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ORIGINAL ARTICLE

Breastfeeding's protection against illness-induced anorexia is mediated partially by docosahexaenoic acid

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Objective: To test whether breastfeeding's protection against anorectic responses to infection is mediated by $n-3$ fatty acids' attenuation of interleukin (IL)-1β and tumor necrosis factor (TNF)α.
Design: Experimental and observational studies.
Setting: A hospital-based study was conducted.
Subjects: Five groups of infants were followed; three in the experimental and two in the observational study.
Methods: Breast-fed- (BF-1), DHA-supplemented formula- (SFF-1), and non-DHA-supplemented formula-fed (FF-1) infants were studied before and after immunization against diphtheria, tetanus, pertussis and haemophilus influenzae type b. Pre- and post-immunization energy intakes (EI) and serum IL-1β and TNFα were measured. The two other groups, breast-fed (BF-2) and formula-fed (FF-2) infants with pneumonia were followed throughout hospitalization. EI, IL-1β and TNFα were measured at admission and discharge. Baseline erythrocyte fatty acid contents were determined.
Results: Both cytokines increased following immunization in all feeding groups. Post-immunization reductions in EI of SFF-1 infants ($-11.8\pm5\%$, $CI_{95}=-23.3$, 1.4%, $P=0.07$) were intermediate to those observed in BF-1 ($-5.2\pm4.2\%$, $CI_{95}=-15.2$, 5.9%, $P=0.27$) and FF-1 infants ($-18\pm4.4\%$, $CI_{95}=-29\%$, -5.4%, $P=0.02$). In the observational study, TNFα (17.2 ± 8.3 vs 3.4 ± 3.0 ng/l, $P=0.001$) and decreases in EI (-31 ± 43 vs $-15\pm31\%$, $CI_{95}=-34\%$, 0.001%, $P=0.056$) were greater in FF-2 than in BF-2 infants at admission. Breastfeeding duration was associated positively with docosahexaenoic acid (DHA) erythrocyte contents, and negatively with admission TNFα. Decreases in EIs were associated with IL-1β and TNFα concentrations.
Conclusion: Reductions in EI following immunologic or infectious stimuli were associated with increases in IL-1β and TNFα. Those reductions were attenuated by breastfeeding, and mediated in part by tissue DHA.
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Introduction

Breast-fed infants receive bioactive factors from human milk that putatively benefit them (Goldman *et al*., 1982). Among human milk's bioactive factors are the essential fatty acids linoleic and α-linolenic and their metabolites arachidonic (AA), docosahexaenoic (DHA) and eicosapentaenoic acids (EPA). These are assumed to be in physiologically balanced proportions (Sellmayer and Koletzko, 1999). Among the benefits ascribed to breast feeding, and presumably, in part, to human milk constituents, is an attenuation of anorectic responses associated with infectious illness. Infants who are

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fed various types of animal or artificial milks and solid foods (Hoyle *et al*., 1980; Brown *et al*., 1990) or are predominantly formula fed (López-Alarcón *et al*., 2002) suffer greater reductions in energy intake than do those who are breastfed. The mechanisms underlying this reported benefit are not understood.

The pro-inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα) appear to influence many of the metabolic responses to infections, including appetite loss (Klasing, 1988). Among the mechanisms through which cytokines may act is the release of eicosanoids derived from polyunsaturated fatty acids, mainly AA. Published reports claim that derivatives of α-linolenic acid (DHA and EPA) produce eicosanoids with less potent bioactivity than those that are produced from AA. DHA and EPA appear to ameliorate cytokine associated effects because they elicit lowered cytokine production (Blok *et al*., 1996; Calder, 1997).

Studies of healthy adults before and during dietary vegetable and fish oil supplementation reported the reduced release of eicosanoids, IL-1β and TNFα following an *in vitro* lipopolysaccharide (LPS) challenge of blood monocytes collected during the supplementation phase (Endres *et al*., 1989; Caughey *et al*., 1996). These and related observations in human and animal studies (McCarthy *et al*., 1985; Socher *et al*., 1988; Michie *et al*., 1989) also suggest that DHA and EPA supplementation may attenuate anorectic responses to infectious illness.

We have reported previously results of a study designed to evaluate anorectic responses to an immunologic challenge, that is, to the first or second diphtheria, tetanus, pertussis and haemophilus influenzae type b (DPTH) immunization dose in breast- and formula-fed infants (López-Alarcón *et al*., 2002). Results demonstrated that although post-immunization energy intakes of breast-fed infants remained unchanged from baseline, those of formula-fed infants decreased significantly (mean 12%). Reductions in energy intake were related to whether infants received the first or second DPTH dose. Post-immunization mean energy intakes decreased 18% (range 13–25%) in formula-fed infants who received the first DPTH dose. No reductions were noted in formula-fed infants who received the second DPTH dose (López-Alarcón *et al*., 2002).

This paper reports the results of studies designed to investigate mechanisms underlying breast feeding's protective effects against anorectic responses to immunologic challenges and infectious episodes. Anorectic responses of infants fed a formula supplemented with DHA were compared with previously published responses of breast- and non-DHA formula-fed infants who received the first DPTH dose. Investigations of the DHA-supplemented formula group overlapped partially with the previously reported investigations (López-Alarcón *et al*., 2002). Also reported in this paper are results of an observational study of infants hospitalized with pneumonia.

Methods

This study was part of a larger investigation designed to evaluate potential mechanisms that account for anorectic responses to infection. Methods were described in detail elsewhere (López-Alarcón *et al*., 2002). Briefly, infants were selected from a pediatric population in Mexico city who received their primary care through the Mexican Institute of Social Security hospital system (IMSS). The right of all subjects to leave the study at any time without prejudicing their health care was explained carefully. Human use approvals were obtained from the IMSS Ethics Committee and Cornell University's Human Subject Use Institutional Review Board.

Subjects and procedures

DHA formula supplementation study. Twenty-one infants who had not received their first DPTH immunization were studied; six were breastfed (BF-1), five received a non-DHA supplemented formula (FF-1) and 10 were fed a DHA-supplemented formula (SFF-1). All non-breastfed infants had been weaned previous to enrollment and caretakers of ten infants agreed to alter the infant's formula to one supplemented with DHA (Aptamil 1 Milupan Powder, Nutricia, Kasdorf, Argentina, containing 0.17–0.21 g of DHA per 100 g of fat). Infants remained on this formula for at least 1 month before DPTH immunization and are referred to below as the SFF-1 group. Infants in group FF-1 were fed a similar, but non-DHA supplemented formula (Nutrilon Premium, Nutricia, Kasdorf, Argentina), and the breast-fed infants (BF-1) never had received formula (López-Alarcón *et al*., 2002).

Group SFF-1 underwent the same protocol as described previously (López-Alarcón *et al*., 2002). Briefly, subjects were hospitalized for 48 h to measure energy intakes 24 h before and 24 h after DPTH immunization. Peripheral blood samples were obtained at admission and several times after immunization to determine serum IL-1β and TNFα concentrations. Fatty acids profiles were measured in erythrocytes obtained from baseline blood samples.

There were no statistical differences in mean ages among the three feeding groups (2.9 ± 0.9, 2.6 ± 0.9 and 2.2 ± 0.4 for BF-1, FF-1 and SFF-1, respectively; $P = 0.17$). None of the infants received solid foods. Two infants in the SFF-1 group had weight-for-length values below -2.0 standard deviations of the World Health Organization/National Center for Health Statistics reference.

Observational pneumonia study. Seventeen formula-fed and 10 breast-fed infants were recruited at admission in the emergency room with a diagnosis of pneumonia. All infants who were formula-fed at enrollment had been breast fed previously for varying durations. All were younger than 1 year of age. All were followed throughout hospitalization. Mothers were asked to stay in the hospital to feed and

provide general care to their infants. Peripheral blood samples were obtained at admission and at discharge to determine serum IL-1β and TNFα concentrations. Fatty acids were measured in erythrocytes obtained at admission. Energy intakes were measured daily.

Energy intake determinations. The 24-h test weighing procedure was used to estimate energy intakes. Formula, liquids other than human milk and solid foods were weighed before and after each feeding. Weight differences were assumed to represent the amount of food consumed. Samples of duplicates of all foods consumed other than human milk and artificial formulas were combusted for calorimetric determinations of energy content (Parr Instruments Co Model 1266, Moline, Ill). Energy intakes from formula were derived from information reported by the manufacturer. Energy content of human milk was assumed to be 0.65 kcal/ml, the average of energy provided by human milk in developing countries (Jensen, 1995). The volumes of human milk consumed were estimated by weighing infants before and after each feeding on an electronic balance with a precision of 1 g (model 3862MP8, Sartorius, Gottingen, FRG). Weight differences were assumed to represent the amount of milk consumed, that is, weight differences were not corrected for respiratory losses.

Laboratory determinations. For the DHA-supplementation studies, an indwelling catheter was placed in a peripheral vein at admission to obtain serial blood samples. The catheter was kept open with a heparin lock to avoid coagulation. Blood samples collected at admission were analyzed for basal IL-1β and TNFα concentrations. Serial blood samples were collected at 90, 180 and 240 min following immunization, and when a rise in body temperature was detected to identify cytokine surges. The highest observed post-immunization serum IL-1β and TNFα concentrations were used in the analyses of post-immunization response. For the observational-pneumonia study peripheral blood samples were obtained at admission and at discharge.

All blood samples were centrifuged within 30 min of collection. Erythrocytes were separated from baseline blood samples to determine fatty acid profiles. Plasma was removed immediately after centrifugation. Erythrocyte pellets were washed twice with 0.9% saline and stored at -20°C until analysis. Total fat was extracted from 0.5 g of frozen erythrocytes with 4.5 ml of isopropanol; butylated hydroxytoluene was added as an antioxidant (10 μg/ml final volume). Tubes were shaken for 15 min and centrifuged for 5 min at 1200 *g* at 4°C, the clear supernatant was poured off and dried at 60°C under a stream of nitrogen. Fatty acids were methylated with 3N methanolic HCl. The methyl esters were extracted from the mixture with hexane and analyzed by gas chromatography (Hewlett Packard 5890 Series II. Avondale, PA, USA) using a flame ionization detector, and a 100 m $\times$ 0.2 mm inside diameter fused silica column coated with 0.2 μm CP Sil 88 (Chrompac. The Netherlands). Fatty acids were identified from chromatograms by comparison with known standards. Fatty acid concentrations were calculated by using a response factor of standard fatty acids. Heptadecanoic acid was added to the samples as an internal standard. Results were expressed as weight percentages of total fatty acids with carbon chain lengths from 14 to 24 carbon atoms.

Serum IL-1β and TNFα concentrations were determined in duplicate with an ultra-sensitive ELISA commercial kit designed to read in the range of 0.31 and 20 ng/l (Cytoscreen, Immunoassay, Biosource International, Inc. Camarillo, CA, USA).

Data analyses. Minitab statistical software (Minitab 14, State College, PA, USA) was used for statistical analyses. Differences were considered significant at $P \leqslant 0.05$. Data are presented as unadjusted means and standard deviations. Values of energy intake were normalized per body weight (kJ/kg day). Fatty acid content was expressed as the weight percentage of total fatty acids. Non-normally distributed values were transformed logarithmically for statistical analyses.

For the DHA-supplementation studies pre- and post-immunization energy intakes and serum cytokine concentrations were compared within each group by paired *t*-tests. One-way analysis of variance was used to compare fatty acid profiles obtained at baseline, pre- and post-immunization energy intake differences and serum cytokines concentrations in the BF-1, FF-1 and SFF-1 groups.

For the observational study, energy intakes and serum cytokine concentrations at admission and discharge were compared within each group by paired *t*-tests. Fatty acid profiles obtained at baseline and differences in energy intakes and serum cytokines concentrations between admission and discharge of BF-2 and FF-2 infants were compared by Student's *t*-tests.

Pearson and univariate correlation analyses were conducted to assess relationships among duration of breastfeeding, erythrocyte fatty acid contents, serum cytokine concentrations and energy intakes. Relative duration of breastfeeding was estimated by dividing the time an infant was breast-fed by the infant's age.

Multiple regression analyses were used in the DHA supplementation and observational studies to evaluate associations among decreases in energy intake, erythrocyte specific fatty acid contents and increases in cytokine concentrations; feeding mode was treated as a covariate.

Results

Fatty acid profiles

Forty-eight infants were studied: 21 infants in the DHA-supplementation study and 27 in the observational study. In

Table 1 Erythrocyte fatty acid profile, stratified by feeding mode, in infants exposed to the first dose of DPTH immunization[1] and infants hospitalized with pneumonia[2]

	Experimental[1]			*Observational*[2]	
Fatty acid[3]	BF	FF	SFF	BF	FF
Monosaturated	19.6±1.1	23.2±1.2[a,b]	17.8±1.1	14.51±0.75	15.20±1.49
Polyunsaturated	30.8±1.1	25.7±1.2[a,b]	31.9±3.3	32.68±2.07	28.93±4.61[a]
Linoleic acid	12.6±1.2	14.3±1.1	11.5±1.8	13.08±2.38	12.45±2.57
Linolenic acid	0.28±0.51	0.27±0.8	0.85±1.0	0.17±0.40	0.09±0.14
EPA	0.40±0.3	0.20±0.3	0.61±0.31	0.17±0.35	0.09±0.25
DHA	4.58±1.6	0.70±1.9[a,b]	5.37±2.26	3.89±0.7	2.34±0.9[a]
AA	11.8±1.3	10.0±1.3	13.3±3.7	14.90±1.8	13.43±2.3
Total *n*–6	24.8±1.1	24.4±1.6	24.9±1.2	28.48±1.79	26.42±4.38
Total *n*−3	5.3±1.7	1.2±2.2[a,b]	6.6±1.7	4.20±0.84	2.51±1.02[1]

Abbreviations: AA, arachidonic acid; ANOVA, analysis of variance; BF, breast-fed; DHA, docosahexaenoic acid; DPTH, diphtheria, pertussis, tetanus and haemophilus influenzae type b; EPA, eicosapentaenoic acid; FF, formula-fed; SFF, DHA-supplemented formula-fed.
Mean±s.d.
[3]Percentage of total fatty acids. ANOVA.
[a]Compared with BF.
[b]Compared to SFF in the DHA-supplementation study. Student's *t*-test.
[a]Compared with SFF in the pneumonia observational study.

general, FF-1 and FF-2 infants presented lower proportions of PUFA, total *n*−3 fatty acids, and DHA than did BF-1 and BF-2 infants. Fatty acids profiles of SFF-1 infants were similar to those of BF-1 and BF-2 infants, but differed from those of FF-1 and FF-2 counterparts (Table 1).

DHA-formula supplementation study

Baseline serum IL-1β (0.84.±4.62, 0.61±2.53 and 0.71±2.24 pg/ml), and TNFα (8.83±3.56, 8.26±2.19 and 5.46±1.93 pg/ml) concentrations were similar among all three groups, that is, BF-1, FF-1 and SFF-1. Post immunization serum IL-1β (139±73%, $P<0.01$) and TNFα (61±57%, $P<0.01$) concentrations increased significantly from pre-immunization values. Serum IL-1β tended to increase least among BF-1 infants compared with FF-1 and SFF-1 infants (Figure 1), but increases did not differ statistically among feeding groups. Post-immunization concentrations of serum IL-1β correlated positively with post-immunization concentrations of TNFα ($r=0.70$, $P=0.001$) when results from all three feeding groups were pooled. Feeding mode did not account for a statistically significant portion of the observed variation in responses.

Relative changes in energy intakes following immunization are presented in Figure 2. Only FF-1 decreased significantly their energy intake (−18±4.4%, $CI_{95}=-29\%$, −5.4%, $P=0.02$); decreases in SSF-1 (−11.8±5%, $CI_{95}=-23.3\%$, 1.4%, $P=0.07$) were intermediate to those observed in FF-1 and BF-1 (−5.2±4.2%, $CI_{95}=-15.2\%$, 5.9%, $P=0.27$) A multivariate analysis indicated that compared with BF-1 infants post-immunization energy intakes fell only in FF-1 infants after adjusting for post-immunization increases in serum TNFα concentration and erythrocytes DHA/AA ratio (Table 2), that is, as observed in BF-1 infants no significant decrease in energy intakes was detected among the SFF-1 group.

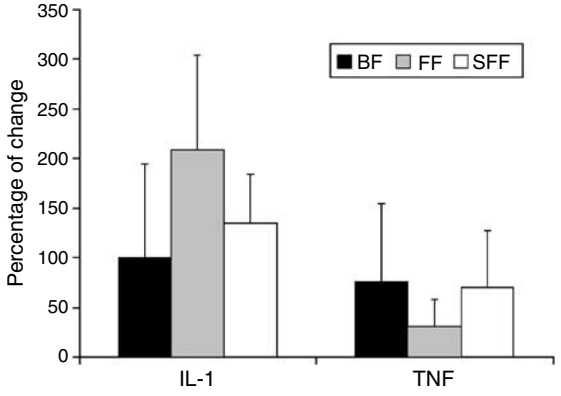

Figure 1 Relative changes of IL-1β and TNFα serum concentrations after the first dose of DPTH immunization in infants BF, non-DHA supplemented FF and DHA-supplemented formula-fed SFF were not different among feeding groups ($P=0.46$ and 0.53, respectively).

Observational pneumonia study

Twenty-seven infants with pneumonia were followed. Ten were breast-fed and 17 were formula-fed at admission. BF-2 infants were younger than FF-2 infants. No differences were detected in the feeding groups' nutritional status (by weight/age, length/age, weight/length and hemoglobin concentration) nor in illness severity as assessed by the duration of hospitalization (days), leukocytes count or the proportion of infants with positive C-reactive protein on admission (Table 3). All infants were treated with antibiotics. None required assisted ventilation or presented with severe complications such as cardiac failure.

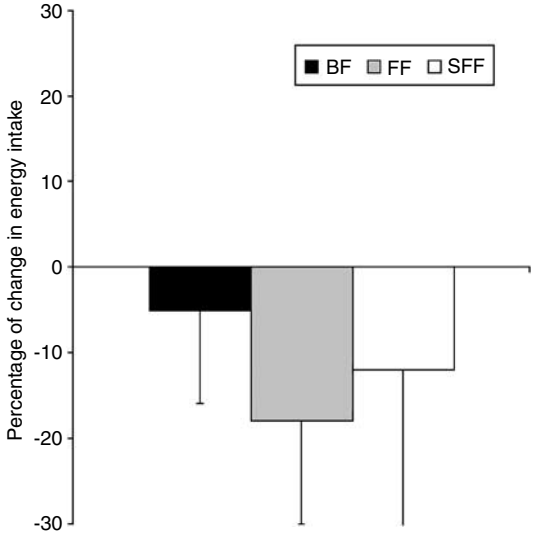

Figure 2 Relative change of energy intake after the first dose of the DPTH immunization. All three groups decreased their energy intake after immunization. Decreases of SFF infants were intermediate to those presented by BF and FF infants. Decreases in energy intake of FF infants were significantly different than those in BF infants ($P=0.03$); but decreases of SFF were not different than those of BF and FF infants ($P=0.22$).

Table 2 Effect of erythrocytes fatty acids, cytokine increases, and feeding mode on changes in energy intake after the first administration of DPTH immunization[a]

Variable	*Coefficient*	*S.E.*	P-*value*
Changes in energy intake (kJ/kg day)			
Constant	0.005	0.029	0.86
Changes in TNFα (ng/l)	−0.140	0.072	0.07
Erythrocytes DHA/AA ratio	−0.085	0.061	0.18
FF-1[b]	−0.088	0.039	0.04
SFF-1[b]	0.028	0.025	0.26

Abbreviations: AA, arachidonic acid; BF-1, breast-fed; DHA, docosahexaenoic acid; DPTH, diphtheria, pertussis, tetanus and haemophilus influenzae type; bFF-1, non-DHA supplemented formula fed; TNF, tumour necrosis factor.
[a]General linear model, logarithmic transformation was used.
[b]Compared to BF infants.

Energy intakes were 25% ($P=0.001$) lower and serum IL-1β concentrations were 20% higher ($P=0.001$) in the acute phase of infection (admission) compared with values observed in the convalescence phase (discharge). Admission and discharge TNFα concentrations did not differ ($P=0.40$). When subdivided by feeding groups, FF-2 infants had significantly lower energy intakes at admission than during convalescence, that is $-31\pm43\%$ ($CI_{95}=-43.3$, -16.4%, $P=0.001$), whereas in BF-2 infants such difference did not reach statistical significance (-15 ± 31, $CI_{95}=-30.1$, 2.6%, $P=0.08$). Within variation in energy intake was borderline higher in FF-2 than in BF-2 infants ($P=0.056$). FF-2 infants presented also significantly higher serum IL-1β at admission

Table 3 Nutritional status and illness-severity characteristics of infants hospitalized with pneumonia[a]

	BF (n = 10)	*FF* (n = 18)	P-*value*
Age, month	3.20 ± 2.9	6.01 ± 3.9	0.04
Nutritional status[b]			
Weight for age	−0.99 ± 1.3	−0.96 ± 1.2	0.95
Length for age	−0.79 ± 0.9	−0.78 ± 1.5	0.99
Weight for length	−0.58 ± 1.2	−0.70 ± 1.0	0.79
Illness severity			
Hospitalization, days	3.9 ± 1.8	3.9 ± 1.6	0.96
Temperature, Celsius	36.9 ± 1.01	37.7 ± 0.86	0.07
Hemoglobin, g/dl	11.6 ± 2.2	10.9 ± 1.6	0.38
Leucocytes, cell/mm[c]	12.150 ± 3.960	13.810 ± 3.410	0.27
C-reactive protein[c]	80 (8)	67 (10)	0.46

Abbreviations: BF, breast-fed, FF, non-DHA supplemented formula fed.
[a]Mean ± s.d.
[b]Z-scores (NCHS reference).
[c]Percentage (counts).

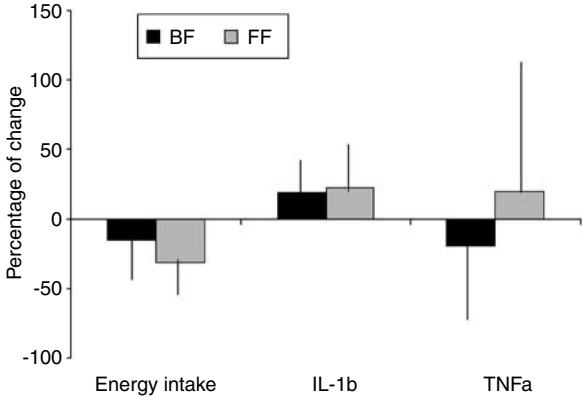

Figure 3 Relative differences of energy intake, IL-1β and TNFα concentrations at admission compared with discharge of infants hospitalized with pneumonia. Energy intake decreased, and TNFα increased, more in FF than in BF infants ($P=0.05$).

than at discharge ($22.2\pm25.8\%$, $CI_{95}=7.5$, 38.9%, $P=0.005$); equivalent values in BF-2 infants were not statistically significant ($16.7\pm39\%$, $CI_{95}=-6.6\%$, 45.8%, $P=0.15$). Within variations in IL-1β were not different between FF-2 and BF-2 infants ($P=0.35$) (Figure 3).

Despite serum TNFα concentrations at admission and discharge did not differ, admission TNFα concentrations were higher in FF-2 than in BF-2 infants (17.18 ± 8.3 vs 3.41 ± 3.0 ng/l, $P=0.001$). Serum TNFα concentrations remained higher at discharge in FF-2 than in BF-2 infants (14.45 ± 7.8 vs 3.10 ± 4.8 ng/l, $P=0.02$). Relative changes of TNFα between admission and convalescence differed significantly between feeding groups ($20\pm113\%$ vs $-18.6\pm52\%$, for FF-2 and BF-2 respectively, $P=0.05$) (Figure 3).

Univariate correlations demonstrated that relative duration of breastfeeding was associated positively with the

Table 4 Predictors of decreases in energy intake (kJ/kg day) in infants hospitalized with pneumonia

	Coefficient	*Standard error*	P-*value*	r^2
			<0.01	0.79
Intercept	105.52	50.65	0.06	
Solid food, yes	−41.44	28.82	0.17	
Hospitalization, days	−37.93	7.32	<0.01	
IL-1β, (ng/l)	−54.39	30.52	0.09	
EPA, (%)	92.56	45.86	0.06	
DHA, (%)	−16.74	15.88	0.31	
FF-2[a]	−71.76	37.13	0.07	

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acids; FF, non-DHA supplemented formula fed; IL-1, interleukin-1.
[a]Compared with BF-2.

proportion of erythrocyte DHA ($r=0.60$, $P=0.005$), and negatively with serum TNFα concentration at hospitalization ($r=-0.48$, $P=0.01$) and discharge ($r=-0.51$, $P=0.007$). Decreases in energy intakes were associated weakly with serum IL-1β ($r=-0.35$, $P=0.07$ and $r=-0.41$, $P=0.03$) and TNFα ($r=-0.37$, $P=0.06$ and $r=-0.39$, $P=0.04$) concentrations at admission and discharge, respectively.

In multivariate analyses, a model including decreases in energy intakes as the response variable demonstrated a negative association with formula feeding after adjusting for serum IL-1β concentrations at admission, duration of hospitalization, EPA and DHA erythrocyte contents, and whether solid foods was included in the infant's diet (Table 4). In a second model, decreases in energy intake were associated negatively with serum IL-1β and TNFα concentrations at admission, but only in infants with positive C-reactive protein ($R=0.65$, $P=0.002$). C-reactive protein concentrations did not differ between BF and FF infants ($P=0.46$).

Discussion

These findings are consistent with the view that anorexia accompanying infections in young infants is mediated by increases in circulating concentrations of pro-inflammatory cytokines. Breastfeeding's protective effect against such reduction in energy intake appears to be mediated, in part by $n-3$ polyunsaturated fatty acids. However, this protection can be attenuated by the challenge's severity.

The association between reduced energy intakes and serum TNFα was demonstrated previously in animal models. Inhibition of food intake that persisted for 2 days was reported in mice given exogenous TNFα by either bolus injection or continuous infusion (Socher *et al*., 1988). Few studies of comparable design have been conducted in humans. In one, an almost complete cessation of food intake was reported following the intravenous infusion of TNFα in adult elective surgical patients (Michie *et al*., 1989). The association between serum IL-1β and reduced energy intake also was described in animal studies. Intracerebral injections of IL-1β decreased food intake by 30–45% in rats (McCarthy *et al*., 1985). Similar studies of TNFα or IL-1β, however, have not been reported in infants, either in humans or in animal models. Nonetheless, increases in cytokines and reductions in energy intakes observed in these investigations are consistent with the limited available animal and human studies and other reports associating proinflammatory cytokines to anorectic response to infections (McCarthy *et al*., 1985; Socher *et al*., 1988; Michie *et al*., 1989).

Interestingly, in the DHA-supplementation study, IL-1β and TNFα increased after the DPTH immunologic challenge, but those increases were similar among all three feeding groups. This suggests that DHA supplementation had no effect on these cytokine responses. This interpretation is supported by observations made in the pneumonia study. Although concentrations of IL-1β and TNFα were higher in FF-2 than in BF-2 infants at admission, no statistically significant associations were detected among these cytokines and the proportion of erythrocyte DHA. This also suggests that the proportion of DHA in tissues did not affect cytokine production during the index infectious episode. It is possible, however, that sample sizes limited the study's ability to detect differences.

The similarity in cytokine responses among feeding groups in our studies are in agreement with observations reported by Granot *et al*. (2000). These authors compared IL-1β and TNFα *in vitro* production by immunocompetent cells obtained from breast- and formula-fed infants. They detected no differences in the release of IL-1β and TNFα in whole blood culture between groups after *in vitro* stimulation with LPS. These and other reports also suggest that eicosanoids derived from $n-3$ fatty acids have less proinflammatory potency than those derived from $n-6$ fatty acids (Blok *et al*., 1996). We thus may speculate that differences in fatty acids do not influence cytokine production, but instead may affect eicosanoid responses. Decreases in energy intake after an infectious or immunologic challenge may not depend on the amount of cytokines released, but on the type of eicosanoids produced. Another explanation is a possible protection conferred by the DHA-derived resolvins, whose role is a rapid resolution of inflammation (Serhan, 2006).

It also is noteworthy that although no differences in cytokine responses were detected among the groups in the DHA-supplementation study, the SFF-1 group presented an intermediate anorectic response relative to BF-1 and FF-1 infants. This supports the possibility that long-chain polyunsaturated fatty acids in human milk may account in part for the differential anorectic responses of BF and FF infants. DHA-supplemented and non-supplemented formulas contained similar amounts of linoleic, α-linolenic and AA, and both formulas were EPA free but similar in all other components. This design feature supports the hypothesis that DHA is among the mediators of the anorectic response to infections. This finding also is supported by

the pneumonia observational study in which we found that anorectic responses to infection were related to tissue DHA contents. That is, the longer the relative duration of breastfeeding, the greater the erythrocyte DHA contents and the lower the observed concentration of TNFα; secondarily, the higher the concentration of serum TNF at admission, the greater the observed reductions in energy intake.

These observations collectively support the possibility that dietary $n-3$ fatty acids participate in the attenuation of anorectic responses to immunologic and infectious stimuli. However, a more detailed understanding of the complex relationships between the ontogeny of the breast- and formula-fed infants' immune systems and the utilization of $n-3$ fatty acids is needed, especially in light of the increased use of these biologically active molecules in infant formulas. Such statement becomes essential after the release of recent evidence about a protective role of DHA on the nutritional status of infants. According with a recent study, DHA-supplemented neonates presented increases in body mass (50 g) and fat mass (70 g) during an episode of sepsis, whereas comparable neonates that received placebo did not demonstrate any change; even though both groups received comparable amounts of energy (López-Alarcón *et al*. 2006).

It is also worthwhile to contrast anorectic responses of breastfed infants challenged by immunization or pneumonia. Unlike the near-total protection of breastfeeding against reductions in energy intakes among those challenged by immunization, protective responses appeared to be partially overwhelmed by the severity of the challenge presented by pneumonia. Importantly, however, reductions in energy intake also were less pronounced among infected breastfed infants compared to formula-fed counterparts. The observation that decreases in energy intake were noted only among infants with elevated C-reactive protein, a marker for severity of illness (Chiesa *et al*., 2003) is also consistent with this view.

Finally, it has been reported that mothers who breastfed their infants may have better bonding with their infants and consequently might spend more time feeding them; if this is true a longer suckling period would result in a better energy intake. However, we measured the duration in minutes of each feeding episode (data not shown). There was no difference in the mean duration at admission ($P=0.30$) and discharge ($P=0.47$) between groups.

In summary, reductions in energy intake following infectious or immunologic stimuli in infants are associated with peripheral increases of the pro-inflammatory cytokines IL-1β and TNFα. Reductions in energy intake are attenuated in BF infants, but this attenuated response is dependent on the magnitude of the stimulus and mediated, in part, by DHA.

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