Stellingen

1. Vanuit diergezondheidsperspectief zou het verstandig zijn om naast een minimum behoefte aan linolzuur ook een minimumbehoefte aan α-linoleenzuur voor pluimvee te definiëren.
   (dit proefschrift)

2. Extrapolatie van studies aan nutrient-immuunsysteem interacties van zoogdieren naar gevogelte leidt heel makkelijk tot de verkeerde conclusies.
   (dit proefschrift)

3. Het is wenselijk om in zoötechnisch onderzoek, naast de effecten van b.v. voeding, huisvesting, stress e.d. op immunologische parameters te bestuderen, een grotere onderzoeksinspanning te leveren naar de betekenis van deze parameters onder de omstandigheden waaronder ze gemeten zijn.

4. Het idee dat mensen cultivering dan wel religie behoeven teneinde moraliteit te ontwikkelen berust op aangeleerd cultuurpessimisme.
   (vrij naar Frans de Waal, Good Natured)

5. Het is aannemelijk dat het gemiddelde intelligentieniveau van "primitieve" volken hoger is dan dat van Nederlanders gezien de geringere noodzaak intelligentie aan te wenden in onze samenleving.
   (vrij naar Jared Diamond, Guns, Germs and Steel: the Fates of Human Societies)

6. Het fenomeen hoogleraar als hoofd van een leerstoel, met wetenschappelijke staf, is schadelijk voor de kwaliteit van het onderzoek aan universiteiten. Een systeem zonder dergelijke hiërarchie, waarin een universitair onderzoeker alleen op basis van eigen prestaties i.p.v. de beschikbaarheid van een positie promotie tot associate- dan wel full professor kan maken, zou beter zijn.

7. De grote populariteit die Máxima onmiddellijk genoot toont het ongelijk aan van het correcte denken dat uiterlijk niet belangrijk zou zijn.

Behorende bij het proefschrift: **"Modulation of the chicken immune cell function by dietary polyunsaturated fatty acids"**

John Sijben, 18 januari 2002

Modulation of the chicken immune cell function by dietary polyunsaturated fatty acids

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Modulation of the chicken immune cell function by dietary polyunsaturated fatty acids

John Sijben

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. ir. B. Speelman,
in het openbaar te verdedigen
op vrijdag 18 januari 2002
des namiddags te vier uur in de Aula

Sijben, J.W.C. Modulation of the chicken immune cell function by dietary polyunsaturated fatty acids.

PhD-Thesis Wageningen University - With ref. - With summary in Dutch

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Abstract: Polyunsaturated fatty acids (PUFA) possess a wide range of biological properties, including immunomodulation. The amount, type, and ratio of dietary PUFA determine the types of fatty acids that are incorporated into immune cell membranes. Consequently, the physiological properties of immune cells and their potential to produce communication molecules, such as eicosanoids, can be modulated. This thesis aims to describe the (interaction) effects of n-3 and n-6 PUFA after challenge with antigens of various nature, and thereby to identify PUFA requirements for optimal immune responses in chickens. Antibody responses to model antigens known to induce T helper-1 and T helper-2 type responses in mice were increased or decreased by n-6, and increased or not affected by n-3 PUFA, depending on the antigen, and levels of other PUFA. Cutaneous hypersensitivity, an index for in vivo T cell reactivity, was increased by n-3 and decreased by n-6 PUFA or not affected. In vitro T cell reactivity was increased in chickens fed n-3 PUFA enriched diet. Evidence that vitamin E interacts with PUFA effects on immune cell function, particularly at high PUFA levels, was not found. Cytokine mRNA levels early after LPS challenge were increased by long-chain n-3 PUFA or not affected by dietary PUFA. The present thesis indicates that dietary PUFA have the potential to modulate chicken immune cell function, but that most effects are the contrary of what is usually found in mammals. The differences are possibly due to differences in chickens and mammals with regard to the metabolism and effector functions of PGE_2, and the effects of dietary n-3 on cytokine production. It is hypothesized that inclusion of 1-2 % of n-3 and inclusion of no more than 3-4 % of n-6 in the diet, is optimal for Ab responsiveness, enhances T cell reactivity, and possibly improves chicken's disease resistance.

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Voorwoord

Eindelijk is het boekje dan af. Hoewel het promoveren zelf een nogal eenzame gebeurtenis kan zijn, is het traject dat daar naartoe leidt dat zeker niet. Bij het tot stand komen van dit proefschrift zijn een groot aantal mensen betrokken geweest. Henk en Johan, jullie bijdrage was zonder twijfel onmisbaar. Jullie verschillende inzichten en achtergronden hadden voor mij een perfecte synergie. Bas, gelukkig dat je er halverwege nog bij kwam, zonder promotor is het lastig promoveren. Mike en Ger, zonder jullie inzet waren de bloedtapsessies ongetwijfeld nachtwerk geworden, om maar te zwijgen over het daaropvolgende labwerk. Jan, Roel en Aard zonder jullie goede zorgen over de kippen was ik nu misschien wel wat meer volleerd kippenboer, mijn dank is er desondanks niet minder om. Kirk, thanks for the opportunity to come over to Davis to work in your lab. It has been an experience of great significance to me. Raymond, Tanya and the rest of the Davis guys, thanks for all the help and good time. Jan, zonder je hulp en gastvrijheid was het heel wat lastiger geweest mijn nieuwe kunstjes thuis te vertonen. Pete and the rest at the IAH, although the many pipetting nearly inflicted RSI, Compton was great fun. Alle overige co-auteurs in dit boekje, Martin, Anton en Huub, Harry en Ajay, en Robert, jullie bijdragen in de vorm van wijze woorden, goede afstudeervakken, en heel veel vetzuuranalyses, was zeer divers maar niet minder belangrijk. Mijn overige collega eerst GenRers en later ADFers, ik had het heel wat slechter kunnen treffen qua sfeer bij de koffie en de borrels. Tenslotte zijn er een aantal mensen die de moeite namen helemaal naar Californië te vliegen om met me te hiken in Yosimite, en een bijzondere vermelding verdienen: Tom en Hans, mijn paranimfen, en maatjes op de vrijdag namiddagen die steeds meer avonden werden, mam en pap, heel bijzonder dat jullie daar ook waren, en Leonie, je kipje heeft met deze fotogenieke pose voor de omslag toch wel een beter lot verdiend dan een zomeravondse barbeque !

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Contents

Chapter 1

General Introduction

Introduction

Nutrition as a tool for improving animal health has been subjected to an increased interest in animal research programs in the past decade. Several grounds are attributable for this. The knowledge of nutritional requirements for production and their efficiency has been well developed, however, a large amount of nutrients have specific functions in maintenance processes which are addressed under conditions of exposure to e.g. environmental or pathogenic stresses. Such dynamic requirements are less well known. Moreover, increased pressure of scientific and public origin (Health Counsel of the Netherlands, 1998; Witte, 1998) entailed National and European Union legislation leading to restrictions in the application of antibiotics for prophylactic use and growth promotion in intensive livestock farming.

The use of anti-microbial growth promoters in livestock farming accounts for a considerable proportion of the total amount of antibiotics used the Netherlands. It has been shown that the use of various antibiotics, such as avoparcin, bacitracin, tylosin, carbadox and olaquindox as growth promoters can produce resistance to these substances within livestock. Humans can be infected by both resistant human pathogenic bacteria (*Salmonella* spp., *Campylobacter* spp.) and resistant commensal bacteria (*E. coli* and enterococci) from the intestinal flora of livestock animals. The possibility of the transfer of resistance genes in plasmid- and bacteriophage-DNA from the bacteria of livestock to human pathogenic micro-organisms has been shown in both laboratory and field research (references in Health Counsel of the Netherlands, 1998).

Already in 1969 this risk of unbridled use of antibiotics in animals farming was recognized by the Swann-committee (Swann Committee, 1969). Their recommendations resulted in the prohibition for the use of tetracyclin and penicillin as growth promoter in the European Union by 1974. By 1996 the EU prohibited the widely used antibiotic avoparcin as growth promoter. Avoparcin is related to vancomycin, the only available anti-biotic effective in the treatment of patients infected by methicillin-resistant *Staphylococcus aureus* (MRSA). Resistance to vancomycin has already been identified in relation to another cause of hospital infections: *Enterococcus* spp. Though adequate information about the relative contribution from the reservoir of resistance genes in animals to the prevalence of resistant micro-organisms in humans is not available, generally further restrictions in the use of antibiotics as were expected.

Such policy might have undesired side effects. Some of the consequences are indicated by the Swedish situation, where a total ban on the use of antibiotics for

growth promotion became effective in 1986. In The Netherlands approximately 100 and 10% of the feeds for broilers and (growing) layer hens, respectively, are supplemented with antibiotics. In addition, approximately 65 and 40% of the feeds are supplemented with coccidiostatics. For poultry farming in the Netherlands, implementation of the Swedish situation (i.e. permission for limited use anti-coccidia) would result, without the use of alternatives, in a decrease in income of 59, 18 and 14% for broiler-, growing layer hen-, and layer farming respectively. A total ban on both antibiotics and coccidiostatics would result, without the use of alternatives, in a decrease in income of 170, 108 and 14% for broiler-, growing layer hen-, and layer farming respectively (IKC, 1998).

These economical losses are associated with, and partially the result of, impaired animals health. Restricted use of antibiotics as feed additives might facilitate colonization of the gastro-intestinal tract of poultry by potential pathogenous microbes or other parasites, and their systemic translocation. It follows that there is a great significance for the development of alternatives as substitutes for antibiotics that complicate infection, such as pro-biotics, pre-biotics, organic acids, enzymes and specific nutrients that can support the immune system. In the present thesis effects of polyunsaturated fatty acids (PUFA), a class of nutrients that possess a wide range of biological properties, including immunomodulation, are studied.

Synthesis of Polyunsaturated Fatty Acids

Pathways illustrating the desaturation and chain elongation steps in liver for *de novo*-synthesized and consumed fatty acids are shown in Figure 1. All mammals, and presumably also Aves, can synthesize fatty acids *de novo* from acetyl-CoA on the cytoplasm membrane in the endoplasmatic reticulum. The end product of the fatty acid synthetase enzyme is palmitic acid (16:0), which may, in turn, be elongated to stearic acid (18:0). Because cell membranes require unsaturated fatty acids to be provided to maintain their structure, fluidity and function, a mechanism for the introduction of double bounds (desaturation) exists. The introduction of a single double bound between C-9 and C-10 is catalyzed by the enzyme Δ^9-desaturase, which is universally present in both plants and animals. This enzyme results in the conversion of stearic acid to oleic acid (18:1*n-9*). Animals, unlike plants, lack the capability to insert additional double bounds into oleic acid between the existing double bound at the 9-position and the methyl terminus of the carbon chain; in plants a Δ^{12}-desaturase converts oleic acid into linoleic acid (18:2*n-6*) while Δ^{15}-desaturase converts linoleic acid into α-linolenic acid (18:3*n-3*).

Animal cells can carry out chain elongation and further desaturation of linoleic acid to arachidonic acid (20:*4n-6*), via γ-linolenic (18:3*n-6*) and dihomo-γ-linolenic (20:3*n-6*) acids. By a similar series of reactions, α-linolenic acid is used to yield n-3 PUFA eicosapentaenoic acid (20:5*n-3*) and docosahexaenoic acid (22:6*n-3*). Many marine plants, especially the unicellular algae in phytoplankton, also carry out synthesis of long-chain n-3 PUFA. The formation of these long-chain n-3 PUFA by marine algae and their transfer through the food chain to fish accounts for the abundance of eicosapentaenoic and docosahexaenoic acid in marine fish oils.

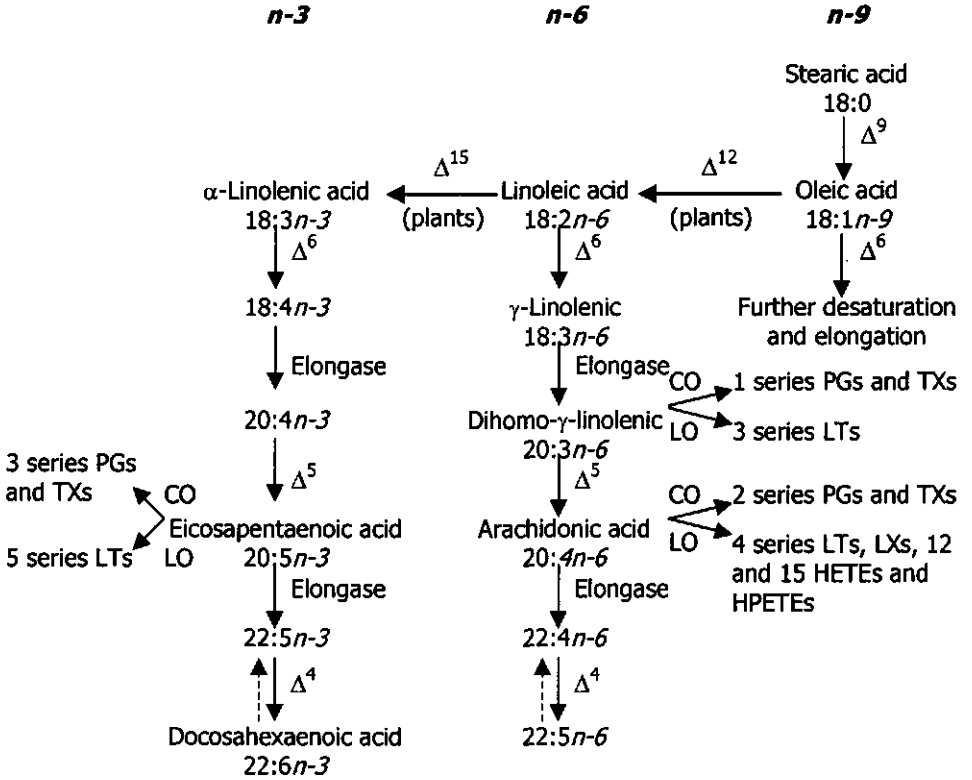

Figure 1. Pathways for the biosynthesis of polyunsaturated fatty acids and eicosanoids. Δ^{15}, Δ^{12}, Δ^{9}, Δ^{6}, Δ^{5} and Δ^{4} indicate the desaturase enzymes. CO and LO indicate cyclooxygenase and lipoxygenase respectively. PG, TX, LT, LX, HETE and HPETE indicate prostaglandin, thromboxanes, leukotrienes, lipoxins, hydroxyeicosatetranoid acid and hydroperoxyeicosatetranoid acid respectively (after Calder, 1996c).

As a result of the actions of the Δ^{9}-, Δ^{12}- and Δ^{15}- desaturase enzymes, two families of PUFA, the n-6 and n-3 family, exist; these PUFA families are not metabolically interconvertible in mammals (Calder, 1996a). The formation of long-chain PUFA depends on the availability of the elongases and Δ^{9}-, Δ^{6}-, Δ^{5}- and Δ^{4}-

desaturase enzymes. The two families of PUFA compete with each other, and also with n-9 monounsaturated fatty acid family, as substrates for these enzymes. The preferred substrates are α-linolenic acid > linoleic acid > oleic acid. During this conversion, the rates of elongation are generally faster than those of for desaturation. Particularly the Δ^6- desaturase step is rate-limiting in mammals (Brenner, 1989). However, it is unclear whether e.g. this rate-limiting step is the same for poultry, since the body of literature describing enzymatic control of desaturation and elongation of fatty acids in poultry is minimal compared to that in mammalian models. Although desaturase acitivities regulate tissue concentrations of fatty acids, especially of PUFA (Sprecher, 1989), dietary lipid can dictate fatty acid composition in poultry.

A large quantity of literature provides evidence that varying the type and amount of dietary unsaturated fatty acid dramatically modifies the fatty acid composition of lipids in the tissue, including lymphoid organs, of growing chicks (Phetteplace and Watkins, 1989, 1990; Fritsche et al., 1991; Friedman and Sklan, 1995; Applegate and Sell, 1996). Briefly, these studies indicate that feeding linseed oil, rich α-linolenic acid, depresses the amount of arachidonic acid and total n-6 PUFA and raises the levels of eicosapentaenoic and docosahexaenoic acid in organ lipids, presumably by enhancing long-chain n-3 PUFA formation. Also feeding fish oil, rich in long-chain n-3 PUFA is effective in lowering total n-6 PUFA in many, including lymphoid tissues. In general these data indicate that the amount of each PUFA deposited into the membrane phospholipids largely depends on the dietary PUFA intake.

Polyunsaturated fatty acid requirement

As indicated before, cell membranes require unsaturated fatty acids to maintain their structure, fluidity and function. Since animal tissues are unable to synthesize linoleic and α-linolenic acid, these fatty acids must be consumed in the diet and so are termed essential fatty acids (Calder, 1998). At present, standard nutritional requirement tables give a fatty acid requirement of 1% of total diet, being linoleic acid, for growing pullets, of both layer and broiler type strains (NRC, 1994). For adult poultry some nutritionists feel that laying hens may have two requirements for linoleic acid: 0.9% for physiological purposes (Balnave, 1971) and an additional 2-4% for maximum egg size (Whitehead, 1984). Inadequacies of linoleic acid result in increased water consumption, reduced resistance to disease, enlarged liver with increased lipid content and elevated concentrations of eicosatrienoic acid but decreased amounts of linoleic and arachidonic acids in many tissues. (Balnave,

1970). A deficiency of linoleic acid in the male can impair spermatogenesis and affect fertility. A dietary need for α-linolenic acid has yet to be demonstrated for the fowl (NRC, 1994).

In modern intensive livestock farming the NRC requirement is met or exceeded by feeding industrial produced corn-soybean-based diets. These plants are rich in n-6 fatty acids but poor in n-3 fatty acids. Studies on fatty acid ratios in chicken eggs, reflecting the diet, pointed out that the n-6/n-3 ratio was 19,9 in US supermarket eggs, compared with 1,3 in range-fed Greek chickens (Simopoulos and Salem, 1989). The total n-3 concentration in the latter eggs was also ten times higher in the range-fed chickens compared to the industrial fed chickens. The large amounts of n-3 consumed by these range-fed chickens derived from plants in the wild that contain more n-3 fatty acids then do cultivated feed plants (Simopoulos and Salem, 1986). Significant amounts of α-linolenic acid are found in chloroplast membranes of terrestrial plants rather than in the seed oils that are usually processed commercially to make feed. It is presumed that, compared to the industrial fed chicken, the conditions under which the chickens producing these Greek eggs are fed is more similar to that of the wild ancestor of modern production poultry. These conditions include free ranging and consuming green leafy vegetables, fresh and dried fruit, insects and occasional worms. So, Pleistocenic nutrition of chickens most probably included much more α-linolenic acid compared to present-day industrial chicken feed. This indicates that from an evolutionary point of view it seems likely that also n-3 fatty acid is required, because pre-domestication chicken must have been adapted well to it's natural habitat, including the available nutrients, in order to survive as a species.

Since the start of domestication of the fowl in China some 10 thousand years ago, followed by intensive poultry breeding programs in modern times, specific nutritional requirements might have changed as a consequence of the genetic changes by domestication and breeding. However, indications that n-3 fatty acids are still important are e.g. the presence of significant concentrations of docosahexaenoic acid (22:6*n-3*) in lipids of the retina (Rezanka, 1989) and nervous tissue (Anderson et al., 1989; Budowski and Crawford, 1986). The proportion of docosahexaenoic acid in the phospholipids of the embryo's brain can be compelled to vary from as little as 2% in the absence of dietary n-3, to as much as 30% by feeding fish oil enriched diet (Speake et al., 1998). Analysis of the fatty acid profiles of the initial yolk and the embryonic brain of 7 avian species (moorhen, pheasant, goose, duck, kestrel, gull and king penguin) living in the wild indicated that the proportion of docosahexaenoic acid in the yolk varied over 12 fold

between these species, however, the proportion of docosahexaenoic acid in the embryonic brain phospholipids was identical for all these species, 17% of the fatty acids (Speake and Thompson, 1999). This lack of interspecies variability displayed by birds on the wild on their natural diets suggests that there is an optimal brain docosahexaenoic acid level which provides the ideal degree of flexibility in the neuronal membranes (Speake and Surai, 2000). It also follows that a minimal amount of dietary n-3 in feed of chickens kept in captivity is required to reach the avian norm.

In addition to a need for α-linolenic acid for normal development of brain and retina, also n-6 and n-3 requirements for other maintenance processes such as eicosanoid production, might be different from the standard tables (Watkins, 1991). The subsistence of specific functions of n-3 deriving eicosanoids indicates that the capability of synthesizing them might be essential. The role of eicosanoids as regulators of the immune system is described in detail in the following paragraphs. In regard of the importance of their function, immune responses and disease resistance may be improved by optimizing their precursor levels, i.e. PUFA, supplied in the diet.

Mechanisms by which dietary PUFA affect immune cell function

The principal roles of PUFA are as energy sources and as membrane constituents. The mechanisms by which dietary (n-3) PUFA might affect immune cell function have been extensively described by many authors and reviewed by Calder (1996a,b,c, 1998). Most important modulator pathway of PUFA on immune cell function is by acting as precursors of eicosanoid synthesis. Eicosanoids are a family of oxygenated derivatives of dihomo-γ-linolenic, arachidonic acid and eicosapentaenoic acids. Eicosanoids include prostaglandins, thromboxanes, leukotrienes, lipoxins, hydroxyeicosatetranoid- and hydroperoxyeicosatetranoid acids. In most conditions the principal precursor of these compounds is arachidonic acid (Figure 1) and these eicosanoids seem to have more potent biological functions than others. The precursor PUFA is released from membrane phospholipids by the actions of phospholipase enzymes. The amounts and types of eicosanoids synthesized are determined by the availability of precursor fatty acids and by the activities of phospholipase, cyclooxygenase and lipoxygenase enzymes. Eicosanoids have a short half-life time and therefore act locally to the cell from which they are produced. Their production is initiated by particular stimuli, such as cytokines, endotoxins and antigen-antibody complexes, and once produced, they themselves are able to modify the response to the stimulus. In poultry, a limited

amount of studies have shown that eicosanoid synthesis is altered by changing the dietary PUFA content (Craig-Smith et al., 1987; Watkins and Kratzer, 1987; Fritsche and Cassity, 1992).

Different eicosanoids have different, and sometimes opposite effects. Eicosapentaenoic and arachidonic acids competitively act as substrates for both cyclo-oxygenase and lipoxygenase. Thus, the ingestion of n-3 rich diet results in a decrease in membrane arachidonic acid level and a concomitant decrease in the capacity to synthesize n-6 derived eicosanoids and an increase in n-3 deriving eicosanoids. In addition, n-3 deriving prostaglandin (PG)E_3 and leukotriene (LT)B_5 are biologically less active then n-6 deriving PGE_2 and LTB_4. Among immunocompetent cells, it seems that macrophages are the principal, and perhaps the only source of eicosanoids, but that an interaction between lymphocytes and macrophages exists. Lymphocytes contain similar amounts of arachidonic acid in their membrane phospholipids compared to macrophages, and mitogen-stimulated lymphocytes release arachidonic acid extracellularly. There is evidence that macrophages use this arachidonic acid for eicosanoid synthesis (see Calder 1996a for references). The best-documented eicosanoid effects are those of PGE_2. In mammals, PGE_2 has a number of pro-inflammatory effects, plays a role in regulating the differentiation of T and B-lymphocytes and can influence the antibody production. In general, the age and type of target cell and the concentration of PGE_2 determine the nature of the response. Other eicosanoid's effects on immune cells are studied less extensive, even in mammals, but are probably just as diverse. Published studies describing the biosynthesis, biological effects and degradation of eicosanoids in poultry are few relative to those conducted in mammalian species. Eicosanoids, unlike most other communication molecules, are not species specific, and much of the metabolism and actions of eicosanoids in poultry are presumed to follow what is reported for other animals. Few studies confirm the legitimacy of this proposition. Both dietary fish oil and lofrin, a potent, reversible and selective 5-lipoxygenase inhibitor (LO pathway in Figure 1), decreased the growth suppressing effects of coccidial infection compared with dietary corn oil (Korver et al., 1997). This observation supports the efficacy of modifying eicosanoid metabolism, i.e. inhibiting the production of LTB_4. In addition, this observation suggests that, similar to mammals, LTB_5 is a less potent chemotactic agent compared to LTB_4 in terms of stimulating chemotaxis and recruiting of pro-inflammatory cells to the site of inflammation.

Apart from influencing the pattern of eicosanoids produced, fatty acids can also elicit their effects by eicosanoid-independent mechanisms (Figure 2). The

composition of phospholipids in cell membranes is usually characteristic for the cell type, but may change in response to stimuli or with changes in the diet. Changing the proportions of different types of fatty acids in cell membranes may alter the fluidity of those membranes. Both the fatty acid composition of plasma-membrane phospholipids and the fluidity of the plasma membrane are known to affect membrane activities, such as ion transport and substrate transport, receptor functioning and the activities of membrane-bound enzymes. However, the mechanisms by which membrane lipids modulate enzyme activity or receptor function are not fully understood. The stimulation of lymphocytes is accompanied by *de novo* synthesis and turnover of membrane phospholipids. Within minutes after stimulation, the substitution of saturated fatty acids in phospholipids by PUFA begins (Ferber et al., 1975). This change in fatty acid composition is accompanied by an increase in membrane fluidity (Calder et al., 1994). In contrast, macrophage plasma membrane fluidity decreases after stimulation (Grimble and Tappia, 1995). The effect of the dietary fat composition on the fluidity of immune cells rather depends on relative the amounts of saturated- versus (poly)unsaturated fatty acids than on the relative amounts of n-3 versus n-6 PUFA.

Some membrane phospholipids, such as phosphatidylinositol-4,5-biphosphate, are involved in intracellular signaling mechanisms since they act as precursors for second messengers such as inositol-1,4,5-triphosphate and diacylglycerol. These phospholipids contain fatty acyl chains attached to the glycerol moiety. Thus, changing the fatty acid present may alter the precise properties of these compounds with regard to their function in signal transduction. In addition to these effects due to changes of the phospholipid composition, unsaturated fatty acids themselves may have direct effect on intracellular signaling mechanisms (Sumida et al., 1993).

More recently, evidence increases that fatty acids directly affect the expression of genes, including genes important in immune cell function. The molecular mechanisms of PUFA regulation of gene expression are still poorly understood (review by Sessler and Ntambi, 1998). Studies on Δ^9 desaturase gene in lymphocytes have shown that the effect of PUFA on gene expression can be at the level of both gene transcription and mRNA stability. For example, the half life time of Δ^9 desaturase mRNA is 67% lower after treatment with arachidonic acid (Sessler et al., 1996). PUFA might also act by possessing intracellular receptors, which directly influence transcription. One idea of PUFA regulation of gene transcription is based on the idea that a *cis*-acting PUFA responsive element is located in the promoter region of the PUFA-regulated genes. To alter gene

transcription, a transcription factor (PUFA binding protein) could bind to a PUFA responsive element and block or enhance transcription. Another idea of PUFA regulation of gene transcription is based on PUFA binding to peroxisome proliferator-activated receptor and then activating or repressing transcription of genes by interacting with the peroxisome proliferator responsive element. Numerous studies already indicated that dietary PUFA alter the mRNA levels of cytokines in mammals. Particularly interesting in this respect was that the lower IL-1β mRNA level in mice stimulated splenocytes of mice fed a fish oil rich diet was not due to accelerated degradation but to impaired synthesis (Robinson et al., 1995). However, so far it is not clear to which extent modulations in the expression of genes involved in immune cell function are due to direct influence on transcription or by altered levels of other mediators, e.g. eicosanoids.

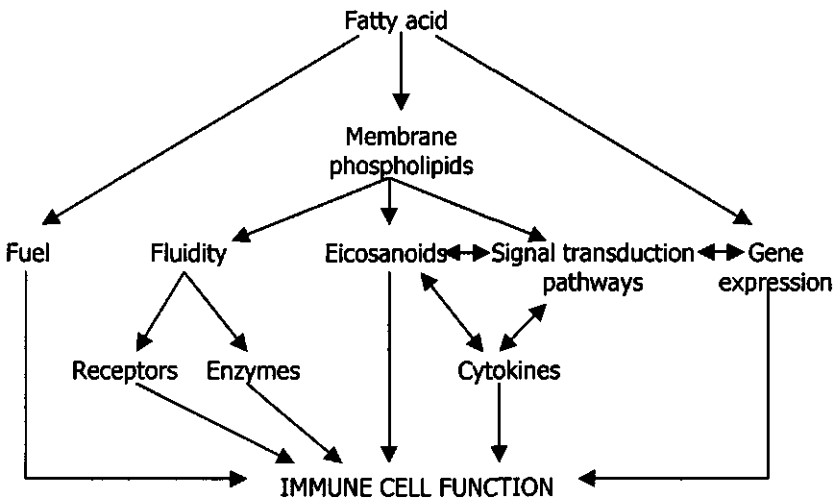

Figure 2. Mechanisms by which fatty acids might affect immune cell function (after Calder 1996a,b).

Effects of dietary PUFA on the immune cell function

In most literature the marked effects of changing the amounts of dietary PUFA on inflammation and immunity are attributed to changes in the amounts and types of eicosanoids. Starting point in such literature usually is the fatty acid content of typical Western diets that is suspected to have become deformed, i.e. too much linoleic acid and too little α-linolenic or long-chain n-3 acids. Intake of higher levels of n-3 PUFA is associated with lower incidence of cardiovascular diseases, type-2-diabetes, arthritis, other inflammatory and autoimmune disorders, and cancer.

Replacement of membrane arachidonic acid by n-3 PUFA is accompanied by decreased capacity of inflammatory cells to produce eicosanoids from arachidonic acid, such as PGE_2, LTB_4 and thromboxane $(TX)A_2$. Prostaglandin E_2 is a well known pro-inflammatory and immunoregulatory eicosanoid. Leukotriene B_4 also induces inflammation and is a powerful inducer of leukocyte chemotaxis and adherence. Thromboxane A_2 is a potent platelet aggregator and vasoconstrictor. Ingestion of n-3 PUFAs leads to decreased formation of PGE_2, LTB_4 and TXA_2 and increased formation of their functionally weaker eicosanpentaenoic acid deriving counterparts PGE_3, LTB_5 and TXA_3. By this means modulation of the potency to produce eicosanoids by immune cells changes the "steady state" of the immune system, resulting in marked effects in cytokine profiles during e.g. inflammation or a T-helper cell mediated responses.

The effects of dietary n-3 PUFA on inflammation and the production of pro-inflammatory cytokines are reviewed by Blok and co-workers (1996). Briefly, dietary n-3 decreased IL-1β, IL-2, IL-6 and TNF-α in human peripheral blood mononuclear cells, but increased IL-1β and TNF-α in peritoneal macrophages in mice. In general, the effects of dietary n-3 appear to depend on the cytokine-producing cell type and the health status of the individual. The role of eicosanoids in this cascade is still inconclusive. Studies on the role of PGE_2 and LTB_4 in the alteration of cytokine production by PUFA show divergent results. It seems that reduced production of these two eicosanoids is not the only mechanism responsible for modulated cytokine production by dietary n-3 PUFA.

The effects of dietary PUFA in T-helper cell mediated responses remain to be established. However, it has been proposed that PGE_2 has an important role in the regulation of specific immune responses (reviewed by Phipps et al., 1991). The effects from PGE_2 result from its binding to a PGE receptor that then stimulates production of the second messenger cyclic-3',5' adenosine monophosphate (cAMP). Prostaglandin E_2 can affect B cell differentiation resulting in enhanced IL-4 induced class switch from IgM to IgG1 and IgE. In addition, PGE_2, as well as other agents that elevate cAMP decrease the T helper-1 subset production of IL-2 and IFN-γ (Betz and Fox, 1991). In addition to inhibiting the production of the Th-1 associated cytokines in murine and human CD4+ T cells, PGE_2 is also associated with up-regulating the production of Th-2 associated cytokines IL-4 and IL-5 (Hilkens et al., 1995). These findings gave rise the concept that PGE_2 may tip the TH-1/TH-2 balance in favor of the T-helper type 2 cells, possibly also leading to B-cell production of IgG1 and IgE. The effect of PGE_2 as a co-stimulatory factor contributing to the development of naive T helper cells towards Th-2 cells is

suggested to be the result of inhibition of dendritic cells to produce IL-12 (reviewed by Kalinski et al., 1999). The latter implies that the presence of PGE_2 might selectively support immune cell function, depending on the nature of the antigen i.e. whether the response directed to the antigen is dominated by either Th-1 or Th-2 cells. This way the protective immunity against typical Th-1 pathogens, such as intracellular parasites, might be inhibited by high PGE_2 production, whereas the immunity against typical Th-2 pathogens, such as metazoan parasites, might be supported by PGE_2. In chickens a similar definition of subsets of helper T lymphocytes based on their cytokine secretion patterns has not been made. Nevertheless, also in avian species different types of antigens might result in a selective boost of cytokines followed by different sorts of immune responses. Whether these responses can be modulated by dietary PUFA, via eicosanoid mediation or by any other mechanism, remains to be established.

Aim and scope of this dissertation

The above-mentioned outline of all the possible mechanisms by which dietary PUFA composition might affect cells of the immune system indicates that this is an extremely complex combination of factors and mechanisms to deal with. By far most of the literature describing these mechanisms is based on studies in mammalian species. In Aves such as chickens, it is presumed that these mechanisms are somewhat similar. The presented studies are an attempt to gain more insight in specific PUFA requirements for immune responses for growing chickens of laying strains, with the ultimate aim to define PUFA requirement for optimal immune cell function. Special emphasis is put on whether this requirement is different for different types of immune responses, such as humoral and cellular responses, or T helper cell-1 and T helper cell-2 like responses. In addition, attention is paid to the mechanisms underlying these effects in chickens to investigate whether these are similar to what is described in mammals.

In more detail, first, the effects of various levels of dietary n-3 and n-6 PUFA on several immune parameters were compared. In *Chapter 2* effects of one diet enriched with linoleic acid, one diet enriched with α-linolenic acid and a diet rich in saturated fatty acid on immune responses directed to antigens known to induce T-helper-1 and T-helper-2 responses in mice are described. Because n-3 and n-6 metabolisms are intertwined, in *Chapter 3* special emphasis is put on interactions of n-3 and n-6 PUFA. In this chapter effects of 16 diets, stepwise enriched with linoleic and/or α-linolenic acid, on immune responses directed to the same antigens are described. High levels of PUFA might require extra protection from

oxidation to maintain their stability and biological properties. Therefore, in *Chapter 4* special emphasis is put on whether extra vitamin E is required to maintain the immunomodulating effects at high levels of dietary PUFA.

Second, to test the hypothesis that dietary PUFA, via PGE_2 mediation, divergently affects immune responses, an experiment in which the role of this potential important eicosanoid is described in *Chapter 5*.

Third, in the cascade from dietary PUFA to differences in read-out parameters of the immune system cytokine regulation may play a pivotal role in the orchestration of these responses. Therefore, in *Chapter 6 and 7* effects of four dietary fat sources, different in PUFA content, on splenic mRNA levels of several cytokines after an inflammatory challenge are presented.

In *Chapter 8*, the results described in this dissertation are discussed.

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Chapter 2

Dietary Polyunsaturated Fatty Acids Divergently Affect Immune Responsiveness of Growing Layer Hens

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Abstract: The effects of linoleic acid (LA) and α-linolenic acid (LNA) enriched diets on humoral and in vivo cellular immune responses to keyhole limpet hemocyanin-dinitrophenyl (KLH-DNP) and *Mycobacterium butyricum* were studied in growing layer hens. Pullets were fed one of three diets: a control, LA-enriched, or LNA enriched. Pullets were assigned to one of three immunization treatments: KLH-DNP, *M. butyricum*, or PBS. The LA enriched diet enhanced the antibody response to KLH in pullets immunized with KLH-DNP. On the other hand, the antibody response to *M. butyricum* in *M. butyricum* immunized birds was decreased by feeding an LA-enriched diet. In vitro lymphocyte proliferation in the presence of Concanavalin A was affected by the interaction between diet and immunization. Neither cutaneous hypersensitivity to KLH nor to *M. butyricum* was affected by the diet. The BW gain before immunization was not affected by the diet, but after immunization, the LA-enriched diet enhanced growth in birds immunized with *M. butyricum*. Diets had various effects on organ weights. We concluded that dietary linoleic acid enrichment of the diet has an antigen-dependent divergent effect on the antibody response. The dietary LNA effect on the antibody response is less pronounced and is opposite to that of the LA effect.

Introduction

Polyunsaturated fatty acids (PUFA) are the precursors of immunoregulatory eicosanoids such as prostaglandins (PG) and leukotrienes (LT). The PUFA induce immunomodulatory actions after intermediate incorporation into lymphoid tissues. PUFA composition of the bursa of Fabricius, thymus (Fritsche et al., 1991b; Friedman and Sklan, 1995), and bone marrow (Friedman and Sklan, 1995) were determined by diet.

Linoleic acid (LA; $C_{18:2n-6}$) is the principal precursor of all n-6 PUFA and derived eicosanoids, whereas linolenic acid (LNA; $C_{18:3n-3}$) forms the principal precursor of longer n-3 PUFA and their eicosanoids. The metabolic pathways of principal n-6 and n-3 PUFA to form eicosanoids are characterized by competition for the binding sites of desaturase and elongase enzymes, and in the last stage of eicosanoid synthesis, of cyclooxygenase and lipoxygenase (Kinsella et al., 1990). As a consequence, dietary PUFA content is likely to influence the following eicosanoid production and tissue PUFA composition.

Experimental evidence that prostaglandin E_2 (PGE_2) synthesis, a product of the cyclo-oxygenase pathway of n-6 PUFA arachidonic acid (AA; $C_{20:4n-6}$) with a wide range of immunoregulatory actions, is influenced by dietary PUFA composition in chicks has been reported (Craig-Smith et al., 1987). Effects of dietary n-3 or n-6 PUFA on immune response of poultry via eicosanoid intermediates have been reported as well. Fish oil, rich in n-3 PUFA, in the diet of broilers had an inflammatory suppressive effect and decreased plasma tumor necrosis factor (TNF) (Korver et al., 1997), and interleukin (IL)-1 release by peritoneal macrophages (Korver and Klasing, 1997). Dietary LA and LNA affect antibody (Ab) responses in growing layer hens, which might be mediated by PGE_2 synthesis (Parmentier et al., 1997).

In mice, inflammatory responses are regulated by T-helper (TH)-1 cells, whereas TH-2 cells underlie Ab responses (Mossmann and Coffman, 1989). The production of TH-1 cytokines (e.g., IL-12 and interferon-gamma) in vitro is downregulated by PGE_2 (Demeure et al., 1997), whereas the release of TH-2 cytokines, (such as IL-4) is enhanced. TH-2-dependent (IgG 1 and IgE) Ab responses in vivo are enhanced by PGE_2. The net effect of PGE2 produced by antigen-presenting cells, such as a macrophgages, would be to promote stimulation of PGE_2–resistant, IL-4 and IL-5 producing TH-2 cells at the expense of the TH-1 subset (Phipps et al., 1991), which suggests that type and magnitude of the immune response of mice can be selectively affected by dietary PUFA via PGE_2 intermediation.

To our knowledge, in birds the TH-1/TH-2 paradigm has not been yet established. Nevertheless, defence against different sorts of pathogens might be supported by a selective increase in humoral or cellular immunity. To establish the effect of dietary n-6 and n-3 PUFA on humoral or cellular immune response in poultry, antigens known to induce TH-2 (keyhole limpet hemocyanin-dinitrophenyl (KLH-DNP)) (Bradley et al., 1995; Dohorty et al., 1995; Bliss et al., 1996) or TH-1 (*Mycobacterium* protein) (Mosmann and Sad, 1996) responses in mice were used. Simultaneously, effects of dietary PUFA on growth performance were measured.

Materials and Methods

Birds and Housing

In the present study ISA-Warren cross hens (medium-heavy layers) were used. The birds were from a randombred control line, as part of a continuous selection experiment (Van der Zijpp and Nieuwland, 1986), in which birds were selected for high or low Ab response to SRBC. From the 17th generation, 167 pullets from one hatch were used. Chicks were housed in battery cages (50 × 100 cm) with a maximum bird density of 10 chicks per cage. The birds had free access to feed and water. The chicks were vaccinated against Marek's disease and Newcastle disease on the day of hatch, against infectious bronchitis at 2 d of age, and against infectious bursal disease at 15 d of age. On the day of hatch, birds were randomly assigned to the experimental treatments.

Experimental Design

Effects of dietary PUFA were studied using a 3 × 3 factorial design of treatments. Factors were diet and immunization treatment. The diets used had the same composition as in a previous study (Parmentier et al., 1997). The three experimental diets had a similar composition, except for their fat composition. The control diet was low in LA and LNA content, whereas LA-rich diet was high in LA and low in LNA, and the LNA-rich diet was low in LA and high in LNA. The differences in LA and LNA contents among diets were created by varying the ratio of animal fat, sunflower oil, and linseed oil (Table1). The analyzed content of LA was 2.9, 6.2, and 3.0%, and of LNA was 0.7, 0.5, and 3.9% for the control, LA-enriched, and LNA-enriched diets, respectively. From hatch until 5 wk of age, the birds were fed the starter diet. Thereafter, pullets were fed the grower diet. Diets were formulated to meet nutritional requirements of layer pullets of a medium-heavy strain (NRC, 1994).

Table 1. Composition of the experimental diets[1]

Ingredients and content	Control diet	LA-enriched diet	LNA-enriched diet
Fat ingredients:		(%)	
Animal fat	6	0	0
Sunflower oil	1	7	1
Linseed oil	1	1	7
Analyzed content:			
Linoleic acid (LA)	2.9	6.2	3.0
α-Linolenic acid (LNA)	0.7	0.5	3.9
LNA/LA ratio	0.24	0.08	1.30

Basal diet (92%)	Starter diet	Grower diet
Maize (8.7% crude protein (CP))	20	20
Wheat (11.9% CP)	5	5
Peas (22.9% CP)	5	5
Wheat middlings (15.7% CP)	8	10
Soybean meal (44.9 % CP)	21.9	15
Sunflower meal (34% CP)	6	8
Maize glutenfeed (20.9% CP)	7	7
Lucerne (16.9% CP)	2	3
Meat and bone meal (58.4 % CP)	2	1
Tapioca (65% starch)	9.05	12.4
Cane molasses	1	1
Soybean oil	1	1
Vitamin mineral mix[2]	1	1
Salt	0.27	0.25
Limestone ($CaCO_3$)	1.54	1.2
Monocalciumphosphate	1.14	1.1
DL-Methionine	-	0.06
Calculated contents (%)		
CP	19.9	17.5
ME, kcal/kg	2,954	2,947
Ca	0.94	0.90
P	0.43	0.40
Lysine	1.03	0.85
Methionine + cystine	0.76	0.65

[1] on as-fed basis

[2] Supplied per kilogram of diet: vitamin A (retinol acetate), 10,000 IU; cholecalciferol, 2,000 IU; vitamin E (DL-α-tocopherol acetate), 20 mg; riboflavin, 4 mg; D-pantothenic acid, 12 mg; niacinamide, 40 mg; choline chloride, 500 mg; biotin, 0.1 mg; folic acid, 0.75 mg; B12, 15 μg; K, 5 mg; $CoSO_4.7H_2O$, 1 mg; $NA_2SeO_3.5H_2O$, 0.15 mg; KJ, 5 mg; $FeSO_4.7H_2O$, 300 mg; $Cu\text{-}SO_4.5H_2O$, 100 mg; MnO_2, 100 mg; $ZnSO_4.4H_2O$, 150 mg; ethoxyquin, 100 mg; and carrier, corn, dosage 1%.

At 35 d of age, birds were immunized with KLH-DNP (Cal Biochem - Novabiochem Co., La Jolla, CA), heat-killed *Mycobacterium butyricum* dry cells (Difco Laboratories, Detroit, MI), or PBS as a control. The antigens were administered by an i.m injection in the left breast with 1 mL PBS containing 1 mg

KLH-DNP, 1 mg *M. butyricum* protein, or 1 ml PBS. Dose of KLH-DNP and *M. butyricum* that were used to initiate antibody responses were derived from previous pilot experiments (data not published). The BW was recorded twice per week from hatch until euthanasia with 1 mL T61[3] (Hoechst Roussel Vet N.V., Brussels, Belgium) i.v. at 62 d of age. Weights of heart, spleen, intestinal tract, cecal tonsils, and bursa were determined. Weight of the intestinal tract included chyme but excluded proventriculus, gizzard, and cecal tonsils.

Humoral Immune Response to KLH, DNP and M. butyricum

Total Ab titers to KLH and *M. butyricum* in serum of all birds were determined by ELISA. Briefly, 96-well plates were coated with 1 μg/mL KLH (Cal Biochem - Novabiochem Co., La Jolla, CA) or 4 μg/mL *M. butyricum*. After subsequent washing with tap water containing 0.05% Tween, the plates were incubated for 90 min with serial dilutions of serum. Binding of Ab to KLH and *M. butyricum* was detected by using 1:20,000 diluted rabbit anti-chicken IgG_{H+L} labeled with peroxidase (RαCh/IgG_{H+L}/PO) (Nordic, Tilburg, The Netherlands). After washing, tetramethylbenzidine and 0.05% H_2O_2 were added and incubated for 10 min at room temperature. The reaction was stopped with 2.5N H_2SO_4. Extinctions were measured with a Multiskan (Labsystems, Helsinki, Finland) at a wavelength of 450 nm. Titers were expressed as the $\log_2$ values of the highest dilution giving a positive reaction. Positivity was derived from the extinction values of a positive control serum present on every microtiter plate.

Cutaneous Hypersensitivity to KLH and M. butyricum

Cutaneous hypersensitivity (CH) to KLH and *M. butyricum* was measured as described by Parmentier et al. (1993). Briefly, 30 KLH-DNP-sensitized birds and 26 unsensitized birds were challenged s.c. with 0.1 mg KLH in 0.1 mL PBS into the flat surface of the webs of both wings at day 22 postsensitization. The same treatment using 0.1 mg *M. butyricum* in 0.1 mL PBS was performed with 30 *M. butyricum*-sensitized birds and 26 unsensitized birds. Triplicate measurements were made of the thickness of the web of both wings before, 4-h after, and 24 h after challenge with a paper thickness micrometer (Käfer, Schwenningen, Germany). A 4 h response might be based on precipitating antibodies leading to Type-III hypersensitivity with characteristics of passive CH as indicated previously (Parmentier et al., 1993), whereas a 24-h response represents type IV delayed-type hypersensitivity. The wing-web response at 4 and 24 h after challenge was calculated per bird as the difference between the average of six wing-web

thickness measurements at 4 and 24 h and the average thickness of the six wing-web thickness measurements before challenge.

In Vitro Lymphocyte Proliferation to Concanavalin A

An in vitro lymphocyte stimulation test was performed to determine effects of dietary PUFA on in vitro T-cell proliferation capacity. At day 15 after initial sensitization with antigen, peripheral blood leukocytes (PBL) from 90 birds, 10 birds per treatment, were obtained from heparinized blood using Ficoll density gradient centrifugation. One mL blood, 1:1 diluted with RPMI, was layered on 0.5 mL Ficoll-Paque (Pharmacia, Uppsala, Sweden) and centrifuged for 1.5 min at 11,500 rpm in an Eppendorf centrifuge (Eppendorf, Hamburg, Germany), after which PBL were collected from the interphase. After thorough washing, PBL were tested for proliferation in a final concentration of 1×10^7 cells/mL with 5 μg/mL Concanavalin A (ConA) (Sigma Chemical Co., St. Louis, MO) in RPMI culture medium supplemented with 2 mM L-glutamine, 1% fetal calf serum, 1% chicken plasma, 100 μg/mL streptomycin, and 100 IU/mL penicillin, in 96-well flat-bottomed plates. The cultures were set up in triplicate with ConA (stimulated) and without ConA (unstimulated). After incubation for 48 h at 41° C and 5% CO_2 in humified atmosphere, the cultures were labeled with 0.5 μCi methyl-^{3}H-thymidine (ICN Pharmaceuticals Inc., Cesta Mesa, CA). After 18 h, cultures were harvested. ^{3}H-Thymidine uptake was determined with a Beckman β-scintillation counter. Results were expressed as stimulation indices (SI) per animal. The SI were calculated as: SI = mean counts per min in ConA-stimulated cultures/mean counts per min in unstimulated cultures.

Statistical Analysis

Effects of diet and immunization treatment on Ab titers and growth were tested by repeated measurements with the following model:

$$Y_{ijkl} = \mu + F_i + I_j + e_{1,ijk} + T_l + (T \times F)_{il} + (T \times I)_{jl} + (T \times F \times I)_{ijl} + e_{2,ijkl}$$

where Y_{ijkl} = dependent variable; μ = overall mean; F_i = fixed effect of feed i (i = 1,2,3); I_j = fixed effect of immunization treatment j (j = 1,2,3); $e_{1,ijk}$ = error term 1, which represents the random effect of animal k within the feed i and immunization j level; T_l = fixed effect of time (l = 1,...,4 for Ab titers, l = 1,...,10 for growth before immunization, or l = 1,...,7 for growth after immunization); and $e_{2,ijkl}$ = error term 2, representing the random effect within groups between time

periods. The effects of feed and immunization and their interaction were tested against error term 1. The effect of time and the interactions with time were tested against error term 2. Because of the presence of the interaction among time, diet, and immunization treatment for Ab titers and growth, these parameters were also analyzed for the effect of diet and immunization by two-way ANOVA at each sample moment. Effects of diet and immunization on CH per measuring moment and organ weight and on ConA-induced SI in LST were analyzed by two-way ANOVA. All ANOVA were tested using the GLM procedures of SAS programs (1990).

Results

Antibody Responses to KLH and M. butyricum

Averages of serum Ab titers to KLH and *M. butyricum* during 3 wk after primary sensitization are shown in Table 2.

KLH: The kinetics of anti-KLH Ab titers in serum during 3 wk after sensitization are shown in Figure 1. The birds injected with PBS and *M. butyricum* did not mount an Ab response to KLH, moreover, the anti-KLH Ab titers in these birds were not different between diets (Table 2). Therefore, Ab titers of these six treatments were combined to one baseline (see Figure 1). The Ab titers against KLH were affected by an interaction between diet and immunization ($P < 0.05$). In the KLH-immunized birds, Ab titers against KLH differed between diets, whereas in the control birds (PBS- and *M. butyricum*-injected) diets had no effect (Table 2). During the complete period, in KLH-immunized birds, Ab titers to KLH were higher in birds fed with the LA-enriched diet compared with birds fed the control diet ($P < 0.05$) and were numerically higher in birds fed the LA-enriched diet than in birds fed the LNA-enriched diet (Table 2). The interaction between diet and immunization altered with time after immunization but not significantly ($P < 0.1$; Figure 1). Anti-KLH titers of birds fed the LNA-enriched diet initially rose equally compared with titers fed the LA-enriched diet, but from day 7 after immunization the Ab titers of these birds, in contrast with birds fed LA-enriched or control diet, did not increase further.

M. butyricum: The kinetics of anti-*M. butyricum* protein Ab titers during the 3 wk after sensitization are shown in Figure 2. The birds injected with PBS and KLH-DNP did not mount an Ab response to *M. butyricum.* Moreover, the *M. butyricum* Ab titers in these animals were not different between diets (Table 2). Therefore, titers of these six treatments were combined to one baseline (see Figure 2). Responses to *Mycobacterium* protein were affected by diet, immunization, and

time interactions ($P < 0.001$) and by the interaction between diet and immunization ($P < 0.05$) (Table 2). *M. butyricum*-sensitized birds that were fed LA-enriched diet had lower titers to *M. butyricum* compared with birds fed with the control- or LNA-enriched diet, and this effect increased over time. Titers of birds fed the LNA-enriched diet were highest from Day 7 after immunization onward.

Table 2. Total serum antibody titers to keyhole limpet hemocyanin (KLH) and *Mycobacterium butyricum* after immunization at 35 wk of age in chickens fed one of three diets differing in dietary fatty acids during the 3-wk experimental period

		Antigen[1]	
Immunization	Diet	KLH	*M. butyricum*
KLH-DNP	Control	3.83^{b}	0.80^{c}
	Linoleic rich	4.57^{a}	0.95^{c}
	Linolenic rich	4.10^{ab}	1.15^{c}
M. butyricum	Control	2.47^{c}	2.12^{ab}
	Linoleic rich	2.28^{c}	1.25^{bc}
	Linolenic rich	2.57^{c}	2.29^{a}
PBS	Control	2.49^{c}	1.08^{c}
	Linoleic rich	2.50^{c}	0.80^{c}
	Linolenic rich	2.65^{c}	0.81^{c}
SEM[2]		0.16	0.21
P value:			
Diet		NS[3]	*
Immunization		***	***
Diet × Immunization		*	*
Time		***	***
Diet × Time		***	***
Immunization × Time		***	***
Diet × Immunization × Time		#	***

[1]Means within a column with no common superscript differ significantly ($P < 0.05$)
[2]Average ± SEM of measurements at 0, 7, 15 and 22 days after immunization
[3]NS=not significant, # $P < 0.1$, * $P < 0.05$, *** $P < 0.001$

Cutaneous Hypersensitivity to KLH and M. butyricum

Subcutaneous challenge of KLH-DNP-sensitized birds in the wing-webs with KLH resulted in an acute swelling of the wing-web 4 h after challenge. Wing-web responses to KLH at 4 h were not significantly affected by treatments. However, birds fed the LA-enriched diet had nonsignificantly thicker swellings in the KLH-DNP- and PBS-sensitized group compared with both other diets (Table 3). At 24 h postchallenge, wing-web thickness decreased compared with the 4 h thickness. The 24 h wing-web response was higher in the KLH-DNP- sensitized birds compared with the PBS-sensitized chicks ($P < 0.05$). In the KLH-DNP-imminized

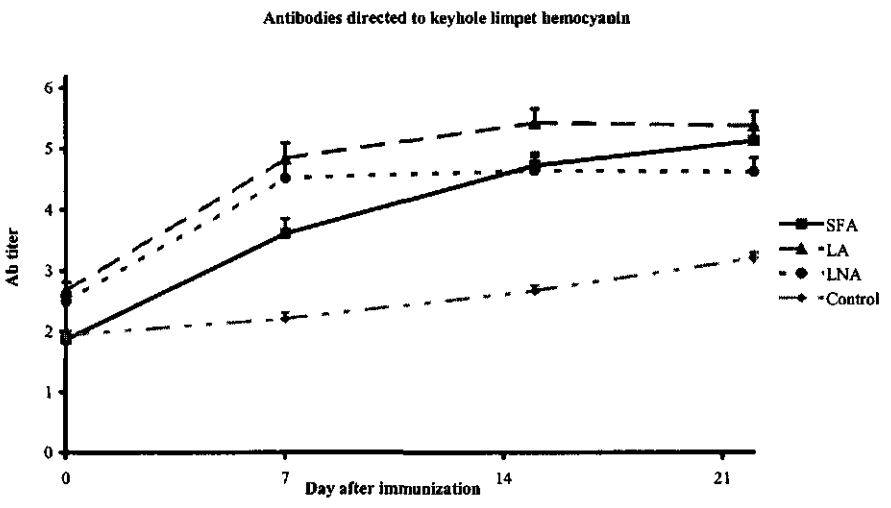

Figure 1. Mean total antibody titers ± SEM to KLH in serum of chicks immunized with KLH-DNP, and fed with control saturated fatty acid rich diet (■, SFA), or linoleic acid-enriched diet (▲, LA), or linolenic acid-enriched diet (●, LNA), or the combined total anti-KLH Ab titers of all *M. butyricum* or PBS immunized chicks (◆, Control)

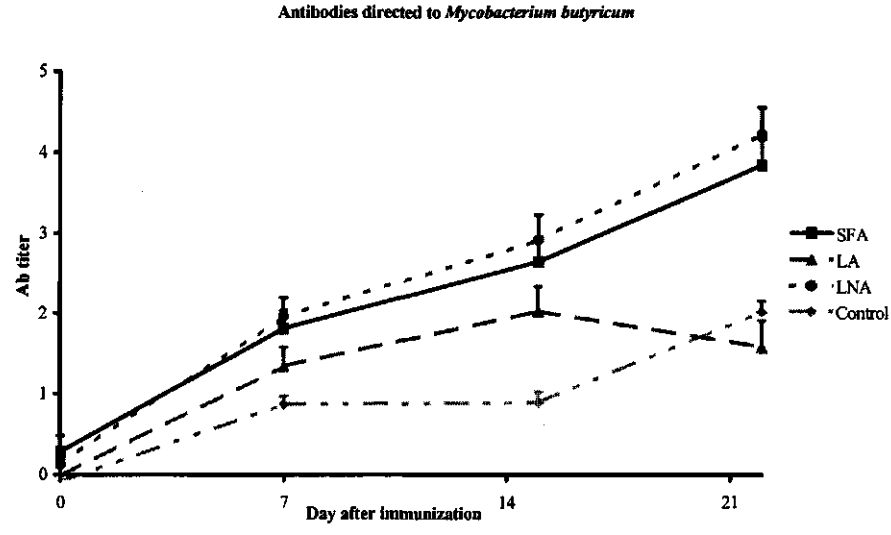

Figure 2. Mean total antibody titers ± SEM to *Mycobacterium butyricum* protein in serum of chicks immunized with *M. butyricum*, and fed with control saturated fatty acid rich diet (■, SFA), or linoleic acid-enriched diet (▲, LA), or linolenic acid-enriched diet (●, LNA), or the combined total anti-*M. butyricum* titers of all KLH-DNP or PBS immunized chicks (◆, Control)

birds, the decrease in wing-web thickness from 4 to 24 h after secondary sensitization was relatively small in the birds fed LNA-enriched diet.

Challenge of the wing-webs with *M. butyricum* also resulted in an acute 4-h wing-web swelling followed by a late 24-h wing-web swelling in birds sensitized 22 days previously with *M. butyricum*. At 4 h after challenge, neither a feed effect nor an immunization effect was found. Unlike the 4-h response, the 24-h response was affected by immunization ($P < 0.001$). By this time the swelling in the unsensitized birds had largely disappeared. At 24-h post-sensitization, unchallenged birds fed the LNA-enriched diet had a notable slight wing-web thickness compared with the other birds.

Table 3. Increase of wing web thickness ($\times 10^{-1}$ mm) of sensitized and unsensitized birds in the primary response to keyhole limpet hemocyanin-dinitrophenyl (KLH-DNP) or *Mycobacterium butyricum* at Day 22 postsensitization

		KLH[1,2]		*M.butyricum*[1,2]	
Immunization	Diet	4 h	24 h	4 h	24 h
KLH-DNP	Control	3.32	1.27	ND	ND
	Linoleic rich	5.21	1.63	ND	ND
	Linolenic rich	3.90	2.36	ND	ND
M. butyricum	Control	ND[3]	ND	4.84	4.63[a]
	Linoleic rich	ND	ND	3.34	3.99[a]
	Linolenic rich	ND	ND	4.11	3.68[a]
PBS	Control	2.52	0.88	3.36	1.82[ab]
	Linoleic rich	4.15	0.48	4.22	1.29[ab]
	Linolenic rich	3.00	0.78	2.93	0.59[b]
SEM		0.93	0.61	0.78	0.67
P value:					
Diet		NS[4]	NS	NS	NS
Immunization		NS	*	NS	***
Diet × Immunization		NS	NS	NS	NS

Means within a column with no common superscript differ significantly ($P < 0.05$)
[1] Average ± SEM.
[2] n = 56 chicks per cutaneous hypersensitivity test (column)
[3] ND = not determined.
[4] NS = not significant, * $P < 0.05$, *** $P < 0.001$

In Vitro Responses to Concanavalin A

Mitogenic challenge with ConA of the cell cultures resulted in 113-fold more counts per minute compared to the unstimulated cultures. Diet interacted with immunization as regards SI ($P < 0.05$) (Table 4). In birds previously challenged with either KLH-DNP or *M. butyricum,* the groups fed LNA-enriched diet had the largest SI. In birds previously injected with PBS, however, the group fed with LA-

enriched diet had the highest SI. Immunization affected SI; stimulated cell cultures of chicks immunized with *M. butyricum* had a lower SI compared with chicks immunized with KLH-DNP or PBS ($P < 0.05$). The cultures of *M. butyricum* immunized birds also had lower SI then the cultures of PBS injected pullets, but not significantly.

Table 4. Stimulation indices of lymphocytes from antigen-sensitized and unsensitized birds fed with one of three diets differing in fatty acid content after stimulation in vitro with ConA at 15 D postsensitization

Immunization	Diet	Stimulation index[1,2,3]
KLH-DNP	Control	151[ab]
	Linoleic rich	71[ab]
	Linolenic rich	224[a]
M. butyricum	Control	31[b]
	Linoleic rich	63[ab]
	Linolenic rich	83[ab]
PBS	Control	114[ab]
	Linoleic rich	171[ab]
	Linolenic rich	107[ab]
SEM		37
P value:		
Diet		NS[4]
Immunization		*
Diet × Immunization		*

Means within a column with no common superscript differ significantly ($P < 0.05$)
[1] Average ± SEM
[2] n = 90 birds
[3] Average counts per minute of unstimulated cultures = 204
[4] NS = not significant, * $P < 0.05$

Growth

At hatching, birds assigned to the 3 dietary treatment did not differ in BW. In the period from hatching until immunization, diet affected growth only in the first 3-d period. Chicks fed the control diet grew more rapidly than chicks fed LA-enriched diet ($P < 0.05$). After 3 days, until immunization at day 35, growth did not differ between diet groups (data not shown). At day 34, 1 day before immunization, the BW of chicks did not differ due to diet, but did differ due immunization ($P < 0.05$). One d before immunization, birds that were to be immunized with PBS were heavier than birds in the KLH-DNP or *M. butyricum* groups. In the entire period after immunization, until the end of the experiment, a diet by immunization interaction affecting growth was observed ($P < 0.05$). Chicks fed the LA-enriched

diet and immunized with *M. butyricum* grew faster than birds fed control- or LNA-enriched diets. In the period from 37 days until 41 days of age, immunization affected growth ($P < 0.05$). During this period, birds injected with KLH-DNP or *M. butyricum* showed compensatory growth compared with the PBS-injected birds. From 41 days until 44 days of age, there was a nonsignificant difference in BW gain of birds immunized with *M. butyricum* compared to KLH-DNP-immunized birds. From 55 until 58 days of age, chicks immunized with KLH-DNP grew slower than PBS- and *M. butyricum*-immunized chicks ($P < 0.05$). Diet affected growth from 44 to 48 days ($P < 0.05$) and from 48 to 51 days ($P > 0.05$). Birds fed LA-enriched feed grew faster than birds fed either the control or LNA enriched diet. Body weights at 58 days of age, the last measurement before euthanasia, were not affected by treatments.

Table 5. Organ weights relative to BW at 58 days of age of birds immunized with keyhole limpet heamocyanin-dinitrophenyl (KLH-DNP), *Mycobacterium butyricum*, or PBS and fed one of three diets differing in fatty acid content

Immunization	Diet	Heart[1]	Spleen	Intestine[2]	Caecal Tonsils
				%	
KLH-DNP	Control	0.66^{a}	0.34^{a}	4.48^{ab}	0.82
	Linoleic rich	0.62^{a}	0.33^{ab}	4.63^{ab}	0.91
	Linolenic rich	0.47^{b}	0.22^{b}	4.39^{ab}	0.78
M. butyricum	Control	0.64^{a}	0.38^{a}	4.51^{ab}	0.87
	Linoleic rich	0.66^{a}	0.32^{ab}	4.40^{ab}	0.90
	Linolenic rich	0.59^{a}	0.36^{a}	4.85^{a}	0.91
PBS	Control	0.68^{a}	0.32^{ab}	4.86^{a}	0.94
	Linoleic rich	0.64^{a}	0.32^{ab}	4.47^{ab}	0.86
	Linolenic rich	0.63^{a}	0.32^{ab}	4.31^{b}	0.78
SEM		0.02	0.03	0.11	0.05
P value:					
Diet		***	#	NS[3]	NS
Immunization		**	*	NS	NS
Diet × Immunization		*	*	***	NS

Means within a column with no common superscript differ significantly ($P < 0.05$)
[1] average ± SEM
[2] full intestine (exclusive of proventriculus, gizzards and cecal tonsils)
[3] NS = not significant, # $P < 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Organ Weights

Weights of the heart, spleen, intestine, and cecal tonsils were expressed as a percentage of total BW at day 58 (Table 5). The weight of the heart was affected by the interaction between diet and immunization ($P < 0.05$). Heart weight was

lower in birds immunized with KLH-DNP and fed the LA-enriched diet compared with other treatments. Both interactions were main effects. The weight of the spleen was affected by the diet by immunization interaction ($P < 0.05$). The weight of the spleen was lower in birds immunized with KLH-DNP and fed the LNA-enriched diet. Birds immunized with KLH-DNP had lighter spleens compared with the *M. butyricum*-immunized birds ($P < 0.05$). A diet by immunization interaction effect was also observed for relative intestinal weight ($P < 0.001$). Heavier intestines were found in birds immunized with *M. butyricum* fed the LNA-enriched diet and in birds immunized with PBS and fed control feed. Low intestinal weights were found in birds immunized with PBS and fed the LNA-enriched diet. Weights of cecal tonsils were not affected by experimental treatments.

Discussion

Dietary PUFA affect immune responses in mammals (Kinsella et al., 1990; Fritsche et al., 1992) and in poultry (Fritsche et al., 1991b, Phetteplace and Watkins, 1992; Friedman and Sklan, 1995). Previously we had found that a diet enriched with LA enhanced Ab responses to BSA and SRBC in a layer line that was genetically selected for enhanced humoral responsiveness, whereas a LNA-enriched diet decreased the Ab response to BSA in another (normal) layer line (Parmentier et al., 1997). The present study extended these findings, i.e. the LA-enriched diet enhanced Ab responses to KLH in the normal layer line immunized with KLH-DNP, an antigen that is known to induce (TH-2 dependent) Ab responses in mice (Bradley et al., 1995; Dohorty et al., 1995; Bliss et al., 1996). Preliminary analysis of Ab titers to DNP (conjugated to BSA) showed an enhancing effect of dietary LA in KLH-DNP immunized birds as well (unpublished data). The increase in Ab titers to KLH by the addition of LA into the diet (Figure 1) suggests TH-2 supporting properties of n-6 PUFA. On the other hand, the LA-enriched diet lowered Ab responses to *M. butyricum* (Figure 2). Mycobacteria proteins induce TH-1-dependent inflammatory responses in mice (Mosmann and Sad, 1996). Murine TH-1-dependent Ab responses are predominantly of the IgG 2 isotype. Murine TH-1 T cells are PGE_2 sensitive; the AA metabolyte PGE_2 inhibits IL-2 and γ-interferon production (Betz and Fox, 1991; Hilkens et al., 1995), favoring TH-2-like cytokine secretion profiles (IL-4 and IL-5) in murine and human TH cells. The PGE_2 primes human naïve T cells in a dose-dependent fashion for production of high levels of IL-4, IL-10, and IL-13 and very low levels of IL-2 and γ-interferon (Demeure et al., 1997). PGE have been demonstrated to upregulate IL-1 receptor expression on peripheral blood mononuclear cells. Binding of IL-1 to its receptors stimulates PGE

production (Kunkel et al., 1986). Such a feedback system may promote PG synthesis and favor the TH-2 subset (Phipps et al., 1991), which led us to speculate that dietary PUFA, such as LA and LNA may affect Ab and cellular immune responses in chickens differently via PG metabolism. TH-1 or TH-2 T-cell subsets and cytokines have not been identified as such in poultry. Also the isotype of Ab responses towards KLH and *M. butyricum* have not been determined; however, our results support the concept of a TH-2-enhancing effect of LA in the diet and that via AA and PGE_2, LA may enhance Ab responses to specific antigens in poultry. It is tempting to speculate that a similar divergence of TH cell-dependent immune responses exists in poultry, because TH-2-dependent Ab responses were enhanced by LA (Figure 1), whereas LA attenuated the Ab response to a TH-1 dependent antigen (Figure 2). Effects of LNA on Ab titers were less clearly observed however. The current results indicate that LNA modulates the Ab response in the opposite direction to that of LA, supporting the preceding speculation. Docosahexanoic or eicosapentaenoic acids may be stronger competition for PG synthetase than LNA. The Ab response to SRBC was affected by diets containing high levels of fish oil rich in these fatty acids (Fritsche et al., 1991b).

In the present experimental design, a group immunized with PBS was included as a control for immunization with KLH or *M. butyricum*. Analysis of Ab titers demonstrated that Ab titers against KLH were equally low in the PBS- and *M. butyricum*-immunized groups. In addition, Ab titers to *M. butyricum* were equally low in the PBS- and KLH-DNP-immunized groups. These results demonstrate that cross-reactivity with these experimental antigens does not occur. Hence, in future experimental designs with the same antigens, a PBS control is redundant. KLH and *M. butyricum* immunization could serve as controls for each other when studying Ab responses.

The effects of dietary PUFA on cellular immunity in vivo and in vitro was less pronounced compared with Ab titers. The LA-enriched diet did not significantly enhance the early component of CH to KLH, nor did LA significantly attenuate the 4-h CH to *M. butyricum*. The inconsistent effects of dietary PUFA on proliferation of T cells in vitro in the presence of ConA are difficult to explain. Variations of immune conditions, and of the T-cell populations from birds, may underlie the different effects of dietary PUFA on T-cell activation in vitro in the current study. PGE_2 favors TH-2 cytokine release of human T cells in vitro depending on the level of endogenous IL-2 production. When IL-2 production is low, PGE_2 inhibits secretion of TH-1 and TH-2 cytokines when IL-2 release is high, PGE_2 up-regulates

IL-4 and IL-5 secretion (Hilkens et al., 1995). Furthermore, the net production of γ-interferon by human T cells is determined by the concentration ratio of PGE_2 and the pro-TH-1 cytokine IL-12 both being produced by accessory cells (e.g., activated monocytes) in response to various stimuli. Susceptibility of T cells to PGE_2 (and indirectly) IL-12 changes in time, whereas the relative contributions of PGE_2 and IL-12 also may shift in time (Hilkens et al., 1996). Additional studies are required on the effects of dietary PUFA on cellular immunity at various moments during the immune response. Alternatively, our data suggest that LNA may affects cellular immunity but only in antigen-sensitized birds.

Similar to previous results (Parmentier et al., 1997), we found no pronounced effects of dietary PUFA on growth of the layer strain studied. These observations suggest that within the range of the present experiment, requirements for growth were met. Relative organ weights were influenced negatively by LNA enrichment of the diet, particularly the weights of heart and spleen. Low spleen weights after feeding an LNA rich diet have been reported previously in rats (Koga et al., 1997). Although spleen weight is a very nonspecific measure, low spleen weight could be interpreted as an indicator of low immune activity because it is a major lymphoid organ in poultry.

In conclusion, the diets supplemented with LA or LNA had no negative effects on performance, e.g., no negative effects on BW (gain) were recorded. Moreover, we found a divergent modulating effect of dietary LA on type and magnitude of specific immune responses in a layer line. The divergent aspect may be provoked by PGE_2 regulation. A mammalian TH-1/TH-2 analogous mechanism may be underlying; however, this has yet to be established in birds. The consequence of such an LA sensitive immunoregulation would be that the optimal dietary LA content for immune responsiveness depends on the nature of the pathogens to which chicks are most likely exposed. The effect of the n-3 PUFA LNA on Ab titers was less intense, and generally was opposite to the LA effect. The effect of LNA on the cellular immune response was clearly enhanced. Moreover, the ratio of n-3/n-6 PUFA appears to be more important in modulating eicosanoid biosynthesis than the absolute concentrations of n-3 PUFA in the diet (Broughton et al., 1991; German et al., 1988). Therefore, subsequent studies should focus on the effects of different ratios of LA and LNA in the diet on humoral and cellular immunity and the role of PGE_2 in the initiation of immune responses in poultry.

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Chapter 3

Interactions and Antigen Dependence of Dietary n-3 and n-6 Polyunsaturated Fatty Acids on Antibody Responsiveness in Growing Layer Hens

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Abstract: Effects of four levels of dietary linoleic acid (LA), an n-6 fatty acid, and four levels of α-linolenic acid (LNA), an n-3 fatty acid, and their interactions on immune responses in growing layer hens were studied. Immune responses were induced by injection with keyhole limpet hemocyanin (KLH) or *Mycobacterium butyricum* particles at 35 d of age. Antibody (Ab) responses were measured until 21 d after immunization. In addition, delayed-type hypersensitivity, lymphocyte proliferation, weekly feed intake, and BW gain were studied. At 7 days after immunization, anti-*M. butyricum* titers in the *M. butyricum*-immunized hens were decreased by the increase of dietary LA ($P < 0.05$). In the period from 10 to 14 d after immunization, anti-KLH Ab titers in KLH-immunized animals were affected by the interaction of dietary LA with LNA ($P < 0.01$). High dietary levels of LA or LNA increased the anti-KLH Ab response. However, at high levels of dietary LA and LNA, anti-KLH Ab titers were not increased. In the same period, anti-*M. butyricum* Ab titers of *M. butyricum*-immunized hens were affected by the interaction of dietary LA with LNA ($P < 0.05$). At low levels of LA and LNA, increased LA levels decreased the Ab response, whereas increased LNA levels at low LA levels hardly affected the anti-*M. butyricum* response. At a high level of LA, increased dietary LNA increased the anti-*M. butyricum* response. In vitro proliferation of peripheral blood leukocytes after stimulation with concanavalin A (ConA) was higher in chickens with a high level of dietary LNA. Feed intake decreased when the dietary levels of LA or LNA increased. However, BW gain was not affected by dietary treatments. Feed conversion was more efficient in birds fed high levels of LA and LNA. The present study indicates that various factors affect the Ab responses. First, the interaction of n-6 and n-3 polyunsaturated fatty acids (PUFA) is more important than the separate effects of n-3 or n-6. Second, the actions of dietary PUFA were different between antigens of a different nature. Third was the nature of the antigen affects when dietary PUFA exert their actions and the persistence of these effects. The presence of these multiple factors affecting immune responses should be considered when comparing effects of dietary PUFA on immune responses.

Introduction

Much of the interest in nutrients in relation to immunity has focused on dietary polyunsaturated fatty acids (PUFA). Two principal classes of immunomodulating PUFA can be distinguished: n-6 and n-3 fatty acids. The principal precursor of n-6 PUFA is linoleic acid, abundant in many plant oils such as sunflower, soybean, corn, and safflower. The principal precursor of n-3 PUFA family is α-linolenic acid, found in green plant tissue and linseed oil. In animal tissues, linoleic acid (LA) is converted to arachidonic acid, and α-linolenic acid (LNA) is converted to eicosapentaenoic and docosahexaenoic acids. N-6 and n-3 PUFA compete for the enzymes metabolizing them. The fatty acid composition of tissue and membrane lipids is largely determined by the relative activities of desaturases (Sprecher, 1989). Thus, the quantity of elongated PUFA not only depends on the quantity of their own precursor but also of the quantity of their competitor. Arachidonic acid is the precursor of 2-series prostaglandins and 4-series leukotrienes, whereas eicosapentaenoic acid is the precursor of 3-series prostaglandins and 5-series leukotrienes. These eicosanoids are important regulators of various immune responses (Kinsella, 1993).

In poultry, previous studies have shown that eicosanoids (Craig-Smith et al., 1987; Watkins and Kratzer, 1987) as well as cytokine profiles (Korver et al., 1997; Korver and Klasing, 1997) can be modulated by varying dietary PUFA. Moreover, the immune response was affected by the dietary PUFA composition. Antibody responses to different antigens were increased by dietary n-3 (Fritsche et al., 1991) but also were decreased by dietary n-3 (Parmentier et al., 1997). Similarly, Ab responses were increased (Parmentier et al., 1997; Sijben et al., 2000) or decreased (Friedman and Sklan, 1995; Sijben et al., 2000) by feeding high levels of n-6 PUFA. These apparent discrepant observations may be the result of the different antigens that the antibody (Ab) responses were directed against, and different levels were defined as high and low in n-3 and n-6. Because the metabolisms of n-3 and n-6 are intertwined, the measured effect of varying one PUFA might depend on the level of other PUFA and thus, on the interaction of n-3 with n-6.

In mammals, prostaglandin synthesis affects the balance between T helper (TH)-1 and TH-2 cytokine profiles (Betz and Fox, 1991). Prostaglandin E_2 (PGE_2) may tip the TH-1/TH-2 balance in favor of a TH-2 type response (Phipps et al., 1991). In birds, a similar definition of subsets of helper T lymphocytes, based on their cytokine secretion patterns, has not been made. Nevertheless, in avian

species, different types of antigens might also result in a selective boost of cytokines followed by different sorts of immune responses. In the present study the interactions of four levels of n-3 and n-6 PUFA on humoral or cellular immune responses against two different types of antigens were studied. In mice these soluble antigens are known to induce TH-2 (keyhole limpet hemocyanin-dinitrophenyl) (Bradley et al., 1995; Dohorty et al., 1995; Bliss et al., 1996) or TH-1 (*Mycobacterium* particles) (Mosmann and Sad, 1996) responses. Simultaneously, effects of dietary PUFA on growth performance were measured.

Materials and Methods

Birds and Housing

In the present study ISA Warren cross hens (medium-heavy layers) were used. Birds were from the randomly bred control line, which is a part of a continuous selection experiment (Van der Zijpp and Nieuwland, 1986), in which birds are selected for high or low Ab response to SRBC. From the 17th generation, 450 pullets from one hatch were used. Chicks were housed in 64 battery cages (50 × 100 cm), with 7 or 8 chicks per cage. The birds had free access to feed and water. The chicks were vaccinated against Marek's disease and Newcastle disease at the day of hatch, against infectious bronchitis at 2 d of age, and against infectious bursal disease at 15 d of age. On the day of hatch, birds were randomly assigned to the experimental treatments.

Experimental Design

Effects of dietary PUFA were studied using a 4 × 4 × 2 factorial design of treatments. Factors were dietary LA ($C_{18:2n-6}$), dietary LNA ($C_{18:3n-3}$), and immunization treatment. Four doses of LA, based on calculation, were used: 1.8, 2.8, 3.8, and 4.8% of total diet. Four doses of LNA based on calculation, were also used: 0, 0.9, 1.8, and 2.7% of total diet. Diets comprised a 90% constant basal diet component and a 10% varying oil mixture component (Table 1). Four oils were used, in varying amounts, to establish the calculated doses of LA and LNA: safflower oil, sunflower oil, linseed oil, and a palm oil fraction. The compositions of each of 16 oil mixtures and their calculated and analyzed LA and LNA contents are shown in Table 2. Diets were formulated to meet or exceed the nutrient recommendations for poultry of the NRC (1994) for all nutrients. The experimental diets were supplied from 6 days of age until the end of the experiment at 11 weeks of age. Before switching to the experimental diet, a standard starter diet

was fed. To prevent auto-oxidation of the oil mixtures in the diets, feeds in stock were stored at -20°C. One 25-kg bag of each diet was stored at 5°C for instantaneous use. The pullets were provided with fresh diet every other day with any remaining diet being discarded. All provided and discarded feed was weighed.

At 35 d of age, birds were immunized with keyhole limpet hemocyanin (KLH) (Cal Biochem - Novabiochem Co., La Jolla, CA) or heat-killed *Mycobacterium butyricum* dry cells (Difco Laboratories, Detroit, MI). The antigens were administered by an i.m. injection in the breast with 1 mL PBS containing 1 mg KLH or 1 mg *M. butyricum* particles. These immunizations were used as controls each other because a previous study pointed out that no cross reactivity between these antigens, with respect to Ab responses, occurs (Sijben et al., 2000). Blood samples were taken on days 0, 3, 10, and 17 from half of the birds and on days 0, 3, 7, 14, and 21 after immunization from the other half of the pullets. These times of blood sampling were according to regulations of the university's animal welfare committee. We recorded BW weekly from hatch until 9 wk of age.

Table 1. Composition of the experimental diets[1]

Ingredients and content	%
Corn (8.7 % crude protein (CP))	20
Wheat (11.9 % CP)	5
Peas (20.7 % CP)	5
Wheat middlings (15.7 % CP)	10
Soybean meal (44.9/5.3)	21.5
Sunflower meal (34 % CP)	7.5
Corn glutenmeal (20.9 % CP)	7.5
Lucerne (16/9 % CP)	4
Meat and bone meal (42.9/6.4)	1
Tapioca (65 % starch)	4.69
Vitamin and mineral mix[2]	1
Limestone ($CaCO_3$)	1.15
Monocalcium phosphate	1.3
Salt	0.3
DL-Methionine	0.06
Oil (variable)	10
Calculated contents	
CP, %	20.064
ME (layers), kcal / kg	3,024 – 3,110[3]
Ca, %	0.994
P, %	0.854
Lysine, %	1.014
Methionine + cystine, %	0.725

[1]On an as-fed basis.
[2]As in Chapter 2, apart from vitamin E (DL-α-tocopherol acetate), 40 mg
[3]Based on ME values for layers of 9,202, 10,349, 10,349, and 10,349 kcal/kg for palm-, sunflower-, linseed-, and safflower oils, respectively.

Table 2. Fat composition of each of 16 experimental diets. Fat mixtures consisted of a palm oil fraction (PO58)[1], sunflower oil (SuO)[2], safflower oil (SaO)[2], and linseed oil (LO)[2]

	Calculated %						Analyzed %		Analyzed
Diet #	PO58	SuO	SaO	LO	LA[3]	LNA	LA	LNA	LNA:LA
1	7.5	2.5	0	0	1.8	0	2.64	0.09	0.034
2	6.25	2.08	1.67	0	2.8	0	4.45	0.11	0.025
3	5.0	1.67	3.33	0	3.8	0	4.84	0.10	0.021
4	3.75	1.25	5.0	0	4.8	0	6.24	0.11	0.018
5	6.25	2.08	0	1.67	1.8	0.9	3.77	0.95	0.252
6	5.0	1.67	1.67	1.67	2.8	0.9	4.78	0.86	0.180
7	3.75	1.25	3.33	1.67	3.8	0.9	5.01	0.72	0.144
8	2.5	0.83	5.0	1.67	4.8	0.9	5.44	0.70	0.129
9	5.0	1.67	0	3.33	1.8	1.8	3.43	1.58	0.461
10	3.75	1.25	1.67	3.33	2.8	1.8	4.12	1.38	0.335
11	2.5	0.83	3.33	3.33	3.8	1.8	4.70	1.25	0.266
12	1.25	0.42	5.0	3.33	4.8	1.8	5.38	1.18	0.219
13	3.75	1.25	0	5.0	1.8	2.7	3.03	2.25	0.743
14	2.5	0.83	1.67	5.0	2.8	2.7	3.80	2.43	0.639
15	1.25	0.42	3.33	5.0	3.8	2.7	4.18	2.16	0.517
16	0	0	5.0	5.0	4.8	2.7	4.97	2.06	0.414

[1]Loders Croklaan B.V., 1520 AA Wormerveer, The Netherlands
[2]Chempri B.V., 4940 AD Raamsdonksveer, The Netherlands
[3]Percentages of dietary linoleic acid (LA) and linolenic acid (LNA) are given as preliminary calculated percentages and as analyzed percentages on an as fed basis.

Antibody Response to KLH and Mycobacterium butyricum

Total Ab responses to KLH and *M. butyricum* were determined by ELISA as described before (Sijben et al., 2000).

Cutaneous Hypersensitivity to KLH, Mycobacterium butyricum and Concanavalin A

Cutaneous hypersensitivity (CH) was measured in all chicks of four diet groups: 1, 4, 13, or 16 (Table 2). These were the four diets that represented the largest contrasts in n-3 and n-6 PUFA content. Fifty-seven KLH-immunized birds and 57 *M. butyricum*-immunized birds were challenged s.c. with 0.1 mg KLH or *M. butyricum* particles in 0.1 mL PBS into the flat surface of the left wing-web at Day 42 after immunization. Both sensitizations served as each others controls. Simultaneously these birds were sensitized with 0.1 mg ConA in 0.1 mL in their right wing-web. Further details concerning measurement of CH are described by Parmentier et al. (1993) and Sijben et al. (2000).

In Vitro Lymphocyte Proliferation to KLH, Mycobacterium butyricum, and ConA

An in vitro lymphocyte stimulation test (LST) was performed to determine effects of dietary PUFA on in vitro T-cell proliferation capacity. At 7 d after immunization,

peripheral blood leukocytes from 226 birds, half of the birds from all treatments, were used for the LST with ConA stimulation. For antigen-specific LST with KLH and *M. butyricum*, on 28 d after immunization lymphocytes from 114 birds were used. These were the same hens as in the CH test; those hens fed diet 1, 4, 13, or 16 (Table 2). Peripheral blood leukocytes were tested for proliferation in a final concentration of 1×10^7 cells/mL in the presence of 5 μg/mL ConA (Sigma Chemical Co., St. Louis, MO), 10 μg/mL KLH, or 10 μg/mL *M. butyricum* particles. The cultures were set up in triplicate with and without the presence of the stimulus. Further details concerning isolation procedure and culture media are given in Sijben et al. (2000). Results were expressed as a stimulation index (SI), with SI = mean counts per minute in stimulated cultures / mean counts per minute in unstimulated cultures.

Statistical Analysis

The Ab responses to experimental antigens were split into three phases for analysis: an early response on Day 7 after immunization, a middle response represented by the mean of Day 10 and Day 14 responses, and a late response, represented by the mean of Day 17 and Day 21 responses. Preliminary analysis showed that Ab titers on Days 0 and 3 after immunization were at an equally low basal level. The effect of immunization treatment on Ab responses to the experimental antigens were tested on data clustered per phase. Effects of dietary LA and LNA on Ab responses to KLH or *M. butyricum*, on growth and feed conversion efficiency, and on SI were tested by regression analysis using analyzed dietary contents of LA and LNA (Table 2). The following model was used:

$$Y_i = \mu + \beta_1(LA_i - \overline{LA}) + \beta_2(LNA_i - \overline{LNA}) + \beta_{1,2}[(LA_i - \overline{LA}) \times (LNA_i - \overline{LNA})] + \beta_3(D_i - \overline{D}) + e_i$$

where Y_i = dependent variable; μ = intercept; β_1 = regression coefficient for the effect of LA; LA_i = LA content; $\overline{LA}$ = average LA content being 4.42 %; β_2 = regression coefficient for the effect of dietary LNA; LNA_i = LNA content; $\overline{LNA}$ = average LNA content being 1.12 %; $\beta_{1,2}$ = regression coefficient for the interaction effect of LA and LNA; β_3 = regression coefficient for the effect of day within the same phase; D_i = day after immunization; $\overline{D}$ = average number of days after immunization in the phase analyzed, being 12 for the middle phase and 19 for the late phase; and e_i = error term. Effects of dietary PUFA on Ab responses directed against the experimental antigens were tested per phase of the Ab response.

Effects of dietary PUFA on growth, feed intake, and feed conversion efficiency were tested on average numbers for the pre- and postimmunization periods, with omission of the factor time. Preliminary analysis showed that immunization treatment did not have an effect on these parameters; therefore, average numbers for the entire experimental period were presented without the factor immunization.

Effects of dietary PUFA and immunization on wing-web thickness at 4 and 24 h after ConA, *M. butyricum*, or KLH stimulation were analyzed by a two-way-ANOVA. All analyses were tested using GLM procedures of SAS software (1990).

Results

Antibody Responses to KLH and Mycobacterium butyricum

In the following presentation, the responses obtained with the lowest levels of dietary LA and LNA will be taken as the reference points to describe effects of varying PUFA levels on Ab titers.

KLH: Antibody titers against KLH in hens immunized with KLH are shown in Table 3. The accompanying regression equations are given in Table 4. In all phases, anti-KLH Ab titers of these hens were increased compared with the control (*M. butyricum*-immunized) hens ($P < 0.001$). In the early phase of the Ab response to KLH, at 7 d after immunization, Ab titers to KLH in KLH-immunized birds were not affected by dietary LA and LNA levels or their interaction (Table 4). In the control hens, anti-KLH titers were positively correlated with dietary LNA ($P < 0.01$) on Day 7 after immunization (data not shown). Anti-KLH-Ab titers in KLH-immunized hens from the middle phase, on Days 10 and 14 after immunization, were affected by the interaction of LA with LNA ($P < 0.01$). Increased dietary LA or LNA increased the anti-KLH Ab response, however, increased dietary LA combined with increased LNA level resulted in a lower increase of Ab titers. The regression equation describing these effects is shown in Figure 1. In the late phase of the Ab response to KLH, on Days 17 and 21 after immunization, Ab titers directed to KLH were not affected by dietary LA and LNA levels.

M. butyricum: Antibody titers against *M. butyricum* in hens immunized with *M. butyricum* particles are shown in Table 3. The accompanying regression equations are shown in Table 4. In all three phases of the Ab response, anti-*M. butyricum* titers of these hens were increased by immunization with *M. butyricum* particles compared with the control (KLH immunized) hens ($P < 0.001$). Seven days after immunization, anti-*M. butyricum* titers in the *M. butyricum*-immunized hens were negatively correlated with dietary LA level ($P < 0.05$). In the middle phase of the

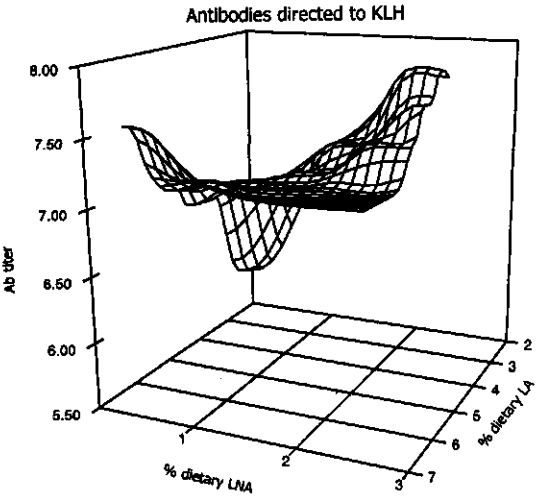

Figure 1. Calculated anti-KLH Ab titers in laying hens from 10 to 14 days after s.c. immunization at 35 d of age with 1 mg KLH in 1 mL PBS. Pullets were fed with 1 of 16 experimental diets, different in dietary linoleic acid (LA) and linolenic acid (LNA). The regression equation describing these titers is as follows: $Y_i = 6.99 + 0.04(LA_i - \overline{LA}) + 0.25(LNA_i - \overline{LNA}) - 0.39[(LA_i - \overline{LA}) \times (LNA_i - \overline{LNA})]$ (Table 4). The X-, Y-, and Z axes represent the analyzed level of dietary LA, LNA, and anti-KLH Ab titers, respectively.

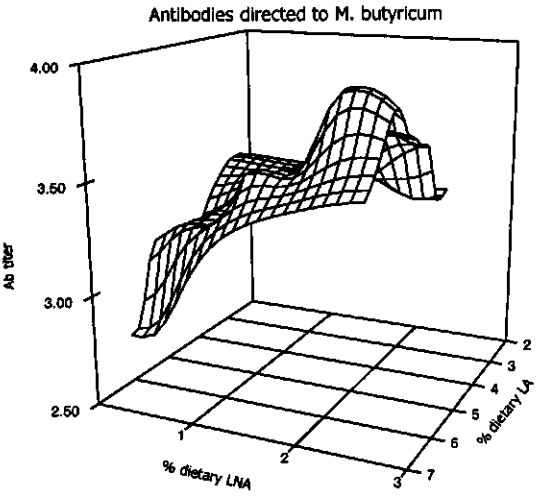

Figure 2. Calculated anti-*Mycobacterium butyricum* Ab titers in laying hens from 10 to 14 days after s.c. immunization at 35 d of age with 1 mg *M. butyricum* particles in 1 mL PBS. Pullets were fed with 1 of 16 experimental diets different in dietary LA and LNA. The regression equation describing these titers is as follows: $Y_i = 3.40 + 0.08(LA_i - \overline{LA}) + 0.33(LNA_i - \overline{LNA}) + 0.25[(LA_i - \overline{LA}) \times (LNA_i - \overline{LNA})]$ (Table 4). The X-, Y-, and Z axes represent the analyzed level of dietary LA, LNA, and anti-*M. butyricum* Ab titers, respectively.

Ab response, we found a positive correlation between anti-*M. butyricum* titers in *M. butyricum*-immunized hens and the dietary LNA level ($P < 0.01$). Moreover, the effects of LA and LNA interacted with each other ($P < 0.05$); increasing the level of LA and LNA increased anti-*M. butyricum* Ab titers, whereas increasing the level of LA at a constant low level of LNA decreased the Ab response. The regression equation describing these effects is shown in Figure 2. In the late phase of the Ab response, this interaction was not significant ($P < 0.1$). In the early and middle phases of the Ab response, anti-*M. butyricum* titers in control (i.e., KLH-immunized) hens were not affected by dietary PUFA.

Table 3. Least square mean values of total antibody (Ab) titers against keyhole limpet hemocyanin (KLH) and *Mycobacterium butyricum* particles in layer pullets immunized *i.m.* with 1 mg antigen in 1 mL PBS at 35 d of age[1]

Diet	% LA[2]	% LNA	Anti-KLH Ab titers			Anti-*M. butyricum* Ab titers		
			Day 7	10 to 14	17 to 21	Day 7	10 to 14	17 to 21
1	2.64	0.09	7.60	5.84	5.94	3.00	3.11	4.14
2	4.45	0.11	6.87	6.62	6.06	2.29	3.34	4.67
3	4.84	0.10	8.30	7.84	6.49	2.53	1.99	3.20
4	6.24	0.11	6.67	6.74	5.90	2.47	2.69	3.85
5	3.77	0.95	7.50	6.51	5.88	3.51	4.03	4.37
6	4.78	0.86	9.10	7.03	6.19	3.90	3.39	4.89
7	5.01	0.72	6.86	7.16	5.79	2.83	3.80	4.86
8	5.44	0.70	7.49	7.62	6.72	3.81	3.47	5.11
9	3.43	1.58	6.53	7.34	6.09	4.21	3.86	4.45
10	4.12	1.38	6.83	6.64	6.39	2.90	3.04	4.89
11	4.70	1.25	7.21	6.94	6.61	2.27	3.31	4.22
12	5.38	1.18	6.93	7.41	6.04	2.61	3.52	3.91
13	3.03	2.25	8.40	8.02	6.58	3.76	3.44	3.91
14	3.80	2.43	8.34	8.35	6.54	3.51	2.96	3.93
15	4.18	2.16	8.17	6.21	5.76	4.14	3.37	3.85
16	4.97	2.06	7.37	7.15	5.76	2.54	4.08	4.64
Pooled SEM			0.61	0.40	0.30	0.32	0.22	0.26

[1]The numbers represent the average Ab titers of 14 (Day 7) or 28 (Days 10 to 14 and Days 17 to 21) pullets immunized with the antigen they were tested against. The Ab titers of the reciprocal control birds are not shown in the table.
[2]Analyzed percentages of dietary linoleic acid (LA) and linolenic acid (LNA) on an as fed basis.

Cutaneous Hypersensitivity to KLH, M. butyricum, and ConA

KLH-immunized chickens: Chickens previously immunized with KLH were re-challenged with KLH in the wing web or challenged with *M. butyricum* particles (control). After 4 and 24 h, wing-web thicknesses were compared with thicknesses before (re-)challenge. At 4 h and 24 h after (re-)challenge, the wing-web thicknesses increased by 0.59 and 0.96 mm, respectively (Table 5). The wing-web thickness at 4 h was affected by the administered antigen ($P < 0.01$) and by the

interaction antigen administration and dietary LNA level ($P < 0.05$). Hens rechallenged with KLH had less thickened wing webs as compared with the control hens challenged with *M. butyricum*, and this effect was particularly present in the birds fed the low dietary LNA. Further, at 4 h after challenge, wing-web swelling thickness was affected by the interaction of dietary LA and LNA ($P < 0.05$) and by dietary LNA level ($P < 0.05$). The high level of LNA increased the wing-web swelling, but only in hens fed the high level of LA. At 24 h after challenge, wing-webs had thickened compared with wing web thicknesses at 4 h; however, dietary treatments did not affect swelling thicknesses at this time.

M. butyricum immunized chickens: Birds previously immunized with *M. butyricum* particles were rechallenged with *M. butyricum* particles in the wing web or challenged with KLH (control). At 4 h and 24 h after (re-)challenge, wing webs thickened compared with the thicknesses prior to (re-)challenge by an average of 0.61 and 0.94 mm, respectively (Table 5). No antigen effects on the 4-h or 24-h response were observed. At 4 h after challenge, no significant effects of experimental treatments were observed, and feeding LA-enriched diets did not significantly decrease swelling thickness ($P < 0.1$); however, at 24 h after challenge the effect was pronounced ($P < 0.05$). At 24 h after challenge no other effects were observed.

Table 5. Effects of dietary LA and LNA on the increase of wing web thickness ($\times 10^{-1}$ mm) at 4 and 24 h after challenge of birds sensitized with KLH, *Mycobacterium butyricum (M.b.)*, or ConA at 42 d after immunization with KLH or *M. butyricum*.

Antigen	Calculated % LA	Calculated % LNA	KLH sensitized 4 h	KLH sensitized 24 h	*M.b.* sensitized 4 h	*M.b.* sensitized 24 h	ConA sensitized 4 h	ConA sensitized 24 h
KLH	1.8	0	4.5	11.8	8.1	8.1	2.9	16.2
	1.8	3	6.2	10.8	6.1	10.0	3.8	20.1
	4.8	0	0.3	7.3	6.5	9.0	3.1	18.0
	4.8	3	7.3	10.2	7.9	9.6	3.5	16.7
pooled SEM			1.2	1.6	1.2	1.6	0.9	1.4
M.b.	1.8	0	5.9	10.6	6.0	9.6	4.0	17.3
	1.8	3	7.6	8.7	7.7	12.2	4.6	18.7
	4.8	0	5.4	7.0	5.2	7.4	3.5	15.5
	4.8	3	4.6	10.4	6.1	9.0	4.9	17.1
pooled SEM			1.2	1.3	1.2	1.3	0.9	1.4

[1]Fifty-seven KLH immunized birds were sensitized in the left wing web with KLH or *M. butyricum* particles (control) or with ConA in the right wing-web. Fifty-seven *M. butyricum*-immunized birds were sensitized in the left wing web with KLH (control) or *M. butyricum* particles or with ConA in the right wing web. The birds were fed diet 1, 4, 13, or 16, which represented the largest contrasts in n-3 and n-6 PUFA in the present experiment.

ConA Average wing-web thicknesses after challenge with ConA increased by 0.38 mm at 4 h and by 1.74 mm at 24 h after administration of ConA. However, at 4 h and at 24 h after challenge, the experimental treatments did not affect the increase in wing-web thickness.

In Vitro Lymphocyte Proliferation

Stimulation with KLH: The mean SI was 1.85 in the KLH-immunized birds and 1.54 in the control birds (immunized with *M. butyricum*). Because the SI of KLH-immunized birds was less than 2, and not significantly higher than the SI of control birds, stimulation was considered as negative in all birds; therefore effects of diet on SI were not observed.

Stimulation with M. butyricum: The SI was higher in birds immunized with *M. butyricum* compared to the KLH-immunized controls ($P < 0.001$), being 6.5 and 1.8, respectively. The SI was affected by dietary LA content ($P < 0.05$). Lymphocyte proliferation was higher for birds fed diets low in LA (SI = 5.3) compared with birds fed with diets high in LA (SI = 3.0). This effect was particularly present in lymphocytes from birds immunized with *M. butyricum*, in which the SI was 8.3 and 4.4 for the hens fed the diets low and high in LA, respectively; however, the interaction between immunization and dietary LA was not significant ($P < 0.1$).

Stimulation with ConA: Lymphocyte proliferation was enhanced by stimulation with ConA ($P < 0.001$). No interactions among dietary LA and LNA level on SI were observed. SI was positively correlated with dietary LNA ($P < 0.05$); however, dietary LA did not affect lymphocyte proliferation. The effect of dietary LA and LNA on SI after ConA stimulation is reflected by:

$$SI = 90.1 + 1.5(LA-\overline{LA}) + 22.9(LNA-\overline{LNA}) + 11.0[(LA-\overline{LA}) \times (LNA-\overline{LNA})]$$

BW gain, Feed Intake, and Feed Conversion

Feed intake, BW gain, and their ratio were not affected by the immunization treatments ($P > 0.1$); therefore, this factor was omitted after preliminary analysis, and pre- and postimmunization data were merged. The regression equations describing the effects of the level of dietary LA and LNA on these parameters are shown in Table 6. Feed intake decreased as dosages of dietary LA ($P < 0.01$) and dietary LNA ($P < 0.001$) increased. The lower feed intake associated with increasing dosages of dietary PUFA did not result in lower growth. BW gain tended to increase with increasing dietary LA. Consequently, the feed efficiency for growth

Table 4. Equations describing effects of dietary linoleic- (LA) and linolenic acid (LNA) on antibody responses to KLH and *M. butyricum* in three phases of the antibody response[1]

Day after immunization	Regression equation for Ab titers in immunized birds
anti-KLH Day 7	$Y_i = 7.49^{***} - 0.18(LA_i - \overline{LA}) + 0.16(LNA_i - \overline{LNA}) - 0.08[(LA_i - \overline{LA}) \times (LNA_i - \overline{LNA})]$
anti-KLH Days 10 to 14	$Y_i = 6.99^{***} + 0.04(LA_i - \overline{LA}) + 0.25^{\#}(LNA_i - \overline{LNA}) - 0.39^{**}[(LA_i - \overline{LA}) \times (LNA_i - \overline{LNA})] - 0.14^{**}(D_i - \overline{D})$
anti-KLH Days 17 to 21	$Y_i = 6.13^{***} - 0.08(LA_i - \overline{LA}) - 0.02(LNA_i - \overline{LNA}) - 0.16[(LA_i - \overline{LA}) \times (LNA_i - \overline{LNA})] + 0.09^{*}(D_i - \overline{D})$
anti-*M.butyricum* Day 7	$Y_i = 3.10^{***} - 0.30^{*}(LA_i - \overline{LA}) + 0.23(LNA_i - \overline{LNA}) - 0.20[(LA_i - \overline{LA}) \times (LNA_i - \overline{LNA})]$
anti-*M.b.* Days 10 to 14	$Y_i = 3.40^{***} + 0.08(LA_i - \overline{LA}) + 0.33^{**}(LNA_i - \overline{LNA}) + 0.25^{*}[(LA_i - \overline{LA}) \times (LNA_i - \overline{LNA})] + 0.27^{***}(D_i - \overline{D})$
anti-*M.b.* Days 17 to 21	$Y_i = 4.33^{***} + 0.13(LA_i - \overline{LA}) + 0.07(LNA_i - \overline{LNA}) + 0.21[(LA_i - \overline{LA}) \times (LNA_i - \overline{LNA})] + 0.32^{***}(D_i - \overline{D})$

[1]The equations were calculated with multiple regression analysis. Only the equations of anti-KLH titers in KLH-immunized chickens, and of anti-*M. butyricum* (*M.b.*) titers in *M. butyricum* immunized chickens, are given. Time was a factor only in the middle (Days 10 to 14) and late (Days 17 to 21) phases of the response. The values for average $\overline{LA}$ and $\overline{LNA}$ levels were 4.42 and 1.12, respectively. The values for the average days after immunization ($\overline{D}$) were 12 and 19 for the middle and late phases of the response, respectively.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 6. Equations describing the effects of dietary LA and LNA level on the weekly growth, feed intake, and feed conversion[1]

Performance parameter	Regression equations for BW gain, feed intake, and feed conversion
BW gain (g/bird per wk)	$Y_i = 108^{***} + 1.25(LA_i - \overline{LA}) + 0.80(LNA_i - \overline{LNA}) + 0.35[(LA_i - \overline{LA}) \times (LNA_i - \overline{LNA})]$
Feed intake (g/bird per wk)	$Y_i = 350^{***} - 7.16^{**}(LA_i - \overline{LA}) - 10.11^{***}(LNA_i - \overline{LNA}) - 3.64[(LA_i - \overline{LA}) \times (LNA_i - \overline{LNA})]$
Feed conversion	$Y_i = 3.14^{***} - 0.11^{*}(LA_i - \overline{LA}) - 0.12^{*}(LNA_i - \overline{LNA}) - 0.05^{*}[(LA_i - \overline{LA}) \times (LNA_i - \overline{LNA})]$

[1]The equations were calculated with multiple regression analysis. Data of birds immunized with keyhole limpet hemocyanin and *Mycobacterium butyricum* are pooled because immunization treatment did not affect these parameters. The values for average $\overline{LA}$ and $\overline{LNA}$ levels were 4.42 and 1.12, respectively.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

was higher if the fat component of the diet was more unsaturated. The latter was illustrated by the regression equation for the feed conversion, which negative regression coefficients between this ratio and dietary LA ($P < 0.05$), dietary LNA ($P < 0.05$), and their interaction ($P < 0.05$) were found.

Discussion

The present study was designed to examine effects of dietary n-6 and n-3 PUFA and their interaction on Ab responses of growing layer hens after challenge with different types of experimental antigens. Effects of these dietary treatments on cellular immune responses in vitro and in vivo were also measured. In several previous studies high levels of n-3 and n-6 PUFA were found to increase or decrease Ab response (Fritsche et al., 1991a; Friedman and Sklan, 1995; Parmentier et al., 1997; Sijben et al., 2000). These apparently discrepant findings might be associated with the doses of PUFA used in each of these studies, because the n-3 and n-6 metabolisms are intertwined. In addition, some of these apparently discrepant findings might be attributed to the nature of the antigen to which the antibodies were directed. Therefore, in the present design we used four doses of n-3 LNA and four doses of n-6 LA to investigate the interactions between n-3 and n-6 PUFA on Ab responses to antigens known to induce TH-1 and TH-2 responses in mice. We hypothesized that n-3 and n-6 dietary PUFA have opposite effects. A high level of n-6 PUFA is expected to favor a TH-2-like response at the expense of a TH-1-like response, whereas n-3 PUFA was expected to have reverse effects. Experimental indications for this hypothesis were provided by a previous study in our laboratory (Sijben et al., 2000). When levels of n-3 and n-6 PUFA were raised, the effects of n-3 were expected to be dominant because desaturases have higher affinity for the n-3 PUFA substrate than for n-6 PUFA. The relative importance of desaturation is α-linolenic acid > linoleic acid > oleic acid, and their ratio 10:3:1 (Brenner and Peluffo, 1966; Lokesh et al., 1988; Brenner, 1989). Moreover, the increase in tissue (n-3):(n-6) ratios exceeded these ratios in the diet, indicating that incorporation of n-3 in membranes was preferred to n-6 PUFA (Korver et al., 1997).

The present study indicates that three factors affect the Ab responses to the experimental antigens. First, the responses are affected differently by dietary LA and LNA, and the effects of LA and LNA are interactive. Second, the actions of dietary PUFA were different between antigens of different natures (KLH vs. *M. butyricum*). Third, the nature of the antigen affects the time at which dietary PUFA

exert their actions and the persistence of the effects. These factors will be discussed below.

In the first phase of the Ab response anti-KLH titers were not affected by the dosage of dietary PUFA. However, there was a negative correlation between Ab titers to *M. butyricum* and dietary LA content in the primary phase of the Ab response. The first observation does not support our preset hypothesis, but the second observation does support the hypothesis that n-6 PUFA downregulate the response to a TH-1 antigen. This observation supports the concept that PGE_2 is a "third signal" costimulating factor in the environment of the antigen-presenting cell at the initiation of the primary immune response (Kalinski et al., 1999). Although the TH-2-like enhancing effect by PGE_2 was not detected in the present trial, PGE_2 is associated with TH-1 downregulation (Betz and Fox, 1991).

In the advanced phase of an Ab response, the role of PGE_2 is not as prominent compared with its costimulatory role in the initial phase, i.e., an eicosanoid-mediated PUFA effect would not be as profound as in the earlier phase of the response. Nevertheless, in the present experiments most effects of dietary PUFA were found between 7 and 14 d after immunization. The present study shows that the interaction of dietary LA and LNA is more important than their individual effects, because the effects of addition of one PUFA depends on the dietary level of the other. The anti-KLH Ab response at Days 10 and 14 after immunization increased by increasing the LA level at low LNA levels but decreased by increasing the LA level at high LNA levels, increasing the LNA level also increased the response at low LA levels but decreased the response at high LA levels. A similar pattern was observed for anti-*M. butyricum* Ab titers at Days 10 to 14 after immunization. If LA and LNA levels were low, increasing the LA level decreased the Ab response, whereas increasing the LNA level at low LA levels hardly affected the anti-*M. butyricum* response. At a high level of LA, greatly increasing the level of dietary LNA increased the anti *M. butyricum* response, whereas to a much lesser extent also increasing the level of dietary LA increased the Ab response against *M. butyricum* at high LNA levels. The effects of dietary PUFA on anti-*M. butyricum* Ab response persist until 17 to 21 d after immunization but to a much lesser extent than at 10 to 14 d after immunization.

The results of the present study are consistent with the results of a previous study; at a low LNA level, a high dietary LA content increased the Ab response against KLH and decreased the Ab response against *M. butyricum* (Sijben et al., 2000). However, the present study also shows that this result does not imply that,

in general, dietary LA increases anti-KLH responsiveness or decreases anti-*M. butyricum* responsiveness. The effects of varying one PUFA is dependent on the level of other PUFA. Moreover, the effects were antigen dependent; the effects on KLH Ab titers were roughly the inverse of these on *M. butyricum* titers. The mechanisms underlying the complex interactions between LA and LNA at separate phases of the humoral immune response and the role of PGE_2 in this mechanism remain to be elucidated. Korver (1997) showed that the release of PGE_2 from chicken splenic leukocytes is not affected in a linear dose-dependent manner. In chicks the release of PGE_2 increases along with increasing total dietary oil and total PUFA level but does not increase with decreasing (n-3):(n-6). In the present study the level of dietary oil was equal in all diets, but as in Korver (1997), the total dietary PUFA varied. Although not much is known about the role of PGE_2 in the advanced stage of an immune response, it is speculated that the Ab response and the PGE_2 release might be determined by the same factor, i.e., total dietary PUFA.

In studies with mammals, high fat diets were found to reduce delayed-type hypersensitivity (DTH) compared with low fat diets, and among high fat diets DTH were lower in diets high in PUFA than at diets high in saturated fatty acids (SFA) (Friend et al., 1980). In PUFA-rich diets fish oil reduced DTH more than safflower oil (Yoshino and Ellis, 1987). Lymphocyte proliferation in rats was lower after being fed large amounts of linseed oil compared with diets high in sunflower oil (Jeffery et al., 1996) or hydrogenated coconut oil (Marshall and Johnston, 1985). In general, in mammals fed high fat diets, the DTH response and lymphocyte proliferation is, in order of magnitude, SFA > n-6 > n-3 (Calder, 1998). Our observations suggest that in chicks this effect is different. Average wing-web thicknesses in birds sensitized with the antigen that they had previously been immunized with was lowest in the birds fed a diet high in n-6 but low in n-3. Diets high in LA and LNA resulted in similar values compared with diets low in LA and LNA. Delayed-type hypersensitivity responses in birds sensitized in the wing-web and also previously immunized with *M. butyricum* were highest in birds fed the diet high in LNA and low in LA. Lymphocyte proliferation after ConA stimulation was enhanced by higher levels of dietary LNA and decreased by dietary LA after *M. butyricum* stimulation in *M. butyricum*-immunized birds. Serum from chicks fed n-3 PUFA significantly enhanced LST to ConA for normally fed chicks (data not published). These data suggest that in chickens DTH and lymphocyte proliferation are, in order of magnitude n-3 > SFA > n-6. The proliferation of lymphocytes depends on the production of IL-2. One may speculate that the effect of dietary

LNA on lymphocyte stimulation is caused by the increased competition of n-3 and n-6 PUFA for the binding sites of cyclo-oxygenase to produce PGE_2. In rats dietary fish oil lowers PGE_2 production by peritoneal macrophages and splenocytes by 70 to 80% compared with dietary corn oil (Fritsche et al., 1992). In mice it has been demonstrated that PGE_2 inhibits the production of IL-2 and INF-γ (Betz and Fox, 1991). However, if this were true, similar results should have been found by Fritsche et al. (1991b) in mammalian lymphocyte proliferation studies. The comparison between the data on Ab responses and cellular-mediated immune parameters in the present study is difficult because the latter are based on lower PUFA levels. Moreover, in the LST, the 4-h DTH and 24-h DTH are based on different mechanisms as indicated previously. Nevertheless the conclusion that effects of n-3 and n-6 PUFA interact and are antigen-dependent finds some support in the data describing cell-mediated immune parameters as well.

In the present study feed conversion decreased if the amount of dietary PUFA increased at the expense of SFA from 3.37 in the diet lowest in PUFA to 2.96 in the diet highest in PUFA. Similar results have been previously observed; diets rich in palm oil decreased growth in turkeys compared with diets higher in PUFA (Friedman and Sklan, 1997), and inclusion of tallow into the diet decreased feed efficiency compared with diets containing oils rich in PUFA (Korver, 1997). In the present study, diets rich in PUFA had a higher energy content than diets lower in PUFA and higher in SFA. The ME content varied in the range from 3,024 kcal/kg to 3,110 kcal/kg in the diets lowest and highest in PUFA, respectively. The absorption of the SFA of palm oil might be decreased compared with the fatty acids of diets higher in PUFA, as previously indicated (Renner and Hill, 1960; Sklan et al., 1973).

In conclusion, the present data indicate that the immunomodulating effects of dietary levels of PUFA are highly dependent on the level of other PUFA, i.e., interaction of n-3×n-6; the phase of the response; and the nature of the challenging antigen. Much of the variation between different studies may be explained by the variation among these factors. The present study implies that all these factors should be considered when comparing effects of dietary PUFA on immune responses.

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Chapter 4

Interactions of Dietary Polyunsaturated Fatty Acids and Vitamin E with Regard to Vitamin E Status, Fat Composition and Antibody Responsiveness in Layer Hens

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Abstract: Effects of dietary polyunsaturated fatty acids (PUFA) and vitamin E (VE) on an immune response may interact because VE may protect PUFA from in vivo oxidation. The present study was designed to study the presence of such interaction in growing layer chickens. Three dietary levels of linoleic acid (LA, 3.3, 6.6 and 10%), in combination with 4 levels of dietary VE (5, 20, 40, and 80 mg/kg) were used. Effects of LA and VE on circulating VE levels, fatty acid composition of bursal and adipose fat, and antibody kinetics against keyhole limpet hemocyanin and *Mycobacterim butyricum* were established. At high dietary LA levels, bursal and adipose LA levels were higher but bursal arachidonic acid and long chain n-3 PUFA levels decreased. The dietary VE level did not consistently affect the deposition of PUFA in tissue. Plasma VE levels were affected by the dietary VE and LA level, but not by their interaction. Antibody responses before- and 7 d after immunisation were affected by the dietary treatments. Ab levels were not affected by tissue fatty acid levels. In conclusion the present study indicates that the interaction effects of dietary PUFA and VE on fat deposition and immune responses are of minor importance compared to separate PUFA and VE effects. This implies that, within the studied range, adding extra VE to preserve or affect the effects of dietary PUFA on Ab responsiveness is unnecessary.

Introduction

In the past decade many studies on polyunsaturated fatty acids (PUFA) in relation to disease resistance and immune responsiveness in poultry have been conducted. A large variety of effects of n-3 and n-6 PUFA on several immune parameters have been described. Linoleic- (LA) and α-linolenic acid (LNA) may induce their immunomodulatory actions after elongation and desaturation to arachidonic acid ($C_{20:4n-6}$) and eicosapentaenoic acid ($C_{20:5n-3}$) which are incorporated into cell membranes. A phospholipase-dependent communication cascade causes the release of these long-chain PUFA and their conversion into metabolically active communication molecules of the immune system, i.e. prostaglandins, leukotrienes and thromboxanes (Kinsella *et al.*, 1990; Klasing, 1998). The effects of dietary fatty acids on the immune response are sometimes opposite. Effects of dietary LA on Ab responses might partially depend on the antigen to which the response is directed e.g. feed enriched with LA increased anti-keyhole limpet hemocyanin (KLH) Ab responses, but decreased anti-*Mycobacterium butyricum* Ab response (Sijben et al., 2000). However, in different studies different effects of similar levels of dietary LA on Ab responses against the same antigens were found. For example, high levels of dietary LA increased the Ab response directed to bovine serum albumin (Parmentier et al., 1997), and decreased Ab responses to bovine serum albumin (Friedman and Sklan, 1995). In a follow up study the effects of dietary LA on these Ab responses were similar, but the effects of dietary LA were also dependent on the dietary level of LNA (Sijben et al., 2001). The intimate metabolic pathways of n-6 and n-3 PUFA might underlie the interaction of effects on immune parameters. Similarly, at constant high dietary levels, effects of PUFA on immune parameters might interact with nutrients that are essential for it's protection against oxidation because the biochemical properties of PUFA change after oxidation.

Vitamin E is a bioregulator with immunomodulatory properties via modulating both the lipoxygenase and cyclooxygenase pathways at the level of arachidonic oxidation (Chan, 1993). In chicks and turkeys high dosages of nutritional VE were found to decrease Ab responses (Friedman et al., 1998). Vitamin E is also a well described antioxidant and stabiliser of biological membranes (reviewed by Surai, 1999). Vitamin E is the principal chain-breaking antioxidant in biological membranes which prevents free radical-induced oxidative damage by trapping reactive oxyradicals. In addition, VE can form complexes with free fatty acids and facilitates the close packing of the PUFA chains in membranes. These actions exert

a membrane stabilising effect by protection against the damaging actions of free fatty acids and by protection of the membrane PUFA from oxidation and phospholipase attack. Because the fatty acid composition of immune tissues reflects the fat composition of the diet (Fritsche et al., 1991; Friedman and Sklan, 1995), feeds with high dosages of PUFA induce higher risks of oxidation of membrane lipids. In rats, the VE requirement for overcoming the detrimental effects of excess oxidation of membrane phospholipids is correlated with the level of dietary PUFA (Leedle and Aust, 1986; Suarez et al., 1999). The effects of dietary PUFA on Ab responsiveness that we previously reported were established at dietary fat levels of about 14 %, and VE levels of 20 and 40 mg/kg diet respectively (Sijben et al., 2000, 2001). At such high dietary PUFA level the effects of fatty acid on immune cell function might interact with the VE level. The present study was performed to investigate this interaction. In more detail, we designed an experiment to elucidate whether 1. the dietary VE level affects the deposition of dietary fatty acids into bursal and adiposal tissue of growing layer hens, 2. the PUFA levels in these tissues are related with Ab responses, 3. the plasma VE concentration affects Ab responses, 4. the effect of dietary PUFA on Ab production is affected by VE. To induce Ab responses two (types of) antigens were used, i.e. KLH and *Mycobacterium butyricum* particles. In mice these antigens are known to induce T helper-2 (Bradley et al., 1995; Doherty et al., 1995; Bliss et al., 1996) and T helper-1 (Mosmann and Sad, 1996) responses, respectively. These antigens were used because Ab responses to these antigens are known to be either increased (KLH) or decreased (*M. butyricum*) by enriching the diet with LA in poultry (Sijben et al., 2000).

Materials and Methods

Birds and Housing

Birds were from one of three selection lines, which originated from an ISA Warren cross (medium heavy layers). Two lines had been selected for either high or low primary Ab responses at d 5 after primary i.m. immunisation with SRBC at 37 d of age. Additionally, a random bred C line has been kept (Van der Zijpp and Nieuwland, 1986). In the present study, from the 18th generation 430 hens from the C line, all from one hatch, were used. Chicks were housed in one of 48 battery cages (50 × 100 cm) with eight or nine chicks per cage. The birds had free access to feed and water. The chicks were vaccinated against Marek's disease and Newcastle disease at the day of hatch, against infectious bronchitis at 2 d of age,

and against infectious bursal disease at 15 d of age. On the day of hatch birds were randomly assigned to the experimental treatments.

Experimental Design

Interactions between dietary PUFA and vitamin E on immune responsiveness were studied using a 3 × 4 factorial design of dietary treatments. Factors were dietary LA (LA, $C_{18:2n-6}$) and VE level. Three doses of LA, approximately 3.3, 6.6 and 10% of total diet, were used in combination with four doses of vitamin E (DL-α-tocopherol acetate), i.e. 5, 20, 40, and 80 mg/kg feed. The level of n-3 PUFA was kept at a constant low level to avert undesired interaction effects with the dietary n-3 level. The experimental diets consisted of a constant basal diet component (90%) as in Chapter 3, and a varying oil mixture component (10%). Approximately 1.8% of the dietary LA derived from the basal diet, the remaining from the varying oil mixture. Three oils were used in varying amounts to establish the calculated different LA doses: safflower oil (Chempri B.V., Raamsdonksveer, The Netherlands), sunflower oil (Chempri B.V., Raamsdonksveer, The Netherlands), and a palm oil fraction (Loders Croklaan B.V., Wormerveer, The Netherlands). The fatty acid composition of the diets is given in Table 1. Diets were formulated to meet or exceed the nutrient recommendations for poultry of the NRC (1994) for all nutrients. Experimental diets were offered from the day of hatch until the end of the experiment. To prevent auto-oxidation of the oil mixtures in the diets, feeds in stock were stored at -20°C. One 25 kg bag of each diet was stored at 5 °C for instantaneous use. The pullets were provided with fresh diet 3 times per week. Feed and feed residuals were weighted and feed residuals were discarded. We assumed that feed residuals had the same composition as fresh feed.

At 35 days of age, birds were immunised with either keyhole limpet hemocyanin-dinitrophenyl (KLH-DNP) (Cal Biochem Co., La Jolla, CA USA), or heat-killed *Mycobacterium butyricum* dry cells (Difco Laboratories, Detroit, MI USA). The antigens were administered by an i.m. injection in the breast with 1 mL PBS containing 1 mg experimental antigen. These immunisations were used as each others controls because a previous study pointed out that cross reactivity between these antigens with respect to Ab responses did not occur (Sijben *et al.,* 2000). Blood samples were taken weekly on 0, 7, 14, 21 days after immunisation. BW was recorded weekly from hatch until 56 days of age. At 63 days of age blood samples of 3 birds of each cage were collected for vitamin E analysis. After blood sampling these birds were killed by i.v. injection with T61 (Hoechst Roussel Vet N.V.,

Table 1. Calculated and analysed fatty acid content of each of 12 experimental diets[1].

Dietary content (g/kg)												
Diet #	1	2	3	4	5	6	7	8	9	10	11	12
Calculated Linoleic Acid	33	33	33	33	66	66	66	66	100	100	100	100
Vitamin E (mg/kg)	5	20	40	80	5	20	40	80	5	20	40	80
Safflower oil[2]	0	0	0	0	50	50	50	50	100	100	100	100
Sunflower oil[2]	25	25	25	25	12.5	12.5	12.5	12.5	0	0	0	0
Palm oil58[3]	75	75	75	75	37.5	37.5	37.5	37.5	0	0	0	0
Crude fat (analyzed)	144.9	144.5	144	146.1	149.5	145.2	144.4	150.8	140.1	148.1	144.6	147.5
Analysed Fatty acid as % of Fatty Acids Methyl Esters												
C18:1n-9	8.49	8.63	8.11	8.61	11.43	11.44	11.03	11.34	14.84	14.84	14.8	14.8
C18:2n-6	22.53	23.11	21.09	23.13	44.3	43.94	41.85	43.38	68.12	68.45	68.66	68.5
C18:3n-3	0.77	0.84	0.76	0.84	0.81	0.78	0.76	0.78	0.85	0.85	0.84	0.84
C20:2n-6	0.3	0.08	0.27	0.16	0.36	0.57	0.54	0.55	0.44	0.39	0.36	0.45
Unknown	0.44	0.01	0.55	0.17	0.89	1.3	1.18	1.36	1.12	0.97	0.93	1.06
Σn-6 PUFA	22.83	23.2	21.35	23.29	44.67	44.51	42.39	43.93	68.55	68.84	69.01	68.94
Σn-3 PUFA	0.77	0.84	0.76	0.84	0.81	0.78	0.76	0.78	0.85	0.85	0.84	0.84
ΣMUFA	9.19	9.08	8.86	9.18	12.52	12.65	12.2	12.54	16.46	16.43	16.37	16.41
ΣSFA	66.77	66.87	68.48	66.52	41.13	40.75	43.46	41.38	13.02	12.92	12.84	12.76

[1]The diets consisted of a constant factor of 90 % of basal diet and a varying factor of 10 % of oil. In addition total amounts of analysed dietary n-6 PUFA, n-3 PUFA, monounsaturated fatty acids (MUFA), and saturated fatty acids (SFA) are given.
[2]Chempri B.V., Raamsdonksveer, 4940 AD, The Netherlands
[3]Loders Croklaan B.V., Wormerveer, 1220 AA, The Netherlands
[4]Not detectable. The lower border of detection was 0.1 % of Fatty Acids Methyl Esters.

Brussels, Belgium) and intra abdominal adipose tissue and bursas were collected for analysis of their fatty acid composition.

Adipose and Bursal Tissue Fatty Acid Analysis

Bursal tissue lipids were extracted using chloroform-methanol (2:1, v/v) (Labscan Limited, Stillorgan, Co. Dublin, Ireland) according to the method of Folch et al. (1957). Adipose and bursal tissue lipids were saponified using methanolic sodium hydroxide and converted into their methyl esters using BF_3 in methanol (Pierce, Rockford, IL USA). Fatty acid analyses were performed by GLC (Chrompack CP 9002) using a flame ionization detector, a Chrompack column (length 25 m, diameter 0.32 mm) and H_2 as a carrier gas (Metcalfe et al., 1966).

Vitamin E Analysis

Serum was deproteinized with HPLC grade ethanol (Merck, Darmstadt, Germany) in the presence of Butylated Hydroxy Toluene (Merck, Darmstadt, Germany), and extracted with HPLC grade hexane (Merck, Darmstadt, Germany). Part of the hexane was taken and evaporated; the residue was dissolved in HPLC grade methanol (Baker, Deventer, The Netherlands). Vitamin E analyses were performed by HPLC (Jasco, Tokyo, Japan) using a fluorescence detector (Separations, Hendrik Ido Ambacht, The Netherlands) an aminotag column (Varian, Walnut Creek, CA USA) and HPLC grade methanol as mobile phase. Vitamin E standards (Fluka, Sigma-Aldrich Group, Zwijndrecht, The Netherlands) were used for calibration (McMurray and Blanchflower, 1979; Dill et al., 1989)

Humoral Immune Response to KLH, and M. butyricum

Total antibody (Ab) titers to KLH and *M. butyricum* in serum of all birds were determined by ELISA as described before (Sijben et al., 2000).

Statistical Analysis

Effects of dietary LA and VE level, and immunisation treatment on the variables plasma VE level, tissue fatty acid level, feed intake, growth, feed conversion, Ab responses at 0, 7, 14 and 21 days after immunisation were tested by the following model:

$$Y_{ijkl} = \mu + LA_i + VE_j + I_k + \text{interactions} + e_{ijkl}$$

where Y_{ijkl} = dependent variable; μ = overall mean; LA_i = fixed effect dietary linoleic acid level i (dose levels i = 1,2,3); VE_j = fixed effect of dietary vitamin E level j (dose levels j = 1,2,3,4); I_k = fixed effect of immunisation treatment k (type of antigen k = 1,2); interactions = all two- and three-way interactions among dietary LA- and VE level, and immunisation treatment; e_{ijkl} = error term. The effects of dietary LA- and VE level, and immunisation treatment were tested against error term e_{ijkl}. For plasma VE level, tissue fatty acid levels, feed intake, growth and feed conversion, there was no effect of immunisation treatment. For these variables data are therefore presented after reanalysis with this model without the factor immunisation treatment. For Ab responses, the factor immunisation treatment was highly significant between the two antigen treatments. Therefore the effects of dietary VE and LA on Ab response were reanalysed within antigen treatment and presented accordingly. In addition, the Ab responses of the birds used for establishing plasma VE and tissue fatty acid levels were tested for correlation with plasma VE and bursal and adiposal fatty acid levels. All analyses were performed using the GLM procedures of SAS programs (1990).

Results

Feed Consumption, BW Gain, and Feed Conversion

Dietary effects on these parameters are presented in Table 2. The level of dietary VE did not affect feed consumption, BW gain and feed conversion. The level of dietary LA affected feed consumption, BW gain, and feed conversion. Higher dietary LA levels were associated with decreasing feed consumption. However, the mean weekly BW gain was higher in the animals fed the highest LA levels compared with animals fed the lowest LA level. The combination of lower feed consumption and higher BW gain in birds fed higher LA levels resulted in a lower feed conversion of these chickens.

Table 2. Weekly feed consumption, BW gain and feed conversion efficiency in the 8 wk experimental period[1], and mean values of analysed vitamin E (VE) in plasma of 9-wk-old hens fed one of 12 diets different in VE and linoleic acid (LA) content[2].

Main effects	Feed consumption	BW gain	Feed conversion	plasma VE
dietary VE (mg/kg)	(g/wk)	(g/wk)		(μg/mL)
5	243	86.7	2.66	8.85^{a}
20	243	85.9	2.67	10.91^{b}
40	243	85.6	2.69	12.07^{bc}
80	243	85.3	2.70	15.27^{d}
SEM[3]	2.80	0.96	0.023	0.38
dietary LA (%)				
3.3	249^{a}	82.3^{a}	2.87^{a}	11.70^{ef}
6.6	244^{ab}	87.6^{b}	2.63^{b}	12.82^{e}
10	236^{b}	87.7^{b}	2.54^{c}	10.80^{f}
SEM	2.42	0.83	0.020	0.33
Main effects				
VE	NS[4]	NS	NS	***
LA	**	***	***	***
LA × VE	NS	NS	NS	NS

[a,b,c,d] Means per main effect in a column with no common superscript differ significantly ($P < 0.05$)
[1]Mean values of birds immunised with either KLH or *M. butyricum* were merged into one feed group because immunisation treatment did not affect these parameters. Mean values in the table are means of 35 or 36 birds. The levels of VE are the amounts as added to the basal diets.
[2]Mean values represent means of 12 birds fed the same diet.
[3]SEM = Standard Error of the Least Square Mean
[4]NS=not significant, ** P < 0.01, ***P < 0.001

Fatty acid concentrations of the bursa and intramuscular fat tissue

Bursal and adipose fatty acid composition as proportion of total fatty acid methyl esters are shown in Table 3 and 4, respectively. For both the bursal and adipose tissue the proportion of n-6 and saturated fatty acids (SFA) were greater when the level of n-6 and SFA in the diet was high. Both long-chain n-3 and n-6 PUFA were below detectable levels in the feed. Bursal, but not adipose arachidonic acid (AA, $C_{20:4n-6}$), was lower at higher levels of dietary LA. The levels of bursal AA and $C_{22:4n-6}$ were affected by the dietary VE levels, i.e. both long-chain n-6 fatty acids had a lower level at VE 40 than at VE 80. The bursal and adipose monounsaturated fatty acid (MUFA) proportions, particularly the major MUFA component oleic acid ($C_{18:1n-9}$, OA), decreased at increasing dietary OA levels. Bursal and adipose total n-3 decreased at increased dietary LA. Bursal total n-3 was lower at higher dietary VE (Table 3). The dietary VE level did not affect the level of any other fatty acid in the bursa or adipose fat.

Table 3. Bursal fatty acid composition of 63-days-old layer hens fed one of 12 diets different in vitamin E (VE) and linoleic acid (LA) content[1].

Calculated dietary content													Statistical significance		
Diet #	1	2	3	4	5	6	7	8	9	10	11	12			
Linoleic Acid (%)	3.3	3.3	3.3	3.3	6.6	6.6	6.6	6.6	10	10	10	10			
Added VE (mg/kg)	5	20	40	80	5	20	40	80	5	20	40	80			
Analysed Fatty acid as % of Fatty Acids Methyl Esters													LA	VE	LA × VE
C18:1n-9	18.6	19.4	19.2	18.3	16.6	15.3	16.4	16.2	16.2	15.8	16.0	15.6	***	NS	NS
C18:2n-6	19.0	17.5	21.5	18.1	27.5	27.3	30.1	28.0	34.9	34.1	35.7	33.8	***	NS	NS
C18:3n-6	0.05	0.09	0.07	0.07	0.13	0.13	0.09	0.08	0.12	0.18	0.14	0.11	**	NS	NS
C18:3n-3	0.26	0.22	0.4	0.15	0.22	0.24	0.28	0.22	0.2	0.24	0.23	0.2	NS	NS	NS
C20:2n-6	0.66	0.48	0.49	0.78	0.83	0.83	0.74	0.65	0.98	1.0	1.05	1.11	***	NS	*
C20:3n-6	1.16	1.15	0.92	1.25	1.07	1.06	0.93	1.04	1.0	1.01	1.04	1.1	NS	NS	NS
C20:4n-6	9.72	9.64	7.66	10.7	8.42	8.86	7.65	8.43	7.43	7.7	7.13	8.21	***	*	NS
C20:5n-6	0.07	0.1	0.02	0.04	ND	ND	ND	ND	ND	ND	ND	ND	***	*	**
C22:2n-6	ND	ND	ND	ND	0.01	0.01	0.01	0.01	0.01	0.03	0.03	0.01	*	NS	NS
C22:4n-6	3.19	3.2	2.41	3.51	2.94	3.15	2.64	2.83	2.84	2.9	2.65	2.98	NS	*	NS
C22:5n-3	0.44	0.43	0.34	0.41	0.23	0.24	0.17	0.18	0.15	0.18	0.11	0.11	***	*	NS
C22:6n-3	0.48	0.5	0.43	0.57	0.29	0.31	0.23	0.25	0.2	0.2	0.17	0.16	***	NS	NS
Unknown	7.33	7.95	5.98	7.33	6.36	6.11	5.61	6.18	5.82	5.94	5.82	6.02	***	NS	NS
Σn-6 PUFA	33.8	32.1	33.0	34.3	40.9	41.4	42.2	41.0	47.3	46.9	47.8	47.3	***	NS	NS
Σn-3 PUFA	1.25	1.25	1.19	1.18	0.74	0.78	0.67	0.66	0.55	0.62	0.51	0.47	***	*	NS
ΣMUFA	23.0	24.4	23.5	22.8	20.3	18.8	19.8	19.7	19.7	19.3	19.2	18.7	***	NS	NS
ΣSFA	34.6	34.3	36.3	34.4	31.7	32.9	31.8	32.4	26.6	27.2	26.7	27.5	***	NS	NS

[1]Mean values represent means of 12 birds fed the same diet. Also, total amounts of analysed bursal n-6 PUFA, n-3 PUFA, monounsaturated fatty acids (MUFA), and saturated fatty acids (SFA) are given.
[2]NS=not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ND = not detectable

Table 4. Fatty acid composition of intramuscular adipose of 63-days-old layer hens fed one of 12 diets different in vitamin E (VE) and linoleic acid (LA) content[1].

Calculated dietary content													Statistical significance		
Diet #	1	2	3	4	5	6	7	8	9	10	11	12			
Linoleic Acid (%)	3.3	3.3	3.3	3.3	6.6	6.6	6.6	6.6	10	10	10	10			
added VE (mg/kg)	5	20	40	80	5	20	40	80	5	20	40	80			
Analysed Fatty acid as % of Fatty Acids Methyl Esters													LA	VE	LA × VE
C18:1n-9	17.1	17.8	17.3	16.7	15.1	14.8	14.0	14.8	16.3	16.3	16.1	16.0	***	NS	NS
C18:2n-6	38.2	36.8	36.3	38.2	52.2	50.9	51.7	51.2	65.8	64.7	66.2	66.1	***	NS	NS
C18:3n-6	ND	ND	ND	ND	0.02	ND	0.01	0.01	0.01	0.02	ND	ND	NS	NS	NS
C18:3n-3	0.86	0.87	0.87	0.83	0.65	0.63	0.66	0.65	0.57	0.58	0.56	0.56	***	NS	NS
C20:2n-6	0.19	0.18	0.13	0.17	0.24	0.19	0.20	0.19	0.25	0.26	0.25	0.26	***	NS	NS
C20:4n-6	0.27	0.31	0.28	0.26	0.26	0.29	0.24	0.28	0.24	0.24	0.25	0.26	NS	NS	NS
C22:4n-6	ND	0.02	ND	0.01	0.01	ND	0.01	0.02	ND	ND	ND	ND	NS	NS	NS
Unknown	0.34	0.41	0.33	0.37	0.43	0.45	0.40	0.44	0.69	0.71	0.66	0.68	***	NS	NS
Σn-6 PUFA	38.7	37.3	36.7	38.6	52.7	51.4	52.2	51.7	66.3	65.2	66.7	66.6	***	NS	NS
Σn-3 PUFA	0.86	0.87	0.87	0.83	0.65	0.63	0.66	0.64	0.57	0.58	0.56	0.56	***	NS	NS
ΣMUFA	18.5	19.3	18.7	18.0	16.2	15.9	16.5	16.0	17.5	17.6	17.4	17.2	***	NS	NS
ΣSFA	41.7	42.1	43.4	42.2	30.0	31.6	30.3	31.3	14.9	15.9	14.7	15.0	***	NS	NS

[1]Mean values represent means of 12 birds fed the same diet. Also, total amounts of analysed adipose n-6 PUFA, n-3 PUFA, monounsaturated fatty acids (MUFA), and saturated fatty acids (SFA) are given.
[2]NS=not significant, ***$P < 0.001$, ND = not detectable

Plasma VE concentrations

The concentration of VE in blood plasma is shown in Table 2. The plasma VE level increased as the dietary VE level increased. Gradual increase of the level of dietary VE, starting at 5 mg/kg, which reflects minimal requirements (NRC, 1994), increased the circulating VE levels compared with the 5 mg/kg level by 6 to 37 % at 20 mg/kg, by 32 to 39 % at 40 mg/kg and by 70-75 % at 80 mg/kg, respectively. The dietary LA content affected circulating levels of VE. Hens fed the feed containing the highest dose of LA had lower plasma VE concentration compared to the hens fed the diet with the middle dose of LA. There was no interaction between the effects of dietary VE and LA on plasma VE levels.

Antibody Responses

Ab response to KLH in KLH immunised chicks: Antibody titers against KLH on 0, 7, 14, and 21 days after immunisation and the significance levels of the experimental treatments are shown in Table 5. On the day of immunisation and on 7 d after immunisation interaction effects between dietary LA and VE on Ab titers were found. On both days the birds fed the diet supplemented with 20 mg/kg VE had the lowest Ab titers within the 3.3 and 6.6% levels of dietary LA and the highest Ab titers within the 10% of dietary LA level. At 14 and 21 days after immunisation no treatments effects on Ab responses to KLH were observed (Table 5).

Ab response to M. butyricum in M. butyricum immunised chicks: Total Ab titers directed to *M. butyricum* at 0, 7, 14, and 21 days after immunisation and the significance levels of the experimental treatments are shown in Table 5. At day 0, anti-*M. butyricum* Ab levels were affected by the dietary VE level. At all LA levels day 0 Ab titers were lowest at 5 mg/kg VE, followed by 40, 80 and 20 mg/kg VE respectively. At 7 days after immunisation Ab titers were affected by the LA level, and and interaction between VE and LA. Antibody titers were lower at the 6 % LA level compared with the 3 and 10% LA level. Ab titers were highest at 20 mg/kg VE within the 3.3 and 6.6% dietary LA levels. At 10% LA, Ab titers were lowest at 20 mg/kg VE and highest at 80 mg/kg VE. At 3.3% LA Ab titers were lowest at 80 mg/kg VE, followed by 5 mg/kg VE. At 6.6% LA, Ab titers were lowest at 40 mg/kg VE, followed by 5 and 80 mg/kg VE. At 14 and 21 days after immunisation no treatment effects on Ab titers were observed (Table 5).

Table 5. Average values of total antibody (Ab) titers against keyhole limpet hemocyanin (KLH), and M. butyricum protein[1].

Diet #	LA (calculated)	VE (mg/kg)	Anti-KLH Ab titers				Anti-*M. butyricum* Ab titers			
Days after immunisation			0	7	14	21	0	7	14	21
1	3.3	5	2.64	7.88	6.88	6.47	0.36	4.70^{bc}	2.99	4.36
2	3.3	20	2.48	7.64	7.29	7.02	1.23	6.16^{c}	2.47	2.99
3	3.3	40	3.03	7.89	6.44	6.12	0.84	6.08^{c}	3.18	3.94
4	3.3	80	2.80	7.84	7.01	5.54	1.03	3.82^{ab}	3.56	3.81
5	6.6	5	2.99	8.91^{a}	6.54	6.83	0.34	4.12^{abc}	2.72	3.39
6	6.6	20	2.55	6.21^{b}	6.42	5.63	1.29	5.55^{bc}	3.04	3.60
7	6.6	40	3.19	8.21	6.39	6.63	0.61	2.44^{a}	3.06	3.39
8	6.6	80	3.22	8.06	5.74	5.54	1.00	4.17^{abc}	2.35	3.05
9	10	5	2.96	8.41	7.28	6.45	0.28	4.32^{abc}	2.42	3.91
10	10	20	3.35	8.72	7.17	6.06	1.19	4.08^{abc}	3.13	3.26
11	10	40	2.63	7.58	6.44	6.29	0.89	4.79^{bc}	3.05	3.27
12	10	80	2.62	7.65	6.32	6.57	1.05	6.19^{c}	3.06	3.68
SEM[2]			0.20	0.46	0.48	0.44	0.28	0.45	0.29	0.36
LA			NS[3]	NS	NS	NS	NS	**	NS	NS
VE			NS	NS	NS	NS	***	NS	NS	NS
LA × VE			**	**	NS	NS	NS	***	NS	NS

[a,b,c] Means per column with no common superscript differ significantly ($P < 0.05$)
[1]The numbers represent the average Ab titers of 18 or 19 35-days-old chickens s.c. immunized with 1 mg antigen in 1 mL PBS. The Ab titers of the control birds are not shown in the table.
[2]SEM = Standard Error of the Least Square Mean
[3]NS=not significant, ** $P < 0.01$, *** $P < 0.001$

Correlations between tissue fatty acid levels, VE status and Ab responses

No significant correlations between tissue fatty acid levels, VE status, and Ab responses were present.

Discussion

The present study was designed to study whether at high dietary PUFA levels, the effects of PUFA on immune cell functions interacts with the dietary VE level. In the following we will discuss the current data on growth performance, circulating VE, fatty acid composition, Ab responses as the result of different dietary LA and VE levels and challenge with two antigens.

The observations reported here with regard to growth, feed intake and feed conversion are consistent with a recent previous study (Sijben et al., 2001). The substitution of dietary SFA by PUFA resulted a lower feed intake and feed conversion, and higher BW gain. These effects might be attributable to a lower absorption of the SFA of palm oil compared to the dietary PUFA (Renner and Hill, 1960; Ketels, 1994).

Two previous studies indicated that increasing the dietary LA level from 3.3 to 6.6%, at the same α-linolenic acid but different VE levels (20 vs. 40 mg/kg) similarly affected Ab responses (Sijben et al., 2000, 2001). In addition, we previously reported that at 0.1% of dietary α-linolenic acid and 40 mg/kg VE, anti-KLH Ab titers increased and *M. butyricum* decreased at increasing dietary LA (Sijben et al., 2001). In the present study the α-linolenic level was 0.1%, and at 40 mg/kg VE, also anti-KLH Ab titers increased and anti-*M. butyricum* decreased. So, the present study confirms that in different studies with similar dietary α-linolenic acid and VE levels fairly similar effects of enrichment of the diet with LA on Ab responses to the same antigens are found. This consistency among different studies indicates that the present model is useful to test the interaction between dietary LA and VE on Ab responsiveness.

A recent study in broilers suggests that the plasma VE level increases linearly from 3 to 32 μg/mL at VE levels from 0 to 200 mg/kg (Leshchinsky, 2000). The present study suggests that in layer breed circulating VE levels increase at a slower rate along with increasing dietary VE levels. One of the important elements that determines the efficiency of VE absorption is the amount and composition of dietary fat (Cohn, 1997). Generally, data concerning the relationship between dietary fat and VE metabolism are still controversial. Increasing the soybean oil level in diets containing 10, 59, and 100 mg α-tocopherol acetate/kg from 0.5 to 3.0%, significantly elevated turkey plasma α-tocopherol (Bartov, 1983). Applegate and Sell (1996) found higher plasma and liver VE levels at increasing dietary LA concentrations from 1.35 to 2.35%, both at 0 and 150 IU VE in turkey poults. In contrast, at higher doses of n-3 (Chautan, et al., 1990) and n-6 (Tijburg et al., 1997) dietary PUFA decreased plasma VE in rats. These data and the present study indicate that dietary LA levels up to 6% benefit VE absorption, but that at even higher PUFA levels circulating VE levels decrease. Possibly, at lower PUFA levels VE absorption is increased by facilitation of the formation of micelles used for VE absorption. Bjorneboe et al. (1990) suggested that at higher levels long-chain PUFA may reduce the absorption of α-tocopherol, and that long-chain PUFA may also enhance the breakdown of α-tocopherol in vivo due to oxidation. Kubo et al. (1997) proposed that in such condition, tissue susceptibility to lipid peroxidation increases because VE is unable to protect tissue membranes rich in long-chain PUFA from lipid peroxidation, even after ingestion of high doses of this vitamin. The present data suggest that in chickens this might be the same for short-chain PUFA like LA.

In most published studies VE supplementation in poultry increased Ab levels (Tengerdy and Nockels, 1973; Marsh et al., 1981; Gore and Qureshi, 1997), but also decreased Ab levels have been reported (Friedman et al., 1998), or no consistent effects (Boa-Amponsem et al., 2000). Gore and Qureshi (1997) suggested that particularly the initial phase (IgM) of an Ab response is affected by VE supplementation above minimal NRC requirement. An increase in Ab levels shortly after challenge might be associated with a relative increase CD4+CD8- T cells by VE supplementation (Erf et al., 1998). The present study is not consistent with this hypothesis because Ab levels were not elevated by VE supplementation after antigen challenge. Vitamin E may modulate an immune response by affecting both lipoxygenase and cyclooxygenase pathways (Chan, 1993). We found the studied interaction of dietary PUFA with VE with regard to Ab response particularly at 7 d post challenge, at the initial (IgM) phase of an Ab response. If the hypothesis that at high dietary PUFA levels the VE requirement were greater to preserve the immunomodulatory effects of PUFA, the effects of increasing the LA level from 3.3 to 6.6% on Ab titers could be expected to be maintained at higher VE intake when the LA level was further increased. However, the VE by LA interaction was not consisted in this interpretation. Therefore, our hypothesis that the effects of dietary LA and VE interact is supported by the current data, and our expectation that this would be particularly notable at high LA and VE levels is not supported.

The present data only partially support what is found many times before, i.e. fatty acid tissue composition of tissues reflects the dietary composition (Fritsche et al., 1991; Friedman and Sklan, 1995; Applegate and Sell, 1996). Our study suggests that at high dietary levels of both LA and OA either the n-6 deposition is favoured above MUFA deposition or that OA is selectively oxidated. The bursal levels of AA and long-chain n-3 are particularly important for immune regulation because they are most relevant for the eicosanoid-producing capacity of this tissue. The decreased bursal AA at increased dietary LA levels suggests the feed forward inhibition of *de novo* synthesised of AA from LA. Such an observation is in contrast with what has been reported before. In turkey poults liver and cerebellum AA levels increased at low and increasing dietary LA levels (Applegate and Sell, 1996). Friedman and Sklan (1995) reported that the dietary LA level did not affect plasma and liver AA levels. Fritsche et al., (1991) found that generally AA levels in serum, bursa, splenocytes and thymus were higher at higher LA levels. These previous studies and the present trial indicate that the inhibition of AA synthesis

and/or deposition may occur only at the high dietary LA levels of the present study and not at lower levels. Only at the highest dietary level, VE might be associated with both high bursal AA and low n-3 fatty acids, both potential important factors in inflammatory processes. Thus, the present experiment does not provide strong evidence that the dietary VE level affects the composition of dietary fatty acids into tissue of growing layer hens. Because tissue eicosanoid precursor AA levels did not increase at increased dietary LA levels and the Ab responses in the present study did not correlate with tissue fatty acid levels, there is no evidence that Ab responses in chickens are affected via AA formation from LA.

In conclusion, the present study indicates that 1. the dietary VE level does not affect the fatty acid composition 2. there is no evidence for the presence of a mechanism that influences Ab responses in chickens via AA formation from LA followed by eicosanoid synthesis, 3. the interaction effects between dietary VE and PUFA on Ab responses is of minor importance and non-consistent. All together we did not find indications that at high dietary PUFA levels higher dietary VE levels affect tissue fatty acid composition and metabolism that might be important for the Ab response. This implies that, within the studied range, adding extra VE to preserve or affect the effects of dietary PUFA on Ab responsiveness is unnecessary.

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Chapter 5

Immunomodulatory Effects of Indomethacin and Prostaglandin E_2 on Primary and Secondary Antibody Response in Growing Layer Hens

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Abstract: Effects of prostaglandin E_2 (PGE_2) and indomethacin, an inhibitor of PGE_2 oxygenase, on primary and secondary antibody (Ab) responses to either *Mycobacterium butyricum* protein or keyhole limpet hemocyanin (KLH) were studied in growing layer hens. Immunizations at 35 and 70 d of age were accompanied by immunomodulating treatments with PGE_2, indomethacin, or PBS. In addition, we studied effects of various doses of indomethacin and PGE_2 on mitogen-induced T-cell proliferation in vitro. Secondary Ab responses to KLH were enhanced by administration of indomethacin at secondary immunization and, to a lesser extent, by PGE_2 administration at secondary immunization. Primary Ab responses to *M. butyricum* tended to decrease by administration of either PGE_2 or indomethacin. Secondary Ab responses to *M. butyricum* were affected by administration of both PGE_2 and indomethacin at primary immunization. Prostaglandin E_2 increased phytohemagglutinin (PHA)-induced lymphocyte proliferation. Indomethacin decreased Concanavalin A (ConA)- and PHA-induced lymphocyte proliferation. The net effect of indomethacin on the Ab response could not be explained by inhibition of PGE_2 oxygenase only. Our data rather suggest an inhibition by indomethacin of other immunosuppressing factors derived from arachidonic acid. We concluded that polyunsaturated fatty acid-derived products might especially affect secondary antibody responsiveness. This finding may depend on inhibition or enhancement of T-cell responsiveness. Consequently, immunomodulation by dietary polyunsaturated fatty acids may have profound effects at secondary rather than at primary exposure to pathogens.

Introduction

Prostaglandins (PG) are important immunoregulators in mammals and possibly also in birds. Prostaglandin E_2 (PGE_2) is a major arachidonic acid (AA) metabolite released by antigen-presenting cells. It plays a role in the initiation and regulation of the antibody (Ab) response. In mammals, PGE_2 up-regulates characteristic T helper-2 (TH2) cytokine profiles at the expense of T helper-1 (TH1) cytokine profile. Prostaglandin E_2 secretion may tip the TH1/TH2 balance in favor of a TH2-type response, leading to B-cell production of IgG1 and IgE (Phipps et al., 1991; Hilkens et al., 1995).

Eicosanoids, such as PG, are synthesized from polyunsaturated fatty acids (PUFA). Prostaglandin E_2 is synthesized by a cyclooxygenase-dependent pathway from n-6 PUFA AA. The initial precursor of AA is linoleic acid, abundant in many plant oils. Previously, we found that a diet rich in linoleic acid increased Ab responsiveness against a known TH2-dependent antigen in mice [keyhole limpet hemocyanin (KLH)]. The same diet, however, reduced the Ab response against a known TH1-dependent antigen (*Mycobacterium butyricum*) in mice (Sijben et al., 2000). A divergent PGE_2 effect initiated by boosted dietary linoleic acid might have underlain these results.

The present experiment was designed to examine the role of PGE_2 in the initiation and regulation of the Ab response in chickens. Prostaglandin E_2 may have long-term effects on Ab responses by modulation of T-cell dependent Ab production cascade at the initiation of the primary Ab response. We investigated effects of administration of PGE_2 or indomethacin, a specific inhibitor of cyclooxygenase pathway (Shen and Winter, 1977), on Ab responses to antigens known to induce TH2 (KLH-dinitrophenyl) (Bradley et al., 1995; Dohorty et al., 1995; Bliss et al., 1996) or TH1 (*M. butyricum* protein) (Mosmann and Sad, 1996) responses in mice. In addition, effects of PGE_2 and indomethacin on in vitro lymphocyte proliferation and growth were tested. Growth may be affected by the experimental treatments by a change of circulating concentrations of interleukin-1 and tumor necrosis factor-α altering the partitioning of dietary nutrients (Klasing and Johnstone, 1991).

Materials and Methods

Birds and Housing

Birds were from one of three selection lines, which originated from an ISA Warren cross (medium heavy layers). Two lines had been selected for high or low primary Ab responses at Day 5 after primary i.m. immunization with SRBC at 37 d of age.

Additionally, a random-bred C line has been kept (Van der Zijpp and Nieuwland, 1986). In the present study, 273 hens were used from the 18th generation of a C line, all from one hatch. Chicks were housed in one of 36 battery cages (50 × 100 cm) with seven or eight chicks per cage. The birds had free access to feed and water. The pullets were fed a regular diet, formulated to meet nutritional requirements of growing layer pullets of a medium-heavy strain (NRC, 1994). The chicks were vaccinated against Marek's disease and Newcastle disease at the day of hatch, against infectious bronchitis at 2 d of age, and against infectious bursal disease at 15 d of age. On the day of hatch birds were randomly assigned to the experimental treatments.

Experimental Design

Effects of indomethacin and PGE_2 on primary Ab response were studied using a 3 × 2 factorial design with three factors for primary immunomodulation and two factors for immunization treatment and forty-five or forty-six birds per treatment. Immunomodulating treatments were administration of PGE_2, indomethacin, or PBS as a control. Indomethacin was administered by s.c. injection in the left femur with 1 mg indomethacin (Sigma Chemical Co., St. Louis, MO) per 100 g BW in 1 mL PBS, 1 d prior to immunization. The dose of indomethacin was based on a report by Johnson et al. (1993), who found that this dose administered in vivo attenuated the reduction in feed intake caused by lipopolysaccharides (LPS). The control injection, with 1 mL PBS, was also administered 1 d prior to immunization. Prostaglandin E_2 (Sigma Chemical Co., St. Louis, MO) was mixed with the experimental antigen at 10^{-7} *M*, and was injected at immunization. This dose of PGE_2 was derived from Demeure et al. (1997), who found human naive CD4 cells in vitro to favor synthesis of TH-2 cytokines at the expense of TH-1 after stimulation at this dose. The animals injected with PGE_2 at immunization were given an extra injection with 1 mL PBS on the day before immunization as a control for the indomethacin or PBS injections in the other immunomodulatory treatments. At 35 d of age, birds were immunized with either KLH-dinitrophenyl (KLH-DNP) (Cal Biochem - Novabiochem Co., La Jolla, CA) or heat-killed *M. butyricum* dry cells (Difco Laboratories, Detroit, MI). The experimental antigens were administered by a s.c. injection in the left femur with 1 mL PBS containing 1 mg KLH-DNP or 1 mg *M. butyricum* protein. These immunizations were used as each others controls because a previous study indicated that no cross-reactivity between these antigens occurred (Sijben et al., 2000).

Thirty-five days after primary immunization (at 70 d of age), birds were reimmunized with the same antigen in the same dose to induce a secondary Ab response. Prior to secondary immunization, each of six groups of birds sharing the same primary treatments was split into three by the secondary immunomodulary treatment, leading to a 3 × 2 × 3 factorial design to examine the effects of indomethacin and PGE_2 on secondary Ab response. Secondary immunomodulation was identical to primary immunomodulation, except that it was given near the moment of secondary immunization.

Blood samples were taken at 7 d before primary immunization and at 7, 14, 21, 28, 35, 42, 49, and 55 d after primary immunization. The primary Ab response is represented by measurements on Day -7 to 35; the secondary Ab response is represented by measurements on Day 35 to 55. Body weight was recorded once a week from hatch until 90 d of age.

Antibody Response to KLH and M. butyricum

Total Ab titers to KLH and *M. butyricum* in serum of all birds were determined by ELISA as described in Chapter 2.

In Vitro Lymphocyte Proliferation to Concanavalin A and Phytohemagglutinin

An in vitro lymphocyte stimulation test was performed to determine effects of PGE_2 and indomethacin on *in vitro* T-cell proliferation capacity. Peripheral blood leukocytes (PBL) from three cocks, others than those used for the in vivo challenge, were tested for proliferation in the presence of 0, 10^{-5}, 10^{-6}, 10^{-7} or 10^{-8} M PGE_2 (doses according to Demeure et al., 1997). Also, PBL were tested for proliferation, at first in the presence of 5, 10 and 20 μg/mL indomethacin and then in the presence of 0.1, 1, or 5 μg/mL indomethacin. The dose of 1 μg/mL was based on Riley et al. (1989). All cultures were set up in triplicate in the presence of 5 μg/mL Concanavalin A (ConA) (Sigma Chemical Co., St. Louis, MO) or 50 μg/mL phytohemagglutinin (PHA) (Difco Laboratories, Detroit, MI) (stimulated) or without mitogen (unstimulated). Details concerning isolation procedure and culture media are given in Chapter 2. ConA or PHA stimulation, with or without PGE_2 or indomethacin, was expressed as stimulation index (SI). The SI was calculated as SI = mean counts per minute in stimulated cultures / mean counts per minute in unstimulated cultures.

Statistical Analysis

Effects of primary and secondary immunomodulation and immunization were tested by repeated measurement analysis. For effects on the secondary Ab responses the following model was used:

$$Y_{ijklm} = \mu + A_i + B_j + I_k + \text{interactions 1} + e_{1,ijkl} + T_m + \text{interactions 2} + e_{2,ijklm}$$

where Y_{ijklm} = dependent variable; μ = overall mean; A_i = fixed effect of primary immunomodulation i (i = 1, 2, 3); B_j = fixed effect of secondary immunomodulation j (j = 1, 2, 3); I_k = fixed effect of immunization treatment k (k = 1, 2); interactions 1 = all two- and three- way interactions among primary and secondary immunomodulation and immunization treatment; $e_{1,ijkl}$ = error term 1, which represented the random effect of animal l within the primary and secondary immunomodulation i and j and immunization k level; T_m = fixed effect of time (l = 1,...,4); interactions 2 = all two-, three- and four way interactions among primary and secondary immunomodultion, immunization, and time; and $e_{2,ijklm}$ = error term 2, representing the random effect within groups between time periods. The effects of primary and secondary immunomodulation, immunization, and their interactions were tested against error term 1. The effect of time and the interactions with time were tested against error term 2. Effects of primary immunomodulation and immunization on primary Ab responses were tested using this model by exclusion of the factor secondary immunomodulation and its interactions. Effects of experimental treatments on BW gain between primary and secondary immunization and after secondary immunization were tested by two-way ANOVA. Effects of PGE_2 concentration on SI after stimulation of PBL with ConA or PHA were tested by regression analysis. All analyses were tested using GLM procedures of SAS (1990).

Results

Primary Ab Response to KLH and M. butyricum

KLH: Antibody titers to KLH of the birds immunized with KLH are shown in Figure 1. Anti-KLH titers in these birds increased compared to birds not immunized with KLH ($P < 0.001$). Administration of indomethacin or PGE_2 did not affect primary Ab responses to KLH in either immunization group.

M. butyricum: Kinetics of anti-*M. butyricum* protein Ab titers in birds immunized with *M. butyricum* are shown in Figure 2. Birds injected with *M. butyricum* mounted a higher anti-*M. butyricum* Ab response compared with the birds not immunized with *M. butyricum* ($P< 0.001$). Primary Ab responses to *M. butyricum* were affected by the interaction among immunization, primary immunomodulation, and time ($P < 0.05$) and by the interaction between immunization and primary immunomodulation ($P < 0.05$). Antibody titers to *M. butyricum* in *M. butyricum*- immunized hens were lower in those hens treated with PGE_2 or indomethacin compared with the PBS control hens (Figure 2). This effect increased toward the end of the primary response. In the KLH control hens, effects of PGE_2 or indomethacin were absent.

Secondary Ab Response to KLH and M. butyricum

KLH: Kinetics of the secondary anti-KLH response of hens immunized with KLH are shown in Figures 1 and 3. Figure 1 shows the effect of PGE_2 and indomethacin administration at primary immunization; Figure 3 shows the effect of PGE_2 and indomethacin administration at secondary immunization. Immunization with KLH increased anti-KLH titers compared with *M. butyricum*- immunized hens ($P < 0.001$). Primary immunomodulation did not affect secondary Ab response in KLH-immunized animals (Figure 1). Anti-KLH Ab titers were affected by the interaction among immunization, secondary immunomodulation, and time ($P < 0.01$) and by the interaction between immunization and secondary immunomodultion ($P < 0.01$). Anti-KLH titers in KLH-injected hens were higher in the birds administered PGE_2 and, moreover, with indomethacin, compared with birds administered PBS at secondary immunization.

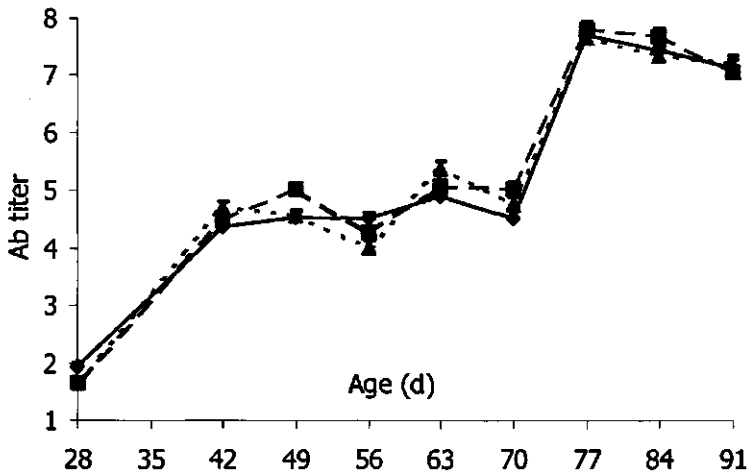

Figure 1. Effects of PGE_2 and indomethacin adminstration at primary immunization on the production of anti-KLH Ab response in layer hens s.c. immunized with 1 mg KLH-DNP in 1 mL PBS at 35 d and 70 d of age. Mean total Ab titers ± SEM to KLH in serum are from 46 or 47 hens per treatment, injected with 1 mg indomethacin per 100 g BW (■), 10^{-7} M PGE_2 (▲), or a control injection with PBS (♦) at primary immunization.

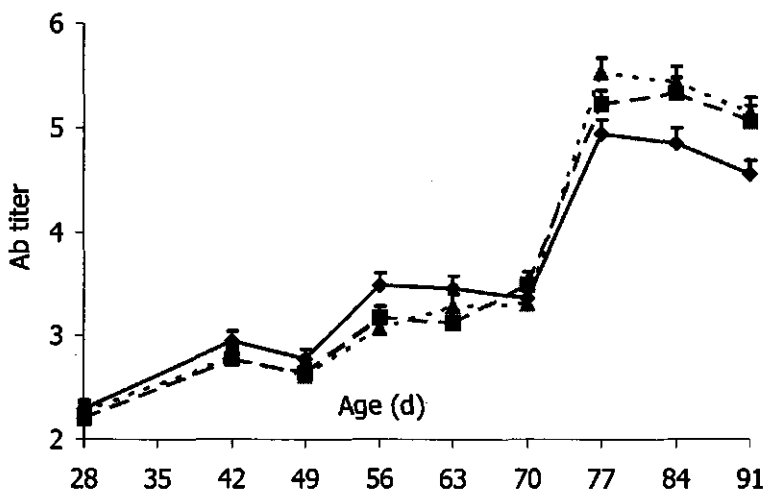

Figure 2. Effects of PGE_2 and indomethacin adminstration at primary immunization on the production of anti-*M. butyricum* protein Ab in layer hens s.c. immunized with 1 mg *M. butyricum* protein in 1 mL PBS at 35 d and 70 d of age. Mean total Ab titers ± SEM to KLH in serum are from 46 or 47 hens per treatment, injected with 1 mg indomethacin per 100 g BW (■), 10^{-7} M PGE_2 (▲), or a control injection with PBS (♦) at primary immunization.

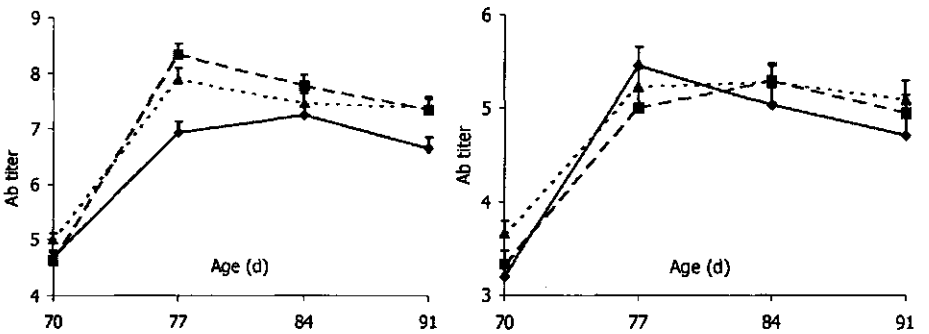

Figure 3 and 4. Effects of PGE_2 and indomethacin administration at secondary immunization on the production of anti-KLH and anti-*M. butyricum* Ab in figure 3 and 4, respectively, after secondary immunization in layer hens s.c. immunized with 1 mg *M. butyricum* or 1 mg KLH-DNP, respectively, in 1 mL PBS at 35 d and 70 d of age. Mean total Ab titers ± SEM in serum are from 46 or 47 hens per treatment, injected with 1 mg indomethacin per 100 g BW (■), 10^{-7} M PGE_2 (▲), or PBS (♦) at secondary immunization. The shown effects of PGE_2 and indomethacin administration at secondary immunization are regardless of treatments at primary immunization.

M. butyricum: Secondary Ab responses to *M. butyricum* protein in *M. butyricum* immunized hens is shown in Figures 2 and 4. Figure 2 shows the effect of PGE_2 and indomethacin administration at primary immunization; Figure 4 shows the effect of PGE_2 and indomethacin administration at secondary immunization. Antibody titers were elevated by secondary immunization ($P < 0.001$). Secondary Ab responses were affected by the interaction among immunization, primary immunomodulation, and time ($P < 0.001$) and by primary immunomodulation ($P < 0.05$). Prostaglandin E_2 and, to a lesser extent, indomethacin administration at primary immunization enhanced the secondary Ab response in *M. butyricum*-immunized hens. Secondary Ab responses were also affected by the interaction among immunization, secondary immunomodulation, and time ($P < 0.05$); by the interaction between secondary immunomodulation and time ($P < 0.05$); and by the four-way interaction among immunization, primary and secondary immunomodulation and time ($P < 0.01$). On Day 7 after secondary immunization, PBS administration had an enhancing effect on anti-*M. butyricum* Ab responses; thereafter, PBS-administered hens had lower Ab titers compared with hens treated with PGE_2 or indomethacin at secondary immunization (Figure 4). Secondary anti-*M. butyricum* antibody titers of *M. butyricum* immunized birds per immunomodulatory treatment are shown in Table 1.

Table 1. Effects of prostaglandin E_2 (PGE_2) and indomethacin administration at primary and secondary immunization on average total anti-*Mycobacterium butyricum* protein antibody (Ab) titers after secondary immunization of layer hens[1]

Primary modulator	Secondary modulator	Days of age			
		70	77	84	91
PBS	PBS	3.17	4.88	4.49	4.32
	Indomethacin[2]	3.37	4.84	5.18	4.24
	PGE_2[2]	3.54	5.11	4.87	5.09
Indomethacin	PBS	3.18	5.73	5.26	4.86
	Indomethacin	3.80	4.97	5.28	5.33
	PGE_2	3.55	4.96	5.44	5.03
PGE_2	PBS	3.25	5.76	5.33	4.96
	Indomethacin	2.81	5.21	5.43	5.32
	PGE_2	3.88	5.62	5.57	5.17
	SEM	0.20	0.24	0.25	0.24
Main effects[3]	Level of significance[4]				
I	***				
M1	*				
M2	NS				
I × M1	NS				
I × M2	NS				
M1 × M2	NS				
I × M1 × M2	NS				
T	***				
I × T	***				
M1 × T	NS				
I × M1 × T	***				
M2 × T	*				
I × M2 × T	*				
M1 × M2 × T	***				
I × M1 × M2 × T	**				
A(I × M1 × M2)	***				

[1]The hens were s.c. immunized with 1 mg *M. butyricum* protein in 1 mL PBS at 35 d and 70 d of age, and administered 1 mg indomethacin per 100 g BW, 10^{-7} M PGE_2, or a PBS control at primary and secondary immunization. Average titers to *M. butyricum* protein in serum are from fifteen or sixteen hens per treatment.

[2]Sigma Chemical Co., St. Louis, MO 63178-9916.

[3]I = Immunization treatment, M1 = Modulator at primary immunization, M2 = Modulator at secondary immunization, T = Time effect, A = Animal effect

[4]NS = not significant, # $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

In Vitro Lymphocyte Proliferation to ConA and PHA

Mean SI of cell cultures of three cocks after stimulation with ConA and PHA, in the presence of PGE_2, are shown in Table 2. Cell proliferation in the presence of PGE_2 was not consistently increased or decreased by the presence of increasing PGE_2 concentrations after ConA stimulation. However, after PHA stimulation, cell proliferation tended to be enhanced by increasing PGE_2 concentrations ($P < 0.1$).

Stimulation indices in the presence of indomethacin dropped from 318 to 312, 142 and 12, respectively, by increasing the concentrations from 0, to 0.1, 1 and 5 μg/mL indomethacin in the ConA stimulated cultures. In the PHA-stimulated cultures SI was 123, 134, 115 and 17 at 0, 0.1, 1, and 5 μg/mL indomethacin, respectively. In the presence of 10 and 20 μg/mL indomethacin, no cell proliferation was found.

Table 2. Average stimulation index (SI) after in vitro stimulation of peripheral blood leukocytes (PBL) with Concanavalin A (ConA) or phytohemagglutinin (PHA) in the presence of prostaglandin E_2 (PGE_2) in different concentrations[1]

	ConA	PHA
PGE_2	Mean SI	Mean SI
0 M	125	68
10^{-8} M	91	87
10^{-7} M	128	127
10^{-6} M	112	158
10^{-5} M	117	179
SEM	13.5	34.3
Main effects		
Cock	***[2]	***
[PGE_2]	NS	#

[1] ^3H uptake by stimulated lymphocytes was expressed as SI, i.e., the ratio between counts per min in stimulated and unstimulated cultures in the presence of the same concentration PGE_2. The proliferation capacity of PBL in the presence of PGE_2 was tested in three, 1-year-old cocks.
[2] NS = not significant, # $P < 0.1$, *** $P < 0.001$

Growth

Weight of the hens did not differ between experimental treatments on either the day of hatch or on the day of primary immunization. Hens immunized with *M. butyricum* and administered PBS control seemed to grow slower than hens in the other treatment groups after primary- and before secondary ($P < 0.05$). However, consistency of this observation with any other observation was not found. No other effects of the experimental treatments on growth were found.

Discussion

The preset hypotheses tested in the current paper were mainly based on research in mice and other mammals. Literature shows that subsets of TH cells that differ in their cytokine secretion patterns have their own effector functions (Mossman and Coffman, 1989). T-helper 1 cells induce phagocyte and T-cell-mediated inflammatory reactions against microbes; TH2 cells induce enhanced humoral (IgG, IgA, and IgE) response. The domination of a TH1 or TH2 response is largely determined by the immunological stimulus. Another factor involved in the

regulation of the TH1/TH2 balance in mice is PGE_2. Prostaglandin E_2 secretion by antigen- presenting cells may tip the balance in favor of a TH2 response (Phipps et al., 1991; Hilkens et al., 1995). Although the TH1/TH2 paradigm has not been established yet in birds, combat against different sorts of antigens may be supported by a selective boost of either humoral or cellular immunity. Recently, we found that dietary n-6 PUFA increased the Ab response against an antigen that induces TH 2 responses in mice, whereas the Ab response against an antigen known to induce TH1 responses in mice was decreased. Dietary n-3 PUFA tended to cause the opposite effect (Sijben et al., 2000). We speculate that elevated PGE_2 synthesis resulting from high dietary n-6 might have underlain this observation. If true, then in the present experiment administration of PGE_2 might have enhanced Ab responses to KLH and attenuated Ab responses to *M. butyricum*. Indomethacin, being a powerful inhibitor of the production of PGE_2 and other AA metabolites, was expected to affect Ab responses conversely.

The results of the present experiments partially confirm and partially reject the postulated hypothesis. Administration of PGE_2 in vivo only enhanced anti-KLH titers in the secondary response, but not in the primary response. As illustrated by the present experiment, in secondary responses, memory T cells mediated enhanced Ab responses as compared with the primary responses (Ahmed and Grey, 1996; Boa-Amponsem et al., 1999). Consequently, the observation that PGE_2 or indomethacin affects the secondary but not the primary anti-KLH response suggests that memory T cells may be the major effector cells of PGE_2 in the underlying mechanism.

The effects of PGE_2 and indomethacin on anti-KLH Ab titers were largely the same. It has been shown previously that aspirin, an indomethacin-like cyclooxygenase inhibitor, depresses endogenous PGE_2 levels in the bursa (Likoff et al., 1981). The present observations suggest that inhibition of cyclooxygenase with indomethacin may block immunosuppressive factors that are more powerful than the immunostimulative properties of PGE_2. If this is the case these products are most likely PG or thromboxanes of the two series or the three series, because their synthesis depends on cyclooxygenase as well. However, information about the nature and the production of eicosanoids in chickens is scarce, and detailed information about the immunological properties of other cyclooxygenase products is lacking.

The primary Ab response to *M. butyricum* was decreased by PGE_2 and indomethacin administration. Similar to the secondary response to KLH, indomethacin and PGE_2 affected Ab responses in the same direction. This finding

supports the observation that the net effect of indomethacin is different from inhibition of PGE_2 oxygenase alone. In accordance with the preset hypothesis, PGE_2 down-regulated Ab response to *M. butyricum*. Previously, PGE_2 has been negatively correlated with anti-LPS Ab titers after infection with *Escherichia coli* (Likoff et al., 1981), LPS being an inducer of TH1 cytokine production in mice. After secondary immunization, the effect of pretreatment at primary immunization with PGE_2, as well as indomethacin, was inverted, which suggests that the immunological memory is primed by initial pretreatment. In mice, PGE_2 enhance IgG1 and IgE production by elevating intracellular cyclic 3', 5' adenosine monophosphate (cAMP), while simultaneously diminishing IgM and IgG3 production in vitro (Phipps et al., 1990; Roper et al., 1990). The mechanism by which this class switch is accomplished in mice is unknown. However, this observation indicates that Ab production for secondary (IgG1 and IgE), rather than primary (IgM and IgG3), responsiveness is typically stimulated by PGE_2.

The effect of PGE_2 on mitogen-induced cell proliferation was different in the PHA-stimulated cultures compared with the ConA-stimulated cultures. This finding may be explained by the possibility that, also in chicken, ConA and PHA stimulate different classes of lymphocytes with different responses to PGE_2. The dramatic decrease in lymphocyte ^3H uptake by increasing concentration of indomethacin may be explained by the toxic properties of indomethacin in higher concentrations.

In conclusion, the observations that PGE_2 and indomethacin affected secondary rather than primary Ab response suggest that eicosanoid synthesis might affect the immune reaction in poultry after secondary rather than after primary exposure to a pathogen. Therefore, when examining effects of dietary PUFA in a disease challenge, it may be worthwhile to include a secondary infection or antigen challenge.

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Chapter 6

Effects of Dietary Polyunsaturated Fatty Acids on In Vivo Splenic Cytokine mRNA Expression in Layer Chicks Immunized with *Salmonella typhimurium* Lipopolysaccharide

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Abstract: Effects of dietary polyunsaturated fatty acids (PUFA) on immune responses in poultry have been reported. However, effects on the underlying mechanisms, such as the role of cytokines, have not been documented because the necessary tools were lacking. Recently, primer sets for chicken interleukin (IL)-1β, IL-2, interferon-gamma (IFN-γ), myelomonocytic growth factor (MGF) and transforming growth factor (TGF)-β_2 became available. Therefore, in the present study we first examined the *in vivo* effects of an inflammatory challenge with *Salmonella typhimurium* lipopolysaccharide (LPS) on cytokine profiles in growing laying type chicks. Secondly, we examined whether dietary fat sources affected the observed cytokine profiles. Two hundred and forty chicks were assigned in a 2 × 4 factorial design of treatments, with injection with LPS or saline, and dietary fat source as factors. Factors were *i.v.* injection with *S. typhimurium* LPS, or saline (control), and 4 dietary fat sources: corn-, linseed-, menhaden oil, and tallow. Two h after injection birds were killed and their spleens removed for RNA extraction. RT-PCR reactions using primer sets for chicken IL-1β, IL-2, IFN-γ, MGF, TGF-β_2, and β-actin were performed with RNA samples pooled by pen. The expression of cytokine mRNA was expressed relative to the level of β-actin mRNA. Interleukin-1 ($P < 0.001$), MGF ($P < 0.0001$), IL-2 ($P < 0.001$), and IFN-γ ($P < 0.001$) mRNA expression was enhanced by challenge with LPS. Immunization treatment had no effect on TGF-β_2 or β-actin expression. Dietary treatment did not affect mRNA expression of IL-1, MGF, IFN-γ, TGF-β_2, or β-actin. Interleukin-2 expression in LPS injected birds fed the fish oil enriched diet was enhanced ($P = 0.05$). The present study indicates that in vivo effects of immune challenge on cytokine mRNA expression can be measured in poultry. The observation that mRNA level of IL-2 but not the mRNA levels of IFN-γ or MGF is enhanced by dietary fish oil at 2 h suggests that dietary PUFA at this moment initially affected naïve T lymphocytes.

Introduction

Numerous studies have demonstrated that dietary PUFA can modulate a wide range of immune responses in poultry. Diets enriched with fish oil rich in long-chain n-3 PUFA have anti-inflammatory properties, increase delayed type hypersensitivity (Korver and Klasing, 1997), increase antibody responses, and decrease lymphocyte proliferation (Fritsche et al., 1991). Diets enriched with linseed oil, rich in short-chain n-3 PUFA also increase delayed-type-hypersensitivity (Korver and Klasing, 1997), decrease lymphocyte proliferation (Fritsche et al., 1991), and decrease antibody responses (Parmentier et al., 1997). Diets enriched with plant oils rich in (short-chain n-6) linoleic acid augment specific anti-vaccine (Friedman and Sklan, 1997) or anti-keyhole limpet hemocyanin antibody responses (Sijben et al., 2000), but decrease anti-bovine serum albumin (Friedman and Sklan, 1995), or heat killed *Mycobacterium butyricum* particles (Sijben et al., 2000) responses.

These immunomodulatory properties of PUFA might be associated with their metabolical function as an eicosanoid precursor (Korver et al., 1997). Besides eicosanoids, cytokines are also important messenger molecules of immune cells. In mammalian studies numerous effects of dietary n-3 PUFA on cytokine production were reported (reviewed by Blok et al., 1996). Dietary n-3 decreased interleukin (IL)-1-β, IL-2, IL-6 and tumor necrosis factor (TNF)-α in human peripheral blood mononuclear cells compared with control diets low in n-3 and not particularly high in n-6. In mice dietary n-3 increased IL-1β and TNF-α in peritoneal macrophages compared with diets low in n-3 and high in n-6. Circulating IL-12 and interferon (IFN)-γ levels, and splenic IFNγ mRNA were lower in mice fed a n-3 PUFA enriched diet compared with a n-6 enriched and low PUFA diet, indicating a shift from a T helper (Th)-1 type to a Th-2 type of immune response (Fritsche et al., 1999).

The mechanisms by which dietary fatty acids modulate cytokine production have not fully been elucidated yet. A widespread concept is that long-chain dietary n-3 PUFA are incorporated into cell membrane phospholipids replacing arachidonic acid as a substrate, and then are converted into prostaglandin (PG)E_3 and leukotriene B_5, which are biologically less active than arachidonic acid metabolites PGE_2 and leukotriene B_4. Moreover, n-3 fatty acids have been shown to be poorly metabolized by cyclooxygenase, thereby reducing the total production of eicosanoids (Lee et al., 1985). In rats, dietary fish oil lowers the production of PGE_2 by peritoneal macrophages and splenocytes by 70-80 % compared with dietary corn oil (Fritsche et al., 1992). Also in mice it was demonstrated that PGE_2 inhibits the production of IL-2 and IFN-γ (Betz and Fox, 1991). These data suggest

that PGE_2 mediates the effects of dietary n-3 PUFA on the level of Th-1 cytokines. In poultry research, studying mechanisms by which dietary PUFA modulate immune responses is still problematic because of the absence of specific antibodies for the detection of cytokines at the protein level. Recently reverse transcription polymerase chain reaction (RT-PCR) assays for the message of chicken IL-1β, IL-2, IFNγ, myelomonocytic growth factor (MGF) and transforming growth factor (TGF)-β have been developed and applied to measure cytokine responses in in vitro cell cultures stimulated with LPS (Leshchinsky, 2000).

In vivo cytokine mRNA responses in Marek's disease herpes-virus infected chickens were reported recently (Xing and Schat, 2000). These assays provide us with the opportunity to study the mechanisms by which nutrients affect immune regulation at the level of cytokine synthesis in poultry. However, the RT-PCR assays have not yet been used to examine effects of challenge with lipopolysaccharide (LPS) on in vivo transcription of chicken cytokines. Therefore, in the present study growing chicks of a layer breed were fed diets different in PUFA content and challenged to induce an inflammatory response in order to investigate whether mRNA production for these cytokines could be measured in vivo, and if so, whether dietary fat source affected the *in vivo* production of specific cytokines.

Materials and Methods

Birds, Housing and Experimental Design

Two hundred and forty 4-d-old male Single Comb White Leghorn chicks were used. These birds were distributed among 48 battery pens (Petersime Incubator Co., Gettysburg, OH) with five chicks per pen stratified by BW to minimize the variability in pen weight. This experiment was designed as 2 × 4 factorial arrangement of treatments, with diet and immunization as factors. The four dietary treatments each consisted of a 95 % constant basal component, and a varying 5 % oil fraction (Table 1). The four oils used in the present study were corn oil (CO) (Mazola, Best Foods, CPC International Inc., Englewood Cliffs, NJ), linseed oil (LO) (ICN, Pharmaceuticals Inc., Costa Mesa, CA), fish (i.e. menhaden) oil (FO) (Omega protein, Inc., Hammond, LA) and beef tallow (BT) (Florin Tallow Co., Dixon, CA). The diets were formulated to meet or exceed the minimum requirements for a growing laying strain (NRC, 1994). The animals had free access to feed and water throughout the trial. Once per week feed intake and BW gain per pen were measured. At 37 or 38 d of age the birds were injected i.v. with 1 mg *Salmonella typhimurium* LPS (Sigma Chemical Co., St. Louis, MO) in 1 mL 0.9 % saline, or with 1 mL 0.9 % saline alone that served as a control. In the four treatments with

a saline control injection, four pens were assigned per treatment because no response, and thus a small variance, was expected in the control birds. A preliminary study showed that the present immunization treatment showed a significantly higher IL-1β, IL-2, IFN-γ response, expressed as cytokine / β-actin (i.e. housekeeping gene) ratio, at 2 h after injection compared with 3, 4, 6, 8, 16, and 24 h after injection (data not shown). Therefore at 2 h after injection, four cockerels per pen were killed by cervical dislocation, and spleens freeze-clamped in liquid nitrogen and stored at −70°C for subsequent RNA extraction. The immunization of birds and sampling of spleens was distributed over 2 consecutive days because of the quantity of birds that had to be processed.

Table 1. Composition of the experimental diets.

Ingredients	g / kg
Corn	467.43
Soybean meal	308.30
Cellulose	137.71
Ground limestone	17.84
Dicalcium phosphate	8.10
Salt	3.52
DL-methionine	1.35
Oil (variable)[1]	50
Choline chloride	0.75
Vitamin mix, carrier corn starch[2]	2.5
Mineral mix, carrier corn starch[2]	2.5
Calculated composition	
ME, kcal/kg	3203
Crude protein %	19.05
Crude fat %	7.08
Crude fiber %	3.60

[1]The variable oil fraction consisted of either corn-, linseed-, fish (i.e. menhaden) oil, or beef tallow.
[2]Practical reference diet (NRC, 1994), except vitamin E was increased to 40 mg/kg

Reverse Transcription and Polymerase Chain Reactions

Total splenic RNA was extracted using the guanidinium thiocyanate-phenol procedure of Chomczynski and Sacchi (1987). Equal amounts of RNA from each cockerel of the same pen were pooled to form one sample per experimental unit (pen). One μg RNA per pooled sample was used for RT reaction, using 200 Units of Moloney Murine Leukemia Virus Reverse Transcriptase, 4 μL accompanying RT-Buffer, 0.5 μg random primers, 20 Units Recombinant RNasin® Ribonuclease Inhibitor (all from Promega Corporation, Madison, WI), and 500 μM dNTP (Perkin Elmer, Applied Biosystmes Division, Foster City, CA) in a 20 μL 0.1% diethyl pyrocarbonate water reaction mix. The RT and PCR reactions was performed using

a GeneAmp PCR system 9600 (Perkin Elmer, Applied Biosystmes Division, Foster City, CA) to form cDNA at 20 C for 15 min, 42 C for 30 min, and 95 C for 5 min.

The PCR reactions were performed in a total volume of 15 μL containing 0.6 μL cDNA from the RT reaction, 1.5 mM $MgCl_2$, 50 mM KCl, 10mM Tris-HCl pH = 8.3, 0.1 % Triton X-100, 0.01 % gelatin, 200 μM dNTP, 0.4 Unit Goldstar polymerase and 150 nM of each primer (both Eurogentec S.A., Ougree, 4102, Belgium). The PCR primer sequences for chicken β-actin, IL-1, IL-2, IFN-γ, and TGF-β_2 were previously developed and certified by Leshchinsky (2000). Before the start of the first cycle all PCR were preceded by a denaturation step of 2 min at 94 C, and the last cycle step was followed by a final elongation step of 7 min at 68 C. The annealing temperatures were 63, 62, 63, 60, 58 and 58 C for the β-actin, IL-1, IL-2, IFN-γ, MGF and TGF-β_2, respectively. The number of cycles was optimized for the present experimental conditions to prevent the amount of PCR product from exceeding the upward slope of a typical cycle number-PCR product curve. For the IL-1, IFN-γ, MGF, and TGF-β_2 assays 30 cycles and for the IL-2 essay 32 cycles were applied, according to Leshchinsky (2000). In the β-actin assay 20 cycles were run. All PCR were performed in triplicate with cDNA from one RT reaction.

Gel Electrophoresis and Quantification of PCR Products

The PCR products were separated on a 1.5 % agarose gel buffered with 0.5 × TBE containing 0.5 μg/mL ethidium bromide at 100 V for 45 min. Simultaneously, a Molecular Mass Standard (Bio-Rad Laboratories, Hercules, CA) was used in duplicate in each row. The Molecular Mass Standard was used according to the manufacturer's instructions and showed five bands of 1000, 700, 500, 200 and 100 bp corresponding with 100, 70, 50, 20, and 10 ng DNA, respectively. The luminescence of several volumes of the PCR product was tested in a preliminary analysis of a pooled sample of approximately eight randomly chosen samples of LPS-injected birds. The volume that fitted best in the range of the Molecular Mass Standards was used to quantify the PCR product. Digital pictures of the gels were taken using a Gel Doc 1000 system (Bio-Rad Laboratories, Hercules, CA). The luminescence of the band of the PCR product was compared with the standard line using the volume analysis option of the Multi-Analyst PC Software (Bio-Rad Laboratories, Hercules, CA) for Gel-Doc 1000 to estimate the mass of the PCR product. Data were expressed as the ratio between the mass of the cytokine mRNA and the mass of β-actin mRNA, which served as an internal control for the initial amount of RNA. Median values of triplicate measurements were used to calculate the cytokine / β-actin ratio.

Statistical analysis

Data for BW gain, feed intake, feed conversion, and cytokine mRNA expression were collected and analyzed per pen. Body weight gain, feed intake and feed conversion were analyzed by one-way ANOVA for effects of dietary fat source. Preliminary analysis of the cytokine mRNA expression data showed that most of these data were not normally distributed (skewness and kurtosis not between –2 and 2). The variance in the saline-injected treatments was smaller than in the LPS immunized treatments. For testing immunization and immunization by diet interaction effects the data were transformed with a square root transformation to achieve a normal distribution. Effects of dietary treatment on mRNA expression were tested by one-way ANOVA within immunization treatments on untransformed data because most data were normally distributed after splitting the data set according to immunization treatment. All analyses were performed using the GLM procedure of SAS (1990). The probability level (P) of ≤ 0.05 was considered significant.

Results

Feed Intake, Growth, and Feed Conversion

Feed intake, growth, and feed conversion were not affected by the diet. Average weekly feed intakes between 4 d and 32 d after hatch were: 179, 170, 176 and 173 g (pooled SEM 4.3) per animal in the birds fed the CO, FO, LO, and BT enriched diets respectively. In the same period the average weekly growth was 61, 60, 60, and 59 (pooled SEM 1.74) gram per animal, respectively. The average feed conversion in this 4-wk period was 2.94, 2.83, 2.94, and 2.96 (pooled SEM 0.08) kg feed per kg growth in the birds fed the CO-, FO-, LO- and BT-enriched diet, respectively. The numerical lower feed conversion in cockerels fed FO enriched diet is mainly the results of a proportionally lower feed intake combined with an equal growth compared with the other feed treatments.

Splenic mRNA expression of β-actin, IL-1β, IL-2, IFN-γ, MGF and TGF-β_2.

Messenger RNA expression of β-actin and the cytokine/β-actin ratios in spleens of saline- and *S. typhimurium*-injected chickens, with data of all diets combined, are shown in Table 2. Immunization treatment highly affected splenic mRNA expression of IL-1β, MGF, IL-2, and IFN-γ ($P < 0.0001$). The cytokine/β-actin ratios were approximately 5, 8, 6, and 13 times higher for IL1β, IL-2, IFN-γ, and MGF, respectively, in LPS-injected chicks compared with saline-injected chicks. Splenic β-actin and TGF-β_2 mRNA expression were not affected by immunization treatment.

The raw data are illustrated in Figure 1, which shows two typical lanes per cytokine: one of a LPS-injected and one of a saline-injected pen of chicks. Significant interaction effects among immunization and dietary treatment on splenic mRNA expression of β-actin, IL1β, IFN-γ, MGF, TGF-β_2, ($P > 0.1$), and IL-2 ($P < 0.1$) were not found.

Messenger RNA expression data of β-actin in splenic tissue and the cytokine/β-actin ratios for each dietary treatment in saline- and LPS-injected chickens are shown in Table 3. In both the LPS- and saline-injected groups, the dietary treatment did not affect the level of β-actin mRNA. In the birds injected with *S. typhimurium* LPS the levels of IL-1β, MGF, IFN-γ, and TGF-β_2 were not affected by the dietary treatment, but the IL-2 / β-actin ratio was affected by the dietary treatment ($P = 0.05$). In cockerels fed the FO-enriched diet, the relative IL-2 mRNA expression level was approximately 58 % higher compared to the average of the other three dietary treatments (Table 3).

In the control birds injected with saline, the cytokine responses, apart from TGF-β_2, were generally much lower compared with the LPS-injected birds. However, in two pens of saline-injected birds, we observed cytokine responses that were close to the levels found in the LPS-injected birds. One of these two pens contained birds fed the CO-enriched diet, and the other one contained birds fed the LO-enriched diet. In the saline control cockerels dietary treatment did not affect the cytokine/β-actin ratios. The numerically high levels in the CO and LO diet groups are the result of the two outlier pens. Also the high SEM values in the saline-treated groups are largely due to these pens.

Table 2. Effect of LPS injection on splenic cytokine expression[1].

PCR product	Saline	LPS	*P* value
β-actin (ng / ng splenic RNA)	2.96 ± 0.30	2.67 ± 0.21	0.516
IL-1β / β-actin ratio	0.99 ± 0.57	5.13 ± 0.40	< 0.0001
MGF / β-actin ratio	0.15 ± 0.16	1.94 ± 0.11	< 0.0001
IL-2 / β-actin ratio	0.09 ± 0.07	0.73 ± 0.05	< 0.0001
IFN-γ / β-actin ratio	0.74 ± 0.39	4.32 ± 0.28	< 0.0001
TGF-β_2 / β-actin ratio	0.37 ± 0.06	0.47 ± 0.04	0.245

[1] Least square mean values ± SEM of cytokine response of laying chicks i.v. injected with either 1 mg *Salmonella typhimurium* lipopolysaccharide (LPS) in 1 mL saline or 1 mL saline only in splenic tissue at 2 h post immunization. Messenger RNA for interleukin (IL)-1β, myelomonocytic growth factor (MGF), IL-2, interferon (IFN)-γ, transforming growth factor (TGF)-β and β-actin were measured by semi-quantitative RT-PCR reaction. The cytokine responses were expressed relative to the β-actin response, which served as an internal control for the initial amount of splenic RNA in the RT reaction. *P* values as given were calculated based on square root transformed data. The table shows the untransformed data of all diets within immunization groups combined.

Table 3. Effect of dietary oil source on splenic cytokine expression[1].

Immunization	Diet	(ng)	Cytokine / β-actin ratio				
		β-actin	IL-1β	MGF	IL-2	IFN-γ	TGF-β_2
LPS	CO	2.46	5.06	2.05	0.58	4.76	0.54
	LO	2.80	4.86	1.76	0.64	4.23	0.37
	FO	2.62	5.11	1.99	1.01	4.40	0.45
	BT	2.78	5.48	1.94	0.69	3.89	0.51
	SEM	0.38	0.94	0.25	0.11	0.63	0.10
P value of Diet		0.92	0.97	0.87	0.05	0.80	0.61
Saline	CO	2.82	1.58	0.26	0.08	1.04	0.40
	LO	2.96	0.85	0.32	0.19	1.34	0.46
	FO	2.98	0.86	0.00	0.05	0.42	0.37
	BT	3.06	0.65	0.00	0.05	0.18	0.27
	SEM	0.71	0.42	0.20	0.05	0.45	0.08
P value of Diet		1.00	0.44	0.56	0.25	0.28	0.37

[1]Least square mean values ± SEM of cytokine response of growing laying chicks i.v. injected with either 1 mg *Salmonella typhimurium* lipopolysaccharide (LPS) in 1 mL saline or 1 mL saline only in splenic tissue at 2 h post immunization. The birds were fed diets enriched with either 5 % corn oil (CO), linseed oil (LO), fish oil (FO), or beef tallow (BT). Messenger RNA for interleukin (IL)-1β, myelomonocytic growth factor (MGF), IL-2, interferon (IFN)-γ, transforming growth factor (TGF)-β_2 and β-actin were measured by semi-quantitative RT-PCR reaction. β-actin responses were expressed in ng PCR product per ng splenic RNA. The cytokine responses were expressed relative to the β-actin response, which served as an internal control for the initial amount of splenic RNA in the RT reaction. Effects of oil source were tested by one-way ANOVA within immunization treatments.

Discussion

The present experiment was performed to study effects of dietary PUFA on immune regulation. In order to induce an immune response, chickens were challenged with LPS, which should result primarily in the production of pro-inflammatory cytokines. In addition, chickens were fed with diets different in levels of n-3 and n-6 PUFA. The purpose of the present study was twofold. First, we evaluated whether in vivo mRNA cytokine expression after LPS injection was detectable by RT-PCR assays. Second, the induced response was used to evaluate the effect of the dietary fat source on the expression of cytokine mRNA.

Interleukin-1 is an acute phase protein that is early released by macrophages after antigenic challenge. Therefore enhanced IL-1β mRNA expression was expected within a few hours after challenge. Transforming growth factor-β_2 is produced by many cell types, including macrophages and T and B lymphocytes. This cytokine is known as a promoter of connective tissue growth and collagen formation, and as a feedback regulator that dampens immune reactions. Mammalian IL-2 is produced by activated naïve T cells (Th 0 cells). Activated naïve $CD4^+$ T cells produce mainly IL-2 upon initial encounter with antigen (reviewed by Abbas et al., 1996). Following stimulation there is transcriptional activation of the

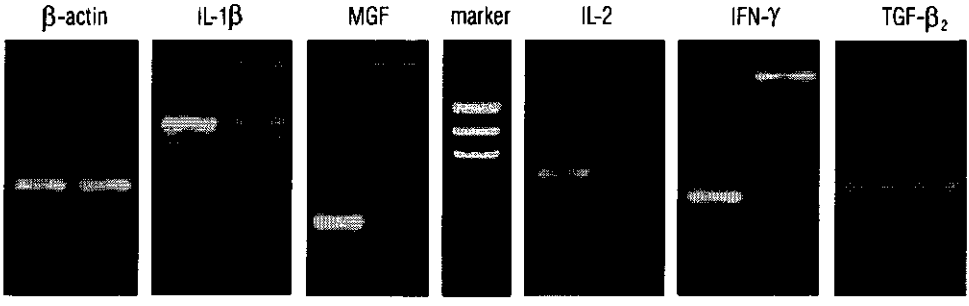

Figure 1. Effect of LPS injection on splenic cytokine expression in chicks at 2 h after injection with *S. typhimurium* LPS or saline. Five weeks-old laying chicks were injected i.v. with 1 mg LPS or a saline control. The RT-PCR with splenic RNA was performed using primer sets for chicken β-actin, interleukin (IL)-1β, myelomonocytic growth factor (MGF), IL-2, interferon-gamma (IFN-γ) and transforming growth factor (TGF)-β_2, resulting in PCR product of 322, 797, 147, 390, 251, and 316 bp respectively. Each pair of lanes represents the effect of immunization on the expression of β-actin and the cytokines, in which the first lane represents the birds injected with LPS and the second lane represents the birds injected with saline. The mass of DNA of each sample was calculated using the molecular mass standard shown in the middle lane. The Molecular Mass Standard shows five bands at 1000, 700, 500, 200 and 100 bp corresponding with 100, 70, 50, 20, and 10 ng DNA respectively. Data were expressed as the ratio between the mass of cytokine mRNA and the mass of β-actin mRNA. Injection with LPS increased the level of IL-1β, MGF, IL-2 and IFN-γ mRNA ($P < 0.0001$), but did not affect the level of β-actin and TGF-β_2 mRNA.

IL-2 gene over 24-48 h. Interleukin-2 and IFN-γ are also produced by differentiated T cells classified as Th 1 cells (Mosmann et al., 1986). Interleukin-2 is the main autocrine growth factor, and IFN-γ is an activator of macrophages and natural killer cells. Furthermore, IFN-γ promotes opsonization, phagocytosis, and MHC class II expression. Other sources of mammalian IFN-γ are CD8+ lymphocytes and natural killer cells. Chicken MGF is related to mammalian IL-6 and granulocyte colony stimulating factor, and cMGF expression can be induced by macrophages and myelomonocytic cells (Leutz et al., 1989). Increases in IL-2, IFN-γ, and MGF mRNA expression was expected following an increase in IL-1β transcription.

The injection with LPS enhanced the in vivo splenic expression of IL1-β, IL-2, IFN-γ, and MGF, but not TGF-β_2, at 2 h after challenge. Our choice to measure at 2 h after LPS injection was based on a preliminary study in which we measured IL-1β, MGF, IL-2, and IFN-γ mRNA expression at 0, 2, 3, 4, 6, 8, 16 and 24 h after i.v. injection with 1 mg LPS. In this pilot study we found the highest transcription level of all cytokines at 2 h after injection. The high speed of the response might be attributed to the systemic LPS injection. The mRNA transcription at 2 h after systemic LPS injection in the present study is probably the gene expression in macrophages (IL-1 and MGF), natural killer cells (IFN-γ), and naïve T cells (IL-2) because a second message like IL-2 and IFN-γ from chicken Th-1 cell equivalents would likely require more time. The expression of the TGF-β_2 mRNA in LPS- and saline-injected birds was at a similar but easily detectable level, as illustrated in Figure 1. This observation indicates that the TGF-β_2 mRNA levels in the present study were the basal levels of preformed TGF-β_2 mRNA in chicken spleens.

In the present experiment, the levels of IL-1β mRNA in birds injected with *S. typhimurium* LPS were not affected by the dietary treatment. Previously, dietary fish oil was found to decrease the release of IL-1 by peritoneal macrophages as compared with corn oil, after i.p. immunization with a lower dose of LPS (Korver and Klasing, 1997). Also, in cultured human mononuclear cells the production of IL-1 bioactivity and TNF was lower after consumption of higher levels of long-chain n-3 PUFA from fish oil (Endres, 1989). This possible discrepancy between mRNA and protein production levels suggests that PUFA may affect cytokine production at the level of protein release instead of at the level of transcription. Previously it has been shown that IL-1 mRNA expression can be induced unaccompanied by substantial production of bioactivity (Frendl et al., 1990). In previous studies (Korver and Klasing, 1997; Endres, 1989) n-3 PUFA may have interfered with the release of the high levels of early de novo synthesized IL-1 as found in mammals (Binns, 1990, 1992), whereas IL-1 transcription may have been unaffected. Further studies should examine differences in kinetics of IL-1 mRNA and protein production after challenge with LPS, and whether dietary effects may be found at later times after immunization.

In the present study, the level of IL-2 mRNA was elevated in LPS-injected birds fed the FO enriched diet relative to the other diets. The incorporation of long-chain n-3 PUFA from dietary FO into the membrane phospholipids at the expense of n-6 PUFA, may inhibit cyclooxygenase and the synthesis of PGE_2 from arachidonic acid. Prostaglandin E_2 is associated with downregulation of IL-2 production by Th1 cells (Betz and Fox, 1991). In clinical studies data on effects of

n-3 PUFA on Th-1 cytokines are conflicting. In healthy volunteers the proliferative response of T lymphocytes and IL-2 levels were decreased by dietary supplementation with n-3 fatty acids (Meydani et al., 1991, 1993). However, in certain patient groups T cell responses were higher following n-3 fatty acids supplementation, and IFN-γ production increased (Kemen et al., 1995). Mice infected with *Listeria monocytogenes* had two-to five-fold higher concentrations of IFN-γ in their blood when fed a fish oil enriched diet compared with mice fed diets low in n-3 PUFA (Fritsche et al., 1997a). These studies indicate that T lymphocytes may behave diffently after n-3 supplementation in healthy individuals compared with certain patient groups (Blok et al., 1996), and by analogy, in challenged and unchallenged animals. Also in poultry, dietary n-3 PUFA has been found to either enhance or decrease immune responses in various studies (Korver and Klasing, 1997; Fritsche et al., 1991; Parmentier et al., 1997; Friedman and Sklan, 1995, 1997; Sijben et al., 2000). The present observation, that IL-2 mRNA, but not IFN-γ, is enhanced by dietary FO at 2 h after LPS injection, suggests that dietary n-3 PUFA initially affects naïve T-cell precursors (Th-0) rather then differentiated Th-1-like cells. We hypothesize that 2 h after challenge would be too early for the expression of a second message of differentiated T-cells. In addition, if Th-1-like cells would have been affected by dietary FO, IFN-γ mRNA expression was expected to be affected similarly as IL-2 transcription. It has been proposed that the level of intracellular IL-2 mRNA corresponds well with the level of secreted IL-2 (Kaempfer et al., 1987). Therefore, the rise in IL-2 mRNA in the present study may initially facilitate both subsequent cellular or humoral immune responses. However, the effect on the kinetics and the magnitude of the effector functions of such an increase of IL-2 has yet to be established. Murine studies showed that dietary n-3 might enhance circulating IFN-γ (Fritsche et al., 1997a) but delayed bacterial clearance as well as decreased survival to a challenge with *Listeria* (Fritsche et al., 1997b) and reduced IFN-γ cytokine expression (Feng et al., 1999).

The i.v. injection of 1 mg LPS per chick induced hypothermia and inactivity in the first hours after this injection, but no mortality, even after 24 h in the preliminary study. Such a challenge may facilitate measurement of cytokines, but the relevance of this model to measure cytokine transcription and bioactivity after immunization with protein antigens remains to be established. To improve the applicability of immunization treatment as a model the present methodology of immune challenge needs to be refined, e.g., doses might be lowered. Recently Leshchinsky and Klasing (2000) reported increased IL-1β, IFN-γ and MGF expression after i.v. injection with 0.5 μg LPS in broiler chicks. Also the route of

antigen administration might be modified. In Japanese quail intra-abdominal injection with 7.5 mg LPS per kg body weight increased splenic IL-1β mRNA expression (Koutsos and Klasing, 2000). These recent data indicate that under more physiological conditions increased splenic levels of IL-1β mRNA are detectable in avian species. The responses to other (model) antigens remain to be investigated.

In summary, the present study indicates that an increase of in vivo cytokine mRNA expression after LPS challenge can be measured in poultry. In addition, in LPS challenged chickens splenic IL-2 mRNA expression was increased by dietary fish oil. We conclude that the increase in IL-2 mRNA expression is most probably caused by activated naïve T cells. In general, these results indicate that the present methods could increase insight into the relationship between nutrition and immunity in poultry at the regulatory level.

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Chapter 7

Early *in vivo* cytokine genes expression in chickens after challenge with *Salmonella typhimurium* lipopolysaccharide and dietary n-3 polyunsaturated fatty acids.

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Abstract: We studied the effects of *Salmonella typhimurium* LPS on *in vivo* cytokine mRNA levels in poultry, and investigated whether these levels could be altered by different nutrients. Two-hundred-and-forty chicks were assigned in a 2 × 4 factorial design of treatments. Factors were *i.v.* injection with *S. typhimurium* LPS, or saline (control), and four dietary fat sources: corn oil, linseed oil, menhaden oil and beef tallow. Two h after injection birds were killed and their spleens removed for RNA extraction. Quantitative real-time RT-PCR assays for mRNA of chicken IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-15, IL-18 and rRNA of 28S were used to obtain the *in vivo* splenic cytokine profiles. Expression levels of IL-1β, IL-2, IL-6, IL-8, IL-18 and IFN-γ mRNA increased, but IL-15 mRNA decreased 2 h after challenge with LPS compared with saline controls. In saline-injected control chickens, the dietary oil source did not affect the splenic mRNA level of any cytokine. In LPS challenged chickens IFN-γ mRNA was significantly higher in the chickens fed the fish oil enriched diet compared with the linseed oil, corn oil and beef tallow enriched diets. In mammals, IL-15 is enhanced by stimulation with LPS, and dietary enrichment with fish oil decreases the expression and protein levels of pro-inflammatory cytokines, and as a consequence decreases inflammation. The present data imply that avian IL-15 has, at least partially, a different function compared to its mammalian counterpart, and in addition, chicken innate immune responses might be affected different by n-3 compared to mammals.

Introduction

Human salmonellosis is a disease that can be caused by a range of serotypes including *Salmomella enteritidis* and *Salmonella typhimurium*. These two serotypes are responsible for the majority of *Salmonella* food-borne enteritis in man. Salmonellosis is often associated with the consumption of contaminated poultry meat or eggs (Humphrey, 1999). With the exception of very young chicks, *S. enteritidis* and *S. typhimurium* rarely cause clinical disease, but can colonise the gut of poultry (Barrow et al., 1987; Barrow and Lovell, 1990). Legislation by the European Union is leading to restrictions in the application of antibiotics for prophylactic use and growth promotion in intensive livestock farming. These restrictions might facilitate the colonisation of the gastro-intestinal tract of poultry by potential hazardous microbes such as *Salmonellae*, and their systemic translocation, resulting in higher risks for contamination of meat and eggs. An alternative way to improve disease control in food animals is by immunomodulation via improved nutrition.

Numerous studies have demonstrated the immunomodulatory properties of dietary polyunsaturated fatty acids (PUFA) (reviews by Blok et al., 1996; Calder, 1998). In some mammalian species, dietary n-3 PUFA decreased interleukin (IL)-1-β, IL-2, IL-6 and tumor necrosis factor (TNF)-α in human peripheral blood mononuclear cells compared with control diets low in n-3 and not particularly high in n-6. In mice, dietary n-3 increased IL-1β and TNF-α in peritoneal macrophages compared with diets low in n-3 and high in n-6. Circulating IL-12 and interferon (IFN)-γ levels, and splenic IFN-γ mRNA were lower in mice fed a n-3 PUFA enriched diet compared with a n-6 enriched and low PUFA diet, indicating a shift from a T helper (Th)-1 type to a Th-2 type of immune response (Fritsche et al., 1999). The mechanisms by which dietary fatty acids modulate cytokine production have not yet been fully elucidated. One widespread concept is that long-chain dietary n-3 PUFA are incorporated into cell membrane phospholipids replacing arachidonic acid as a substrate, and then are converted into prostaglandin (PG)E_3 and leukotriene B_5 which are biologically less active than arachidonic acid metabolites PGE_2 and leukotriene B_4. Moreover, n-3 fatty acids have been shown to be poorly metabolized by cyclooxygenase, thereby reducing the total production of eicosanoids (Lee et al., 1985). In rats dietary fish oil lowers the production of PGE_2 by peritoneal macrophages and splenocytes by 70-80 % compared with dietary corn oil (Fritsche et al., 1992). Also in mice it was demonstrated that PGE_2 inhibits the production of IL-2 and IFN-γ (Betz and Fox, 1991). These data suggest that PGE_2 mediates the effects of dietary n-3 PUFA on the level of Th-1 cytokines. In

avian species several studies have demonstrated effects of dietary PUFA on immune cell function (Fritsche et al., 1991; Friedman and Sklan, 1997; Korver and Klasing, 1997; Parmentier et al., 1997; Sijben et al., 2000). However, these studies are inconsistent. Research in Aves has usually investigated regulatory mechanisms, though one study suggests that the anti-inflammatory properties of n-3 PUFA are associated with its metabolical function as an eicosanoid precursor (Korver et al., 1997). Information on effects of PUFA on cykine profiles in poultry is lacking. The detection of avian cytokines at the protein level is hampered by the absence of specific antibodies. Recently several avian cytokines have been cloned and sequenced, and reverse transcription-polymerase chain reaction (RT-PCR) assays for avian cytokines were developed (Xing and Schat, 2000; Leshchinsky, 2000; Kaiser et al., 2000).

The present study was designed to quantify the *in vivo* level of cytokine expression in poultry after *S. typhimurium* lipopolysaccharide (LPS) stimulation, and to investigate whether differences in these levels caused by nutrients could be detected. Previously, we reported such effects using semi-quantitative RT-PCR on the expression of splenic mRNA of IL-1β, IL-2, IFNγ, myelomonocytic growth factor (MGF) and transforming growth factor (TGF)-β (Sijben et al., 2001a). We detected increased *in vivo* expression of IL-1β, IL-2, IFNγ and MGF following LPS challenge, and increased IL-2 mRNA in LPS-challenged chickens fed a diet rich in long-chain n-3 PUFA compared with other LPS-challenged chickens. Since this study, more quantitative and repeatable techniques, such as real-time RT-PCR, have been developed and are expected to be more useful for discriminating small, physiological changes in message expression due to dietary changes. In this paper, quantitative real-time RT-PCR assays were used to obtain more quantitative *in vivo* cytokine profiles for IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-15 and IL-18 in the same samples as described before (Sijben et al., 2001a).

Materials and Methods

Chickens, Housing and Experimental Design

The birds, their housing, and the design used in the present study have previously been extensively described (Sijben et al., 2001a). Briefly, in a 4 × 2 factorial arrangement of treatments, with diet and immunisation as factors, two hundred and forty 4-d-old male chicks were distributed among 48 battery pens. To a 95% constant basal diet, 5% of one of the oil fractions corn oil (CO), linseed oil (LO), fish (menhaden) oil (FO) or beef tallow (BT) was added. The diets met or exceeded the minimum requirements for a growing layer strain (NRC, 1994) and

were supplied *ad libitum*. Feed intake, body weight gain and feed conversion per pen were established and were not affected by the oil source. At 37 or 38 d of age the birds were injected i.v. with either 1 mg *Salmonella typhimurium* LPS in 1 ml 0.9% saline, or with 1 ml 0.9% saline as a control. Per LPS treatment, eight pens were assigned, and four pens were assigned per saline control treatment because no response, and thus a small variance, was expected in control birds. A preliminary study indicated that the present immunisation treatment showed a higher IL-1β, IL-2, IFN-γ response at 2 h after injection compared to 3, 4, 6, 8, 16, and 24 h after injection. Therefore at 2 h after injection 4 cockerels per pen were euthanized by cervical dislocation, and spleens freeze-clamped in liquid nitrogen and stored at −70 °C for subsequent RNA extraction.

Real-time quantitative RT-PCR

Total splenic RNA was extracted using the guanidinium thiocyanate-phenol procedure of Chomczynski and Sacchi (1987). Purified RNA was eluted in RNase-free water and the yield of total RNA obtained from each individual chick was determined spectrophotometrically at 260 nm. Equal amounts of RNA from each cockerel of the same pen were pooled to form one sample per experimental unit (pen) of 0.2 μg of single stranded RNA/μL and stored at −70°C.

Cytokine mRNA levels in chicken spleens was quantified using a method based on that of Moody et al. (2000) as described by Kaiser et al. (2000). Briefly, cytokine and 28S rRNA-specific amplification primers and probes were designed using the Primer Express software program (PE Applied Biosystems). Details of probes and primers are given in Table 1. All cytokine probes were designed, from the sequence of the relevant genes, to lie across intron-exon boundaries. Cytokine probes were labelled with the fluorescent reporter dye 5-carboxyfluorescein (FAM) at the 5′ end and the quencher N,N,N,N′-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3′ end. The 28S probe was labelled with the fluorescent reporter dye VIC (PE Applied Biosystems) at the 5′ end and TAMRA at the 3′ end.

RT-PCR was performed using the Taqman EZ RT-PCR kit (PE Applied Biosystems). The RT-PCR mixture consisted of 1 × EZ RT-PCR buffer [including 60 nM 6-carboxy-x-rhodamine (a fluorescent reference dye)], 3 mM manganese acetate, 300 μM dATP, dCTP, and dGTP, 600 μM dUTP, 600 nM each primer, 100nM probe, 0·1 U *rTth* polymerase, 0·01 U AmpErase UNG (uracil-*N*-glycosylase), 5 μL 10 × diluted total RNA, made up to 25 μl RNase-free water. Amplification and detection of specific products were performed using the ABI PRISM 7700 Sequence Detection

Table 1. Real-time quantitative RT-PCR probes and primers.

RNA	Probe/primer sequence		Exon	Acc. No.[1]
28S	Probe	5′-(VIC)-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3′		X59733
	F	5′-GGCGAAGCCAGAGGAAACT-3′		
	R	5′-GACGACCGATTTGCACGTC-3′		
IFN-γ	Probe	5′-(FAM)-TGGCCAAGCTCCCGATGAACGA-(TAMRA)-3′	3/4	Y07922
	F	5′-GTGAAGAAGGTGAAAGATATCATGGA-3′		
	R	5′-GCTTTGCGCTGGATTCTCA-3′		
IL-1β	Probe	5′-(FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)-3′	5/6	AJ245728
	F	5′-GCTCTACATGTCGTGTGTGATGAG-3′		
	R	5′-TGTCGATGTCCCGCATGA-3′		
IL-2	Probe	5′-(FAM)-ACTGAGACCCAGGAGTGCACCCAGC-(TAMRA)-3′	2/3	AJ224516
	F	5′-TTGGAAAATATCAAGAACAAGATTCATC-3′		
	R	5′-TCCCAGGTAACACTGCAGAGTTT-3′		
IL-6	Probe	5′-(FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)-3′	3/4	AJ250838
	F	5′-GCTCGCCGGCTTCGA-3′		
	R	5′-GGTAGGTCTGAAAGGCGAACAG-3′		
IL-8	Probe	5′-(FAM)-TCTTTACCAGCGTCCTACCTTGCGACA-(TAMRA)-3′	1/2	AJ009800
	F	5′-GCCCTCCTCCTGGTTTCA G-3′		
	R	5′-TGGCACCGCAGCTCATT-3′		
IL-15	Probe	5′-(FAM)-AAGTTGCAAATCTTGCATTTCCATTTTTCCA-(TAMRA)-3′	4/5	AJ416937
	F	5′-TAGGAAGCATGATGTACGGAACAT-3′		
	R	5′-TTTTTGCTGTTGTGGAATTCAACT-3′		
IL-18	Probe	5′-(FAM)-CCGCGCCTTCAGCAGGGATG-(TAMRA)-3′	4/5	AJ276026
	F	5′-AGGTGAAATCTGGCAGTGGAAT-3′		
	R	5′-ACCTGGACGCTGAATGCAA-3′		

[1]Acc. No. refers to the genomic DNA sequence.

System (PE Applied Biosystems) with the following cycle profile: 1 cycle of 50 °C for 2 min, 96 °C for 5 min, 60 °C for 30 min and 95 °C for 5 min, and 40 cycles of 94 °C for 20 s, 59 °C for 1 min.

Quantification was based on the increased fluorescence detected by the ABI PRISM 7700 Sequence Detection System due to hydrolysis of the target-specific probes by the 5' nuclease activity of the *rTth* DNA polymerase during PCR amplification. The passive reference dye 6-carboxy-x-rhodamine, which was not involved in amplification, was used to correct for fluorescent fluctuations resulting from changes in the reaction conditions, for normalisation of the reporter signal. Results are reported in terms of the threshold cycle value (C_t), the cycle at which the change in the reporter dye (ΔR_n) passes the significance threshold. In this work, the threshold values of ΔR_n are shown in Table 2 for all reactions described.

To generate standard curves for the cytokine and 28S rRNA specific reactions, total RNA extracted from stimulated splenocytes was serially diluted from 10^{-1} to 10^{-5}. Each RT-PCR experiment contained three no-template-controls, triplicates of 24 test samples and a $\log_{10}$ dilution series. Regression analysis of the mean values of two replicate RT-PCRs was used to generate standard curves and to calculate the slopes of the 28S rRNA and cytokine mRNA $\log_{10}$ dilution series. To correct for differences between RNA levels between samples within the experiment, first the Difference Factor for each sample was calculated by dividing the mean C_t value for 28S rRNA-specific product of a sample by the mean C_t value for 28S rRNA-specific product from all samples. Second, the corrected cytokine mRNA per sample was calculated using the following formula:

[(40 – mean cytokine C_t sample) × cytokine slope] / [Difference Factor sample × 28S slope]

To calculate the fold changes per sample the following formula was used: fold change sample = [(corrected cytokine mRNA sample – basal mRNA level cytokine) × 10] / cytokine slope.

Basal levels were defined as the average corrected cytokine mRNA of all saline injected control chickens apart from two samples that were considered as being outliers (see results section). These two outliers were not excluded from the statistical analysis.

Table 2. Standard curve data from real-time quantitative RT-PCRs on total RNA extracted from stimulated splenocytes

Target	ΔR_n* significance threshold	Log dilutions	C_t values†	R^2‡	Slope
28S	0.03	10^{-1}-10^{-5}	7-19	0.9382	2.976
IFN-γ	0.02	10^{-1}-10^{-5}	20-34	0.9787	3.315
IL-1β	0.02	10^{-1}-10^{-5}	15-29	0.9755	3.520
IL-2	0.02	10^{-1}-10^{-5}	24-36	0.9005	2.873
IL-6	0.02	10^{-1}-10^{-5}	19-34	0.9967	3.763
IL-8	0.03	10^{-1}-10^{-5}	13-24	0.9636	2.675
IL-15	0.02	10^{-1}-10^{-5}	27-39	0.9963	3.135
IL-18	0.02	10^{-1}-10^{-5}	17-30	0.9854	3.195

* ΔR_n = change in the reporter dye.
† C_t = threshold cycle value, the cycle at which the change in reporter dye levels detected passed the ΔR_n.
‡ R^2 = coefficient of regression.

Statistical analysis

In order to test for the presence of an LPS effect and interaction between LPS challenge and dietary oil source, corrected cytokine mRNA values were analyzed by two-way ANOVA. To test for the presence of diet effects within the LPS-challenged treatments and the saline controls, corrected cytokine mRNA levels were analyzed by one-way ANOVA within control and LPS treatments. The variance in the saline injected treatments was different from the variance in the LPS-immunized treatments, and therefore the cytokine expression data were considered as two different normal distributions. All analyses were performed using the GLM procedure of SAS (1990). The probability level (P) of < 0.05 was considered significant.

Results

Figure 1 shows the standardised data for cytokine levels expressed as fold changes in mRNA levels compared to basal mRNA levels. mRNA levels of IL-1β, IL-2, IL-6, IL-8, IL-18 and IFN-γ increased by 9.9, 15.0, 29.2, 10.4, 12.2 and 18.7 fold compared with basal levels respectively (all $P < 0.0001$) 2 h following LPS injection. Interleukin-15 mRNA decreased by 5.1 fold compared with basal levels at 2 h following LPS injection. In saline-injected control animals, the dietary oil source did not significantly affect the splenic mRNA level of any cytokine. However, Figures 1 indicates that mRNA levels in all cytokines except IL-15, are strikingly higher in the corn oil and linseed oil treatments compared with the fish oil and beef tallow treatments. Also the SE values are much larger in these treatments. Further data analysis indicated that this was largely attributable to two outliers in

these treatments. In LPS-challenged chickens, a diet effect was found for IFNγ mRNA ($P < 0.05$) (Figure 1A). The level of IFN-γ mRNA was highest in the chickens fed the fish-oil enriched diet, followed by the chickens fed the linseed-oil, corn-oil and beef tallow enriched diet respectively. IL-8 mRNA levels were also substantially (though not significantly – $P > 0.05$) increased in LPS-injected chicks fed the fish oil diet. In the LPS-injected birds, the higher IFN-γ ($P < 0.05$) and IL-8 ($P > 0.05$) mRNA levels in chicks fed the diet enriched with fish oil are 20.2 and 11.2 fold higher respectively compared to basal levels. These alterations correspond to average basal level increases of 1.5 and 0.8 times respectively for all LPS injected chicks.

Discussion

The cytokine responses to *S. typhimurium* in chickens are poorly described and their role in the pathogenesis in such infections have not yet been extensively studied. In this study, we investigated whether *in vivo* differences in cytokine mRNA were detectable, how they were affected by LPS challenge, and whether modulation of these levels by the dietary fat source could be detected by real-time quantitative RT-PCR. All mRNAs for cytokine genes were up-regulated at 2 h upon LPS stimulation by 10-30 fold, apart from IL-15 mRNA, that was down-regulated 5-fold as compared to basal levels. The IFNγ mRNA level in LPS injected chickens was increased by dietary fish oil.

Mammalian IL-15 is a monocyte deriving cytokine that shares many biological activities with IL-2, such as the induction of T cell and NK cell proliferation and the co-stimulation of B cells for growth and immunoglobulin synthesis, as a consequence of their co-utilisation of the β and γ chains of the IL-2R. Differences in IL-15 and IL-2 activities might be associated with their receptor-specific α chains. The major function of IL-15 production by macrophages is support of innate immunity, e.g. selective activation of natural killer (NK) cells (review by Ma, 2000). Interleukin-15 may promote NK cell activity during innate immune responses before the activation of T lymphocytes and subsequent production of IL-2. IL-15 substitutes for IL-2 through its use of the IL-2R β and γ chains in promoting NK cell proliferation and as a co-stimulus for IL-12-dependent secretion of IFNγ by NK cells (Carson et al., 1994). As IL-15 is produced by macrophages following stimulation with bacterial components in vitro, it has been proposed to have a role in the early innate pro-inflammatory response to infection (Doherty et al., 1996). Several studies indicate that IL-15 mRNA expression in human monocytes (Carson-William et al., 1995; Qian et al., 1997) is elevated

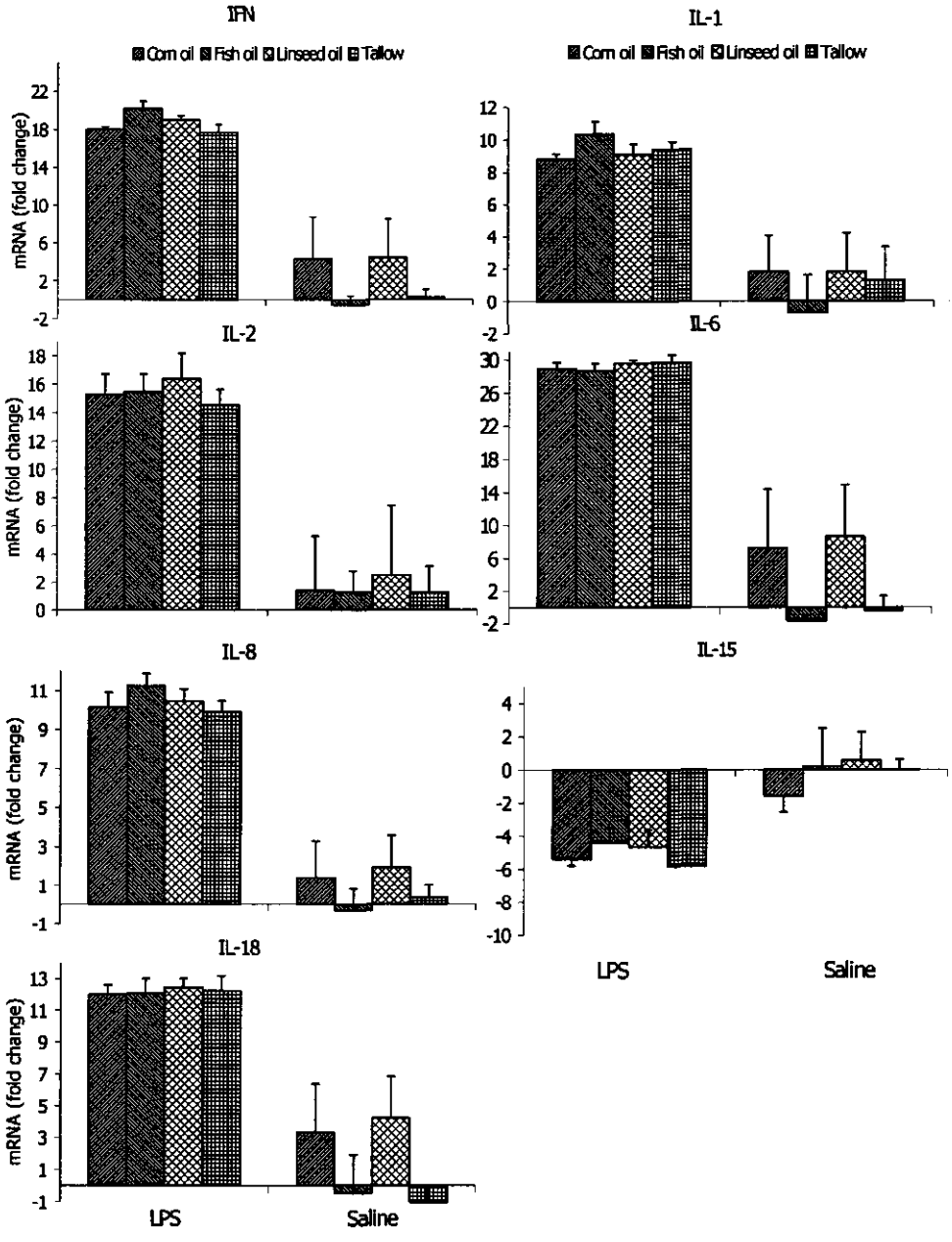

Figure 1. Quantification of cytokine mRNA in RNA extracted from LPS and saline injected chickens at 2 h after injection and fed with 95% basal diet enriched with 5% of either Corn oil, Linseed oil, Fish oil or Beef tallow. The bars represent standardized values for cytokine mRNA corrected for variation in input RNA measured by 28S rRNA levels, expressed as fold change in cytokine mRNA levels, when compared to those of the average level of saline injected chickens, apart from 2 outliers. Error bars show SE for the values within one treatment group.

within 5 and 12 h after stimulation with LPS. According to Kirman and Nielsen (1996), LPS induces a non-specific production of this cytokine by human PBMC. In addition to several mammalian IL-15 genes, the chicken homologue of IL-15 has recently been cloned and characterised (Kaiser et al., unpublished data). The present observation that chicken IL-15 mRNA was 5 fold lower at 2 h after LPS stimulation seems in contrast with the important role of IL 15 in the innate immunity because in at the same time the IFNγ mRNA levels was 18 fold higher. Several causes may underlie this seeming discrepancy. IL-15 mRNA expression might have been elevated in other tissue such as in endothelian cells and non-lymphoid stromal cells. In mice IL-15 has an important role in the early activity of γδ T cells and their IFNγ production in response to infection with *Salmonella* (Nishimura et al., 1996). In addition, it has been suggested that physiological delivery of IL-15 to memory T lymphocytes predominantly occurs within on lymphoid stroma (review Waldmann et al., 1999). The decrease of splenic IL-15 mRNA after LPS injection might be the result of selective LPS induced apoptosis of splenic IL-15 expressing macrophages. In the present study the increased IFNγ expression at 2 h after LPS challenge is most probably caused by innate immune cells such as NK cells or CD8+ T cells because a T cells produced IL-2 stimulus would likely require more time. The possibility that the gene identified as chicken IL-15 may be functionally different from mammalian IL-15 with regard to its role in the innate immunity implies that other co-stimulatory factors induced the increased IFNγ mRNA levels. In mice, IL-18 has been found to be a powerful co-stimulatory factor of the synthesis of IL-12 driven IFNγ gene transcription in NK cells (Walker et al., 1999). Also in the present study the 12 fold higher IL-18 expression might be associated with increased IFNγ gene transcription.

Previously we reported elevated IL-1β, IL-2, MGF and IFNγ expression after LPS injection, but no increased IFNγ mRNA levels following LPS challenge and consumption of the fish oil diet in the same samples as described in the present study (Sijben et al., 2001a). These data were obtained by end-point-analysis of semi-quantitative RT-PCR and cytokine mRNA level expressed relative to β-actin mRNA levels. We assume that the present log-phase-analysis by quantitative real-time RT-PCR with cytokine mRNA levels expressed relative to 28S rRNA is more accurate, and therefore, that the diet effect on IFNγ is realistically estimated.

Studies with mammals suggest that n-3 PUFA consumption reduces the production of phagocyte-derived pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and T-cell-derived IL-2, IL-12 and IFN-γ (Blok et al., 1996; Fritsche et al., 1999). The current observation of increased IFN-γ mRNA levels in chickens fed fish oil diet

seems inconsistent with this. In mice, increases in IFNγ protein production following dietary fish oil enrichment was reported (Fritsche et al., 1997). We did not observe any other statistically significant alteration in cytokine mRNA as a result of diets. Also no difference in IL-18 mRNA, a potent stimulator of IFNγ production (Okamura et al., 1995), was observed. Possibly most of the increased IFNγ transcription was induced by IL-18, and the extra IFNγ mRNA in the chickens fed fish oil diet was related to differences in the levels of eicosanoids, e.g. prostaglandin E_2, in the presence of stimulated IFNγ mRNA producing NK cells. In chickens, dietary PUFA can alter the release of PGE in stimulated splenocytes (Fritsche and Cassity, 1992). Prostaglandin E_2 inhibited iNOS activity in mouse macrophages (Marotta et al., 1992). IFN-γ may supports protective innate immunity against salmonellosis in chickens (Farnell et al., 2001). Maximal avian macrophage iNOS activity is induced by a combination of IFNγ and LPS (Kaspers et al., 1995: Suresh et al., 1995). Because NO acts as an innate immune effector molecule, dietary fish oil might enhance chicken innate immunity. Previously we found that n-3 as compared with n-6 PUFA elevated Ab responses against *Mycobacterium butyricum* protein (Sijben et al., 2000) and that n-6 and n-3 effects are interactive, with minimal amounts of n-3 being essential for optimal Ab responses (Sijben et al., 2001b). Together, these data indicate that n-3 PUFA in chickens enhance both innate and adaptive immunity, depending on the phase of the immune response and the presence of other immunomodulating nutrients, e.g. n-6 PUFA.

In summary, chickens may react differently compared to mammals two ways. First, LPS stimulation inhibits the expression of chicken IL-15 mRNA, whereas in mammals increased IL-15 mRNA levels are found. This implies that the gene characterized as avian IL-15 has, at least partially, a different function compared to its mammalian counterpart. Second, the n-3 PUFA increased mRNA expression of IFNγ in LPS-stimulated chickens indicates that the innate response can be modulated albeit differently compared to mammals.

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Chapter 8

General Discussion

Introduction

The dramatically increased interest of the animal feed industries and the scientific world in specific functions of nutrients with regard to health in the past decade is an attempt to anticipate on altered legislation regarding the use of antibiotics in livestock farming and consumer's demands concerning safe nutrition. The present thesis is an attempt to evaluate the effects of dietary PUFA on the immune response of growing layer chickens. A better understanding of how dietary PUFA affect the immune system facilitates the formulation of a more balanced diet in terms of fatty acid requirements for immune cell function. Consequently, such diet might improve the resistance of a chick at pathogen exposure, and thereby decrease the need for the use of anti-biotics. In the following general discussion the effects of dietary PUFA on immune responses described are evaluated.

First, it is discussed if the effects of n-3 and n-6 PUFA on indices of specific immunity correspond with the hypothesized mechanism in *Chapter 1*. Second, the possible role of PGE_2 in the modulation of the chicken immune cell function is discussed. Third, the current immune parameters are discussed in order to elucidate the practical significance of the scientific data on PUFA and immune responses. Fourth, the effects of dietary PUFA in the present thesis and in avian literature are compared and evaluated in order to identify and discuss the analogs and differences. Fifth, general conclusions are drawn.

Effects of dietary PUFA on the responses to TH-1 and TH-2 like antigens

The studies described in *Chapter 2 and 3* indicate that dietary PUFA have the potential to modulate chicken immune cell function. In these chapters, the extent to which indices of specific immunity, such as antibody (Ab) levels, in vitro lymphocyte proliferation capacity, and cutaneous hypersensitivity are affected by modulation of the dietary PUFA levels is described in detail. In *Chapter 1* it was hypothesized that these responses might be affected in an antigen dependent fashion by PUFA via eicosanoid mediation, with PGE_2 in a key role. The following discussion evaluates whether the current data correspond with this hypothesis or not.

In *Chapter 2,* Ab levels were affected divergently by dietary PUFA: the Ab level directed to KLH was increased and the Ab level directed to *M. butyricum* was decreased by increasing the linoleic acid (LA) level from 3 to 6.2%. In the same study the Ab levels directed to the same antigens following α-linolenic acid (LNA) enrichment from 0.6 to 3.9% were not affected significantly, but tended to react opposite compared to the chickens fed the LA enriched diet. In mice these

antigens are known to induce TH-2 and TH-1 responses respectively. Presuming that these antigens have similar characteristics in chickens, this observation supports the concept that LA supports a TH-2 like response and inhibits a TH-1 like response in chickens. In addition, it is temping to speculate that the numerical differences following LNA enrichment also support this concept. Further evidence to support or reject this from data on cutaneous hypersensitivity is lacking in *Chapter 2* because no effects on cuteneous hypersensitivity were found. Lymphocyte proliferation capacity in KLH and *M. butyricum* immunized birds was highest in the birds fed the LNA enriched diet, and in the PBS injected birds the LNA enriched diet fed birds had the lowest response to concanavalin A (ConA). So these in vitro data do not support divergence between antigens but they indicate divergence between immunized and unimmunized control birds. Thus, the data from *Chapter 2* are not unambiguously supporting the preset hypothesis. Further evidence should come from the more extensive study described in *Chapter 3.*

If the preset hypothesis were correct, typical n-3 effects, i.e. high response to *M. butyricum*, were expected to be most profound at the highest n-3 and lowest n-6 level. If the n-6 level increased, the n-3 effect would be expected to decrease in a rate that depends on the relative affinities for n-3 and n-6 fatty acids of the enzymes in the metabolic pathways of fatty LA and LNA to long-chain PUFA (*Chapter 1*). For typical n-6 effects, i.e. high response to KLH, reverse effects would be expected. The Ab responses in *Chapter 3* were too whimsical to easily identify such patterns, but they did clearly indicate that LA and LNA interaction is important. The interactions were particularly found in the period from 10 to 14 days after immunization (*Chapter 3*, Figure 1 and 2). These observations reject that high levels of anti-KLH Ab are a typical n-6 phenomenon, but they suggest that for optimal Ab response to KLH, LA and LNA can replace each other. However, high levels of dietary LA depressed Ab levels directed to *M. butyricum* in the first 14 days after challenge, particularly at low n-3 levels. The highest Ab levels were found in the birds fed the highest levels of LNA. Thus, high anti-*M butyricum* Ab levels are a typical n-3 phenomenon. In addition, lymphocyte proliferation after stimulation with *M. butyricum* was lower in birds fed a diet high in LA, and *Mycobacterium butyricum* induced wing-web swellings, in birds previously injected with this antigen, were higher at 2.1% compared to 0.1% dietary LNA. These data indicate that cellular responses to the T helper-1 antigen might be enhanced by n-3 and suppressed by n-6 PUFA. In vivo cellular responses directed to the T helper-2 were suppressed at the highest (i.e. 6.2%) LA and lowest (i.e. 0.1%) LNA level, but were restored by increased LNA level up to 2.1%. The ConA induced

lymphocyte proliferation and the ConA induced wing-web swelling were also increased by high levels of dietary LNA. Thus, these data indicate that for cellular responsiveness the type of PUFA is more determinative for the effect than the (nature of the) antigen the response is directed to.

In conclusion, the data in *Chapter 2 and 3* indicate that dietary PUFA have the potential to modulate immune cell function. Evidence is provided that these modulations are partially antigen-dependent. PUFA effects on antibody responsiveness are particularly antigen dependent, whereas cellular responses are less antigen-dependent. Cellular responses are supported by higher levels of n-3 PUFA or not affected by modulations of the dietary PUFA levels. The preset hypothesis, that effects of PUFA on adaptive responses would be mediated by PGE_2 suggests that the effect of PUFA would be largely determined by the antigen the response were directed to, via domination of either T helper-1 or T helper-2 regulated response. Therefore, this hypothesis is not supported by the present (indirect) experimental evidence. First, the observation that, in some cases, dietary LA and LNA can replace each other to maintain the Ab response is in contrast with this hypothesis. Second, the observation that cellular responses directed to KLH, *M. butyricum* and ConA are supported by n-3 PUFA rejects this hypothesis. However, it should be noted that an important assumption, that *M. butyricum* and KLH are TH-1 and TH-2 like antigens in chickens too, is unverified. The present observations that chickens react different to these antigens provide only indirect evidence that these antigens are of different nature to the chicken immune system. Cytokine profiles following immunization should be identified to make these necessary characterizations. The experiment to yield these data has been performed but the conclusive (mRNA) cytokine profiles remain to be analyzed. Furthermore, in these experiments data on the isotypes in the Ab responses are lacking. Though features of isotypes can not be extrapolated across species, different "behavior" of IgM versus IgG responses might have given additional information on the TH1/TH-2 nature of the responses to these antigens.

The role of PGE_2 in the modulation of the chicken immune cell function

The previous paragraph indicates that the type and levels of dietary PUFA affect a range of immunnological parameters in chickens. In *Chapter 1* the possible mechanisms by which such affects might be induced are outlined. Most of the possible mechanisms have not been studied in the present dissertation, but in *Chapter 2 and 3* one of these mechanism is studied indirectly. In order to further support or reject the hypothesis that PGE_2 plays a key role, in *Chapter* 5 this

hypothesis was tested more directly. Briefly, chickens were challenged with KLH or *M. butyricum* and simultaneously PGE_2 or indomethacin were administered. Indomethacin is a powerful inhibitor of cyclooxygenase pathway, inhibiting the synthesis of PGE_2, as well as other 1, 2 and 3 series prostaglandins and thromboxanes (*Chapter 1*, Figure 1).

The hypothesis on the possible role of PGE_2 in T helper cell mediated Ab response is only to some extent supported by the data of *Chapter 5*, i.e. lower primary anti-*M. butyricum* Ab response and higher secondary anti-KLH Ab response following PGE_2 administration. The lack of PGE_2 effects on primary anti-KLH Ab titers and the unexpected indomethacin effects make the test of the hypothesis on the role of PGE_2 inconclusive. The observation that PGE_2 administration at primary challenge with *M. butyricum* protein results in decreased primary Ab response and increased secondary Ab response suggests that at primary challenge, the presence of PGE_2 shifts the differentiation of naive B cells to memory B cells at the expense of plasma cells. However, evidence from anti-KLH Ab kinetics to support this hypothesis is lacking. The similar effects of PGE_2 and indomethacin administration on Ab responses indicate that inhibition of PGE_2 synthesis is not the major perceptible effect of indomethacin. This observation suggests that indomethacin either inhibits (a) factor(s) with effects opposite to PGE_2 or has autonomous "PGE_2 like" effects in poultry. In addition to the indomethacin effect, direct evidence that the effect of PGE_2 on immune cell function differs in poultry compared to mammals is found. In vitro lymphocyte proliferation after PHA stimulation increased at increasing levels of PGE_2 added to the culture, but in mammals PGE_2 suppresses lymphocyte proliferation (Calder et al., 1992).

In conclusion, *Chapter 5* indicates there might be important differences between the production and effector function of avian and mammalian PGE_2. Evidence that eicosanoids are produced in chickens, and also that their production is affected by the dietary PUFA composition is abundant (Craig-Schmidt, 1987; Watkins and Kratzer, 1987; Olomu and Baracos, 1991; Vericel et al, 1991; Watkins et al., 1997). However, differences in macrophage eicosanoid metabolism in avian species compared to mammals have been identified before (review Dietert and Golemboski, 1998). For example, chicken macrophages did not produce detectable amounts of PGE_2, PGI or LT under the same conditions that mouse macrophages produce substantial amounts of these eicosanoids. Similarly, chicken TXA_2 was only produced early in an inflammatory challenge, whereas murine TXA_2 was produced

at constant levels throughout the response. Thus, chicken macrophages require different conditions than murine macrophages to produce eicosanoids.

In vivo evidence that chicken eicosanoids are involved in immune cell function is scarce and indirect. In one study, the release of eicosanoids by stimulated and unstimulated splenocytes and peripheral blood leukocytes, Ab response and Ab-dependent T cell cytotoxicity in chickens fed diets different in dietary PUFA was reported (Fritsche and Cassity, 1992). Ab responses were unaffected by the diet, but Ab-dependent cellular cytotoxicity of isolated splenocytes was lower in chickens fed n-3 rich diet, and also the release of LTB, PGE and TXB (probably mainly LTB_4, PGE_2 and TXB_2) was numerical or significantly lower in n-3 fed chickens. These data suggest that comprised cellular cytotoxicity might be the result of impaired eicosanoid production, but there is no evidence for a causal correlation of these parameters. However, if the decreased PGE_2 production and impaired cytotoxicity were associated with each other, this would be in contrast with what is usually found in mammalian studies, where PGE series have been shown to suppress cytotoxicity (references in Hwang, 2000).

In summary, evidence for the hypothesis that PGE_2 increases the response to a TH-2 type antigen and decreases the response to a TH-1 type antigen in chickens is inconclusive. Differences in PGE_2 metabolism and function in chickens and mammals might be associated with the observations in the previous paragraph that reject the initial hypothesis that effects of PUFA on adaptive responses would be divergent via selective support of TH-1 or TH-2 antigens by PGE_2 mediation. The data of the present thesis rather indicate that both linoleic acid and PGE_2 inhibited TH-1 mediated primary responses but do not affect TH-2 mediated responses to the same extent.

Significance of the current parameters

Antibody response

B-lymphocytes are continually released from the primary lymphoid organs into the periphery, equipped with surface immunoglobulins that enables them to bind antigens. These B cells can differentiate into either memory B cells or antibody-secreting plasma cells. Primary B cell activation by antigen-binding, typically results in a transient wave of proliferation, followed by a burst of antibody secretion by plasma cells. Secondary challenge with the same antigen results in a more rapid and expanded antibody production due to activation of memory B cells carrying this specific antibody as surface receptor. Antibodies block the antigenic sites of an antigen and mucosal adhesive molecules to protect the host from various

infections and to facilitate the clearance of blood-borne antigens. Antibodies also induce hypersensitivity, activation of the complement system, and antibody dependent-cell cytotoxity to facilitate clearance of pathogens from infectious sites.

Evidence that higher Ab levels are associated with higher resistance and survival after challenge with several pathogens is found. Survival percentage after challenge with *Escherichia coli* was higher in chickens with higher Ab titers directed to *E. coli* (Leitner et al., 1990). In addition, selection for the ability to produce high Ab response to *E. coli* (Leitner et al., 1992), or nonpathogenic, multideterminant antigens (Gross et al.,1980; Yamamoto et al., 1991; Pinard et al., 1993) showed that the ability to produce high Ab levels may efficiently improve disease resistance to a wide range of pathogens in commercial flocks. Therefore it is assumed that antibody production is an index for the potential humoral immune response and the part of disease resistance that is associated with the humoral responsiveness.

In vitro lymphocyte proliferation

Lymphocytes can be classified as either T or B-lymphocytes by the presence of CD3 or CD19, respectively, on their surface. When T lymphocytes are presented with antigen, they become activated, secrete cytokines and ultimately enter the cell cycle and divide. This proliferation of lymphocytes leads to an increase in the number of antigen-specific lymphocytes and, as such, is a key-component of the regulation, amplification and memory of the cell mediated immune response (after Thies et al., 2001). In cell culture, the stimulation and subsequent proliferation of T lymphocytes can be achieved by mitogens, such as concanavalin A (ConA), which bind to the T cell receptor CD3 complex (Licastro et al., 1993).

Because mitogen-stimulated lymphocyte proliferation is relatively simple to measure in vitro and because it is believed to be a reliable measure of lymphocyte function in vivo, this test is used in many studies to assess the effects of dietary lipids upon lymphocytes. However, it is now apparent that the results of lymphocyte proliferation measurements ex vivo is strongly influenced by the anatomical site of origin of the lymphocytes and the cell culture conditions, particularly the nature of the serum used (reviewed by Calder, 1995). The use of a whole-blood culture system (which is not used in the studies in the current thesis) would probably better reflect in vivo conditions, and is therefore desirable. Such system avoids the removal of erythrocytes, the loss of non-cellular physiological components normally in contact with the cells in the circulation and the need for the addition of serum. In addition, evidence that in vitro lymphocyte proliferation capacity is an index for resistance to pathogens, bacterial clearance, or host

survival is not available. It has been suggested that the capacity to produce high levels of antibodies is associated with impaired in vitro lymphocyte proliferation capacity (Parmentier et al., 1994). Thus, conclusions from modulations in lymphocyte proliferation capacity due to dietary changes on the consequences for disease resistance should be drawn with caution.

Cutaneous hypersensitivity

Cutaneous delayed-type hypersensitivity using T-cell dependent mitogens or antigens is often used as a model for in vivo T-cell reactivity. In fact, it is the only parameter for in vivo cell-mediated immunity in chickens. Local increase in thickness of a challenged wing-web or wattle is due to local influx of T cells that recruit inflammatory cells to the site of challenge. In the Chapter 2 and 3 cuteneous hypersensitivity was established at 4 and 24 h after a wing-web challenge. The 4 h response represents an intermediate response, preceded by an acute response at 1-2 h (Parmentier et al., 1993). This intermediate 4 h response might be based on precipitating antibodies leading to granulocyte recruitment by complement activation i.e. Type-III hypersensitivity. The 24 h response represents type IV delayed type hypersensitivity based on the activation of memory T cells.

In several studies it has been suggested that the extent of cutaneous hypersensitivity might be a relevant index for cellular immunity and health status in general, but the evidence to support this is scarce and inconsistent. In chickens Afraz et al. (1994) reported increased incidence and mortality following challenge with Marek's disease virus in White Leghorn chickens selected for high delayed-type hypersensitivity wattle reaction to BCG antigen, compared to the low line of the same selection. At the same time the birds of the low line scored higher on other indices of cellular immunity, i.e. competence of splenomegaly in graft-versus-host reaction, wattle swelling following PHA challenge, and phagocitic activity. Also in cattle (Burton et al., 1989), feline (Weiss and Cox, 1988) and pig (Wilkie and Mallard, 1999) research attempts have been made to associate indices of specific cutaneous hypersensitivity with disease resistance in general, but these attempts remain inconclusive. The study by Afraz illustrates that high delayed-type hypersensitivity following challenge with a pathogen may very well increase cellular immunity directed to that pathogen, but at the same time decrease reactivity or resistance to other pathogens. Due to the high specificity of such tests, they can only be used as an index for whether or not in vivo T cell reactivity can possibly be modulated and cannot serve as an index for disease resistance.

Cytokine mRNA

In the cascade from dietary PUFA to differences in read-out parameters of the immune system such as Ab responses, lymphocyte proliferation and delayed-type-hypersensitivity, cytokine regulation plays a pivotal role in the orchestration of these responses. Cytokines are produced upon recognition of an antigen by antigen-presenting cells and exert their actions in a paracrine or autocrine fashion. Their effects on various cells of the immune system are highly pleiotropic and depending on the type of target cell or cellular process involved, the actions of cytokines may be either stimulatory or inhibitory, and different cytokines may act either synergistically or antagonistically. The complex network of interactions between soluble cytokines and specific receptors on select sets of immune cells determines the course of an antigen specific immune response. Therefore cytokines provide a tool by which the regulation of the effects of dietary PUFA on immune responses can be studied. However, the detection of avian cytokines at the protein level is still problematic owing to the absence of specific antibodies. Only very recently the first monoclonal Ab to detect a chicken cytokine has been produced and characterized (Miyamoto et al., 2001). Due to this complicating factor, in Chapter 6 and 7 of the current thesis effects of dietary PUFA on mRNA levels of some cytokines were studied by using reverse transcription-polymerase chain reaction (RT-PCR) assays to obtain cytokine profiles. Because we were lacking the experience of in vivo cytokine mRNA detection in response to the model protein antigens used in the first chapters, we used an inflammatory challenge that was known to increase mRNA levels to detectable levels.

An inflammatory response is the first line of defense against novel pathogens, so increased levels of inflammatory cytokines may suggest increased defense. However, cells and mediators of the inflammatory responses have been implicated in the pathology of many poultry diseases, e.g. coccidiosis (Trout and Lillehoj, 1993) and *S. enteritidis* (Tellez et al., 1994; Kogut et al., 1995). An optimal inflammatory response may be a response resulting in the first place in an effective attack of the invading pathogen, and in the second place minimal tissue damage for the host. The levels of cytokines (mRNA) that realize such response are not known, and probably dependent on the degree of infection, the nature of the parasite and the site of invasion. Therefore experimental evidence that mRNA of inflammatory cytokines is modulated by PUFA cannot be interpreted as ameliorated or deteriorated physiological status without all such information about the cytokine levels under the experimental conditions. The only information that such modulated cytokine levels alone can provide is on the direction into which the

immune response is modulated, e.g. more or less inflammatory, T helper-1, or T helper-2 type response, at a given moment after challenge.

Review of PUFA effects on immune cell functions

The results of the present thesis have partially been discussed above. In the following paragraph the data of these and other results of the current thesis and the available literature based on avian studies are compared in an attempt to identify a "bigger picture". Such overview of the most important effects of dietary PUFA on immune parameters might provide indications on what is most likely a good feed regimen for immune cell function of chickens. The effects of dietary PUFA on immune indices reported in the chapters of the present thesis and found in literature are summarized in Table 1. These studies are difficult to compare due to different PUFA levels and different antigens the responses were directed to. In addition, *Chapter 3* illustrates the importance of interaction of n-3 and n-6 PUFA on Ab responses, but in avian literature other studies on these interactions are lacking.

The overview in Table 1 indicates that in most studies in which effects of fish oil on Ab responses were studied, fish oil increased Ab levels. In most studies without the inclusion of fish oil but with linseed oil, LNA increased Ab levels compared to LA rich fat sources. In some studies increased dietary LA levels increased Ab levels. In most cases the n-3 levels of these diets were very low (at the most 0.5% but mostly around 0.1 to 0.2%). So it is concluded that in general dietary n-3 increased Ab responses, with long chain n-3 being more potent compared to short-chain n-3. N-6 can also increase Ab responses, particularly in the absence of n-3 rich dietary sources, but can also depress Ab responsiveness, even down to levels lower than in unimmunized controls (*Chapter 2*). For maximal Ab response there might be a dietary α-linolenic acid requirement of approximately 2% or a long-chain n-3 PUFA requirement of 1-2%. In case of a dietary LNA content of less than 0.4%, a linoleic acid content of at least 2-3% might partially substitute for the n-3 requirement to prevent depressed Ab responses. Dietary LA levels of more than 3-4% might suppress these responses. These percentages are general indications, they are likely to depend on the type of challenge a chicken is exposed to.

In *Chapter 4* it has been proposed that at the high dietary PUFA levels, which are used to realize the immunomodulation, additional vitamin E might be required to maintain PUFA stability and its biological properties. However in the current thesis no evidence was found that at PUFA levels as high as 6-10% vitamin E

levels higher than NRC requirement of 5 mg/kg influences the effects of PUFA on Ab responses.

Dose-response type of studies are not performed in studies on indices of cellular immune response, such as in vitro lymphocyte proliferation and cutaneous hypersensitivity. Therefore the only indication an overview of literature might give is whether enrichment with particular oil sources enhances these parameters or not. The data in Table 1 indicate that in all studies performed by others, lymphocyte proliferation induced by ConA or pokeweed mitogen was impaired in chickens fed n-3 rich diets. In contrast, in the studies of the present thesis, lymphocyte proliferation following ConA stimulation was higher in birds fed LNA rich diets. In addition, in the presence of non-autologous serum of birds fed LNA enriched diet, the lymphocyte proliferation capacity of birds fed a standard commercial diet also increased (unpublished data). These discrepant results might be associated with the presence of autologous serum in the cell culture or not. In rats, suppressive effects of dietary n-3 PUFAs were demonstrated when the cells were cultured in autologous serum, but were lost if the cells were cultured in fetal calf serum (Yaqoob et al., 1994). It has been shown that the changes in lymphocyte fatty acid composition induced by dietary manipulations are better maintained if the cells are cultured in autologous rather than in fetal calf serum (Yaqoob et al., 1995). These observations illustrate the importance of the use of conditions as similar as possible to the in vivo physiological conditions, as indicated in the previous paragraph. The results of the studies with autologous serum rather than non-autologous or fetal calf serum are consistent with what is generally found in mammals. Mammalian studies indicate that high-fat diets in general decrease T-lymphocyte proliferation compared with low-fat diets. Among high fat diets the order of potency to reduce proliferation is: saturated fat < n-6 PUFA rich-oils < olive oil < linseed oil < fish oil (Calder, 1998). The data in Table 1 suggest that, with the use of autologous serum, in chickens this order might be: n-6 PUFA rich-oils < saturated fatty acids rich oils < fish oil < linseed oil.

In contrast to in vitro lymphocyte reactivity, in vivo T cell reactivity, measured as cutaneous hypersensitivity, was more consistently affected by PUFA in the few published studies. These studies indicate that short-chain as well as long-chain n-3 PUFA might support in vivo T cell reactivity in layer and broiler chicks. These observations are in contrast with what is generally found in mammalian studies. In mammals high-fat diets reduce the delayed-type hypersensitivity response with the order of potency: saturated fat < n-6-rich oils < fish oil (reviewed by Calder, 1998).

Table 1. Effects of dietary polyunsaturated fatty acids on immune parameters in studies with poultry.

Reference	type birds	dietary contrast	challenge	parameter	effect
Craig-Schmidt et al., 1987	broilers	10% soybean-, 5% soybean- + 5% linseed-, and 10% linseed oil		PGE, $PGF_{1+2\alpha}$ and TXB_2	↓ by LO
Fritsche et al., 1991	layer pullets	7% lard, fish-, canola- corn oil	SRBC i.v.	Ab at 7 d	↑ by FO
		with autologous serum in cell culture	ConA, PWM in vitro	splenocyte proliferation	↓ by FO and LO
		without autologous serum in cell culture			no effect
Fritsche and Cassity, 1992	broilers	7% lard, fish-, linseed-, corn oil	SRBC i.v. two times	Ab at 6 or 7 d	no effect
			SRBC in vitro	Ab dependent cellular cytotoxicity	↓ FO and LO
			calcium ionophore in vitro	eicosanoids	↓ FO and LO
Friedman and Sklan, 1995	broilers	increase LA from 2.0 to 4.6, 5.7 and 7% at expense of oleic and palmitic acid	BSA ?	Ab at d 11	↓
Friedman and Sklan, 1997	turkeys	increase LA from 1.2, 2.2, 2.7, 3.3 and 4.0% at expense of α-linolenic (0.5-0.1%), palmitic and oleic acid	NCD oral, turkey pox, and - necrotic enteritis i.m. vaccines	Ab at 7 d	↑ until 3.3% LA, then ↓
Parmentier et al., 1997	3 lines of layer pullets	7% sunflower-,or linseed oil, or animal fat	two times SRBC i.m.	Ab kinetics	no effect
			two times BSA i.m.	Ab kinetics	↑ LA in high line
			two times BSA i.m.	Ab kinetics	↓ LNA low line
Korver et al., 1997	broilers	4% fish oil or corn oil	*Eimeria tenella* oral	hemopexin	↑ by FO
				TNF like activity	↓ by FO
				cecal inflammatory cells	no effect
Korver and Klasing, 1997	broilers	0.5, 1.0 or 2.0% fish oil vs. 0.5, 1.0 or 2.0% corn oil	PHA s.c.	wattle swelling	↑
			PHA in vitro	IL-1 activity	↓
			LPS i.p.	febrile, hemopexin, methallothionein	↓
			IBV vaccine i.m.	Ab at d 14	no effect

Wang et al., 2000	layer pullets	5% fish-, sunflower-, animal-, linseed oil	-	IgG	↑ by fish oil
			ConA in vitro	splenocyte	↓ by FO and LO
			PWM in vitro	proliferation	↑ by SO
			ConA in vitro	thymocyte proliferation	↑ by SO
				IgM^+ lymphocytes	↑ by FO and LO
				$CD8^+$ T cells	↑ by LO
Sijben et al., 2000, i.e. chapter 2	layer pullets	7% animal fat, sunflower-, or linseed oil	KLH s.c.	Ab kinetics	↑ by SO
			M. butyricum s.c.	Ab kinetics	↓ by SO
			KLH, *M. butyricum* s.c.	PBL proliferation	↑ by LO
			KLH, *M. butyricum* s.c.	wing-web swelling	no effect
Sijben et al., 2001a, i.e. chapter 3	layer pullets	all combinations of 4 levels safflower oil and 4 levels linseed oil with LA and LNA content from 2.6-6.2 and 0.1-2.4 respectively	KLH, *M. butyricum* s.c.	Ab kinetics	interactions
			M. butyricum s.c.	PBL proliferation	↓ SFO
			ConA s.c.	PBL proliferation	↑ LO
			KLH, *M. butyricum* s.c.	wing-web swelling	↓ by SFO ↑ by LO
Sijben et al., 2001b, i.e. chapter 6	layer pullets	5% tallow, fish-, linseed-, or corn oil	LPS i.v.	splenic IL-1β, 2, MGF, $TGF\beta_2$ and IFNγ mRNA	IL-2 ↑ by FO no other effect
Sijben et al., submitted, i.e. chapter 7	layer pullets	5% tallow, fish-, linseed-, or corn oil	LPS i.v.	splenic IL-1β,2,6,8,15, 18 and IFNγ	IFNγ ↑ by FO no other effect

Abbreviations used: Ab = antibody, BSA = bovine serum albumin, ConA = concanavalin A, FO = fish oil, IBV = infectious bronchitis virus, IL= interleukin, IFN = interferon, i.m. - intra muscular, i.p. = intra peritoneal, i.v. = intra venous, KLH = keyhole limpet hemocyanin, LA = linoleic acid, LNA = α-linolenic acid, LO = linseed oil, LPS = lipopolysaccharide, *M. butyricum* = *Mycobacterium butyricum*, MGF = myelomonocytic growth factor, NCD = Newcastle disease, PBL = peripheral blood leucocytes, PHA = phytohemagglutinin, PWM = pokeweed mitogen, s.c. = sub cutaneous, SFO = safflower oil, SO = sunflower oil, SRBC = sheep red blood cells, TGF = transforming growth factor

Dietary fish oil significantly increased the mRNA level of IL-2 (*Chapter 6*) and IFN-γ (*Chapter 7*), and non-significantly increased IL-1β and IL-8 (*Chapter 7*) at 2 h after LPS challenge. The differences in results between *Chapter 6 and 7* might be associated with the different methods, different primer sets, or degradation of some mRNA over storage. In theory the real-time RT-PCR of *Chapter 7* is more quantitative compared to the semi-quantitative method in *Chapter* 6, particularly because the real-time RT-PCR compares RNA quantities in the log-phase, whereas the semi-quantitative method is based on end-point analysis. Therefore it is assumed that the effects reported in *Chapter 7* are most reliable. The possible discrepancy between inhibition of IL-1 activity (Korver and Klasing, 1997 and in Table 1) and the slight increase of IL-1β mRNA by dietary fish oil might be the result of the differences in methodology (bio-active IL-1 vs. IL-1 mRNA) and time after challenge.

The response at 2 hours after LPS challenge first of all may represent innate immunity, which is not T cell dependent. It has been suggested in *Chapter 7* that fish oil may support innate mechanisms of protective immunity by IFN-γ and LPS dependent induction of maximum iNOS activity. iNOS is the LPS- and cytokine-inducible isoform of the enzyme NOS producing the highly reactive metabolite NO, known as an innate effector molecule (Dietert et al., 1993). In mammals, LPS stimulates NK cells to secrete IFN-γ, which in turn stimulates macrophages to produce TNF-α, IL-1 and IL-8 (Scott and Kaufmann, 1991). In *Chapter 7*, besides IFN-γ, also IL-1 and IL-8 were non-significantly higher in the fish oil treatment upon LPS stimulation. So these data suggest potential enhancing properties of dietary fish oil for the chicken innate immune cells function. Significant effects of linseed oil were not detected in these studies, but Figure 1 in *Chapter 7* shows that for many cytokines mRNA expression was numerical highest in the fish oil and linseed oil diet. In avian literature no evidence that supports or undermines the suggested effects of dietary PUFA on innate immunity is found. In mammals, no similar studies are performed with specific focus on effects of dietary PUFA in early cytokine mRNA responses. As indicated in the previous paragraph, much more information is required to assess whether raised pro-inflammatory cytokine levels due to dietary fish oil are beneficial or detrimental to the chickens.

In conclusion, the optimal dietary PUFA content for specific immunity cannot easily be defined owing to difficulties in comparison of different studies and translation of immune parameters to health status or disease resistance. Nevertheless some of the current general conclusions might be helpful to overview the effects of dietary PUFA on health parameters. As indicated before, the effect of

PUFA modulation on Ab levels is highly antigen dependent and n-3 and n-6 PUFA might be interreplaceable. In general, this overview indicates that n-6 enrichment higher than 3% entails the risk of impaired Ab responses and n-3 enrichment of n-3 up till 2% in most cases enhances Ab responses, without the risk of impaired responses. Supplementation of the diet with up to 5% of n-3 rich oils such as linseed-, or fish oil provides this level of n-3 PUFA. Such supplementation is also effective in up-regulating in vivo T cell reactivity, modulating in vitro lymphocyte proliferation capacity of chickens, and increasing cytokine mRNA expression. The supporting effect of n-3 PUFA on Ab responsiveness might be an index for increased health status, whereas the effects of dietary PUFA on the remaining parameters are mainly indices of immunomodulation which might work out both beneficial of detrimental, dependent on the circumstances. As indicated, most of the effects of n-3 PUFA in poultry are opposite to what is generally found in mammals. In such mammalian studies, diminished host defense following n-3 PUFA include impaired wound-healing (Albina et al., 1993), impaired resistance to *S. typhimurium* (Chang et al., 1992), and increased persistence of experimental tuberculosis (Mayatepek et al., 1994). It follows that possibly also such indices of resistance might be opposite in chickens, which implies that host defense would be strengthened by n-3 supplementation.

General Conclusions

The studies described in the present thesis provide ample evidence that the chicken immune cell function can be modulated by dietary polyunsaturated fatty acids. Generally, the effects of n-3 PUFA are immuno-enhancing compared to the effects of n-6 PUFA or saturated fatty acids. These results are the contrary of what is usually found in mammals. Some differences in the mechanisms underlying the immunomodulating properties in chickens and mammals that might be (partially) responsible for the differences are identified. First, the metabolism and effector functions of eicosanoids in general, and PGE_2 in particular, might be different. Second, the effects of dietary n-3 enrichment on cytokine production might be different compared to mammalian species. In addition to differences in the avian and mammalian immune system that were already known, another important difference identified in this thesis, might be the function of IL-15.

For poultry nutrition these observations imply that dietary PUFA are a useful tool with the potential to support immune cell function. The fatty acid requirement for all types of poultry is set on 1% of linoleic acid. Although α-linolenic acid is recognized as an essential fatty acid, minimal requirements are not defined. The

present studies suggest that 1-2% dietary n-3 might be beneficial for antibody responses, which potentially ameliorates disease resistance. The effects of PUFA on Ab responsiveness were antigen dependent, indicating that a universal optimal fatty acid intake for optimal resistance might be non-existent. N-6 PUFA might suppress Ab responsiveness or support Ab responsiveness at minimal n-3 levels. The consequences of the dietary modulation of Ab levels, and particularly T cell reactivity and cytokine production, at exposure to various pathogens remain to be established. Studies on PUFA supplementation at infection with intra- and extra-cellular parasites are recommended to further investigate the potential of dietary PUFA to contribute to improved chicken's disease resistance.

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Summary

Polyunsaturated fatty acids (PUFA) are well known immunomodulatory nutrients. Two families of PUFA are distinguished, the n-3 and n-6 families. The principal precursors of the n-3 and n-6 PUFA are α-linolenic and linoleic acid, respectively. PUFA are important components of cell membranes in which they are deposited in membrane phospholipids. The fatty acid composition of membrane phospholipids is largely determined by the dietary fatty acid intake. In animal tissues, the short-chain PUFA α-linolenic and linoleic acid can be converted into long-chain eicosapentaenoic and arachidonic acid, respectively. Upon antigen encounter, the latter can be released from the membrane phospholipids and serve as precursors of eicosanoids, a class of potent immune regulators. In these metabolic pathways, n-3 and n-6 PUFA compete for the binding sites of the same enzymes. Thus, the eicosanoid production following antigenic challenge or infection may depend on the availability of precursors, and thereby on the dietary PUFA composition. The eicosanoids deriving from n-3 and n-6 PUFA have different biological properties. In general, n-6 deriving eicosanoids, such as prostaglandin (PG) E_2 and leukotriene (LT) B_4 are considered pro-inflammatory, whereas n-3 deriving eicosanoids are expected to decrease inflammation compared to n-6 eicosanoids. In many mammalian studies dietary n-3 PUFA decreased the level of pro-inflammatory cytokines such as IL-1β, IL-2, IL-6 and TNF-α. Whether these effects are mediated by eicosanoid production, or other mechanisms, such as by affecting immune cell membrane fluidity, intracellular signal transduction, or gene expression, is still inconclusive. In addition, eicosanoid production, particularly PGE_2, may affect T cell-dependent immune responses by affecting the balance between T helper(TH)-1 and T helper 2 cells. In mammals, PGE_2 can affect B cell differentiation, resulting in enhanced interleukin(IL)-4 induced class switch from IgM to IgG1 and IgE. In addition, PGE_2 decreases the T helper-1 subset production of IL-2 and IFN-γ and is associated with up-regulating the production of TH-2 associated cytokines IL-4 and IL-5. These findings gave rise the concept that PGE_2 may tip the TH-1/TH-2 balance in favor of the TH-2 type cells. The latter implies that the presence of PGE_2 might selectively support immune cell function, depending on the nature of the antigen i.e. whether the response directed to the antigen is dominated by either TH-1 or TH-2 cells. This way the protective immunity against typical TH-1

pathogens, such as intracellular parasites, might be inhibited by high PGE_2 production, whereas the immunity against typical TH-2 pathogens, such as metazoan parasites, might be supported by PGE_2. In chickens a similar definition of subsets of helper T lymphocytes based on their cytokine secretion patterns has not been made yet. Nevertheless, also in avian species different types of antigens might result in a selective boost of cytokines followed by different sorts of immune responses. Whether these responses, as well as inflammatory responses, can be modulated by dietary PUFA, via eicosanoid mediation or by any other mechanism, is studied in this dissertation.

In *Chapter 2 and 3*, the model antigens keyhole limpet hemocyanin (KLH) and *Mycobacterium butyricum* were used to induce TH-2 and TH-1 like responses, respectively. Enrichment of the diet with linoleic acid (LA) increased antibody (Ab) levels directed to KLH and decreased Ab levels directed to *M. butyricum*. Enrichment of the diet with α-linolenic acid (LNA) tended to have opposite effects. In *Chapter 3,* special emphasis is put on interactions of n-3 and n-6 PUFA. In this chapter effects of 16 diets, stepwise enriched with linoleic and/or α-linolenic acid, on immune responses directed to the same antigens are described. The data lack a consistent stepwise correlation of Ab levels and dietary LA and LNA, but they indicated that, besides the individual effects of LA and LNA, their interaction is also important. In addition, the effects of dietary α-linolenic and linoleic acid were antigen dependent. It is concluded that in general dietary n-3 increase Ab responses. N-6 can also increase Ab responses, particularly in the absence of n-3 rich dietary sources. For optimal Ab response there might be a dietary α-linolenic acid requirement of approximately 2%. In case of a dietary LNA content of less than 0.4%, a linoleic acid content of at least 2-3% might partially substitute for the n-3 requirement to prevent depressed Ab responses. Dietary LA levels of more than 3-4% might suppress these responses. These percentages are general indications, they are likely to depend on the type of challenge a chicken is exposed to. In the same studies effects of diets on in vitro and in vivo T cell reactivity were studied by establishing lymphocyte proliferation capacity and cutaneous hypersensitivity. High dietary levels of α-linolenic acid increased lymphocyte proliferation after stimulation with ConA or *M. butyricum* protein. Wing-web swellings induced by secondary challenge with KLH or *M. butyricum,* or primary challenge with ConA were increased by high dietary α-linolenic acid levels. It is concluded that cellular responses are enhanced by dietary n-3 PUFA and the PUFA effects are less antigen dependent for cellular responses compared to Ab responses.

In *Chapter 4*, it has been proposed that at dietary PUFA levels several percents higher than NRC requirements, additional vitamin E might be required to maintain PUFA stability and biological properties by protecting PUFA from oxidation. However in the current thesis no evidence was found that at PUFA levels as high as 6-10%, vitamin E levels higher than NRC requirement of 5 mg/kg influences the effects of PUFA on Ab responses, or consistently affects tissue fatty acid composition.

The role of PGE_2 in the Ab responses directed to KLH and *M. butyricum* was studied in *Chapter 5* by administration of PGE_2 or indomethacin, a potent cyclo-oxygenase inhibitor, at primary and secondary immunization with these antigens. It was hypothesized that PGE_2 and indomethacin administration would have opposite effect. PGE_2 decreased anti-*M. butyricum* Ab levels after primary challenge, but increased these levels after secondary challenge, indicating that the presence of PGE_2 shifts the differentiation of naive B cells to memory B cells at the expense of plasma cells. The effects of PGE_2 and indomethacin were similar and most effects on Ab responses were found after secondary challenge. In vitro lymphocyte proliferation after PHA stimulation increased at increasing levels of PGE_2 added to the culture. These observations indicate that PGE_2 metabolism and function in chickens might be different from mammals, and that PGE_2, just like dietary LA, inhibited TH-1 antigen mediated primary Ab responses but did not affect TH-2 antigen mediated Ab responses to the same extent.

In *Chapter 6 and 7*, effects of four dietary fat sources on splenic cytokine mRNA levels following an inflammatory challenge with *Salmonella typhimurium* lipopolysaccharide (LPS) are reported. At 2 hours after LPS injection, mRNA levels of IL-1β, IL-2, IL-6, IL-8, IL-18, MGF and IFNγ had increased, mRNA levels of TGFβ had not changed, and IL-15 mRNA levels had decreased. In LPS injected chickens, dietary fish oil, rich in long-chain n-3 PUFA, increased IL-2 mRNA levels (*Chapter 6*) and IFNγ mRNA levels (*Chapter 7*) compared to dietary linseed oil, corn oil and beef tallow. The 2 hour response may represent innate immunity, indicating that high dietary levels of long-chain n-3 PUFA up-regulates innate immune responsiveness in chickens.

In conclusion, the present thesis provides ample evidence that the chicken immune cell function can be modulated by dietary PUFA. In general, the effects of n-3 PUFA are immuno-enhancing compared to the effects of n-6 PUFA or saturated fatty acids, which is the contrary of what is usually found in mammals. These differences are possibly due to differences in the mechanisms underlying the immunomodulating properties in chickens and mammals. Firstly, the metabolism

and function of PGE_2 on other eicosanoids, and secondly, the effects of dietary n-3 enrichment on cytokine production might be different. In addition, evidence that the function of avian IL-15 is different from mammalian IL-15 is found. These observations imply that dietary PUFA are a useful tool in poultry nutrition to support immune cell function. The present studies suggest that 1-2% dietary n-3 might be beneficial for antibody responses, which potentially ameliorates disease resistance. The effects of PUFA on Ab responsiveness were antigen dependent, indicating that a universal optimal fatty acid intake for optimal resistance might be non-existent. N-6 PUFA might suppress Ab responsiveness or support Ab responsiveness at minimal n-3 levels. The consequences of the dietary modulation of Ab levels, and particularly T cell reactivity and cytokine production, at exposure to various pathogens remain to be established. Studies on PUFA supplementation at infection with intra- and extra-cellular parasites are recommended to further investigate the potential of dietary PUFA to contribute to improved chicken's disease resistance.

Samenvatting

In het afgelopen decennium is de interesse in voedingscomponenten in relatie tot diergezondheid sterk toegenomen. Hieraan ten grondslag liggen enerzijds de steeds hogere eisen die aan diergezondheid en welzijn worden gesteld, en anderzijds de toenemende maatschappelijke en wetenschappelijke druk om het gebruik van antibiotica in de dierhouderij terug te dringen. Door het aanbieden van een voer dat beter aansluit bij de specifieke behoefte aan nutriënten van het immuunsysteem, kan mogelijk de natuurlijke weerstand van pluimvee worden verbeterd. Meervoudig onverzadigde vetzuren zijn nutriënten met potentiële mogelijkheden in dit opzicht.

Op basis van verschillen in de molecuulstructuur kunnen twee typen meervoudig onverzadigde vetzuren onderscheiden worden: de z.g. n-3 en n-6 vetzuren. Belangrijke bronnen van het n-3 vetzuur α-linoleenzuur zijn groene bladerrijke groenten, lijnzaad, raapzaad en walnoten. Andere belangrijke n-3 vetzuren, die kunnen worden gesynthetiseerd uit α-linoleenzuur, zijn eicosapenteenzuur en docosahexeenzuur. Deze laatste twee worden voornamelijk aangetroffen in vette zout water vis, die ze opnemen uit algen. Het belangrijkste n-6 vetzuur in voedingsmiddelen is linolzuur. Linolzuur wordt in grote hoeveelheden gevonden in plantaardige oliën zoals zonnebloem-, soja-, en maïsolie. N-3 en n-6 vetzuren hebben beide specifieke eigenschappen en moeten bovendien concurreren om dezelfde enzymen die ze nodig hebben om hun eigenschappen tot uiting te laten komen. Hieruit volgt dat niet alleen de absolute hoeveelheden van deze vetzuren in het voer van belang zijn, maar dat ook hun onderlinge verhouding belangrijk is. De minimum behoefte aan vetzuren voor pluimvee wordt in standaard voedertabellen op 1% linolzuur gesteld. Hoewel ook α-linoleenzuur als een essentieel vetzuur wordt gezien gaat men hieraan voorbij bij het definiëren van de minimale behoefte aan vetzuren. Daarnaast zijn er, zoals beschreven in hoofdstuk 1, goede redenen om aan te nemen dat de optimale vetzuuropname anders is dan de gedefinieerde minimumbehoefte, met name onder specifieke omstandigheden zoals bij blootstelling aan ziekteverwekkers.

Voor het goed functioneren van cellen van het immuunsysteem zijn vetzuren onder meer van belang als energiebron en als structuurmolecuul voor de celmembraan (d.w.z. celwand). Daarnaast fungeren met name meervoudig

onverzadigde vetzuren als voorlopermoleculen van belangrijke boodschapperstoffen van het immuunsysteem, de z.g. eicosanoiden, zoals prostaglandines, leukotrieëen en thromboxanen. N-3 en n-6 vetzuren zijn de voorlopermoleculen van verschillende klassen eicosanoiden, met verschillen in biologische eigenschappen. Om het verband tussen eicosanoidenproductie met het immuunsysteem te verduidelijken moet er onderscheid gemaakt worden tussen niet-specifieke en specifieke immuniteit, of, met andere woorden, de aangeboren en verworven immuniteit.

Belangrijke cellen van de aangeboren afweer zijn macrofagen. Macrofagen hebben als belangrijke eigenschappen het "opeten" van lichaamsvreemd materiaal en het stimuleren en sturen van andere cellen van het immuunsysteem. Wanneer macrofagen in aanraking komen met een lichaamsvreemd deeltje zullen ze onder meer vetzuren die opgeslagen zijn in hun celmembraan vrij maken en omzetten in eicosanoiden, welke vervolgens uitgescheiden worden. De eicosanoiden die op deze wijze worden geproduceerd door macrofagen kunnen andere macrofagen, maar ook andere cellen in hun omgeving, aanzetten tot deling en productie van signaalstoffen zoals cytokinen. De mate en de verhouding waarin allerlei signaalstoffen worden afgegeven is bepalend voor de sterkte van de opgewekte immuunreactie. Belangrijk voor de niet-specifieke afweer is dat de eicosanoiden die uit n-3 vetzuren gesynthetiseerd worden veel minder pro-inflammatoir (d.w.z. ontstekingsbevorderend) zijn dan n-6 eicosanoiden. Dit komt doordat ze in mindere mate ontstekingscellen naar de locatie waar de immuunreactie zich afspeelt aantrekken.

Het specifieke immuunsysteem bestaat in belangrijke mate uit T en B cellen, dit zijn witte bloedlichaampjes, evenals macrofagen. Er worden drie typen T cellen onderscheiden met elk een eigen specialisatie. Geheugen T cellen vormen samen met geheugen B cellen het immunologische geheugen. Cytotoxische T cellen spelen een belangrijke rol bij het aanvallen van pathogene (d.w.z. ziekteverwekkende) deeltjes. Helper T cellen zijn de regulatoren van de specifieke afweer. Ze produceren de belangrijkste boodschapper moleculen van het immuunsysteem, de cytokinen. De specifieke afweer wordt mogelijk door het vetzuurpatroon beïnvloed door middel van een sturende werking van prostaglandines op de balans tussen subtypen T helper cellen, de T helper-1 en T helper-2 cellen. T helper cellen zijn van belang bij de initiatie van een immuunrespons, waarbij het type pathogeen bepalend is voor het activeren van T helper-1 dan wel T helper-2 cellen. Met name de immuunrespons tegen een infectie met virussen en intracellulaire microben is T helper-1 cel gereguleerd,

terwijl de respons tegen een infectie met extracellulaire microben of wormen T helper-2 cel gereguleerd is. Een specifieke immuunrespons bestaat onder meer uit de productie van antilichamen. Antilichamen, ofwel immunoglobulinen, zijn eiwitten die in grote hoeveelheden geproduceerd worden door gespecialiseerde plasma cellen, die afstammen van B cellen. Antilichaampjes gaan bindingen aan met antigene (d.w.z. lichaamsvreemde) of pathogene deeltjes waarmee ze het opruimen van deze ongewenste elementen vergemakkelijken. In dit proefschrift staat beschreven hoe verschillen in de vetzuursamenstelling in het voer van opgroeiende leghennen verschillende parameters van het immuunsysteem beïnvloed, van zowel de specifieke als niet-specifieke afweer.

In de experimenten die zijn beschreven in hoofdstuk 2, 3 en 4 zijn opgroeiende leghennen geïnjecteerd met modelantigenen waarvan bekend is dat ze in muizen een T helper-1 dan wel T helper-2 geïnduceerde immuunrespons opwekken. Immunizatie met deze antigenen werd gecombineerd met het voeren van voeders met verschillende n-3 en n-6 samenstelling. In hoofdstuk 2 werden de effecten van een controle voer vergeleken met een voer verrijkt met linolzuur en met een voer verrijkt met α-linoleenzuur. Uit deze vergelijking kwam naar voren dat het linolzuur verrijkt voer leidde tot een verhoogde productie van antilichamen gericht tegen het T helper-2 antigeen, en een verlaagde productie van antilichamen gericht tegen het T helper-1 antigeen. Het effect van verrijking van het voer met α-linoleenzuur op de antilichaamproductie was minder sterk, maar leek het omgekeerde effect te hebben ten opzichte van linolzuur. In hoofdstuk 3 werden de effecten van vier linolzuurniveaus en vier α-linoleenzuurniveaus en alle combinaties van deze niveaus op immunologische parameters bestudeerd. Hieruit kwam naar voren dat de effecten van linolzuur afhankelijk zijn van de hoeveelheid α-linoleenzuur, en andersom. Daarnaast waren de vetzuureffecten verschillend voor de verschillende antigenen waartegen de respons gericht was. Wanneer deze resultaten gecombineerd worden met resultaten die in de wetenschappelijke literatuur te vinden zijn, lijkt een combinatie van 2 % α-linoleenzuur met 3-4 % linolzuur een voersamenstelling waarbij voor de antigenen in onze proeven en voor andere antigenen uit de literatuur een redelijk tot optimale antilichaamrespons verwacht kan worden. Naast de effecten op antilichaamproductie werden eveneens effecten van vetzuren *in vivo* en *in vitro* (d.w.z. binnen en buiten het lichaam) op T cel reactiviteit bestudeerd. De *in vitro* delingscapaciteit van T cellen was groter indien deze afkomstig waren van hennen gevoerd met n-3 verrijkte voeders. Deze waarneming is in contradictie met wat in de wetenschappelijke literatuur gevonden wordt. Waarschijnlijk is dit verschil toe te schrijven aan het niet toevoegen van

lichaamseigen serum aan het kweekmedium in onze proeven. Indien dat in vergelijkbare studies met pluimvee wel gebeurde leidde dit tot een afname in de delingscapaciteit. De in hoofdstuk 3 beschreven in vivo T cel reactiviteit was hoger bij hennen gevoerd met een voer verrijkt met 2% α-linoleenzuur in vergelijking met hennen gevoerd met een dieet dat slechts 0.1% α-linoleenzuur bevatte. Deze waarneming wijst op een verhoogde in vivo T cel respons als gevolg van een verhoogde opname van n-3 vetzuren.

In hoofdstuk 4 werden de effecten van extra vitamine E in voer rijk aan onverzadigde vetzuren bestudeerd. Vetzuren zijn kwetsbaar voor oxidatie, en verliezen bij oxidatie hun biologische eigenschappen. Vitamine E heeft een anti-oxidatieve werking en zou daardoor bij hoge gehaltes aan onverzadigde vetzuren een conserverende werking op de vetzuureffecten kunnen hebben. Indicaties dat de vitamine E behoefte verhoogd was als gevolg van hoge vetzuurgehaltes werden echter niet gevonden in hoofdstuk 4. Daarnaast werd in dit hoofdstuk getoetst of de vitamine E dosering effect had op de vetzuursamenstelling van lichaamsweefsel, wat belangrijk zou kunnen zijn voor de beschikbaarheid van vetzuren voor het immuunsysteem. Ook hiervoor werden geen sterke aanwijzingen gevonden. Deze resultaten impliceren dat voor het conserveren van de invloed van onverzadigde vetzuren op de antilichaamrespons het toevoegen van extra vitamine E boven de 5 mg/kg, onnodig is, met name bij onverzadigde vetzuren gehalten tot 6%.

De belangrijkste boodschappermoleculen van het immuunsysteem zijn cytokinen. Ergens in de metabole route van meervoudig onverzadigd vetzuur in voer naar gemeten immuunrespons spelen deze eiwitten hoogstwaarschijnlijk een sturende rol in de verschillen tussen de gemeten immuunrespons als gevolg van voerverschillen. Het is bij gevogelte zoals pluimvee echter vooralsnog onmogelijk cytokinen te detecteren omdat de hiervoor benodigde technieken alleen nog zijn ontwikkeld voor een aantal zoogdieren. Recentelijk zijn wel een aantal cytokinen van de kip gekloneerd en "gesequenced", d.w.z. de nucleotidenvolgorde op het genoom van de kip, waar de informatie die codeert voor de synthese van het eiwit is vastgelegd, is bekend. Deze informatie maakt het mogelijk om, in plaats van de hoeveelheid van het eiwit zelf te meten, de mate waarin het gen dat voor dit eiwit codeert tot expressie is gebracht te kwantificeren. Kwantificering van gen expressie wordt gedaan middels meting van de hoeveelheid mRNA, oftewel boodschapper RNA. Dit mRNA is het molecuul dat als boodschap de genetische code van het cytokine bevat die in de cel als een recept voor het maken van het eiwit wordt afgelezen.

In de hoofdstukken 6 en 7 wordt beschreven in welke mate het tot expressie komen van de genen die coderen voor een aantal cytokinen wordt beïnvloed door het opnemen van meervoudig onverzadigde vetzuren. De cytokinerespons die hiervoor werd gemeten werd opgewekt door toediening van *Salmonella typhimurium* lipopolysaccharide, een toxine dat door deze bacterie wordt geproduceerd en een ontstekingsreactie veroorzaakt. Twee uur na toediening bleek dat de mRNA niveaus van de cytokinen interleukine-1, 2, 6, 8 en 18, interferon-γ, en myelomonocytic growth factor waren toegenomen, het niveau van transforming growth factor-β_2 gelijk was gebleven, en dat van interleukine-15 was afgenomen, als gevolg van de lipopolysaccharide toediening. De afgenomen expressie van interleukine-15 (hoofdstuk 7) was een verrassende waarneming omdat op basis van analogie met zoogdieren verwacht werd dat er een toename zou zijn gevonden. Dit verschil duidt erop dat interleukine-15 van kippen functioneel verschilt van interleukine-15 van zoogdieren. Daarnaast werd een verhoogde expressie van interleukine-2 (hoofstuk 6) en interferon-γ (hoofdstuk 7) gevonden in kuikens die lipopolysaccharide toegediend gekregen hadden en gevoerd waren met een dieet verrijkt met 5% visolie, rijk aan eicosapenteenzuur en docosahexeenzuur, in vergelijking met kippen gevoerd met een dieet verrijkt met 5% maïsolie (linolzuurrijk), lijnzaadolie (α-linoleenzuurrijk) of dierlijk vet (rijk aan verzadigde vetzuren). Een respons op twee uur na toediening van een dergelijk toxine is, vanwege de snelheid, een niet-specifieke immuunrespons, dat wil zeggen dat de respons niet verloopt door de activatie van een specifiek stukje immunologisch geheugen, maar door middel van een niet-specifieke herkenning van pathogeen. Deze waarnemingen duiden er op dat het verrijken van het voer met visolie leidt tot een verhoogde reactie van de niet-specifieke immuniteit. Of deze verhoogde reactie een verbetering of een verslechtering voor het dier is valt uit deze proef niet af te leiden.

De mechanismen die ten grondslag liggen aan de hierboven beschreven effecten van vetzuren op parameters van het immuunsysteem zijn niet geheel duidelijk. In hoofdstuk 5 staat een experiment beschreven waarin de mogelijke rol van prostaglandine E_2, een metaboliet van linolzuur, bestudeerd is in de immuunrespons tegen antigenen uit hoofdstuk 2, 3, en 4. Prostaglandine E_2 of indomethacine, een stof die de productie van prostaglandine E_2 afremt, werden toegediend in combinatie met de antigenen, waarna zowel de primaire als secundaire antilichaamrespons werd gevolgd. Uit hoofdstuk 5 bleek onverwacht dat prostaglandine E_2 en indomethacine dezelfde effecten hadden, die zich met name tijdens de secundaire respons manifesteerden. De resultaten waren slechts

deels in overeenstemming met de voorafgestelde hypothese dat prostaglandine E_2 leidt tot een verhoging van de respons tegen het T helper 2 antigeen, en een verlaging van de respons tegen het T helper 1 antigeen. Toevoeging van prostaglandine E_2 leidde daarnaast tot een hogere *in vitro* delingscapaciteit van witte bloedcellen, het tegengestelde vergeleken met wat in zoogdieren gevonden wordt. Uit deze proef blijkt dus dat er, voor wat betreft eicosanoid metabolisme en functie, belangrijke verschillen lijken te bestaan tussen pluimvee en laboratoriumdieren zoals muizen en ratten.

Concluderend kan gesteld worden dat de resultaten beschreven in dit proefschrift erop wijzen dat het heel goed mogelijk is het immuunsysteem van pluimvee te beïnvloeden door te variëren in het aanbod van meervoudig onverzadigde vetzuren via het voer. De meeste effecten zijn echter tegenovergesteld aan wat op basis van studies aan zoogdieren verwacht werd. Voor zoogdieren wordt aan n-3 vetzuren voornamelijk een onderdrukkend effect op het immuunsysteem toegeschreven, terwijl in dit proefschrift een toename van de meeste immunologische parameters als gevolg van het voeren van extra n-3 werd gevonden. Verschillen in de mechanismen die ten grondslag liggen aan de effecten van vetzuren op de immuunrespons zijn hiervoor mogelijk (deels) verantwoordelijk. Ten eerste zouden verschillen in het metabolisme en de functies van eicosanoiden, en in het bijzonder prostaglandine E_2, hierin een belangrijke rol kunnen spelen. Ten tweede zou de productie van cytokinen in pluimvee en zoogdieren verschillend beïnvloed kunnen worden, met name door n-3 vetzuren. De verhoogde antilichaam respons, die wordt gevonden als gevolg van het voeren van extra n-3 vetzuren, wijst mogelijk op een verbetering van de weerstand. Een algemeen geldende optimale vetzuurdosering voor het immuunsysteem bestaat waarschijnlijk echter niet omdat het optimum afhangt van het antigeen of pathogeen waar de respons tegen gericht is. Of de verhoogde T cel reactiviteit en cytokine productie eveneens duidt op een verhoogde weerstand is minder duidelijk. Om hierover meer duidelijkheid te verschaffen worden studies aanbevolen waarin verrijking van het voer met verschillende soorten vetzuren wordt gecombineerd met infectie van zowel intracellulaire als extracellulaire parasieten.

List of Publications

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Curriculum Vitae

John (Johannes Wilhelmus Christina) Sijben werd op 21 januari 1972 geboren op de boerderij in het Limburgse Maasdorp Neer. In 1990 haalde hij het VWO-diploma aan de Scholengemeenschap Sint Ursula te Horn. In hetzelfde jaar begon hij met studeren aan de toenmalige Landbouwuniversiteit Wageningen, eerst Agrarische Economie, en in 1991, Dierwetenschappen. Deze studie werd in 1996 afgerond na een doctoraalonderzoek in fysiologie en ethologie, een stageonderzoek in immunologie bij het CSIRO in Armidale (New South Wales, Australië), en een doctoraalonderzoek in gezondheid & voeding. In 1996 trad hij in dienst als toegevoegd onderzoeker bij het Departement Dierwetenschappen van de Wageningen Universiteit. In oktober 1997 ging deze aanstelling over in een aanstelling als AIO, wat leidde tot het in dit proefschrift beschreven promotieonderzoek. Een gedeelte hiervan werd uitgevoerd gedurende een verblijf aan de Universiteit van Californië in Davis in 1999. In oktober 2001 trad hij in dienst bij Numico Research, waar hij werkt aan de ontwikkeling van voedingssupplementen.

John Sijben was born on January the 21st 1972 in Neer, in the Dutch province Limburg. In 1990 he graduated from secondary education. In the same year he started to study at the Wageningen University, first Agricultural Economy, and in 1991, Animal Science. In 1996 he finished his masters after MSc research in physiology, ethology, health & nutrition, and a practical training in immunology at the CSIRO in Armidale, New South Wales, Australia. In 1996 he was appointed as researcher at the Department of Animal Science at the Wageningen University. In October 1997 he started his PhD research at the same department, leading to the current dissertation. A part of this research was performed during a stay at the University of California in Davis in 1999. In October 2001 he joined Numico Reseach to work on the development of food supplements.