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Fatty Acid Formula Supplementation and Neuromotor Development in Rhesus Monkey Neonates

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ABSTRACT

Docosahexaenoic acid (DHA) is an omega-3 fatty acid that is highly concentrated in CNS tissues. Although breast milk contains the fatty acids DHA and arachidonic acid, infant formulas marketed in North America do not contain these nutrients. The potential deleterious effects of rearing infants with formulas devoid of these nutrients was assessed by comparing nursery-reared rhesus macaque infants (*Macaca mulatta*) fed standard formula with infants fed standard formula supplemented with physiologically relevant concentrations of DHA (1.0%) and arachidonic acid (1.0%). Neurobehavioral assessments were conducted on d 7, 14, 21, and 30 of life using blinded raters. The 30-min assessment consisted of 45 test items measuring orienting, temperament, reflex capabilities, and motor skills. Plasma concentrations of DHA in standard formula-fed infants were significantly lower than those fed supplemented formula or mother-raised (breast-fed) infants; however, infants fed the supplemented formula exhibited higher arachidonic acid levels than either mother-reared infants or infants fed standard formula. Infant monkeys fed the supplemented formula exhibited stronger orienting and motor skills than infants fed the standard formula, with the differences most pronounced during d 7 and 14. This pattern suggests an earlier maturation of specific visual and motor abilities in the supplemented infants. Supplementation did not affect measures of activity or state control, indicating no effect on temperament. These data support the assertion that preformed DHA and arachidonic acid in infant formulas are required for optimal development. **(*Pediatr Res* 51: 273–281, 2002)**

Abbreviations

DHA, docosahexaenoic acid
AA, arachidonic acid
BHT, butylated hydroxytoluene
LC-PUFA, long-chain polyunsaturated fatty acid
NBAS, Neonatal Behavioral Assessment Scale
NICHD, National Institute of Child Health and Human Development
NIAAA, National Institute on Alcohol Abuse and Alcoholism

There has been great interest in understanding the role of the long-chain fatty acids in promoting optimal cognitive and neurologic development. Although these nutrients are present in all mammalian breast milks, commercially available infant formulas do not support the tissue requirement for these nutrients in developing infants (1–4). The LC-PUFAs AA (20:4 n-6) and DHA (22:6 n-3) are selectively concentrated in the cellular membranes of neural and retinal tissues (5). Although human infants are able to synthesize AA and DHA from the precursor molecules linoleic acid (18:2 n-6) and α-linolenic acid (18:3 n-3) (6–8), the rate of synthesis appears to be inadequate to meet the developmental demands of infants (7). In addition, uptake of DHA into brain tissue is more efficient than formation of DHA from α-linolenic acid (9). Human breast milk from women in all countries studied delivers both preformed AA and DHA to the developing infant (10). In contrast, commercially available infant formulas in the United States are virtually devoid of DHA and AA but contain the precursor fatty acids 18:2 n6 and 18:3 n3 (11). Thus, formula-fed infants exhibit lower levels of AA and DHA in blood and brain tissues relative to their breast-fed counterparts [blood (12, 13); brain (1–4)]. These findings have prompted efforts to determine the physiologic benefits of LC-PUFA, particularly DHA, in infant nutrition and development.

Several studies have shown that breast-fed infants exhibit superior performance relative to formula-fed infants on visual task performance (14), psychomotor development (15), intel-

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lectual capability (16, 17), and achievement scores (18). However, it is difficult to isolate a deficiency of DHA and AA in formulas as the causal variables in these studies. Comparisons between breast-fed and formula-fed infants include a large number of confounding variables including absence of other nutrients and biologically active factors in formula (19), and differences in maternal factors including socioeconomic status, education, and maternal infant interactions (20, 21). In contrast, randomized supplementation trials have been able to isolate AA and DHA as critical variables. These trials have demonstrated that LC-PUFA supplementation can support plasma concentrations of AA and DHA at levels that are comparable to those observed in breast-fed infants and higher than standard formula-fed infants (11, 22, 23).

Several randomized clinical trials have also provided evidence that LC-PUFA supplementation improves cognitive development and visual function. Studies of both preterm and term infants have reported improvements in mental developmental scores assessed by the Bayley Scales of Infant Development [(24, 25) term infants only; however, findings of no effects of supplementation have also been reported, see (26)], the Brunet-Lezine Test (27), the Infant Planning Test (28), and shorter look duration to novel stimuli (29). Several studies have also demonstrated that these improvements persist beyond the period of supplementation (25, 28, 30) although some do not (31). Improvements in visual acuity and function resulting from LC- PUFA supplementation in both term and preterm infants have also been described (14, 23, 32, 33) and have been supported by meta-analyses (34, 35). Outcome measures used to assess cognitive development and visual acuity in infants often have high inherent variability (36, 37). Thus, the studies that have described no differences (38, 39) must be carefully examined for adequate power, control of the precursor fatty acid composition, and environmental variances of the study populations (36, 40). Finally, the concentrations of DHA and AA used for supplementation in the negative studies should be evaluated to determine whether they were adequate (36, 40).

One particularly valuable approach that reduces variability in the study population has been the use of nonhuman primates. Among monkeys deprived both pre- and postnatally of LC-PUFAs, dietary depletion of n-3 fatty acids causes lower DHA levels in both retina and brain (41). These n-3 fatty acid–deficient animals exhibited deficiencies in visual physiology (41, 42), as well as behavioral effects. The n-3 fatty acid–deficient monkeys exhibited polydipsia, polyuria, and more locomotor stereotypies compared with control animals (43, 44). Most previous nonhuman primate models have invoked n-3 fatty acid dietary deficiency throughout the life span, including during pregnancy. In contrast, we conducted this study using a controlled animal model that more closely resembles patterns of LC-PUFA intake in formula-fed human infants; prenatal LC-PUFA sufficiency with postnatal LC-PUFA deficiency. In this study we provided a higher amount of supplementation than any prior human infant study [*i.e.* 0.36 wt % DHA and 0.72 wt % AA (25)]. By supplementing the formula with 0.9 wt % DHA and 1.0 wt % AA, we were able to provide a level of supplementation that is physiologically relevant and ecologically valid. For example, this level of supplementation provides DHA and AA levels similar to those found in Japanese mothers' milk (45), and to DHA levels in macaque colostrum (46). Because our groups of animals were small, and the dependent measures had high variability, we endeavored to use concentrations of DHA and AA in the high end of the range of concentrations found in humans. By isolating DHA and AA as variables in two formulas and by rigorously controlling these environmental and nutritional conditions, we are able to test the hypothesis that a DHA and AA insufficiency occurring selectively during the postnatal period would affect neonatal behavioral outcomes.

We used a neurodevelopmental battery specifically designed to test nonhuman primate infants (47) to assess the effects of formula supplementation on neonatal behavioral development. The instrument used, a nonhuman primate adaptation of the NBAS (48), is sensitive to a variety of intrinsic and environmental factors in primate neonates (47, 49–53). The advantages of studying nonhuman primate infants include the similarity of physiologic and basic behavioral characteristics to human infants, the ease of access to subjects for repeated testing, and the close relatedness to humans. In addition, environmental conditions were strictly controlled in the nursery, to eliminate confounding factors such as maternal diet or infant treatment. To the best of our knowledge, this is the first study comparing LC-PUFA-supplemented and -unsupplemented formula-fed infants on NBAS outcomes.

METHODS

Subjects

Twenty-eight nursery-reared rhesus macaque infants (*Macaca mulatta*) served as study subjects. The study was conducted using three age cohorts for 3 consecutive y. Cohorts 1 and 2 each contained eight infants; cohort 3 consisted of 12 infants. In each cohort, half the infants were provided standard infant formula (described below), and half were fed the standard formula supplemented with a DHA/AA blend. The study was conducted in accordance with regulations governing the care and use of laboratory animals, and was approved by the NICHD and NIAAA Animal Care and Use Committees.

The sample size of 28 infants was derived based on the following considerations. We reviewed previous data from 97 nursery-reared infants from six birth cohorts (1991–1996) to obtain SD measures on each day for each of the dependent measures. The standard deviations for each cluster, averaged across test days, ranged from 0.31 to 0.58. In a previous study examining genotypic differences on these neonatal assessment outcomes (52), for two of the dependent measures a mean difference of 0.44 was obtained in each case. Although there were no mean differences between genotype groups for the other two dependent measures, for the purpose of this study we assumed a potential mean difference of 0.44 for these measures as well. With an expected power of 80%, an alpha error of 0.05, and an SD of 0.58 (the largest SD of the four dependent measures from the historical data), the projected sample size to detect a statistically significant effect would be 27 animals. Because we rear our nursery animals in groups of four we could have elected to include either 24 or 28 animals in the

study. Concerns about the potential genetic variability resulting from using animals from two facilities prompted us to include the larger number of monkeys in the study. This sample size is comparable to those used in prior studies using this neonatal assessment in between-group comparisons to assess genetic and environmental effects on behavior [*e.g.* 23 infants (49); 42 infants (52)].

Housing

Infants were separated from their mothers at birth and reared in a neonatal nursery according to previously published procedures (54). From d 1 through 14 of age, animals were individually housed in 51 × 38 × 43-cm plastic cages. Each cage contained a 25-cm-high inanimate surrogate mother composed of a 16.5-cm-circumference polypropylene cylinder anchored to an 11.5-cm-wide circular metal base by a flexible metal component that allowed the surrogate to rock. The surrogate mother was covered with an inner layer consisting of an electric heating pad, and the heating pad was covered with fleece fabric. Loose pieces of fleece fabric, which was typically used as a blanket, covered the floor of the cage. The internal temperature of the cage was maintained at approximately 27°C. Infants could see and hear, but not physically contact, other infants. At 15 d of age, the heating pads were removed and the infants were moved with their fleece and surrogates into individual wire mesh cages measuring 64 × 61 × 76 cm. As in the earlier housing condition, animals were in visual, auditory, and olfactory, but not tactile, contact with other infants. At approximately d 37 of age, animals entered social groups with similar-aged peers. Lights were on in the nursery from 0700 h to 2100 h. Room temperature was maintained between 22° and 26°C, and humidity was maintained at 50 to 55%.

Feeding

The standard formula was composed of a 1:1 mixture of Similac (Ross Laboratories, Columbus, OH, U.S.A.) and a commercial monkey formula (Primilac, Bio-Serv, Inc., Frenchtown, NJ, U.S.A.). The supplemented formula consisted of 1 L of standard formula with 1 mL of DHA/AA (46% DHASCO and 54% ARASCO, Martek Biosciences Co., Columbia, MD, U.S.A.) blended in with a hand mixer. Supplemented formula was mixed as needed, at least once per day. Animals were hand-fed until they were old enough to independently feed themselves (usually by d 3 of life). Between 0800 h and 2000 h, 50 mL of formula was provided, and formula intake was assessed at 2-h intervals. Animals were provided formula until 6 mo of age. Small pieces of monkey chow (Purina, Allied Mills, Chicago, IL, U.S.A.) were provided daily to the monkeys from d 14. Fatty acid composition of monkey chow is provided in Table 1. Chow contained DHA and AA at higher concentrations than formula, although well below rhesus milk. However, it is unlikely that chow consumption was a significant factor in the study of 7- to 30-d-old infants, as nursery infants rarely consume monkey chow at this age. There is no compelling *a priori* reason to hypothesize differential chow consumption by the older infants in the different formula feeding conditions.

Table 1. *Fatty acid composition of monkey chow*

Total fatty acids	12,938
Saturated	
14:0	732
16:0	2,336
18:0	1,026
Monounsaturated	
14:1	18
16:1	201
18:1n9	3,612
18:1n7	237
Polyunsaturated	
18:2n6	4,275
18:3n6	3
20:3n6	6
20:4n6	16
22:4n6	5
22:5n6	3
18:3n3	256
20:5n3	41
22:6n3	35

Results are in micrograms per gram ($n = 3$).

AA and DHA Content of Formula/Milk

Formula samples were collected for measurement of LC-PUFA levels, and, for comparison purposes, samples of rhesus breast milk were also obtained. Supplemented formula ($n = 7$) and standard ($n = 7$) formulas were sampled on three separate occasions. Samples were removed directly from the refrigerated containers holding the premixed, ready-for-consumption formulas. Samples of rhesus monkey milk were collected from four females from other research protocols at the Laboratory of Comparative Ethology. All mothers were nursing infants at the time of sample collection; ages of infants ranged from 2 to 6 mo. Mothers were anesthetized with 15 mg/kg ketamine hydrochloride intramuscularly; samples were collected by gently manually expressing approximately 2 mL of milk into a plastic vial. Milk and formula samples were stored at −70°C until analysis.

After collection throughout the study, the supplemented formula contained the highest concentration of AA of all tested samples (1.0 ± 0.4 wt%); rhesus breast milk contained intermediate levels (0.2 ± 0.1 wt%), and standard formula contained very small amounts of AA (0.04 ± 0.006 wt%). These three groups differed ($H = 20.8$, $p < 0.0001$) with Kruskal-Wallis testing. DHA was undetectable in standard formula; supplemented formula contained 1.0 ± 0.4 wt% DHA and mothers' milk 0.4 ± 0.1 wt% DHA. The DHA weight percent differed when comparing mothers' milk and standard formula ($U = 88.0$; $p < 0.0003$) using the Mann-Whitney U test. The concentration of total fatty acids was higher in breast milk (61.7 ± 26.1 mg/mL) compared with both standard (16.5 ± 2.5 mg/mL, $U = 87.9$, $p < 0.0004$) and supplemented formulas (16.7 ± 2.5 mg/mL, $U = 87.9$, $p < 0.0004$). Table 2 contains a detailed description of fatty acid contents of breast milk, supplemented formula, and unsupplemented formula.

Differences among Replications

Procedures were identical among replications with the following exceptions: in the first year of the study, subjects were

Table 2. *Fatty acid composition of breast milk, standard, and supplemented infant formulas*

Fatty acid (wt%)	Standard formula ($n = 7$)	Supplemented formula ($n = 7$)	Breast milk ($n = 11$)
8:0	1.9 ± 0.1	1.8 ± 0.05	3.2 ± 1.0*†
10:0	1.6 ± 0.03	1.6 ± 0.03	4.1 ± 1.5*†
12:0	13.6 ± 1.4	12.7 ± 0.9	1.2 ± 0.5*†
14:0	5.9 ± 0.6	6.2 ± 1.4	1.5 ± 0.3*†
16:0	11.5 ± 0.8	11.8 ± 2.2	13.4 ± 10.7†
18:0	4.2 ± 0.2	3.9 ± 0.9	11.9 ± 8.7*†
20:0	0.3 ± 0.0	0.3 ± 0.0	1.3 ± 1.7
22:0	0.3 ± 0.1	0.3 ± 0.0	2.7 ± 3.7
16:1	1.0 ± 0.1	1.1 ± 0.1	3.6 ± 0.9*†
18:1n9	29.7 ± 5.0	28.2 ± 8.8	27.8 ± 1.7
18:1n7	0.9 ± 0.1	4.0 ± 9.2	2.2 ± 1.7
20:1n9	0.2 ± 0.01	0.2 ± 0.1	0.5 ± 0.3*†
24:1n9	0.1 ± 0.01	0.1 ± 0.003	0.1 ± 0.1
18:2n6	27.4 ± 3.4	25.8 ± 2.4	15.7 ± 12.5
18:3n6	0.03 ± 0.008	0.1 ± 0.0†	0.04 ± 0.04
20:2n6	ND	ND	0.3 ± 0.1*†
20:3n6	ND	ND	0.3 ± 0.1*†
20:4n6	0.04 ± 0.006	1.0 ± 0.4†	0.2 ± 0.1*†
22:4n6	ND	ND	0.1 ± 0.04*†
22:5n6	ND	ND	0.1 ± 0.04*†
18:3n3	2.0 ± 0.5	2.1 ± 0.7	0.7 ± 0.5*†
20:5n3	ND	0.1 ± 0.1†	0.2 ± 0.05*†
22:5n3	ND	0.02 ± 0.01†	0.2 ± 0.1*†
22:6n3	ND	1.0 ± 0.4†	0.4 ± 0.1*†

* $p < 0.01$ compared with supplemented formula.
† $p < 0.01$ compared with the standard formula.
All comparisons used Mann-Whitney nonparametric testing.
ND, not detected.

obtained opportunistically and it was not possible to match groups for sex or birth weight. The standard-formula group in cohort 1 contained two males and two females; the supplemented-formula group consisted of three males and one female. In the second and third replications a larger subject pool was available, and animals were matched for sex and birth weight among groups. Cohorts 2 and 3 therefore contained equal numbers of males and females in the standard-formula and supplemented-formula conditions. Animals in the first cohort were born at the Laboratory of Comparative Ethology (NICHD) breeding facility in Poolesville, MD, U.S.A., and the animals in cohorts 2 and 3 were obtained from Laboratory Animal Breeders Services (LABS, Yemassee, SC, U.S.A.).

Neonatal Assessment

A 30-min developmental assessment battery was administered on d 7, 14, 21, and 30 of life. This test was derived from the Brazelton Neonatal Assessment Scale used in human newborns (48) and has been described in detail elsewhere (47, 49). Raters were trained to a reliability criterion of 0.90 before collecting data (Pearson product-moment correlation) according to a rigorous training protocol (55). The author of the assessment directly trained M.C. to a criterion of reliability exceeding 0.90; M.C. then trained additional raters. All individuals were highly trained, performing the assessment reliably on both mother- and nursery-reared infants for more than 1 y before the onset of this study. Two individuals who were blind to the experimental condition of the animals conducted the majority of the assessments (99 of 111 assessments; 89%). Two additional observers who were not blind to the experimental treatment conducted 12 assessments: nine in cohort 1 and three in cohort 2. This circumstance was unavoidable owing to the necessity to obtain all assessments on the appropriate day of life, and the resulting inability of the blind observers to perform all the required assessments on any given day. The nonblinded observers were unaware of the experimental hypotheses being tested, and performed seven assessments on control infants and five assessments on supplemented infants. Assessment scores from blinded and nonblinded observers did not differ significantly for either feeding condition or any of the clusters. One supplemented-formula infant in cohort 1 was not tested on d 21 owing to scheduling conflicts.

The test was administered between 1100 h and 1300 h. Test items were presented in invariant order following a predetermined sequence. Initially, orientation abilities and attention to visual and auditory stimuli were assessed. This was followed by measurement of a variety of reflex and sensorimotor functions, including tactile responsiveness, postural adjustment capabilities, and muscle tone. In addition, the response to a brief challenge was assessed during a 6-min session in which the animal was placed into a small cage (see above for description of the housing cage used during d 1–14). The test cage was empty except for an absorbent liner pad and the stimulus used for the visual orienting items. The cage was not unfamiliar for the animals, but was devoid of comfort items such as blankets, toys, surrogates, and bottles. The first minute of the 6-min session was devoted to obtaining a count of emitted vocalizations; a 5-min focal behavioral observational period then ensued in which behavioral inactivity, fine and gross motor activity, and coordination were assessed. Temperament characteristics were rated after administration of the orienting and neuromotor items, based on the infant's behavior throughout the test period. These measures included the tester's impressions of the animal's fearfulness, tendency to struggle, consolability, irritability, ability to self-soothe, cuddliness, and overall state of arousal. With the exception of 60-s vocalization count, all items were scored on a scale of 0–2, with scores of 0.5 and 1.5 allowable. As in previous studies (49), some of the individual test items were aggregated into four clusters representing orientation, state control, motor maturity, and activity. Cluster constituents are listed in Table 3.

Blood Collection

Blood samples were collected for determination of plasma DHA and AA levels. Samples were obtained from all study infants at 2, 4, 8, 12, 16, and 20 wk of age. For comparison purposes, equivalent samples were also collected from 14 mother-reared (breast-fed) infants from the breeding colony: four mother-reared infants each were sampled in y 1 and 2, and six infants were sampled in y 3, of the study. Mother-reared infants were matched to study infants on sex of infant and birth date of infant, as closely as possible. All samples were collected between 1130 h and 1430 h. Animals were immobilized with ketamine hydrochloride (intramuscularly, 15 mg/kg) before sample collection. Two milliliters of blood was collected

Table 3. *Neonatal assessment item definitions*

Item	Definition
Orientation cluster	
Visual orientation	Eyes oriented toward toy (Mickey Mouse face) held in four positions in infant's periphery
Visual following	Eyes following moving toy (same as above) in horizontal and vertical directions
Duration of looking	Examiner rating of length of looks on orienting items
Attention	Examiner rating of attention on orienting items
State control cluster	
Irritability	Amount of distress noted during the entire examination
Consolability	Ease of consoling infant following distress
Predominant state	State of infant during examination
Struggle	Amount of squirming during examination
Motor maturity cluster	
Coordination	Quality of motor activity rated during the 5-min observation period
Head posture prone	Ability to hold head up when held in air prone
Head posture supine	Ability to hold head up when held in air supine
Labyrinthian righting	Realignment of head when body is tilted 45° sideways
Response speed	Examiner rating of speed of responding
Activity cluster	
Passive	Duration of time spent inactive during the 5-min observation period
Coordination	Quality of motor activity rated during the 5-min observation period
Motor activity	Observation of amount of motor activity during the 5-min observation period
Spontaneous locomotion	Quality of locomotion rated during the 5-min observation period

from the femoral vein into an EDTA anticoagulant-treated Vacutainer collection tube. After centrifugation, a 500-μL aliquot of plasma was placed into a plastic vial and stored at −70°C until assay.

Analysis of AA and DHA Content of Plasma and Formula/Milk

Fatty acids were extracted from 100 μL of plasma using a modification of the Folch method (56). Samples were aliquoted into 2 mL of $CHCl_3$, 1 mL of BHT-MeOH, and a known quantity of 23:0 fatty acid as an internal standard. One milliliter of 0.2 M Na_2HPO_4 was added after a brief vortexing. The samples were capped under nitrogen and vortexed again. After centrifugation, the $CHCl_3$ solvent layer was extracted and placed under nitrogen stream. The extraction procedure was repeated a second time with an additional 2 mL of $CHCl_3$. The two $CHCl_3$ layers were combined and evaporated to dryness under nitrogen. Samples were methylated with BF_3-MeOH for 60 min (57). Gas chromatography was performed on the methylated samples using a Hewlett Packard 5890 series II with a flame ionization detector, an autosampler, and a DB-FFAP capillary column (J &W Scientific, Folsom, CA, U.S.A.), using previously described methods (58). Peaks were identified using authentic standards (NuCheck Prep, MN, U.S.A.). Fatty acids were quantified by comparison to peak areas of the 23:0 internal standard. The BHT peak was selectively excluded by adjusting peak integration variables.

Fatty acid compositions of milk and formula samples were determined without extraction using direct transesterification methods (59). All samples were appropriately protected from UV light during storage and protected from oxidation during analysis with the use of nitrogen blankets and cold conditions. A 40-μL quantity of each milk or formula sample was aliquoted into 2 mL of 4:1 methanol-hexane solvent containing 50 μg/mL BHT and 10 μg of 23:0 fatty acid internal standard. After addition of sample, tubes were vortexed briefly and 200 μL of acetyl chloride reagent was added slowly, over ice. Sample reaction mixtures were then heated at 100°C for 60 min. After heating, the reaction mixtures were neutralized with 5 mL of 6% K_2CO_3 buffer, and the hexane layers were extracted for gas chromatographic analysis. Gas chromatography was performed on the methylated samples using the same equipment and methods as the plasma fatty acid quantification procedures (see above). The within run and between run coefficients of variation were 0.3% and 1.0%, respectively.

Statistical Analysis

Milk and formulas. Comparison among breast milk and formulas was conducted using nonparametric Mann-Whitney *U* test, unpaired two-group comparisons, and Kruskal-Wallis three-group comparisons.

Plasma samples. Initial univariate analyses of variance were conducted with cohort and condition (control, supplemented, mother-reared) as between-groups factors and time point (wk 2, wk 4) as a within-groups factor. Because there were some missing data points owing to inability to collect sufficient blood for assay, univariate ANOVAs, which compensate for missing data, were conducted using the statistical program SuperANOVA (Abacus Concepts, Berkeley, CA, U.S.A.). Because initial analyses revealed statistically significant effects of cohort for both AA ($F_{2,31} = 74.79$; $p < 0.001$) and DHA ($F_{2,31} = 69.65$; $p < 0.001$), additional analyses were conducted. AA and DHA values were converted to standard scores within each cohort; ANOVAs were then conducted on the standardized scores.

Neonatal assessments. One male infant from cohort 3 was removed from analyses because, even though he had been allocated to the standard-formula group, he had not been removed from his mother until d 3 postpartum. Therefore, all analyses were conducted comparing 13 standard-formula infants with 14 supplemented-formula infants. Mother-reared comparison infants were not incorporated into these analyses, as substantial rearing condition differences between mother- and nursery-reared infants on the response to testing could potentially confound the comparison with supplemented formula–fed nursery-reared infants.

Data from each of the four clusters were analyzed separately. Data were analyzed by three-way mixed design ANOVAs with formula type (standard, supplemented) and cohort (y 1, y 2, y 3) as between-group factors and test day (d 7, d 14, d 21, d 30) as within-groups factors. The statistical program SuperANOVA (Abacus Concepts) was used for all analyses.

RESULTS

AA and DHA content of plasma. Analyses of plasma AA and DHA levels converted into standardized scores within each cohort demonstrated statistically significant effects of condition (AA: $F_{2,37}$ =12.45; $p < 0.001$; DHA: $F_{2,37} = 61.35$; $p < 0.001$). For AA, *post hoc* comparisons indicated that infants fed supplemented formula exhibited higher plasma *z*-scored AA values than mother-reared infants and infants fed standard formula ($p < 0.01$ for both comparisons). Mother-reared infants did not differ significantly from infants fed standard formula. For DHA, mother-reared infants exhibited higher *z* scores of plasma DHA than infants fed both formula types, and supplemented formula–fed infants exhibited higher values than standard formula–fed infants ($p < 0.001$ for all comparisons). Plasma AA and DHA levels (expressed as raw values, micrograms per milliliter) in the three conditions are depicted in Figures 1 and 2, respectively.

Neonatal assessment. There were statistically significant main effects for group, with infants fed the supplemented formula exhibiting higher scores than infants receiving the standard formula on the motor maturity cluster ($F_{1,21} = 11.83$; $p < 0.01$; Fig. 3) and on the orientation cluster ($F_{1,21} = 5.10$; $p < 0.05$; Fig. 4). In addition, the analysis revealed a significant interaction of group and test day for the motor maturity cluster ($F_{3,62} = 3.11$; $p < 0.05$), which indicated that group differences in motor maturity were most pronounced on test d 7 and 14. No group differences were detected on either the state control ($p = 0.38$; Fig. 5) or activity ($p = 0.51$; Fig. 6) clusters.

As in previous findings (47), significant effects of test day were obtained for the orientation cluster ($F_{3,62} = 8.01$; $p < 0.01$), the motor maturity cluster ($F_{3,62} = 6.48$; $p < 0.01$), and the activity cluster ($F_{3,62} = 9.55$; $p < 0.01$). In all three clusters, scores increased as the subjects matured. No effect of test day was detected for the state control cluster.

Significant effects of cohort were demonstrated for the orientation cluster ($F_{2,21} = 11.60$; $p < 0.01$) and for the motor maturity cluster ($F_{2,21} = 4.03$; $p < 0.05$). In both cases, cohort 3 animals exhibited lower scores than either cohorts 1 or 2; cohorts 1 and 2 did not significantly differ from each other. However, significant group by cohort effects were not obtained for either cluster. Cohort effects were not observed for either the state control ($p = 0.07$) or activity ($p = 0.61$) clusters. Table 4 depicts the cohort averages for each cluster.

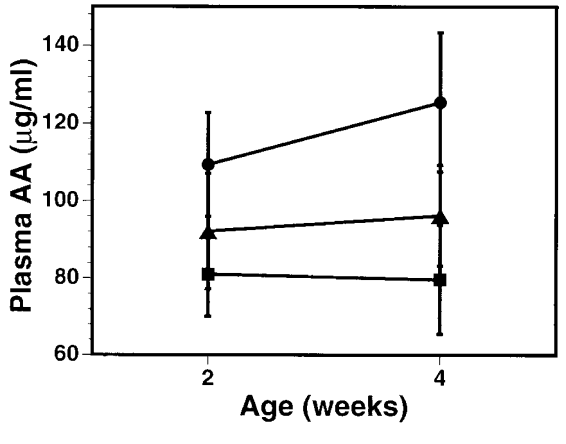

Figure 1. AA levels (μg/mL; mean ± SEM) in rhesus monkey infant plasma. *Triangles*, breast-fed infants ($n = 14$); *squares*, infants fed standard formula ($n = 14$); *circles*, infants fed supplemented formula ($n = 14$). Graph depicts average across three cohorts. Infants fed supplemented formula exhibited higher AA values than breast-fed infants and infants fed standard formula. Mother-reared infants did not differ from infants fed standard formula ($F_{2,37} = 12.45$; $p < 0.001$).

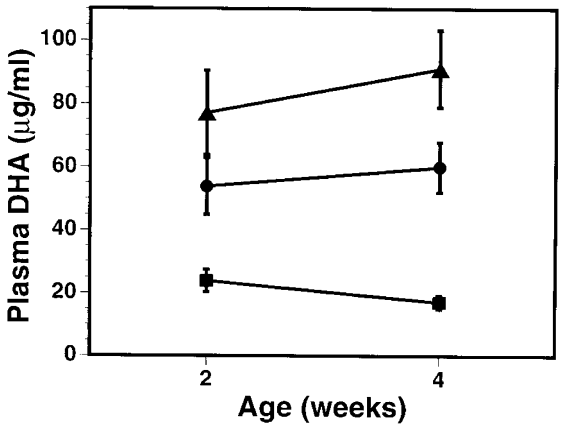

Figure 2. DHA levels (μg/mL; mean ± SEM) in rhesus monkey infant plasma. *Triangles*, breast-fed infants ($n = 14$); *squares*, infants fed standard formula ($n = 14$); *circles*, infants fed supplemented formula ($n = 14$). Graph depicts average across three cohorts. Breast-fed infants exhibited higher levels of plasma DHA than infants fed both supplemented and unsupplemented formula, and supplemented formula–fed infants had higher levels than standard formula–fed infants ($F_{2,37} = 61.35$; $p < 0.001$).

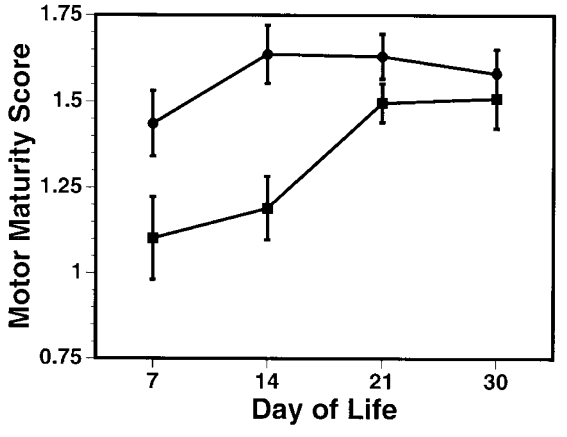

Figure 3. Motor maturity cluster (mean ± SEM) in rhesus monkey infants fed supplemented formula (*circles*, $n = 14$) and standard formula (*squares*, $n = 13$). Graph depicts average across three cohorts. Infants fed supplemented formula exhibited higher scores than infants fed the standard formula ($F_{1,21} = 11.83$; $p < 0.01$), with differences being most pronounced on d 7 and 14 (group × day interaction effect, $F_{3,62} = 3.11$; $p < 0.05$).

DISCUSSION

Rhesus neonates consuming LC-PUFA–supplemented formula obtained higher scores on motor maturity and orientation items than infants receiving standard formula. Because these findings were most pronounced during d 7 and 14 of life, and because these abilities typically improve during the first month of life using this assessment (55), our findings suggest an earlier maturation of specific visual and motor abilities in

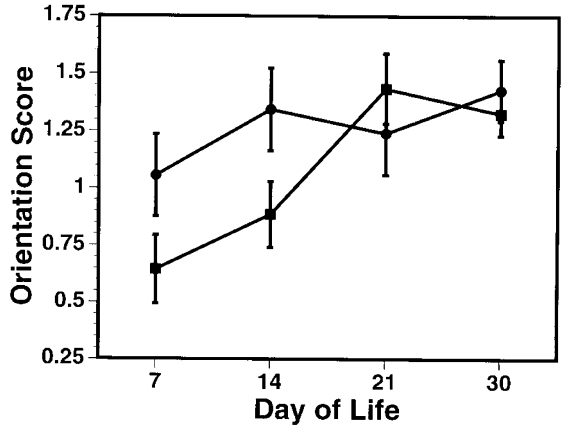

Figure 4. Orientation cluster (mean ± SEM) in rhesus monkey infants fed supplemented formula (*circles*, $n = 14$) and standard formula (*squares*, $n = 13$). Graph depicts average across three cohorts. Infants fed supplemented formula exhibited higher scores than infants fed the standard formula ($F_{1,21} = 5.10$; $p < 0.05$).

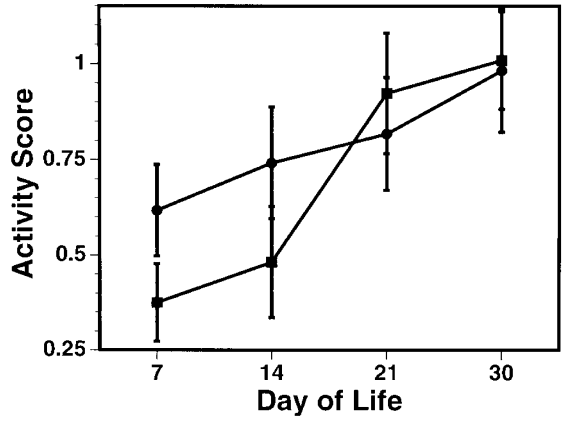

Figure 5. Activity cluster (mean ± SEM) in rhesus monkey infants fed supplemented formula (*circles*, $n = 14$) and standard formula (*squares*, $n = 14$). Graph depicts average across three cohorts. No group differences were detected on this measure.

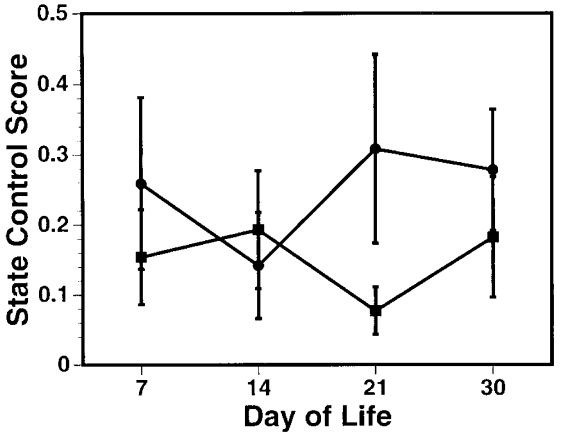

Figure 6. State control cluster (mean ± SEM) in rhesus monkey infants fed supplemented formula (*circles*, $n = 14$) and standard formula (*squares*, $n = 13$). Graph depicts average across three cohorts. No group differences were detected on this measure.

Table 4. *Cohort results for neonatal assessment clusters*

Cluster	Study cohort		
	Cohort 1	Cohort 2	Cohort 3
Motor maturity*	1.486 ± 0.075	1.577 ± 0.049	1.334 ± 0.049
Orientation*	1.494 ± 0.102	1.282 ± 0.107	0.867 ± 0.075
State control	0.097 ± 0.035	0.109 ± 0.045	0.338 ± 0.061
Activity	0.806 ± 0.098	0.805 ± 0.108	0.656 ± 0.072

Results are presented as mean ± SEM.
* $p < 0.05$.

infants fed the supplemented formula. In human infants, differences in developmental quotient scores between LC-PUFA–supplemented and –unsupplemented 4-mo-old infants were noted, using a global neurodevelopmental assessment scale (27, 60). The results in human infants are consistent with the present findings: many of the Brunet-Lézine test items for 4-mo-old infants parallel those used in the primate examination (*e.g.* head posture, visual attention, and tracking). Additionally, the test ages of the subjects in the two studies are similar, as a 4-mo-old human infant is developmentally comparable to a 30-d-old rhesus infant (61). It is noteworthy, however, that in studies comparing breast-fed and formula-fed human infants, there were no differences in orientation and motor scores on the NBAS (62, 63).

No effects of LC-PUFA supplementation were observed for the activity and state control clusters. In contrast to the orientation and motor maturity clusters, which assess specific visual or motoric skills, the activity and state control clusters reflect temperament and behavioral characteristics. In particular, two of the items in the activity cluster (passivity, spontaneous locomotion) measure the behavioral response to placement into an empty cage. Familiar attachment or comfort objects (inanimate surrogate, human caretaker, and fleece blankets) are absent or unobtainable during this portion of the examination, implying that the infant is undergoing an enforced separation experience. The nonhuman primate infant response to separation exhibits considerable individual variation, and is believed to be (at least partially) under genetic control. Although environmental factors can and do affect the behavioral response to separation (64), this usually occurs in response to profound manipulations such as rearing with mother *versus* rearing with peers. In contrast, less potent manipulations of the early rearing environment often fail to impact separation responses [*e.g.* controllable *versus* uncontrollable environments (65); adoption (66)]. Hence it is not surprising that environmental manipulations such as formula supplementation with LC-PUFA do not influence the response to separation.

The state control cluster represents the infant's emotional state during the assessment procedure. Although evidence indicates that breast-fed infants exhibit more distress than formula-fed infants during the NBAS (63) and during neonatal examinations (67, 68), this may reflect a disparity in breast-feeding and bottle-feeding styles and not LC-PUFA content of feeds *per se* (*i.e.* breast-fed infants require more frequent feedings and therefore may have been more hungry during examination). There is no direct evidence linking LC-PUFA intake with temperament characteristics in human neonates. In monkeys, as in humans, breast-fed (mother-reared) infants are fussier than bottle-fed (nursery-reared) infants. However, this

contrast most likely reflects differences in infants' response to handling by humans rather than nutritional factors. The state control cluster also appears to possess substantial genetic underpinnings in rhesus monkeys: recent data (69) indicate the values for this cluster at 14 and 30 d of age exhibit modest, but statistically significant, heritabilities.

Examination of the data indicates that the differences between feeding conditions for both orientation and motor maturity items appeared most pronounced during test d 7 and 14. This pattern suggests an earlier maturation of specific visual and motor abilities in the supplemented infants. Because unsupplemented monkeys exhibited the expected maturational increase in motor maturity and orientation on d 21 and 30 (47), the disparity between groups was not as pronounced on those days. This ceiling effect represents a limitation of the test instrument. Although it is possible that the effects of LC-PUFA supplementation dissipate by the third week of life in the monkey infant, this cannot be determined without use of a more challenging or sophisticated instrument. It is plausible to consider that the persistent differences in plasma DHA and AA levels in supplemented and unsupplemented infants until 5 mo of age could potentially contribute to behavioral or cognitive differences between these groups between ages 1 and 5 mo.

Owing to time and space constraints it was only possible to study a limited number of animals in each year; hence the requirement to conduct the project in three cohorts. Furthermore, sufficient animal numbers were not available from one breeder to enable all infants to be obtained in one cohort. There is a possibility that there may have been genetic differences between cohort 1 and cohorts 2 and 3, which would influence our test outcome. Nonetheless, cohorts 1 and 2 did not differ significantly despite coming from different facilities. However, the marked differences in the infants' orientation and motor maturity scores in cohort 3 require explanation. We hypothesize that the lower scores of cohort 3 monkeys may in part reflect the outcome of unavoidable procedural differences in the third year of the study. Unlike cohorts 1 and 2, which were reared in dedicated nursery facilities, cohort 3 was raised for 4 to 11 d in a room containing monkeys of wide age ranges, including adults and groups of juveniles. Several infants received their d 7 assessment in that location. In addition, many of the cohort 3 monkeys were tested on d 7 and 14 in the morning immediately after overnight transport to the Poolesville facility. These testing conditions were clearly less than optimal. It should also be noted that although the comparison did not reach statistical significance ($p = 0.07$), the animals in cohort 3 exhibited a high value for the state control cluster, indicative of more distress, relative to the values for infants in cohorts 1 and 2. It is possible that the distress during the examination related to the lower orientation scores exhibited by y 3 infants. Previous studies (52) have demonstrated that high arousal levels during the examination are associated with low orienting abilities because of competing motor responses.

As expected, plasma concentrations of AA were higher in the group which consumed supplemented formula containing 1.0 wt% AA in comparison to the group fed mother's milk that contained 0.2 wt% AA. We note that the plasma concentrations of AA in the mother-reared and standard formula groups did not differ although the standard formula contained only 0.04 wt% preformed AA. However, this result is consistent with the plasma concentrations in phospholipid predicted by the Lands equation (70) because the standard formula also contained relatively high concentrations of linoleic acid (27.4 wt%) in comparison to α-linolenic acid (2.0 wt%). With little competition for elongation and desaturation from α-linolenic acid and virtually no feedback inhibition from EPA and DHA (both nondetectable), plasma levels of AA were supported by linoleic acid at levels higher than expected by examining the amount of preformed AA alone. Supplementation of standard formula with 1.0 wt% DHA was not effective in equating plasma DHA levels in formula-fed infants with mother-reared infants. However, supplemented formula–fed infants exhibited higher plasma DHA concentrations than did infants fed standard formula.

In summary, our findings add to a growing body of evidence indicating a benefit in neurodevelopmental capabilities in infants fed LC-PUFA–supplemented formulas. Because all animals were reared in identical environments, and the only distinction between groups was in LC-PUFA availability, the differences in motor and visual functioning in this study can be directly and solely attributed to LC-PUFA intake. Future studies should address the effects of early divergence in LC-PUFA intake on developmental outcomes by using more extensive neurobehavioral test batteries, as well as assessments appropriate for older infants (49, 71–73).

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