

NATURAL ANTIOXIDANTS IN AVIAN NUTRITION AND REPRODUCTION

Peter F. Surai

 **NOTTINGHAM**
University Press

Nottingham University Press
Manor Farm, Main Street, Thrumpton
Nottingham, NG11 0AX, United Kingdom

NOTTINGHAM

First published 2002
© PF Surai

All rights reserved. No part of this publication may be reproduced in any material form (including photocopying or storing in any medium by electronic means and whether or not transiently or incidentally to some other use of this publication) without the written permission of the copyright holder except in accordance with the provisions of the Copyright, Designs and Patents Act 1988. Applications for the copyright holder's written permission to reproduce any part of this publication should be addressed to the publishers.

British Library Cataloguing in Publication Data
Poultry Environment Problems: A Guide to Solutions

ISBN 1-897676-95-6

Disclaimer

Every reasonable effort has been made to ensure that the material in this book is true, correct, complete and appropriate at the time of writing. Nevertheless the publishers, the editors and the authors do not accept responsibility for any omission or error, or for any injury, damage, loss or financial consequences arising from the use of the book.

Typeset by Nottingham University Press, Nottingham
Printed and bound by Bookcraft, Bath, England

CONTENTS

Preface	xv
1. Antioxidant systems in the animal body	
Introduction	1
Free radicals and reactive oxygen and nitrogen species	1
Three levels of antioxidant defence	4
Antioxidant-pro-oxidant balance in the body and stress conditions	14
Conclusions	18
References	19
2. Vitamin E	
Introduction	27
Chemical structure and biological activity	28
Occurrence in nature and feed sources	32
Absorption and metabolism	36
Tocopherol and tocotrienol discrimination in the chicken embryo	50
Absorption and metabolism impairment	54
Tocopherol binding proteins	62
Molecular mechanisms of action	65
Membrane stabilising effect of vitamin E	65
Antioxidant properties of vitamin E	70
Non-antioxidant action of vitamin E	77
Vitamin E deficiency	77
Vitamin E excess	86
Vitamin E requirement and supplementation	92
Conclusions	95
References	96
3. Carotenoids	
Chemical structure and properties	129
Occurrence in nature and feed sources	131
Carotenoid absorption and bioavailability	134
Molecular mechanisms of action	140

Carotenoids and vitamin A	141
Other carotenoid functions	145
Antioxidant properties of carotenoids	147
Evaluation of the antioxidant activity of carotenoids	150
Antioxidant action of carotenoids <i>in vivo</i>	154
Carotenoid-vitamin E interactions	163
Modulation of antioxidant systems by carotenoids	168
Carotenoids in avian plasma and egg yolk	170
Carotenoids and chick embryo development	184
Carotenoid discrimination in avian tissues	194
Conclusions	199
References	200

4. Selenium

Introduction	233
Occurrence and feed sources	234
Absorption, availability and metabolism	236
Molecular mechanisms of action: selenoproteins	240
Glutathione peroxidase	240
Thioredoxin reductase	244
Iodothyronine deiodinases	246
Other selenoproteins	248
Selenium deficiency	250
Selenium toxicity and selenosis	252
Pro-oxidant properties of selenite and possible antioxidant protection by SeMet	254
Selenium and chicken embryo development	259
Selenium and semen quality	270
Selenium requirement for poultry breeders	272
Selenium and egg freshness	273
Selenium and meat quality, broiler performance and feathering	275
Practical approaches to improve selenium status of poultry	280
Conclusions	282
References	283

5. Developing optimal egg status for a viable chick

Introduction	305
--------------	-----

Lipids and polyunsaturated fatty acids	305
Natural antioxidants	311
Vitamin E in egg yolk	311
Vitamin E and hatchability	316
Antioxidant systems and avian embryo development	318
Digestive and immune system development	340
Conclusions	344
References	344

6. Antioxidant enriched eggs: from improvement of egg composition to functional food

Introduction	359
The egg and cholesterol	360
Designer eggs as a source of essential nutrients in the human diet	362
Iodine enriched eggs	363
n-3 designer eggs	364
Other types of designer egg	367
Organoleptic quality of modified eggs	367
Health benefit of modified eggs	369
Commercial production of modified eggs	371
Super egg development and evaluation	373
Conclusions: eggs are on the way to functional food	378
References	380

7. Fatty acid composition and antioxidant protection of avian semen

Introduction	391
Vitamin E and fertility	391
Fatty acid composition of avian semen	392
Lipid peroxidation in avian semen	396
Antioxidant protection in avian semen	404
Superoxide dismutase	405
Selenium and glutathione peroxidase	407
Vitamin E	408
Ascorbic acid	411
Glutathione	413
Antioxidant properties of seminal plasma	415

Dietary effects on fatty acid composition and antioxidant capacity of semen	422
Polyunsaturated fatty acids	422
Seleni	425
Vitamin E	428
Conclusions	434
References	435

8. Natural antioxidants and mycotoxins

Introduction	455
Mycotoxins and lipid peroxidation	456
Ochratoxin A	456
T-2 toxin	459
Fumonisin B1	462
Aflatoxin B1	464
Aurofusarin	466
Other mycotoxins	471
Effects of mycotoxins on antioxidant systems	472
Mycotoxins and apoptosis	478
T-2 toxin	479
Fumonisin B1	480
Ochratoxin A	481
Other mycotoxins	482
Protective effects of antioxidants against mycotoxicoeses	482
Conclusions	490
References	491

9. Natural antioxidants and immunity

Introduction	511
The avian immune system and its evaluation	511
Antioxidants and antibody production	517
Antioxidants and lymphocyte function	527
Antioxidants and phagocyte function	531
<i>In vitro</i> effects of antioxidants on the immune system	533
Antioxidants and disease resistance	536
Mechanisms of immunostimulating properties of antioxidants	539
Protection of cell membranes and receptors	540

Effect on immunomodulator production	540
Prostanoid synthesis regulation	541
Effect on signal transduction	541
Apoptosis regulation	542
Conclusions	543
References	545

10. Natural antioxidants: from the animal feed industry to human health

Introduction	565
Diet optimisation and human health	565
Polyunsaturated fatty acids	565
Natural antioxidants	568
Vitamin E	570
Carotenoids	574
Selenium	578
Are there ideal diets?	583
Hunter-gatherer diet	584
Mediterranean diet	585
Japanese diet	587
Antioxidants and functional food	589
Conclusions	591
References	592
Index	605

NATURAL ANTIOXIDANTS AND IMMUNITY

“Best defence is attack”

Introduction

All animals protect themselves from invasion of microorganisms, parasites, fungi, viruses and any foreign molecules. This protective capacity is based on the effective immune system which is considered to be an important determinant of animal health and wellbeing. In that sense, a remarkable ability of components of the immune system to distinguish between self and non-self is a great achievement of animal evolution.

Commercial poultry production is based on balanced feed, providing requirements in major nutrients and optimised environmental conditions. However, it is very difficult to avoid various nutritional or environmental stresses which are responsible for immunosuppression and increased susceptibility to various diseases and as a result decreased productive and reproductive performance of birds. For example, mycotoxins are among the major immunosuppressive agents in a poultry diet. In such situations immunomodulating properties of certain macro and micronutrients are of great importance for the poultry industry. Information has been actively accumulated for the past 10 years indicating that vitamin E, Se and carotenoids are among major immunostimulating agents and their requirements for such action are usually higher than those for bird growth and development.

The avian immune system and its evaluation

Chicken defence against various diseases depends on the efficacy of the immune system responsible for elimination of foreign substances (e.g., parasites, bacteria, moulds, yeast, fungi, viruses and various macromolecules) or the creation of specific inhospitable conditions within the host for a wide range of pathogens. There are two major types of immune function: natural and acquired immunity (Figure 9.1). Natural immunity, called the innate immune system, includes physical barriers (e.g., skin, mucus coat of the GI tract), specific molecules (e.g., agglutinins, precipitins,

acute phase proteins, lysozyme), phagocytic function of phagocytes (macrophages and heterophils), and lysing activity of a class of lymphocytes called natural killer (NK) cells. These cells were originally described as a population of granular lymphocytes with the ability to lyse tumour and virally infected target cells. They differ from classic lymphocytes being larger in size, containing more cytoplasm and having electron dense granules. The mechanism of killing is mediated through release of its granule contents (perforins and granzymes) onto the surface of the infected cell (Lydyard *et al.*, 2000).

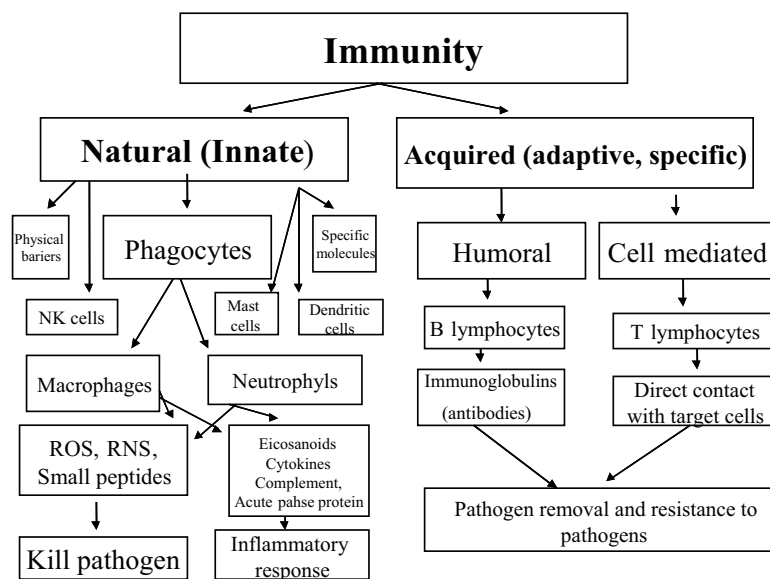


Figure 9.1 General scheme of the immune system.

Macrophages perform a range of functions, including phagocytosis of foreign particles, destruction of bacterial or tumour cells, secretion of prostaglandins and cytokines and as a result regulating activity of lymphocytes and other macrophages (Qureshi, 1998). In fact, phagocytosis is the major mechanism by which microbes are removed from the body and is especially important for defence against extracellular microbes. As a result of stimulation (for example by microbes) monocytes differentiate into macrophages that are more powerful in mediating host defence (Klasing, 1998a). The phagocytic process includes several stages (Lydyard *et al.*, 2000):

- Movement of phagocyte towards the microbe using chemotactic signals

- Physical contact of the micro-organisms with macrophage and attachment to the phagocyte surface with a nonspecific or receptor-mediated binding.
- Endocytosis (engulfment) of microbe resulting in a phagosome by invagination of surface membrane
- Fusion of the phagosome with a lysosome
- Killing of microbes by bombarding them with oxidants (superoxide and hydroxyl radicals, hydrogen peroxide, nitric oxide, hypochlorous acid etc.).

Macrophage activation and phagocytosis of foreign particles are regularly accompanied by a so called “respiratory burst”, an increase in the production of reactive oxygen species (ROS), exerted by the enzyme complex NADPH oxidase (Figure 9.2). Therefore macrophages as well as other phagocyte leukocytes (e.g., neutrophils, monocytes and eosinophils) can synthesise toxic oxygen metabolites such as superoxide anion (O_2^-), hydroxyl radical (OH^*), singlet oxygen (1O_2), H_2O_2 , nitric oxide (NO) and peroxynitrite ($ONOO^-$) during the respiratory burst (Zhao *et al.*, 1998). For example, a bacterium coming into contact with the plasma membrane is enclosed in a plasma membrane vesicle containing NADPH oxidase, and exposed to an intensive flow of superoxide radical (Giller and Sigler, 1995). Superoxide radical can disproportionate to H_2O_2 which penetrates into the bacterium with a production of hydroxyl radical, which is ultimately a deadly weapon able to damage any biological molecules. In general, the production of ROS and reactive nitrogen species is a characteristic for both mammalian and avian macrophages (Qureshi *et al.*, 1998). It is interesting that people whose phagocytes possess no functional NADPH oxidase, are shown to suffer from chronic infections of the skin, lung, liver and bones leading to a premature death (Halliwell and Gutteridge, 1999).

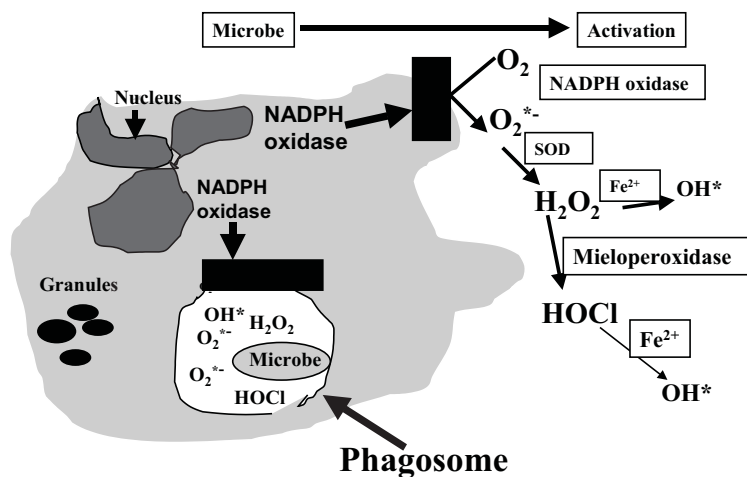


Figure 9.2 Respiratory burst in neutrophils (adapted from Kettle and Winterbourn, 1997; Nordberg and Arner, 2001).

In general ROS, RNS and eicosanoids (e.g., leukotrienes and prostaglandins) have recently received substantial attention as major metabolites produced by macrophages (Dietert and Golemboski, 1998). Because of this powerful weapon, macrophages bind, internalize, and degrade foreign antigens (e.g. bacteria) quite quickly. It takes only 15 minutes for chicken macrophages to kill more than 80% of the internalised *Salmonella* (Qureshi *et al.*, 1998). Therefore natural immunity works rapidly, gives rise to the acute inflammatory response. They contain various substances (including enzymes producing free radicals and small peptides with an antibiotic activity) involving in microbial killing. They also have receptors for chemoattractive factors released from microbes. In addition to ROS, RNS and eicosanoids mentioned above, macrophages also synthesise and secrete a great number of such communicational molecules as cytokines, including the proinflammatory cytokines called interleukin 1 (IL-1), interleukin 6 (IL-6), and tumour necrosis factor- α (TNF). They also produce cytokine inhibitors, endocrine hormones, neurotransmitters (Klasing, 1998a) which regulate specific immunity (initiating and directing the immune and inflammatory responses) and many other related physiological responses. Therefore these cells are important amplifiers of the immune response, both by cytokine production and by serving to present parasite derived peptides to T cells.

The toxic metabolites produced by macrophages are used as an important weapon in their microbicidal activities. However, they also can cause a series of harmful effects, including killing host cells, injuring cells and tissue directly via oxidative degradation of essential cellular components and damaging cells indirectly by altering the protease/ antiprotease balance that normally exists within the tissue interstitium (Conner and Grisham, 1996). They also can cause cell mutations and sister chromatid exchanges (Weitzman and Stossel, 1981). Therefore, overproduction of ROS or impaired antioxidant defence can result in oxidative damage to host macromolecules (McBride *et al.*, 1991) and extensive pathology and disease (Dietert and Golemboski, 1998). For example, in the case of disease challenge, the overproduction of ROS may occur and they can leak from fagolysosomes into the surrounding cytoplasm and intracellular space. A number of antioxidant enzymes are expressed at the same time to protect the cells from the cytotoxic effects of ROS directed against engulfed microorganisms. However, low efficiency of these antioxidant mechanisms, for example due to Se deficiency, could damage the cell's microbicidal and metabolic functions (Larsen, 1993). Indeed, macrophage function is disturbed, if there is a lack in antioxidant enzyme activity (Ebert-Dumig *et al.*, 1999). It was shown that TrxR activity as well as GPx activity in these cells can be upregulated by the addition of Se *in vitro* and *ex vivo*.

Heterophils are the major granulated leukocytes in the acute inflammatory response in birds (Harmon, 1998). They interact with endothelial receptors in the vessel, emigrate through the vascular wall and are accumulated in the site of

inflammation. Heterophils are activated by cytokines and chemokines and it is believed that they use mainly non-oxidative microbial mechanisms with lack of myeloperoxidase (MPO; Harmon, 1998). However, this point of view was challenged by Lam (1997) presenting evidence that chicken heterophils contain a DNA sequence that is homologous to segment 10 of the human MPO gene; histochemical staining and microplate assay also showed that heterophils contain MPO activity. Furthermore, stimulation of chicken and turkey heterophils with ionomycin resulted in significant increases in adherence, percentage of cells with a shape change, phagocytosis, intracellular calcium concentration, and oxidative burst (Kogut *et al.*, 1998a). Regardless of which killing mechanisms heterophils employ, they are highly effective in killing bacteria.

Once a pathogen enters to the host, the initial non-specific response is an inflammatory reaction. It results in a series of behavioral, immunologic, vascular and metabolic responses (Korver and Klasing, 2001). As a result growth rate could be slowed, appetite decreased, muscle protein degradation increased with possible increased morbidity and mortality and decreased productivity. Therefore a price for this defence against pathogens could be quite high.

Acquired or specific immunity includes humoral immunity and cell mediated immunity. There are two major types of lymphocytes, B cells and T cells. Humoral immunity is mediated by antibodies that are released by B lymphocytes into the bloodstream. The bursa of Fabricius is the site of B lymphocyte development and differentiation. This immunity is based on the production of immunoglobulins. They are responsible for specific recognition and elimination of various antigens: they bind and remove from the host invading organisms/substances.

Cell mediated immunity is based on specific antigen recognition by thymus derived T lymphocytes. Due to this immunity cells infected with a foreign agent, for example virus, are destroyed via a direct contact between an activated T cell and target (infected) cell (Qureshi *et al.*, 1998). Cell mediated immunity is responsible for delayed type hypersensitivity reactions, foreign graft rejections, resistance to many pathogenic microorganisms and tumor immunosurveillance (Wu and Maydani, 1998).

In birds, both T cell and B cell precursors originate in the bone marrow. Actual development of T cells takes place in the thymus and B cells develop in the bursa Fabricius (Lahtala, 1998). Interactions between T and B cells, as well as antigen presenting cells, are responsible for the development of specific immunity. These defence mechanisms of specific immunity are induced or stimulated by exposure to foreign substances, are specific for distinct macromolecules and increase in magnitude with each successive exposure to a particular macromolecule (Miles and Calder, 1998). In comparison to natural immunity, specific immunity takes longer to develop, but is highly specific for antigens and has memory. These two parts of the immune system work together through direct cell contact and through

interactions involving such chemical mediators as cytokines and chemokines. Therefore the chicken immune system requires the cooperation of macrophages, bursa derived B lymphocytes and thymus derived T lymphocytes with various other types of cells.

Therefore, the immune response involves cellular proliferation (T lymphocytes), enhanced protein synthesis (including immunoglobulin synthesis by B lymphocytes and acute phase protein synthesis by liver) and inflammatory mediator production. Physiological changes resulting from stimulation of the immune system include fever, anorexia and loss of tissue components (Grimble, 1997).

When analysing data related to immunomodulating properties of various nutrients it is necessary to pay special attention to methods used to assess immunological functions. For example, *in vivo* methods of immune function assessment are based on two main approaches: antibody response to vaccine or delayed-type hypersensitivity (DTH) reaction. In the first method immunisation with appropriate antigens (viral or bacterial) can elicit serum antibodies. So called haemagglutination (HA) assay measures serum antibody concentration (titre) against antigens. Sheep red blood cells (SRBC) are often used as antigens. This assay provides information about humoral immunity (B cell responsiveness) and its association with cell mediated immunity (T cell co-operation). The second (DTH) method is used to assess cell mediated immune function.

In vitro indices of immune function include (Wu and Meydani, 1999):

- *Lymphocyte proliferation assay.* Lymphocyte proliferation assay provides information about cell mediated immune response and includes measuring the number of cells in culture with or without addition of stimulatory agent (mitogen). In this assay, isolated lymphocytes are incubated with mitogens which activate division of either T-or B-lymphocytes. Various mitogens are used in such assays, but most often they include concanavalin A (conA, T cell mitogen), phytohemagglutinin (PHA, T cell mitogen), lipopolysaccharide (LPS, B cell mitogen) and pokeweed mitogen (PWM, T and B cell mitogen) (Hayek *et al.*, 1996). Decreased proliferation may indicate impaired cell mediated immunity.
- *Measuring cytokine production.* T cells produce a range of protein mediators called cytokines which regulate cell activation, growth, differentiation, inflammation and immunity.
- *Cytotoxicity assay.* This assay assesses activity of cytotoxic T lymphocytes (a group of T cells that kill other cells by recognising their cell surface antigens) and natural killer (NK) cells (a group of non T and non-B lymphocytes that kill virus infected and tumour cells).
- *Flow cytometric analysis.* The assay deals with identifying the cells with different surface markers. The results can be used for understanding the cellular basis of immune response.

- *Plaque-forming cell (PFC) test*, which shows the number of antibody producing cells.

Effect and role of the chicken immune system in modern poultry production is difficult to overestimate. Banning feed grade antibiotics in Europe will make immune system competence the major factor determining efficiency of poultry production. Molecular immunology is developing very quickly and mechanisms of chicken immunocompetence recently have received substantial attention (McCorkle, 1998; Saif and Swayne, 1998) and nutritional modulation of resistance to infectious diseases in poultry (Klasing, 1998) is a front-line for future research.

Among different nutrients playing an important role in modulating immune response (Klasing, 1998) are natural antioxidants. Selenium (Turner and Finch, 1991; Larsen, 1993; MacPherson, 1994; McKenzie *et al.*, 1998), vitamin E (Kelleher, 1991; Wu and Meydani, 1998) and carotenoids (Bendich, 1989; Moller *et al.*, 2000) are shown to be very promising. However, antioxidant roles in avian immune system modulation received only limited attention. Mechanisms of nutritional modulation of resistance to infectious diseases were divided by Klasing (1998) into seven categories. Vitamin E was included into two categories (“direct regulatory action of nutrients on the immune system” and “reduction of pathology”). Carotenoids and Se were not mentioned there but both these antioxidants can be included in the same categories as vitamin E.

It is well known that several indicators of immune responsiveness are depressed when chicks are Se and/or vitamin E deficient. Decreases in both cellular and humoral immune function in man, laboratory and farm animals and chickens are also observed (Combs and Combs, 1986). Since these nutrients serve as antioxidants, membrane integrity may be affected by a deficiency. However, cellular integrity is very important for receiving, and responding to the messages needed to coordinate an immune response (Latshaw, 1991). Therefore the antioxidant status of the host is a critical consideration in the optimal functioning of the immune system. Furthermore, modulating immune responses of birds by nutritional means in many cases targets specific immunity (Korver and Klasing, 2001).

Antioxidants and antibody production

Research data accumulated for the past few years clearly indicate that antioxidant deficiency is associated with impaired immune system in human and laboratory and farm animals. For example, when 2 week old chickens were maintained on Se or Se-vitamin E deficient diets from hatching, they had reduced antibody titres to SRBC (Marsh *et al.*, 1981). In the same experiment 3 week old chicks had decreased antibody production only in combined Se-vitamin E deficiency. Therefore

it was suggested that Se and vitamin E may be important components of immune function, and their effects depend on antigen concentration, sex, and the age (Larsen, 1993).

In contrast to vitamin E and/or Se deficiency, antioxidant supplementation of the diet is shown to have immunostimulating effect and the level of vitamin E or Se required for optimal immune function may be higher than that required for growth and other physiological functions. The simplest tests with antibody production against SRBC clearly showed a beneficial effect of vitamin E and Se. For example Tengerdy *et al.* (1972) demonstrated that feeding vitamin E (60 mg/lb) to chickens increased antibody production in response to intraperitoneal injections of SRBC. Similarly, in mice vitamin E (60-180 mg/kg) increased the HA response against intraperitoneal injections of SRBC and tetanus toxoid (Tengerdy *et al.*, 1973). When challenged at 7 days after posthatching, total anti-SRBC and IgM antibodies were higher in poult treated *in ovo* with vitamin E (10 IU) than in controls (Gore and Qureshi, 1997).

Newcastle disease challenge is often used to assess immunostimulating properties of various antioxidants. For example, the immune response of chicks vaccinated with living Newcastle disease vaccines was significantly improved by selenium and vitamin E. Diets were supplemented 14 days before vaccination, at a ratio of 0.25 ppm and 300 mg/kg respectively and improvement in haemagglutination inhibition (HI) antibody titres and protection rate against challenge with velogenic Newcastle disease virus were observed (Bassiouni *et al.*, 1990). When Se was added to the feed of White Leghorn chickens (between 0.1 and 0.8 mg/kg) before challenge with either *E coli* or sheep erythrocyte antigen an antibody titre against sheep erythrocytes increased by 77% (Larsen *et al.*, 1997). Following chilling, antibody titre response was substantially reduced and the titre reduction was prevented with dietary additions of Se between 0.1 and 1.2 mg/kg (Larsen *et al.*, 1997). Furthermore, significantly higher antibody titres at 10 days post immunisation for Newcastle disease virus were attributed to 0.06 mg/kg and 150 IU/kg Se and vitamin E, respectively (Swain *et al.*, 2000). The antibody immune response in White Leghorn chicks was also significantly improved for the Se enrichment diet in the *Salmonella* or *Salmonella* and aflatoxin inoculated groups (Hegazy and Adachi, 2000). In another experiment the starter and finisher diets of three groups of chickens contained vitamin E 60 and 50, 90 and 75, and 120 and 100 IU/kg, respectively (Mazija *et al.*, 1992). Each chicken was vaccinated at 14 days of age against Newcastle disease. Immune responses of the three groups were compared with those of a control group given starter and finisher diets containing vitamin E 30 and 25 IU/kg. The highest immune response, measured by haemagglutination titres of specific antibodies, was in the group given the highest amount of vitamin E.

Vitamin E supplementation (300 mg/kg diet) had beneficial effects on chicken serum antibody development against Newcastle virus disease vaccine, phagocytic index and delayed hypersensitivity index as well as on live weight gain, weight of lymphoid organs, feed intake, feed conversion efficiency (Raza *et al.*, 1997). At the same time vitamin E deficiency adversely affected all these parameters. Antibody titres to killed Newcastle disease vaccine increased by the vitamin E dietary supplementation (up to 100 mg/kg diet) or direct injection into eggs and, in ovo administration (2.5-5 mg vitamin E/egg) was more efficient in increasing the immune response (Hossain *et al.*, 1998). In an experiment conducted by Blum *et al.* (1992) from hatching to slaughter, 3600 male and female broiler chickens were fed on diets supplemented with vitamin E (20, 40, 80 and 160 mg/kg diet). At 6 weeks of age, 52 broilers from each group were vaccinated against Newcastle disease. Serum antibodies increased gradually, independently of sex. After 7 days low titres were observed with the lowest value in high vitamin E groups. However, antibody titre increased significantly after 14 days with the highest value in the highest vitamin E group. These data clearly showed that time of immune system assessment after its stimulation is also crucial.

Selenium can also be used with drinking water. For example, Deng *et al.* (1999) studied effects of sodium selenite solution on the humoral immunity and erythrocyte immunity function against Newcastle disease in chicks. Their results showed that the HI titre of the control group was much lower than that of the four Se supplemented groups. At the same time, the erythrocyte immune function of the control group was lower than that of the tested groups. Similarly, when chickens were vaccinated with attenuated strain C30-86 of Newcastle disease virus via drinking water at 3 and 21 days of age supplementation with Se through water increased HI antibody titre by stimulating antibody production (Yang Hong *et al.*, 2000).

In an experiment by Panda and Rao (1994), 90 day old male chicks from a single hatch were infected with infectious bursal disease (IBD) virus by intraconjunctival inoculation with a stimulation with a subcutaneous injection of *Brucella abortus* antigen. As a result, they showed an enhancement of immune responses (increased antibody titre) due to vitamin E Se supplementation. When vitamin E was given to chickens, the efficacy of immunisation with an infectious bursal disease virus vaccine was improved (Shadaksharappa *et al.*, 1998) with an increased antibody production.

Poults treated with vitamin E had higher numbers of Sephadex elicited inflammatory exudate cells, as well as a greater percentage of phagocytic macrophages (Gore and Qureshi, 1997). As mentioned in chapter 5, increased vitamin E supplementation of the maternal diet could be an effective means of improved immunocompetence in newly hatched chicks. In fact, chicks hatched

from hens fed on high vitamin E supplemented (300 mg/kg) diet had significantly higher antibody titres at 1 and 7 days of age than chicks from the control group (Haq *et al.*, 1996).

Results of immune system assessment also depend on the tests used. For example, high levels of vitamin E (5, 10 and 25 times the recommended level) in the turkey diet reduced total antibody titres to SRBC and the levels of IgM, whilst IgG levels and macrophage function were enhanced (Ferket *et al.*, 1995). In an experiment conducted by Heffels-Redmann *et al.* (2001) one day old turkey poults were fed on the diet supplemented with different levels of vitamin E (40, 100, 200, 400 and 800 mg/kg feed). The poults were inoculated with the test antigens Newcastle disease virus (NDV) and injected with SRBC, and were tested for antibody production using the haemagglutination inhibition (HI) and haemagglutination test (HA), respectively. While the development of HI antibodies against NDV was not improved, the groups with 400 and 800 mg vitamin E/kg feed were characterised by improved antibody production in response to SRBC.

When considering immunostimulating properties of antioxidants it is necessary to take into account interactions between antioxidants and other nutrients in particular PUFAs. For example, comparisons were made among control cockerels and those fed diets containing safflower or maize oils or beef tallow at 3, 6 or 9% with each of these diets supplemented with 0 or 300 mg of all-rac- α -tocopheryl acetate (Nockels *et al.*, 1992). In this experiment chick antibody titres to sheep red blood cells were markedly stimulated by 6% tallow without added vitamin E. However, supplemental vitamin E depressed antibody titres in the 6% tallow group. Therefore vitamin E did not enhance antibody titres and they were actually depressed when tallow was fed. Clearly there are significant interactions between vitamin E and fatty acids that affect the immune system. For example in rats, antibody production against sheep red blood cells was highest in rats fed on the fish oil diet supplemented with vitamin E at 900 mg/kg diet (Fritsche *et al.*, 1992).

It is worth mentioning some controversy among results of immunostimulating properties of vitamin E. For example, immunological responses of cockerels fed diets containing either 10 or 300 mg/kg of vitamin E were measured in three commercial broiler nuclear lines designated as A, B, and C (Boa-Amponsem *et al.*, 2000). For cockerels of lines A and B, the higher level of vitamin E maintained primary and secondary SRBC antibody levels, whereas for Line C, levels were depressed. The effect of the higher vitamin E diet on IgG and IgM was also stock dependent. In another experiment chickens were fed four diets with added vitamin E (0, 10, 30, and 150 mg/kg) and turkeys were fed three diets with added vitamin E (0, 50, and 150 mg/kg; Friedman *et al.*, 1998). Significantly higher responses were measured in chickens that received low (0 and 10 mg/kg feed) vitamin E supplementation, whereas in chickens receiving 30 and 150 mg/kg, antibody production was significantly lower. Similarly in turkeys, antibody titres to Newcastle

disease and turkey pox vaccines were highest in groups receiving no additional vitamin E, whereas titres in groups receiving 150 mg/kg were significantly lower. These results are in conflict with many other observations indicating a stimulating effect of vitamin E in similar doses on the immune system of birds. It is necessary to underline that immunological response depends on time when it was assessed. An inconsistency in dose dependent stimulation of the immune system by vitamin E was observed by Jackson *et al.* (1978) who reported that passively transferred antibody levels were significantly increased in plasma of 2 and 7 day old chicks when maternal diet was supplemented with 150 and 450 mg/kg vitamin E prior to immunisation with *Brucella abortus*. However, other vitamin E doses (90, 400 and 900 mg/kg) were not effective, indicating a non-linear antibody response by the reticuloendothelial system to vitamin E. These discrepancies indicate a requirement for more research in this field. It is important to remember that various stress conditions could have a detrimental effect on the immune system and in such conditions natural antioxidants could be beneficial. For example, feeding oxidised fat for 14 days to broiler chicks resulted in depressed growth rate, feed utilisation, plasma α -tocopherol and ascorbic acid concentrations, but increased TBARS in plasma and the ratio of heterophils to lymphocytes in blood, reduced microsomal cytochrome P-450 content in liver and primary antibody production to *Brucella abortus* (Takahashi and Akiba, 1999).

Immunostimulating properties of antioxidants are not restricted to avian species as information is quickly accumulating indicating that this phenomenon has important applications to other farm animals. For example, increased Se supplementation was also beneficial to swine immunity. In particular, the most consistent trend was seen in the humoral immune response to lysozyme, where diets with 0.9 mg/kg Se and various concentrations of vitamin E invariably produced higher titres than diets which contained 0.3 mg/kg Se (Blodgett, *et al.*, 1988). However, cell mediated immunity as measured by the *in vivo* skin response to phytohaemagglutinin was similar among diets. Piglets receiving the higher doses of vitamin E (200 ppm) and Se (0.3 ppm) had more antibodies present against Aujeszky vaccine in the 3rd week postweaning than piglets receiving the lower doses (30 and 0 ppm) (Daza *et al.*, 2000). Also in pigs, haemagglutination assays indicated that vitamin E (220 IU/kg diet) and Se (0.5 mg/kg diet) independently increased the immune response, particularly during the latter weeks of the experiment (Peplowski *et al.*, 1980). The combination of both nutrients provided in the diet or by injection, resulted in a further increase in haemagglutination titres, suggesting an additive response.

Vitamin E (476 mg/kg of feed) enhanced the primary immune response of 6 month old lambs to haemocyanin and lambs given 3 g vitamin E by mouth developed higher antibody titres to *Brucella ovis* than untreated animals (Ritacco *et al.*, 1986). Similarly, immunisation of lambs against *Clostridium perfringens* was

more effective with increased vitamin E supplementation causing increased HA titres of animals (Tengerdy *et al.*, 1983). The results of Daniels *et al.*, (2000) indicate that, although supplemental vitamin E to the ewe (400 IU/kg feed) increased lamb serum vitamin E concentration, it had no effect on measures used in this study to assess humoral immunity in the ewe or passive immunity to the lamb. However, Se (0.2 mg/kg diet) and vitamin E (20 mg/kg diet) independently increased the immune response of lambs challenged with a viral pathogen in comparison to animals fed on the diet without Se or vitamin E supplementation (Reffett *et al.*, 1988). Rainbow trout given vitamin E dietary supplementation (450 mg/kg diet) increased the antibody response to vaccination against enteric redmouth disease while antibody titres were decreased in those not given vitamin E (Verlhac *et al.*, 1993).

Selenium is toxic at high doses so it is necessary to be careful choosing the correct dose of this trace element for an experiment. For example, mice were supplemented parenterally with Se at 1.5-1.9 mg/kg body weight, vitamin E at 500-600 IU/kg body weight or Se plus vitamin E (25-100 mg and 7.5-30 IU/mouse, respectively) 24 hours before primary (day 1) and secondary (day 22) challenge with ovalbumin (OVA) 0.2 ml (intraperitoneally) (Jones and Stevenson, 1993). Treatment with Se alone or the Se/vitamin E combination significantly decreased circulating anti-OVA IgG antibody responses compared with those in unsupplemented controls. Mice treated with vitamin E alone gave results comparable with controls. Therefore, over supplementation with Se, in the presence or absence of vitamin E and in amounts insufficient to cause clinical toxicity, may impair vaccination responses.

β -Carotene was shown to be effective in increasing antibody titres in cockerels (McWhinney *et al.*, 1989), wild gulls (Blount *et al.*, 2001) and rainbow trout (Amar *et al.*, 2000). Similarly lutein (Jyonouchi *et al.*, 1994), astaxanthin (Jyonouchi *et al.*, 1993; 1995; 1995a; Okai and Higashi-Okai, 1996), canthaxanthin (El-Haffez *et al.*, 2000) and zeaxanthin (Jyonouchi *et al.*, 1996) were effective in increasing antibody production in various *in vitro* systems (Tables 9.1-9.3). Furthermore, lutein partially restored decreased *in vivo* antibody production in response to T dependent antigen in mice (Jyonouchi *et al.*, 1994).

One more important option in using vitamin E to enhance the immune response is its adjuvant role. In general, an adjuvant is considered to be a substance which, when mixed with an antigen, enhances the immune response against the antigen (Hayek *et al.*, 1996). Franchini *et al.* (1986; 1991; 1995) using different quantities of vitamin E as adjuvants for vaccines demonstrated the enhancing effect of this vitamin to viral and bacterial antigens. In fact, vaccines with vitamin E, especially when it replaced 20-30% of mineral oil, induced a more rapid and higher antibody response than control vaccines (Franchini *et al.*, 1995). Positive effects of vitamin E as an adjuvant for *Clostridium toxoid D* vaccine were recorded for sheep

Table 9.1 Effect of b-carotene on immunity

<i>Birds</i>	
Antibody titres in response to Newcastle disease in cockerels ↑	McWhinney <i>et al.</i> , 1989
Ig concentration in eggs of wild gulls ↓ (dietary supplementation: mixture of lutein, zeaxanthin, canthaxanthin and b-carotene)	Blount <i>et al.</i> , 2001
T lymphocyte proliferation in day old chicks in response to mitogens ↑ as a result of maternal diet supplementation	Haq <i>et al.</i> , 1996
Alleviated detrimental effect of aflatoxin B1 on duck lymphocyte proliferation <i>in vitro</i>	Cheng and Pang, 1996
Number of macrophages in ducks ↑	Cheng <i>et al.</i> , 2001
Phagocytosis and haemotaxis of Sephadex-50 activated peritoneal macrophages ↑ and cellular immunity (skin swelling test) ↑ in ducklings	Cheng <i>et al.</i> , 1999
Disease protection ↑ and hepatomegaly caused by <i>E coli</i> infection ↓ in chickens	Tengerdy <i>et al.</i> , 1990
<i>Farm animals</i>	
Neutrophilic granulocyte percentage ↓; lymphocyte percentage ↑; lymphocyte blastogenesis induced by PHA and Con A ↑ in weaned pigs	Zomborszky-Kovacs <i>et al.</i> , 2000
Mitogen-induced lymphocyte proliferation ↑ but killing ability by PMN ↓ in piglets	Hoskinson <i>et al.</i> , 1992
Mitogen-induced lymphocyte proliferation ↑; phagocytic activity of blood neutrophils and intracellular killing by blood neutrophils ↑ in cows	Michal <i>et al.</i> , 1994
Phagocytosis after dry off and kill of <i>Staphylococcus aureus</i> before dry off ↑ in cows	Tjoelker <i>et al.</i> , 1988
Lymphocyte proliferation in response to Con A, PH or PWM ↑; phagocytic activity of neutrophils and intracellular killing by neutrophils ↑ in cows	Michal <i>et al.</i> , 1994
Serum complement activity and total plasma immunoglobulin ↑ in rainbow trout	Amar <i>et al.</i> , 2000
Survival rates of b-carotene supplemented larvae of parrotfish ↑; spleen lymphocyte proliferation in response to mitogens ↑	Tachibana <i>et al.</i> , 1997
<i>Laboratory animals</i>	
PHA-induced lymphoblastogenesis ↑ in mice	Chew <i>et al.</i> , 1999
IL-2 secretion by lymphocytes ↑, mitogen-induced proliferation of T cells ↑ in mice	Buiuklinskaia <i>et al.</i> , 1992
No effect on susceptibility to UV immune suppression in mice	Noonan <i>et al.</i> , 1996

Table 9.1 (contd)*Laboratory animals (contd)*

T cell proliferation in response to ConA ↑; T-cell and macrophage cytotoxicity ↑ in mice	Abronina <i>et al.</i> , 1993
Prevented radiation induced thymus involution and lymphopenia in mice	Seifter <i>et al.</i> , 1984; Bendich, 1989
T and B lymphocyte responses to mitogens ↑ in rats	Bendich and Shapiro, 1986
Monocyte numbers ↑ in rats with a positive correlation between BC intake and monocyte numbers	Brevard, 1994
Number of mast cells ↑ in rat colorectal tissue	Brevard <i>et al.</i> , 1985
CD4+ cell numbers ↑; CD4:CD8 ratio ↑; plasma IgG ↑; DTH response to PHA ↑ in dogs	Kim <i>et al.</i> , 2000 Chew <i>et al.</i> , 2000
<i>Humans</i>	
Prevented a suppression of DTH test responses as a result UV treatment	Fuller <i>et al.</i> , 1992;
Suppression of the DTH-test responses as a result of UV treatment ↓	Herraiz <i>et al.</i> , 1998
NK cell cytotoxicity ↑ by 34%	Wood <i>et al.</i> , 2000
Percentage of cells expressing natural killer cell markers ↑ in human PBMC	Prabhala <i>et al.</i> , 1991
Percentage of lymphoid cells with surface markers for T helper and NK cells and cells with IL-2 receptors in peripheral blood mononuclear cells ↑	Watson <i>et al.</i> , 1991; Prabhala <i>et al.</i> , 1989
Frequency of helper/inducer T cells ↑ after 7 days and all T cells ↑ after 14 days in human volunteers.	Alexander <i>et al.</i> , 1985
Plasma level of TNF-α ↑ in oral leukoplakia patients	Prabhala <i>et al.</i> , 1993
IL-2R+ T lymphocytes and CD4+ lymphocytes ↑ in patients with colonic polyps or cancerous lesions	Kazi <i>et al.</i> , 1997
Loss of macrophage receptors following exposure to ROS ↓ in human PBMC	Gruner <i>et al.</i> , 1986
Number of monocytes and eosinophils ↑ in healthy volunteers	Brevard, 1989
CD4-CD8 ratio ↑ in human blood	Murata <i>et al.</i> , 1994
Percentages of monocytes expressing the major histocompatibility complex class II molecule HLA-DR and the adhesion molecules intercellular adhesion molecule-1 and leukocyte function associated antigen-3 ↑; TNF-α secretion ↑ by human blood monocytes	Hughes <i>et al.</i> , 1997

Table 9.1 (contd)

<i>In vitro</i>	
Maximum mitogenic proliferative responsiveness of lymphocytes ↓	Kramer <i>et al.</i> , 1997
Splenocytes and thymocytes proliferative response ↑; release of IL-1 alpha and tumor necrosis factor-alpha ↑	Okai and Higashi-Okai, 1996
ConA induced lymphocyte proliferation ↑ in cow at wk 1, but was suppressive (10 ⁻⁸ M) at week 4	Daniel <i>et al.</i> , 1991
Potentiated interferon and prevented inhibition of stimulatory action of IFN on monocyte membrane function caused by retinoic acid and retinol or tumour in <i>in vitro</i> system	Rhodes, 1983; Rhodes <i>et al.</i> , 1984
Prevented stress induced thymic involution and lymphopenia; graft versus host response ↑; regression of virally induced tumours ↑; cytotoxic macrophage and T cell activities ↑ in tumour models	Bendich, 1987
NK cell lysis of tumour cells ↑	Leslie and Dubey, 1982
Neutrophil killing of <i>Candida</i> ↑	Kanofsky <i>et al.</i> , 1987
Cell proliferative response of mice splenocytes and thymocytes ↑, release of IL-1 and TNF-α ↑	Okai and Higashi-Okai, 1996
Alleviated detrimental effect of aflatoxin B1 on duck lymphocyte proliferation	Cheng and Pang, 1996
The cytotoxicity of porcine lymphocytes ↑ and superoxide production by peritoneal macrophages ↑; prevented the inhibitory action of CLA on the phagocytic activity of macrophages.	Chew <i>et al.</i> , 1997

Table 9.2 Effect of lutein and astaxanthin on immunity

<i>Lutein</i>	
DTH response to PHA and vaccine ↑; lymphocyte proliferative response to PHA, ConA and PWM ↑, percentages of cells expressing CD5, CD4 and CD8 ↑, IgG production ↑ in cats	Kim <i>et al.</i> , 2000a
pim-1 mRNA expression in splenocytes stimulated with Con A ↑ in mice	Park <i>et al.</i> , 1999
Partially restored decreased <i>in vivo</i> Ab production in response to T dependent antigen in mice	Jyonouchi <i>et al.</i> , 1994
PHA induced lymphocyte proliferation ↑ in mice; tumour latency ↑, mammary tumour growth ↓	Chew <i>et al.</i> , 1996
<i>In vitro</i> Ab production in response to T dependent antigens ↑ in mice	Jyonouchi <i>et al.</i> , 1994

Table 9.2 (Contd)

<i>Astaxanthin</i>	
Phagocytosis and haemotaxis of Sephadex-50 activated peritoneal macrophages ↑ and cellular immunity (skin swelling test) ↑ in ducklings	Cheng <i>et al.</i> , 1999
Number of macrophages in ducks ↑	Cheng <i>et al.</i> , 2001
PHA induced lymphoblastogenesis ↑; Lymphocyte cytotoxic activity ↑ in mice	Chew <i>et al.</i> , 1999
Bacterial load and gastric inflammation ↓ as a result of treatment of <i>H pylori</i> infected mice with astaxanthin in the form of algal cell extract	Bennedsen <i>et al.</i> , 1999
<i>In vitro</i> antibody (Ab) production to T cell dependent antigen ↑ in mice	Jyonouchi <i>et al.</i> , 1993
Spleen cell production of plaque-forming cells ↑, immunoglobulins M and G ↑; [³ H]thymidine incorporation by thymocytes ↑ <i>in vitro</i>	Jyonouchi <i>et al.</i> , 1991
IgM and IgG production by spleen cells ↑ <i>in vitro</i>	Okai <i>et al.</i> , 1996
IgM and IgA production in response to various stimulants ↑ <i>in vitro</i>	Jyonouchi <i>et al.</i> , 1995
Augmented the number of IgG Ab secreting cells when primed B cells and Th cells clones were stimulated with suboptimal doses of Ag specific for each clone <i>in vitro</i>	Jyonouchi <i>et al.</i> , 1995a
Augmented the number of IgM Ab-secreting cells <i>in vitro</i>	Jyonouchi <i>et al.</i> , 1996
Splenocytes and thymocytes proliferative response ↑; release of IL-1 α and tumour necrosis factor-α ↑ <i>in vitro</i>	Okai <i>et al.</i> , 1996
IFN-gamma production ↓; number of Ab secreting cells ↑ <i>in vitro</i>	Jyonouchi <i>et al.</i> , 1996

Table 9.3 Effect of canthaxanthin and other carotenoids on immunity

Canthaxanthin	
Antibody titres to the Newcastle disease virus, weight of bursa, spleen and thymus ↑ with increased lymphocytic population in chickens	El-Hafeez <i>et al.</i> , 2000
Were not effective in enhancing <i>in vitro</i> bursal lymphocyte proliferation in chickens	Haq <i>et al.</i> , 1996a
T- and B-lymphocyte responses to mitogens ↑ in rats	Bendich and Shapiro, 1986
Maintained macrophage receptors for antigens	Gruner <i>et al.</i> , 1986)
Cell proliferative response of mice splenocytes and thymocytes ↑, release of IL-1 and TNF-alpha - <i>in vitro</i>	Okai and Higashi-Okai, 1996

Table 9.3 Contd

Splenocytes and thymocytes proliferative response ↑; release of IL-1 alpha and tumor necrosis factor-alpha ↑ <i>in vitro</i>	Okai <i>et al.</i> , 1996
<i>Other carotenoids</i>	
Resistance of lymphocytes to oxidative DNA damage ↑ as a result of consumption of lycopene in the form of tomato puree by adult women	Porrini and Riso, 2000
Resistance of mice to bacterial infection with <i>Klebsiella</i> <i>pneumoniae</i> ↑ by lycopene intraperitoneally	Lingen <i>et al.</i> , 1959
Zeaxanthin augmented the number of IgM Ab secreting cells <i>in vitro</i>	Jyonouchi <i>et al.</i> , 1996

(Tengerdy *et al.*, 1983) or rams vaccinated against *Brucella ovis* (Afzal *et al.*, 1984). Adjuvant administration has a far greater effect than dietary supplementation (Tengerdy, 1989).

Therefore Se and/or vitamin E deficiencies are associated with a compromised humoral immune response not only in chickens but also in mice, horses, pigs, cattle, calves, lambs and sheep (Larsen, 1993) and inclusion of increased doses of these nutrients into the diet improved the immune response (Turner and Finch, 1991; Larsen, 1993; MacPherson, 1994).

Antioxidants and lymphocyte function

It has been suggested that Se and vitamin E deficiencies affected T lymphocytes to a greater extent than B lymphocytes (Larsen, 1993). This was a result of higher levels of PUFAs in T lymphocytes and higher membrane fluidity. In fact, vitamin E and Se deficiencies may affect both the maturation of specific lymphocyte subpopulations and the functional and proliferative capabilities of the peripheral lymphocytes. A combined Se and vitamin E deficiency in chicks showed a more severe depression of T cell response than just Se deficient chicks. For example in an experiment by Chang *et al.* (1994) the dietary deficiencies in vitamin E and Se (basal diet without vitamin E and Se supplementation starting from hatching) resulted in a significant inhibition of T lymphocyte proliferation. In particular a decreased proportion of peripheral T cells and more specifically a decreased number of CD4(+) peripheral blood leukocytes were observed. The proliferative response to both ConA and PHA was impaired by the vitamin E and Se dietary deficiencies. However, the proliferative response could be fully reconstituted after vitamin E and Se supplementation for periods longer than 1 week.

Selenium deficiency in growing chickens was associated with impaired bursal growth and Se vitamin E deficiency caused inhibition in thymus growth. (Marsh *et al.*, 1986). In this experiment a reduced number of lymphocytes were seen in both the thymus and bursa in combined Se-vitamin E deficiency. On the other hand, a significant increase in relative weight of the bursa of Fabricius was observed in broiler chicks at a level of 0.10 mg of Se and 150 IU of vitamin E (Swain *et al.*, 2000). It is interesting to note that bursectomy in chickens caused a small but significant fall in spleen Se concentration, but not in that of other tissues (Abdel-Ati *et al.*, 1984). It has been shown that Se deficiency alone or in combination with vitamin E is associated with depressed splenocyte ability to proliferate in culture (Marsh *et al.*, 1987). Such a depression was not due to reduced lymphocyte viability in culture. Marsh *et al.* (1986) suggested that the primary lymphoid organs are major targets of Se and vitamin E dietary deficiencies and provide a possible mechanism by which immune function may be impaired. In their experiment with chickens specific deficiencies of Se or vitamin E significantly impaired bursal growth. Thymic growth was impaired only by the combined vitamin E-Se deficient diet. Severe histopathological changes in the bursa resulted from the combined deficiency and these were detectable by 10-14 days after hatching (Marsh *et al.*, 1986). These changes were characterised by a gradual degeneration of the epithelium and an accompanying depletion of lymphocytes. On the other hand, chicks receiving Se (1 mg/kg) and vitamin E (300 IU/kg) had significantly higher cellular immune responses in terms of per cent leukocyte migration inhibition (Swain *et al.*, 2000). Dietary vitamin E has been shown to significantly affect T cell differentiation in the thymus of broiler chicks (Erf *et al.*, 1998).

Inclusion of Se in the chicken diet (0.3-0.6 mg/kg) for 2 months significantly increased the response of peripheral blood lymphocytes to phytohaemagglutinin and NK cell activities, accelerating the development of cell mediated immunity in chickens (Huang and Chen, 1999). Therefore, Se could significantly increase the response of peripheral blood lymphocytes to phytohaemagglutinin and NK cell activity, accelerating the development of cell mediated immunity in chickens (Huang and Chen, 1999). Vitamin E supplementation has been found to be effective in preventing age related decreases in NK activity in old mice challenged with antigen (Gogu and Blumberg, 1992). Vitamin E also prevented retrovirus induced suppression of splenocyte proliferation and natural killer cell activity in mice (Wang *et al.*, 1994). Since NKs are susceptible to oxidant injury vitamin E may affect NK function via altering peroxide tone or preventing lipid peroxidation events.

The results of Colnago *et al.* (1984) indicate that dietary Se supplementation modifies the number of peripheral blood leukocytes in chickens infected with coccidia. In particular, Se significantly increased blood leukocyte number 8 hours after challenge in one experiment and produced numerically higher leukocyte number in three other experiments. Immunisation of chickens against coccidiosis

can be enhanced by Se supplementation (Colnago *et al.*, 1984a). During heat stress lymphocyte proliferative responses to ConA and LPS were greater in hens fed 45 and 65 IU of vitamin E/kg in comparison to birds fed on a diet supplemented with vitamin E at a concentration of 25 IU/kg (Puthongsiriporn *et al.*, 2001). Chicks receiving Se (1 mg/kg diet) and vitamin E (300 IU/kg diet) had significantly higher cellular immune responses in terms of per cent leukocyte migration inhibition (Swain *et al.*, 2000). The effect of vitamin E on the cellular immune system was examined in turkeys by lymphocyte transformation assay (LTA) using Con A as mitogen. The vitamin E supplemented chicken group (up to 800 mg/kg) revealed a significant higher lymphocyte stimulation rate than the control group (Heffels-Redmann *et al.*, 2001).

There are marked differences in susceptibility to antioxidant deficiency among species (Turner and Finch, 1991). Both T and B cell responses were significantly depressed in vitamin E deficient guineapigs compared with responses from guinea pigs fed on diets containing vitamin E (Bendich *et al.*, 1984). As mentioned above, Se is proved to have an important function in maintaining proliferative capacity of T and B cells (Turner and Finch, 1991). It has been suggested that Se may modulate the expression of IL-2 receptors on the cell surface, which could lead to the altered ability of lymphocytes from Se deficient animals to respond to mitogen and antigens (Larsen, 1993). Again, this phenomenon is a characteristic not only for chickens but also for laboratory and farm animals (Larsen, 1993; Turner and Finch, 1991; MacPherson, 1994; Jacques, 2001).

The effect of vitamin E deficiency on the proliferative response of canine lymphocytes was studied by Langweiler *et al* (1981), using the lymphocyte blastogenesis assay, with PHA, Con A, and pokeweed as mitogenic stimulants. Lymphocytes from pups fed deficient diets were poorly responsive to mitogen induced blastogenesis, compared with the lymphocytic response seen in the controls. Lymphocytes of lambs on a low Se/vitamin E diet were isolated from peripheral blood, and mitogenic responses to PHA tested in the presence of different doses of sodium selenite and emulsified vitamin E added *in vitro* (Finch and Turner, 1989). An enhancing effect of Se was observed at doses of 1 ng/ml or less, and reached a plateau at about 10 ng/ml. Optimal doses of vitamin E added to culture (0.15-1.5 mg/ml) elevated responses beyond those seen with Se. Similar results were obtained when lymphocytes from deficient, myopathic lambs were cultured with serum from lambs supplemented *in vivo*, and when PHA responses of untreated and treated lambs were compared. Tests with other phytolectins (Con A and pokeweed mitogen) suggested that the two micronutrients exert a differential influence on lymphocyte sub populations. Three groups of lambs from a flock in which white muscle disease had been diagnosed were used (Ramos *et al.*, 1998). Group 1 was inoculated with 6 mg of sodium selenite and 200 mg of vitamin E, group 2 was inoculated with 200 mg of vitamin E, and group 3 was the untreated

control group. Lambs in group 1 treated with Se and vitamin E showed a greater response to the delayed hypersensitivity test compared to the other groups. It is concluded that supplementation of lambs with Se and vitamin E has a positive effect, especially on cell mediated immunity.

When 32 Holstein-Friesian heifer calves, eight per group, were given 0, 125, 250, or 500 IU/day of supplemental vitamin E/calf, from birth to 24 weeks of age, overall mean lymphocyte blastogenic responses to various T cell and B cell mitogens were higher in supplemented calves than in control animals (Reddy *et al.*, 1987). However, there are also data indicating that vitamin E deficiency did not impair humoral or cell mediated immune responses of calves to bovine herpes virus live vaccine, tetanus toxoid or *Mycobacterium paratuberculosis* vaccine (Anderson *et al.*, 1986). In the same experiment feed supplemented with vitamin E (200 mg/kg of feed) did not enhance the immune responses.

Usually combined deficiency of several antioxidants has a more detrimental effect on the immune system in comparison to a single antioxidant deficiency. For example, compared with controls, a vitamin E and Se deficient diet in sows during gestation until day 4 of lactation decreased the mitogenic responses of lymphocytes of peripheral blood and colostrum, phagocytic activity of blood and colostrum polymorphonuclear cells (PMN), and the microbiocidal activity of blood, colostrum, and milk PMN (Wuryastuti *et al.*, 1993). The vitamin E deficient diet reduced mitogenic responses of peripheral blood lymphocytes and colostrum, and the phagocytic activity of the lymphocytes. The Se deficient diet reduced phagocytic activity of PMN.

Mitogen induced lymphocyte proliferation in fish was stimulated by supplemental vitamin E with three of the four mitogens tested at days 60 and 90. Fish not given vitamin E had a lower stimulation index with all mitogens tested. Similarly, rainbow trout on a diet deficient in α -tocopherol had significantly reduced immune responses, although appearing healthy and growing normally (Blazer and Wocke, 1984).

Natural killer cell mediated cytotoxicity in mice was depressed after eight weeks on diets deficient in Se and/or vitamin E (Meeker *et al.*, 1985). T lymphocyte mediated cytotoxicity was found to be depressed by combined Se-vitamin E deficiency after 7 weeks on diets. In contrast, antibody dependent cell mediated cytotoxicity was not affected in such conditions. The findings of Tanaka *et al.* (1979) suggested that vitamin E stimulated the helper activity of T lymphocytes in mice. In mice fed a high level of vitamin E (500 IU/kg diet), the production of interleukin-12 (IL-12) and tumour necrosis factor- α was significantly lower and IL-2 was significantly higher than in animals fed a lower (75 IU/kg diet) level of vitamin E (Venkatraman and Chu, 1999). Furthermore, vitamin E lowered the levels of proinflammatory cytokines and lipid mediators.

Immunostimulating properties of vitamin E are age dependent. For example, Wakikawa, *et al.* (1999) studied the effect of vitamin E (500 IU/kg/diet) given to

young and old male mice on the immune system before and after the exposure to restraint stress. Vitamin E slightly increased the percentage of splenic T cells and enhanced Con A response of spleen cells, enhanced the production of IL-2 and IFN-g and further improved the recovery after the stress in young but not in old mice. It has been shown (Fuente and Victor, 2000) that antioxidants preserve adequate function of lymphocytes against homeostatic disturbances such as those caused by endotoxic shock and ageing.

Clearly, vitamin E has the ability to improve the decreased cellular immune functions caused by aging, and appears to be associated with the enhancement of macrophage functions and lymphocyte responsiveness. For example, the proliferation of splenic lymphocytes with PHA or Con A was lower in old rats fed on the regular diet (vitamin E, 50 mg/kg diet) as compared to that of young rats (Sakai and Moriguchi, 1997). In great contrast, the proliferation of splenic lymphocytes in old rats fed on the high vitamin E (585 mg/kg) diet was similar to that of young rats. Furthermore, the high vitamin E diet induced an increase in IL-2 production from splenocytes in young rats and old rats following *in vitro* stimulation with Con A for 48 hours. High vitamin E diet (585 mg/kg diet) could restore decreased cellular immune function (proliferation of thymocytes and splenocytes with PHA and Con A) in spontaneously hypertensive ageing rats (Moriguchi *et al.*, 1993; 1995). This effect was attributed to the stimulation of production of IL-2 and inhibition of production of natural thymocytotoxic autoantibody from thymocytes following vitamin E supplementation.

Lymphocyte proliferation is shown to be affected by various carotenoids in avian and mammalian species (Tables 9.1-9.3) *in vivo* and *in vitro*. Since lutein and other carotenoids which are not converted to vitamin A are also active in modulating lymphocyte function, this immunostimulating effect of carotenoids is not related to provitamin A activity of carotenoids. It is especially important because *b*-carotene is not accumulated in chicken tissues (Surai *et al.*, 2001).

Antioxidants and phagocyte function

As mentioned above phagocytes play an important role in natural immunity. Among six major specific rationales for modulating macrophage function in poultry analysed by Klasing (1998a), mitigation of immunosuppression arising from infectious diseases, dietary toxins or stress could be affected by dietary antioxidants. In fact the availability of certain substrates and enzymatic cofactors can greatly influence the capacity for metabolite production by macrophages. In particular, dietary deficiencies of Se and/or vitamin E have been shown to impair neutrophil and macrophage activities in chickens including a decrease in peritoneal macrophages and decreased phagocytosis of red blood cells (Dietert *et al.*, 1990). The intracellular

killing of yeasts and bacteria by neutrophils and macrophages from chickens with Se deficiency is reduced (Larsen, 1993). Similar effects were observed in rats, goats and cattle (for review see Larsen, 1993). In contrast, natural antioxidants, for example vitamin E (Gore and Qureshi, 1997) can improve macrophage function. In fact, in ovo vitamin E treated chickens had more Sephadex elicited abdominal exudate cells, greater macrophage phagocytic potential and increased production of nitrite by macrophages (Gore and Qureshi, 1997). Since various mycotoxins are shown to reduce macrophage viability and effector functions (Bondy and Pestka, 2000) a protective immunostimulating effect of antioxidants, including Se, could be especially important in commercial conditions associated with mycotoxicoses (Hegazy and Adachi, 2000). Furthermore, optimised management of macrophage metabolite production by nutritional means is a crucial factor for bacterial resistance of poultry (Dietert and Golemboski, 1998).

Beneficial effects of vitamin E and Se have been shown in experiments with farm and laboratory animals. When calves, aged 3 to 8 weeks, received (with an interval of 2 weeks) two intramuscular injections, each of which contained 5.75 mg Se and 75 mg α -tocopheryl acetate they showed higher blood leukocyte counts (with less variation), a greater phagocytosis index and more NBT positive granulocytes than untreated animals (Bednarek *et al.*, 1996). In the experiment of Politis *et al.* (1996) dairy cows were supplemented with vitamin E from 4 weeks before and up to 8 weeks after parturition at the rate of 3000 IU/cow/day plus an injection of vitamin E (5000 IU) 1 week before the expected date of parturition. It has been shown that vitamin E supplementation enhanced by 30 to 83% chemotactic responsiveness of dairy cow blood neutrophils beginning 2 weeks before to 4 weeks after parturition, compared with unsupplemented controls.

The effect of oral administration of high dosages of vitamins C and E on the innate immune system of the seabream was investigated by Ortuno *et al.* (2001). Gilthead seabream (*S. aurata* L) were fed four different diets for 45 days: a commercial diet as control, a 3 g/kg vitamin C-supplemented diet, a 1.2 g/kg vitamin E supplemented diet or a diet containing both vitamin supplements. The fish fed the vitamin C supplemented diet showed an enhanced respiratory burst activity, while fish fed the vitamin E supplemented diet exhibited increased complement and phagocytic activities. For 80 days rainbow trout were fed on semipurified diets containing supplementary vitamin E (0, 28 or 295 mg/kg feed; Clerton *et al.*, 2001). Phagocytic activity of gut leukocytes and head kidney enriched macrophages was studied, as well as head kidney cell pinocytosis and serum lysozyme activity. Gut leukocyte phagocytosis was enhanced in trout given high vitamin E. Phagocytosis was impaired in unsupplemented trout. In Atlantic salmon phagocytic chemiluminescent responses were positively affected by vitamin E after 14 and 23 weeks, but serum complement mediated haemolysis and lysozyme activity in serum and in head kidney were unaffected (Lygren *et al.*, 1999). The

highest macrophage oxidative burst activity, measured by chemiluminescence, was found in rainbow trout receiving vitamins C and E at high levels (2000 and 800 mg/kg feed respectively) (Wahli *et al.*, 1998).

Increased vitamin E levels were shown to be beneficial for macrophage function in mice experimentally infected with *Diolococcus pneumonia* type I infection. In this experiment a phagolytic index was increased four fold in comparison to control animals (Heinzerling *et al.*, 1974). In mice, increased vitamin E supplementation was associated with stimulated mitogen response to ConA, PHA and LPS (Corwin and Schloss, 1980). A similar response was observed in rats (Bendich *et al.*, 1986).

The effect of vitamin E (intraperitoneal injections of 5 mg vitamin E/rat for six successive days) on macrophage migration inhibitory factor (MIF) production in macrophages in response to stimulation by calcium ionophore A23187 and LPS was studied (Sakamoto *et al.*, 1999). Vitamin E enriched macrophages without stimulation and after stimulation showed less MIF content than the controls. However, there was no alteration of intracellular MIF content of vitamin E macrophages, suggesting a suppression of MIF secretion into the culture medium by vitamin E, possibly as a result of modulating macrophage-membrane architecture.

In an experiment by Fuente *et al.* (2000) 3 week old guinea pigs were fed vitamin E at levels of 15, 150 or 1500 mg/kg feed during 5 weeks. With respect to the medium vitamin E diet, low ingestion of vitamin E caused a decrease in chemotaxis and production of superoxide anion by macrophages and an increase in the phagocytic capacity. However, with the high vitamin E diet an increase in macrophage and lymphocyte chemotaxis, superoxide anion production and lymphoproliferative capacity, as well as a decrease in phagocytosis were observed.

Phagocyte function was shown to be effected by carotenoids (Tables 9.1-9.3). In particular in birds b-carotene or astaxanthin dietary supplementation increased the number of macrophages in ducks (Cheng *et al.*, 2001) and increased phagocytosis and chemotaxis of activated macrophages in ducklings (Cheng *et al.*, 1999). Phagocytic activity of neutrophils can also be increased in cows by b-carotene supplementation (Tjoelker *et al.*, 1988; Michal *et al.*, 1994). In humans b-carotene or canthaxanthin decreased the loss of macrophage receptors following exposure to ROS (Gruner *et al.*, 1986).

***In vitro* effects of antioxidants on the immune system**

Natural antioxidants are essential nutritional factors that affect the development and expression of cell mediated immune responses. Treatment of peritoneal exudate cells (PECs) from normal chickens or HD11 cells *in vitro* with RRR-a-tocopheryl

succinate at 0.1, 1, 5 or 10 mg/ml, before activation with LPS activated serum as a source of chemotactic complement fragment C5a, significantly enhanced chemotactic responses. The antioxidants Trolox, BHA and BHT also enhanced PECs and HD11 chemotaxis (Romach *et al.*, 1992). Pretreatment of PECs obtained from avian erythroblastosis virus infected chickens with RRR- α -tocopheryl succinate enhanced chemotaxis of PECs obtained during the preleukaemic phase, but had no effect on migration of PECs obtained during the erythroleukemic phase of tumorigenesis (Romach *et al.*, 1992). Supernatants from PECs exposed to vitamin E for 45 minutes exhibited a 256% increase in prostaglandin E₂ (PGE₂) levels compared with supernatants from replica cultures of PECs not exposed to the vitamin (Romach *et al.*, 1993). Pretreatment of PECs with vitamin E before exposure to avian erythroblastosis virus maintained PGE₂ levels at normal control levels. Vitamin E treatment also enhanced IL-1 production by avian and murine macrophage cells. It is concluded that down regulation of retrovirus induced PGE₂ production and/or upregulation of IL-1 production by vitamin E are potential mechanisms for vitamin E amelioration of retrovirus induced immune suppression.

It is interesting that ascorbic acid, known as an effective antioxidant *in vitro* and *in vivo* is also effective in stimulating phagocytes. For example, significant increases in bacterial killing were found in heterophils treated with ascorbic acid. No significant differences were found in phagocytosis, but ascorbic acid tended to decrease random migration (Andreasen and Frank, 1999). The *in vitro* effects of varying concentrations of vitamin E, ascorbic acid, glutathione, N-acetylcysteine on the phagocytic process of peritoneal macrophages were examined in macrophages from mice (Rio *et al.*, 1998). The results showed an antioxidant induced stimulation of the phagocytic process of macrophages. Adherence to substrate was increased after a short incubation with vitamin E and ascorbic acid. Random migration, chemotaxis, ingestion and superoxide anion production were increased by all the antioxidants used. The *in vitro* effect of vitamin C and/or E was also studied on the functions and ultrastructure of gilthead sea bream head kidney phagocytic leukocytes (Mulero *et al.*, 1998). Different concentrations of vitamins C and/or E were added to the incubation medium and leukocytes were incubated for 48 hours. Leukocyte migration and phagocytosis increased when vitamin C or E were added, while the respiratory burst was synergistically enhanced when both vitamins were used together. Brennan *et al.* (2000) showed the protective effect of vitamin C or E supplementation *in vivo* against H₂O₂ induced DNA damage in human peripheral blood lymphocytes.

It has been shown that dietary (2 ppm for 8 weeks) or *in vitro* (1x10⁻⁷M) supplementation with Se (as sodium selenite) results in a significant enhancement of the proliferative responses of spleen lymphocytes from C57B1/6J mice in response to stimulation with mitogen or antigen. In contrast, Se deficiency (0.02 ppm for 8 weeks) had the opposite effect. The alterations in the ability of cells to

proliferate were apparently related to the ability of Se to alter the kinetics of expression of high affinity IL-2 receptors on the surface of activated lymphocytes. This was associated with enhanced or delayed clonal expansion of the cells. The changes in tumour cytotoxicity were paralleled by changes in the amounts of lymphotoxin produced by the activated cells. The results also suggested that Se exerts its effect 8-24 hours after stimulation, and that it most likely affects processes in the cytoplasmic and/or nuclear compartments of activated lymphocytes (Kiremidjian-Schumacher *et al.*, 1992).

The impairment in lymphocyte proliferative response to antigens in Se and/or vitamin E deficiency could be a result of lipid peroxidation and damage to membrane structures, and more importantly to membrane receptors (Larsen, 1993). As a result cell to cell communication could be compromised. In this case a protective effect of antioxidants could be a crucial factor in immune system competence. For example, it was found that lipid peroxidation in lymphocytes before Con A stimulation was lower than that after stimulation and that superoxide dismutase promoted lymphocyte proliferation dose dependently. The addition of Na₂SeO₃ to lymphocyte culture or supplementation in drinking water to mice decreased lipid peroxidation in lymphocyte response to Con A. In the presence of Se, there was an inverse correlation between the levels of LPO in lymphocytes and the stimulated proliferation (Sun *et al.*, 1995).

As mentioned above there are principal differences in metabolism of organic and inorganic Se. This could be a reason of differences in stimulating activity of different forms of Se on the immune system. For example, sodium selenite and selenomethionine were tested in parallel, and their capability to inhibit or to increase antibody production by human lymphocytes *in vitro* was investigated (Borella *et al.*, 1996). Low doses of Se (0.5-2.0 µM) added as sodium selenite or selenomethionine did not alter the secretion of antibodies. When Se was added at higher levels, instead, an inhibitory effect was found using selenite, whereas a progressive increase in immunoglobulin production was observed after exposure to selenomethionine. Therefore there is an advantage in using organic Se for immune system stimulation in comparison to selenite.

When rat alveolar macrophages were isolated from rats fed 50 mg vitamin E/kg diet and induced by LPS, they increased superoxide and H₂O₂ production by 2.44-2.54-fold and 2.1-2.3-fold respectively. However, an increase in vitamin E supplementation to 250 or 1250 mg/kg prevented such a stimulation in ROS production (Vandana P. *et al.*, 1999). *In vitro* vitamin E was also effective in stimulating the mitogen response of murine spleen cells in culture (Corwin and Gordon, 1982). Stimulating effects of vitamin E on the mitogen response were also shown in pigs (Larsen and Tollersrud, 1981) and calves (Reddy *et al.*, 1986). When whole and macrophage depleted rat splenocytes were preincubated with vitamin E (2 mg/ml) for 24 hours, the proliferation of whole splenocytes was

significantly enhanced compared to that of whole splenocytes preincubated with medium alone (Oonishi *et al.*, 1995). Supplementation of cow macrophages *in vitro* with vitamin E (1 mM) and Se (0.5 mg/ml) enhanced the production of neutrophil chemotaxins by macrophages and enhanced the proliferation of lymphocytes in response to stimulation with concanavalin A, but not to phytohaemagglutinin or pokeweed mitogen (Ndiweni and Finch, 1995). Anderson *et al.* (1992) demonstrated that vitamin E at threshold concentrations of 0.1-1 mg/ml inhibited the generation of superoxide by neutrophils activated with soluble stimuli, by mechanisms independent of α -tocopherol antioxidant activity. It has been suggested that vitamin E could interfere with the activity of NADPH oxidase. However, different results were obtained in an experiment with channel catfish. Intracellular superoxide anion production of macrophages was higher in fish fed a diet fortified with Se (0.8 vs 0.2 mg/kg) and vitamin E (240 versus 60 mg/kg feed) in comparison to the fish fed a control diet (Wise *et al.*, 1993).

Antioxidants and disease resistance

The final goal in improvement of the immune system is to increase resistance to various diseases. Indeed, this option has been extensively studied with chickens. Heinzerling *et al.* (1974) showed that broiler chicks infected with *E coli* and fed vitamin E (40 mg/kg feed) resulted in total mortality of 25% and increased level of vitamin E to 150 and 300 mg/kg resulted in mortality reduction to 9.3 and 5.4% respectively. In fact, the supplementation of vitamin E (300 mg/kg diet) to a standard chick ration increased the protection of 6 week old immunised chickens against *E coli* infection, decreasing mortality from about 40% to 5%. In that experiment vitamin E did not protect chicks from weight loss and severe morbidity due to infection, but increased the rate of recovery (Tengerdy and Nockels, 1975). Protection was attributed to increased antibody production and increased phagocytosis with a significant increase in vitamin E level in the spleen (Tengerdy and Brown, 1977). Therefore, chicks and turkeys infected with *E coli* and fed supplemental vitamin E had reduced mortality and increased HA titres (Nockels, 1979; Tengerdy, 1978).

A combination of vitamin E with Se resulted in reduced mortality and increased body weight gain in chickens infected with *Eimeria tenella* (Colnago *et al.*, 1984). The same authors showed that dietary supplementation with Se or vitamin E reduced mortality and increased body weight gain of non-immunised chickens infected with *E tenella* in three of four experiments. When chicks were inoculated with virulent Marek's disease (MD) virus at 10 days of age, Se (0.6 mg/kg) decreased the morbidity and mortality from MD, increased the ability to remove oxygen free radicals and lipid peroxide, and alleviated the degree of tissue damage caused by

oxygen free radicals (Huang and Chen, 1996). In another experiment, from 1 day of age chicks were given a basal diet containing Se at 0.086 mg/kg (group I) or the basal diet supplemented with Se at 0.3 mg/kg (group II) or 0.6 mg/kg (group III). The chickens were infected with infectious bursal disease virus at 39 days of age. Ten days later the mortality rates in groups I, II and III were 33.3, 12.4 and 10.6%, respectively, and the infection induced inhibition of T lymphocyte transformation was less in the Se supplemented birds (Bu *et al.*, 1996). When Se was added to the feed of White Leghorn chickens prior to challenge with either *E coli* or sheep erythrocyte antigen, the incidence of death or lesions was reduced from 86 to 21% at the optimal dose of Se (0.4 mg/kg feed; Larsen *et al.*, 1997). Lower Se values were measured in chickens infected with *Ascaridia galli* compared with controls, and this was related to a lower degree of microelement absorption and the regeneration of the intestinal mucosa in infected birds (Damyanova *et al.*, 1995).

Effects of vitamin E on immunity are species and genotype specific. For example, adding 300 IU of vitamin E/kg diet did not alleviate the adverse effects of *E coli* infection in young turkeys (Sell *et al.*, 1997). Yang *et al.* (2000) used chicks selected for 24 generations for high (HAS) or low (LAS) antibody responses to SRBC. The chickens were fed on a diet supplemented with vitamin E at 10 or 300 IU per kg feed. The HAS chicks were more susceptible to *E coli* infection than LAS chicks. Although dietary vitamin E had no effect on lesion scores in either line, body weight loss at 24 hours after *E coli* inoculation was significantly reduced in HAS chicks fed the higher concentration of vitamin E but in LAS chicks vitamin was not effective.

The immunostimulating effect of vitamin E is also dose dependent. For example, in an experiment of Macklin *et al.* (2000) vitamin E was supplemented in a chicken diet at 8.82 mg, 41.89 mg, 74.96 mg, 108.03 mg, or 141.10 mg vitamin E/kg feed. At 4 weeks of age, the birds were scratched on the breast and placed onto avian cellulitis *E coli* seeded litter. One week later, the birds were examined and a positive correlation was seen between vitamin E and the inhibition of cellulitis formation when the birds were fed a diet containing 74.96 mg vitamin E/kg feed. However 141.1 mg vitamin E/kg feed increased the incidence of cellulitis.

It is interesting that in combination with vitamin E, b-carotene, which is known not to be efficient in tissue accumulation in chickens (Surai *et al.*, 2001) significantly increased disease protection and reduced hepatomegaly caused by *E coli* infection (Tengerdy *et al.*, 1990). b-Carotene alone increased disease protection and decreased hepatomegaly caused by *E Coli* infection (Tengerdy *et al.*, 1990) but this effect was much lower in comparison to vitamin E supplemented birds.

It is interesting that an immunostimulating effect of natural antioxidants could be seen even in conditions without disease challenge. For example, the inclusion of vitamin E (10, 100 or 300 IU/kg) in a chicken diet for the first 12 days post-

hatching with the following diet supplemented with vitamin E at 10 IU/kg was associated with a significant decrease in chicken mortality at day 22 (5.4, 4.8 and 1.1% respectively) (Siegel *et al.*, 2000). At day 41 mortality of chicks in the same groups were 8.6; 7.5 and 5.4 % respectively. There were some sex specific differences in regard to vitamin E action. In the same experiment the high level of vitamin E supplementation had positive effects on body weight and breast yield of females, but had no effect on males. These data clearly show that increased vitamin E supplementation in the first 2 weeks posthatch could have a positive effect on chicken viability. Similarly, decreased mortality was observed in chickens (3600 male and female broiler chickens); at the lowest vitamin E supplementation (20 mg/kg diet) mortality was 3.2 and 2.9% and at the highest vitamin E levels (160 mg/kg diet) it was 1.7 and 1.5%, for cocks and hens, respectively (Blum *et al.*, 1992). Therefore, vitamin E and/or Se as a dietary supplement increase humoral and cell mediated immunity and disease resistance in chickens.

Immunostimulating and disease preventing effects of natural antioxidants are not restricted to avian species, but obvious with other farm animals. For example, in pigs challenged with *E coli* increased vitamin E supplementation was associated with increased agglutination response (Ellis and Vorhies, 1976). Vitamin E alone or in combination with Se was shown to have a protective effect against swine dysentery infection, when challenged with *Trichoepionema hyodysenteriae* (Teige *et al.*, 1982). When lambs were experimentally infected with *Chlamidia*, increased vitamin E supplementation was beneficial in terms of less extensive pneumonia, greater post-infection feed consumption and significantly higher weight gain (Stephens *et al.*, 1979). It has been shown that supplementation of high levels of vitamin E (at least 1000 IU per day) for dairy cows during the dry period and early lactation can reduce the incidence of mastitis, possibly because of an increase in immune system activity and function, but there was little benefit of supplementation on infectious diseases other than mastitis (Allison and Laven, 2000). Vitamin E added to calf diets to supply more than 400 IU/animal daily seemed beneficial for increasing gain and decreasing bovine respiratory disease morbidity (Galvayan *et al.*, 1999). It is interesting that survival rates were greatest in fish receiving vitamins E and C at high levels. For example, highest mortalities in fish infected with *Y. ruckeri* occurred in rainbow trout fed double deficient or double low diets. Similarly, fish exposed to *I. multifiliis* had a higher survival rate if the diets were high in one vitamin or both vitamins. Vitamin E (2500 mg/kg feed) significantly enhanced the ability of channel catfish macrophages to phagocytose virulent *E ictaluri* (Wise *et al.*, 1993a). Carotenoids can also enhance disease resistance of birds and mammals (Tables 9.1-9.3). Survival rates of b-carotene supplemented larvae of parrotfish was also increased (Tachibana *et al.*, 1997) and resistance of mice to bacterial infection with *Klebsiella pneumoniae* was enhanced by lycopene (Linmgem *et al.*, 1959).

The results presented above clearly show that various components of the immune system as well as general animal health are improved when vitamin E and/or Se have been added to deficient diets or supplemented at levels far above those required for growth. The benefit of vitamin E and/or Se supplementation would be greatest in situations when animals are infected with a particular pathogen. In this case clinical signs of infection could be reduced. Clearly, vitamin E could significantly enhance chicken immunity to coccidiosis (Colnago *et al.*, 1984), Newcastle disease (Franchini *et al.*, 1995), infectious bursal disease (McIlroy *et al.*, 1993) and *Ea coli* infection (Tengerdy and Brown, 1977). Furthermore, for optimising the animal's resistance to disease, vitamin E and Se requirements are higher than those for adequate growth, feed efficiency, egg production or even reproduction (Nockels, 1988). The optimal doses of vitamin E and Se for maximum protection depends on many factors and need further elucidation (Tengerdy, 1990).

It is well known that vitamins A, E, C, Se and carotenoids are able to protect cells from free radical oxidation, reduce the detrimental effects of certain eicosanoids, and enhance humoral and cellular immune responses in disease (Nockels, 1988). Vitamin E supplementation enhances humoral and cell mediated immunity, and augments the efficiency of phagocytosis in laboratory animals, farm animals, and humans. During disease protection vitamin E interacts with other nutrients in the diet. However, vitamin A excess, known to decrease vitamin E and compromise antioxidant systems in chickens (Surai *et al.*, 1998; 2000; Surai and Kuklenko, 2000) led to increased susceptibility of chicks of *E coli* infection and this was accompanied by depressed immune responses (Friedman *et al.*, 1991).

An improved immune system could ultimately lead to higher resistance of chickens to various diseases. It is important to remember that during disease challenge nutrient assimilation from the diet could be compromised and result in absorption impairment or decreased feed consumption. This could lead to decreased efficiency of the antioxidant system and as a result the immune system could be compromised. For example Hao Yan Hong *et al.* (1999) studied the activities of Se-GSH-PX and lipid peroxidation in central and peripheral immune organs and main visceral organs of broilers experimentally infected at 1 day of age with virulent Marek's disease virus (vMDV). They showed that in infected birds the Se-GSH-PX activity was significantly decreased and lipid peroxidation was enhanced. Therefore preventing these changes in antioxidant defence systems is believed to be an effective means to maintain immune system efficiency and this could be associated with better survival of infected chicks.

Mechanisms of immunostimulating properties of antioxidants

Several mechanisms are involved in antioxidant-stimulation of immune system (Wu and Meydani, 1998):

PROTECTION OF CELL MEMBRANES AND RECEPTORS

Antioxidants prevent oxidative stress induced damage to immune cells. As mentioned above, phagocytosis is the major mechanism for microbe removal from the body. As a result of stimulation (e.g., by microbes) monocytes differentiate into macrophages that are more powerful in mediating host defence (Klasing, 1998a). The immune system generates ROS as part of its defence function and these ROS are an important weapon in killing pathogens. However, chronic overproduction of ROS can cause damage to immune cells and compromise their function (Wu and Meydani, 1998). In fact immune cells are rich in PUFAs, which are very susceptible to free radical attack. In this respect vitamin E can substantially increase membrane resistance against lysis by activated neutrophils (Sepe and Clark, 1985). It is well recognised that many immunological functions are membrane dependent. These are antigen recognition, receptor expression, secretion of antibodies and cytokines, lymphocyte transformation, and contact cell lysis (Wu and Meydani, 1998). In particular, the receptors are important for antigen recognition and the secretion of various chemical mediators such as interferon, tumour necrosis factor, prostaglandins and interleukins. Therefore lipid peroxidation can change membrane structure and properties (e.g., fluidity, permeability and flexibility) which would affect immune cell functions. In contrast antioxidants are able to prevent the damaging effects of ROS and maintain immune function. For example, H₂O₂ depressed lymphocyte proliferation (Metzger *et al.*, 1980), while vitamin E decreased H₂O₂ formation by PMN (Baehner *et al.*, 1977). In fact, the suppressive effect of carotenoids on the respiratory burst of macrophages is considered to be a way by which carotenoids *in vivo* protect host cells and tissues from harmful effects of oxygen metabolites overproduced by macrophages (Zhao *et al.*, 1998).

EFFECT ON IMMUNOMODULATOR PRODUCTION

There is a range of regulatory molecules produced by immune cells. For example, IL-2, a lymphocyte growth factor, is recognised as an important immunomodulatory molecule. Oxidative stress suppresses IL-2 production and antioxidants can help to overcome this suppression. Therefore antioxidants (e.g. Se, vitamin E and carotenoids) upregulate the expression of the T cell high affinity IL-2 receptor and provides a vehicle for enhanced T cell responses. For example, recently it has been suggested that there is a specific requirement for vitamin E in total T and T helper cell proliferation (Lee and Wan, 2000). Furthermore, it has been suggested that Se can increase the inducibility of IL-2 receptor whereas vitamin E may counteract the down regulatory effect of cAMP on IL-2 activity (McCarthy, 1997).

PROSTANOID SYNTHESIS REGULATION

Antioxidants alter the production of immunomodulatory molecules such as prostaglandins and leukotrienes altering the ratio between immunosuppressive and immunostimulating eicosanoids. The relationship between antioxidants and inflammatory reactions are shown in Figure 9.3. It is clear that poor antioxidant defence is associated with enhanced inflammation, overproduction of PGE₂ resulting in suppression of lymphocyte activity (Grimble, 1997). For example, at low concentrations, PGE₂ is essential for cellular immunity; however, increased PGE₂ concentration is associated with a suppression of cellular and humoral immunity, including antibody formation, DTH, lymphocyte proliferation, and cytokine production. In this regard, it is interesting that vitamin E deficiency was shown to increase eicosanoid production, while vitamin E supplementation decreased their formation (Meydani *et al.*, 1986). Therefore it has been hypothesised that vitamin E protects chickens from a lethal *E coli* infection by inhibiting the biosynthesis of prostaglandins, thereby activating humoral immunity and phagocytosis. In fact, vitamin E decreases PGE₂ concentration in the bursa of chicks infected with *E coli* (Lawrence *et al.*, 1985). Vitamin E as an antioxidant protects the cells of the immune response from peroxidative damage; possibly through a modulation of lipoxygenation of arachidonic acid, vitamin E alters cell membrane functions and cell-cell interactions (Tengerdy, 1989). When chickens were fed supplemental vitamin E at the level of 300 mg/kg diet, endogenous PGE₁, PGE₂, and PGF₂ a levels decreased in the immunopoietic organs, bursa, and spleen. Antibody titres to *E coli* lipopolysaccharide and phagocytosis increased at the same time. Aspirin, a known prostaglandin inhibitor acted synergistically with vitamin E in depressing endogenous PG levels in the bursa and decreasing mortality from *E coli* infection (Likoff *et al.*, 1981). In an *in vitro* coculture system vitamin E improved T cell responsiveness by reducing PGE₂ production by macrophages (Beharka *et al.*, 1997). *In vitro* vitamin E caused a concentration dependent inhibition of arachidonic acid induced aggregation of PMNs and mononuclear leukocytes (Villa *et al.*, 1986). Furthermore, vitamin E supplementation of old mice decreased the age related increase in PGE₂ production by macrophages (Wu *et al.*, 1998). It is interesting to mention that canthaxanthin inhibited while b-carotene stimulated PGE₂ formation in a dose related manner (ElAttar and Lin, 1991).

EFFECT ON SIGNAL TRANSDUCTION

Essential nutrients such as Se and vitamin E may protect against oxidant mediated inflammation and tissue damage by virtue of their ability to scavenge free radicals and by their ability to inhibit the activation of NF-*κ*B (and possibly other oxidant

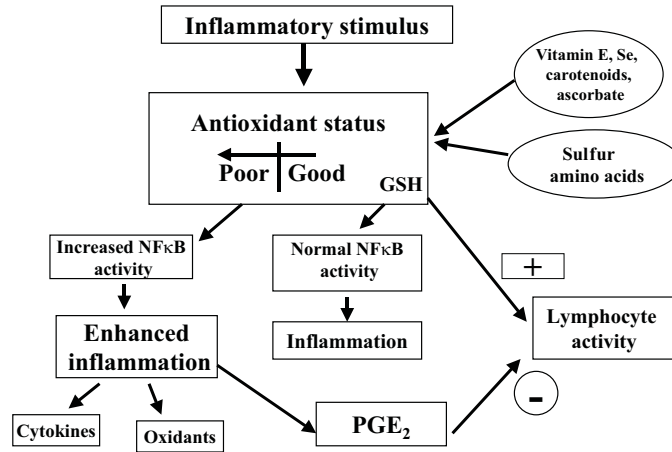


Figure 9.3 Antioxidants and inflammation (adapted from Grimble, 1997).

sensitive transcription factors). In fact, NF- κ B is required for maximal transcription of many inflammatory cytokines and adhesion molecules (Hughes, 1999). Thus, maintaining adequate antioxidant status may provide a useful approach in attenuating the cellular injury and dysfunction observed in some inflammatory disorders. (Conner and Grisham, 1996). It is necessary to underline that non-toxic concentrations of reactive metabolites of oxygen and nitrogen play an important role in regulating the expression of genes involved in the inflammatory response and in modulating apoptosis (Jourdeuil *et al.*, 1997). At the same time an immune response requires extensive communication between a wide range of cell types (Klasing, 1998) and special cell receptors are of great importance in this communication. Therefore protective effect of antioxidants in prevention of membrane and receptor damages due to peroxidation could provide an important way of enhancing the immune system. Furthermore carotenoids are considered to play a specific role in activation of the gap junction in cells and this could substantially improve cell to cell communications.

APOPTOSIS REGULATION

Antioxidants are considered to prevent apoptosis caused by oxidative stress. This could have a great effect on immune cell apoptosis, preventing immunosuppression.

Conclusions

When considering immunostimulating properties of antioxidants it is necessary to take into account several points:

- Individual antioxidants in the body interact with each other (vitamin E, C, carotenoids, Se) and pro-oxidants (iron, high level of PUFAs, mycotoxins) which themselves have immunostimulating or immunosuppressive effects. Therefore in every experiment the results reflect a sum of all these interactions and if background dietary concentrations of those nutrients differ results could be completely different. This could explain inconsistency of some results published for the past 20 years. Furthermore antioxidants can suppress respiratory bursts; however, it is not clear at present if there is a limit of this suppression after which the phagocyte antimicrobial activity would be compromised.
- The immunostimulating effect of antioxidants is shown to be maximal at their supplementation usually above the requirement for maximal growth and maintenance of reproduction. It could well be that the Se and vitamin E doses that are adequate for maximal productivity in healthy, unchallenged birds are not optimal for immunocompetence and disease resistance.
- There are no data available on the effect of antioxidants on intestinal immune structures which could be crucial barriers to external pathogens (Qureshi *et al.*, 1998). Since free radicals can be damaging to intestinal structures (Hoerr, 1998) antioxidant functions of Se can be doubled and would include prevention damages to intestinal lymphoid structure as well as damages to intestinal enterocyte membranes. This could be especially important in relation to digestive immunosuppression caused by toxins/mycotoxins, nutritional deficiencies and infectious agents (Hoerr, 1998). The evidence is accumulating indicating a non-immunological protective effect of Se from various toxic agents including cadmium (Zasadowski *et al.*, 1997), monensin (Yarsan, 1998), salinomycin (Zarski *et al.*, 1995) and mercury (Maretta *et al.*, 1995) in chickens.
- The selenium source (organic versus inorganic) seems to be an important element of its immunostimulating properties. Organic Se appears to be at an advantage because it is better assimilated from feed and better accumulated in tissues. Indeed with the same dose of supplementation organic Se can deliver more element to the target tissues and because of toxicity of high Se levels this could be a solution to avoid adverse effect of Se overdose. For example, selenium supplementation in organic form could have a beneficial effect during the acute phase response in many infectious diseases. The acute phase response is characterised by the synthesis of acute phase

544 *Natural Antioxidants in Avian Nutrition and Reproduction*

proteins, fever, accelerated whole body protein turnover, high rates of hepatic gluconeogenesis (Klasing, 1998) and decreased Se concentration in plasma (Sattar *et al.*, 1997). It is interesting that acute infections decrease serum Se levels regardless of the infective agent (Sammalkorpi *et al.*, 1988). Therefore if the body has Se reserves in the form of SeMet in muscles, during the acute phase response it would be liberated as a result of skeletal muscle protein catabolism by proteosome action and would be used for resynthesis of new selenoproteins which could decrease oxidative stress.

- There is a need for further experiments to study various combinations of n-3 fatty acids and natural antioxidants (Se, vitamin E, vitamin C and carotenoids), all of them are proved to have immunostimulating properties. However, analyses of recent experiments on the effect of n-3 PUFAs on the immune system (Surai and Sparks, 2001) showed that vitamin E supplementation was 20-60 ppm, which is even lower than recent recommendations for broiler breeders (Surai, 1999). Therefore, optimal combination of those nutrients could be more beneficial than their individual use.
- The first 2 weeks post-hatching represent the most important period of immune system development and maternal diet is shown (Klasing, 1998; Surai and Sparks, 2001) to have a profound effect on this process. In particular, the first week of chick life is a period of rapid expansion of leukocyte population, seeding of lymphoid organs and other events ultimately leading to the production of unique clones of lymphocytes that will mediate immunity in postnatal development (Klasing, 1998). In this respect effects of various combinations of natural antioxidants and n-3 PUFA await investigation.
- Because of complexity of regulation of the immune response and lack of understanding of molecular mechanisms involved in such a regulation there is a reasonable suggestion (Klasing, 1998a) to direct immunomodulation firstly toward the correction of dysfunctional situation created by immune system immaturity, stress, immunosuppressive disease, or genetics. In this respect natural antioxidants, especially Se, could have a prominent role.
- As a result of antioxidant (Se) deficiency, increased oxidative stress of a host can lead to increased virus mutation rate and change in a viral pathogen (Beck, 1999) resulting in emerging viral pathogens with new pathogenic properties. Therefore, Se deficiency was associated with a change to the viral genotype, converting the virus from a benign to a virulent strain (Beck, 1998). This possibility was not exploited in poultry production, but it seems important to study this more extensively.

There is a need to study antioxidant composition and fatty acid profile of immunocompetent tissues depending on chicken age and nutritional supplementation of antioxidants and n-3 PUFAs.

References

- Abdel-Ati, K.A., Latshaw, J.D. and Donahoe, J.P. (1984). Distribution of selenium in chicken tissues as affected by bursectomy. *Poultry Science* **63**: 518-523
- Abronina, I.F., Ramanauskaite, R.I., Andreichuk, T.N., Rytchenko, A.N. and Korostelev, S.A.. (1993). The beta-carotene stimulation of cellular immunity reactions in mice. *Bulletin of Eksperimental Biology and Medicine* **116**: 295-298
- Alexander, M., Newmark, H. and Miller, R.G. (1985). Oral beta-carotene can increase the number of OKT4+ cells in human blood. *Immunology Letters* **9**: 221-224
- Afzal, M., Tengerdy, R.P., Ellis, R.P., Kimberling, C.V. and Morris, C.J. (1984). Protection of rams against epididymitis by a Brucella ovis-vitamin E adjuvant vaccine. *Veterinary Immunology and Immunopathology* **7**: 293-304
- Allison, R. D. and Laven, R. A. (2000). Effect of vitamin E supplementation on the health and fertility of dairy cows: a review. *Veterinary Record* **147**: 703-708
- Amar, E. C., Kiron, V., Satoh, S., Okamoto, N. and Watanabe, T. (2000). Effects of dietary b-carotene on the immune response of rainbow trout *Oncorhynchus mykiss*. *Fisheries Science* **66**: 1068-1075
- Anderson, R., Theron, A. J., Myer, M. S., Richards, G. A. and Savage, J. E. (1992). Vitamin E is a potent inhibitor of superoxide generation by neutrophils activated with soluble, but not particulate stimuli of membrane-associated oxidative metabolism. *Journal of Nutritional Immunology* **1**: 43-63
- Anderson, P. H., Hartley, P. and Berrett, S. (1986) Effect of dietary vitamin E concentrations on calves' response to vaccination. *Proceedings of the 6th international conference on production disease in farm animals*, Belfast, pp.203-206
- Andreasen, C.B. and Frank, D.E. (1999). The effects of ascorbic acid on in vitro heterophil function. *Avian Diseases* **43**: 656-663
- Baehner, R.L., Boxer, L.A., Allen, J.M. and Davis, J. (1977). Autooxidation as a basis for altered function by polymorphonuclear leukocytes. *Blood* **50**: 327-335

Lipids and polyunsaturated fatty acids Natural antioxidants. Vitamin E in egg yolk Vitamin E and hatchability Antioxidant systems and avian embryo development Digestive and immune system development Conclusions References. 6. Antioxidant enriched eggs: from improvement of egg composition to functional food.Â Introduction Vitamin E and fertility Fatty acid composition of avian semen Lipid peroxidation in avian semen Antioxidant protection in avian semen. Superoxide dismutase Selenium and glutathione peroxidase Vitamin E Ascorbic acid Glutathione Antioxidant properties of seminal plasma. 305 311 311 316 318 340 344 344.Â 30/04/02, 09:44. 518 Natural Antioxidants in Avian Nutrition and Reproduction.