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 Day 7 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.

(*Key words*: layer, polyunsaturated fatty acid, antibody response, lymphocyte proliferation, growth)

2001 Poultry Science 80:885–893

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 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)

Received for publication July 24, 2000.

Accepted for publication February 25, 2001.

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Abbreviation Key: Ab = antibody; CH = cutaneous hypersensitivity; ConA = concanavalin A; DTH = delayed type hypersensitivity; KLH = keyhole limpet hemocyanin; LA = linoleic acid; LNA = linolenic acid; LST = lymphocyte stimulation test; PGE_2 = prostaglandin E_2; PUFA = polyunsaturated fatty acids; SFA = saturated fatty acid; SI = stimulation index; TH = T-helper cell.

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 found to be affected by 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 seven or eight 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.

[2]Cal Biochem-Novabiochem Co., La Jolla, CA 92039-2087.

TABLE 1. Composition of the experimental diets[1]

Ingredients and content	%
Corn (8.7% 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]Supplied per kilogram of diet: vitamin A (retinol acetate), 10,000 IU; cholecalciferol, 2,000 IU; vitamin E (DL-α-tocopherol acetate), 40 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; B_{12}, 15 μg; K, 5 mg; $CoSO_4 \cdot 7H_2O$, 1 mg; $NA_2SeO_3 \cdot 5H_2O$, 0.15 mg; KI, 5 mg; $FeSO_4 \cdot 7H_2O$, 300 mg; $Cu\text{-}SO_4 \cdot 5H_2O$, 100 mg; MnO_2, 100 mg; $ZnSO_4 \cdot 4H_2O$, 150 mg; Ethoxyquin, 100 mg; carrier, maize; dosage 1%.

[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.

Experimental Design

Effects of dietary PUFA were studied using a 4 × 4 × 2 factorial design of treatments. Factors were dietary LA ($C_{18:2n\text{-}6}$), dietary linolenic (LNA) ($C_{18:3n\text{-}3}$), 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 d of age until the end of the experiment at 11 wk 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)[2] or heat-killed *Mycobacterium*

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

	Calculated %						Analyzed %		Analyzed
Diet	PO58	Sunflower oil	Safflower oil	Linseed oil	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.

butyricum dry cells.[3] 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 for each others 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.

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 Concanavalin A (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,[4] 10 μg/mL KLH,[2] or 10 μg/mL *M. butyricum*[3] 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 per 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

[3]Difco Laboratories, Detroit, MI 48232-7058.

[4]Sigma Chemical Co., St. Louis, MO 73178-9916.

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 of 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 of 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.

Keyhole Limpet Hemocyanin. 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 in 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 the 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.

Mycobacterium 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 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.

Cutaneous Hypersensitivity to KLH, M. butyricum*, and ConA*

KLH-Immunized Chickens. Chickens previously immunized with KLH were rechallenged 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 of 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

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]

			Anti-KLH Ab titers			Anti-*M. butyricum* Ab titers		
Diet	% LA[2]	% LNA	Day 7	Days 10 to 14	Days 17 to 21	Day 7	Days 10 to 14	Days 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.

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.

Mycobacterium 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.

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 contols ($P < 0.001$), 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,

TABLE 4. Equations describing effects of dietary linoleic acid (LA) and linolenic acid (LNA) on antibody responses to keyhole limpet hemocyanin (KLH) and *Mycobacterium butyricum* in three phases of the antibody (Ab) 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	$Y_i = 6.99^{***} + 0.04(LA_i - \underline{LA}) + 0.25^{\#}(LNA_i - \underline{LNA}) - 0.39^{**}[(LA_i - \underline{LA}) \times (LNA_i - \underline{LNA})] - 0.14^{**}(D_i - \overline{D})$
anti-KLH Days 17 to 21	$Y_i = 6.13^{***} - 0.08(LA_i - \underline{LA}) - 0.02(LNA_i - \underline{LNA}) - 0.16[(LA_i - \underline{LA}) \times (LNA_i - \underline{LNA})] + 0.09^{*}(D_i - \overline{D})$
anti-*M. butryicum* 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. butyricum* Days 10 to 14	$Y_i = 3.40^{***} + 0.08(LA_i - \underline{LA}) + 0.33^{**}(LNA_i - \underline{LNA}) + 0.25^{*}[(LA_i - \underline{LA}) \times (LNA_i - \underline{LNA})] + 0.27^{***}(D_i - \overline{D})$
anti-*M. butyricum* 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.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

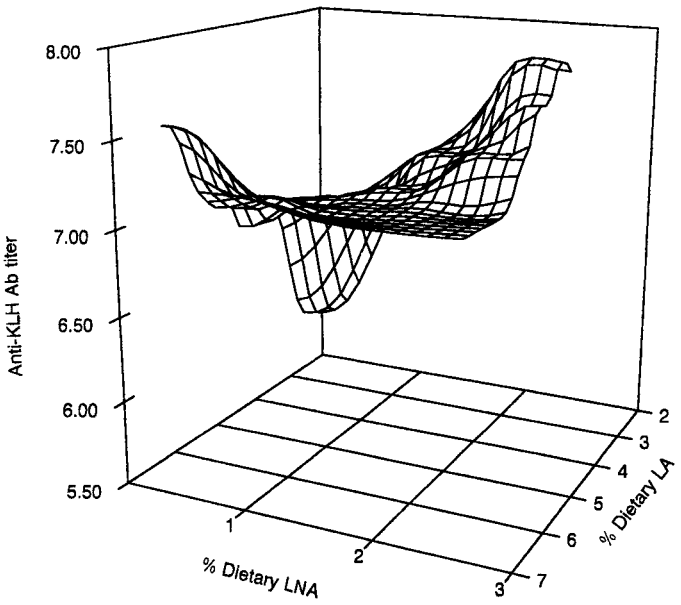

FIGURE 1. Calculated anti-keyhole limpet hemocyanin (KLH) antibody (Ab) titers in laying hens from 10 to 14 d after s.c. immunization at 35 d of age with 1 mg KLH in 1 mL PBS. Pullets were fed with 1 of 16 experimental diets, different in dietary linoleic acid (LA) and linolenic acid (LNA). The regression equation describing these titers was based on Ab titers measured by ELISA and 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.

the interaction between immunization and dietary LA was not significant ($P < 0.1$).

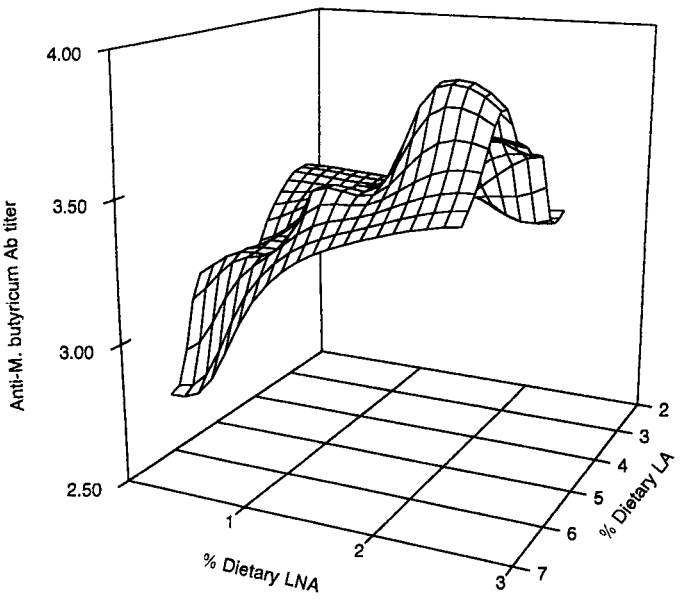

FIGURE 2. Calculated anti-*Mycobacterium butyricum* antibody (Ab) titers in laying hens from 10 to 14 d after s.c. immunization at 35 d of age with 1 mg *M. butyricum* particles in 1 mL PBS. Pullets were fed with 1 of 16 experimental diets, different in dietary linoleic acid (LA) and linolenic acid (LNA). The regression equation describing these titers was based on Ab titers measured by ELISA and 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.

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 the following equation:

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

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 was higher if the fat component of the diet was more unsaturated. The latter was illustrated by the regression equation for the feed conversion, in 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 α-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.

TABLE 5. Effects of dietary linoleic acid (LA) and linolenic acid (LNA) on the increase of wing web thickness ($\times 10^{-1}$ mm) at 4 and 24 h after challenge of birds sensitized with keyhole limpet hemocyanin (KLH), *Mycobacterium butyricum*, or concanavalin A (ConA) at 42 d after immunization with KLH or *M. butyricum*[1]

Immunization treatment	Calculated % dietary LA	Calculated % dietary LNA	KLH sensitized		*M. butyricum* sensitized		ConA sensitized	
			4 h	24 h	4 h	24 h	4 h	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. butyricum	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 polyunsaturated fatty acids in the present experiment.

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,

TABLE 6. Equations describing the effects of dietary linoleic acid (LA) and linolenic acid (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.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

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 interleukin-2 and interferon-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, in the present study, 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.

ACKNOWLEDGMENTS

The authors thank Reggy van der Wielen (Loders Crocklaan B.V., Wormerveer, The Netherlands) for gener-

ously providing fat blend PO58, Jan Veldhuis and Ger de Vries Reilingh (Department of Animal Science, Wageningen University) for skillful technical assistance, Anton Beynen (Faculty of Veterinary Medicine, University of Utrecht, The Netherlands) for helpful comments, and Martin Verstegen (Department of Animal Science, Wageningen University) for critically reading of the manuscript.

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