Fish Oil Supplementation Modulates Immune Function in Healthy Infants[1]

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Abstract

(n-3) PUFA influence immune function in adults and may also affect immune maturation during development. This randomized trial is, to our knowledge, the first to investigate whether fish oil supplementation in late infancy modifies immune responses. The study was a 2×2 intervention in 64 healthy Danish infants, who received cow's milk or infant formula alone or with fish oil (FO) (3.4 ± 1.1 mL/d) from 9 to 12 mo of age. Before and after the intervention, fatty acid composition of erythrocyte membranes, plasma IgE, C-reactive protein, and soluble IL-2 receptor concentrations were measured. TNF-α, INF-γ, and IL-10 concentrations in whole-blood cultures, stimulated for 22 h with LPS+phytohemaglutinin (PHA) or *Lactobacillus paracasei,* were also determined. IgA was measured in feces when infants were 10 mo of age. FO supplementation effectively raised erythrocyte (n-3) PUFA ($P < 0.001$), increased *L. paracasei*-induced INF-γ ($P = 0.05$) and tended to reduce LPS+PHA-induced IL-10 ($P = 0.08$). The FO intervention did not affect any of the other analyzed immune variables. The erythrocyte content of eicosapentanoic acid was negatively associated with LPS+PHA-induced IL-10 ($r = -0.38$, $P = 0.02$). Feeding milk rather than formula did not affect cytokine production, but plasma soluble IL-2 receptor concentration was greater in the formula group than in the cow's milk group ($P = 0.03$). Since the capacity to produce INF-γ has been proposed as a maturation marker for the immune system in early life, this study suggests a faster immune maturation with FO supplementation with no apparent reduction in immune activation. The implications for later health need further investigation.   J. Nutr. 137: 1031–1036, 2007.

Introduction

(n-3) PUFA are thought to have immunosuppressive effects in adults, but their immunomodulatory potential in infancy has been sparsely elucidated. The possible effects on innate immunity and general inflammation and the possible capacity to polarize T helper (Th)[4] lymphocytes toward a Th1-response or a Th2-response are key points of interest.

Most trials among healthy adults and patients have shown a suppression of cytokine production with fish oil (FO) supplementation (1–5), whereas others have not found any effects (6–8). The production of TNF-α, IL-6, and IL-1β from peripheral blood mononuclear cells was especially affected, particularly in older subjects (9,2). An anti-inflammatory effect of (n-3) PUFA in early life may impair infant protection and defense against infections, but to our knowledge, no randomized trials have investigated the effect of FO supplementation in infancy on immune function.

Infants are born with an immature immune system, characterized by a reduced ability to produce a number of cytokines (10) and likely a Th2-polarization (11). Immune maturation during infancy seems to be characterized by a Th1-polarization and an improvement in the capacity to produce cytokines such as INF-γ and IL-2 (10). Therefore, a Th-modulating effect of (n-3) PUFA in infancy could possibly affect immune maturation and susceptibility to allergies. Immune maturation occurs faster in breast-fed than in formula-fed infants and is enhanced by the addition of long-chain PUFA (LCPUFA) to infant formula (10), indicating an early immunomodulatory effect of these fatty acids. Recently, a randomized trial conducted by our group showed that FO supplementation during lactation affected cytokine production at 2.5 y of age in a manner that indicated faster immune maturation (12). Increased intake of (n-3) PUFA has also been hypothesized to protect against atopy and allergic diseases (13,11).

Fish, fish oils, and breast milk are important sources of (n-3) LCPUFA, but infant consumption of these long-chain fatty acids is likely to be limited after the cessation of breast-feeding. In many Nordic countries there is a tradition for giving young children a daily spoonful of cod liver oil, which is rich in (n-3) LCPUFA and vitamins A and D. However, the implications of this early supplementation on immune function and health have not been thoroughly investigated.

[4] Abbreviations used: CRP, C-reactive protein; DHA, docosahexaenoic acid 22:6(n-3); EPA, eicosapentaenoic acid 20:5(n-3); FO, fish oil; LCPUFA, long-chain PUFA; PBMC, peripheral blood mononuclear cell; PHA, phytohemaglutinin; RBC, erythrocyte; sIL-2R, soluble IL-2 receptor; Th, T helper lymphocyte; TLR, toll-like receptor.

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We performed a 2 × 2 randomized trial in infants 9 mo of age to test whether 3 mo of FO supplementation affects general immune activation, determined by ex vivo production of cytokines from whole-blood cultures, plasma C-reactive protein (CRP), soluble IL-2 receptors (sIL-2R) and fecal IgA, and immune maturation, primarily assessed by the production of INF-γ from the whole-blood cultures. Although the main focus of this paper was the effect of FO, the milk intervention was included in all the analyses, insofar as it may also affect infant immune status.

Materials and Methods

Study design and subjects. The details of the study design, recruitment procedure, subjects, and interventions have been reported in detail elsewhere (14) and are described briefly here. The study had a randomized, unmasked 2 × 2 factorial design in which infants were randomized to FO (5 mL/d) or no supplement from 9 to 12 mo of age. In each of the groups infants were further randomized to drink either standard infant formula or whole cow's milk. Ninety-four infants were recruited from the National Danish Civil Registry during May–October 2003. The inclusion criteria were singleton infants born at term ($\geq$37 wk of gestation) with a birth weight >2500 g and above the 5th percentile for gestational age (15), a 5-min Apgar score $\geq$7, no major complications at birth or in fetal life, and no chronic diseases. Only infants with a daily consumption of cow's milk or infant formula, whose parents agreed to the principle of randomization, were included. If the child was allotted to FO, five 105-mL bottles of Eskimo-3 (Cardinova; a kind gift from Anjo A/S) were provided from our department. The parents were asked to give their infant 5 mL (1–2 teaspoonfuls)/d, to keep open bottles refrigerated, to return remaining bottles to our department, and to report any waste. The completion rate was 88% and not significantly different among the intervention groups ($P = 0.149$). Sixty-four of the 83 completing infants were included in this study, based on successful blood sampling at 12 mo. The reported mean FO consumption was 3.4 mL/d (range 0.8–5.0 mL/d), providing 571 mg eicosapentaenoic acid (EPA) and 381 mg docosahexaenoic acid (DHA) daily. If infants were randomized to formula, their parents were instructed to use one without LCPUFA and with a ratio between 18:2(n-6) and 18:3(n-3) of ~8:1.

The protocols for the intervention trial were approved by the Ethical Committee of the Municipalities of Frederiksberg and Copenhagen. After the study had been explained orally and in writing, both parents of all participating children gave written consent to participate.

Data collection. As previously described (14), examination visits were conducted in our department before the beginning of the intervention at the age of 9 mo $\pm$ 3 wk and at the end of the intervention period of 3 mo $\pm$ 3 wk. At these visits infant weight and length were measured (14), and parents were interviewed about infant diet, growth, and allergy diagnoses, using questions that were validated for atopic dermatitis (16). Only allergic tendencies (itchy rash, wheezing, or food allergy), reported as verified by a doctor, were used for categorization. Infant diet was recorded by the parents for 7 consecutive days before each examination visit by use of a precoded dietary record developed for children (17), and, as previously described, there were no major dietary differences between the FO intervention groups (14) (data not shown).

At both visits, blood samples were collected from the infants into ice-cold lithium-heparinized tubes (3 mL) and EDTA-tubes (1 mL) by forearm venipuncture. If parents desired, local anesthesia of the skin was given by EMLA patch (Astra Zeneca AB). Median fasting time before blood sampling was 2.5 h (range 0.5–5.0 h), except from one infant who, at 12 mo, was deprived of food for 13 h overnight. In 49 of 64 infants who were successfully blood sampled at 12 mo, a blood sample was also obtained at 9 mo. Within 1 h after sampling, erythrocytes (RBC) were separated from plasma and leukocytes by centrifugation (3000 × g for 10 min). The plasma was frozen at $-80°$C and RBC, isolated from 1 mL heparinized blood, was washed twice, reconstituted 1:1 in physiological saline with 1 mmol/L EDTA and 0.005% butylated hydroxytoluene, and

kept at $-80°$C until analysis of fatty acid composition (maximum storage time, 16 mo).

Cytokine production in whole-blood cultures. Within 30 min after sampling, 40-μL aliquots of heparinized whole-blood were cultured for measurement of cytokine production. Heparinized blood was diluted 6.25 × in RPMI 1640 medium with 0.1% fetal calf serum and 30 kIU/L Na-heparin. Diluted whole-blood cultures were set up in 96-well culture and stimulated with either LPS (from *Escherichia coli* O26:B6; Sigma L2654; final concentration 1 mg/L) and phytohemaglutinin (PHA; Sigma L9132; 23 mg/L) in quadriplicate or with UV-killed *Lactobacillus paracasei* (CRL431, Chr. Hansen A/S; 20 mg/L) in duplicate. Two control wells contained medium alone. After 22 h (range 21.5–24.5 h) of culture at 37°C, 5% CO_2 supernatants were harvested and frozen at $-20°$C.

Supernatant cytokine concentrations were determined by ELISA. INF-γ and TNF-α were measured with commercial kits (Duosets DY285 and DY210, R&D Systems) in accordance with manufacturer's instructions, and IL-10 was measured with matched antibodies (clone JES3–19F1, 2 mg/L and clone JES3–12G8, 1 mg/L) from BD Pharmingen. Limits of detection were 0.03 μg/L (TNF-α), 31 ng/L (INF-γ), and 60 ng/L (IL-10). Cytokines were detected in <2% of the control (unstimulated) samples and these values were subtracted from the concentrations in the stimulated samples. Nondetectable values were set to 0.5 × the limit of detection. Accordingly, cytokine production was determined in 43, 55, and 39 infants at 9 mo, 12 mo, and at both occasions.

Plasma analyses. Concentrations of IgE, sIL-2R, and CRP were measured in plasma from heparinized blood by chemiluminescent immunometric assay with commercial kits (LKIE1, LKIP1, and LKCR1, respectively) on an Immulite 1000 analyzer (all from Diagnostics Products). All samples were analyzed within the same day. The limits of detection were 2 μg/L, 5 × 10^{-3} MU/L, and 0.1 mg/L and the interassay variations were 3.4, 2.6, and 4.9% CV, for IgE, sIL-2R, and CRP, respectively.

Fecal IgA. Fecal samples were only sampled from 40 infants whose parents collected the material from the diaper and returned it by mail in the provided containers. Infants were 10 mo of age, not ill, and not on nonsteroidal anti-inflammatory drugs at the time of collection. The fecal samples were immediately frozen at $-20°$C and freeze-dried at $-50°$C, 0.3 mm Hg for 72 h. For IgA analysis, 0.5 g material was suspended in 50 mL PBS, 0.1% Tween 20, incubated for 2 h at 4°C and centrifuged at 280 × g for 5 min at 4°C. One and one-half mL supernatant was isolated and centrifuged at 10,000 × g for 10 min at 20°C, after which the supernatant was frozen at $-80°$C. The IgA concentration was determined by ELISA using matched antibodies (clone G18–1, 1 mg/L and clone G20–359, 1 mg/L) from BD BioSciences.

RBC fatty acid analysis. Thawed RBC from heparinized blood samples were hemolyzed in redistilled water and the lipids extracted by the Folch procedure (18). Lipids were methylated with boron trifluoride in methanolic NaOH (19) and the resulting fatty acid methyl esters were extracted by heptane and separated by GLC, as previously described (14). All peaks from 12:0 to 22:6(n-3) were identified from retention times of commercial standards (Nu-Chek-Prep). The fatty acid compositions of all RBC samples were determined in duplicate and results were expressed as the area percentage of each fatty acid relative to fatty acid peaks together. Sufficient blood for successful analysis of RBC fatty acid composition was obtained from 49, 58, and 46 infants at 9 mo, 12 mo, and at both occasions.

Statistics. Data were analyzed with SPSS for Windows, version 13.0. CRP, sIL-2R, INF-γ, and INF-γ:IL-10 were logarithmically transformed and IgE and LPS+PHA-induced TNF-α were square-root transformed to obtain normality. Baseline differences were tested using 1-way ANOVA with Tukey's post hoc test or by Chi-square test. After checking for interaction, outcome variables were tested with 2-way ANOVA or ANCOVA including the interventions, baseline values, and covariates associated with the outcome variable ($P < 0.15$). Sex, parity, and breast-feeding duration were tested as covariates because they are known to

TABLE 1 Baseline characteristics of the infants in the 4 intervention groups[1]

	No fish oil		Fish oil	
	Cow's milk	Formula	Cow's milk	Formula
Age at intervention start, *mo*	9.13 ± 0.26 (20)	9.05 ± 0.32 (11)	9.05 ± 0.36 (13)	9.03 ± 0.32 (20)
Gestational age, *wk*	40.4 ± 1.3 (20)	40.2 ± 1.6 (11)	39.8 ± 1.5 (13)	40.2 ± 1.3 (20)
Birth weight, *g*	3726 ± 495 (20)	3838 ± 289 (11)	3586 ± 583 (13)	3642 ± 436 (20)
Weight at 9 mo, *g*	8952 ± 739 (20)	9386 ± 1005 (11)	9258 ± 1175 (13)	9339 ± 740 (20)
Length at 9 mo, *cm*	72.8 ± 2.1[a,b] (20)	73.7 ± 1.9[a] (11)	71.3 ± 2.1[b] (13)	72.5 ± 1.6[a,b] (20)
BMI at 9 mo, *kg/m²*	16.9 ± 1.0[a] (20)	17.3 ± 1.6[a,b] (11)	18.1 ± 1.6[b] (13)	17.8 ± 1.3[a,b] (20)
Sex, *% girls*	60[a] (20)	9[b] (11)	54[a] (13)	50[a] (20)
Siblings, *n*	0 (0–1) (20)	0 (0–1) (11)	1 (0–1) (11)	0 (0–2) (20)
Breast-feeding duration, *mo*	7.7 ± 4.1 (16)	8.2 ± 1.8 (9)	6.9 ± 3.9 (9)	6.0 ± 3.1 (17)
Eczema or wheezing at 9 mo,[2] *%*	5 (20)	18 (11)	8 (13)	23 (20)
Feces IgA at 10 mo, *log₂titer*	18.8 ± 2.0 (12)	20.0 ± 0.8 (6)	18.4 ± 2.5 (9)	19.1 ± 1.8 (13)

[1] Values are means ± SD (*n*) or medians (range) (*n*). Frequencies are shown as % (*n*). Comparisons were done with 1-way ANOVA or chi-square test. Means in a row with superscripts without a common letter differ, *P* < 0.05 (Tukey's post hoc test).

[2] Includes only children with an atopic diagnosis made by a physician. None of the infants was diagnosed with food allergy.

affect immune function and the development of allergies (20,10). To explore possible dose-response associations, we performed Pearson correlation and multiple linear regression analyses between immune variables and the RBC content of EPA, which is a good marker of FO dose in this population (14). Values in the text are given as means ± SEM, median (range), or adjusted means (95% CI). Significance was established at *P* < 0.05.

Results

Apart from slight differences in length, BMI, and sex distribution, the intervention groups did not differ at baseline (**Table 1**). FO supplementation had a pronounced effect on the fatty acid composition of RBC with increases in (n-3) PUFA and reductions in (n-6) PUFA (**Table 2**). The slight difference in 22:5(n-3) at baseline was probably due to chance and was much smaller than the effects of FO (Table 2). The milk intervention had only minor effects on RBC composition with slightly higher (n-6) PUFA (30.5 ± 0.4 vs. 29.2 ± 0.4%, *P* = 0.04) and lower (n-3) PUFA (11.1 ± 0.4 vs. 12.5 ± 0.4%, *P* = 0.02) in the formula group than in the cow's milk group at 12 mo.

The FO intervention did not affect plasma CRP, IgE, or sIL-2R concentrations at 12 mo (**Table 3**) or fecal IgA at 10 mo (Table 1). None of these variables were correlated with RBC EPA content (data not shown). The milk intervention affected plasma concentrations of sIL-2R with higher values in the formula group [1.9 (1.8; 2.2) MU/L] than in the cow's milk group [1.7 (1.5; 1.8) MU/L, *P* = 0.03] but did not affect any of the other immune variables (data not shown).

L. paracasei induced a detectable production of both TNF-α and IL-10 in all infant whole-blood cultures at both 9 and 12 mo, whereas LPS+PHA-induced IL-10 was only detectable in 67 and 89% of the infant samples at 9 and 12 mo, respectively. INF-γ production was detectable in 53 and 65% of the infant cultures at 9 and 12 mo after LPS+PHA-stimulation and in 96 and 98% after stimulation with *L. paracasei*. The FO intervention resulted in higher levels of *L. paracasei*-induced INF-γ compared with those in the unsupplemented group *(P* = 0.05) and tended to reduce the levels of LPS+PHA-induced IL-10 (*P* = 0.08) (Table 3). FO did not affect TNF-α, LPS+PHA-induced INF-γ, *L. paracasei*-induced IL-10, or the ratio between INF-γ and IL-10, but the *L. paracasei*-induced INF-γ:IL-10 was twice as high in the FO group than in the unsupplemented group [3.1 (1.6; 5.7) vs. 1.5 (0.8; 3.0), *P* = 0.15].

The tendency of lower LPS+PHA-induced IL-10 was supported by regression analysis and showed a negative association between RBC EPA and LPS+PHA-induced IL-10 at 12 mo following adjustment for 9-mo values (*r* = −0.38, *P* = 0.02) (**Fig. 1**). Furthermore, *L. paracasei*-induced IL-10 production was negatively associated with breast-feeding duration (*r* = −0.32, *P* = 0.03). There was no dose-response relation between RBC EPA content and *L. paracasei*-induced INF-γ at 12 mo (*P* = 0.38, data not shown).

LPS+PHA-induced IL-10 production at 9 mo was correlated with plasma IgE concentrations (*r* = 0.28, *P* = 0.04) and plasma

TABLE 2 Fatty acid composition of the erythrocytes of infants with or without fish oil supplementation before (9 mo) and after (12 mo) the intervention, with pooled data from the milk and formula groups[1]

Fatty acid	Age	No fish oil	Fish oil
	mo	*Area %*	
SFA[2]	9	43.0 ± 0.7	41.2 ± 0.6
	12	40.9 ± 0.1	41.0 ± 0.1
MUFA[3]	9	18.4 ± 0.5	17.2 ± 0.5
	12	16.8 ± 0.2	16.8 ± 0.2
18:2(n-6)	9	10.6 ± 0.3	10.8 ± 0.3
	12	10.8 ± 0.2	9.2 ± 0.2*
20:4(n-6)	9	15.0 ± 0.6	16.3 ± 0.5
	12	16.7 ± 0.3	13.8 ± 0.3*
20:5(n-3)	9	0.5 ± 0.1	0.6 ± 0.1
	12	0.6 ± 0.2	3.4 ± 0.2*
22:5(n-3)	9	1.9 ± 0.1	2.1 ± 0.0*
	12	2.4 ± 0.0	3.6 ± 0.1*
22:6(n-3)	9	5.0 ± 0.4	5.5 ± 0.4
	12	5.4 ± 0.3	7.7 ± 0.3*
(n-6) PUFA	9	30.7 ± 0.9	32.7 ± 0.9
	12	33.0 ± 0.4	26.7 ± 0.4*
(n-3) PUFA	9	7.5 ± 0.5	8.4 ± 0.5
	12	8.7 ± 0.4	14.9 ± 0.4*
(n-6):(n-3) PUFA	9	4.7 ± 0.3	4.2 ± 0.3
	12	3.9 ± 0.1	1.9 ± 0.1*

[1] Values are means ± SEM, *n* = 24–30 (2-way ANOVA). *Different from the no fish oil group, *P* < 0.05.

[2] SFA, saturated fatty acids.

[3] MUFA, monounsaturated fatty acids.

TABLE 3 Plasma concentrations of IgE, CRP, sIL-2R and ex vivo cytokine production in 22-h whole-blood cultures stimulated with LPS+PHA or *L. paracasei* from infants with or without fish oil supplementation, with pooled data from the milk and formula groups[1]

	No fish oil	Fish oil
IgE, *μg/L*		
9 mo	15 (2–163) (24)	12 (2–463) (23)
12 mo	12 (2–128) (28)	16 (2–475) (30)
12 mo adj.[2]	23 [17; 26] (23)	21 [16; 26] (21)
CRP, *mg/L*		
9 mo	0.4 (0.1–22.1) (24)	0.3 (0.1–2.9) (25)
12 mo	1.8 (0.1–17.4) (29)	0.8 (0.1–28.6) (30)
12 mo adj.[3]	1.7 [0.9; 3.5] (23)	1.7 [0.9; 3.3] (24)
sIL-2R,[4] *MU/L*		
9 mo	1.9 (1.2–5.6) (23)	1.9 (1.2–3.1) (23)
12 mo	1.8 (1.2–4.5) (28)	1.8 (0.9–2.8) (29)
12 mo adj.[3]	1.9 [1.7; 2.1] (22)	1.7 [1.6; 1.9] (21)
TNF-α – LPS+PHA, *μg/L*		
9 mo	3.5 (0.5–8.3) (22)	3.3 (1.6–14.5) (21)
12 mo	5.9 (0.5–1.5) (24)	5.4 (1.8–13.0) (31)
12 mo adj.[5]	7.0 [5.2; 9.1] (20)	5.4 [3.9; 7.1] (19)
TNF-α – *L. paracasei*, *μg/L*		
9 mo	19.0 ± 2.6 (22)	22.3 ± 2.5 (22)
12 mo	25.8 ± 1.9 (24)	23.0 ± 1.3 (31)
12 mo adj.[3]	26.6 [21.9; 31.3] (19)	23.0 [18.6; 27.3] (21)
INF-γ – LPS+PHA, *ng/L*		
9 mo	75 (16–1120) (21)	32 (16 - 1312) (22)
12 mo	73 (16–1028) (24)	65 (16–1336) (31)
12 mo adj.[3]	71 [40; 126] (18)	82 [49; 136] (21)
INF-γ – *L. paracasei*, *ng/L*		
9 mo	559 (157–2450) (21)	785 (16–2819) (22)
12 mo	593 (16–3893) (24)	640 (48–3461) (31)
12 mo adj.[3]	447 [281; 710] (18)	845 [559; 1279]* (21)
IL-10 – LPS+PHA, *ng/L*		
9 mo	632 ± 75 (22)	713 ± 64 (22)
12 mo	1291 ± 101 (24)	1117 ± 95 (31)
12 mo adj.[6]	1312 [1057; 1567] (17)	989 [765; 1213] (20)
IL-10 – *L. paracasei*, *ng/L*		
9 mo	114 ± 22 (22)	111 ± 23 (21)
12 mo	413 ± 71 (24)	341 ± 50 (31)
12 mo adj.[6]	518 [344; 693] (17)	355 [200; 511] (19)

[1] Values are means ± SEM (*n*) or median (range) (*n*). Adjusted 12-mo values are given as mean [95% CI] (*n*), by ANCOVA. Nondetectable values were set to 0.5 × the limit of detection. *Different from the no fish oil group, $P < 0.05$.
[2] Adjusted for milk group, 9-mo values, sex, and parity.
[3] Adjusted for milk group and 9-mo values.
[4] sIL-2R was higher in the formula group than in the cow's milk group, $P = 0.03$.
[5] Adjusted for milk group, 9-mo values, and parity.
[6] Adjusted for milk group, 9-mo values, and breast-feeding duration.

IgE was positively associated with parity at 9 mo ($r = 0.38$, $P < 0.01$) and 12 mo ($r = 0.46$, $P < 0.01$) following adjustment for the effects of the interventions. Furthermore, 9 mo values were correlated ($P < 0.05$) with 12 mo values for plasma IgE and sIL-2R concentrations, *L. paracasei* and LPS+PHA-induced INF-γ, and LPS+PHA-induced IL-10 production.

Seven infants (11%) were reported to have a diagnosis of eczema or wheezing before the study. These children were equally distributed among groups (Table 1) and the pattern did not change during the study. Total plasma IgE was not higher in children with a diagnosis of eczema than in nondiagnosed
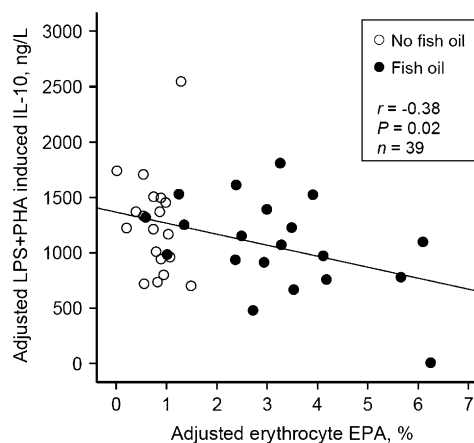
Figure 1 Erythrocyte EPA content and LPS+PHA-induced IL-10 production in 22 h whole-blood cultures from infants after 3 mo with or without fish oil supplementation. Data from the milk and formula groups were pooled. Data were adjusted for LPS+PHA-induced IL-10 production at 9 mo.

children ($P = 0.37$), but the one infant with wheezing had a very high value (463 $\mu g/L$) compared with the rest of the infants [13 (2–163) $\mu g/L$] at 9 mo.

Discussion

In the present study, a daily teaspoonful of FO given to healthy infants for 3 mo markedly increased the (n-3) PUFA content of their RBC membranes. Although the FO did not affect any of the measured markers of general inflammation, we observed a higher INF-γ production and a tendency for lower IL-10 production in the whole-blood cultures after FO supplementation. The latter was supported by dose-response analyses and by a significant negative association between *L. paracasei*-induced IL-10 and breast-feeding duration, which provides (n-3) LCPUFA in early infancy.

We are unaware of other randomized trials assessing the effects of (n-3) PUFA supplements in early childhood on ex vivo cytokine production. Although we cannot rule out the risk of a chance finding in the case of INF-γ, the results agree with our earlier findings that FO supplementation during lactation increased ex vivo INF-γ production and INF-γ:IL-10 in whole-blood cultures from the children at 2.5 y of age (12,10). Vaisman et al. (21) observed higher ex vivo production of both pro- and anti-inflammatory cytokines after FO supplementation in children 8–12 y of age, but the authors did not adjust for baseline levels. In splenocytes from young rats, the addition of (n-3) LCPUFA to the diet increased the production of INF-γ and IL-2 (22). The effects of FO on Th-polarization have not been thoroughly investigated in humans. Among multiple sclerosis patients, INF-γ production was reduced after FO administration (23), and maternal FO supplementation during pregnancy was shown to reduce allergen-induced production of IL-10 in blood from the neonate (24).

Children are born with a Th2-polarized immune system (11). Cytokine responses are low in infants and early immune maturation is thought to be characterized by a favoring of a Th1-response (10). INF-γ is produced by Th1-cells and promotes the differentiation of Th-cells into Th1-cells. IL-10 is now considered to be anti-inflammatory and appears to drive the differentiation of regulatory T-cells (25). IL-10 is, however, secreted by a wide variety of cell types, and results from our previous study indicate that IL-10 in young children may be used as a Th2-marker (12). This

could also be hypothesized in the present study, insofar as LPS+PHA-induced IL-10 was positively associated with plasma IgE before the start of the intervention. The indicated increase in INF-γ production and the tendency of an IL-10 lowering with FO could therefore be speculated to indicate an acceleration of immune maturation. In a study on preterm infants, Field et al. (26,12) observed a slower maturation of the immune response in formula-fed infants than in breast-fed infants, which disappeared with the addition of LCPUFA to the formula.

Cytokine production after polyclonal stimulation, as measured in the present study, examines the underlying predisposition of cytokine production. Atopy is characterized by increased B-lymphocyte IgE-production, regulated by cytokines produced by Th2-cells according to the Th1/Th2 hypothesis (26,27), and FO has been hypothesized to reduce the risk of atopy and allergic disease (24). Studies of FO supplementation in adults tend to show limited efficacy once the allergic immune responses are established (13). However, atopic sensitization occurs early in life, and therefore, (n-3) PUFA exposure in infancy may affect immune maturation in infants and risk of subsequent disease. Two large Scandinavian cohort studies showed positive associations between early fish intake and low risk of allergic diseases (28,29), and, in an Australian case-control study, the dietary ratio between (n-6) and (n-3) PUFA was positively associated with the risk of asthma (30). No differences in atopy or plasma IgE were observed between the groups in the present study, but the study was not statistically powered to look at atopic sensitization.

FO supplementation did not affect the plasma concentration of sIL-2R, which is thought to reflect in vivo T-cell activation (26). The addition of LCPUFA to infant formula decreased the sIL-2R concentration in peripheral blood mononuclear cells from preterm infants (26), but the responsiveness of older, term infants may be different. Our observation, that infants receiving formula had higher plasma sIL-2R concentrations than infants allocated to cow's milk, is difficult to explain. Differences between the 2 milk types, such as the content of vitamin A and D, iron, PUFA, and a number of bioactive proteins may account for the effect. The milk intervention also affected RBC PUFA, probably because of the higher PUFA content and the higher ratio of (n-6) to (n-3) PUFA in the formula (~8:1) compared with cow's milk (~3:1).

Our study was limited by the small sample size and the low rate of completion with blood samples. This is an inevitable consequence of doing research among healthy infants and may indicate that some borderline effects could have become significant if the study had been larger. In some infants, cytokine production was close to the limit of detection, which is a general problem in studies in young children (31,32). This may explain why differences in INF-γ were only seen after stimulation with *L. paracasei* and not with LPS+PHA, because with the latter, INF-γ could be detected in only 55–60% of the infant cultures. Ex vivo stimulation explores only a small potential of the immune response, but we measured both plasma and fecal markers of immune function and used 2 different toll-like receptor (TLR) stimulation regimes for the whole-blood cultures, which resulted in different immune responses. Both *L. paracasei* and LPS are activators of innate immunity, but whereas *L. paracasei* binds to TLR2 on antigen-presenting cells (33), LPS signals through TLR4 (34). PHA is a mitogen shown to activate T-cells by binding to the T-cell receptor (35). Cytokine production induced by the 2 different stimulation regimens were correlated for all cytokines (data not shown) showing consistency. The induced cytokine responses varied, possibly due to differences in cell counts of mononuclear cells between samples, but we did not assess this. This is, however, unlikely to give rise to bias between the groups.

Furthermore, we adjusted for baseline (9 mo) values in all our models. This is essential, because cytokine production is known to show large interindividual variation. Our 9- and 12-mo values were correlated for both INF-γ and IL-10, indicating biological rather than random variation.

The study was not blinded, because control oil was not used, since we wanted to examine the effect of advising parents to give their infants FO. Theoretically, we would expect a slightly higher fat intake in the FO group, but this was not indicated by the dietary or anthropometric data [see (14)]. The differences in length and BMI between the groups at 9 mo persisted throughout the study and are therefore thought to have happened randomly. The milk intervention could theoretically have affected the results of the FO analysis, but we minimized this risk by carefully adjusting for the milk intervention in all outcome analyses.

This study showed that a relatively high (n-3) LCPUFA dose from 9 to 12 mo of age did not affect markers of innate immunity or general inflammation in healthy infants. Therefore, infant protection against invading micro-organisms was unlikely to be impaired with this high (n-3) LCPUFA consumption. The tendency to an increase in INF-γ production after FO supplementation may relate to accelerated immune maturation. The accompanying tendency of reduced IL-10 production may support this or could indicate a dampened Th2 response with possible implications for the development of allergies. Larger long-term randomized infant trials are needed to further explore the immuno-modulating effects of (n-3) LCPUFA and the exact role of the different cytokines in early life.

Literature Cited

1. Endres S, Ghorbani R, Kelley VE, Georgilis K, Lonnemann G, Van der Meer JW, Cannon JG, Rogers TS, Klempner MS, et al. The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. N Engl J Med. 1989;320:265–71.

2. Meydani SN, Endres S, Woods MM, Goldin BR, Soo C, Morrill-Labrode A, Dinarello CA, Gorbach SL. Oral (n-3) fatty acid supplementation suppresses cytokine production and lymphocyte proliferation: comparison between young and older women. J Nutr. 1991;121: 547–55.

3. Trebble T, Arden NK, Stroud MA, Wootton SA, Burdge GC, Miles EA, Ballinger AB, Thompson RL, Calder PC. Inhibition of tumour necrosis factor-alpha and interleukin 6 production by mononuclear cells following dietary fish-oil supplementation in healthy men and response to antioxidant co-supplementation. Br J Nutr. 2003;90:405–12.

4. Caughey GE, Mantzioris E, Gibson RA, Cleland LG, James MJ. The effect on human tumor necrosis factor alpha and interleukin 1 beta production of diets enriched in n-3 fatty acids from vegetable oil or fish oil. Am J Clin Nutr. 1996;63:116–22.

5. Abbate R, Gori AM, Martini F, Brunelli T, Filippini M, Francalanci I, Paniccia R, Prisco D, Gensini GF, Neri Serneri GG. n-3 PUFA supplementation, monocyte PCA expression and interleukin-6 production. Prostaglandins Leukot Essent Fatty Acids. 1996;54:439–44.

6. Blok WL, Deslypere JP, Demacker PN, van der Ven-Jongekrijg J, Hectors MP, Van der Meer JW, Katan MB. Pro- and anti-inflammatory cytokines in healthy volunteers fed various doses of fish oil for 1 year. Eur J Clin Invest. 1997;27:1003–8.

7. Kew S, Mesa MD, Tricon S, Buckley R, Minihane AM, Yaqoob P. Effects of oils rich in eicosapentaenoic and docosahexaenoic acids on immune cell composition and function in healthy humans. Am J Clin Nutr. 2004;79:674–81.

8. Kew S, Banerjee T, Minihane AM, Finnegan YE, Muggli R, Albers R, Williams CM, Calder PC. Lack of effect of foods enriched with plant- or marine-derived n-3 fatty acids on human immune function. Am J Clin Nutr. 2003;77:1287–95.

9. Rees D, Miles EA, Banerjee T, Wells SJ, Roynette CE, Wahle KW, Calder PC. Dose-related effects of eicosapentaenoic acid on innate immune function in healthy humans: a comparison of young and older men. Am J Clin Nutr. 2006;83:331–42.

10. Field CJ, Clandinin MT, Van Aerde JE. Polyunsaturated fatty acids and T-cell function: implications for the neonate. Lipids. 2001;36:1025–32.

11. Field CJ. The immunological components of human milk and their effect on immune development in infants. J Nutr. 2005;135:1–4.

12. Lauritzen L, Kjaer TM, Fruekilde MB, Michaelsen KF, Frokiaer H. Fish oil supplementation of lactating mothers affects cytokine production in 2 1/2-year-old children. Lipids. 2005;40:669–76.

13. Prescott SL, Calder PC. N-3 polyunsaturated fatty acids and allergic disease. Curr Opin Clin Nutr Metab Care. 2004;7:123–9.

14. Damsgaard CT, Schack-Nielsen L, Michaelsen KF, Fruekilde MB, Hels O, Lauritzen L. Fish oil affects blood pressure and the plasma lipid profile in healthy danish infants. J Nutr. 2006;136:94–9.

15. Greisen G, Michaelsen KF. [Perinatal growth. A practical perinatal growth curve] Ugeskr Laeger. 1989;151:1813–5.

16. Benn CS, Benfeldt E, Andersen PK, Olesen AB, Melbye M, Bjorksten B. Atopic dermatitis in young children: diagnostic criteria for use in epidemiological studies based on telephone interviews. Acta Derm Venereol. 2003;83:347–50.

17. Ulbak J, Lauritzen L, Hansen HS, Michaelsen KF. Diet and blood pressure in 2.5-y-old Danish children. Am J Clin Nutr. 2004;79:1095–102.

18. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem. 1957;226:497–509.

19. Hamilton S, Hamilton RJ, Sewell PA. Extraction of lipids and derivative formation. In: Hamilton RJ, Hamilton S, editors. Lipid analysis - a practical approach. Oxford: IRL Press; 1992. p. 13–64.

20. Benn CS, Melbye M, Wohlfahrt J, Bjorksten B, Aaby P. Cohort study of sibling effect, infectious diseases, and risk of atopic dermatitis during first 18 months of life. BMJ. 2004;328:1223.

21. Vaisman N, Zaruk Y, Shirazi I, Kaysar N, Barak V. The effect of fish oil supplementation on cytokine production in children. Eur Cytokine Netw. 2005;16:194–8.

22. Robinson LE, Clandinin MT, Field CJ. R3230AC rat mammary tumor and dietary long-chain (n-3) fatty acids change immune cell composition and function during mitogen activation. J Nutr. 2001;131:2021–7.

23. Gallai V, Sarchielli P, Trequattrini A, Franceschini M, Floridi A, Firenze C, Alberti A, Di BD, Stragliotto E. Cytokine secretion and eicosanoid production in the peripheral blood mononuclear cells of MS patients undergoing dietary supplementation with n-3 polyunsaturated fatty acids. J Neuroimmunol. 1995;56:143–53.

24. Dunstan JA, Mori TA, Barden A, Beilin LJ, Taylor AL, Holt PG, Prescott SL. Fish oil supplementation in pregnancy modifies neonatal allergen-specific immune responses and clinical outcomes in infants at high risk of atopy: a randomized, controlled trial. J Allergy Clin Immunol. 2003;112:1178–84.

25. Thompson C, Powrie F. Regulatory T cells. Curr Opin Pharmacol. 2004;4:408–14.

26. Field CJ, Thomson CA, Van Aerde JE, Parrott A, Euler A, Lien E, Clandinin MT. Lower proportion of CD45R0+ cells and deficient interleukin-10 production by formula-fed infants, compared with human-fed, is corrected with supplementation of long-chain polyunsaturated fatty acids. J Pediatr Gastroenterol Nutr. 2000;31:291–9.

27. Romagnani S. T cells in allergy and asthma. Curr Opin Allergy Clin Immunol. 2001;1:73–8.

28. Nafstad P, Nystad W, Magnus P, Jaakkola JJ. Asthma and allergic rhinitis at 4 years of age in relation to fish consumption in infancy. J Asthma. 2003;40:343–8.

29. Kull I, Bergstrom A, Lilja G, Pershagen G, Wickman M. Fish consumption during the first year of life and development of allergic diseases during childhood. Allergy. 2006;61:1009–15.

30. Oddy WH, de Klerk NH, Kendall GE, Mihrshahi S, Peat JK. Ratio of omega-6 to omega-3 fatty acids and childhood asthma. J Asthma. 2004;41:319–26.

31. Smart JM, Kemp AS. Increased Th1 and Th2 allergen-induced cytokine responses in children with atopic disease. Clin Exp Allergy. 2002;32:796–802.

32. Dunstan JA, Mori TA, Barden A, Beilin LJ, Taylor AL, Holt PG, Prescott SL. Maternal fish oil supplementation in pregnancy reduces interleukin-13 levels in cord blood of infants at high risk of atopy. Clin Exp Allergy. 2003;33:442–8.

33. Galdeano CM, Perdigon G. The probiotic bacterium Lactobacillus casei induces activation of the gut mucosal immune system through innate immunity. Clin Vaccine Immunol. 2006;13:219–26.

34. Underhill DM, Ozinsky A. Toll-like receptors: key mediators of microbe detection. Curr Opin Immunol. 2002;14:103–10.

35. Licastro F, Davis LJ, Morini MC. Lectins and superantigens: membrane interactions of these compounds with T lymphocytes affect immune responses. Int J Biochem. 1993;25:845–52.