifically an epitope that binds IgE (an IgE epitope). To elicit an IgE allergic response, allergens must be able to cross-link one IgE antibody with another.

### Table 15.3. Some of the major food allergens.

<table>
<thead>
<tr>
<th>Allergen source</th>
<th>Systematic and original names</th>
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<tbody>
<tr>
<td><strong>Bos domesticus</strong>: domestic cattle</td>
<td>• <em>Bos d</em> 4; α-lactalbumin</td>
</tr>
<tr>
<td>• Cow’s milk</td>
<td>• <em>Bos d</em> 5; β-lactoglobulin</td>
</tr>
<tr>
<td><strong>Gallus domesticus</strong>: hen</td>
<td>• <em>Gal d</em> 1; ovomucoid</td>
</tr>
<tr>
<td>• Egg</td>
<td>• <em>Gal d</em> 2; ovalbumin</td>
</tr>
<tr>
<td><strong>Gadus callarias</strong>: cod</td>
<td>• <em>Gad c</em> 1; allergen M</td>
</tr>
<tr>
<td><strong>Metapenaeus ensis</strong>: shrimp</td>
<td>• <em>Met e</em> 1; tropomyosin</td>
</tr>
<tr>
<td><strong>Arachis hypogea</strong>: peanut</td>
<td>• <em>Ara h</em> 1; vicilin</td>
</tr>
<tr>
<td></td>
<td>• <em>Ara h</em> 2; conglutinin</td>
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IgE antibody (both IgE antibodies need to be bound to a mast cell/basophil). Therefore, the allergen needs to be at least bivalent (as far as IgE binding is concerned), with the binding sites appropriately situated (Ishizaka and Ishizaka, 1984). However, it has been observed in mice that some monovalent allergens (e.g. an allergen found in venom, called mellitin) are able to trigger the release of mediators from mast cells or basophils or generate anaphylaxis (see Taylor and Lehrer, 1996). Such allergens can act as haptens (small molecules that elicit an immune response when bound to large molecules, such as bovine serum albumin), and thus are able to achieve cross-linking of IgE antibodies when part of a large complex. The significance of this mode of cross-linking in allergic disease has yet to be determined.

With the gradual elucidation of the biochemical structure of some common food allergens, comparisons between structures have been made in an attempt to identify common features. However, there does not appear to be any pattern of consistency between structures. For example, comparison of linear amino acid sequences and secondary and tertiary structures of these allergens has not revealed any general features. Such observations do not necessarily mean that general biochemical features between food allergens are not present, but that such features, which may cause food allergens to differ from other food proteins, have yet to be identified. Research on the biochemical and immunochemical characteristics of food allergens, specifically the properties of IgE epitopes – both B-cell epitopes and T-cell epitopes – in an attempt to identify unique features is ongoing (see Taylor and Lehrer, 1996; Bush and Hefle, 1996; Shin et al., 1998).

### Allergen cross-reactivity

Different allergens may share the same or similar IgE epitope structure, which may result in cross-reactivity. A well-documented example is that of individuals with pollen allergies – for example, birch, oak and mugwort – who may also suffer from adverse reactions to certain fruits and vegetables, including apple, pear, hazelnuts, kiwi, carrots and celery (Bruijzeel-Koomen et al., 1995; Kazemi-Shirazi et al., 2000). Another example of allergen cross-reactivity is

<table>
<thead>
<tr>
<th>Table 15.4. General properties of food allergens.</th>
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<tbody>
<tr>
<td><strong>Immunological/biochemical</strong></td>
</tr>
<tr>
<td>• Possess IgE epitope</td>
</tr>
<tr>
<td>• Possess at least two IgE-binding sites to elicit an IgE response</td>
</tr>
<tr>
<td><strong>Physical/chemical</strong></td>
</tr>
<tr>
<td>• MW between 10 and 70 kDa</td>
</tr>
<tr>
<td>• Glycosylated</td>
</tr>
<tr>
<td>• Stable at high temperatures</td>
</tr>
<tr>
<td>• Resistant to digestion</td>
</tr>
<tr>
<td>• Resistant to proteolysis</td>
</tr>
<tr>
<td>• Resistant to hydrolysis</td>
</tr>
<tr>
<td>• Resistant to acidic environment</td>
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Ig, immunoglobulin; MW, molecular weight.
latex and banana (see Perkin, 2000). Cross-reactivity also exists between food allergens. For example, cross-reactivity between allergens present in foods that belong to the legume family and between different fish species has been documented (see Bush and Hefle, 1996). The magnitude of the clinical manifestation of cross-reactivity does vary between individuals. For example, not all individuals with pollen allergies present symptoms following the ingestion of certain fruits and vegetables. This variation in clinical symptoms has also been observed between proteins in related foods – for example, the legume family – that cross-react. In general, the manifestation of symptoms is rare, but very severe reactions have been reported (See Bush and Hefle, 1996).

*Physical and chemical properties*

**Molecular weight and carbohydrate moieties.** Most known food allergens have a molecular weight of between 10 and 70 kDa. However, smaller molecules may be able to elicit an allergic response and some food allergens, such as some of the peanut allergens, are very large (200–300 kDa) in their native form. In addition, most food allergens are glycosylated and have acidic isoelectric points. However, many proteins that are not allergens possess these properties, and thus molecular weight, glycosylation and isoelectric point are not useful indicators of allergenicity (see Taylor and Lehrer, 1996).

**Heat stability.** Most, but not all, food allergens are resistant to high temperatures. Heat causes denaturation of allergenic proteins and the loss of conformational IgE epitopes. However, some food allergens still retain their allergenicity, indicating that epitope conformation is not always essential for IgE binding to certain allergens (although appropriate epitope conformation is essential for cross-linking). Cow’s milk, fish, soybean and peanut allergens have all been shown to be resistant to heat. However, these food allergens are not always 100% resistant to high temperatures. For example, the IgE-binding capacity of β-lactoglobulin, a cow’s-milk allergen, is diminished but not completely eliminated by heating at 80–100°C. The IgE-binding capacity of the peanut allergen *Ara h 1* is unaffected by temperatures as high as 100°C (see Taylor and Lehrer, 1996).

**Resistance to digestion, proteolysis, hydrolysis and low-pH environment.** Digestion, proteolysis and hydrolysis may destroy both conformational and linear epitopes. However, a number of common food allergens, including cow’s milk, egg, peanut, fish and soybean allergen, have been shown to be resistant to:

- Specific enzymes that are involved in gastric and intestinal digestion of proteins, such as pepsin and trypsin.
- Simulated gastric and intestinal fluids over time *in vitro*.
- Chemicals, such as cyanogen bromide and hydrochloric acid, that hydrolyse proteins (see Taylor and Lehrer, 1996).

Generally, the allergenicity of the above, assessed by IgE-binding capacity, was fully retained or diminished but not eliminated. However, the resistance to
digestion may not be consistent: a study on the effects of pepsin digestion on the allergenicity of peanut showed that pepsin eliminated its IgE-binding capacity (Hong et al., 1999).

A few studies have indicated that digestion may enhance the allergenicity of food proteins, since digestion may unravel hidden epitopes. One such study reported that IgE from the serum of some patients with cow’s-milk allergy is more reactive with digests of β-lactoglobulin than with the undigested form (see Taylor and Lehrer, 1996).

Fruit and vegetable allergens are completely heat-labile and are more sensitive to digestion, proteolysis and hydrolysis than the allergens discussed above. Reactions to fruit and vegetable allergens occur primarily in the mouth and cause oral allergy syndrome (see Table 15.2).

**Effects of food processing**

Since certain food allergens are resistant to high temperatures and proteolysis, food-processing techniques (such as thermal processing and enzymatic proteolysis) have, in general, very little effect on their allergenicity. However, a recent study reported that the binding of roasted peanut to IgE from individuals with peanut allergy was approximately 90-fold higher than that of raw peanut to IgE (Maleki et al., 2000). The study also reported that two major peanut allergens (Ara h 1 and Ara h 2) bound higher levels of IgE and were more resistant to heat and digestive enzymes once they had undergone the Maillard reaction. The Maillard reaction is a reaction between amino acids in protein and reducing sugars – glucose, fructose, lactose and maltose – which causes browning of food and the production of compounds that impart a burnt and caramel-like flavour. The impact of roasting on the allergenicity of peanut proteins is further supported by an investigation by Beyer et al. (2001) of the effect of cooking methods on peanut allergenicity. This study showed that, in contrast to frying and boiling, the roasting of peanuts increased the allergenic properties of Ara h 1 and Ara h 2, indicated by IgE binding. Thus, thermal processing (i.e. roasting) may play a role in the enhancement of the allergenic properties of peanut protein.

Some food processes, specifically phenolic browning and lyophilization, have been shown to diminish IgE-binding capacities of apple and fish allergens, respectively. However, such effects are said to be rare (see Taylor and Lehrer, 1996).

Over the past several years, the effect of novel food techniques, such as genetic modification and protein microparticulation (the heating and shearing of food proteins to cause coagulation) on the allergenicity of food proteins has become a major food-safety issue. However, assessing the impact of such techniques on allergenicity is not always straightforward. One reason for this is that some novel techniques, specifically genetic modification, utilize proteins for which the allergenic history is unknown and thus the determination of IgE-binding capacities is difficult because IgE antibodies with an appropriate specificity are unavailable. For further information on assessing the potential allergenicity of novel foods, see Lehrer et al. (1996), Metcalfe et al. (1996),

Development of Food Allergy

There are a number of factors that may contribute to susceptibility to IgE-mediated food allergies:

- A genetic predisposition.
- Early exposure to foods when the gut mucosal barrier is immature.
- The high consumption of certain foods.
- The introduction of new foods to the diet.

Genetic predisposition

The development of food allergy may be influenced by a familial history of atopy. Atopy is a genetic predisposition towards mounting IgE antibody responses. Thus, atopic individuals make IgE constantly and have unusually high levels of IgE in their serum (unlike non-atopic individuals, in which an IgE response can only be elicited via the mechanism described above). Atopy is associated with allergic diseases, such as asthma, rhinitis, atopic dermatitis (eczema) and also food allergies. Atopic individuals are commonly defined, in practice, as those who exhibit sensitization to two allergens or more.

Studies on children have shown that the risk of developing an IgE-mediated food allergy ranges from 13.5 to 58% when one parent is atopic (uniparental history of atopy) and up to 100% when both parents are atopic (biparental history of atopy) (see European Commission, 1997).

Overall, work in the area of the genetics of food allergy is limited. A study carried out in the UK suggests that the MHC genes that code for class II human leucocyte antigen (HLA) proteins may play a role in determining susceptibility to peanut allergy (Howell et al., 1998). The same study also suggests that there is a higher rate of peanut allergy among siblings compared with the general population of the UK. A study from the USA (Sicherer et al., 2000) investigated the role of genetic and environmental factors in the development of peanut allergy by looking at twins – identical and non-identical – in whom one or both siblings had peanut allergy (not all their parents were peanut-allergic). The study reported a significantly higher concordance rate of peanut allergy among identical twins than among non-identical twins. In addition, common (e.g. parental style and eating habits) and specific (e.g. separate peer groups) environmental factors were shown not to influence the development of peanut allergy. Thus, in this study, the genetic influence on the development of peanut allergy is seen to be significant. Whether this genetic influence is conferred via the action of genes that code for HLA II proteins was not investigated. However, the authors of this study highlighted the need for further work to identify the genes that are likely to influence food allergy. However, given that the prevalence of allergic disorders has increased over the recent past, it is
Early exposure to foods

Early exposure to foods is believed to be a major influence on the development of food allergy in young children. One of the main reasons for this is the immaturity of the gut mucosal barrier in young children (see Brandtzaeg, Chapter 14, this volume). The gut mucosal barrier does not mature fully until the age of approximately 9–10 years. The gut mucosa of young children exhibits a greater permeability than that of older children and adults, and thus allows more protein, including food allergens, through. As a result, systemic exposure of the ingested allergen to the child's immune system occurs. Thus, due to mucosal-barrier immaturity, early exposure of new foods may influence the development of IgE-mediated food allergy in infants and children. With maturation, the mucosal barrier becomes less permeable and more efficient as a barrier and studies have shown that with this maturation many cases of allergic reactions to certain foods (e.g. egg and milk) cease (Dannaeus and Inganaes, 1981; Høst and Halken, 1990).

An immature mucosal barrier may also be linked to the duration of peanut allergy. A study on the resolution of peanut allergy suggests that peanut allergy in a small proportion of young children who were sensitized to peanut early in life resolves in a similar way to egg and milk allergies (Hourihane et al., 1998). However, peanut allergy rarely resolves in older children and adults, in whom severe peanut allergy is common. One possible explanation for the resolution of peanut allergy focuses on epitope structure, IgE binding and gut-mucosa maturity. Studies suggest that children who develop a tolerance to peanut may possess IgE that binds more to conformational epitopes than to linear epitopes; children who do not develop tolerance to peanuts possess IgE that binds mostly to linear epitopes. Conformational epitopes are generally more labile, whereas linear epitopes are more stable. Thus, as the gut mucosal barrier matures more linear than conformational epitopes pass through the mucosal barrier and elicit an IgE-mediated response (Berger, 1998).

Early exposure of young children in the USA and Scandinavia to peanuts and fish, respectively, has been attributed to the development of both allergies (Aas, 1966; Sampson, 1996). Whether this is due to gut mucosal immaturity is unclear. However, the development of peanut allergy in young children following early exposure may be linked to atopy. The Department of Health (1998) report on peanut allergy advises that, in an attempt to reduce the risk of developing peanut allergy, the introduction of peanuts and products containing peanuts into the diets of infants from families with a history of atopy should be avoided.

Early exposure to foods and therefore food allergens may also occur via the maternal diet. Studies have shown that cow’s milk allergen, β-lactoglobulin and egg allergen, ovalbumin, can be transmitted to infants via breast milk in variable amounts (1–1000 μg l⁻¹) (Department of Health, 1998). In addition, Vadas et al. (2001) reported the presence of two peanut allergens (Ara h 1 and...
Ara h 2) in the breast milk of lactating women following the ingestion of 50 g of roasted peanuts. Collectively, these studies demonstrate the potential for sensitization of at-risk infants via breast milk. Sensitization may also occur in utero. Fetal blood mononuclear cells responded to allergens following exposure to house-dust mite, birch pollen, milk and egg allergens during pregnancy (Jones et al., 1996; Warner et al., 2000). In addition, mothers from atopic families who consumed peanuts more than once a week during pregnancy have been shown to be more likely to have a child who is allergic to peanut than mothers who consumed peanuts less than once a week (Marian et al., 1999) (see also section on 'Prevention, Management and Treatment of Food Allergy').

The high consumption of certain foods

Persistent exposure to foods that are allergenic may also play a role in the development of IgE-mediated food allergy. Peanut and fish allergy are more common in the USA and Scandinavia, respectively (Aas, 1966; Sampson, 1996). The frequent consumption of these foods may to some extent determine the likelihood of the development of their respective allergies. On the other hand, the high consumption of these foods may only be linked to the prevalence of their respective food allergies and not to development (see section on 'Prevalence of Allergic Reactions to Food').

Introduction of new food into the diet

The introduction of soybean to the French diet in the mid-1980s led to an increase in the incidence of soybean allergy (Moneret-Vautrin, 1986). In addition, the introduction of kiwi fruit into the USA in the 1980s was followed by reports of allergic reactions to kiwi in the literature (Fine, 1981).

In light of the association between the introduction of new foods into a diet and the development of food allergy, the possibility that the introduction of foods generated using novel techniques, such as genetically modified foods, may influence the development of new food allergies is real. One prime example of this is the transgenic soybean that contained a gene (which expressed the protein 2S albumin) isolated from Brazil nut, a known allergenic food. Tests on this transgenic soybean revealed that individuals allergic to Brazil nut, but not to soybean, were allergic to transgenic soybean (Nordlee et al., 1996). This finding highlights the importance of assessing potentially allergenic new foods. However, as stated earlier, the difficulty in attempting to broach this area of food allergy is that many genetically modified foods contain proteins of unknown allergenic history. In addition, such foods may contain proteins that are not recognized as allergenic in their modified form or may become allergenic due to the modification that the food has undergone. Research is ongoing to develop new approaches to tackle the potential problem of allergenicity and genetically modified foods.
Prevalence of Allergic Reactions to Food

The prevalence of food allergy is unclear. One reason for this lack of clarity is that many food allergies are perceived and not real. As many as 20–30% of the UK population think they have a food allergy. However, the prevalence of true food allergy in the UK is estimated to be between 1 and 3% and possibly less (Department of Health, 1998; Committee on Toxicity, 2000; British Nutrition Foundation, 2001).

A number of factors influence the prevalence of food allergy. These include:

• Geography. A number of studies on cow’s milk allergy carried out in the USA, Sweden, Canada, England, Denmark and Holland estimate prevalence to range from 0.1 to 7.5% (Høst, 1994). The prevalence of peanut allergy also appears to vary based on location: peanut allergy affects 0.6% of the US population and 0.4% of US children (Sicherer et al., 1999). However, estimation of the prevalence of peanut allergy in Australian children is reported to be 1.9% (Hill et al., 1997) (to the author’s knowledge, no data are available for prevalence of peanut allergy in Australian adults). In Asian and African countries, peanut allergy is reported to be rare; however, few data on the incidence of peanut allergy in these countries are available (Department of Health, 1998).

• Cross-reactivity. Similarities in the IgE epitope structures between food allergens and between pollen and food allergens can influence prevalence. For example, in areas where allergy to birch and mugwort pollen is prevalent, 30–50% of individuals with these allergies present with symptoms following the ingestion of fruits and vegetables that share the same or similar IgE epitope structures with those of both pollens (De Blay et al., 1991). However, as stated previously, the magnitude of the clinical manifestation of cross-reactivity between food allergens can vary between individuals.

• Genetic predisposition.

• Resolution of food allergy over time.

• Exposure to new and novel foods.

• The high consumption of allergenic foods. Generally, in countries where the consumption of a commonly allergenic food (such as peanuts and fish) is high, the incidence of allergic reactions to that food is more common. For example, as stated earlier, a high rate of peanut consumption has been linked to peanut allergy being more common in the USA. However, the rate of peanut consumption in China is also high, but the prevalence of peanut allergy in China is much lower than that in the USA. This difference in prevalence may be linked to the methods used in the cooking of peanuts. The methods of boiling and frying peanuts are common practices in China and both boiling and frying have been reported to decrease the allergenicity of peanut protein (Beyer et al., 2001). In contrast, roasting, which is the main way in which peanuts are cooked in the USA, has been reported to increase the allergenicity of peanut proteins (Maleki et al., 2000; Beyer et al., 2001). Thus, modes of cooking may also have an impact on prevalence.
In addition to the factors listed above, differences in the approaches utilized in the study of food-allergy prevalence might be important (Høst, 1994).

**Diagnosis of Food Allergy**

Since many food allergies are perceived and not real, any study on food allergy using humans requires confirmation of the allergy. There are a number of techniques available for the diagnosis of food allergy:

- Clinical history and physical examination.
- Elimination diets.
- Double-blind, placebo-controlled food challenge (DBPCFC): an *in vivo* test.
- Skin-prick test: an *in vivo* test.
- Radioallergosorbent test (RAST): an *in vitro* test.

**Clinical history and physical examination**

The clinical history of an individual who has reported suffering from a food allergy is taken to identify the suspect food(s) and thus to determine whether the food(s) is(are) the likely cause(s) of allergic symptoms (Committee on Toxicity, 2000). A clinical history includes:

- A description of the symptoms caused by the food.
- The time between ingestion of the food(s) and the onset of symptoms.
- The quantity of food that generates a response.
- The consistency of symptoms.
- The presence of other factors that generate the same symptoms – for example, exercise (see section on ‘Other Immunological Reactions to Food’).
- The length of time of the reaction.

A standard physical examination can be used to support a clinical history. In addition to the above, a review of family history for atopic individuals (particularly when diagnosing food allergy in children) may help identify individuals at risk of developing food allergies.

**Elimination diets**

Verification of the suspect food can be achieved by eliminating the food from the diet, since then symptoms caused by the food would no longer occur. For such diets to be successful:

- One needs to be aware of any other foods that may have an adverse effect.
• The diets need to be based on thorough and accurate dietary history and knowledge of foods and their components.

**Double blind, placebo controlled food challenge**

If a clinical history indicates that an individual is suffering from a food allergy, a DBPCFC can be carried out to confirm the food allergy (see Bruijnzeel-Koomen et al., 1995; European Commission, 1997; Committee on Toxicity, 2000; British Nutrition Foundation, 2001). This test is considered to be the ‘gold standard’ for diagnosing food allergy and involves giving an individual the suspect food. To prevent a subjective response, the smell and taste of the food are masked, although this can sometimes be difficult due to the quantity of food needed for the challenge and/or the strong flavours and odours that the food of interest may possess. This test is not always practical, since severe reactions to the food can arise, and it must therefore be carried out under medical control. In addition, interpretation of results from DBPCFC is not always straightforward, since a positive response to a food challenge indicates a cause-and-effect relationship but will not provide information about the underlying mechanism that caused the response (Watson, 1995). Such information may be obtained from the results of other diagnostic techniques, which are discussed below.

**Skin-prick test**

This test involves pricking an extract of the suspect food on to an individual’s skin (normally on the back of the forearm; sometimes on the back); positive control substances (e.g. histamine) and negative control substances are also tested. The appearance of a wheal-and-flare reaction peaking in 15–25 min and disappearing in 1–2 h is a positive response. Late reactions with skin-prick tests are rare. Although a skin-prick test can demonstrate sensitization to food allergens, a positive skin-prick test cannot be used to determine whether the suspect food gives rise to clinical symptoms and only indicates that the food and the individual’s symptoms (wheal and flare) are related. To determine whether the predictive value of the skin-prick test is high, a DBPCFC would need to be carried out.

Although the skin-prick test is practical and commonly used to screen for food allergy, the use of different forms of the same food can generate varied responses. For example, commercial extracts of the same food allergen from different manufacturers have been shown to have varied allergenic activities. One possible reason for this is that the process of extraction may alter the activity of the allergen. For example, labile allergens from fruit and vegetables are unlikely to resist the extraction. Thus, skin-prick tests with commercial extracts of fruit and vegetable allergens are variable and probably unreliable. Fresh food can be used instead of commercial extracts and its use in skin-prick tests has been shown to be more sensitive and reproducible than using commercially
available extracts. In addition, skin-prick tests using fresh foods have shown greater concordance with DBPCFC than those using commercial extracts (see Bruijnzeel-Koomen et al., 1995; European Commission, 1997; Committee on Toxicity, 2000).

Radioallergosorbent test

RAST is the most common in vitro diagnostic test for food allergy and may be used as an alternative to the skin-prick test. RAST involves measuring the levels of allergen-specific IgE in the sera of allergic individuals. However, as with the skin-prick test, RAST cannot provide a definitive diagnosis of food allergy and thus a DBPCFC would need to be carried out to ascertain its predictive power. RAST is generally believed to be less sensitive than the skin-prick test and is more expensive. In addition, the results of RAST are not immediately available. There are also problems relating to the quality of the food allergens used for this test (see Bruijnzeel-Koomen et al., 1995; European Commission, 1997; Committee on Toxicology, 2000).

Despite the drawbacks associated with measuring allergen-specific IgE, investigations have indicated that quantifying the levels of allergen-specific IgE may be a viable alternative to the DBPCFC. Using children and adolescents (most (90%) with a family history of atopic disease), Sampson and Ho (1997) and Sampson (2001) demonstrated that the concentrations of IgE specific to cow’s milk, egg, peanut and fish, measured using an enzyme-linked immunosorbent assay, could be used to predict the likelihood of a clinical response to these foods and thus eliminate the need for DBPCFC.

Other diagnostic tests

Another in vitro test involves measuring the release of mediators from mast cells or basophils following exposure to the suspected food. These tests vary in sensitivity and specificity, and they show varying degrees of concordance with other tests. However, since such tests require the preparation and use of serum and mast cells/basophils, they are time-consuming and cannot be used routinely for the diagnosis of food allergy.

A number of other methods have been used in the diagnosis of food allergy. However, there is no scientific evidence to suggest that such tests are in any way useful (Committee on Toxicity, 2000; British Nutrition Foundation, 2001). These tests include:

1. Electroacupuncture, in which the electrical activity of the skin is determined at points considered appropriate for the detection of food allergy.
2. Applied kinesiology, which is based on muscle strength.
3. Autologous urine injections, in which urine from a sensitive individual is said to cause a positive skin reaction in another individual with the same sensitivity.
Prevention, Management and Treatment of Food Allergy

Prevention

Based on research on the impact of early nutrition on the immune system of infants, advice on the prevention of food allergy is focused on reducing risk of development in infants and young children, particularly those who are related to atopic individuals. Much of this preventive advice is linked to reducing or eliminating the presence of common allergenic foods from the diets of atopic mothers/mothers-to-be and their children.

Dietary modification (i.e. eliminating common allergenic foods from their diets) by pregnant women with a history of allergy in the family has been shown to be beneficial in terms of allergy outcome in the infant (see Hampton, 1999; Chandra, 2000). However, studies indicate that, for these elimination diets to be useful, they (the diets) must begin before week 22 of pregnancy, since sensitization may occur as early as the second trimester (Jones et al., 1996) and that these diets should continue until the end of lactation. Such diets may put stress on the mother, which may be detrimental to the fetus and thus any gain in respect of preventing the development of atopic disease and allergy may be lost (Hampton, 1999).

Research has also shown that the early introduction of weaning foods and the greater diversity of these foods, particularly those that are highly allergenic, such as milk, eggs, soybean, fish and nuts, may result in an increased incidence of food allergy in infants from atopic families (Hampton, 1999; Chandra, 2000).

Based on these studies, recommendations to reduce the risk of development of food allergies highlight the need for mothers with a history of atopy to:

- Avoid allergenic food during pregnancy and lactation.
- Delay introduction of solid foods, particularly those that are highly allergenic, such as milk, dairy products, eggs, fish, soybean, nuts and peanuts, until after 4 months, limiting variety until at least 6 months.
- Breast-feed exclusively and for a prolonged period. Breast-feeding may provide partial protection against the development of food allergy (Chandra, 2000). Reasons for this include reduced exposure to food proteins that would be present in formulas, improved maturation of the gut mucosal barrier, reduced infection and the presence of anti-inflammatory and immunological factors in human breast milk (Peat et al., 1999; see also Brandtzaeg, Chapter 14, this volume).

In its report on peanut allergy, the advice of the Department of Health (1998) on reducing the risk of developing peanut allergy is directed at pregnant mothers 'who are themselves atopic, or those for whom the father or any sibling of the unborn child has an atopic disease'. These individuals are advised to avoid eating peanuts, peanut products and foods containing peanuts. The same advice is directed at atopic breast-feeding mothers. The report goes on to advise that infants with a parent or sibling with atopic disease should be breastfed for 4–6 months and that, during weaning and until at least 3 years of age, their diets should not include peanuts, peanut products and foods containing
peanuts. However, in spite of the above recommendations/advice, it must be stressed that not all studies support maternal avoidance of highly allergenic foods during pregnancy and/or during lactation (Hattevig et al., 1999; Zeiger, 2000; British Nutrition Foundation, 2001). Thus, researchers have highlighted the need for further studies to facilitate a clearer understanding of the role of maternal diet in the occurrence of atopic diseases and sensitization to allergenic foods (Zeiger, 2000; British Nutrition Foundation, 2001).

Maternal avoidance of peanuts and other highly allergenic foods during pregnancy and lactation also raises the issue of tolerance. In its report on peanut allergy, the Department of Health (1998) states that the rarity of peanut allergy in African and Asian countries may be due to peanut allergy going unrecognized, genetic differences or the development of tolerance to peanut. Differences in cooking practices may also be a factor. Concerning tolerance, it may be that exposure of infants to peanut allergens – for example, via breast milk – may allow the child to build up a tolerance to peanut. However, until data on the consumption and prevalence of peanut and peanut allergy, respectively, in African and Asian countries are obtained, such a possibility cannot begin to be confirmed.

The use of hydrolysed milk formulas to prevent the development of cow’s milk allergy has also been investigated. Hydrolysed milk formulas, which are nutritionally adequate, vary in their degree of hypoallergenicity; however, they are not completely non-allergenic, since some can still cause allergic reactions in individuals with cow’s milk allergy. Studies have shown that such formulas reduce the incidence of cow’s milk allergy, atopic dermatitis and specific cow’s milk IgE in infants. Furthermore, studies note that these effects are specific for cow’s milk allergy and are limited to infants when given before 6 months of age (Zeiger, 2000). However, since many of these studies had technical and methodological limitations, such as inadequate sample size, further work is needed.

Alternative formulas, specifically soya formula, have also been investigated. Although soybean is itself allergenic, studies have reported that in infants of atopic parents fed soya formula from birth or early in life, the incidence of soya allergy is very low. In these studies, soya allergy was diagnosed using DBPCFC. However, in studies where DBPCFC was not used, the results do not suggest a low soya allergy in such infants. Soya formulas may be used as a safe alternative for children with IgE-mediated cow’s milk allergy (Zeiger, 2000; see section on ‘Other Immunological Reactions to Food’). However, concerns have been raised about the bioavailability of nutrients, specifically the micronutrients calcium, zinc and iron, in soya drinks and soya-based infant formulas, due to the presence of phytates in these products (Sandstrom et al., 1983; Lonnerdal et al., 1984; Hurrell et al., 1992; Reddy et al., 1996; Couzy et al., 1998; Heaney et al., 2000).

Management and treatment

Exclusion diets

Currently, the only way to manage food allergy is by exclusion of the offending food/s. Expert dietary advice on exclusion diets is essential to ensure (Baker and David, 1996):
1. Dietary compliance – complete avoidance of the offending food/s.
2. That the nutritional requirements of the patient are being met.
3. That the patient has knowledge of how to read and interpret food labels (although food labels do not always contain the information required to make decisions about the suitability of foods).
4. That patients are able to lead as near as possible to a normal life in spite of the obstacles placed by dietary compliance and are aware of whole foods that are hidden sources of the offending food.

**Novel approaches to the management and treatment of food allergy**

**PROBIOTICS.** One of the recent novel approaches involves the use of probiotics (microbial cell preparations or components of microbes that are reported to maintain health via their action on gut flora (see Gill and Cross, Chapter 13, this volume)). Studies have linked the development of atopy and subsequent atopic disease with the composition of gut flora (Björksten et al., 2001; Kalliomäki et al., 2001; Ouwehand et al., 2001). In addition, studies focusing on the therapeutic role of probiotics have provided clinical evidence for their potential use in the management of food allergy. Trapp et al. (1993) reported that volunteers given yoghurt had decreased concentrations of IgE in their serum and a lower frequency of allergies. Matsuzaki et al. (1998) showed that oral feeding of the probiotic bacterium *Lactobacillus casei* inhibited ovalbumin-induced IgE production in mice. Furthermore, Majamaa and Isolauri (1997) showed that probiotics significantly improved the clinical symptoms associated with food allergy in infants with atopic eczema and cow’s milk allergy. However, information on the efficacy of probiotics in the treatment of food allergy is limited and thus more research is needed (Isolauri et al., 1999; Kirjavainen et al., 1999).

**GENE THERAPY.** Another novel approach, which is in its infancy, is gene therapy. A report by Roy et al. (1999) described the use of gene therapy in the treatment of peanut allergy. The study involved cloning the major peanut allergen *Ara h* 2 and administering it in a murine model of peanut allergy and anaphylaxis. Subsequent dosing with *Ara h* 2 was associated with a decrease in IgE levels and also reduced the severity of anaphylaxis, indicating that the mice had built up a degree of tolerance. However, this approach is far from being used clinically and further studies are required on the efficacy of this therapy in mice that are already sensitized to peanuts, as is the case in humans.

**Other Immunological Reactions to Food**

**Non-IgE-mediated allergic reactions**

There are three non-IgE-mediated allergic reactions:

1. Type II. Antibody-dependent cytotoxic hypersensitivity reactions involve the binding of antibodies (IgG or IgM) to cell-bound antigen/allergen and the
subsequent destruction of the cell-bound antigen by complement proteins, macrophages or killer cells.

2. Type III. Immune-complex-mediated hypersensitivity reactions involve the generation of large insoluble antibody (IgG or IgM) antigen/allergen complexes. Their presence activates complement proteins, which leads to cellular (neutrophil, mast cell, basophil and eosinophil) infiltration and activation and ultimately tissue damage.

3. Type IV. Delayed T-cell-mediated hypersensitivity reactions are not antibody-mediated but T-cell-mediated and involve the proliferation of antigen/allergen-specific T-cells, which normally results in a delayed (after 24 h) inflammatory response.

There is very little information on the role of non-IgE-mediated reactions to food (British Nutrition Foundation, 2001). Some foods do give rise to non-IgE mediated reactions, but the specific mechanisms are not generally known. However, both type III and type IV reactions have been associated with gluten, cow’s milk and soybean allergies and are associated with the manifestation of gastrointestinal and cutaneous symptoms (Høst, 1994).

Exercise-induced anaphylaxis

Cases of anaphylaxis induced by exercise following the ingestion of food have been reported in the literature. The first case involved an individual who developed anaphylactic reactions (cutaneous and respiratory reactions, including urticaria and airway obstruction) when running 12 h after ingesting shellfish; each time the individual went for a run following the earlier ingestion of shellfish, anaphylactic reactions ensued. The relationship between exercise-induced anaphylaxis and the earlier ingestion of food remains controversial and the immunology behind it is unclear, since it may or may not be mediated by IgE (Tilles and Schocket, 1997).

Conclusion

Food allergy is believed to be on the increase and its symptoms can sometimes be life-threatening. Why the immune system reacts to certain foodstuffs as if they were pathogens is still unclear. However, research into this area of nutrition and immunity has provided an increased understanding of the immune mechanisms that are generated in response to food and the proteins that cause them. Continuing research into the properties that confer allergenicity on to food proteins and into the development of food allergy, in particular the role that genes may play in predisposing to adverse responses to some foods, may bring us closer to finding out why these reactions occur.
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Food Allergy

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Exercise and Immune Function – Effect of Nutrition

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Introduction

Regular recreational exercise is generally understood to be beneficial to health, whereas total inactivity is detrimental. Regular exercise may increase resistance to infections such as the common cold, whereas hard training is associated with increased respiratory-tract infections (Brines et al., 1996). Indeed, as early as 1902, the Boston marathon was used as an experiment in violent exercise to demonstrate post-exercise leucocytosis, and, in more recent years, it has been shown that elite athletes may become more immune-suppressed through over-training (see Hoffman-Goetz and Pedersen, 1994). The physiological basis of altered resistance to infections is not well understood, but exercise-induced changes in the cellular immune system are among the possible explanations. The relationship between nutritional factors and resistance to infections has generated considerable interest over the past several decades. It is possible that the exercise-induced immunological changes can be modulated by nutritional factors and that dietary factors influence resting levels of immune activity in athletes (Pedersen et al., 1998, 1999).

Exercise and Immune Function

Effects of acute exercise

Acute exercise induces dramatic changes in the immune system (for an extensive review, see Pedersen and Hoffman-Goetz, 2000). The neutrophil concentration in the bloodstream increases during exercise and continues to increase post-exercise. The lymphocyte concentration increases during exercise but falls below pre-exercise values following intense long-duration exercise, although not after moderate exercise.
Several reports describe exercise-induced changes in subsets of blood lymphocytes (for references, see Pedersen and Hoffman-Goetz, 2000). Increased lymphocyte concentration is probably due to the recruitment of all lymphocyte subpopulations (CD4+ T-cells, CD8+ T-cells, B-cells and natural killer (NK) cells) to the vascular compartment. During exercise, the CD4/CD8 ratio of circulating lymphocytes decreases, reflecting the greater increase in CD8+ lymphocytes than in CD4+ lymphocytes. CD4+ and CD8+ T-cells contain both CD45RO+ (memory) and CD45RA+ (naive) cells. It has been shown that it is mainly memory cells that are mobilized to the circulation in response to acute physical exercise (Bruunsgaard et al., 1999). It has also been shown that the lymphocytes recruited to the blood have short telomere lengths (Bruunsgaard et al., 1999), which strongly indicates that ‘old’ lymphocytes with a long replicative history are recruited to the blood. Thus, the initial increase in CD4+ and CD8+ cells after exercise is not due to repopulation by newly generated cells but may be a redistribution of activated cells. It is therefore likely that the cells are mobilized from peripheral compartments, such as the spleen, and it has indeed been demonstrated that splenectomized patients are not able to mount a normal lymphocyte response to exercise (Nielsen et al., 1997).

Following intense long-duration exercise the functions of NK, T- and B-cells are suppressed (for references, see Pedersen and Hoffman-Goetz, 2000). Thus, NK-cell activity (the ability to kill target tumour cells) and lymphocyte proliferation are inhibited. Furthermore, B-cell function is inhibited and the local production of secretory immunoglobulin (IgA) in saliva decreases (Gleeson and Pyne, 2000).

There are few studies that document immune function in vivo in relation to exercise. However, impairment of in vivo cell-mediated immunity, but not specific antibody production, could be demonstrated after intense exercise of long duration (triathlon race) (Bruunsgaard et al., 1997). The cellular immune system was evaluated as a delayed-type hypersensitivity (DTH) ‘skin’-test response to seven recall antigens. The response was significantly lower in subjects who performed a triathlon race compared with triathletes and untrained controls who did not participate in the triathlon. However, no differences in specific antibody titres to pneumococcal, diphtheria or tetanus vaccines were found between the groups (Bruunsgaard et al., 1997).

In summary, during moderate as well as intense exercise, lymphocytes are mobilized to the circulation. However, following intense exercise, the lymphocyte concentration declines, T-cell proliferation is impaired, NK-cell function is decreased and the level of IgA in saliva is suppressed. During the post-exercise immune impairment, also called ‘the open window’ in the immune system, it has been suggested that microorganisms may invade the host and establish infections (Fig. 16.1).

Strenuous exercise also induces increased circulating levels of several cytokines in the blood (for a review, see Pedersen, 2000). Interleukin (IL)-6 has been found to be markedly enhanced in several studies. Thus, after a marathon, the concentration of IL-6 in the bloodstream has been shown to increase 100-fold (Ostrowski et al., 1999). Recent studies demonstrate that IL-6 is produced locally in contracting skeletal muscles and that the net-IL-6 release
from skeletal muscles can solely account for the exercise-induced increase in arterial plasma IL-6 (Steensberg et al., 2000). It is likely that IL-6 mediates some of the exercise-related metabolic responses (Steensberg et al., 2001). A full cytokine cascade develops in response to exercise, including tumour necrosis factor (TNF)-α, IL-1β, IL-1 receptor antagonist (IL-1ra), IL-8, IL-10, TNF-receptors (TNF-R) and macrophage inhibitory proteins (MIP)-1 (Pedersen et al., 2001; Fig. 16.2).

**Effects of chronic exercise**

Although exercise has an acute effect on circulating lymphocyte numbers, on immune cell functions and on plasma cytokine concentrations (see above), many immune functions are similar in resting athletes and non-athletes (see Nieman, 2000). Components of the adaptive immune system (measured in resting athletes) seem to be largely unaffected by intensive and prolonged exercise training. The innate immune system appears to respond differentially to the chronic stress of intensive exercise, with NK-cell activity tending to be enhanced, while neutrophil function is suppressed.

Based on epidemiological studies a relationship between exercise and upper respiratory-tract infections (URTI) has been modelled in the form of a 'J-shaped' curve. This model suggests that while the risk of URTI may decrease below that of a sedentary individual when one engages in moderate exercise training, the risk may rise above average during periods of excessive amounts

![Fig. 16.1. Generalized scheme of the effects of moderate and intense exercise on immune function and susceptibility to infection. During moderate and intense exercise, the immune system is enhanced (as shown by mobilization of lymphocytes to the circulation), but intense exercise is followed by a period of immune impairment (decreased natural killer cell activity, lymphocyte proliferation and levels of salivary immunoglobulin A), during which there is an 'open window' of opportunity for pathogens.](image-url)
of high-intensity exercise. The link between exercise-associated immune changes and sensitivity to infections may be explained by the so-called ‘open window’ of altered immunity. It is hypothesized that viruses and bacteria may gain a foothold, increasing the risk of sub-clinical and clinical infection. However, it remains to be demonstrated if athletes showing the most extreme immunosuppression following heavy exertion are those that contract an infection within the following 1–2 weeks.

**Exercise, Nutrition and Immune Function**

The mechanisms underlying exercise-associated immune changes are multifactorial and include neuroendocrinological factors, such as adrenalin, noradrenalin, growth hormone, cortisol and beta-endorphin (see Pedersen and Hoffman-Goetz, 2000; Fig. 16.3). It has been suggested that altered protein metabolism, such as decreased glutamine concentration in the plasma, influences lymphocyte function and that decreased plasma glucose concentration increases stress-hormone levels and thereby reduces immune function. Furthermore, as a consequence of the catecholamine- and growth-hormone-induced immediate changes in leucocyte subsets, the relative proportions of these subsets change and activated leucocyte subpopulations may be mobilized to the blood. Free radicals and prostaglandins (PG) released by the elevated number of neutrophils and monocytes may influence the function of lymphocytes and contribute to the impaired function of the latter cells. Thus, nutritional supplementation with glutamine, carbohydrate, antioxidants or PG inhibitors may, in principle, influence exercise-associated changes in immune function.

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**Fig. 16.2.** Changes in plasma cytokine concentrations in relation to strenuous exercise. TNF, tumour necrosis factor; IL, interleukin; IL-1ra, IL-1 receptor antagonist; MIP, macrophage inhibitory protein.
Glutamine

Skeletal muscle is the major tissue involved in glutamine production and is known to release glutamine into the bloodstream at a high rate (see Calder and Newsholme, Chapter 6, this volume). It has been suggested that the skeletal muscle plays a vital role in maintenance of the key process of glutamine...
utilization in the immune cells. Consequently, the activity of the skeletal muscle may directly influence the immune system. It has been hypothesized that during intense physical exercise, or in relation to surgery, trauma, burn and sepsis, the demand on muscle and other organs for glutamine is such that the immune system may be forced into a glutamine debt, which temporarily affects its function (see Calder and Newsholme, Chapter 6, this volume). Thus, factors that directly or indirectly influence glutamine synthesis or release could theoretically influence the function of immune cells (see Calder and Newsholme, Chapter 6, this volume). Following intense long-term exercise and other physical stress disorders, the glutamine concentration in plasma declines (Rohde et al., 1998a). In four placebo-controlled glutamine intervention studies (Rohde et al., 1998b, c; Krzywkowski et al., 2001a, b), it was found that glutamine supplementation abolished the post-exercise decline in plasma glutamine without influencing post-exercise impairment in immune function. Thus, these studies do not support the hypothesis that the post-exercise decline in immune function is caused by a decrease in the plasma glutamine concentration.

Carbohydrate

Earlier research has established that a reduction in blood glucose levels is linked to hypothalamic–pituitary–adrenal activation, increased release of adrenocorticotropic hormone and cortisol, increased plasma growth hormone, decreased insulin and a variable effect on blood adrenalin level. Given the link between stress hormones and immune responses to prolonged and intensive exercise, increased availability of carbohydrate should maintain plasma glucose concentrations and attenuate increases in stress hormones and thereby diminish changes in immunity. This hypothesis has been tested in a number of studies, using double-blind, placebo-controlled randomized designs. Carbohydrate beverage ingestion before, during and after 2.5 h of exercise was associated with higher plasma glucose levels, an attenuated cortisol and growth-hormone response, fewer perturbations in blood immune cell counts, lower granulocyte and monocyte phagocytosis and oxidative-burst activity and a diminished pro- and anti-inflammatory cytokine response (for a review, see Nieman, 1998). Overall, the hormonal and immune responses in the subjects taking carbohydrate were less affected by exercise than in subjects in the placebo groups. Some immune variables were affected slightly by carbohydrate ingestion (e.g. granulocyte and monocyte function), while others were strongly influenced (e.g. plasma cytokine concentrations, blood immune cell numbers) (see Nieman, 1998). The clinical significance of these carbohydrate-induced effects on the endocrine and immune systems awaits further research. At this point, the data indicate that athletes ingesting carbohydrate beverages before, during and after prolonged and intensive exercise should experience lowered physiological stress. Research to determine whether carbohydrate ingestion will improve host protection against viruses in endurance athletes during periods of intensified training or following competitive endurance events is warranted.
Lipids

There are two principal classes of polyunsaturated fatty acids (PUFA): the n-6 and the n-3 families (see Calder and Field, Chapter 4, this volume). The precursor of the n-6 family is linoleic acid, which is converted to arachidonic acid, the precursor of PG and leucotrienes (LT), which have potent pro-inflammatory and immunoregulatory properties (see Calder and Field, Chapter 4, this volume). The precursor of the n-3 family of PUFA is α-linolenic acid. If the ratio of n-6 to n-3 PUFA in the diet is decreased by administration of a diet rich in n-3 fatty acids, PGE₂-mediated immunosuppression may be abolished (see Calder and Field, Chapter 4, this volume).

The possible interaction between intense acute exercise, immune function and PUFA was examined in inbred female C57Bl/6 mice. The animals received either a natural ingredient diet or a diet supplemented with various fats, such as beef tallow, safflower oil (rich in the n-6 PUFA linoleic acid), fish oil (rich in long-chain n-3 PUFA) or linseed oil (rich in the n-3 PUFA α-linolenic acid) for an 8-week period. Linseed oil abolished post-exercise suppression of the IgM plaque-forming cell response (Benquet et al., 1994). Although some experiments show that the increase in IL-1 and TNF following administration of endotoxin is reduced when the animals are pretreated with n-3 PUFA as fish oil (see Calder and Field, Chapter 4, this volume), a recent study showed that n-3 PUFA did not influence the exercise-induced elevation of pro- or anti-inflammatory cytokines (Toft et al., 2000).

Antioxidants

During exercise, the enhanced oxygen utilization leads to production of reactive oxygen species, as indicated by the blood glutathione redox status. Antioxidants may in theory neutralize the reactive species that are produced by neutrophilic leucocytes during phagocytosis (see Hughes, Chapter 9, this volume). The effect of vitamin C supplementation on lymphocyte function and stress-hormone levels after exercise has been studied. Supplementation with vitamin C did not influence circulating leucocyte subsets, NK-cell activity, lymphocyte proliferative responses, granulocyte phagocytosis or activated burst or circulating concentrations of catecholamines and cortisol (Nieman et al., 1997; Petersen et al., 2001).

Conclusion

There is recruitment of lymphocytes to the circulation during exercise. Following strenuous exercise, the lymphocyte count declines, natural immunity and T-cell proliferation are impaired and the level of secretory IgA in saliva is low. Few studies have addressed the potential protective role of dietary supplementation in exercise-induced immunosuppression. Exercise-related immunosuppression in animals was prevented by a diet rich in n-3 fatty acids, but there
is a lack of human studies. Antioxidant supplementation has no effect on exercise-induced changes in NK and T-cell functions, but vitamin C supplementation has been shown to decrease the incidence of post-race URTI symptoms in some studies. Glutamine supplementation has not been effective in abolishing post-exercise suppression of the immune system, whereas carbohydrate loading diminishes the hormonal and immune responses to exercise. The clinical consequences of carbohydrate loading remain to be determined. At this point in time, it is premature to make recommendations regarding nutritional supplementation to avoid exercise-induced immune changes.

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References


Introduction

Many experimental and clinical data gathered over a number of years have demonstrated a marked decline in many immune responses with the ageing process (Makinodan and Kay, 1980; Lesourd, 1990a; Miller, 1992). In contrast, in recent years it has emerged that some immune responses do not decline, and can even increase, with age (Kubo and Cinader, 1990, Ershler et al., 1993; Barrat et al., 1997). As a consequence, the influence of ageing on the immune system is generally described nowadays as a progressive occurrence of dysregulation, rather than as a general decline in function (Weksler, 1995; Cakman et al., 1996). In addition, it has also been shown that many decreased immune responses described as age-related are actually linked to other factors, such as poor nutritional status (Lesourd, 1990a, 1999; Lesourd and Mazari, 1999). In fact, in carefully selected, very healthy subjects, decreased immune responses are observed only in the very oldest (> 90 years of age) (Mazari and Lesourd, 1998).

Most investigations into ageing in human subjects rely on data from apparently healthy aged individuals without checking for the possibility of underlying factors, such as ongoing disease, which is not clinically apparent, or poor nutritional status. However, some studies have attempted to identify such confounding factors. The first was the use of the Senieur protocol (Ligthart et al., 1984), but later studies added additional factors for the selection of very healthy elderly subjects (Reibnegger et al., 1988; Lesourd et al., 1994; Lesourd and Mazari, 1999). In such very carefully selected aged individuals, the immune changes observed are due to the ageing process alone, and not to environmental (including nutritional) influences. In fact, we reported that changes due to
nutritional factors in aged subjects are similar to what was previously described as immune ageing (Lesourd and Meaume, 1994; Lesourd et al., 1994). Furthermore, the changes of immune responses in aged individuals are proportional to the intensity of the observed nutritional deficits (Lesourd et al., 1992).

This chapter will describe:

1. The primary ageing immune deficiency. This is observed in the very carefully selected healthy elderly (VCSH elderly), in whom all known influence of external factors has been eliminated. We focus here on studies comparing VCSH elderly persons of different ages, referred to as ‘young elderly’ (60–80 years of age) and ‘old elderly’ (> 90 years of age), with young adults. These subjects fit the Senieur criteria for healthiness and, in addition, have ‘normal’ nutritional status (as defined by the EURONUT/SENECA European study (Haller et al., 1996)) and a serum albumin concentration > 39 g l\(^{-1}\).

2. The secondary nutritionally induced immune deficiency in the elderly. This can be observed in VCSH elderly persons with poor nutritional status but without any detectable acute and/or chronic disease. These subjects are referred to as ‘apparently healthy elderly’. These subjects fit the same criteria as the VCSH elderly except that they have a serum albumin concentration of 30–39 g l\(^{-1}\).

3. The tertiary immune deficiency in the elderly. This corresponds to the immune responses measured in diseased elderly subjects, in whom not only nutrient deficiencies, but also diseases, induce changes in immune responses.

**Primary (Ageing) Immune Deficiency**

**Decreases in T-cell generation and maturation**

Stem cells are generated in bone marrow and mature as T lymphocytes in the thymus. Although stem cell generation does not appear to decline with age, the ability of stem cells to undergo clonal proliferation declines with age (Tyan, 1981), as does thymocyte maturation in relation to thymus involution (Steinmann et al., 1985; Hirokawa et al., 1994). Such involution starts relatively early in life (at puberty) and the major changes are mostly related to the end of the maturation period and to early adulthood, rather than to ageing. Thymic function is almost lost by the age of 60 years.

**Changes in peripheral T-cell subsets and functions**

Circulating lymphocyte numbers are decreased in aged persons (Lesourd et al., 1994; Huppert et al., 1998), but the decline is usually quite small (10–15%), even in the ‘old elderly’ when they are very healthy (Mazari and Lesourd, 1998), and indeed the decline is not always observed in ‘young elderly’ (Wick and Grubeck-Loewenstein, 1997). Although T-cell numbers show little change with ageing, T-cell subsets do change: there are decreases in the number of fully mature T-cells (CD3+) and parallel increases in the number of ‘immature’ T-
cells (CD2+ CD3−) (Lesourd et al., 1994; Table 17.1). Equivalent changes are observed in both ‘young elderly’ and ‘old elderly’, indicating that they mainly occur earlier in life, probably at middle age (Mazari and Lesourd, 1998). It appears that this change is simply a marker of the end of the T-cell maturation period, rather than a phenomenon linked to old age. As a consequence, peripheral T lymphocytes from aged subjects exhibit lower proliferative ability, since the CD2+ CD3− subset has a lower proliferative response than the CD3+ subset (Alès-Martinez et al., 1988). The inability of the thymus to generate new T lymphocytes is partly compensated, in aged animals (Abo, 1992) and in humans (Lesourd et al., 1994), by the occurrence of new T-cells, which are not fully mature, in other primary immune organs, such as the liver (Nakayama et al., 1994). As a result, aged individuals have difficulty in generating fully mature T lymphocytes and, as a consequence, cause lymphopenia in the elderly (Proust et al., 1986), since the elderly are unable to rapidly generate new T-cells.

Two other changes in T-cell subsets occur with ageing. There is a decrease in naive T-cell (CD45RA+) numbers, with a parallel increase in memory T-cell (CD45RO+) numbers (Cossarizza et al., 1992) and a decrease in cytotoxic T-cell (CD8+) numbers (Lesourd and Meaume, 1994; Lesourd et al., 1994; Wick and Grubeck-Loewenstein, 1997). The switch from CD45RA to CD45RO occurs mainly within the first three decades of life, when the individual is inducing new immune responses to antigens not previously encountered. Within the third decade, about 65% of peripheral T lymphocytes bear CD45RO (Cossarizza et al., 1992; Mazari and Lesourd, 1998; Table 17.1). Later in life, the switch towards CD45RO continues, but at a far slower rate (10% increase from 30 to 80 years of age). Therefore, this important change is not an ageing phenomenon but rather a component of the maturation of the immune system (Lesourd, 2000).

The change in CD8+ T-cell numbers mainly occurs during adulthood (20–25% decrease from age 20 to 70) and is associated with decreases in the subset exhibiting the highest level of expression of CD8 molecules on the cell surface (Lesourd et al., 1994). This is probably related to changes in T-helper (Th) cells (see below) and is partly compensated for by an increase in the CD8+ subset exhibiting a low level of expression of CD8 (Lesourd et al., 1994; Mazari and Lesourd, 1998), probably generated within the liver. Both changes result in lower cell-mediated immune functions. CD45RO memory cells are poor interleukin (IL)-2 secretors and therefore exhibit lower proliferative levels (Nagelkerken et al., 1991; Hobbs and Ernst, 1997). Thus, the change in the naive/memory ratio with ageing will result in lower lymphocyte proliferative responses. Nevertheless, these changes are probably not of very great influence on the immune system, since it has recently been shown that ‘young elderly’ have comparable proliferative responses and similar IL-2 secretion to those of young adults (Mazari and Lesourd, 1998; Myslinska et al., 1998; Table 17.1). The decline in IL-2 production and proliferation occurs only in the very old (Lesourd and Mazari, 1999). The decrease in the number of CD8+ cells is...
Table 17.1. Subsets and functions of peripheral blood T lymphocytes from healthy elderly subjects. All subjects were recruited using the Senieur criteria (Ligthart et al., 1984) and additional criteria previously reported (Mazari and Lesourd, 1998).

<table>
<thead>
<tr>
<th>Subset</th>
<th>Very healthy young adults (25–34 years) (n = 57)</th>
<th>Very healthy ‘young elderly’ (65–85 years) (n = 41)</th>
<th>Very healthy ‘old elderly’ (&gt; 90 years) (n = 19)</th>
<th>Apparently healthy ‘young elderly’ (70–85 years) (n = 51)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± Standard deviation</td>
<td>Mean ± Standard deviation</td>
<td>Mean ± Standard deviation</td>
<td>Mean ± Standard deviation</td>
</tr>
<tr>
<td>Age (years)</td>
<td>29.1 ± 3.2</td>
<td>79.6 ± 5.3</td>
<td>94.3 ± 3.2</td>
<td>78.9 ± 6.2</td>
</tr>
<tr>
<td>Albumin (g l⁻¹)</td>
<td>43.5 ± 2.8</td>
<td>42.4 ± 4.1</td>
<td>41.7 ± 3.7</td>
<td>37.2 ± 3.9</td>
</tr>
<tr>
<td>Lymphocytes (number μl⁻¹)</td>
<td>2248 ± 456</td>
<td>1993 ± 568</td>
<td>1817* ± 598</td>
<td>1705** ± 434</td>
</tr>
<tr>
<td>CD2+ cells (number μl⁻¹)</td>
<td>2014 ± 321</td>
<td>1794** ± 397</td>
<td>1586** ± 434</td>
<td>1511***± 427</td>
</tr>
<tr>
<td>CD3+ cells (number μl⁻¹)</td>
<td>1897 ± 264</td>
<td>1565** ± 311</td>
<td>1323***± 356</td>
<td>1207***± 299</td>
</tr>
<tr>
<td>CD2+ CD3− cells (number μl⁻¹)</td>
<td>117 ± 105</td>
<td>214* ± 224</td>
<td>254* ± 242</td>
<td>283** ± 241</td>
</tr>
<tr>
<td>CD4+ cells (number μl⁻¹)</td>
<td>1267 ± 209</td>
<td>1136* ± 243</td>
<td>997* ± 264</td>
<td>812***± 271</td>
</tr>
<tr>
<td>CD8+ cells (number μl⁻¹)</td>
<td>672 ± 144</td>
<td>437*** ± 174</td>
<td>378*** ± 211</td>
<td>387*** ± 196</td>
</tr>
<tr>
<td>CD45RA cells (number μl⁻¹)</td>
<td>1234 ± 314</td>
<td>658*** ± 228</td>
<td>404***± 197</td>
<td>464***± 213</td>
</tr>
<tr>
<td>CD45R0 cells (number μl⁻¹)</td>
<td>846 ± 201</td>
<td>1222*** ± 365</td>
<td>1192*** ± 462</td>
<td>1057* ± 497</td>
</tr>
<tr>
<td>IL-2 production (ng l⁻¹)</td>
<td>2.01 ± 0.35</td>
<td>1.84 ± 0.34</td>
<td>1.21***± 0.44</td>
<td>1.11***± 0.38</td>
</tr>
<tr>
<td>IL-6 production (ng l⁻¹)</td>
<td>1.37 ± 0.16</td>
<td>1.82* ± 0.22</td>
<td>1.99*** ± 0.34</td>
<td>1.48 ± 0.40</td>
</tr>
<tr>
<td>Lymphocyte proliferation</td>
<td>152 ± 40</td>
<td>114 ± 35</td>
<td>75** ± 32</td>
<td>54***± 34</td>
</tr>
</tbody>
</table>

IL, interleukin.
Significant differences from very healthy young adult controls: *P < 0.05, **P < 0.01, ***P < 0.001.
Significant differences from very healthy ‘young elderly’: †P < 0.05, ††P < 0.01.
§ Determined using 5 μg mitogen 10⁶ cells⁻¹.

IL₂ production‡ (ng l⁻¹)
IL-6 production (ng l⁻¹)
Lymphocyte proliferation§

(10³ cpm 10⁶ cells⁻¹)
associated with decreased cytotoxic T-cell function (Bruley-Rosset and Payelle, 1987; Mbawuike et al., 1997). This may be because the cells bearing a low level of CD8 on their surface have poor cytotoxic function.

Since the early 1990s immune ageing has also been described as a change in the ratio of the Th1 to the Th2 phenotype (see Devereux, Chapter 1, this volume). There is a progressive decrease in Th1 function and a relative preservation and/or increase in Th2 function with age (Cakman et al., 1996; Shearer, 1997). Decreased IL-2 secretion, a Th1 function, with age was described some time ago (Rabinowich et al., 1985; Nagel et al., 1988). Nevertheless, such a decline is not observed in all mouse strains (Engwerda et al., 1996). More recently, it was shown that lymphocytes from ‘young elderly’ subjects produce similar amounts of IL-2 to those from young adults (Mazari and Lesourd, 1998; Myslinska et al., 1998; Lesourd and Mazari, 1999; Table 17.1).

Another Th1 function, interferon-γ (IFN-γ) secretion, has been described as declining with ageing (Chen et al., 1987). This was considered to be a major phenomenon of the ageing immune system, although it could be reversed by exogenous IL-12 supply in a cell-culture setting (Mbawuike et al., 1997), suggesting that decreased IFN-γ production was due to the absence of a stimulating factor rather than to an inability of Th1 cells to respond. Indeed, other reports have shown than IFN-γ production does not decline with age and might even increase (Sindermann et al., 1993; Barrat et al., 1997). IFN-γ is secreted by memory T-cells, as well as Th1 cells (Sanders et al., 1998), which may explain these contradictory reports. In summary, it is not obvious that Th1 functions decline in all aged persons. The influence of ageing on Th1 function may be very different between individuals, linked to differences in genetic background (Proust et al., 1982; Yong-Xing et al., 1997).

Most reports show increases in the release of Th2 cytokines (IL-4, IL-5, IL-6, IL-10) with ageing (Kubo and Cinader, 1990; Daynes et al., 1993; Ershler et al., 1993; Barrat et al., 1997; James et al., 1997). This change starts in middle adulthood (Myslinska et al., 1998) and continues progressively thereafter. Thus, ageing is characterized by a progressive switch towards Th2-type responses. Antigenic pressures throughout life may be responsible for this change. Indeed, it has been reported that a similar change occurs during the evolution of human immunodeficiency virus (HIV) infection and that, when Th2 becomes the dominant response, significant immune deficiency occurs and then the clinical signs of the disease, such as opportunistic infections, start to occur (Clerici et al., 1992).

In summary, if cell-mediated immunity declines with age, this probably occurs at a very old age in very healthy persons, but may occur sooner in some individuals, due to intense and accumulative antigenic pressures throughout life.

**Changes in humoral immune responses**

Humoral immune responses are less severely affected by the ageing process than cell-mediated immune responses are (Lesourd, 1990a). There are reports
of an age-related increase in immunoglobulins G and A (Batory et al., 1984; Moulias et al., 1984), a change that has been linked to the relative increase in Th2 function (Lesourd, 1997). In contrast, primary antibody responses to vaccine are decreased with ageing, while booster responses are comparable to those seen in earlier adulthood (Moulias et al., 1985). The decreased antibody responses have been associated with age-related decreases in Th-cell function (Miller, 1996) and to the increased production (or accumulation) of anti-idiotypic antibodies (Arreaza et al., 1993), which leads to synthesis of antibodies with lower antigen affinities (Muller et al., 1986). The lower affinity of the antibodies produced after vaccination also results from a dysregulation of B-cell subsets with ageing: decreases in the CD5− subset, which produces antibody against foreign antigens, and relative increases in the CD5+ subset, which produces autoantibodies, are observed (Weksler, 1995). Therefore, even though the secondary responses produce similar levels of antibodies (Huang et al., 1992), the antibodies produced exhibit lower antigen specificity and antibody responses are less adapted to the stimulus (Lesourd, 1997).

Changes in monocyte–macrophage functions

The functions of monocytes and macrophages are preserved, or even enhanced, with the ageing process (Lesourd, 1999; Table 17.2). Antigen processing and presentation are comparable in young and old mice (Goidl, 1987; Doria, 1988). IL-1 release is sustained in old mice (Goldberg et al., 1991) and in elderly humans (Nafziger et al., 1993; Mazari and Lesourd, 1998). IL-6 release is increased by cells from 'young elderly' compared with those from young adults (Mazari and Lesourd, 1998; Table 17.2). Macrophages from aged animals and humans exhibit greater production of prostaglandin (PG)E₂ and free radicals (Meydani et al., 1986, 1995; Hayek et al., 1997). There may be a continuous activation of these cells. This is certainly of great importance, since PGE₂ induces suppression of T-cell functions and since lymphocytes from aged individuals are more susceptible to PGE₂ than those from younger individuals (Goodwin, 1992). Therefore, ageing may be characterized by a continuous ongoing monocyte activation, which induces a permanent suppression of T-cell functions. This represents another age-related dysregulation of the immune system.

Secondary Immunodeficiency in the Elderly: Role of Nutritional Factors

This section will review the immune responses of the healthy and apparently healthy elderly (i.e. those without ongoing disease), in whom immune responses reflect both the ageing process and nutritional status.

Lower nutritional status, indicated by lower albumin levels or by low folate status (although still within the normal range), is associated with lower lymphocyte proliferation (Lesourd and Meaume, 1994; Lesourd et al., 1994; Mazari...
Table 17.2. Functions of peripheral blood monocytes from elderly subjects. Groups were selected, using previously described criteria (Mazari and Lesourd, 1998), as being very healthy, self-sufficient home-living apparently healthy (frail and/or undernourished) or profoundly undernourished old subjects with undernourishment due only to insufficient nutrient intakes (with mild inflammatory process: CRP < 30 mg l\(^{-1}\)).

<table>
<thead>
<tr>
<th></th>
<th>Very healthy</th>
<th>Apparently healthy</th>
<th>Undernourished,</th>
<th>Very undernourished</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>'young elderly'</td>
<td>frail 'young elderly'</td>
<td>self-sufficient</td>
<td>hospitalized</td>
</tr>
<tr>
<td></td>
<td>(65–85 years)</td>
<td>(70–85 years)</td>
<td>(70–85 years)</td>
<td>'young elderly'</td>
</tr>
<tr>
<td></td>
<td>(n = 41)</td>
<td>(n = 51)</td>
<td>(n = 25)</td>
<td>(n = 17)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>Mean 79.6</td>
<td>Mean 78.9</td>
<td>Mean 78.7</td>
<td>Mean 79.6</td>
</tr>
<tr>
<td></td>
<td>Standard deviation 5.3</td>
<td>Standard deviation 6.2</td>
<td>Standard deviation 5.9</td>
<td>Standard deviation 6.4</td>
</tr>
<tr>
<td>Albumin (g l(^{-1}))</td>
<td>Mean 42.4</td>
<td>Mean 37.2</td>
<td>Mean 29.4*** †</td>
<td>Mean 22.3***††</td>
</tr>
<tr>
<td></td>
<td>Standard deviation 4.1</td>
<td>Standard deviation 3.9</td>
<td>Standard deviation 3.2</td>
<td>Standard deviation 2.8</td>
</tr>
<tr>
<td>CRP (mg l(^{-1}))</td>
<td>&lt; 6</td>
<td>7.1</td>
<td>16.8***†</td>
<td>26.4***†††</td>
</tr>
<tr>
<td>IL-1 production (ng ml(^{-1}))</td>
<td>Spontaneous release ND</td>
<td>ND</td>
<td>1.4</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Using 25 µg LPS 10(^6) cells(^{-1}) 2.6</td>
<td>2.4</td>
<td>1.2* †</td>
<td>0.7***††</td>
</tr>
<tr>
<td>IL-6 production (ng ml(^{-1}))</td>
<td>Spontaneous release ND</td>
<td>ND</td>
<td>0.1</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Using 25 µg LPS 10(^6) cells(^{-1}) 1.7</td>
<td>1.9</td>
<td>0.35</td>
<td>0.24</td>
</tr>
</tbody>
</table>

CRP, c-reactive protein; IL, interleukin; LPS, lipopolysaccharide.  
Significant differences from very healthy 'young elderly': *P < 0.05, **P < 0.01, ***P < 0.001.  
Significant differences from apparently healthy 'young elderly': † P < 0.05, †† P < 0.01, ††† P < 0.001.  
ND, not detectable.
and Lesourd, 1998). In addition, the changes in T-cell subsets related to ageing (decreased numbers of CD3+ cells with a parallel increase in numbers of CD2+ CD3− cells; decreased numbers of CD8+ cells) are greater in such apparently healthy elderly subjects than in those with better nutritional status. Treatment of such elderly individuals with folic acid induces an increase in immune responses to levels similar to those seen in young adults, without a change in T-cell subsets (B. Lesourd unpublished data). Therefore, even in the apparently healthy elderly, low nutritional status (although still within the ‘normal’ range) is associated with lower immune responses. Interestingly, the effects of lower nutritional status on immune functions in the apparently healthy elderly were not observed in young adults (Mazari and Lesourd, 1998), indicating that the immune system of the elderly may be more sensitive to nutritional status than that of young adults.

The association between lower nutritional status and lower immune responses in apparently healthy elderly subjects has been the basis of a number of studies aimed at improving immune function with nutritional intervention. Talbott et al. (1987) showed that decreased immune responses in the elderly are linked to lower vitamin B₆ status and that supplementation with vitamin B₆ induces an increase in lymphocyte proliferation, but only in individuals with low vitamin B₆ status. Subsequently, other studies, using either a single micronutrient (e.g. zinc (Boubaïka et al., 1993; Prasad et al., 1993), vitamin E (Meydani et al., 1990, 1997), vitamin B₆ (Meydani et al., 1991)), a few antioxidant micronutrients (Penn et al., 1991; Fortes et al., 1993) or several micronutrients (Bogden et al., 1990, 1994; Chandra, 1992a; Pike and Chandra, 1995), have shown increased immune responses with supplementation. Such increases in immune responses with micronutrient supplementation have also been observed in institutionalized elderly subjects (Galan et al., 1997). These effects are related to correction of micronutrient deficiencies (Bogden et al., 1990; Chandra, 1992a; Galan et al., 1997). Micronutrient deficiencies are quite common in the elderly: half of apparently healthy elderly subjects have low intakes of at least one micronutrient (Amorin-Cruz et al., 1996) and a third have low status of at least one micronutrient (Haller et al., 1996; Lesourd and Mazari, 1999). Some of the lowest intakes or the lowest status, quantified in apparently healthy elderly subjects are reported for micronutrients known to influence the activity of the immune system: vitamin B₆ (6–26% of subjects deficient), folic acid (9–16%), vitamin C (5–10%) (Haller et al., 1996; Lesourd et al., 1998). All supplementation studies concern micronutrients for which nutritional deficit is linked to immune deficit: zinc (Keen and Gershwin 1990; Cunningham-Rundles et al., 1991; see also Prased, Chapter 10, this volume), vitamin B₆ (Chandra and Sudhakaran, 1990; Rall and Meydani, 1993), antioxidants (Meydani et al., 1995; see also Hughes, Chapter 9, this volume). In some reports, it has been shown that long-term (1 year) supplementation may reduce infection rate and/or length of infections in independent (Chandra, 1992a) and institutionalized (Girodon et al., 1997) elderly subjects. These findings demonstrate that nutritional deficiency may be of great significance to the health and well-being of elderly persons, even if they are apparently healthy. They also show the close association between nutritional status, immune responses and clinical state in such elderly subjects.
In addition to enhanced immune responses upon supplementation in apparently healthy self-sufficient elderly who exhibit decreased micronutrient status, vitamin E supplements improved immune responses of healthy elderly subjects who apparently did not have vitamin E deficiency (Meydani et al., 1990, 1997; see also Hughes, Chapter 9, this volume). Vitamin E deficiency is uncommon, affecting less than 1% of such elderly subjects (Amorin-Cruz et al., 1996; Lesourd et al., 1998). The effect of vitamin E was obtained using a high dose (50–800 mg α-tocopherol acetate day$^{-1}$), four to 80 times the recommended intake in many Western countries (e.g. Cynober et al., 2000). Vitamin E supplementation increased lymphocyte proliferation, IL-2 production, delayed-type hypersensitivity and antibody responses to some vaccines (Meydani et al., 1990, 1997). Furthermore, vitamin E decreased PGE$_2$ and free-radical production by macrophages (Meydani et al., 1986, 1997). These are two changes that are considered part of 'normal' ageing (Lang et al., 1992). Thus, the effect of vitamin E may be important in slowing the effect of ageing. If some ageing phenomena, such as permanent macrophage activation and decreased T-cell responses, can be reversed by high-dose micronutrient supplementation (e.g. with vitamin E), then the recommended intakes for such micronutrients may be too low.

The findings from these studies are of great importance. First, they show that micronutrient deficiencies are deleterious for immune responses. Second, they indicate that recommended micronutrient intakes are probably too low for the elderly, at least for some micronutrients with antioxidant properties. Therefore, they point to a new direction for slowing the ageing process: higher recommendations for intakes of some micronutrients. Since these nutrients influence the immune system, the measurement of T-cell and macrophage functions is probably a sensitive functional marker to quantify the consequences of nutrient deficiencies and the ageing process.

However, before recommending nutritional supplementation for the elderly, it should be noted that there are reports of high doses of some micronutrients (e.g. zinc) having deleterious effects on immune responses. For example, one study showed that immune recovery occurs faster when a supplement does not contain zinc (Bogden et al., 1990), while Chandra (1984) showed a deleterious immunological effect of high zinc supplements (300 mg d$^{-1}$) in young adults. This indicates that the use of such supplements should be approached cautiously and adapted properly to the micronutrient status of the individual concerned.

**Tertiary Immunodeficiency in the Elderly: Role of Protein-Energy Malnutrition**

Protein–energy malnutrition (PEM) exerts a strong influence on immune responses, particularly at the extremes of life (Chandra, 1989, 1992b; Lesourd, 1990a, b, 1995, 2000; see also Chandra, Chapter 3, this volume). PEM affects all types of immune responses in elderly subjects leading to decreased cell-mediated immunity (Chandra, 1989; Lesourd, 1990b, 1995, 2000), decreased
humoral immunity (Chandra et al., 1984) and decreased innate immunity (Rudd and Banerjee, 1989; Lesourd and Mazari, 1997, 1999). PEM accentuates the age-related decline of immune responses so that undernourished elderly subjects have further changes in peripheral blood lymphocyte counts, and further decreases in CD3+ cell numbers, in lymphocyte proliferation and in cytokine release (Lesourd and Meaume, 1994; Lesourd et al., 1994; Lesourd and Mazari, 1999; Table 17.3). PEM and ageing exert cumulative effects on T-cell responses, so that the degree of the immunodeficiency in the elderly patient is linked to the degree of PEM (Chandra, 1989; Lesourd et al., 1992). Besides this cumulative effect, PEM induces other changes in immune responses of the elderly: in aged persons with PEM, the number of CD4+ cells is decreased (Lesourd and Meaume, 1994; Lesourd et al., 1994; Table 17.3) in line with the degree of the nutritional deficit (Lesourd, 1995). PEM is also associated with decreased humoral responses, measured as lower antibody responses to vaccine (Chandra et al., 1984; Lesourd 1995) and by decreased non-specific immunity either of polymorphonuclear (Lipschitz and Udupa, 1986) or macrophage functions (Rudd and Banerjee, 1989; Lesourd and Mazari, 1997; Table 17.2). Undernutrition and ageing exert cumulative effects on non-specific immune responses in animals (Lipschitz and Udupa, 1986) and in humans (Lesourd and Mazari, 1997).

The influence of nutritional status on macrophage functions is of great importance for elderly subjects. Indeed, ageing induces a disequilibrium between macrophage functions, which are preserved in the apparently healthy elderly, while T-cell functions start to decline. If any disease occurs, the disequilibrium leads macrophages to release more cytokines in order to stimulate an efficient T-cell response. This can have detrimental effects on the host (Lesourd, 1996; Lesourd et al., 1996). Macrophage-derived cytokines are responsible for the mobilization of body nutritional reserves in order to provide activated cells with the nutrients they need for their high metabolic activity (Klasing, 1988; Lesourd, 1992). In the elderly, nutritional reserves are lost at a similar rate to that in younger adults, but they are not fully restored after recovery from disease (Lesourd, 1996; Lesourd et al., 1996). This is due to the age-related disequilibrium between muscle catabolism, which is preserved with ageing (Fereday et al., 1997), and muscle anabolism, which declines in aged individuals (Welle et al., 1993; Yarasheski et al., 1993). Therefore, during any disease, the rate of muscle catabolism exceeds that of muscle anabolism (Fereday et al., 1997), so that aged persons recover after the disease with muscle and bone deficits that are never fully rebuilt (Lesourd, 1996; Lesourd et al., 1996). Therefore, any disease process pushes the elderly to a more frail state and this is a common situation in elderly patients who exhibit continuous increases in monocyte/macrophage cytokines (Cederholm et al., 1997).

If such disease occurs in combination with PEM in the elderly, cytokine release from macrophages is lower and then the stimulation of defence mechanisms is less effective (Nafziger et al., 1993). Hypercatabolism lasts longer and is quite permanent (Cederholm et al., 1997) and the decline in body nutritional reserves is more pronounced (Fereday et al., 1997). Therefore, undernutrition is a factor that pushes the elderly to a more frail state (Lesourd, 2000).
Table 17.3. Subsets and functions of peripheral blood T lymphocytes from elderly subjects with different nutritional and health status. Groups were selected, using previously described criteria (Mazari and Lesourd, 1998), as being very healthy, self-sufficient home-living apparently healthy (frail and/or undernourished) or profoundly undernourished old subjects with undernourishment due only to insufficient nutrient intakes (with mild inflammatory process: CRP < 30 mg l⁻¹).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Very healthy 'young elderly' (65–85 years) (n = 41)</th>
<th>Apparently healthy 'young elderly' (70–85 years) (n = 51)</th>
<th>Undernourished, self-sufficient 'young elderly' (70–85 years) (n = 25)</th>
<th>Very undernourished hospitalized 'young elderly' (70–85 years) (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>Mean 79.6 ± 5.3</td>
<td>Mean 78.9 ± 6.2</td>
<td>Mean 78.7 ± 5.9</td>
<td>Mean 79.6 ± 6.4</td>
</tr>
<tr>
<td>Albumin (g l⁻¹)</td>
<td>42.4 ± 4.1</td>
<td>37.2 ± 3.9</td>
<td>29.4 ± 3.2</td>
<td>22.3 ± 2.8</td>
</tr>
<tr>
<td>Lymphocytes (number µl⁻¹)</td>
<td>1993 ± 568</td>
<td>1705 ± 434</td>
<td>1356 ± 287</td>
<td>859 ± 214</td>
</tr>
<tr>
<td>CD2+ cells (number µl⁻¹)</td>
<td>1794 ± 397</td>
<td>1511 ± 427</td>
<td>1264 ± 336</td>
<td>864 ± 217</td>
</tr>
<tr>
<td>CD3+ cells (number µl⁻¹)</td>
<td>1565 ± 311</td>
<td>1207 ± 299</td>
<td>935 ± 221</td>
<td>517 ± 167</td>
</tr>
<tr>
<td>CD2+CD3⁻ cells (number µl⁻¹)</td>
<td>214 ± 224</td>
<td>283 ± 241</td>
<td>335 ± 148</td>
<td>356 ± 203</td>
</tr>
<tr>
<td>CD4+ cells (number µl⁻¹)</td>
<td>1136 ± 243</td>
<td>812 ± 271</td>
<td>461 ± 188</td>
<td>354 ± 271</td>
</tr>
<tr>
<td>CD8+ cells (number µl⁻¹)</td>
<td>437 ± 174</td>
<td>387 ± 196</td>
<td>395 ± 165</td>
<td>256 ± 178</td>
</tr>
<tr>
<td>CD45RA cells (number µl⁻¹)</td>
<td>658 ± 228</td>
<td>464 ± 213</td>
<td>517 ± 245</td>
<td>549 ± 276</td>
</tr>
<tr>
<td>CD45R0 cells (number µl⁻¹)</td>
<td>1222 ± 365</td>
<td>1057 ± 497</td>
<td>831 ± 497</td>
<td>372 ± 349</td>
</tr>
<tr>
<td>IL-2 production¹ (ng l⁻¹)</td>
<td>1.84 ± 0.34</td>
<td>1.11 ± 0.38</td>
<td>0.77 ± 0.28</td>
<td>0.45 ± 0.33</td>
</tr>
<tr>
<td>IL-6 production¹ (ng l⁻¹)</td>
<td>1.82 ± 0.22</td>
<td>1.48 ± 0.40</td>
<td>1.10 ± 0.44</td>
<td>0.77 ± 0.31</td>
</tr>
<tr>
<td>Lymphocyte proliferation⁶ (10^5 cpm 10^6 cells⁻¹)</td>
<td>114 ± 35</td>
<td>54 ± 34</td>
<td>43 ± 29</td>
<td>21 ± 29</td>
</tr>
</tbody>
</table>

CRP, C-reactive protein; IL, interleukin.
Significant differences from very healthy 'young elderly': *P < 0.05, **P < 0.01, ***P < 0.001.
Significant differences from apparently healthy 'young elderly': †P < 0.05, ††P < 0.01, †††P < 0.001.

¹Determined using 5 µg mitogen 10⁶ cells⁻¹.
⁶Determined using 1 µg mitogen 10⁶ cells⁻¹.
Conclusion

There is now strong evidence that ageing exerts less influence on the immune system than environmental factors. In VCSH elderly subjects, ageing induces changes in T-cell and B-cell subsets, but most immune functions are preserved until very old age. Furthermore, it appears that non-specific immunity is even increased, perhaps in relation to a continuous activation of macrophages. Undernutrition, irrespective of the nutrient concerned, induces immunodeficiency even in apparently self-sufficient, non-diseased elderly subjects. Indeed, chronic low intakes of some nutrients, even though they are within the normal range, induce lower cell-mediated immune responses. As chronic low intakes are quite common in the apparently healthy elderly population, it is possible that many immune changes that were previously reported to be age-related are, in fact, due to the influence of undernutrition. Treatment of such nutritional deficits with supplements enhances immune responses in relation to the correction of the deficit. Vitamin E supplements also enhance immune responses, even though the elderly do not exhibit vitamin E deficiency, as defined by current recommendations. Such vitamin E supplementation induces immune changes that partly reverse immune ageing. Thus, it is possible that current recommendations for intakes of some micronutrients are insufficient to prevent or to slow the ageing process.

Nutritional influences on immune responses seem greater in elderly subjects with disease. PEM lowers non-specific and specific immune mechanisms, induces longer hypercatabolic responses and then, in combination with the age-related changes in protein metabolism, pushes the elderly towards a more frail state. Nutrition, through its action on such process, is an efficient way to slow the ageing process, even in diseased aged patients.

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Sanders, M.E., Maggoba, M.W., Sharrow, S.E., Stephany D., Spriger A., Young, H.A. and Shaw, S. (1998) Human memory T lymphocytes express increased level of three cell adhesion molecules (LFA3, CD2, and LFA1) and three other molecules (UCHL-1, CDw29, and Pgp-1) and have enhanced IFN-γ production. *Journal of Immunology* 140, 1401–1407.


Nutrition, Infection and Immunity: Public Health Implications

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Introduction

Infection accounts for the deaths of millions of children and contributes to the deaths of nearly a million mothers each year globally. With new strains of pathogens emerging and antibiotic resistance developing, there is an urgent need to look for new strategies for infection control. There is now solid evidence for a role for nutrition interventions to achieve reduction of child and maternal mortality and morbidity. Nutrition policies need developing and implementing. Nutrition researchers and practitioners can play a major role if they apply their science to the various stages of the policy process used by ministries of health, agriculture and community development. Evidence for a nutritional impact needs to be assembled using biological plausibility, clinical studies, randomized controlled trials, effectiveness studies and a review of what promotes the sustenance of nutrition intervention. The process of establishing an evidence base that is sufficiently robust to stimulate the development of new nutrition interventions that are sustainable even in poor populations is examined. This chapter examines several nutritional deficiency syndromes and focuses on the particular roles of vitamin A, zinc and selenium and examines the issues around iron supplementation in malarious populations.

General Overview

The importance of preventing and treating malnutrition as a strategy to reduce the prevalence, severity and mortality associated with infectious disease is now well recognized. Around 13 million children worldwide die every year, mostly from infectious diseases, including pneumonia, diarrhoea, malaria, measles, meningitis and septicaemia. In a high proportion of these cases, malnutrition is
a major contributing factor; the World Health Organization (WHO) and the United Nations Children’s Fund (UNICEF) estimate that almost 60% of child deaths are malnutrition-associated (Pelletier et al., 1995). As a result, training in the early recognition and treatment of malnutrition has been incorporated into the Integrated Management of Childhood Illness (IMCI) Programme of WHO/UNICEF, which is being implemented globally among less developed countries (World Health Organization, 2001). Over 600,000 women die every year from pregnancy-related causes (Tomkins, 2001a). A recent landmark study showing an almost 50% reduction in maternal mortality in Nepal as a result of vitamin A or \( \beta \)-carotene supplements (West et al., 1999) emphasizes the importance of nutritional strategies to prevent mortality. Nutritional interventions now need to be included more actively within the Safe Motherhood programmes of WHO/UNICEF, which have been introduced in most less developed countries, as this is where nearly all the maternal mortality occurs.

With increasing longevity in many industrialized countries, there is greater interest in the role of micronutrients in preventing infection among the elderly (e.g. see Hughes, Chapter 9, this volume). With increasing sophistication of intensive care, after surgical interventions in particular, there is more awareness of the importance of improving nutrition as a means of improving surgical outcome (e.g. see Duff and Daly, Chapter 5 and Calder and Newsholine, Chapter 6, this volume).

Despite the strong evidence base for nutrition interventions, they are less often included in policies for public health or clinical management by national and international agencies than are vaccines, anti-microbial therapy, anti-retroviral therapy, impregnated mosquito-nets, improved sanitation, clean water and better personal hygiene. There are various reasons for the omission of nutrition interventions from health strategies. First, there has been a tendency to focus on dietary interventions as the key means of improving nutrition – often to the exclusion of other means, such as food fortification or supplements. The result is an assumption by many that improving nutrition can only be achieved by improving household food security and this depends on improving economic status, often a slow process. Second, nutrition has not been ‘marketed’ as aggressively as other interventions, such as antibiotics, which have been strongly promoted by pharmaceutical companies with the skills and resources to ensure that their products are made widely available. Third, until recently, there have been few examples of clear nutritional management regimes; without their inclusion within the curriculum of training programmes for health or community workers, nutritional interventions have not been taken seriously. The challenge is to review and present the benefits of nutritional interventions more effectively. This is even more important now that new microbial pathogens are emerging and anti-microbial resistance is more prevalent. It is extremely unlikely that new effective anti-microbials will be sufficiently available in poor communities, even if they are developed by pharmaceutical companies. Nutritional interventions could come to occupy the key position that antimicrobials occupied until they failed.

There is now strong evidence for cost-effective nutrition interventions to prevent and manage infectious disease in both industrialized and less developed countries. The recent studies have focused on three areas. First, there is
susceptibility to infection – this includes studies on different components of the host immune system, the pathogenicity of individual organisms and the replication rates of the organisms at different stages of disease. In addition there is new information on genetic susceptibility, which makes certain individuals more susceptible to micronutrient deficiency during infection (Delanghe et al., 1998). Second, there is severity of disease – this includes data on the degree of tissue invasion, clinical indicators of disease severity and speed of recovery, which may relate to a combination of the speed of elimination of the infection causing the organism and/or the immune response by the host to the organism. Important studies show how nutritional interventions also reduce mortality. Third, there is disease prevention – there is new information on the ways in which interventions influence the epidemiology of infection, indicating that improved nutrition may have just as much impact as traditional public health measures, if not more.

Despite the strong evidence for a preventive or therapeutic role for nutrition interventions, there are key operational issues that have delayed the introduction and maintenance of nutritional interventions. If a reduction in the prevalence of infection is to be achieved through the introduction of nutritional interventions, several hurdles need to be overcome more efficiently.

First, which government agency should be responsible for championing nutrition as an infection control strategy? Intersectoral collaboration is difficult to achieve, but this is improving. Officials in ministries of health often see nutrition as being the responsibility of the ministry of agriculture. They in turn see their role as increasing national household food security, but hope that nutrition will increase as a result of improving economic purchasing power and see nutrition as the responsibility of the ministry of economic affairs or planning. There have been strong pressures by proponents for the 'food-based approaches are good; supplement-based approaches are bad' philosophy. The artificial polarization towards either/or for the choice of which nutrition interventions to use has created confusion among health and agriculture professionals alike. The lack of clarity among nutrition experts who have taken 'polar positions' has not been helped by Western donors, who failed to include nutrition as an intervention within the health sector. Many donors, often in concert with the large international banks, argued that improved nutrition would only result from economic improvement, which was supposed to accompany structural adjustment, fiscal reforms and free-market economies (World Bank, 2001). There are now welcome recent signs of change. For example, the latest policy of the UK's Department for International Development places a key importance on specific, focused nutrition interventions, especially with micronutrients, as a strategy for improving child health, survival and development (Department for International Development, 2001). There have been considerable efforts to clarify the nature of nutritional interventions during such international activities as the country case-studies following the United Nations World Summit for Children in 1990 (UNICEF, 2001), the Micronutrient Conference on Ending Hidden Hunger in 1991 and the International Conference on Nutrition in 1992 (World Health Organization, 2001). The results of improved collaboration between partner organizations over the last 10 years have been encouraging.
There are now many more opportunities for collaboration between different ministries, each recognizing and respecting the crucial role of the other’s activities. Programmes such as the Prevention of Low Birth Weight of UNICEF now include ministries of health, agriculture and community development.

Second, how do nutritional interventions fit within the ‘life-cycle’ approach that is increasingly espoused by international agencies (Tomkins, 2001b)? There is now greater emphasis on public health and societal interventions from ‘conception to the grave’. The new data on the benefits of nutrition interventions at critical phases of the life cycle, such as pregnancy, the early neonatal period and among infants, school-age children and adolescents, make it possible to identify specific activities that have a short- and long-term impact on infection. For instance, strategies that improve the height and nutritional status of primiparous women are likely to lead to improved pregnancy outcome and birth weight, resulting in improved immune status of the fetus, now recognized to be so important for immunological programming (Moore et al., 1999). The benefits of improved birth weight for subsequent immunity have been recognized for years, but other aspects of fetal malnutrition are now increasingly recognized as contributing towards immunity in childhood and possibly even adult life (Barker, 1997). The life-cycle approach provides an opportunity for nutritional interventions to be planned that will have an immediate impact, for example, on the developing fetus in present pregnancies (e.g. through malarial prophylaxis, deworming, infection control and multiple micronutrient supplements) and pregnancies in future generations (e.g. through interventions that will enhance the weight, height and micronutrient status of girls/women at their first pregnancy).

Third, how widespread does malnutrition have to be across the community before public health interventions should be implemented for all the population? Ideally, policy-makers need to know what the benefits are before they can divert scarce resources towards nutrition interventions. Calculations of the cost of nutrition interventions have been most persuasively made for vitamin A supplementation, results of which have been used to estimate the cost of saving lives or preventing serious infections (Murray and Lopez, 1994). However, there is increasing interest in other micronutrients, such as zinc, iron and selenium (see also Prasad, Chapter 10, Kuvibidila and Baliga, Chapter 11, and McKenzie et al., Chapter 12, this volume). Precise cost–benefit calculations cannot yet be made because the precise impact of interventions with these and other nutrients has not yet been fully evaluated. In the meantime, it is valuable to review the types of information that governments need before they implement nutrition interventions.

Establishing biological plausibility is vital. A case can be made by compiling data from apparently disparate studies and synthesizing the responses in different parts of the immune system during experimental malnutrition in human volunteers or animals. The strength of such data may be enhanced by an analysis of the impact of nutritional interventions on immune function or susceptibility to infection. One of the difficulties facing investigators seeking to identify the significance, in terms of susceptibility to infection, is the widespread finding that an acute or chronic infection may decrease serum levels of
micronutrients. This leads to an incorrect assumption of a causal relationship where a low serum level of a particular nutrient predisposes towards increased risk of becoming infected or developing a more rapid progression of disease. This is a particular problem in human studies, where infection often coexists with nutritional deficiencies. Similarly, individuals with low serum levels of micronutrients may well be deficient because of poverty; the poor physical and social environment that such people live in may be a key factor explaining their susceptibility to infection. Thus, however strong the evidence is for biological plausibility, a case for intervention can only really be made if a randomized controlled clinical trial can be established. These are invaluable and have been pivotal in identifying the impact of vitamin A supplementation, for example (see also Semba, Chapter 8, this volume). However, such a trial is not always easy to establish. There may be technical issues as to what the ‘placebo’ should contain and doubts over whether it is possible to prepare a nutritional intervention that is really a ‘placebo’. Sometimes there are additional logistical and political issues, where it is felt that a ‘placebo’ is not acceptable. In such circumstances, where it is desirable, but impossible, to perform a randomized controlled trial, a careful analysis of changes as a result of interventions introduced in a phased manner may be performed. The impact of phased dietary supplementation on birth weight and perinatal mortality was carefully assessed in communities in the Gambia (Ceesay et al., 1997), where provision of additional food for some but not all communities would have been unacceptable. However strong the design of the epidemiological study and however rigorous the peer review process, it needs more than one or more published papers to promote the development and implementation of a new policy.

International working groups and agencies play a vital role by publicizing and synthesizing data from all studies available. The use of meta-analysis allows a stronger conclusion than is possible from individual studies. From these, expert working groups can produce generic policies that are applicable for countries with a reasonably defined level of deficiency, using community-based clinical and biochemical surveys, even if no specific research on the nutrition intervention in question has been performed in the country concerned. Unlike many health interventions, such as vaccinations, where it is assumed that most citizens are at risk, nutritional deficiencies may not be nationwide. Governments need to decide what proportion of the population is deficient and whether there are enough citizens at risk to merit regional or national interventions.

Even if some form of national nutrition survey is performed, information on nutritional status alone is not enough to make a case for a nutrition intervention, especially when there are competing demands for resources. This requires a careful analysis of what benefits a nutritional intervention will bring over and above or even instead of other public health interventions. While almost all nations are signatories to the Convention on the Rights of the Child, which contains a strong commitment to the provision of an adequate diet, many less developed countries have to make painful decisions about what can be afforded and what cannot. This needs a critical review of how nutrition fits into national or regional health policies – information on costs, resources, train-
ing and community acceptability is necessary. Arguing an evidence-based case for nutrition interventions may seem perfectly logical, but there are competing demands for resources and sometimes considerable difficulties in establishing change, however logical that may appear.

In many less developed countries, the provision of resources for the introduction or maintenance of primary health care/public health programmes is often ‘kick-started’ by supplies and training provided by bilateral or multilateral donors. Indeed, such activities may sometimes antedate the development of a national policy. Once the donor supply ceases, a government needs to decide whether to continue with the intervention or not. This can create problems if sufficient resources have not been allocated from government funds in the first place. There are many reasons for the continuation or discontinuation of nutrition programmes; they are often influenced by central and local politics. As many governments now tend to decentralize their planning and delivery of health services, allowing regional authorities to decide on priorities for expenditure, it is quite possible for regions to have different priorities for nutrition interventions. It is difficult for senior health staff to make judgements on these comparative issues. They have a difficult task in deciding what to focus on. The availability of well-argued policy documents in which the benefits of nutrition interventions are clearly displayed and ‘marketed’ can be very influential.

Finally, no strategy for improving nutritional status will succeed unless there is a strong sense of conviction among the communities that the intervention proposed is worth the additional effort. Case studies are needed to document whether there has been sufficient ‘social marketing’ of nutritional interventions. This is particularly important where changes in food-production practices are being promoted or where supplements need to be bought, however cheaply. Research-funding agencies tend not to be interested in such ‘applied’ knowledge. Yet all the biochemical arguments in the world may be of less importance than what citizens think about the significance of a nutritional deficiency and what is suggested that they and their government might do about it.

How then can governments or international agencies make evidence-based reviews of the importance of nutritional interventions as an infection-control strategy in their own situation? Despite the value of meta-analyses and expert working groups, it is not always possible to extrapolate from one environment to another. For instance, the very favourable effects of micronutrient supplementation in Nepal need to be examined among women in sub-Saharan Africa, where human immunodeficiency virus (HIV) and malaria are endemic. There may need to be different infant-feeding policies for different communities. For instance, similar rates of post-natal transmission of HIV from mothers to children in South Africa are found among exclusively breast-feeding women and women who use infant formulas (Coutsoudis et al., 1999). These results are particularly important in areas where feeding infant formulas is associated with higher mortality (UNICEF, 1998a). Thus, infant-feeding policies that have been developed for HIV-positive mothers in Europe and North America (where breast-feeding is always discouraged) are not appropriate for women living in poor environments.

There are ethical issues facing researchers and policy-makers as they con-
duct studies and analyse published data in seeking to develop their own country-specific nutrition policies. The design of such interventions needs to take account of current changes in ethical climates and considerations, such as in the Helsinki declaration. However, many of the principles developed by modern ethics committees relate to the problems of performing clinical trials among individuals with a disease who are allocated to a standard compared with a novel treatment. There is a deficiency of ethical guidelines suitable for research in public health nutrition.

The development of the evidence base for the design and implementation of public health nutrition interventions is a complex process. It needs to take account of biological plausibility, the cost-effectiveness of the impact in comparison with other interventions and an analysis of the social factors affecting the sustenance of a programme. This chapter seeks to provide an analytical approach to enable policy-makers to reach informed conclusions. It uses the above framework to examine particular nutritional deficiencies.

The difference between the effects of a large dose of a micronutrient, the regular consumption of a better diet or smaller doses of supplements needs clarification. Some micronutrients appear to have a particularly profound effect on immunity – vitamin A, selenium and zinc (see Semba, Chapter 8, Prasad, Chapter 10, and McKenzie et al., Chapter 12, this volume). Their impact is so striking that pharmaconutrient immunological responses may well be the cause for a change in immunity rather than repletion of a deficiency state. Furthermore, there are differences in the effect of micronutrients on different diseases. For instance, vitamin A benefits pneumonia associated with measles (Hussey and Klein, 1990), but not other types of pneumonia (Fawzi et al., 2000). Zinc supplements seem effective against the prevalence and severity of both diarrhoea and respiratory infection (Bhutta et al., 1999a; see also Prasad, Chapter 10, this volume). This information is sufficient to generate global programmes of vitamin A supplementation and some interesting possibilities for supplementation programmes for zinc. Selenium has an increasingly recognized anti-infective property, but data on its role in public health are lacking. Now that the immunological benefits of individual micronutrients are recognized, it seems intuitively sensible to combine these into a multiple micronutrient preparation. Interestingly, there has been almost no regulatory control on multiple micronutrients. Any company can produce or market micronutrients and their advertising claims have rarely been subjected to any form of evaluation. A rare but notable exception has been the recommendations to avoid toxic levels of vitamin A during pregnancy. Despite the widespread lack of published evaluation of their efficacy, many people in industrialized countries consume multiple micronutrients on a daily basis. The finding of deficient serum levels of antioxidants in apparently well-nourished subjects who smoke has increased concerns in smokers, who are increasingly taking antioxidants to ‘protect’ themselves against the toxic effects of tobacco smoking. There are widespread public concerns that ‘modern’ diets are not adequate nutritionally and the shelves of pharmacies in many industrialized countries are stocked with single and multimicronutrient supplements. Many pregnant and lactating women take them. Parents frequently give them to their children. However, there are almost no data on their efficacy.
The interaction of individual micronutrients on the absorption and metabolism of other micronutrients is well recognized. For that reason, most preparations contain up to two times the recommended intake. It is at present uncertain as to whether there is an additive or interactive effect of micronutrients that individually have been shown to have profound immunological effects. Will a multiple micronutrient capsule containing vitamin A, zinc and selenium produce the same immunological and anti-infective benefit as when these have been given individually?

New analytical approaches are necessary if the potential benefits of micronutrient interventions in particular are to be achieved. With the escalating costs of the development of new antibiotics and anti-retrovirals, giving very high patient costs for treatment of an infection, the relative cheapness of nutritional interventions is becoming increasingly attractive, especially as they are not associated with the development of disease-resistant strains and are rarely associated with patient toxicity.

Malnutrition and Infection

There are clear descriptions of the criteria for classifying individuals as underweight, thin or short (World Health Organization, 2001). However, such individuals may also be micronutrient-deficient independently of their anthropometric status.

Many studies have identified the impaired immunity and high infection rates among children with severe malnutrition (Waterlow et al., 1992). Undoubtedly, the high rates of mortality among such children were attributable to the high rates of infections, such as septicaemia, pneumonia, diarrhoea and meningitis. It is often difficult to know which came first – the infection or the malnutrition – in the severely malnourished, heavily infected child. However, comparison of the low rates of mortality during nutrition rehabilitation of children in the Caribbean, where malaria, measles and tuberculosis were uncommon (Waterlow et al., 1992), and the high rates of mortality among malnourished children in sub-Saharan Africa (Kessler et al., 2000), even when similar nutrition rehabilitation rates were used (Prudhon et al., 1997), indicates the importance of the additional burden of infection on the mortality. Rates as low as 1 or 2% mortality or less are commonly achieved in the management of severe malnutrition, such as children with kwashiorkor or marasmus, in Jamaica. Rates of mortality among severely malnourished children in Africa may be as high as 50%. Intermediate results are obtained in Asia (Khanum et al., 1998). Ashworth and Khanum (1997) identified key nutritional and infection-control interventions, demonstrating that it is possible to bring down mortality to around 10% or even less, even in difficult circumstances in Africa, showing the importance of improving nutritional and infection management together (Schofield and Ashworth, 1996).

The interactions between immunity and the presence of severe metabolic disturbances associated with systemic and intestinal infection have been reviewed (Waterlow et al., 1992; Tomkins, 2000). Despite the importance of severe malnu-
Nutrition as a challenge for health care professionals and community workers alike, it should be recognized that, for every one severely malnourished child, there are at least another 20 with moderate malnutrition. It is estimated that around 150 million children are underweight, with particularly large numbers in South Asia (over 85 million) and sub-Saharan Africa (over 30 million) (UNICEF, 2001). Using prospective observational studies, in which measurements of weight and height are accompanied by environmental and socio-economic assessments, it has been possible to assess the significance of mild to moderate malnutrition on subsequent risk of infection or mortality. These studies have controlled for the differences in physical, socio-economic and caring environments that malnourished children often live in. A study of almost 5000 preschool children in Uganda showed that there was an increasing mortality risk according to decreasing nutritional status, allowing for socio-economic factors that also increased the risk of mortality (Vella et al., 1994). A study of malnutrition and risk for infection, as assessed by regular home visits, showed an increase in the prevalence of several types of infection; the greatest impact, however, was on the duration of illness (Pickering et al., 1987). The use of the mid upper arm circumference as an indicator of malnutrition has been promoted over the years (UNICEF, 2001) and data from Uganda demonstrate the mortality risk for individual infections, such as respiratory and diarrhoeal disease, against mid upper arm circumference (Vella et al., 1992, 1993). Moreover, the data demonstrate that different cut-off levels may be selected for identifying individuals at high risk of mortality. The precise cut-off chosen is a trade-off between a high sensitivity or prediction (resulting in a high proportion of children needing to be seen by a health/nutrition professional) with a lower sensitivity but a smaller number of individuals being selected as being below the cut-off for referral. The importance that nutrition within infection-control strategies has been given in recent years is best seen in the programme for IMCI by WHO/UNICEF. In this programme, careful guidelines are provided for basically trained health workers for the recognition and management of malnutrition (WHO/UNICEF, 1997). There are specific opportunities for the introduction of micronutrient interventions, such as vitamin A, within the under-5 child health programme, seeing the visit to a clinic by a sick child as an opportunity for ensuring that prophylactic micronutrient administration is up to date.

The overall conclusion is that severely malnourished subjects are at great risk of infection, such that infective diseases are probably a more common cause of death than metabolic failure. Many studies have demonstrated that micronutrient deficiency is common among severely malnourished children, but there is very little published evidence of the benefit of supplementation in terms of infection outcome. Interestingly, despite the widespread notion that vitamin A should be given to all children with severe malnutrition, there have not been randomized controlled trials investigating the benefits in terms of mortality, though the benefits in terms of the prevention of xerophthalmia are well established (Sommer, 1993). Similarly although zinc is now known to prevent infection and reduce the severity of infection, there have been few randomized clinical trials investigating the precise impact of zinc in severe malnutrition (see also Prasad, Chapter 10, this volume).
**Vitamin A and Infection**

**Vitamin A and childhood morbidity and mortality**

Early studies showed a high risk of mortality among children with clinical and biochemical evidence of vitamin A deficiency (Humphrey et al., 1992). Data on the effects of vitamin A on immunity are now available through preventive and therapeutic studies (see also Semba, Chapter 8, this volume). Several randomized controlled clinical trials showed that regular administration of vitamin A (in capsules) reduces mortality by over 20% among infants and young children in vitamin-A-deficient areas (Sommer, 1997). There was also a reduction in morbidity, though not necessarily mortality, in the neonatal and early infant periods if vitamin A was given in the post-natal period (West et al., 1995; Humphrey et al., 1996). In Ghana, regular supplementation with vitamin A reduced by around 15% the prevalence of episodes of infection that were severe enough to require a clinic attendance (Ghana VAST, 1993). This was particularly noticeable for children presenting with severe, dehydrating episodes of diarrhoea. Regular supplementation with vitamin A also has an impact on malaria, according to a recent study in Papua New Guinea (Shankar et al., 1999). There was a 30% decrease in the number of febrile episodes with a high parasite count. Parasite density was also reduced. In the Ghana study of vitamin A supplementation and child survival, there was no evidence of a decrease in morbidity or mortality from malaria, as assessed by reports of febrile episodes, but there was a 20–30% reduction in the prevalence of malaria slide-positive episodes in the vitamin-A-supplemented group (Ghana VAST, 1993). The sample size was not large enough to know whether there was any impact on malaria-related mortality in the Ghana or Papua New Guinea studies.

The impact of vitamin A as a treatment has been evaluated, with a special focus on the clinical outcome of diarrhoea and pneumonia in children (Semba, 1999a). There has been a variable response to the use of high-dose vitamin A in the treatment of diarrhoea. In Dhaka, Bangladesh, a randomized controlled trial with factorial design compared the use of vitamin A and/or zinc with placebo in children with diarrhoea of > 3 days’ duration (Faruque et al., 1999). A dose of 4500 IU of vitamin A daily for 15 days had no effect on the mean duration of diarrhoea. However, there was a trend towards a reduction in the risk of prolonged diarrhoea (> 7 days in this group). A study from New Delhi, India, analysed the effect of 60 mg vitamin A on the mean duration of diarrhoea and stool frequency (Bhan and Bhandari, 1998). There were no differences between intervention and control groups as a whole, although there was a significant lower risk of persistent diarrhoea in the vitamin A-treated group. However, in a subgroup analysis of children who were not breast-fed, there was a significant reduction in all the main outcome measures of the study among the children receiving vitamin A. This included a 16% reduction in the length of the average diarrhoeal episode, 27% reduction in the mean stool frequency and 60% reduction in the proportion of children who passed watery stools. These benefits were not seen in breast-fed children. Another study from New Delhi evaluated the role of vitamin A supplementation (100,000 or...
200,000 IU, according to age) on the clinical outcome of acute diarrhoea of < 3 days’ duration (Dewan et al., 1995). There was no effect of vitamin A on the group as a whole, but in those children who had pre-existing vitamin A deficiency, as defined by abnormal conjunctival impression cytology, there was a significant reduction in the duration of diarrhoea. A further study from Bangladesh (Henning et al., 1992) found no effect of vitamin A on the duration of acute watery diarrhoea and no reduction in the subsequent number of episodes with persistent diarrhoea. A study of children with acute shigellosis in Bangladesh given 200,000 IU as a single dose showed an increased proportion of children who were clinically cured at 5 days, even though there was no difference in the bacteriological cure rate (Faruque et al., 1999). A study in South Africa comparing early and late administration of a large dose of vitamin A showed no impact on clinical outcome or the extent of intestinal damage as assessed by lactulose and mannitol excretion in the urine after an oral dose; vitamin A levels assessed by relative dose response and plasma retinol levels were the same 8 weeks later (Rollins et al., 2000). Overall, it seems that, with the exception of dysentery, vitamin A does not have a therapeutic role in acute diarrhoea, although there are insufficient data to enable conclusions on its role in persistent diarrhoea.

Stephensen et al. (1998) gave vitamin A to children with pneumonia in Peru. Those receiving vitamin A had a longer duration of clinical signs and a greater need for supplemental oxygen. These adverse effects were not so severe as to require longer hospitalization. Among children hospitalized with pneumonia in Mozambique, there was no benefit overall in giving vitamin A, but there was a reduction in the number of children still requiring hospitalization after day 5 from those given vitamin A if they were < 1 year of age (Julien et al., 1999). Overall, therefore, it seems as though vitamin A treatment in non-measles-associated pneumonia is not beneficial and may even have some side-effects.

Vitamin A treatment has been evaluated in measles-associated pneumonia. The evidence for a role of vitamin A in the treatment of measles is strong. A study from Tanzania showed a mortality of 7% in the vitamin A-supplemented group, compared with 13% in the placebo group (Barclay et al., 1987). The largest decrease in mortality was seen in the under-2-year-olds and in those children who had measles-related complications, especially croup. A similar study in South Africa showed significant reductions in mean duration of stay, rates of admission to the intensive care unit and mortality in children > 15 months old (Klein and Hussey, 1990). A further study, also in South Africa, of measles-associated pneumonia showed a significant improvement in recovery and in morbidity assessed by a local integrated score system (Hanekom et al., 1997). In a study of Zambian children with measles-associated pneumonia who were insufficiently ill to require hospital admission, there was no benefit from prescribing vitamin A during the acute stage of the illness (Gernaat et al., 1998). However, when analysed at 4 weeks, there was a significant reduction in cough and pneumonia in the vitamin A group. Overall, there seems to be a very strong effect of vitamin A in measles-associated pneumonia. The reasons for the difference between the effect in measles-associated pneumonia and
non-measles-associated pneumonia have not been clarified, but the profound immune suppression in measles may be a factor that is amenable to response to vitamin A supplementation.

Vitamin A and HIV-associated infection in children

A study in Tanzania showed that vitamin A supplementation reduced acquired immune deficiency syndrome (AIDS)-specific mortality in Tanzanian children (Fawzi et al., 1999). A South Africa study has similarly shown a reduction in morbidity among HIV-positive children with pneumonia and given vitamin A (Bobat et al., 1999). A recent study showed that antenatal vitamin A has an effect on the intestinal tract of HIV-positive, but not HIV-negative, infants in the first few weeks of life (Filteau et al., 2001). These studies suggest that vitamin A has a selective response, exerting a strong preventive activity against infection and mortality in childhood if the infections are associated with measles or HIV. This compares with the somewhat less striking effect in HIV-negative children reviewed above.

Vitamin A and clinical outcome in adults

In view of the well-recognized effect of vitamin A deficiency on epithelial surfaces, it is interesting that shedding of HIV from the reproductive tract is greater among women with low serum retinols in Kenya (Mostad et al., 1997). There is also an association between low levels of vitamin A and high rates of transmission of HIV from mothers to their infants in Malawi (Semba et al., 1994). It is difficult to know whether these relationships between low serum retinol and pathological change are causal, as low levels of plasma retinol are often indicative of an acute-phase response. Thus, the women with the highest viral load and clinical manifestations of HIV-related disease, and therefore more likely to shed or transmit the virus, are also likely to have low serum retinols. It was hoped that intervention with vitamin A in pregnant HIV-positive women would reduce the prevalence of mother-to-child transmission of the virus (Nduati et al., 1995). Unfortunately, preliminary results of such studies have failed to show such a beneficial effect of vitamin A supplementation among pregnant women in South Africa (Coutsoudis et al., 1997). Results of studies in other countries are awaited.

Vitamin A and maternal morbidity and mortality

A recent review outlines some aspects of the interaction between vitamin A and causes of maternal mortality, emphasizing biological plausibility (Faisel and Pittrof, 2000). Women who had low serum vitamin A levels during the second trimester of pregnancy and throughout the post-partum period had an increased risk of puerperal infection (West et al., 1999). This study also showed
that pregnant women with night blindness were twice as likely to develop geni- 
tourinary infections as women without night blindness (Christian et al., 2000).
However, the impact of systemic infection and metabolic stress on vitamin A is 
well documented and it is almost impossible to know which came first – the 
metabolic/infective stress or the low serum vitamin A status. The Nepal study 
also showed a remarkable reduction in maternal mortality as a result of supple-
mentation with β-carotene or vitamin A (West et al., 1999): 44,646 married 
women, of whom 20,119 became pregnant 22,189 times, were included in the 
study. The women were randomized to receive weekly a single oral supplement 
of placebo, vitamin A (7000 μg retinol equivalents) or β-carotene (42 mg or 
7000 retinol equivalents) for over 3.5 years. All causes of mortality in women 
during pregnancy and up to 12 weeks post-partum (pregnancy-related mortal-
ity) and mortality during pregnancy to 6 weeks post-partum, excluding deaths 
apparently related to injury (maternal mortality), were used as the main out-
come measures. Mortality related to pregnancy in the placebo, vitamin A and 
β-carotene groups was 704, 426 and 361 deaths per 100,000 pregnancies, 
respectively. Combined, vitamin A or β-carotene lowered mortality by 44% 
and reduced the maternal mortality-rate ratio from 645 to 385 deaths per 
100,000 live births. Important causes of maternal mortality, in order of fre-
quency, were obstetric-related causes, eclampsia, haemorrhage, sepsis and 
injury. There was not sufficient statistical power to ascribe the mortality impact 
of vitamin A or β-carotene to their effect on one cause of mortality rather than 
another. However, an analysis of the impact of the supplements on symptoms 
of illness was performed (Christian et al., 1998). There was no impact of either 
supplement on morbidity rates reported up to 28 week of gestation. However, 
in late pregnancy (> 28 weeks), symptoms of nausea, faintness and night 
blindness were reduced with vitamin A, but not β-carotene, supplementation. 
Vitamin A supplementation shortened the length of labour by 1.5 h and 50 min 
among nulliparous and multiparous women, respectively. Both interventions 
reduced the post-partum prevalence of diarrhoea (at least four loose stools) 
and night blindness. β-Carotene supplementation also reduced symptoms of 
high fever post-partum. The mean number of days of any reported illness 
symptoms was 3–4 per week throughout pregnancy. Among women receiving 
vitamin A, the total number of days of illness symptoms accrued over the last 
12 weeks of pregnancy was lower by 5 days compared with the placebo recipi-
ents. There are very few publications on rates of morbidity among pregnant 
women and the above study is the only one published to date describing the 
impact of a micronutrient supplementation on morbidity.

A study of 1075 HIV-positive women in Dar es Salaam, Tanzania, exam-
ined the impact of vitamin A, with or without micronutrients (20 mg vitamin 
B1, 20 mg vitamin B2, 25 mg vitamin B6, 100 mg niacin, 50 mg vitamin B12, 
500 mg vitamin C, 30 mg vitamin E and 0.8 mg folic acid), on pregnancy out-
come (Fawzi et al., 1998). Women were recruited at between 12 and 27 weeks’ 
gestation and received their regular supply of ante-natal iron and folic acid. 
They were randomized to receive placebo, vitamin A, a mixture of multivita-
mins without vitamin A or a mixture of multivitamins, including vitamin A. 
There were 30 fetal deaths in the women receiving multivitamins compared
with 49 among those not receiving multivitamins. The prevalence of low birth weight (< 2500 g) was 15.8% in those receiving placebo and 8.8% in those receiving the multivitamins. The prevalence of severe prematurity (< 37 weeks) was 10.2% in those receiving placebo and 6.2% in those receiving multiple micronutrients. Interestingly, vitamin A did not affect any of these variables, and multivitamins, but not vitamin A, resulted in an increase in the number of CD4, CD8 and CD3 cells in the bloodstream. This is the only published study on the effect of multivitamins on such outcomes to date.

A review of the impact of vitamin A supplements as ‘anti-infective’ interventions (Semba, 1999b) describes the earlier work of supplementation with cod liver oil as a protection against puerperal sepsis in the UK. Using various criteria for puerperal sepsis, there was a lower incidence among women receiving cod liver oil than among those who did not. In view of the recent interest in the immunological effect of vitamin D (Wilkinson et al., 2000), the high concentration of vitamin D in cod liver oil may also have been important; it would be interesting to explore this in future studies.

An extensive review of nutritional interventions for the prevention of maternal morbidity concluded that there is an urgent need for trials such as the above with sufficient statistical power in order to determine what micronutrients could usefully achieve in terms of improved immune function and morbidity (Kulier et al., 1998). The benefits of regular doses of vitamin A should not overshadow the many opportunities there are for increasing intake of dietary vitamin A and integrating supplementation with improved dietary intake (Filteau and Tomkins, 1999). However, the problems in achieving satisfactory improvement in vitamin A status using fruits and vegetables have been highlighted recently (de Pee et al., 1999). The development of the new data on the effects of vitamin A and its incorporation into policies and programmes has been a very good ‘model’ for evidence-based public health nutrition policy.

**Selenium and Infection**

There is increasing interest in the role of selenium deficiency in infection (see also McKenzie et al., Chapter 12, this volume). Initially, the interest came because of the recognized value of selenium as an antioxidant. More recently, there seems to be increasing evidence for a role in cellular immunity (see McKenzie et al., Chapter 12, this volume). The presence of selenium is important within the enzyme glutathione peroxidase, which catalyses the reduction of peroxides as part of an antioxidant response to infection.

A dietary supplementation study in young volunteers using 200 µg day⁻¹ of sodium selenite for 8 weeks showed a 118% increase in cytotoxic lymphocyte-mediated tumour toxicity and an 82.3% increase in natural killer cell activity, as compared with baseline values (Kiremidjian-Schumacher et al., 1994). There was also a significant augmentation of the ability of peripheral blood lymphocytes to respond to stimulation with mitogen and to express the high-affinity interleukin (IL)-2 receptor on their surface (Roy et al., 1994). These changes occurred despite the failure of the supplementation regime to increase plasma...
selenium levels. A study of elderly Italian subjects showed an association between the percentage of natural killer cells in the circulation and serum selenium concentrations (Ravaglia et al., 2000). Overall, there seems to be a good case for including selenium within supplements for improving immunity.

Keshan disease, an epidemic form of myocarditis occurring in certain parts of China, is particularly prevalent in areas with a low selenium content of soil and food and is associated with low plasma selenium levels (Ge and Yang, 1993). Sodium selenite, given as an oral dose once a week, reduced the prevalence and mortality from this cardiac disease, which seemed extremely likely to be precipitated by a virus in view of its epidemiology (Blot et al., 1993). The pathophysiology of Coxsackie virus and selenium deficiency has been investigated in great detail by an elegant series of experiments, which demonstrated that certain strains of this virus induced more severe pathological damage in the myocardium of selenium-deficient animals (Beck and Levander, 1998). There was an interaction with vitamin E deficiency, which increased the severity of the disease (Beck et al., 1994; Beck, 1999). Of great interest was the demonstration that passage of the virus through certain strains of selenium-deficient animals resulted in change in the viral RNA genome (Beck, 1997). This demonstration that host malnutrition might actually change the structure of a viral genome has major public health nutrition implications for infectious disease.

The interaction between selenium and viral infection has been explored in other infections in China. It was noted that hepatitis B virus was more common in populations with selenium deficiency (Yu et al., 1989); regular selenium supplementation resulted in a decrease in its carriage rate. Interestingly, subjects with HIV have decreased plasma selenium levels (Cirelli et al., 1991); these are associated with increased indicators of lipid peroxidation, such as plasma lipid peroxides and breath pentane and ethane output (Aghdassi and Allard, 2000). However, there are also low plasma levels of other antioxidants, including vitamin E, β-carotene and zinc, in HIV-infected subjects. There is an association between selenium concentrations and severity of HIV, as measured by erythrocyte sedimentation rate and haemoglobin concentration (Cirelli et al., 1991), indicating that low plasma selenium levels may be part of the impact of an inflammatory response on plasma micronutrient levels. Low serum selenium was a good predictor of clinical deterioration. Among children with HIV, a low serum selenium had a particularly strong predictive power for risk of mortality (Baum et al., 1997). Daily supplements of 100 μg selenium alone failed to show an impact on CD4 count or incidence of opportunistic infections in the supplemented group, even though the serum selenium was maintained, whereas the placebo group experienced a decrease in serum selenium (Constans et al., 1995, 1999). When comparing micronutrient levels and risk of mortality among HIV-infected drug users, serum selenium was more predictive than vitamin A (Baum and Shor-Posner, 1997). The only other published result of selenium supplementation describes a combination of selenium (500 μg day⁻¹) and N-acetylcysteine in HIV-positive subjects. There was an effect on the CD4–CD8 ratio but no impact on viral load; no clinical impact was reported (Look et al., 1998). Overall, there is strong biological plausibility for a
role of selenium in immunity but, so far, there is insufficient evidence that selenium supplements influence the severity or outcome of infection.

**Zinc and Infection**

Zinc has many effects on immunity and the host response to infection (see Prasad, Chapter 10, this volume). It plays a pivotal role in many hundreds of enzymes, stabilizes cell membranes, modulates humoral and cell-mediated immunity and is increasingly recognized to be responsible for the control of apoptosis and oxidative capacity.

Studies of experimental zinc deficiency in humans showed that the functions of T-helper (Th) 1 cells, as evidenced by production of interferon-γ, IL-2 and tumour necrosis factor alpha, were decreased (Beck *et al.*, 1997). Functions of Th2 cells (as evidenced by production of IL-4, IL-6 and IL-10) were unaffected by zinc deficiency. This imbalance between Th1 and Th2 cells and the decreased percentage of cytotoxic T-cells may account for the decreased cell-mediated immune functions in zinc-deficient subjects. Antibody titres measured after elderly subjects had been vaccinated with influenza vaccine were higher in apparently healthy subjects who received a mixture of zinc and selenium (Turk *et al.*, 1998). Zinc supplementation has been studied in patients with sickle-cell disease, who are often zinc-deficient due to increased requirements resulting from haemolysis: zinc supplements resulted in an increase in B lymphocyte and granulocyte zinc levels and in IL-2 production (Prasad *et al.*, 1999). This was accompanied by a decreased incidence of documented bacteriologically positive infections and a decreased number of hospitalizations. The authors concluded that zinc deficiency results in decreased production of IL-2, as a result of decreased activation of nuclear factor kappa B and subsequent decreased expression of IL-2 and IL-2 receptor genes (Prasad, 2000). In a placebo-controlled study, preschool children in India were supplemented with zinc gluconate, providing 10 mg of elemental zinc daily unless they had diarrhoea, in which case they were given 20 mg. Zinc supplementation resulted in a decrease in the percentage of children who were anergic or hypoergic (using the induration score after a Mantoux test) (Sazawal *et al.*, 1997). The zinc-supplemented group had a significantly higher increase in the numbers of CD3 and CD4 cells and in the CD4-to-CD8 ratio, but there was no difference in the numbers of CD8 or CD20 cells. These data show clearly that zinc supplements have wide-ranging effects on the immune system, even in apparently well-nourished subjects. This emphasizes the fact that zinc deficiency may occur independently of malnutrition assessed by anthropometric indices.

The effects of zinc deficiency on the infection, growth and survival of children with severe malnutrition are well described (Golden and Golden, 1979). Zinc supplements are now recommended for the routine management of children with severe malnutrition. Zinc supplementation improves tissue function and integrity. Experimental studies of zinc deficiency show a marked effect on intestinal morphology and function, producing villous atrophy and a heightened secretory
response to diarrhoeal pathogens, such as the toxin from *Vibrio cholerae* (Tomkins, 2000). Studies of the effect of zinc supplementation among malnourished children in Bangladesh showed a reduction in loss of intestinal fluid among children with acute diarrhoea and a shortening of the duration of diarrhoea (Roy et al., 1997). Similarly, among malnourished Bangladeshi children with persistent diarrhoea syndrome, there was a beneficial effect of zinc supplementation on the duration and severity of diarrhoea (Roy et al., 1998); there was also a lower mortality among the zinc-supplemented group, but the sample size was small. Improvement in the intestinal barrier, as assessed by urinary excretion of lactulose and mannitol after an oral test dose, occurred following zinc supplementation in both studies (Roy et al., 1992). Regular follow-up to the homes of these children showed that the zinc-supplemented children had fewer episodes of diarrhoea and respiratory infection. More recent studies have examined the effect of daily doses of zinc syrup among malnourished children in an urban slum in India. Children who received daily doses of zinc experienced a decrease (around 45%) in the incidence of respiratory infection (Sazawal et al., 1998). Among low-birth-weight full-term infants in Brazil, there was a 28% decrease in the prevalence of diarrhoea and a 33% decrease in the prevalence of cough in a zinc-supplemented group (Lira et al., 1998). A cohort of children aged 12–59 months and recovering from acute diarrhoea was studied in India. Children with a low initial plasma zinc concentration (≤ 8.4 μM) had significantly more episodes of diarrhoea and severe diarrhoea than did children with a normal plasma zinc. The mean prevalence rate of diarrhoea was four times higher in the zinc-deficient group and the mean prevalence rate of acute lower respiratory-tract infections was 3.5 times higher in children with low plasma zinc (Bahl et al., 1998). A study of preschool children in Mexico showed that those supplemented with 20 mg of zinc had fewer episodes of disease overall and, in particular, had fewer episodes of diarrhoea (Rosado et al., 1997). A study among malnourished children in Bangladesh examined the long-term effects of giving a 2-week course of elemental zinc (20 mg day⁻¹) on morbidity episodes over the following 8 weeks (Roy et al., 1999). Zinc-supplemented children had significantly fewer episodes of diarrhoea and acute lower respiratory-tract infections compared with the control group. The impact of zinc supplementation on underweight children (< 71% weight for age) on diarrhoeal episodes was even more striking. Among preschool children in Guatemala, those receiving a zinc supplement for 7 months (given as 10 mg elemental zinc day⁻¹) had a 20% lower median incidence of diarrhoea, with an even more marked reduction among those who had low weight-for-length at baseline (Ruel et al., 1997). Zinc supplementation also produced a 67% reduction in the percentage of children who had one or more episodes of persistent diarrhoea. Interestingly, in this study, zinc did not change the incidence of respiratory infections. This contrasted with the experience in India, where, using zinc gluconate (10 mg day⁻¹ unless the children had diarrhoea, in which case they were given 20 mg day⁻¹), a significant reduction (45%) in the number of respiratory infections among zinc-supplemented children was observed (Sazawal et al., 1998).

Many of these studies have been subject to a pooled analysis of randomized controlled trials for the effect of zinc on acute and persistent diarrhoea.
Overall, zinc-supplemented children had a 15% lower probability of continuing diarrhoea on a given day in the acute diarrhoea trials, a 24% lower probability of continuing diarrhoea and a 42% lower rate of treatment failure or death in the persistent diarrhoea trials. In none of the subgroup analyses were the two subgroups of each pair significantly different from each other. However, in persistent diarrhoea, there tended to be a greater effect of zinc in subjects aged < 12 months who were male or who had wasting or lower baseline plasma zinc concentrations (Bhutta et al., 2000). Zinc-supplemented children had a 41% lower probability of developing pneumonia. Despite a large number of trials investigating the effect of zinc on the clinical outcome of the common cold, a careful meta-analysis performed recently showed that the data are inconclusive for a therapeutic role for zinc (Marshall, 2000). Overall, there is a very strong case for improvement in zinc status as a means of preventing diarrhoea and pneumonia.

Zinc deficiency may also contribute to decreased immunity and increased morbidity and mortality during pregnancy. There are several reviews of the association of zinc deficiency and a series of complications in pregnancy and their contribution to maternal mortality (Caulfield et al., 1998). Rather few supplementation studies have been performed and most of the published data have been from studies among women with marginal zinc deficiency. Several studies show an improvement in birth weight following zinc supplementation. Other studies show an association between zinc deficiency and increased risk of pregnancy-related morbidity, such as prolonged labour, toxæmia and blood loss (Goldenberg et al., 1995), but there are no solid data on the impact of zinc supplementation on these aspects of maternal health and morbidity. How much of the pathophysiology is due to immune change is uncertain. The importance of including an adequate dietary zinc intake for pregnant and lactating women is recognized, but studies from Malawi show the considerable problem in obtaining zinc from high-phytate cereals (Gibson et al., 1998; Huddle et al., 1998). There are many theoretical possibilities for a key role for zinc in HIV by its effect on the immune system, viral replication and clinical responsiveness. A study of adults with HIV/AIDS in Zambia failed to show an effect of a combination of micronutrients, including zinc, on clinical outcomes, but these patients were studied late in their disease (Kelly et al., 1999). A study of late-stage HIV patients receiving anti-retroviral therapy in Italy showed that the provision of 45 mg of elemental zinc daily for 30 days resulted in decreased incidents of infection with Pneumocystis and Candida (Mocchegiani and Muzzioli, 2000). In a study examining the rate of progression from HIV to AIDS and mortality in adults in the USA, the consumption of high doses of zinc supplements was associated with an increased rate of progression to clinical AIDS and mortality during the follow-up of the study (Tang et al., 1993). This study did not, however, conform to standard practice for conducting randomized controlled trials. A recent study of adults with HIV showed low serum zinc levels in 23% of HIV subjects (Wellinghausen et al., 2000). This was associated with a low CD4 count, a high viral load and increased neopterin and immunoglobulin A levels. The mean serum zinc level was highest in stage C and lowest in stage A, suggesting that, even if anti-retroviral triple therapy is available, zinc deficiency may well be of clinical importance.
There has been rather little focus on the impact of zinc deficiency in relation to susceptibility to nematode parasitic infections, but experimental and some clinical studies suggest that zinc deficiency may produce profound effects on the gut mucosal immune system (Scott and Koski, 2000). However, it is important to recognize that not all studies of zinc supplementation show benefit. A study of severely malnourished Pakistani children who were receiving a rehabilitation diet of lentils, rice and milk did not show any benefit in terms of diarrhoea morbidity or weight gain if zinc supplements were also given (Bhutta et al., 1999b). It may be that the addition of zinc salts to the local high-phytate diet impairs their bioavailability or that the zinc content of the rehabilitation diet might have been sufficient anyway. Severely malnourished children in a nutrition rehabilitation centre in Bangladesh showed an increased mortality when given large doses of oral zinc (Doherty et al., 1998). Several explanations are possible. Copper deficiency is recognized during large-dose zinc supplementation – zinc impairs copper absorption. Copper deficiency may cause a severe leucopenia, with a consequent decrease in immune function (Percival, 1998).

Overall, there is a strong case for improving zinc status as a means of preventing diarrhoea and pneumonia. Its impact on other infections has not been sufficiently evaluated. Its impact on established disease is well demonstrated for diarrhoea, but there has been no evaluation of it in pneumonia, sepsicaemia or malaria. Its potential role in HIV is intriguing but unproved. A key challenge facing those who wish to improve zinc status is the need to provide small quantities on a regular basis; in this regard, it has a disadvantage when compared with vitamin A. However, the administration of zinc on a daily basis is quite possible – many citizens in industrialized countries do it regularly without any scientific basis. It seems very important to examine the effect of such doses among deficient subjects in environments with a high risk of infection.

Iron and Infection

Iron deficiency is frequently present in subjects with immune deficiency and high loads of infection. Severe iron deficiency causes suppression of several aspects of the immune system (Bhaskaram and Reddy, 1975; see also Kuvibidila and Baliga, Chapter 11, this volume), but the level of immune suppression is less than that experienced in zinc deficiency. Experimental studies on iron deficiency have shown some effect on immunity, but this is mild, certainly in comparison with the effects of zinc or vitamin A. Periodontal disease, due to *Actinobacillus*, is associated with low levels of mucin MG2 (Groenink et al., 1999). Subjects with periodontal disease were noted to have a low level of lactoferrin in the bloodstream, suggesting that this predisposed the subjects to the infection. The significance of low levels of lactoferrin in iron-deficient children in developing countries, in whom periodontal disease is common, has not been explored. There are associations between indicators of iron status, such as ferritin levels, and infection. High levels of ferritin and intense iron stores in bone-marrow macrophages have been associated with shorter survival times in
patients with HIV, and studies of anaemia in pregnancy in Malawi have shown high mortality among HIV-infected women who have high levels of iron in their bone-marrow examinations (van den Broek and Letsky, 2000). Non-transfer-rin-bound iron is increased in the lower respiratory tract of patients with *Pneumocystis* pneumonia, who show eight to ten times higher levels in bronchoalveolar lavage fluid, compared with controls (Mateos et al., 1999). The authors suggested the use of iron-chelating agents as a rationale for improved management, but the high levels may reflect the chronic inflammatory response rather than a form of iron metabolism that is contributing to the pathology. A study of apparently healthy residents in Lagos, Nigeria, showed an association between high ferritin levels and parasitaemia (Odunukwe et al., 2000). The authors point out the difficulty of assessing iron status using ferritin levels in malaria-endemic regions. Ferritin is certainly very labile in infection and cannot be used as an indicator of iron status. The measurement of transferrin receptors may be more useful, as they are less affected by inflammation (Beesley et al., 2000). HIV-infected patients carrying the haptoglobin 2–2 phenotype show a worse prognosis with a more rapid rate of viral replication and higher mortality (Delanghe et al., 1998; Gordeuk et al., 2001). These patients had higher serum iron, transferrin saturation and ferritin levels and a low vitamin C concentration, suggesting that less efficient protection against haemoglobin/iron-driven oxidative stress may be a direct mechanism for stimulating viral replication. Further evidence of a toxic effect of iron comes from studies of neutrophil dysfunction in haemosiderosis (Cantinieaux et al., 1999). Serum samples from such patients induced a defect in neutrophil function, which was prevented by coincubation with desferrioxamine. The transferrin–albumin fraction of serum had no effect on neutrophils, whereas the ferritin fraction of normal serum was deleterious to neutrophils and the same fraction from thalassaemic serum decreased neutrophil function even further (Cantinieaux et al., 1999). Overall, there is rather little evidence that iron deficiency impairs the immune response and quite strong evidence that iron overload is damaging.

There are two characteristics of iron that are especially important in infection. First, iron induces oxidative stress (Walker and Walker, 2000). This has been demonstrated in volunteers taking low doses of oral iron and it is particularly marked in subjects receiving large doses of iron by intramuscular or intravenous routes (Oppenheimer, 1998). Second, iron is a stimulant for microbial growth, both for free-living bacteria in the blood, such as *Salmonella* and coliforms, and for intracellular organisms, including parasites, such as malaria. There are considerable advantages in improving iron status with regard to features of child development, such as improved growth and cognitive development. However, in communities where infection loads are high, the desire to improve iron status may enhance the risk of infection. There has been considerable interest in iron status in relation to hepatitis C. Several studies indicate that high-dose iron may actually enhance the infection (Bassett et al., 1999). Iron supplementation increases growth of the hepatitis C virus in culture (Kakizaki et al., 2000). However, there was no improved liver function following iron depletion of subjects with hepatitis C (Herrera, 1999). These studies certainly indicate that there is a risk from iron therapy in terms of oxidative
damage or stimulation of microbial growth. There seems to be some form of
dose relationship, with lower doses being non-toxic and higher doses being
toxic, especially if injected. This has led to the proposal for iron chelation dur-
ding certain infections (Thuma et al., 1998). A particularly extensive review of
this complex relationship between iron and infection has been produced by

The increased mortality associated with severe anaemia is well established
among children and pregnant women (Brabin et al., 1990). Many public health
programmes include iron supplementation of pregnant women, but very few
provide iron supplementation for infants and children. There have been several
theoretical or empirical reasons why iron has not been advised in populations
where infections are endemic. First, experimental studies show an increased
bacterial growth when iron is added to the culture medium (Andrews, 1998;
Brochu et al., 1998). Second, there were initial reports of increased intestinal
parasitic infection during refeeding of refugees, though these were observa-
tional studies and there were confounding variables (Murray et al., 1978).
Third, there was increased respiratory morbidity and mortality when iron was
given intramuscularly to anaemic infants in Papua New Guinea (Oppenheimer
et al., 1986). Fourth, there have been reports that iron supplementation
increased susceptibility of infants and children to malaria (see Oppenheimer,
2001). Thus, iron deficiency has often been portrayed as a ‘protective mechani-
ism’.

However, there is conflicting evidence and several recent studies show no
deleterious effects of iron supplementation, even in malarious areas. A rigorous
study of supplementation of Tanzanian infants in an area that is endemic for
malaria has compared different prophylactic regimes (Menendez et al., 1997).
Those subjects who received daily iron supplements had a lower rate of
anaemia than those who received malarial prophylaxis alone. The data showed
a protective efficacy for iron of 28.8%, compared with the control population
that did not receive iron. The attack rate for anaemia, as assessed by regular
anaemia surveillance, was 0.62 cases per child year \(^{-1}\), compared with 1.00
case per child year \(^{-1}\) in the controls. The groups did not experience different
attack rates of clinical malaria. The frequency of malaria episodes in unsupple-
mented vs. supplemented children was 0.87 vs. 1.00 cases per child year \(^{-1}\). A
study of Tanzanian children aged 5 months to 3 years examined the impact of
providing a low-dose micronutrient supplement, including iron, three times per
week (Ekvall et al., 2000). The mean haemoglobin level was 8 g l \(^{-1}\) higher
among supplemented children during the 5-month period of the study. In a
group of supplemented children who also received sulphadoxine-
pyrimethamine, the mean level of haemoglobin increased by 22 g l \(^{-1}\).
Supplementation with iron did not affect malaria incidence. Supplementation
of older children with a mixture of several micronutrients, including iron,
improved biochemical status but did not increase the frequency or severity of
clinical episodes of malaria (Bates et al., 1987; Fuller et al., 1988). A study of
the effect of weekly iron supplements in adolescent girls in Tanzania showed
benefits of supplementation in terms of raised ferritin levels and growth
(Beasley et al., 2000). There was slightly increased malaria parasitaemia, but
there were no adverse clinical effects. These recent studies indicate that the provision of regular oral iron supplements contributes to the prevention of anaemia, with little or no increased risk of malaria. Although there are dangers if parenteral iron is given to children in malarious communities, as reviewed above, the benefits of improving iron status in anaemic communities appears to outweigh the risk of enhancing infection in children. The effects of iron on HIV infection and associated opportunistic infection are not yet clarified.

### Mixed Micronutrients and Infection

In view of the evidence that individual micronutrients have quite marked effects on morbidity and mortality from infectious disease, there is increasing interest in combining micronutrients. There are important interactions between micronutrients, such that large doses of one may inhibit the absorption of another, and these need to be considered in interpreting the results of multiple micronutrient intervention. Mixtures of micronutrients have been investigated in many studies where the focus has been on nutritional outcomes (Ndossi and Taylor, 1999). Variable responses in haemoglobin, plasma zinc and retinol, with increases in linear growth, have been observed in Vietnam (Thu et al., 1999), though effects on morbidity were not recorded. Indeed, at present, there are remarkably few studies on the effect of multiple micronutrient interventions on immunity or morbidity in women and children. One of the most recent is an evaluation of the impact of multiple micronutrients on the pregnancy outcome of HIV-positive women in Tanzania (Fawzi et al., 1998). Mothers received either a placebo, vitamin A alone or vitamin A with a range of additional micronutrients. These consisted of vitamins B₁, B₂, B₆, B₁₂, C and E, niacin and folic acid. There were several important findings. First, there was an increase in numbers of circulating CD3, CD4 and CD8 lymphocytes among the mothers receiving mixed micronutrients, whereas supplementation with vitamin A had no effect. Similarly, there was no effect of vitamin A supplementation on pregnancy outcome. In contrast, multiple micronutrient supplementation was associated with a significant reduction in the percentage of women who had fetal deaths (9.6 vs. 5.9%), stillbirths (6.1 vs. 3.5%), low birth weight (15.8 vs. 8.8%), a composite of preterm birth or low birth weight (8.8 vs. 3.8%) or an infant who was small for gestation age (17.6 vs. 10.0%). These rather striking findings are important for HIV-positive women, but there is no evidence yet that such improvements occur in women who are HIV-negative.

### Breast-feeding and Infection

The benefits of breast-feeding with regard to infection in the neonate and older infant are extensive and well documented (Victoria et al., 1989). The benefits have mostly focused on the reduction in incidence, severity, duration and mortality from diarrhoea, especially among malnourished children (Brown et al., 1989). Several studies have also emphasized the protective effect of breast-
feeding against pneumonia, which is fast becoming the most important global cause of death among children under 5 years of age. A recent study from Brazil showed that infants who were not being breast-fed were 17 times more likely to be admitted to hospital for pneumonia (Cesar et al., 1999). The benefits of breast-feeding were considerably reduced if infant formula was used as well as breast milk. Rates of pneumonia were much higher in those receiving solids, fluid supplements and/or formula milk. The excess risk was particularly pronounced in infants less than 3 months of age but was still present among older infants. These data support the promotion of exclusive breast-feeding, especially during the first 3 months of life.

The demonstration of transmission of HIV from mother to infant presents a tragic dilemma. Millions of children have now been infected with HIV; most of them live in sub-Saharan Africa (Newell, 1999). Transmission can be attributed in about equal proportions to infection in utero, during delivery or from breast milk, though the relative contribution to each of the opportunities for infection varies considerably (Dunn et al., 1992; Bertolli et al., 1996).

Whereas in industrialized countries mother-to-child transmission of HIV has been dramatically reduced by the use of anti-retroviral agents during pregnancy, appropriate obstetric care and exclusive formula feeding after delivery, these options are usually not available or feasible for women in less developed countries. The cost of anti-retroviral therapy, such as AZT, in pregnancy is still so high and its availability so low that only a minute fraction of the population will be able to have access to it. Some of the alternative options have been reviewed (Kuhn and Stein, 1997). Recent data on the use of nevirapine, a much cheaper drug, given as two single doses – one in labour and the other to the infant – show a striking reduction in transmission (UNICEF, 1998b). However, even with nevirapine, transmission by breast milk is still significant. Unfortunately from the perspective of transmission, the cost and safety of infant formula in poor socio-economic conditions are such that the potential benefit of preventing post-natal transmission of HIV by using formula may result in increased mortality from infectious disease as a result of the lack of breast-feeding. An added dilemma is caused by the lack of diagnostic facilities. Currently, the only tests widely available are for HIV antibodies rather than virus; it is not possible to know whether an infant is truly HIV-positive or negative until at least 12 months, and feeding advice based on knowledge of infectious status is not possible on an individual basis.

Although it is estimated that about a third of cases of paediatric HIV globally contract their infection from breast milk, these figures vary considerably from country to country. A meta-analysis showed that transmission was much higher (29%) if the HIV infection was acquired by the mother during lactation than if the mother was already HIV-positive during pregnancy (15%) (Dabis et al., 1993). An important factor is the duration of breast-feeding (Coovadia and Couttsoudis, 2000). If transmission is more common in early lactation, these data would tend to overestimate the proportion of infants with HIV who acquire their infection from breast milk. Recent calculations using pooled data from African children who were known to be HIV-negative at 3 months post-partum indicate a risk of transmission of 3.2 cases per 100 child years of breast-feeding (Leroy et al., 1998).
The HIV load is highest in colostrum (Markham et al., 1994) and in early lactation (Van de Perre, 1999), but breast milk immune factors are also higher at these times. The relationship between viral load in breast milk and transmission via this route needs further study. Other risk factors are likely to be important in the post-natal transmission of HIV. These include deficiency of some of the many immunologically active components of breast milk. The presence of sulphated glycosaminoglycans is of potential importance, because of their inhibition of binding of CD4 cells to the HIV envelope glycoprotein (Newburg et al., 1992). Impaired immunity in the mother, assessed by clinical staging of HIV/AIDS and CD4 and CD8 counts as a result of HIV itself, breast abscess or cracked nipples, systemic maternal infection (from pelvic inflammation or malaria) or increased viral shedding in vitamin A deficiency have each been proposed. There may also be altered immune function and viral uptake by the intestinal mucosa of the infant. This could be influenced by dietary antigen stimulation and malnutrition (including zinc and vitamin A deficiency, which are known to affect immunological competence and mucosal structure and function). Other factors that could enable increased viral uptake include candidal lesions of the buccal mucosa and intestinal damage from infections.

An additional, novel hypothesis has been put forward during studies of breast milk immunology among women in Bangladesh, Tanzania and South Africa (Georgeson and Filteau, 2000; Willumsen et al., 2000). It was noted that around 20% of women in these countries have subclinical mastitis, as assessed by a high ratio of sodium (Na) to potassium (K) and high IL-8 levels in breast milk. This is especially important within the HIV context, because of the association between high numbers of HIV particles and subclinical mastitis (Filteau et al., 1999a). The veterinary literature has recognized subclinical mastitis for several decades. It is known to be associated with a high load of a range of bacteria and is especially common among cattle being fed on antioxidant-deficient pastures. Subclinical mastitis has been noted to be associated with poor milk volume and growth faltering in farm animals and was also present in the study of infant growth in relation to subclinical mastitis in Bangladesh (Filteau et al., 1999b). A recent study in South Africa shows that there are certain patterns of occurrence of subclinical mastitis (Willumsen et al., 2000). Bilateral subclinical mastitis is of a typical mild form, with low Na/K ratios, whereas unilateral subclinical mastitis is more common and is often more severe, with high Na/K ratios and elevated levels of IL-8; there are higher viral loads in samples from women with subclinical mastitis in Durban. The association between subclinical mastitis, viral load and mother-to-child transmission of HIV has been demonstrated in Malawi, though no information on the pattern of subclinical mastitis was provided (Semba and Neville, 1999).

The demonstration that HIV viral load is increased among women with subclinical mastitis has enormous implications for the transmission of HIV in breast milk. It puts great emphasis on the reduction of the prevalence and severity of subclinical mastitis by whatever means possible. A recent study among women in Tanzania shows that the prevalence of subclinical mastitis is lower among women who received dietary supplements with sunflower-seed oil during pregnancy and lactation (Filteau et al., 1999a). Sunflower-seed oil has a
high level of vitamin E; the potential antioxidant capacity of this may be extremely relevant to the decrease in levels of mastitis. Interestingly, the role of deficiency of selenium and vitamin E in the development of mastitis in cattle is well recognized (Hogan et al., 1993).

Among the women studied in South Africa, those who fed their infants with breast milk exclusively had a lower prevalence of subclinical mastitis than those who used mixed feeding. It is postulated that milk stasis, attributable to the introduction of mixed feeding, might contribute to the establishment of subclinical mastitis. Thus, a combination of infection, micronutrient deficiency and mechanical issues, such as placement, may be important in the development of subclinical mastitis (Willumsen et al., 2001).

Rates of mother-to-child transmission of HIV in relation to type of infant feeding have been studied in an urban community in Durban, South Africa (Coutsoudis et al., 1999). Transmission was 18.8% among never-breast-fed children (i.e. those who received infant formula alone). This compared with 14.6% among exclusively breast-fed infants and 24.1% among infants receiving mixed feeding. Even after allowance for potential confounders, such as maternal CD4/CD8 cell ratio, syphilis-screening results and premature delivery, there was a significantly lower risk of HIV transmission (hazard ratio of 0.52) among exclusively breast-fed infants compared with those receiving mixed feeding. There are several possible explanations. First, the protective effect of exclusive breast-feeding may be due to lower levels of subclinical mastitis and therefore a decrease in the accompanying viral load in breast milk (Willumsen et al., 2000). Second, the delay in introduction of dietary antigens may cause less immunological response in the intestinal mucosa; this may decrease the uptake of virus from the gut into the circulation. Third, the addition of microbes in a mixed diet may damage the intestinal mucosa; indeed, this is suggested by previous studies of intestinal permeability in different dietary groups (Udall et al., 1981).

The findings from Durban suggest that the risk of transmission is relatively low if infants are breast-fed exclusively for 3 months. However, the infection rates thereafter, not yet available from the Durban study, are quite high in other studies and, in the absence of more data, it seems that, after 3 months, breast milk should not be given if accompanied by other foods. It is hoped that, with more widespread availability and effectiveness of new anti-retroviral drugs, the mother-to-child transmission rates are likely to fall. Even so, it will still be necessary to promote the reduction of transmission of HIV by dietary means. At present, it seems that the appropriate advice in poor communities is to reinforce existing messages promoting the exclusive use of breast-feeding until 3–4 months of age. Thereafter, the message is not so clear. In the absence of carefully controlled studies examining the post-natal transmission of HIV using different regimes of stopping breast milk, it is not possible to calculate the additional risk of mother-to-child transmission if breast milk is continued after 4–6 months. If early cessation of breast-feeding is promoted, there may be considerable deficiencies of iron, zinc and other micronutrients. Growth faltering, anaemia, impaired immune responses and an increase in the prevalence of severe infection are all possible in the non-breast-fed child. In addition, the loss of the con-
traceptive benefits of breast-feeding increases the risk of early, further pregnancies, with associated detriment to maternal health.

Other infections are also transmitted through breast milk; these include hepatitis B, hepatitis C and cytomegalovirus. It is estimated that 58–76% of cytomegalovirus-positive mothers transmit the infection to their children (Georgeson and Filteau, 2000). Premature babies are at special risk of developing cytomegalovirus, HIV and other infections. It seems advisable that a range of lactation interventions should be tested, including the role of the promotion of exclusive breast-feeding and the correct positioning and attachment of the child to minimize breast trauma, the promotion of antioxidant micronutrient status and improved control of opportunistic infection.

Policy Implications

National governments and international agencies have several options. They can improve dietary intake, fortify certain foods with particular micronutrients or provide supplements. They obviously need to keep their ‘eyes on the ball’ as regards the essential first option, but providing the second and third options is also essential. Government commitments to provide an adequate diet are enshrined within many important international agreements, such as the Convention on the Rights of the Child and the International Conference on Nutrition in Rome. There is further opportunity to rededicate themselves to this goal as a result of the World Summit for Children in 2001.

The demonstration that improved nutritional status has a profound effect on immunity, disease susceptibility, illness severity and mortality should drive governments and civil society to improve dietary intake from all perspectives – ‘human rights’ and a ‘right to the best health status that is possible’ and pragmatic concerns to enhance human development.

The second option – food fortification – is technically possible and logistically feasible. Objections have in the past been raised that those populations eating centrally processed food, such as vitamin A-fortified sugar, iodine-fortified salt or folic acid-fortified wheat, would be predominantly in the better-off urban areas, leaving the rural areas untouched by such strategies. With increasing urbanization globally and the presence of many millions of individuals in very-low-income areas of cities, micronutrient deficiencies will definitely occur in subjects who purchase food rather than growing their own and, for this reason, there are immense benefits available from fortification.

Whether either of the above forms of micronutrient provision enhances dietary intake sufficiently to increase micronutrient status and improve immunity and resistance to infection is uncertain. Whereas a small number of studies have examined the impact of fortified-food provision on nutritional indicators, such as haemoglobin concentrations, there have been no studies on the impact of the provision of fortified food on infection. There is an urgent need to establish such studies, particularly now that reliable and robust technologies exist for fortifying foods with a range of micronutrients.

It will be particularly important to perform efficacy and effectiveness stud-
ies. It is one thing to show that nutritional status and disease prevalence change as a result of the administration of fortified food in controlled circumstances; it is another to see what happens in the free-market situation in which the food industry and malnourished populations interact. New programmes for nutritional supplementation require consideration of what the extra manpower and other necessary resources are in relation to the existing activities in ministries of health and community development; they are already busy with established programmes that were promoted by evidence-based proposals and the ‘champions’ who promoted them. Governments can be persuaded to ‘invest in nutrition’.

Within the health sector, a strong case has been made for providing regular vitamin A capsules, and this has now been adopted as UNICEF/WHO policy globally and increasingly countries are implementing this at national and district level. It is clear that the impressive mortality and morbidity benefits from regular doses of vitamin A are so striking that the supplementation programme for deficient populations should be regarded as essential under any circumstances.

Supplementation with iron is an effective way of improving haemoglobin in malnourished populations. Many studies have shown the benefit of weekly, as opposed to daily, iron. Certain target populations such as school children can be assisted by school-based or parent–teacher-based distribution systems. Among young children, where malarious morbidity and mortality are of greatest risk, the results indicate that the provision of low-dose iron supplements improves haemoglobin without increasing malarious risk. High-dose oral preparations or any intramuscular preparations are not advisable because of the direct toxicity of iron and the possibility of increasing infection.

The benefits of providing daily doses of zinc during carefully controlled field trials are impressive, with well-documented impacts on morbidity reduction. Unfortunately, because of limited body stores of zinc, it is not possible to give infrequent large doses, as it is for vitamin A. Whereas vitamin A given three times a year has profound effects on mortality, daily administration of zinc is necessary if an impact is to be achieved. There are, to date, no data on the effectiveness of encouraging the daily administration of zinc by child carers in a less intensive manner than that of a field trial. Such studies are, however, under way and will provide important data on effectiveness (where an intervention is given in a strongly promoted manner but not intensively supervised), as opposed to efficacy (where the provision of the intervention is closely supervised). Similarly, while there are individual studies showing the benefits of closely supervised supplementation with selenium or vitamin E, there are very few data on the impact of community-based provision of these micronutrients on a regular basis.

Should single micronutrient programmes be continued? In the light of the clear evidence for a benefit in regard to immunity and infection of vitamin A, zinc and possibly selenium, it seems logical to provide these as a multiple micronutrient preparation. There are now many preparations available for adults and some for children. The recently prepared WHO/UNICEF preparation provides about the recommended daily amount for an adult subject. There are currently several studies examining the nutritional, immunological and health
benefits of regular provision of these to women in pregnancy and to young children. When these studies are completed, it will be possible to provide specific information on the degree by which immunity and health indicators are enhanced. Given the remarkable success of the promotion of vitamin A supplementation, largely as a result of data that show an impressive reduction in morbidity and mortality, there is great interest in knowing how much a multiple micronutrient supplementation will change these indicators in children and adults. Nutrition interventions now provide one of the most effective ways of preventing illness, reducing mortality and promoting child development and human capital. The challenge now is whether science can be presented in ways that will persuade governments and civil leaders to take active steps to make a major attack on infectious disease using nutrition interventions.

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Index

Page numbers in **bold** refer to figures and tables

abdominal surgery, glycyl-glutamine 121  
absorbed food antigens 296–298  
absorption, iron 210  
accumulative damage 175  
acquired (adaptive) immune protection 273  
acquired immune deficiency syndrome (AIDS)  
  nuclear transcription factor kappa B (NFκB) 143  
  plasma glutathione reduction 141  
  probiotics 264  
  selenium deficiency, children 31  
  serum vitamin E levels 178  
**Actinobacillus** 393  
activator protein 1 (AP-1) 143–144, 241  
activators 27–28  
active mucosal immunity development 300–301  
acute lower respiratory infections (ALRI) 158–159, 281  
adaptive immune system 2–19, 21, **274**  
adhesion molecules, selenium effects 241–243  
adults 23, 211, 278, 386  
age factors 23, 41, 47, 51, 65  
age groups **181**  
age-related decline T3 content 245  
ageing 23, 171, 357–368  
  see also elderly  
airways, antigen stimulation 293  
alcoholics, zinc supplements 201  
allergens 17, **323, 325–328, 329–330**  
allergy  
  anti-allergy immunoregulatory 262  
  anti-allergy properties, **Lactobacillus** 262  
avitamin E levels 178  
**atopy** 285, 291–292, 293  
**children** 292  
**diseases** 76, 262  
**food** 321–342  
inappropriate Th2 responses 18, 19  
infectious, **Lactobacillus** 285  
nasal mucosa 293  
pathogenesis 285–286  
symptoms **324**  
**alternate T-cell activation pathway** 34  
**amino acids**  
  arginine 93–105  
  creatine biosynthesis **135**  
  glutamine 109–124  
  selenomethionine 234, **235**  
  sulphur 133–147  
  see also cysteine  
anaphylaxis 323, 342  
anaemia, mortality 395  
anaemia, mortality 395  
anaphylaxis 323, 342  
anergy 212  
**animals**  
  arginine and immune function 99  
  dehydroacetic acid (DHA) 62, 64–65  
  eicosapentaenoic acid (EPA) 62, 64–65  
  farm, selenium 238, 241  
  fish oil 62  
  healthy, glutamine feeding 116–117  
  infection models 118–120  
  models, infection and trauma 118–120  
  zinc deficiency 193, 195–196  
  anti-allergy immunoregulatory 262  
  anti-allergy properties, **Lactobacillus** 262
anti-cancer defence 80
anti-inflammatory effects
  fish oil 63, 79–80
  selenium 241–243
anti-retroviral therapy 397
anti-tumour activity, arginine 99
anti-tumour immunity 261
  Lactobacillus 261
antibiotics, newborn 293–294
antibodies
  antigen specificity 4
  binding to bacteria 5–6
  breast milk 276–277, 280, 282, 295
  dietary antigens 281
  general structural features 3–6
  immunoglobulin (Ig)E production
    monoclonal 26
    production suppression 64
  reduction, malnutrition 45
  responses
    influenza vaccine, elderly 47
    inhibition, zinc deficiency 200
    probiotics effects 259
    tetanus toxoid immunization 213, 214
    vitamin A deficiency 156
    secretory 294–296, 298–299
  see also immunoglobulin
antibody-mediated defence, neonate 275–278
antigen
  absorbed food, handling 296–298
  clearing mechanism 296–297
  exclusion 273
  exposure effects, secretory immunity 283–285
  intact 296
  thymus-independent (TI) 14
  transportation 9
  uptake 297
antigen-binding (Fab) sites 4
antigen-presenting cell (APC) 278, 288–291
antigen-specific humoral and cellular immunity, adult
  host response 23
antigenic pressures, ageing 361
antimicrobial factors, breast milk 299
antioxidant
  activity, selenium 230
  defences 138–140, 171–173, 196
  dietary sources 151–152, 173–174
  effect mechanism 142–144
  exercise 353
  immune function improvement 28
  interaction 139
  reactive oxygen species (ROS) 171–174
  smokers 381
  vitamins 171–185
antip-retroviral therapy (ART) 145–146
apoptosis 195–196, 212
applied kinesiology, food allergy 338
appropriate for gestational age (AGA) 47, 51
arachidonic acid
  atopic disease, putative role 78
  decrease, fish oil 71
  dienoic prostaglandins 70
  fish oil, decrease 71, 72
  immune cell function regulating role 69
  intakes 61
  metabolites 76, 236–237
  proportion human immune cells 67
  see also eicosanoids
arachidonic acid-derived eicosanoids, production
  decrease, fish oil 72
Arachis hypogea 328
arginine 93–105
  see also enzymes
arthritis 262–263
ascorbic acid (vitamin C) 142, 174–176
asparaginase, immunosuppressive effect 114
assessment methods 31–34
asthma 76–79, 276, 302
  asthma and related diseases 76–79
  atopy 78, 276, 285, 291–292, 293, 302
autoimmune diseases
  development 1
  fatty acids 75–76
  glutamine 121
  immunoregulating lactic acid bacteria (LAB) 262–263
  inappropriate Th1 responses 18–19
  probiotics 264
  see also deficiency
autologous urine injections, food allergy 338
AZT 397
B-cells
  activation and maturation 14–15
  adaptive immunity 2–3
  fractions enriched, iron status function 214
  homing, mammary glands 280–281
  iron deficiency 212–214
  iron overload 219–220
  proliferation inhibition, zinc deficiency 200
  subset distribution, spleen iron-deficiency mice 212
  suppression 348
  system, local activation 282
B lymphocytes 2–3, 46, 114, 156, 198
babies, formula-fed, salivary IgM 301
bacteria
  adherence, epithelial cells 47
  antibodies binding 5–6
  commensal bacteria role 294
Gram-positive bacterial cell-wall components 255
growth increase, iron 395
lactic acid 252, 256, 262–263
pathogens 259
signalling 258
translocation decrease, glutamine 121
Bacteroids 284
barrier function, zinc deficiency effects 197
β-carotene 179–180, 183, 184, 376, 387
β-lactoglobulin, breast milk 296
β-thalassaemia 220
Bifidobacterium 252, 256, 258, 260, 292
biological effector mechanisms 1–2
biological plausibility establishment 378–379
biosynthesis
creatine 135
glutamine 110
pathway, polyunsaturated fatty acids 60
polyamine 134
precursor, glutamine 123
birth weight 49–50, 392
see also low-birth-weight (LBW)
blood lymphocytes, exercise-induced changes 348
blood lymphocytes proliferation in vitro, glutamine effect 115
bone-marrow transplantation patients, glutamine intravenous 120
Brazil nut allergy 334
breast milk
antibodies 276–277, 280, 282, 295
antimicrobial factors 299
β-lactoglobulin 296
bioactive components 303, 304
glycoproteins 300
immunoregulatory effects 298–302
leucocytes 299–300
oligosaccharides 294
ovalbumin 296
peanut allergens 340
protective effects 298–302
breast-feeding
critical role 276–278
food allergy 339
immunological integration of mother and child 280, 281
infection 396–400
promotion 53
role, mucosal homeostasis 273–304
secretory immunity 301
transforming growth factor (TGF)-β 302
British Nutrition Foundation 322

anti-tumour activity, arginine 99
carotenoids 179
liver 244
lung 183, 184, 185
oesophageal, glutamine 121
prostate cancer 183
selenium anti-cancer properties 229, 230
selenium intake 245–246
see also neoplasms; tumour
Cancer Prevention Study II (CPS-II) 184–185
Candida 392
carbohydrate moieties, food allergens 330
carbohydrates 352
cardiotoxic virus 242
cardiosterevase disease 139, 246
carotenoids 174, 179–185
catabolic stress 117–118, 120–121, 123–124
catalase 173
CD4 T-cells 8, 16–17, 19, 102
CD8+ T-cells 11, 238, 359–361
CD8 T-cells 8, 11–12, 16, 19
CD40 signals 12, 13
cell-mediated immune response 64, 182, 182
cell-mediated immunity, iron deficiency impairment mechanisms 216, 217
cell-mediated immunity impairment 51
cells
biology, zinc 194–196
cycle, zinc 194
death, mechanism, zinc 195
mediating innate immunity, selenium deficiency effects 239, 240
oxidant damage 143
replication, zinc 194–195
signalling, selenium effects 241
surface molecules 182
cells, -mediated immune response initiation 182
survival and proliferation, glutamine 112
characterization, selenoproteins 231–234
Chediak–Higashi syndrome 176, 180
children
acquired immune deficiency syndrome (AIDS), selenium deficiency 31
allergy 292
colostomies defunctioning 284
diarrhoeal disease
breast-feeding 276
iron fortification 222
Lactobacillus 257, 260
tobacco intake 183
treatment 384
vitamin A deficiency 158
gut mucosal barrier, immaturity 333
human immunodeficiency virus (HIV) 386, 389
children continued
immunocompetence 42
testinal microflora 292
iron deficiency 211, 222
malaria 25, 27, 395–396
malnutrition 383, 393
marasmic 199
measles 28, 157–158
morbidity and mortality 152–153, 382, 384–386
nutrition rehabilitation 382
Peyer’s patches 293
pneumonia 384, 385–386
protein–energy malnutrition (PEM) 24, 45, 46
respiratory infections 138, 222
selenium deficiency, acquired immune deficiency syndrome (AIDS) 31
T lymphocytes subsets, protein–energy malnutrition (PEM) 45
tomato intake 183
tuberculosis 27
undernourished 284
vitamin A 386
see also infants; neonates; newborn
chronic inflammatory disease 77
chronic inflammatory diseases 75–76
cigarette smoke 178
ciliary movement reduction, mucosal infection 47, 48
cirrhosis 143
citrulline structure 94
clinical history, food allergy 336
clinical studies, arginine patients at risk of sepsis and septic complications 101–105
clonal expansion, lymphocytes 3, 11
Clostridium spp. 292, 296
cost–benefit calculations, nutritional interventions 378
cooking methods, food allergens 335
copper deficiency 393
cost–benefit calculations, nutritional interventions 378
cow’s milk allergy 340
Coxsackie virus 244, 389
creatine, biosynthesis 135
crohn’s disease 264
cross-reactivity, allergen 329–330, 335
cyclo-oxygenase (COX) 70
cysteine
conversion from cystine 146
conversion to glutathione synthetase (GSH) 135
glutathione synthesis enhancement 144
incorporation 134
inflammation 145
losses 138
metabolism 133
metabolites 136
metabolized 141
residues 230
synthesis 142
toxicity 145
see also amino acids
cytokine
biology 24
concentrations changes, strenuous exercise 350
interleukins 16–17
microenvironment 17
production 46, 289–290
production in vitro, glutamine influence 289–290
release inhibition, human keratinocytes, selenium 242
selenium effects 241–243
selenoproteins expression 243
Th2 release increase 361
cytomegalovirus 400
cytotoxic T-cells 11, 19, 238, 359–361
see also effector cells
daily diet, saturated fatty acids, UK adult survey 60–61
danger signals 293
see also infection; inflammation
defences
antibody-mediated, neonate 275–278
antioxidant 138–140, 171–173, 196
infection 273–304
innate cell, probiotics effects 258
iron 222
vitamin A deficiency 153
zinc 196
deficiency
essential fatty-acid 61, 62
fetal zinc 197
immune deficiency, ageing 358–362
iron 210–217, 222
micronutrient 383
nutritional 47, 151–162
Index

primary (ageing) immune deficiency 358–362
selenium 234–235, 239, 240
vitamin A 153, 155–156, 158, 161
zinc 193–194, 197, 198–200, 202, 390
see also autoimmune diseases; human immunodeficiency virus (HIV)
degenerative disorders, oxidative damage 172
dehydroacetic acid (DHA) 61, 62–63, 64–65, 67, 75, 81
delayed-hypersensitivity skin test response 43, 44
dendritic cells (DCs) 9–11, 278–279, 289–290
Department for International Development, UK 377
diabetes, type 1, pathogenesis, glutamine role 121
diagnosis, food allergy 336–338
diarrhoeal disease
children
breast-feeding 276
iron fortification 222
Lactobacillus 257, 260
tomato intake 183
treatment 384
vitamin A deficiency 158
infant 253
management 54
prevention 393
protein–energy malnutrition (PEM) 43
zinc 381, 391–392, 393
dienic prostaglandins 70
diet
elimination 336–337
exclusion 340–341
modern 381
new food introduction 334, 335
dietary
antigens, antibodies 281
compliance 341
components, beneficial effects assessment problems 175–176
fat 57, 61–62, 63
fatty acids, effect mechanisms 65–75
fish oil supplementation 23
intake 28, 29
sources
antioxidant vitamins 151–152, 153, 173–174
fatty acids 58–61
see also supplementation
digestion epitopes 331
digestion resistance 330
dimeric IgA 295
disease causing agents 252–253
DNA building blocks 112
DNA damage 242–243
DNA synthesis 45
dogs, tracheal-cell cilia, protein–energy malnutrition 48
donor countries 380
double-blind placebo controlled food challenge 337
down-regulatory cytokines 291
Down’s syndrome 201
drugs, enhancing glutathione synthesis 144
DTH responses 180–181
Durban, human immunodeficiency virus (HIV) 399–400
dysfunctions control, probiotics 262–263
eye exposure, food allergy 333–334
eczma 302
effector cells 9–19
see also cytotoxic T-cells
effector mechanisms 18–19, 282, 303–304
eicosanoids 69–73, 76, 183, 236–237
see also arachidonic acid
eicosapentaenoic acid (EPA) 61–65, 67, 73, 74, 81
everly
antibody response, influenza vaccine 47
immune cell function enhancement 180
immune system and nutrition 357–368
immunodeficiency 358, 362–367
low selenium status 245
micronutrients 376
peripheral-blood 360, 363, 367
protein–energy malnutrition (PEM), role 365–367
tomato juice 183
vitamin supplements 175–177, 365
wound healing 101, 201
zinc deficiency 201, 365
see also ageing
electroacupuncture, food allergy 338
elimination diets 336–337
endocrine effects, arginine 98
endotoxaemia 79–80
enteral dietary intake 30
enteral feeding formulae, composition 104
enteral nutrition 103
enterocytes 275, 290
enzymatic food intolerance 322
enzymes 96, 110, 134, 135, 173, 194–195
see also arginine
eosinophils 18–19
epidermal cells, zinc deficiency damage 197
epithelial barrier 275–276
epithelial polymeric Ig receptor 277
epitopes 328, 331
Escherichia coli 257, 284, 296
essential fatty-acid deficiency 61, 62
ethical issues 380–381
eukaryotes 230, 231
European Academy of Allergy and Clinical Immunology (EAACI) 321–322
index of page 19: Nutrition Index 4/9/02 4:07 PM Page 418

European Commission 322
evening primrose oil 67
exclusion diets 340–341
exercise 118, 142, 177–178, 342, 347–354
exogenous glutamine 118–120
experimental zinc deficiency 390

farm animals, selenium 238, 241
fat, diet, acquired immune system 63
fatty acids 57–81
feeding regime, newborn 293–294
ferritin 393–394, 395
fetal growth retardation 47
fetal zinc deficiency, effects on immunological development 197
fish allergy 334, 335
fish oil 67, 73
  anti-inflammatory effects 63, 79–80
  arachidonic acid, decrease 71, 72
  asthma 76, 78–79
  benefits, rheumatoid arthritis 76, 77
  chronic inflammatory diseases 75–76
  effect, PGE2 production 73
  eicosapentaenoic acid (EPA) plus dehydroacetic acid (DHA) 61, 67, 81
human studies 65
laboratory animals 62
linoleic acid 57
lymphocyte phospholipase-Cy activity 69
supplementation 23, 388
flow cytometry 34
follicle-associated epithelium (FAE) 278
Food and Agriculture Organization, allergenic foods list 325–326
food allergy 321–342
food antigenic constituents 283–284
food fortification 400
food intolerance 321–342
formula-fed babies, salivary IgM 301
free secretory component (SC), secretory antibodies 298–299
fruit allergens reactions 331
functional immune response, assessment 32

γ-glutamyl cycle, glutathione role 135
gene expression changes 73–74
gene therapy, food allergy 341
gene transcription, selenium effects 241
genetic predisposition, food allergy 332–333, 335
geography, food allergy factors 335
gestational zinc deficiency 197
glucocorticoid hormones 201
glucose utilization rates 113
 glutathione 123, 133–147, 173
 glycine 138, 145
 glycoproteins, breast milk 300
 glucyl-glutamine, abdominal surgery 121
 Gram-positive bacterial cell-wall components 255
 granulocyte-macrophage colony-stimulating factor (GM-CSF) 292
 growth factor, selenium 230
 growth hormone receptor 98
 growth retardation 47–52
 gut
  barrier improvement, glutamine 121
  closure 275
  mucosal barrier, immaturity, children 333
  neonatal, milk macrophages 300
  oral tolerance, central role 287–288
  gut-associated lymphoid tissue (GALT) 278, 279, 294, 301
  gut-associated systems, glutamine influence 119

haematopoiesis, vitamin A deficiency 155
haemolytic activity total, decreased 46
health benefits, probiotics 257, 259–263
heat stability, food allergens 257, 259–263
helminth infections 18, 19
hepatitis 244, 389, 394, 400
homeostatic immune regulation, importance 291–294
hormones 98, 196–197, 201, 253
human genome 30–31
human immunodeficiency virus (HIV)
  anti-retroviral therapy (ART) 145–146
  breast-feeding 396–400
  children 386, 389
  controls replication 244
  cytokines, Th2 release increase 361
  ferritin, high levels 393–394
  malnutrition 24
  micronutrients 380, 396
  nuclear transcription factor kappa B (NFκB) 143
  nutritional deficiency 52
  paediatric 397
  plasma glutathione reduction 141
  postnatal transmission 380
  reduced vitamin E status 178
  selenium 238
  study design 31
  sulphate excretion 138
  transmission mother–child 397, 399
  vitamin A supplementation 160
  women, vitamin A 387–388
  zinc 392, 393
see also deficiency; viruses
humoral immune response 361–362
humoral immunity 3–6, 212–214
hydrolysed milk formulas 340
hydrolysis, cell-membrane phosphatidyl inositol-4, 5-bisphosphate 217, 219
hydrolysis resistance 330
hygiene hypothesis 285, 292
hypercatabolism 366
hyperinflammatory response 79
IgA 30, 277, 282, 294–298
IgE 323–325, 326, 332–334
IGF-1 98
IgG 4, 30, 46, 51, 238, 297
IgM 238, 277
IL-1 242
IL-2 116
IL-4, cytokine 16–17
IL-6 242, 348–349
IL-10 242–243, 302
IL-12, cytokine 16
IL-14 16–17
Immun-Aid® 105
immune cells 66, 197–200, 299–300
exclusion, IgA-mediated mucosal homoeostasis 294–298
impairment 348, 349, 350
induction, mucosa-associated lymphoid tissue (MALT) 278–281
responder phenotype imprinting, infant 303
stimulation 254, 263–264
immunization 23, 52, 53
immuno-inflammatory diseases 262
immunocyte density, human lactating mammary glands 284
immunodeficiency 44
immunoglobulin (Ig) effector functions 5–6
IgA 30, 277, 282, 294–298
IgE 323–325, 326, 332–334
IGF-1 98
IgG 4, 30, 46, 51, 238, 297
IgM 238, 277
isotypes 4–5
structure 3–4
see also antibodies
immunology 102, 197, 297, 341–342
immunomodulation, probiotics-mediated, health benefits 259–263
immunonutrition 103, 104
immunophenotyping 23
immunoregulation
anti-allergy immunoregulatory 262
breast milk 298–302
eicosapentaenoic acid (EPA) 74
lactic acid bacteria (LAB) 262–263, 264
probiotics 256–259
putative factors 290, 300
roles
LTB4 72
PGE2 71
immunosuppression 123–124, 201, 350
IMPACT® enteral nutrition 80, 103, 104, 105
impaired immunity, mechanisms in iron deficiency 216
improvement strategies 144–146
inappropriate responses, Th2 18
induction of tolerance via gut see oral tolerance
infants
allergy, Lactobacillus 285
appropriate for gestational age (AGA) 47, 51
diarrhoeal disease 253
feeding policies, human immunodeficiency virus (HIV)-positive mothers 380
immune-responder phenotype imprinting 303
immunocompetence 43
innate immunity 23
low-birth-weight (LBW) 51
measles 157–158
mixed feeding 302
preterm 75
small for gestational age (SGA) 41, 47, 51
zinc supplement, oral 52
see also children; neonates; newborn
infection
breast-feeding 396–400
clinical 44
control strategy, nutrition 377
defence 273–304
helminth 18
immune system response, metabolism effects 137
intervention strategies 53
iron 209–223, 393–396
malnutrition 382–383
micronutrients, mixed 396
models, animal 118–120
public health implications 375–402
resistance, impairment correction 201
risk 41–54, 394
selenium 388–390
sulphur amino acid and glutathione metabolism 136–140
susceptibility 222, 349
vitamin A 151–162, 384–388
zinc 193–203, 390–393
see also danger signals; inflammation
infectious diseases 156–161, 202, 260, 396
inflammation 57–81, 142–144, 236
see also danger signals; infection
inflammatory bowel disease (IBD) 291, 293
inflammatory changes, sepsis 79–80
inflammatory diseases 75–76, 139, 262–263
influenza vaccine, antibody, response, elderly 47
injury
arginine 99, 101
exogenous glutamine 118–120
lung 216
sulphur amino acid and glutathione metabolism 136–140
wound healing, arginine supplementation 101
wound healing impairment correction 201
innate cell defences, probiotics effects 258
innate immune system 22, 23, 239, 240, 292
Integrated Management of Childhood Illness (IMCI) 376, 383
intercellular adhesion molecule 1 (CAM-1)-leucocyte function associated antigen (LFA-1) ligand receptor pair 182
interferon (IFN)-γ secretion 361
interleukins
IL-1 242
IL-2 116
IL-4, cytokine 16–17
IL-6 242, 348–349
IL-10 242–243, 302
IL-12, cytokine 16
IL-14 16–17
International Conference on Nutrition, Rome 1992, 377, 400
interorgan transport 109–112
intervention 379
intervention strategies 52–54
interventions 376–378
intestinal parasitic infection increase 395
intestine 251–252, 292, 293
intraepithelial lymphocytes (IELs) 283–284
intracellular virus neutralization 295
intracellular lung 216
luminal
lymphocytes
activity promotion 121, 195
circulating numbers decrease, elderly 358
clonal expansion 3, 11
concentration increases, exercise 347
growth 99
mitogens treatment 194
mobilized to circulation, exercise 348
lactic acid bacteria (LAB) 252, 256, 262–263
Lactobacillus
anti-allergy properties 262
anti-tumour immunity 261
diarrhoeal disease 257, 260
immune stimulating probiotics 263–264
infants allergy 285
mucosal tolerance induction 294
oral delivery 256, 258, 259
probiotics 252, 255, 256
tumour cells 258
lens opacities 175–176
leucocytes 241, 258, 299–300, 351
leucotrienes (LTs) 72, 236–237
life-cycle approach 378
linoleic acid 57, 58–62, 64, 67
lipid peroxides 140, 172
lipids 30, 68–69, 353
lipoic acid 146
lipoplysaccharide, spleen cells, response proliferation 214
lipoxygenase (LOX) 70
liver 111, 135, 244
local immunity, role, mucosal homoeostasis 273–304
low-birth-weight (LBW)
babies, glutamine enriched feeding formula 120
growth retardation and nutritional deficiency 47
infants 51
mortality risk 49–50
multivitamins 388
phagocytic function, deranged 51
Prevention of Low Birthweight programmes, UNICEF 378
see also birth weight
low-pH environment resistance 330
lumen 277
lung cancer 183, 184, 185
lung injury 216
lutein sources 183
lycopene 183, 184
lymphocytes
activity promotion 121, 195
circulating numbers decrease, elderly 358
clonal expansion 3, 11
concentration increases, exercise 347
growth 99
mitogens treatment 194
mobilized to circulation, exercise 348
Keshan disease 244, 389
keyhole limpet haemocyanin (KLH) 286–287
kidney 111
killer cells, selenium effects 238–241
kiwi fruit allergy 334
Klebsiella, adhering, tracheal epithelial cells 48
Kwashiorkor 25, 42, 43
lactating mammary glands, immunocyte density 284
lactating women, infections 161
lactic acid bacteria (LAB) 252, 256, 262–263
juvenile chronic arthritis 262–263
phospholipase-Cy activity, fish oil 69
probiotics effects 256–257
proliferation 99, 115, 184, 362–365
selenium effects 238–241
zinc 195
lymphoepithelial interactions, putative involvement 287–288
lymphoid cells 282
lymphoid organs 112–113
lymphokine-activated killer cells 238, 239
lymphopenia 197–198
lysozyme, decreased levels 47
macrophage
cytokine increases, elderly 366
cytotoxicity increase 101
glutamine 113, 114, 115, 116
gut-associated lymphoid tissue (GALT) 278
immune cells 299–300
iron 215–216, 220
migration inhibitory factor reduction 215
–monocyte functions, changes, ageing process 362
nutritional status influence 366
signals 12
T cells 12–14
transferrin receptor 215
vitamin A deficiency 155–156
major histocompatibility complex (MHC) 7–9, 10, 182
malaria
children 25, 27
children susceptibility increase 395
iron 394
iron status 222
micronutrient supplementation 380
vitamin A 159–160
malnourished children, zinc 383, 393
malnutrition
antibodies reduction 45
children 383, 393
human immunodeficiency virus (HIV) 24
indicator, mid upper arm circumference 383
infection 382–383
intervention strategies 53
lymphocytes subpopulations differentiation 24
prevention and treatment strategy 375–376
see also protein-energy malnutrition (PEM)
mammary glands, B-cell homing 280–281
management, food allergy 340–341
marasmus 25, 42, 43, 199
mast cells 327
maternal antibodies 276–277, 298
see also breast milk; breast-feeding
maternal diet, food allergens 333–334
maternal morbidity and mortality, vitamin A 386–388
measles 28, 157–158, 381
mechanisms
adaptive immune, mucosal surfaces 274
arginine action 99–101
effector 18–19
exercise-associated immune changes 350, 351
fatty acids, exerted effect, immune cell function 66
glutamine action 121–123
impaired cell-mediated immunity 217
iron deficiency, impaired immunity 216–217
membrane protection from peroxidative damage 243
selenium, mutants lack 231
selenium supply to selenoprotein regulation 234
tolerance induction 291
membrane proteins, function alterations 68
membrane structure and composition alterations 66–68
membrane-mediated signals, changes 68–69
memory T-cell (CD45RO+) 359
meta-analysis 379
metabolic disturbances 382–383
metabolism
arginase/ornithine pathways 96–97
ergine 94–96, 98, 102
effects, immune system response, injury 137
eicosanoid, selenium 236–237
glutamine 111–113
glutathione, following infection and injury 136–139
iron 212–214, 215–216
neutrophils, iron deficiency effects 216
selenium pathways 235
sulphur amino acids 133–139
vitamin A 151–152
metabolites production 113
metallic elements see iron; zinc
methionine 133–134, 136, 145
microbe killing, selenium effects 236
microbes, intestinal environment 251–252
microbial growth stimulation 394, 395
microbial invasion response 137
microflora 251–252
Micronutrient Conference on Ending Hidden Hunger, 1991 377
micronutrients
deficiency 383
elderly 376
human immunodeficiency virus (HIV) 380, 396
interaction 382
micronutrients continued
malaria 380
Micronutrient Conference on Ending Hidden Hunger 1991, 377
mixed, infection 396
role, preventing infection, elderly 376
see also vitamins
microorganisms 18, 251
mid upper arm circumference, malnutrition indicator 383
mitogens treatment, lymphocytes 194
modulatory hormones (cytokines) 253
molecular weight, food allergens 330
molecules, mediating innate immunity, selenium deficiency effects 239, 240
monocytes
cytokine increases, elderly 366
function, iron deficiency 215–216
functions 198–199
—macrophage, function changes, ageing process 362
peripheral-blood, elderly 363
vitamin A deficiency 155–156
monovalent allergens 329
mortality rate improvement, immunonutrition 104
mortality risk, low birth weight 49–50
mother and newborn, mucosal immunity integration 281
mucosa
barrier function 275, 276, 284–286, 303
defence 274, 300–301
effector sites, primed lymphoid cells homing 282–283
IgA system, postnatal establishment 284
immune system 28, 273, 274, 279, 281
immunity 153–154, 281, 282–286
induction, tolerance 286–294
infection, ciliary movement reduction 47, 48
tolerance induction, commensal bacteria role 294
mucosa-associated lymphoid tissue (MALT), immune induction 278–281
multimicronutrient supplements 381
murine models 258
murine splenic B-cell proliferation 213, 214
murine splenic lymphocytes, increased capacity 256
muscle 117–118, 137, 338
see also skeletal muscle
Mycobacterium tuberculosis 300
myocarditis 389
n-3 fatty acids 103
n-3 polyunsaturated fatty acids, clinical trials 77
N-acetyl-cysteine (NAC) 141, 143, 145–146
naive immune system 23
naive T-cell (CD45RA) 359
natural killer (NK) cells
activity 62, 181, 216, 351
β-carotene 180
cytokine production 22
cytotoxicity increase 101
defined 6
function 199, 221
functions, suppression 348
iron 216, 221
rat, activity inhibitor 62
response to exercise 351
selenium effects 238–241
suppression 62
vitamin A deficiency 154
nematode parasitic infections 393
neonates
antibody-mediated defence 275–278
gut milk macrophages 300
immune system 28
innate immunity 23
mucosal barrier function 28
mucosal defence 2
non-specific, immunity, iron 216, 220–221
vitamin A deficiency 154
nevirapine 397
new food introduction, diet 334, 335
newborn 281, 292, 293–294
see also children; infants; neonates
neoplasms 252–253
see also cancer; tumours
neuroblastoma, growth, supplemental arginine 100
neutrophils
concentrations, exercise 347
functions 115–116, 198
glutamine utilization 113, 114
innate immunity 2
iron 216, 220–221
vitamin A deficiency 154
night blindness 387
nitric oxide pathway, arginine metabolism 94–96
nitrogen balance 102
nomenclature, dietary sources fatty acids 58–61
non-IgE-mediated allergic reactions 323, 341–342
non-metallic elements see selenium
non-specific, immunity, iron 215
non-specific immunity 216, 217
nuclear transcription factor kappa B (NFκB) 143–144, 178, 200, 241
nucleotides 103
nutrient-immune function interaction, experimental approach 29
nutrients 21–34, 141–142, 144, 151–162
see also iron; micronutrients; probiotics; selenium; supplementation; vitamins; zinc nutritional immunology studies 34 nutritional rehabilitation, children 382 nutritional status 362, 364, 380 nutritional thymectomy 43


putative aberrant immunoregulatory functions, non-professional antigen-presenting cell (APC) 290
putative immunoregulatory factors 300
putative involvement, lymphoepithelial interactions 287–288
radioallergosorbent test (RAST) 338
rat natural killer (NK) cells activity inhibition 62
reactive oxygen species (ROS) 171–174, 241
recommended daily allowance (RDA) enteral feeding formulae 104
immune function tests 26
selenium 244–246
vitamin A 401
vitamin E, elderly 365
zinc 365, 401–402
regulatory mechanisms 1
respiratory burst defined 236
respiratory infections 54, 183, 222, 381
respiratory lymphoid systems, glutamine influence 119
respiratory morbidity increase 395
response evaluation 25–34
response microenvironment 22
rheumatoid arthritis, fish oil benefits 76, 77
ribonucleic acid (RNA) viruses 112, 244
Safe Motherhood programmes 376
salivary immunoglobulins 301
Salmonella 259, 260, 394
secondary immunodeficiency, elderly, nutritional factors, role 362–365
secondary nutritionally induced immune deficiency, elderly 358
secretory antibody system 294–296, 298–299
secretory component (SC) 277, 299
secretory immunity 283–285
secretory immunoglobulin (Ig) 277, 295
selenium acquired immune deficiency syndrome (AIDS) 31
anti-infective property 381, 382
cardiovascular disease 246
closely supervised supplementation 401
deficiency 229–247, 388–390
infection 388–390
intake relationship 244–246
recommended daily allowance 244–246
selenomethionine 234, 235
selenophosphate synthetases 234
selenoproteins 230–234
selonocysteine 230–231
selonocysteine insertion sequence (SECIS) 230
Semliki Forest virus 244
Senieur protocol 357–358
sensitization 324, 325, 327, 334
sepsis 79–80, 101–105, 140
septic complications 101–105
serine decreases, plasma 138
serum 3–6
serum antibody responses, protein–energy malnutrition (PEM) 46
shellfish allergy 342
sickle-cell disease 201, 217–218
signals and signalling
B-cell activation 14
bacterial signalling 258
CD40 12, 13
cells, selenium effects 241
co-stimulation antigen-presenting cells 288–291
danger, infection, inflammation 293
immune activation 27
immunological 256
macrophage 12
membrane-mediated 68–69
orally delivered probiotics 253–255
transduction 68–69, 195
UGA codon 230
Sinbis virus 244
skeletal muscle 109–110, 117, 118, 351–352
skin
cells 241, 242–243, 290
immune system, selenium effects 243
induration 44
test response 43, 44
tests, food allergy 337–338
tumour 243
skin-prick test, food allergy 337–338
small for gestational age (SGA) 41, 47, 51
small intestine 111
smokers 184–185, 215–216, 381
soybean allergy 334, 340
species differences, striking 275–276
specific immunity 292
spleen 64, 212, 213, 214
splenic leucocytes 258
statistical evaluation 34
stimulation, immune system, probiotics 252–253, 256–259
stress
body 28, 99
catabolic 117–118, 120–121, 123–124
oxidative 171, 244, 394–395
stromal immune clearance 295
stunting 25, 27
subclinical mastitis 398–399
substitution therapy 300–301
sulphur amino acids 133–147
superoxide dismutase defined 173
supplementation adverse consequences 159
concerns 174
overload 217–220
therapy response measure 52
trauma-induced thymic involution 99
wound healing 101
see also diet; dietary; micronutrients
suppressivemechanisms 273, 274
surface immune exclusion 295
symptoms, allergic reactions to food 323, 324, 336
synthesis
arginine 93–94
dietary sources fatty acids 58–61
eicosanoids 69–73
glutamine, interorgan transport 109–112
glutathione enhancement 144
selenoproteins 230–231
synthesizing date, publicising 379
T-cells
cell-mediated immunity 6–18
clonal expansion into effector cells 11
differentiation into effector cells 11
generation decrease 358
iron deficiency 210–212
iron overload 217–219
maturation decrease 358
receptor (TCR) 6, 10
–receptor molecule, schematic representation 7
subset distribution, spleen iron-deficiency mice 212
subsets, peripheral, changes 358–361
suppression 348
T-helper (Th) cells 12–19, 289
T lymphocytes
activation 25–26, 102, 195
apoptosis 196
breast milk 299–300
cocultures 46
functional properties 2–3
-glutathione synthetase (GSH) content 140
numbers reduction, zinc deficiency 198
peripheral blood, elderly 360
proliferation in vitro, glutamine influence 114
purified splenic, response proliferation
signal transduction, zinc role 195
subsets, children, protein–energy malnutrition (PEM) 45
supplemental arginine 100
supplementation therapy response measure 52
thymus 358
vitamin A deficiency 155
zinc 195
taurine 133, 134–135, 138, 146–147
tertiary immunodeficiency, elderly 358, 365–367
thioredoxin reductase 238, 241, 243
thromboxane synthesis 237
thymulin 196–197
thymus
atrophy 51, 201, 212
iron deficiency 212
nutritional thymectomy 43
regrowth 24
size reduction 198, 201
size and weight 43
T lymphocytes 358
trauma-induced thymic involution 99
weight, decrease 64
thymus-independent (TI) antigens 14
tissue destruction 18
tissue glutathione synthetase (GSH) content, modulating strategies 144–146
tissues 2–3, 109
tolerance
induction mechanisms 291
mucosal induction 286–294
tomatoes 28, 183
total parenteral nutrition 28, 30
trace elements see iron; selenium; zinc
tracheal-cell cilia, dogs, protein–energy malnutrition (PEM) 48
transcription factors 143, 144, 178
transferrin receptor 212–213, 215, 394
transforming growth factor (TGF)-β 302
gentic soybean allergy 334
transport, iron 210
trauma 79–80, 99, 118–120, 136–140
treatment, food allergy 340–341
treatment regime, newborn 293–294
triacylglycerol, dietary fat 57
tuberculosis 24, 27, 160–161
tumour
Bifidobacterium 258
growth 100, 260–261
Lactobacillus 258, 261
probiotics 252–253, 260–261
skin 243
thioredoxin reductases 238
see also cancer; neoplasms
tumour necrosis factor (TNF)-α 215–216, 242, 244
tumour necrosis factor (TNF) 137
type I allergic responses 322–323
UK population, food allergy 335
ultraviolet B radiation (UVB) 183, 242, 243
ultraviolet (UV) light exposure 180–181
UNICEF/WHO policy 383, 401–402
United Nations World Summit for Children 1990, 377
upper respiratory-tract infections (URTI) 349–350
urine tests, food allergy 338
vaccines development 280
veterinary allergens reactions 331
very carefully selected elderly (VCSH elderly) 358, 368
viruses
cardiotoxic 242
Coxsackie 244, 389
cytomegalovirus 400
hepatitis B 244, 389, 400
hepatitis C 394, 400
intracellular virus neutralization 295
ribonucleic acid (RNA) 244
selenium 244, 389
Semliki Forest 244
shedding 260
Sinbis 244
see also human immunodeficiency virus (HIV)
vitamin A
adults 386
capsules 401
childhood morbidity and mortality 384–386
deficiency 28, 284
HIV-associated infection in children 386
impact 381, 382
infection and immune system 151–162, 384–388
maternal morbidity and mortality 386–388
recommended daily allowance (RDA) 401–402
supplementation 378
supplementation impact 379
supplementation promotion 402
supplements 376
Th2 response 22
vitamin B6 141–142
vitamin C 142, 174–176
vitamin D 388
vitamin E
deficiency, Coxsackie virus 389
dietary sources 173–174
elderly 365, 368
immune function 176–178
mastitis 399
nuclear transcription factor kappa B (NFκB) 143
activation 143
supplementation 401
vitamins
adverse consequences 159
antioxidant 171–185
ascorbic acid 142
dietary sources 151–152, 153, 173–174
see also dietary sources; micronutrients; supplementation
Waldeyer’s pharyngeal ring 280
whey protein 145
whole food 28
World Health Organization (WHO) 375–376, 383, 401–402
World Summit for Children 2001, 400
wound healing 101, 201
zinc
apoptosis 195–196
daily doses 401
depletion 26
elderly 365
high-dose, effects, immune cell functions 200
impact 381–382
infection 390–393
infection and immunity 193–203
intervention costs 378
supplement, oral, preterm infants 52