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Dietary Polyunsaturated Fatty Acids Alter Lymphocyte Subset Proportion and Proliferation, Serum Immunoglobulin G Concentration, and Immune Tissue Development in Chicks

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ABSTRACT The effects of fat source on immune response of the offspring of the Single Comb White Leghorn laying hens were investigated. The laying hens were fed for 6 wk with a wheat-soybean meal basal diet with added sunflower oil (SO), animal oil (AO), linseed oil (LO), or menhaden fish oil (FO) at 5% (wt/wt). Upon hatching, the chicks (30/group) were given the same types of diets for 8 wk. The dietary SO, AO, and LO provided different n-6 to n-3 polyunsaturated fatty acids (PUFA) ratios. The FO and LO had ratios of n-6 to n-3 PUFA that were close but had different components of n-3 PUFA. The results demonstrated that the chicks fed LO or FO had significantly lower ($P < 0.05$) splenocyte proliferative response to ConA than the chicks fed SO or AO at either 4 wk or 8 wk of age, with a stronger ($P < 0.05$) suppressive effect produced by LO at 4 wk. A significantly lower ($P < 0.05$) splenocyte response to PWM was produced by the chicks fed AO, LO, and FO compared with the chicks fed SO at 8 wk. The thymus lymphocyte proliferation in response to ConA at 4 wk was lower ($P < 0.05$) in the chicks fed AO, LO, and FO than in the chicks fed SO. Both LO and FO elevated ($P < 0.05$) the proportion of IgM^+ lymphocytes in spleen, but only FO increased ($P < 0.05$) the serum IgG concentration. The LO elevated ($P < 0.05$) the percentage of $CD8^+$ T-lymphocytes but not the ratio of $CD4^+$ to $CD8^+$ cells ($P > 0.05$) in spleen. Growths of thymus, spleen, and bursa were impacted significantly ($P < 0.05$) by the amount of dietary PUFA, the ratio of n-6 to n-3 fatty acids, and n-3 PUFA components.

(*Key words*: chicks, n-6 to n-3 polyunsaturated fatty acids ratio, specific n-3 polyunsaturated fatty acids, immune responses, immune tissue weight)

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INTRODUCTION

In recent years there has been increased interest in the effects of polyunsaturated fatty acids (PUFA) on the immune responses of animals and humans. It has been reported that feeding rats or mice high levels of n-3 PUFA, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) resulted in marked suppression of in vitro spleen, thymus, lymph node, and peripheral blood lymphocyte proliferation (Alexander and Smythe, 1988; Yaqoob et al., 1994; Sanderson et al., 1995; Yaqoob and Calder, 1995). Feeding rats or mice high levels of n-3 PUFA as linolenic acid (LNA) also suppressed in vitro lymphocyte proliferation (Marshall and Johnston, 1985; Jeffery et al., 1996). Fritsche et al. (1991) reported that n-3 PUFA, LNA or EPA, and DHA significantly suppressed in vitro spleen lymphocyte proliferation in chicks.

Humoral immune response is another important aspect of the immunity and can be assessed by measuring antibody production and activity. Dietary supplementation of n-3 PUFA derived from fish oil decreased antibody production in rats (Pricket et al., 1984), mice (Atkinson and Maisey, 1995), and humans (Virella et al., 1991). It has also been reported that n-3 PUFA from linseed oil or fish oil had no effect on antibody production in rabbits (Kelley et al., 1988) and rats (Kim and Lee, 1992). However, Pricket et al. (1982) reported the production of Ig G and E to alvobumin is enhanced when rats are fed high levels of n-3 PUFA (EPA and DHA) compared with those fed high levels of saturated fatty acids. Yoshino and Ellis (1989) reported that the mouse serum hemagglutinin titer was significantly higher in the group fed a diet with the n-6 (linoleic acid, LA) to n-3 PUFA (LNA) ratio of 0.25 than in the group fed a diet with 2.78 or 100 of n-6 to n-

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Abbreviation Key: AO = 5% animal oil diet; CCM = cell culture medium; ConA = concanavalin A; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; FBS = fetal bovine serum; FO = 5% menhaden fish oil diet; LA = linoleic acid; LNA = α-linolenic acid; LO = 5% linseed oil diet; MUFA = monounsaturated fatty acids; PHA-P = phytohemagglutinin-P; PUFA = polyunsaturated fatty acids; PWM = pokeweed mitogen; SO = 5% sunflower oil diet.

3 PUFA ratio. In chickens, feeding n-3 PUFA from fish oil produced more antibodies to sheep red blood cells than feeding saturated fat (lard) and n-6 PUFA rich oil (maize oil; Fritsche et al., 1991). Antibody production developed more rapidly, reached a higher level, and was more persistent in the chicks fed lower levels of n-6 to n-3 PUFA (LA to LNA) ratio (Friedman and Sklan, 1995).

Lymphocytes, with several phenotypes, play vital roles in determining an immune response. Alteration of the proportion of a lymphocyte subset could change the functions of the immune system. Flaxseed oil (rich in LNA) supplementation does not affect the proportion of $CD4^+$, $CD8^+$, $CD3^+$, and B-cells in human peritoneal blood (Kelley et al., 1991). Fish oil feeding does not change the proportion of $CD4^+$ or $CD8^+$ lymphocytes in spleen, thymus, and lymph nodes in rats (Yaqoob et al., 1994). However, consumption of a low-fat, low-cholesterol, n-3 PUFA-rich diet resulted in a lower proportion of $CD4^+$ and a higher proportion of $CD8^+$ peripheral blood lymphocytes in humans, with the proportion of $CD3^+$ cells (total T-cells) unaffected (Meydani et al., 1993). Huang et al. (1992) observed that dietary fat did not affect total yield or percentage of B-cells or macrophages, but n-3 PUFA feeding increased the percentage of T-cells in noninfected mice. In *Listeria* monocytogen-infected mice, the n-3 PUFA feeding produced the highest percentage of B-cells and the lowest percentages of T-cells and macrophages in the peritoneum.

The chicken immune system is somewhat different from mammals such as rats and mice. The bursa is unique in birds and has been used to study B-lymphocyte development and functional maturation. The thymus and bursa wither when a chicken matures. Thereafter, the immune responses of the birds rely on spleen and peripheral lymph nodes. The antibodies transferred from egg yolk provide the only immune protection of the embryo and newly hatched chicks against various pathogens (passive immunity). The contradictory results obtained from mammalian studies on the effects of dietary n-6 and n-3 PUFA on antibody production and immune cell subset populations might be due to different species, basal diet, fat amount, and ratio of n-6 to n-3 PUFA and different fatty acids of n-3 PUFA used for various studies. Research on the effects of dietary fatty acids on chicken immunity is relatively low. Little is known about the effects of dietary n-3 PUFA on total Ig production and immune cell subset proportions. However, immune responses have been found to impact chicken nutrient utilization and performance (Klasing and Korver, 1997). Modulation of the immune status of chickens may produce beneficial effects and provide a new avenue in improving poultry production. For example, the reduction of the suppressive effect of inflammation by adding fish oil to chicken diets improved the growth and feed efficiency (Korver and Klasing, 1997). Therefore, it is important to know the effects and mechanisms of dietary fatty acids on chicken immune responses. The objective of the current study is to examine the effects of different dietary ratios of n-6 to n-3 PUFA and different n-3 fatty acids on chick immune responses including in vitro lymphocyte proliferation, serum IgG concentration, lymphocyte subset proportion, and immune tissue growth.

MATERIALS AND METHODS

Animals and Diets

The experiment was reviewed and approved by the Faculty of Agriculture and Forestry Animal Policy and Welfare Committee, University of Alberta, and was conducted in accordance with the Canadian Council on Animal Care guidelines. One-day-old chick pullets were used in this study with four dietary treatments of 30 chicks each. The chicks of each group were the progeny of Single Comb White Leghorn laying hens[2] fed, for 6 wk, a wheat-soybean basal diet with addition of 5% (wt/wt) of sunflower oil (SO), animal oil mixture[3] (AO), linseed oil (LO) or menhaden fish oil (FO). The chicks were given the same kind of diets upon hatching until 8 wk of age. The chick diets were formulated to meet the nutrient requirements for early growing chicks (Table 1; National Research Council, 1994). The fatty acid compositions of diets were analyzed using gas chromatography (Cherian and Sim, 1992), and are summarized in Table 2. The diets SO, AO, and LO provided n-6 to n-3 PUFA ratios of 29:1, 9:1, and 0.8:1, respectively. The FO gave a n-6 to n-3 PUFA ratio of 1.1:1, close to that of LO, but the main n-3 PUFA in FO is EPA and DHA instead of LNA, the only n-3 PUFA in LO. The chicks were housed in separate floor pens with free access to feed and water.

TABLE 1. Composition of chick basal diet

Ingredients	g/kg
Wheat	567
Barley	100
Soybean meal	230
Limestone	14
Dicalcium phosphate	25
DL-Methionine	1.8
L-Lysine-HCl	0.2
Layer premix[1]	5
Choline chloride premix[2]	5
Sodium chloride	2.3
Amproline	0.5
ME (kJ/g)	12.3
Crude protein (g/kg)	195

[1]Layer premix provides per kg diet: vitamin A, 12,000 IU; vitamin D3, 3,000 IU; vitamin E, 40 IU; vitamin K, 2.0 mg; pantothenic acid, 14.0 mg; riboflavin, 6.5 mg; folacin, 1.0 mg; niacin, 40.0 mg; thiamine, 3.3 mg; pyridoxine, 6.0 mg; vitamin B12, 0.02 mg; biotin, 0.2 mg; iodine, 0.5 mg; manganese, 75.0 mg; copper, 15.0 mg; zinc, 80.0 mg; selenium, 0.1 mg; iron, 100.0 mg.

[2]50 kg choline chloride premix contains choline chloride (60%) 1.7 kg, wheat shorts 48.3 kg.

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[3]Animal oil mixture is composed mainly of beef fat.

TABLE 2. Fatty acid composition of chick diets

	Diet[1]			
Fatty acid (%)[2]	SO	AO	LO	FO
16:0	10.1	22.2	9.7	20.8
18:0	3.6	10.3	3.8	3.6
16:1	0.1	2.3	0.2	7.5
18:1	17.9	36.9	17.0	16.6
18:2n-6	64.4	22.2	30.8	21.0
20:4n-6		0.1		0.5
18:3n-3	2.2	2.4	37.5	3.4
20:5n-3				7.3
22:5n-3				1.6
22:6n-3				7.4
SAFA	15.1	35.0	14.2	32.4
MUFA	18.3	40.1	17.5	26.0
PUFA	66.6	24.9	68.3	41.6
n-6 PUFA	64.4	22.5	30.8	22.0
n-3 PUFA	2.2	2.4	37.5	19.6
P/S	4.4	0.7	4.8	1.3
n-6:n-3 PUFA	29.1	9.3	0.8	1.1

[1]SO, AO, LO, and FO represent diets containing 5% sunflower oil, animal oil mixture, linseed oil, or menhaden fish oil, respectively.

[2]SAFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; and P/S = ratio of PUFA to SAFA.

Sample Collection

At 4 wk of age, six chicks in each group were selected and anesthetized by CO_2 inhalation. Blood (5 ml) was collected by cardiac puncture and allowed to clot. The sera were separated from the clot by centrifuging at 250 $\times$ *g* for 10 min (Fritsche et al., 1991). An aliquot (1 mL) of each serum sample was sterile-filtered and kept on ice for lymphocyte proliferation assays, the remainder was frozen at –20 C for later IgG analysis. Immediately, the birds were killed by cervical dislocation. The thymus, spleen, and bursa were removed and weighed. The thymus and spleen were put in cell culture medium (CCM) and placed on ice. The CCM comprised RPMI-1640[4] supplemented with 1% (vol/vol) antibiotic and antimycotic mix.[5] Suspensions of spleen and thymus lymphocytes were made as described by Field (1995). Briefly, each tissue was cut into small pieces with sterile scissors and pushed through a tissue sieve equipped with 80-mesh stainless steel. Cell clumps were dispersed by several gentle washings with CCM through the sieve. The red blood cells and dead cells were removed by placing the CCM and cell mixture over lymphocyte separation medium[4] (density, 1.08) and centrifuging at 1,100 $\times$ *g* for 15 min. The cells were washed two to three times with CCM and resuspended in 5 mL of CCM with 5% (vol/vol) fetal bovine serum (5% FBS-CCM). The cells were counted with a hemacytometer, and cell viability was determined by trypan blue exclusion (Field, 1995). The cell suspension was diluted to a final concentration of 1×10^7 cells/mL in 5% FBS-CCM. The same procedure was repeated with chicks (n = 6) at 8 wk.

Mitogenic Responses of Lymphocytes

Lymphocyte proliferation was assayed according to the method reported by Field (1995) with some modifications. Spleen and thymus lymphocytes were cultured (1×10^6 cells/well) in triplicate using 96-well microtiter plates.[6] The plates were incubated in a humidified atmosphere with 5% (vol/vol) CO_2 at 40 C for 72 h. The concentration of concanavalin A (ConA), phytohemagglutinin-P (PHA-P) and pokeweed mitogen (PWM)[4] were 10, 50, and 50 μg/mL for spleen lymphocytes, and 5, 50, and 5 μg/mL for thymus lymphocytes, respectively. The optimum concentration of each mitogen was determined in preliminary studies (data not shown). Assays were conducted in CCM containing 5% (vol/vol) FBS and 5% (vol/vol) chicken serum from the same bird from which lymphocytes were derived (Fritsche et al., 1991). Eight hours before harvesting cells, 1 μCi of [^{3}H] thymidine was added to each well. The cells were harvested on glass fiber filter by using a multiwell harvester[7] and were counted by using Ecolite in a Betacounter.[8] Data were presented by stimulation index, calculated as (amount of [^{3}H] thymidine incorporated by the stimulated cells—the amount of [^{3}H] thymidine incorporated by the unstimulated cells)/amount of [^{3}H] thymidine incorporated by unstimulated cells.

Splenocyte Phenotyping

Lymphocyte subsets from freshly isolated splenocytes and blood mononuclear cells were identified by direct single label (one color) immunofluorescence assay (Robinson and Field, 1997) by using purified mouse monoclonal antibodies[9] specific for the different chicken lymphocyte subsets. CT-4 recognizes T-helper lymphocytes ($CD4^+$), CT-8 recognizes cytotoxic/suppressor T-lymphocytes ($CD8^+$), M-1 recognizes IgM^+ cells, and L-1 recognizes Ig^+ cells. For each sample, 5×10^5 lymphocytes were added to each of the four wells, and then 200 μL of phosphate buffered saline (PBS) with 4% (vol/vol) FBS (the mixture was named as 4% FBS-PBS) was added to each well and centrifuged at 900 $\times$ *g* at 4 C for 2 min. The supernatants were aspirated, and the pellets were broken up by gentle vortexing. Each monoclonal antibody, conjugated with fluorescein isothiocyanate (0.4 μg) in 50 μL of 4% FBS-PBS, was added. An equal amount of 4% FBS-PBS was added into the blank wells. The plates were covered and incubated at 4 C for 30 min. Next, the cells were washed three times with 4% FBS-PBS. Cells were fixed in paraformaldehyde (1% wt/wt in PBS), and all samples were analyzed, within 5 d, by flow cytometry on the same FACScan.[10] The incident/excitation light from the laser light source was set at a wavelength of 488 nm. The fluoresence from the excitation of FITC was

[4]Sigma, St. Louis, MO 63178-9974.
[5]Gibco-BRL, Grand Island, NY 14072.
[6]Corning Inc., Corning, NY 14830.
[7]Skatron Instruments AS, N-3401 Lier, Norway.
[8]Beckman Instruments Inc., Mississauga, Ontario, L5T 1W5, Canada.
[9]South Biotechnology Associates Inc., Birmingham, AL 35226.
[10]BD Pharmingen, San Jose, CA 95131-1807.

detected at a wavelength of 530 nm by using the Lysis II program.[10] The resulting percentages were corrected for background fluorescence determined by incubating cells with 4% FBS-PBS. Unwanted events (dead cells and debris) were detected on the basis of forward scatter and side scatter and were excluded from subsequent phenotype analyses by electronic gating of the viable lymphocyte populations.

Measurement of Serum IgG Concentration

The serum IgG content was determined by the radial immunodiffusion technique as described by Sunwoo et al. (1996).

Statistical Analysis

One-way ANOVA was used to analyze the overall difference of the main effects among the four dietary treatments by using the general linear model procedure of SAS software (SAS Institute Inc., 1990). Significant differences among the means were analyzed by the method of least squares means test after a significant ANOVA. Significance level was set at 0.05.

RESULTS

Mitogenic Response of Splenocytes

Dietary fat source, providing different ratios of n-6 to n-3 PUFA, significantly affected the spleen lymphocyte proliferation in response to ConA (T-cell mitogen) at 4 wk ($P < 0.0003$; Table 3). The chicks fed SO produced a higher response than the chicks fed LO ($P < 0.005$) or FO ($P < 0.0001$). A significantly higher response was also achieved by the chicks fed AO relative to the chicks fed LO ($P < 0.03$) or FO ($P < 0.02$). The response of chicks fed LO was 42.5% lower ($P < 0.05$) than the chicks fed FO. At 8 wk, different fat sources continued to produce significant effects on spleen lymphocyte proliferation to ConA stimulation ($P < 0.0001$). The chicks fed SO had significantly higher responses than the chicks fed LO ($P < 0.0003$) or FO ($P < 0.0003$). The response of the chicks fed AO was also significantly higher than the chicks fed LO ($P < 0.0001$) or FO ($P < 0.0001$).

The PWM (T- and B-cell mitogen)-induced spleen lymphocyte proliferative response did not differ among the four dietary treatments at the age of 4 wk ($P = 0.10$, Table 3). At 8 wk, the response from the chicks fed SO was higher than the chicks fed AO ($P < 0.003$), LO ($P < 0.003$) and FO ($P < 0.0001$). No significant differences were found among the chicks fed AO, LO, and FO ($P > 0.05$). The splenocyte proliferative response to T-cell mitogen PHA-P stimulation was not significantly different among the four dietary treatments at 4 or 8 wk ($P > 0.05$).

Mitogenic Response of Thymocytes

Dietary fat source also significantly influenced the mitogenic response of thymus lymphocytes to ConA at 4 wk ($P < 0.0001$, Table 3). This effect was evident as a 61% decrease in the chicks fed AO ($P < 0.0002$), 86% decrease in the chicks fed LO ($P < 0.0001$), and 79% decrease in the chicks fed FO ($P < 0.0001$) relative to the chicks fed SO. There were no significant differences among the four dietary treatments in the thymus lymphocyte proliferation assay in response to stimulation with PHA-P ($P = 0.11$) or PWM ($P = 0.44$).

IgG Concentration in Serum

There were no significant differences in serum IgG concentration among the four dietary treatments ($P > 0.05$) at 4 wk (Figure 1). However, the serum IgG concentration in the chicks fed FO was 73% higher than in the chicks fed SO ($P < 0.0002$), 37% higher than in the chicks fed AO ($P < 0.005$), and 40% higher than in the chicks fed LO ($P < 0.005$) at 8 wk, with no significant differences observed among the groups fed SO, AO, and LO ($P > 0.05$).

Phenotypes in Spleen

The phenotypes of freshly isolated splenocytes were determined at 4 wk only. Dietary fat source did not affect

TABLE 3. The effect of dietary fat source on spleen and thymus lymphocyte proliferative responses[1] to three polyclonal mitogens

	Spleen lymphocytes[3]						Thymus lymphocytes		
	4 wk			8 wk			4 wk		
Diet[2]	ConA	PHA-P	PWM	ConA	PHA-P	PWM	ConA	PHA-P	PWM
SO	26.4 ± 2.5[a]	5.6 ± 0.7	3.1 ± 0.6	25.1 ± 3.4[a]	9.2 ± 1.9	13.2 ± 0.7[a]	31.1 ± 11.3[a]	13.3 ± 4.6	7.9 ± 2.7
AO	33.8 ± 3.4[a]	5.8 ± 1.1	3.6 ± 1.1	27.5 ± 3.0[a]	6.6 ± 0.6	7.0 ± 1.0[b]	12.0 ± 1.0[b]	7.1 ± 2.2	3.7 ± 1.2
LO	10.6 ± 0.6[c]	5.4 ± 0.9	1.6 ± 0.1	7.3 ± 1.6[b]	7.3 ± 1.3	6.9 ± 1.0[b]	4.3 ± 0.7[b]	3.6 ± 1.3	4.6 ± 1.3
FO	18.5 ± 2.7[b]	6.0 ± 1.2	2.0 ± 0.3	11.4 ± 0.9[b]	8.4 ± 1.7	3.9 ± 1.3[b]	6.5 ± 2.4[b]	4.0 ± 1.5	3.9 ± 1.8

[a–c]For each mitogen, values that do not have common superscripts are significantly different ($P < 0.05$).

[1]Lymphocyte proliferative response to each mitogen is expressed as stimulation index, which is equal to (the amount of [^{3}H] thymidine incorporated by stimulated lymphocytes – the amount of [^{3}H] thymidine incorporated by unstimulated lymphocytes)/the amount of [^{3}H] thymidine incorporated by unstimulated lymphocytes. Each value represents mean ± SEM (n = 6).

[2]SO = diet containing 5% sunflower oil; AO = diet containing 5% animal oil mixture; LO = diet containing 5% linseed oil; FO = diet containing 5% menhaden fish oil.

[3]ConA = concanavalin A; PHA-P = phytohemagglutinin-P; PWM = pokeweed mitogen.

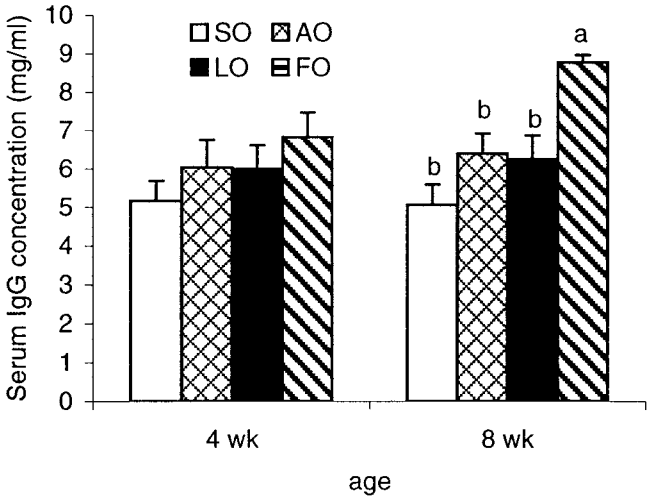

FIGURE 1. The effect of dietary fat source on IgG concentration in chick serum. SO = diet containing 5% sunflower oil; AO = diet containing 5% animal oil mixture; LO = diet containing 5% linseed oil; FO = diet containing 5% menhaden fish oil. Each bar represents mean ± SEM (n = 6). For each age, bars that do not have common letters are significantly different ($P < 0.05$).

($P > 0.05$) the proportion of $CD4^+$ T-lymphocytes and Ig^+ B-lymphocytes in spleen (Table 4). The proportion of $CD8^+$ T-lymphocytes in the spleen of chicks fed LO was higher ($P < 0.05$) than the chicks fed SO or AO. There were no significant differences among the chicks fed SO, AO, and FO ($P > 0.05$) or between the chicks fed LO and FO ($P > 0.05$). The ratio of $CD4^+$ to $CD8^+$ T-cells did not differ ($P > 0.05$) among the four treatments. The chicks fed LO and FO, respectively, had 58% more ($P < 0.05$) and 46% more ($P < 0.05$) spleen IgM^+ B-lymphocytes than the chicks fed SO. No significant differences ($P > 0.05$) were detected in the proportion of IgM^+ B-lymphocytes among the chicks fed AO, LO, and FO or between the chicks fed SO and AO.

Immune Tissue Weight

Body weights were not significantly different ($P > 0.05$) among the four groups at 4 and 8 wk, respectively (Table 5). At 4 wk, the chicks fed the three PUFA-rich diets (SO, LO, and FO) had significantly higher weights of thymus ($P < 0.006$), spleen ($P < 0.002$), and bursa ($P < 0.001$) as a percentage of body weight compared with the chicks fed the diet with a moderate level of PUFA (AO). There were no differences ($P > 0.05$) in the percentages of each tissue to body weight among the groups fed SO, LO, and FO. At 8 wk, the differences in the percentage of thymus or spleen to body weight among the dietary treatments disappeared ($P > 0.05$). The relative weight of the bursa was significantly different among the four treatments ($P < 0.0001$). Contrary to the results obtained at 4 wk, the chicks fed AO had the highest average bursa weight as the percentage of body weight, which was significantly higher ($P < 0.05$) than the chicks fed LO and FO, respectively. There was no significant difference ($P > 0.05$) between the chicks fed SO and LO, which were both significantly higher ($P < 0.01$) than the chicks fed FO.

DISCUSSION

We found that the increases of dietary n-3 PUFA (LNA) in chick diets resulted in marked suppression of the spleen and thymus lymphocyte mitogenic responses to ConA and PWM with n-6 to n-3 PUFA (LA to LNA) ratios ranging from 0.8 to 29. Similar results were reported in chicks in an even larger range of dietary n-6 to n-3 PUFA (LA to LNA) ratios from 0.3 to 59 (Fritsche et al., 1991). Although ConA and PHA-P are both T-cell mitogens, they bind to different parts or determinants on the T-cell (Leca et al., 1986; Benichou et al., 1989; Chilson and Kelly-Chilson, 1989). The amount of PHA-P used resulted in a significantly lower response in both the spleen and thymus lymphocytes, which might explain why no significant diet-induced effects were observed for T-cell response to PHA-P. Many studies in mammalian species also have demonstrated that n-3 PUFA from linseed oil (rich in LNA) and fish oil (rich in EPA and DHA) can suppress lymphocyte proliferation in response to mitogen stimulation (Marshall and Johnston, 1985; Endres et al., 1993; Meydani et al., 1993; Jeffery et al., 1996). Nevertheless, due to different n-3 fatty acid components provided

TABLE 4. The effect of dietary fatty acids on single-labeled immune phenotypes in chick splenocytes

Antibody	Diet[1] SO	AO	LO	FO	$P <$
	(% of the total spleen lymphocytes)				
CT-4 ($CD4^+$ cells)	23.2 ± 1.7	22.6 ± 1.1	24.4 ± 0.8	23.6 ± 0.4	NS[2]
CT-8 ($CD8^+$ + NK[3] cells)	40.4 ± 2.4[b]	40.7 ± 2.7[b]	49.6 ± 1.5[a]	44.4 ± 0.7[ab]	0.01
CT-4/CT-8	0.6 ± 0.1	0.6 ± 0	0.5 ± 0	0.5 ± 0	NS
L-1^+ (Ig^+ cells)	21.8 ± 1.8	20.5 ± 2.7	22.5 ± 1.1	24.3 ± 0.4	NS
M-1^+ (IgM^+ cells)	14.0 ± 1.4[b]	19.0 ± 2.8[ab]	22.2 ± 0.8[a]	20.5 ± 1.0[a]	0.01

[a,b]For each antibody, values that do not have common superscripts are significantly different ($P < 0.05$).

[1]SO = diet containing 5% sunflower oil; AO = diet containing 5% animal oil mixture; LO = diet containing 5% linseed oil; FO = diet containing 5% menhaden fish oil.

[2]Not significant ($P \geq 0.05$).

[3]Natural killer. Values are means ± SEM (n = 6) for freshly isolated splenocytes from the chicks at 4 wk of age.

TABLE 5. The effect of dietary fat source on the relative percentage of immune tissue weight to the body weight in chicks

	4 wk				8 wk			
Diet[1]	BW[2]	Thymus	Spleen	Bursa	BW	Thymus	Spleen	Bursa
	(g)	(% of body weight)			(g)	(% of body weight)		
SO	256 ± 14	7.8 ± 0.2[a]	2.9 ± 0.3[a]	5.6 ± 0.4[a]	820 ± 38	8.8 ± 0.6	4.1 ± 0.3	4.9 ± 0.2[ab]
AO	256 ± 14	4.8 ± 0.6[b]	1.7 ± 0.1[b]	3.8 ± 0.3[b]	831 ± 47	7.6 ± 0.7	4.3 ± 0.4	5.7 ± 0.5[a]
LO	238 ± 19	6.4 ± 0.5[a]	2.4 ± 0.1[a]	5.5 ± 0.2[a]	831 ± 28	7.6 ± 0.7	3.5 ± 0.3	4.3 ± 0.3[b]
FO	262 ± 18	6.8 ± 0.7[a]	2.9 ± 0.2[a]	5.2 ± 0.3[a]	786 ± 36	7.8 ± 0.3	4.2 ± 0.1	2.0 ± 0.2[c]

[a–c]For each tissue, values that do not have common superscripts are significantly different ($P < 0.05$).

[1]SO = diet containing 5% sunflower oil; AO = diet containing 5% animal oil mixture; LO = diet containing 5% linseed oil; FO = diet containing 5% menhaden fish oil.

[2]Each value represents mean ± SEM (n = 6).

by linseed oil and fish oil, the intensities of suppressive effects of 5% linseed oil diet (LO) and 5% fish oil diet (FO) on splenocyte proliferation were different. The LNA had a stronger suppressive effect on splenocyte proliferation in response to ConA at 4 wk than EPA and DHA. This result is opposite that obtained from most studies with mammals (Calder and Newsholme, 1992a,b; Das, 1994; Davi and Das, 1994) that EPA from fish oil appears to be the most inhibitory, but similar to the result obtained with chickens by Fritsche et al. (1991).

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 bloodborne antigens. Antibodies also induce hypersensitivity, activation of the complement system, and antibody dependent-cell cytotoxicity to facilitate clearance of pathogens from infectious sites. We assumed that total Ig might represent the potential humoral immune response of chickens exposed to antigenic challenges. Based on this assumption we made the following comparisons. In this study, the chick serum IgG concentration did not differ among the three groups of chicks fed the diets with the ratio of LA to LNA as 29:1, 9:1, and 0.8:1. The production of antibodies (IgG) to albumin was also not affected when feeding rabbits linseed (Kelley et al., 1988), whereas Friedman and Sklan, (1995) reported in broilers that antibody (IgG) developed more quickly and reached a higher level and was more persistent when dietary LA to LNA ratio was reduced to 12:1 from 31:1, 28:1, or 24:1. Our data showed that feeding a diet containing 1.1:1 of n-6 to n-3 PUFA (5% fish oil) significantly increased IgG concentration in chick serum compared with the chicks fed the other three diets. The enhanced IgG activity was also reported in rats fed a 25% (wt/wt) fish oil diet compared with those fed a 25% tallow diet (Pricket et al., 1982) and in chicks fed a 7% (wt/wt) fish oil diet relative to those fed a diet with 7% lard, corn oil, or canola oil (Fritsche et al., 1991). The data we compared here indicated that alterations of total IgG production induced by dietary fatty acids in chicks without an antigenic challenge might reflect the potential of specific antibody IgG production when chicks were challenged with an antigen. However, further investigations are required.

Diet-induced alterations in immune responses might be determined by assessing changes in the proportions of different lymphocyte subsets (Hoffman-Goetz and Pedersen, 1994). To our knowledge, this is the first study to examine the effect of a dietary LA to LNA ratio on the proportions of lymphocyte subsets in chick spleen. In comparison to chicks fed diets with 29:1 or 9:1 of LA to LNA, the chicks fed the diet with 0.8 of n-6 to n-3 PUFA did not significantly change the ratio of $CD4^+$ to $CD8^+$ T-cells although the proportions of $CD8^+$ lymphocytes were significantly increased. The diet containing LO or FO significantly increased the proportion of IgM^+ B-cells in spleen, but only the fish oil diet increased serum IgG concentration. The proportions of $CD4^+$ and $CD8^+$ cells and the ratio of $CD4^+$ to $CD8^+$ lymphocytes in the chicks fed FO diet did not differ from any others. These results suggested that the differences in lymphocyte proliferation and IgG formation by feeding different ratios of n-6 to n-3 PUFA or different n-3 fatty acids may be attributable to the functional alterations of immune cells rather than the changes of immune cell subset proportions.

Immune tissue development is the basis of immune system functionality. The increase of PUFA in chick diets significantly promoted the growth of thymus, spleen, and bursa before 4 wk. An even greater effect was reported by Elis et al. (1986) that 10 d of feeding PUFA, LA, LNA, or arachidonic acid resulted in a two- to threefold increase of spleen weight. However, the increase of immune tissue weight resulted from feeding high PUFA diets did not produce a consistent effect on immune cell phenotypes, immune cell proliferation, or IgG production. Among the three high PUFA diets, the effect on the ratio of $CD4^+$ to $CD8^+$ T-lymphocytes was not significant. The chicks fed the LO or FO diet had diminished cell proliferation compared with those fed the SO diet although they had similar immune tissue weights. Only FO-fed chicks had increased serum IgG concentration, although the proportion of IgM^+ B-cells was significantly increased by feeding LO and FO diets. Interestingly, the growth-promoting effect of high PUFA diets on immune tissues stopped or even shifted to a suppressive effect in the course of 4 to 8 wk. The bursa weight of chicks decreased with the increase of dietary PUFA and further decreased with the increase

of n-3 PUFA, especially EPA and DHA. Anatomically, the bursa in the chicks fed the FO diet became flat and almost withered. It is not known whether n-3 PUFA, in particular, n-3 PUFA from fish oil promoted chick bursa growth and maturity and then caused withering early or inhibited growth or even damaged the bursa in the course of 4 to 8 wk of age. All these changes in chick immune tissues did not correlate with the functionality of thymus and spleen in terms of lymphocyte proliferation and IgG production up to 8 wk of age.

It is worth noting that the effect of n-3 PUFA on immune responses appears to be dose dependent. In our study, relatively higher levels of oils (5%, wt/wt) in the diet were utilized. An even higher level (7%, wt/wt) was employed by Fritsche et al. (1991) in chicks. Both studies showed that the increase of dietary n-3 PUFA diminished chick lymphocyte proliferative responses to ConA and PWM, and that LNA (linseed oil) showed different potencies from EPA and DHA (fish oil). However, Korver and Klasing (1997) reported that when moderate levels of n-3 PUFA (≤2% fish oil, wt/wt) were applied, increased dietary n-3 PUFA resulted in greater cell-mediated immunity in chickens as determined by the wattle delayed-type hypersensitivity. In humans, inclusion of fish oil at 0.54% of total energy in a low fat diet decreases T-cell proliferation in response to ConA and PHA-P, whereas inclusion of only 0.13% of calories as fish oil in a similar diet results in an increase in the same indices. Delayed-type hypersensitivity is decreased vs. baseline at the higher level of fish oil, but there was no change at the low level of fish oil (Meydani et al. 1993). Fish oil is immunosuppressive in the host vs. graft model in mice only at high concentrations (≥ 10 g/100 g diet; Hinds and Sanders, 1993). It has also been reported that the immunomodulating effect of dietary n-3 PUFA is related to physiological status. For sedentary rats, longer-chain n-3 PUFA augmented cell-mediated immune function and natural killer (NK) cell cytotoxicity. For exercise-trained rats, the effect was opposite (Robinson and Field, 1997).

The results of this experiment give insights into a potential dietary method to modulate chicken immune responses toward improving chicken performance under a given condition. For example, inflammatory response is the first line of defense against novel pathogens, but cells and mediators of the inflammatory responses have been implicated in the pathology of many poultry diseases, including coccidiosis (Trout and Lillehoj, 1993) and *S. enteritidis* (Tellez et al., 1994; Kogut et al., 1995). It has been reported that the indices of an inflammatory (nonspecific cell-mediated immunity) response was lower in fish oil-fed (≤2% fish oil, wt/wt) birds, whereas indices of specific immunity were either unchanged or greater in the chicks fed fish oil diet (Korver and Klasing, 1997). The growth and feed efficiency of the chicks with an inflammatory response were improved when the chicks were given fish oil diet (Korver and Klasing, 1997). Modification of antibody production and activity by dietary fatty acid manipulation may provide an avenue to strengthen chick humoral immunity and protection against various pathogens. However, long-term effects of immunomodulation induced by dietary n-3 PUFA on the resistance of chickens to commercially relevant infectious challenges and chicken performance remain to be investigated.

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