

Effect of Feeding a Formula Supplemented With Long-chain Polyunsaturated Fatty Acids for 14 Weeks Improves the Ex Vivo Response to a Mitogen and Reduces the Response to a Soy Protein in Infants at Low Risk for Allergy

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ABSTRACT

Background and Objective: Feeding long-chain polyunsaturated fatty acids (LCP) influences immunity in adults; however, less is known about their effect during development. The aim of the study was to determine the effect of feeding LCP on immunity in healthy infants during the first 4 months of life.

Patients and Methods: Formula-fed infants were randomized at ≤ 14 days of age to standard term formula (Formula) or formula containing LCP (Formula+LCP). Infants exclusively fed human milk (HM) were included for comparison. At 16 weeks of age, blood was collected and phenotypes, the ability to proliferate and produce cytokines (interleukin [IL]-2, IL-4, IL-6, IL-10, IL-12, interferon [IFN]- γ , tumor necrosis factor [TNF]- α , TGF- β) after incubation with phytohemagglutinin (PHA), β -lactoglobulin, or soy protein were measured.

Results: Feeding LCP resulted in a higher than and more similar proliferation rate to PHA in HM-fed infants, possibly because of a greater T_H1 type cytokine response and a higher percentage of antigen mature (CD45RO+) cells ($P < 0.05$). The response to β -lactoglobulin did not differ among groups. After incubation with soy protein Formula+LCP, compared with Formula produced less IL-2 and more TNF- α and had a higher percentage of CD8+ and a lower percentage of CD20+ (CD20+CD54+) cells poststimulation ($P < 0.05$). Both formula groups produced less IL-2 after PHA, had a lower percentage of CD80+ cells, and a higher percentage of CD54+ cells after incubation with food proteins ($P < 0.05$).

Conclusions: Formula-fed infants, at low risk for allergy, respond differently to mitogen and food proteins ex vivo than those fed HM. Feeding LCP altered some of these differences in the direction that is hypothesized to confer immune benefits.

Key Words: β -lactoglobulin, cytokines, human milk, lymphocytes, soy

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Infancy is an important period for the maturation of the immune system, particularly the acquired immune system. Adverse reactions to cow's milk and other food proteins are frequent in the first year of life. It was recently demonstrated that more than half of the children who demonstrated adverse reactions to milk early in infancy did not show adverse reactions by 1 year of age (1). This suggests that the ability to develop tolerance to dietary antigens also occurs during the first year of life. Human milk (HM) is not only the optimal food for infants in the early postnatal period but it also affects the development of the newborn's immune system (2). Epidemiological data suggest that infants who do not exclusively breast-feed are at higher risk of developing allergies (3,4), thereby implicating a role of early diet in the process of developing tolerance. However, significantly increasing the rates and duration of exclusive breast-feeding in the PROBIT (Promotion of Breast-feeding Intervention Trial) did not reduce the incidence of asthma or allergy (5). Although this does not negate the importance of HM or compounds of HM on immune development, it does support that additional environmental and genetic factors are involved in the etiology of atopic diseases. A better understanding of the effect of some of the compounds in HM that modify immune development is necessary to ensure that the infants not fed HM develop healthy immune systems.

Immune maturation during infancy is characterized by a movement toward T_H1 cytokine polarization and is associated with an improvement in the capacity to produce cytokines such as interferon (IFN)- γ and interleukin (IL)-2 (6). Many other immune functions that may be hypothesized to be involved in the development of tolerance, which are independent of a T_H1 response, have also been reported to be immature during early infancy. These include dendritic cell function (7), the production of antibodies/immunoglobulins (8,9), and the production of cytokines (IL-4 and IL-10) that augment T_H2 responses (10,11). It is often hypothesized that the development of allergic diseases and asthma are initiated and sustained by inappropriate T_H -cell responses of the T_H2 phenotype (12–14). Although there is detailed knowledge of the immunological, molecular, and genetic factors influencing T_H2 differentiation (6), far less is known about how diet may influence the critical immune regulation that maintains the balance between immunity and tolerance.

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HM contains biologically important quantities of long-chain polyunsaturated fatty acids (LCP), arachidonic acid (AA), and docosahexaenoic acid (DHA), as well as their precursors linoleic acid (18:2n-6) and α -linolenic acid (18:3n-3). Supplementation of infant formulas with LCP, AA, and DHA has been demonstrated to favorably modulate the development of the nervous system and the digestive system (15). Extensive immunomodulatory properties of LCP on both the innate and adaptive immune system are well recognized in adults and animal models (16) and provide the rationale that these fatty acids could assist in the development of the infant's immune system. Although considerable evidence has accumulated that T cell–derived cytokines are involved in immunological responses and disease pathogenesis (17), data on normal cytokine production profiles during the neonatal period are limited. Several studies have reported that supplementation of LCP during pregnancy (14,18) and childhood (19) influence the risk of developing atopic diseases in high-risk populations. An imbalance between n-6 and n-3 polyunsaturated fatty acids and an insufficient cellular level of AA may alter thymic T cell development and the balance of T_H1 and T_H2 cytokines, thus affecting the ability to develop tolerance (20). In the preterm infant we have demonstrated that adding AA and DHA to infant formula results in T cell maturation and a cytokine response, after stimulation with a polyclonal mitogen, phytohemagglutinin (PHA)-L, that is closer to the breast-fed infant (21). Similarly, in the term infant, we have demonstrated that feeding a formula containing LCP for 4 weeks influenced the ability of peripheral blood mononuclear cells (PBMC) from young infants to respond to ex vivo challenges to PHA in a manner that is consistent with those of infants fed HM (22) and to food proteins in a more T_H1 -skewed direction (23). To our knowledge, there has not been a study aimed at assessing the relation between neonatal LCP supplementation and immune responses to proteins and mitogen beyond the first 6 weeks of age.

The present study is a 16-week follow-up and presents data on the so-called ex vivo response of PBMC from infants randomized to consume a standard infant formula with or without DHA and AA from 2 weeks of age. The present study was designed to compare the ex vivo response by PBMC with a polyclonal T cell mitogen and 2 common food proteins. We specifically measured changes in immune cell types and the ability of PBMC to proliferate and produce cytokines (IL-2, IL-4, IL-6, IL-10, IL-13, transforming growth factor [TGF]- β , tumor necrosis factor [TNF]- α and IFN- γ) in vitro after stimulation. A similar age group of infants fed exclusively HM was included in the study.

PATIENTS AND METHODS

Subjects

Mothers (from the Regional Program for Newborn Services, Edmonton, Alberta, Canada) who had chosen to switch from breast-feeding to feeding their infants with commercial formula before 14 days of age were approached to enter the study. Infants of consenting mothers ($n = 30$) were randomized to receive either a standard term infant formula (Formula; $n = 14$) or the same formula supplemented with AA (20:4n-6) and DHA (22:6n-3) (Formula+LCP; $n = 16$). A group of infants who were fed exclusively HM ($n = 16$) from birth were included for comparison. The infants were fed exclusively with these treatments for 14 weeks. Infants were enrolled in the study between 7 and 14 days of age. The average age of entry into the study was 11.8 ± 2.2 days for the HM, 10.2 ± 3.0 days for the Formula, and 9.7 ± 2.8 days for the Formula+LCP groups. All of the infants in the formula groups received some HM before entering the study. However, these infants consumed commercial formula for not more than 3 days before

beginning the study, and the number of days of consuming HM did not differ between the 2 formula groups. None of the infants randomized to the formula treatments received a formula containing LCP before entering the study. All of the infants completed the study. Mothers and investigators were blinded to the formula received. Laboratory staff was blinded to all groups. Inclusion criteria for all of the infants stipulated that by 14 days of age, infants were receiving 100% of their intake by mouth from HM or commercial infant formula and that infants were healthy with respect to birth weight, length, and head circumference between the 10th and 90th percentile for gestational age, according to the National Center for Health Statistics growth charts (24). Infants with congenital malformations, documented systemic or congenital infection, significant neonatal morbidity, diagnosed maternal autoimmune disorders, acute illness precluding oral feedings, or conditions requiring infant feedings other than standard formula or HM were excluded. None of the infants had received corticosteroids, red cell or plasma transfusions, or intravenous lipid emulsions before entering the study. Infants were enrolled in the study between 7 and 14 days of age. All of the mothers had healthy pregnancies without complications and all infants were term (gestational age 39.5 ± 1.2 weeks). Infants were categorized as at low risk for allergy, based on a negative family history of atopic diseases and the absence of cow's-milk intolerance was further confirmed by a milk challenge that was administered at 16 weeks of age after the blood sample was collected. Briefly, at 16 weeks, when all of the infants in this study were still exclusively fed 1 of the formula or breast milk, each infant was fed 125 mL of nonfat (skim) cow's milk. Infants were monitored by their mothers for 24 hours and then examined by a neonatologist. All of the infants tolerated the milk feeding without any clinical signs of intolerance. This study was approved by the research ethics committee of the Faculty of Medicine, University of Alberta, and the special services and research committee of the Capital Health Authority, Edmonton, Alberta. Informed written consent was obtained from 1 parent of each infant participating in the study.

Diet Treatments

The commercial formula fed was S-26 (Wyeth Nutrition, Philadelphia, PA) and S-26Gold (Wyeth Nutrition), which differs from S-26 only by the addition of AA (20:4n-6 at 0.34% w/w) and DHA (22:6n-3 at 0.2% w/w) from single-cell triglycerides (Martek Biosciences Corp, Columbia, OH). The complete fatty acid composition of the 2 formulas has been previously reported (22).

Study Population, Anthropometric, and Blood Measures

Infant weight, length, and head circumference were measured at birth and at 16 weeks of age. A 2-mL venous blood sample was drawn from each infant at 16 weeks of age. Standard blood chemistry and hematological measures were made including hematocrit, hemoglobin, white blood cell count (WBC), and differential and mean corpuscular volume. Assessment of standard blood chemistry, hematological measures, plasma phospholipid composition, and immune parameters at 2 and 6 weeks of age have been reported (22,23).

Phenotyping of PBMC

The following anti-human mouse monoclonal antibodies (mAbs) were purchased from Sigma Chemical Co (St Louis, MO) or BD Pharmingen (Mississauga, ON), labeled with fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), biotin (B), or quantum

red (QR): CD3-B (pan T cells), CD4-FITC (T helper/inducer cells), CD8-PE (T cytotoxic/suppressor cells), CD45RO-FITC (antigen exposed T cells, B cells, granulocytes, and monocytes), CD45RA-QR (antigen-naïve T cells, natural killer [NK] cells, B cells), CD14-FITC (monocytes), CD20-FITC (B cells), CD16-PE (NK cells and macrophages), CD28-FITC (costimulatory molecule for T cell activation), and CD44-PE (H-CAM on leukocytes, erythrocytes, platelets). The effect of diet on the phenotypes of cells present in whole blood was determined by flow cytometry using 2-color immunofluorescence assay, as previously described in detail (21). After labeling, cells were washed and fixed in paraformaldehyde (10 g/L in phosphate-buffered saline with sodium azide as a preservative), and all of the samples were acquired (within 3 days) on the same flow cytometer (FACScan; Becton Dickinson, Sunnyvale, CA). Flow cytometry analysis (10,000 cells per mAb combination) was performed on the gated mononuclear cell population, which was set to exclude any remaining erythrocytes. Appropriate isotype controls (Sigma Chemical Co) were used for each labeled mAb and were corrected for background fluorescence ($<1\%$ for all fluorescent tags). Using combinations of 2-color (2 antibodies) dot plots the proportion of single- and double-labeled cells for each antibody combination was determined by setting gates (vertical and horizontal) to separate the cells that stained positive or negative for the antibodies.

Estimation of PBMC Proliferation to Mitogen or Food Protein

PBMC were isolated from whole blood as previously described (21). Although blood was collected from every baby, for some infants, we could not perform all of the immune assays on their blood. Additionally, a few assays did not work on every infant's sample. For this reason, the number of infants used in calculating the group mean for each parameter is indicated with the results. Isolated mononuclear cells (1×10^6 cells per milliliter, 0.2×10^6 per well) were cultured in 96-well microtiter plates with or without the polyclonal T cell mitogen PHA ($5 \mu\text{g/mL}$, Sigma Chemical Co) for 48 hours, or with or without either β -lactoglobulin (BLG, $980 \mu\text{g/mL}$, Sigma Chemical Co), soy protein (SOY) with a significantly reduced isoflavone content (SOY, $643 \mu\text{g/mL}$, Wyeth Nutrition), or a slightly hydrolyzed SOY isolate with normal levels of isoflavones (HSOY, $624 \mu\text{g/mL}$, Wyeth Nutrition) for 120 hours, as previously described (22,23). The 2 different SOYs selected for the study have been previously used in the formulation of infant formula. Preliminary studies confirmed that the maximum rate of ^3H -thymidine uptake by cells stimulated with these proteins at these concentrations was achieved by 120 hours (data not shown). Eighteen hours before harvesting the cells, each well was pulsed with $1 \mu\text{Ci}$ of ^3H -thymidine (Amersham/Pharmacia Biotech, Montreal, Canada) and analyzed as previously described (22). All of the assays were performed in triplicate. The response to PHA is reported as the amount of thymidine incorporated (disintegrations per minute, dpm). For the response to food proteins we reported both the amount of thymidine incorporated and the stimulation index (SI), which was calculated as the amount of ^3H -thymidine (dpm) incorporated by cells in the presence of the protein divided by the amount of ^3H -thymidine (dpm) incorporated by cells incubated in the absence of the protein. Responses are usually compared using an SI (which enables one to look at the relative change with stimulation) when the incorporation of ^3H -thymidine is low and when there is considerable interindividual variability in the unstimulated response.

Cytokine Production by Cultured PBMC

Isolated PBMC (1×10^6 cells) were cultured in duplicate in 1-mL complete culture medium and incubated with or without

PHA ($5 \mu\text{g/mL}$) for 48 hours and with BLG ($980 \mu\text{g/mL}$), SOY ($643 \mu\text{g/mL}$), and HSOY ($624 \mu\text{g/mL}$) for 120 hours at 37°C in a controlled atmosphere ($5\% \text{ CO}_2$, 95% relative humidity). After incubation, tubes were centrifuged at $200g$ at 4°C for 10 minutes to pellet cells to be used for postculture phenotyping. Supernatant was collected and stored at -70°C for subsequent cytokine analysis. The concentrations of IL-2, IL-4, IL-6, IL-10, IL-12, IFN- γ , TNF- α , and TGF- β 1 were determined using enzyme-linked immunosorbent assay kits (OptEIA set from BD Pharmingen) as previously described (22).

Postculture/Stimulation Phenotyping

Cells were phenotyped postculture in an attempt to identify cell types that may have become activated by exposure to PHA or food proteins. Immune cell phenotypes were identified by immunofluorescence technique as previously described. The following mAb (purchased from Sigma Chemical Co or BD Pharmingen) were used in the postculture analysis: CD20-FITC (B cells), CD54-PE (intercellular adhesion molecule [I-CAM]-1), CD80-B (B-cell subset costimulatory signal to T cells), CD16-FITC (NK cells and macrophages), CD11c-PE (monocytes, granulocytes, and macrophages), CD11b-B (granulocytes, monocytes, NK cells, and macrophages), CD14-FITC (monocytes), CD8-PE (T cytotoxic/suppressor cells), and CD4-B (T helper/inducer cells). Analyses (1000 – $10,000$ cells per mAb combination) were performed on the gated lymphocyte population. Appropriate isotype controls (Sigma Chemical Co) were used for each labeled mAb and resultant percentages were corrected for each subject's background fluorescence ($<1\%$). The number of cells remaining in culture was not counted postculture.

Statistical Analysis

Based on an earlier feeding study of preterm infants (21), the sample size for each group was deemed sufficient to establish statistical differences in IL-2 production after PHA stimulation. At 16 weeks of age, the effect of diet was tested by a 1-way analysis of variance. Differences between groups were identified using least squares means. For all measures, a probability of $P < 0.05$ was accepted as statistically significant. For those measures that were not normally distributed, data were log transformed (and normal distribution confirmed) before statistical analysis. All of the statistical analyses were performed using SAS, version 8 (SAS Institute, Cary, NC). Results are presented as mean $\pm$ standard deviation (SD) unless otherwise stated. Data that were not normally distributed were presented as the median and 95% confidence interval.

RESULTS

Anthropometric Measures and Blood Chemistry

There were no significant differences in weight, length, or head circumference among diet groups at birth or at 16 weeks of age (Table 1). Routine blood chemistry values were within normal ranges for healthy term infants at 16 weeks of age and did not differ among diet groups (data not illustrated).

Total and Differential WBC Counts

There were no differences among groups in the concentrations ($n = 46$) of WBC ($10 \pm 3 \times 10^9/\text{L}$), neutrophils ($2 \pm 1 \times 10^9/\text{L}$), lymphocytes ($7 \pm 3 \times 10^9/\text{L}$), or eosinophils ($0.3 \pm 0.2 \times 10^9/\text{L}$) at 16 weeks of age. Compared with the Formula group, the concentration ($0.9 \pm 0.4 \times 10^9/\text{L}$ vs $0.5 \pm 0.2 \times 10^9/\text{L}$) and relative

TABLE 1. Demographic characteristics of infants at birth and at 16 weeks of age*

Variable	HM (n = 16)	Formula (n = 14)	Formula+LCP (n = 16)
Gestational age at birth, wk	39 ± 1	39 ± 2	40 ± 1
Sex (M:F)	6:10	3:11	7:9
Weight, g			
Birth	3690 ± 536	3508 ± 696	3638 ± 595
16 wk	6663 ± 931	6659 ± 762	7118 ± 1097
Length, cm			
Birth	52 ± 3	51 ± 3	51 ± 2
16 wk	64 ± 3	63 ± 3	63 ± 2
Head circumference, cm			
Birth	35 ± 2	35 ± 2	35 ± 1
16 wk	42 ± 1	41 ± 1	42 ± 1

HM = human milk; LCP = long-chain polyunsaturated fatty acids.

*Values are mean ± SD. There were no significant differences between groups for any of the anthropometric measures.

percentage of monocytes ($9\% \pm 3\%$ vs $6\% \pm 3\%$) was higher for the Formula+LCP group.

Compared with the HM group, the mean relative percentage of lymphocytes was lower ($65\% \pm 9\%$ vs $73\% \pm 9\%$) and concentration of monocytes was higher ($0.9 \pm 0.4 \times 10^9/L$ vs $0.7 \pm 0.2 \times 10^9/L$) for the Formula+LCP.

PBMC in Blood (Preculture)

There were no significant differences among groups for the following phenotypes (overall mean ± SD [number of infants]): CD3+ (75 ± 7 [44]), CD4+ (56 ± 7 [44]), CD8+ (19 ± 5 [45]), CD3+CD4+ (72 ± 9 [44]), CD8+CD28+ (13 ± 5 [45]), CD14+ (2.6 ± 2.3 [45]), CD16+ (10 ± 6 [39]), CD20+ (20 ± 6 [43]), CD44+ (97 ± 4 [45]), CD4+CD45RA+ (50 ± 7 [43]), and CD8+CD45RA+ (19 ± 5 [43]). The Formula+LCP group had a higher ($P < 0.05$) proportion and concentration of total CD4+ and CD8+ cells that were CD45RO+ compared with the Formula group (Table 2). The proportion and concentration of CD45RO+ cells (total, CD4+, and CD8+) in Formula infants were significantly lower ($P < 0.05$) than in HM infants. This concentration in the

Formula+LCP group did not differ significantly from HM infants. Compared with the HM group, feeding either formula resulted in a higher proportion of cells expressing the co-stimulatory molecule CD28 in the gated mononuclear cell population (Table 2). The unsupplemented Formula group had a higher ($P < 0.05$) proportion of CD4+CD28+ cells (T_H cells expressing the co-stimulatory marker) than the HM infants, with the Formula+LCP not differing from either group. There was a lower ($P < 0.05$) proportion of total CD45RA+ cells in infants fed Formula+LCP compared with HM, but this did not seem to be because of changes in CD4+ or CD8+ cells expressing CD45RA.

³H-Thymidine Uptake and Cytokine Production by Cells Incubated in the Absence of Mitogen/Protein

At 16 weeks, there were no significant differences among groups in the amount of ³H-thymidine uptake at 48 or 120 hours in the absence of mitogen (Table 3). In culture media from cells incubated in the absence of mitogens/food proteins for 48 hours only IL-6 and TNF-α were produced in detectable amounts. Both formula-fed groups produced more ($P < 0.05$) TNF-α than cells from HM infants (Formula, 769 ± 369 [n = 14]; Formula+LCP, 711 ± 505 [n = 15]; HM, 493 ± 476 [n = 16]). The Formula-fed infants produced more ($P < 0.05$) IL-6 (26 ± 21 [n = 6]) than HM (11 ± 7 [n = 10]) and Formula+LCP (5 ± 6 [n = 9]) groups.

PBMC Response In Vitro to Incubation With PHA

There were no differences among groups in the production (mean ± SD, n = 45) of IFN-γ (1761 ± 1343 pg/mL), TNF-α (3298 ± 1340 pg/mL), IL-5 (25 ± 19 pg/mL), IL-6 (22 ± 13 ng/mL), IL-10 (365 ± 198 pg/mL), or IL-12 (10 ± 10 pg/mL) after 48-hour stimulation with PHA. There were no differences among groups in the following phenotypes (overall mean number of cells ± SD [number of infants]): CD4+ (36 ± 11 [33]), CD16+ (26 ± 14 [39]), CD11C+ (33 ± 12 [36]), CD11b (39 ± 13 [31]), CD16+CD11C+ (14 ± 10 [39]), CD16+CD11b+ (14 ± 10 [28]), or CD80+ (8 ± 6 [39]) all remaining in culture poststimulation with PHA.

Adding LCP to Formula resulted in a significantly higher ($P < 0.05$) response to PHA, as estimated by the amount of ³H-thymidine incorporation (Table 3), a higher ($P < 0.05$) IFN-γ/IL-4 cytokine ratio (Table 4) and a higher ($P < 0.05$) proportion of CD8+ cells (Table 5) after PHA stimulation. Despite a higher

TABLE 2. Peripheral blood cell phenotypes (relative percent and concentration) of cells at 16 weeks of age by dietary group*

Immune cell phenotype	Total gated mononuclear cells, %			Concentration in peripheral blood, $10^7/L$		
	HM (n = 15)	Formula (n = 14)	Formula+LCP (n = 16)	HM (n = 15)	Formula (n = 14)	Formula + LCP (n = 16)
CD4+CD28+	48 ± 10^b	55 ± 6^a	$53 \pm 9^{a,b}$	353 ± 93	402 ± 121	353 ± 93
CD28+	61 ± 11^a	71 ± 7^b	70 ± 10^b	464 ± 106	539 ± 179	543 ± 179
CD45RO+	3.2 ± 1.6^a	1.3 ± 0.7^b	2.8 ± 2.0^a	22 ± 11^a	9 ± 5^b	21 ± 15^a
CD45RA+	87 ± 3^a	$80 \pm 15^{a,b}$	78 ± 13^b	638 ± 140	634 ± 267	628 ± 122
CD4+ CD45RO+	$1.2 \pm 0.7^{a,b}$	0.8 ± 0.4^b	1.9 ± 1.5^a	$9.1 \pm 5.1^{a,b}$	6.5 ± 5.0^b	13.0 ± 9.3^a
CD8+ CD45RO+	0.3 ± 0.2^a	0.2 ± 0.2^b	0.3 ± 0.3^a	2.5 ± 1.5^a	0.8 ± 0.8^b	2.6 ± 2.6^a

HM = human milk; LCP = long-chain polyunsaturated fatty acids.

*Values are mean ± SD (n).[†]For the relative percent (% gated cells) or the concentration, means in a row that do not share a letter are significantly different ($P < 0.05$).

TABLE 3. Ex vivo proliferation of PBMC to PHA and food proteins at 16 weeks of age by dietary group*

Mitogen or protein	HM (n = 14)	Formula (n = 10)	Formula+LCP (n = 12)
48 h			
No mitogen, dpm	1502 ± 564	1160 ± 587	1414 ± 561
PHA, dpm	113685 ± 47705 ^{a,†}	57148 ± 31560 ^b	80575 ± 22113 ^c
120 h			
No mitogen, dpm	4517 (2788–6200)	2227 (1599–4273)	4837 (3212–7110)
HSOY, dpm	24771 ^a (19262–34574)	9417 ^b (7117–18769)	18070 ^{a,b} (13093–28123)
(SI) [‡]	5.4 ^a (5.2–10.1)	4.8 ^{a,b} (3.5–7.4)	4.9 ^b (3.4–5.5)
SOY, dpm	24442 ^a (17198–30151)	11606 ^b (7508–17206)	18362 ^{a,b} (12510–24385)
(SI)	5.1 ^a (4.9–9.8)	4.4 ^{a,b} (2.7–8.0)	4.0 ^b (3.4–4.5)
BLG, dpm	2399 (1968–6105)	2030 (1715–3909)	2589 (2296–5253)
(SI)	1.3 (0.7–1.8)	1.0 (0.8–1.7)	0.7 (0.6–1.1)

BLG = β -lactoglobulin; dpm = disintegrations per minute; HM = human milk; HSOY = hydrolyzed SOY; LCP = long-chain polyunsaturated fatty acids; PBMC = peripheral blood mononuclear cells; PHA = phytohemagglutinin; SD = standard deviation; SI = stimulation index; SOY = soy protein.

* Values are mean $\pm$ SD except when nonparametric, in which case they are presented as a media (95% confidence interval).

[†] Means in a row that do not share a letter are significantly different ($P < 0.05$). Values were nonparametric and normalized (using a log conversion) before statistical analysis.

[‡] SI is calculated as: the rate of ³H-thymidine incorporation after incubation with mitogen/protein (dpm)/the rate of ³H-thymidine incorporation in the absence of mitogen/protein (dpm).

proliferative response to PHA by the Formula+LCP compared with the Formula group, the proliferative response to PHA (Table 3) and the PHA-stimulated production of IL-2 (Table 4) were significantly lower ($P < 0.05$) for both Formula groups compared with the HM group. Compared with the HM group, the production of TGF- β was significantly higher in the Formula group and the production of IL-4 lower (IFN- γ /IL-4 ratio higher) in the Formula+LCP group ($P < 0.05$, Table 4). Compared with the HM group, both formula groups had a lower proportion of CD14+ cells and a higher proportion of CD20+ (CD20+CD54+) cells poststimulation with PHA ($P < 0.05$, Table 5). Compared with the HM group, only the supplemented Formula group had a significantly higher ($P < 0.05$) proportion of CD20+CD80+ and CD54+ cells post-PHA stimulation (Table 5).

Lymphocyte Response In Vitro to Incubation With Food Proteins

Soy Proteins

There was no detectable production of IL-5, IL-4, or TGF- β after stimulation with HSOY. Also, there were no differences

among groups in the production (mean $\pm$ SD, $n = 40$) of IFN- γ (485 ± 388 pg/mL), IL-6 (12 ± 7 ng/mL), or IL-10 (54 ± 46 pg/mL). After HSOY stimulation, there were no differences in the mean proportions (overall mean $\pm$ SD [number of infants]) of CD20+ (14 ± 7 [44]), CD14+ (2 ± 2 [40]), CD16+ (5 ± 4 [43]), CD11B+ (8 ± 8 [37]), CD54+ (82 ± 17 [44]), CD20+CD80+ (2 ± 3 [31]), or CD20+CD54+ (13 ± 6 [44]) cells among groups.

The amount of ³H-thymidine incorporation (dpm) and the SI (response above background) to both SOYs were not significantly different between the 2 formula groups (Table 3). Because the stimulation response did not differ between the 2 SOYs, further in vitro work was done only with the HSOY protein. After stimulation with HSOY there was a lower production of IL-2 and a higher production of TNF- α by cells from the Formula+LCP compared with the Formula group ($P < 0.05$, Table 4). After stimulation with HSOY there was a significantly higher percentage of CD8+, CD11c+, CD16+CD11c+, and a lower percentage of CD80+ cells left in culture for the Formula+LCP compared with the Formula group ($P < 0.05$, Table 5).

Compared with the HM group, the rate of amount of ³H-thymidine incorporation (dpm) was significantly lower for the Formula group and the SI for the Formula+LCP group for both

TABLE 4. Ex vivo cytokine production by PHA- and food protein-stimulated PBMC at 16 weeks of age by dietary group*

Cytokine	HM, ng/mL	Formula, ng/mL	Formula+LCP, ng/mL
PHA (48 h)			
IL-2	3780 ± 1802 (16) ^{a,†}	2391 ± 1706 (14) ^b	2535 ± 1588 (16) ^b
IL-4	39 ± 23 (16) ^a	29 ± 11 (14) ^{a,b}	23 ± 12 (16) ^b
TGF- β	21 ± 9 (8) ^a	55 ± 34 (11) ^b	43 ± 40 (9) ^{a,b}
IFN- γ /IL-4	34 ^a (33,103) (16)	54 ^a (34,81) (14)	79 ^b (66,130) (9)
HSOY (120 h) [‡]			
IL-2	32 ± 20 (16) ^{a,b}	41 ± 24 (10) ^a	20 ± 13 (14) ^b
TNF- α	32 ^{a,b} (30,84) (16)	33 ^a (26,54) (12)	51 ^b (50,132) (13)

HM = human milk; HSOY = hydrolyzed SOY; IFN- γ = interferon-gamma; IL = interleukin; LCP = long-chain polyunsaturated fatty acids; PBMC = peripheral blood mononuclear cells; PHA = phytohemagglutinin; SD = standard deviation; TNF- α = tumor necrosis factor-alpha.

* Values are mean $\pm$ SD (n) except when nonparametric, wherein they are presented as a median (95% confidence interval) (n).

[†] Means in a row that do not share a letter are significantly different ($P < 0.05$). Values that were nonparametric were normalized (using a log conversion) before statistical analysis.

TABLE 5. Changes in postculture PBMC phenotypes after stimulation with PHA or food protein at 16 weeks of age by dietary group*

Immune cell phenotype	Total cells, %		
	HM	Formula	Formula+LCP
PHA (48 h)			
CD8+	21 ± 6 (12) ^{a†}	25 ± 9 (11) ^a	34 ± 10 (12) ^b
CD14+	12 ± 7 (11) ^a	5 ± 3 (12) ^b	5 ± 3 (12) ^b
CD20+	10 ± 5 (13) ^b	17 ± 6 (14) ^a	15 ± 7 (15) ^a
CD20+CD80+	2.2 ± 1.7 (13) ^b	5.2 ± 3.8 (11) ^a	3.8 ± 2.9 (13) ^{a,b}
CD20+CD54+	8 ± 5 (13) ^b	15 ± 8 (13) ^a	13 ± 8 (15) ^a
CD54+	69 ± 10 (13) ^a	81 ± 15 (13) ^b	75 ± 16 (15) ^{a,b}
HSOY (120 h)			
CD8+	24 ± 4 (16) ^a	25 ± 7 (13) ^a	29 ± 5 (16) ^b
CD4+	59 ± 8 (15) ^a	56 ± 13 (13) ^{a,b}	52 ± 6 (14) ^b
CD16+CD11C+	1.8 ± 1.1 (15) ^a	0.8 ± 0.6 (11) ^b	2.1 ± 2.0 (15) ^a
CD16+CD11B+	1.0 ± 1.2 (13) ^a	2.3 ± 2.1 (12) ^{a,b}	2.5 ± 1.8 (16) ^b
CD11c+	3.4 ± 2.3 (16) ^a	1.8 ± 1.4 (14) ^b	4.0 ± 3.1 (14) ^a
CD80+	27 ± 7 (10) ^a	5 ± 2 (9) ^b	3 ± 2 (12) ^c
BLG (120 h)			
CD8+	28 ± 7 (16) ^a	28 ± 8 (12) ^a	35 ± 8 (15) ^b
CD20+	19 ± 8 (14) ^{a,b}	21 ± 6 (13) ^b	15 ± 7 (15) ^a
CD20+CD54+	18 ± 2 (14) ^{a,b}	21 ± 2 (12) ^b	14 ± 8 (15) ^a
CD16+CD11B+	0.7 ± 0.7 (13) ^a	3.3 ± 1.8 (10) ^b	2.3 ± 1.9 (16) ^b
CD80+	22 ± 16 (11) ^a	7 ± 3 (11) ^b	11 ± 5 (9) ^c
CD54+	76 ± 12 (15) ^a	86 ± 9 (12) ^b	86 ± 11 (15) ^b

BLG = β -lactoglobulin; HM = human milk; HSOY = hydrolyzed SOY; LCP = long-chain polyunsaturated fatty acids; PBMC = peripheral blood mononuclear cells; PHA = phytohemagglutinin; SD = standard deviation.

* Values are mean $\pm$ SD (n). Data are the percentage of total gated peripheral mononuclear cells (% of total cells).

[†] Means in a row that do not share a letter are significantly different ($P < 0.05$).

SOYs ($P < 0.05$, Table 3). There was no difference in cytokine production post-HSOY stimulation between the formula groups and HM. Compared with HM there was a significantly lower ($P < 0.05$) percentage of CD11c+, CD16+CD11c+ cells in the Formula but not in the Formula+LCP group (Table 5). Both formula groups had a lower proportion of CD80+ cells remaining postculture, compared with HM ($P < 0.05$, Table 5). The Formula+LCP group but not the Formula group had a lower percentage of CD4+ and a higher percentage of CD8+ and CD16+CD11b+ cells after HSOY stimulation ($P < 0.05$, Table 5).

BLG

Using the amount of ³H-thymidine uptake as a measure of response, we found that cells did not respond to BLG (Table 3) and there was no detectable production of IL-2, IL-5, IL-4, or TGF- β after incubation with BLG. Cells, however, did produce (mean $\pm$ SD, n = 45) TNF- α (326 $\pm$ 217 pg/mL), IL-10 (104 $\pm$ 131 ng/mL), IFN- γ (70 $\pm$ 112 pg/mL), and IL-6 (14 $\pm$ 11 ng/mL) after incubation with BLG, but this did not differ among groups. After stimulation with BLG, there were no significant differences among groups for the following phenotypes (overall mean $\pm$ SD [number of infants]): CD4+ (43 $\pm$ 11 [41]), CD14+ (2 $\pm$ 2 [36]), CD16+ (6 $\pm$ 4 [42]), CD11B+ (12 $\pm$ 14 [38]), CD11C+ (6 $\pm$ 4 [38]), CD16+CD11C+ (2 $\pm$ 1 [38]), and CD20+CD80+ (2 $\pm$ 2 [31]) cells. After stimulation with BLG, there was a significantly higher percentage of CD8+ and CD80 and a lower percentage of CD4+, CD20+ (CD20CD54+), and CD16+CD11b+ cells in the cultures from Formula+LCP compared

with Formula ($P < 0.05$, Table 5). Compared with HM, both formula groups had a higher percentage of CD16+CD11b+ and CD54+ and a lower percentage of CD80+ cells remaining in culture ($P < 0.05$, Table 5).

DISCUSSION

This article presents the effects of feeding a formula supplemented with LCP to infants up to 16 weeks of age on the ability of peripheral mononuclear cells to respond to PHA and 2 common food proteins. We previously reported the effects on immune function in these infants at 6 weeks of age (22,23). At 16 weeks of age, there was no effect of diet on infant growth and few differences were observed in either the concentration or the proportion of different immune cells present in peripheral blood of infants fed the different diets. The infants were randomized into the 2 formula groups, but randomization was not stratified by sex. This resulted in a predominance of female infants in the unsupplemented formula group. There are no data, to our knowledge, in which sex influences the immune parameters measured at this age. When tested there was no difference in any of the immune parameters measured between the sexes; however, we did not have sufficient power to adequately test this and future studies should consider stratification by sex.

CD45 marks the leukocyte common antigen and T lymphocytes express the CD45RA (antigen naïve) isoform, whereas antigen-mature (activated and memory) lymphocytes express the shorter CD45RO isoform. The vast majority of cells in the 16-week infants were antigen naïve (CD45RA+). Although the

antigen-mature population (CD45RO+) represented <3% of total cells, diet significantly altered this population. Feeding a formula supplemented with LCP resulted in a significantly higher proportion of CD45RO+ and lower proportion of CD45RA+ cells in peripheral blood compared with feeding the unsupplemented formula. Similar to our findings at 6 weeks (22), the proportion of CD4+ and CD8+ CD45RO+ cells in infants fed Formula+LCP was not different from HM infants, suggesting that providing LCP supported similar T cell maturation. The physiological importance of the small but higher proportion and concentration of CD45RO+ cells in the infants fed Formula+LCP compared with the unsupplemented formula is not known. CD45RO (antigen-activated T cells), in contrast to naïve (CD45RA) cells, are more easily triggered, respond at lower antigen dose, and are more efficient at helping B cells to differentiate into antibody-secreting cells (25).

Response to PHA

Plant lectins such as PHA are commonly used to mimic microbial superantigens, and response is assessed using a number of parameters, including the amount of ³H-thymidine uptake (estimation of proliferation), amount and pattern of cytokines produced, and expression of activation markers. In the present study, the SI after PHA stimulation was significantly lower for cells from the Formula group compared with the HM group. The SI at 16 weeks was higher than that produced at 6 weeks (22) for all groups. Because the SI did not differ significantly at 6 weeks among groups (22), our data suggest that limiting the dietary supply of LCP may inhibit the developmental increase in the ability of T cells to respond to stimulation. In agreement with a recent study in piglets (26), there was no significant difference in the SI between cells from the infants supplemented with LCP and those fed HM. Cells that are able to respond to proliferate are those that express the CD45RO phenotype (27), and the higher proportion and peripheral blood concentration of total CD45RO+ (both CD45RO+ and CD8+CD45RO+) cells' blood from infants fed the Formula+LCP, LCP, compared with the unsupplemented formula, suggest a possible contributor to this difference.

The amount of IL-4 produced after stimulation was less for the Formula+LCP compared with HM-fed infants. IL-4 is produced primarily by CD4+ cells and NK cells after stimulation. It is a pleiotropic cytokine best known for its ability to direct antigen-activated T cells to differentiate into T_H2 cells (28). Neonatal immune responses are dominated by T_H2 cytokines, and the development of the child's immune system toward adult-like T_H1 cytokine production progresses over time in both healthy (29) and atopic infants (30). The lower production of IL-4 and the subsequent higher IFN- γ /IL-4 ratio for cells from the Formula+LCP group, compared with the Formula group, supports a more predominant T_H1-type response. Previous studies have demonstrated that supplementing mothers at low risk for having a child with atopic disease with fish oil lowered T_H2-related cytokines in cord blood (31). Our results extend this work and suggest that supplementing the infant can also produce a T_H1-skewed response.

Despite the better stimulation response by the Formula+LCP infants, cells from both formula-fed groups produced about 40% less IL-2 than that produced by cells from the HM group. This suggests that the ability to produce a mitogenic T cell cytokine after stimulation is different in infants fed formula compared with HM. Additionally, the production of TGF- β after stimulation with PHA was 2 times higher for cells from formula-fed infants (although only reaching significance for the unsupplemented formula group). Although the differences did not reach statistical significance, the same trend was observed in cells from formula-fed infants at

6 weeks of age (22). TGF- β is an important regulatory cytokine produced by a variety of cell types. It is often classified as a T_H3 cytokine and can suppress T-cell function, including the production of IL-2 (32). Thus, to what extent the lower production of IL-2 by cells from the formula-fed infants represents a form of immunological immaturity or is the consequence of immune suppression by the overactivity of regulatory T cells remains to be established. Additionally, there was a higher proportion of B cells (those expressing markers important for antigen presentation, CD54 [I-CAM] and CD80 [costimulatory signal to T cells]) present poststimulation, suggesting a differential activation of humoral immunity in the formula-fed infants. By including a mitogen that stimulates B and T cells such as pokeweed mitogen or specifically stimulating T cells with anti-CD3 in future studies, this would assist in determining whether the primary effect of LCP is on the T or B cells.

Response to Food Proteins

Dietary antigens, particularly those derived from cow's milk, often are the first triggers of allergic responsiveness in infancy. We have defined tolerance as the response that PMBC make to prevent an "ex vivo" proliferative response to a dietary protein (SI or IL-2 production). Although there is considerable evidence that cytokines are involved in immunological responses and food allergies (33), data on cytokine production to food proteins by immune cells from healthy infants, with no history of allergy (both IgE and non-IgE mediated), are limited. In the present study 2 common food proteins were selected to compare the ex vivo cytokine response between the formula-fed infants. The protein source of the infant formulas used was an α -lactoglobulin-enriched whey protein that contained some BLG but no SOY.

Perhaps the most interesting finding from the postculture phenotyping was the effect of incubation with food proteins on the proportion of CD80+ cells. After stimulation with PHA only about 9% of cells postculture expressed CD80 (there were no detectable cells expressing CD80 preculture) and this did not differ among groups. However, compared with PHA stimulation, after stimulation with HSOY or BLG the proportion of CD80+ cells was about 4 times greater in the cultures from the breast-fed infants but about the same in the cultures from both formula groups. At 6 weeks of age we also observed a higher proportion of CD80+ cells after stimulation with SOY in the HM group (although it was only significantly higher than the Formula group) (23). CD80 is a molecule found on activated B cells and monocytes that provides a co-stimulatory signal necessary for T-cell activation and survival. The proportion of CD20+CD80+ cells did not differ among groups after stimulation with the food proteins and accounted for about 30% of the CD80+ cells in the formula-fed infants but only 9% of the cells in the breast-fed infants. This suggests that the much greater portion of CD80+ cells may be due to another population expressing CD80. Some of this could be monocytes/macrophages, but these represented a small portion of the cells postculture after stimulation with HSOY or BLG. CD80 is also reported to be expressed by T cells upon activation and provides a negative feedback signal that limits the immune responses (34). Although we did not measure CD3+CD80+ cells, the large increase in CD80+ cells in the breast-fed babies suggests that these proteins stimulated CD3+CD80+ cells and that this may be involved in the development of tolerance. After stimulation with BLG or SOY there was a higher proportion of CD16+CD11b+ (Mac-1, an adhesion glycoprotein) cells remaining in the cultures from formula-fed infants. The higher proportion of CD16+CD11b+ cells poststimulation with food proteins suggests that there may be an activation of NK cells after exposure to the food proteins.

SOY

Although there was a low proliferative response (defined by the amount of ^3H -thymidine uptake and IL-2 production) by all of the infants to SOY, cells from infants fed Formula+LCP produced less IL-2 and more TNF- α compared with cells from those fed the unsupplemented formula. No difference in the SI index or the production of IL-2 was observed in these infants at 6 weeks of age. However, similar to our previous report in infants age 6 weeks, infants fed Formula+LCP produced more TNF- α after SOY stimulation (23) and these levels did not differ from the amount produced by the HM group in the present study. Although the amount of cytokines that was produced was low compared with PHA, adding LCP to formula altered the amount produced ex vivo after incubation with SOY. TNF- α has a role in activating antigen-presenting cells and T cells and downregulating the differentiation of dendritic cells in the promotion of oral tolerance to food antigens (35). Allergic infants and those at risk for allergy have been shown to secrete reduced amounts of TNF- α when challenged with food allergens (36–38). It has been suggested that the inability to produce sufficient TNF- α contributes to the greater risk of developing food allergies (IgE mediated) in formula-fed infants with a family history of allergy (38). Thus, formula-fed infants may need to produce more TNF- α to promote oral tolerance because of the absence of HM components, including cytokines and other molecules with immunosuppressive activity (2,39), needed for the development of oral tolerance. The observed overproduction of TNF- α by SOY-stimulated PBMC from the Formula+LCP group may reflect the system's effort to compensate for the lack of HM-derived factors that are needed for the development of tolerance. This hypothesis is supported by the small but lower proliferative response by the cells from the Formula+LCP group compared with Formula and is consistent with the higher proportion of CD8+ cells remaining in culture after stimulation and the lower proportion of CD80+ (an activation marker expressed on activated T cells, B cells, macrophages, and dendritic cells (34)). It has been demonstrated that infants with food protein allergies have a lower number of CD8+ cells (35). Interestingly, there was a higher proportion of CD16+ CD11b+ cells (vs HM) and lower proportion of total CD11c+ cells and CD16+CD11c+ cells left after stimulation in the cultures from Formula+LCP. At 6 weeks of infant age a lower proportion of CD11c (an adhesion molecule expressed on NK cells, monocytes, granulocytes, and macrophages) and CD16+CD11c+ cells after PHA stimulation in infants fed the LCP-supplemented formula compared with the unsupplemented formula was also observed (22). Although these cell populations represent only a small portion of cells present after stimulation, it suggests that there was also a differential response by macrophage and/or NK cells after exposure to SOY.

BLG

BLG is only 1 of many proteins in milk, but it is reported to be a major allergen in cow's milk (40). Cells from all of the groups did not proliferate or produce IL-2 in response to BLG. However, similar to what we reported at 6 weeks of infant age (23) of consuming the diet, measurable amounts of TNF- α , IFN- γ , IL-6, and IL-10 were produced, confirming that the cells do respond. The higher production of TNF- α and IFN- γ by cells from formula-fed infants when exposed to BLG that we observed at 6 weeks of age (23) appeared to have disappeared by 16 weeks of age. Despite no difference in measured response between formula groups, there were a number of changes in the proportion of cells remaining postculture that suggested that the response was not identical

between these groups. Consistent with observations at 6 weeks (23), there was a lower proportion of B cells expressing CD54 (ICAM-1) in the cultures from LCP compared with unsupplemented infants. Interestingly, there was a significantly lower proportion of CD20+CD54+ cells after BLG stimulation for LCP-supplemented compared with unsupplemented formula-fed infants. ICAM-1 is an inducible cell adhesion glycoprotein that is expressed on activated monocytes and B cells (critical to the inflammatory response and participates in antigen-specific T-cell activation) (41).

CONCLUSIONS

The small sample size and large interindividual variation in many of the measures may have limited our ability to detect changes. Despite this, compared with feeding an unsupplemented formula, exclusively feeding infant formula supplemented with LCP resulted in a higher proliferative response to a polyclonal T-cell mitogen that may have been supported by a more predominant production of a $\text{T}_\text{H}1$ -type cytokine pattern and a higher proportion of antigen-mature lymphocytes (CD45RO+). Cells from infants fed formula supplemented with LCP compared with those consuming formula not supplemented with LCP produced less IL-2 in response to SOY, possibly because of the higher production of TNF- α and the higher proportion of CD8+ cells observed postculture. Compared with cells from infants fed HM both formula groups produced less IL-2 after stimulation with PHA, and exposure to food proteins resulted in a lower proportion of CD80+ cells and a higher proportion of CD54+ cells, suggesting that formula-fed infants respond differently to food proteins. The results of this clinical trial add to our understanding of the role of LCP in the response of immune cells to immune challenges and in the tolerance to food proteins in healthy infants.

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