Longer Term Effects of Early Dietary Cholesterol Level on Synthesis and Circulating Cholesterol Concentrations in Human Infants

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De novo endogenous cholesterol synthesis is an integral component of developing human infant adaptive mechanisms that regulate cholesterol homeostasis. Smaller central pools of cholesterol later in life may contribute to reduced low-density lipoprotein-cholesterol (LDL-C) concentrations in the plasma and thus reduce the risk of cardiovascular disease. Early infant adaptive mechanisms may theortically result in altered central pools of cholesterol in later life. To examine the response of cholesterol homeostasis to long-term dietary cholesterol supplementation, endogenous cholesterol synthesis was studied in 81 full-term healthy neonates at 4 months or at 11 and 12 months of age. Thirty-two infants were breast-fed (BF) (6 males, 7 females), fed regular cows milk protein-based formula (RF) (6 males, 3 females) 0.85 mmol cholesterol/L (33 mg cholesterol/L), or fed regular cow milk protein-based formula with the addition of cholesterol (RF+cholesterol) (5 males, 5 females) 3.44 mmol cholesterol/L (133 mg cholesterol/L). Effects of cholesterol supplementation on cholesterol synthesis rates were evaluated at 4 months of age. Forty-nine other infants, BF (11 males, 6 females) fed RF (7 males, 12 females), or RF+cholesterol (6 males, 7 females) until 6 months of age were studied to test the hypothesis of imprinting using a cross-over design study with a 1-month 250 mg cholesterol/day challenge at 11 months of age. The incorporation rate of deuterium in body water into erythrocyte-free cholesterol over 48 hours was used as an index of cholesterol fractional synthetic rate (FSR) at 4, 11, and 12 months of age. Both plasma total- and LDL-C were higher ($P < .04$) in BF compared with RF+cholesterol and RF formula-fed groups at 4 months of age. Plasma cholesterol concentrations for all 3 groups were similar at 11 and 12 months of age. FSR (pools/d) was 4-fold higher ($P < .0001$) in both RF and RF+cholesterol compared with BF groups, but not different between RF and RF+cholesterol formula-fed groups. No differences in FSR before and after cholesterol challenge were observed within the 3 feeding groups at 11 and 12 months of age. However, synthesis rates from 4 months to 12 months increased ($P < .03$) in BF infants and decreased in both RF+cholesterol ($P < .0001$) and RF ($P < .0001$) fed groups. These results demonstrate relative insensitivity of synthesis rates and serum cholesterol concentrations to cholesterol challenge, irrespective of early dietary cholesterol intake in formula-fed and BF infants. These findings support the notion that early dietary cholesterol causes minimal changes in cholesterol metabolism about 6 months after dietary exposure in young infants.

CHOLESTEROL IN MILK has been speculated to endow nursing infants with enhanced ability to metabolize cholesterol later in life. Early rat studies of Reiser and Sidelman[1] demonstrated an inverse relationship between serum cholesterol concentration of adult male offspring and the cholesterol content their mothers' milk. However, results of other "Resier Hypothesis" studies have not been unequivocal; other rat studies were inconclusive,[2] studies in pigs[3] generally support the hypothesis, while those in baboons[4] and guinea pigs[5] do not.

In human infant studies,[6,7] the effects of dietary cholesterol feeding on cholesterol metabolism have similarly been largely inconclusive, hampered by the difficulty in separating effects of dietary cholesterol from those of fatty acids when breast-fed (BF) infants are used as a control group since breast milk and formula differ in both dietary constituents. The cholesterol content of human milk is typically higher (100 to 120 mg/L) compared with regular cow milk protein-based commercial formula (RF) (10 to 30 mg/L).[8] Whether neonatal dietary cholesterol is responsible for the perturbations of cholesterol metabolism[9,10,11] demonstrated at 4 months of age has been a topic of speculation for several decades. The critical period in development when prolonged alteration is possible and the biochemical and physiologic mechanisms responsible are questions that remain.[12]

An additional problem in establishing the effect of early dietary cholesterol on cholesterol metabolism in childhood or adulthood has been the presence of many uncontrolled variables in free living population studies. The few long-term studies completed have shown that BF, compared with formula-fed infants, exhibit lower[13] or higher[14] serum cholesterol concentrations in childhood or adulthood, a response that can be predicted by duration of breast-feeding.[15]

It has been theorized that differences in plasma lipid concentrations and cholesterol synthesis rates in infancy and adulthood may be explained, in part, by changes in endogenous cholesterol synthesis rates that are modulated by the quantity of dietary cholesterol.[16,17] High cholesterol intakes inhibit de novo cholesterol synthesis, while low cholesterol intakes stimulate synthesis; alterations in synthesis rates theoretically might remain for a long period, ie, metabolic imprinting.[18] Such early modulation of cholesterol fractional synthesis rate (FSR) might theoretically be of benefit later in life when challenged with dietary cholesterol. The present study therefore determined whether dietary cholesterol intake in early life influences lipid levels and endogenous cholesterol FSR and assessed whether addition of cholesterol to regular cow milk-based formula

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Submitted August 31, 2000; accepted June 4, 2001.

Supported by Wyeth-Ayerst Laboratories.

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0026-0495/02/5101-0030$35.00/0

doi:10.1053/meta.2002.28154

results in FSRs similar to human milk-fed infants. We hypothesized that after challenge with a high-cholesterol diet at 12 months of age, infants formerly BF or fed regular cow milk-based formula + cholesterol similar to breast milk during the first 6 months of life will demonstrate cholesterol FSRs similar to their respective fractional synthetic rates at 4 months, and such FSRs will be less than those of infants formerly fed regular cow milk-based formula, (schematically [FSR] BF $\gg$ RF+cholesterol $<$ RF). We further hypothesized that when infants fed regular cow milk-based formula + cholesterol from birth are switched to regular cow milk-based formula and infants fed regular cow milk-based formula from birth are switched to regular cow milk-based formula + cholesterol at 6 months of age, FSRs will be similar.

MATERIALS AND METHODS

Subjects

A total of 168 full-term healthy infants were recruited from the normal newborn nurseries of the University Hospital of Cincinnati (UHC) and other area hospitals, of whom 107 completed the study. The study itself was conducted at UHC. Study infants were appropriate for gestational age (37 to 41 weeks), as determined from last normal menstrual cycle and confirmed by physical assessment using the standard Ballard score.[19] Appropriate birth weight for age was determined by intrauterine growth curves of Usher and Maclean.[20] Only mothers who had planned to exclusively breast-feed for at least 4 months were invited to participate in the BF group. Only infants whose parents were both black or both white were invited to participate. Infants with a family (father, mother, or sibling) history of hypercholesterolemia or hypertriglyceridemia (as assessed by questionnaire) were excluded from the study. Parents were asked if there was any history of high cholesterol or high trigylceridemia. Exclusion criteria also included any evidence of cardiac, respiratory, hematologic, gastrointestinal, or other systemic disease in the infants; infants initially recruited who subsequently developed formula intolerance; infants of mothers who were strict vegetarians or vegans.

Study Design

A prospective, double-blind, partially randomized, crossover study compared 30 BF to 51 formula-fed infants, all recruited in the first week of life and followed until 4 months or 1 year of life. The major variables in these infants was type of diet: BF, RF, and regular formula with added cholesterol (RF+cholesterol).

This study was comprised of 2 test periods. The first test period was directed towards evaluating the effect of diet, specifically, cholesterol supplementation, on FSR at 4 months of age. This specific age was chosen because this is the age when the infants would most likely not have had a prior exposure in the first 4 months of life to any other supplemental food items, which may have affected FSR, since their diets consisted predominantly of milk. Thus, any detected differences in this test period should be related directly to differences in dietary composition. The participation of the infants enrolled in arm 1 ended after completion of the test period at 4 months of age.

The second test period, arm 2, conducted with a different set of infants than arm 1, was directed towards testing the hypothesis of metabolic imprinting, using a crossover design. The type of milk intake was as follows (Fig 1); the duration of breast feeding after 4 months was decided by the infant's mother. If breast-feeding mothers decided to wean their infants to formula, the infants were given RF+cholesterol until the end of the study. In this manner, the cholesterol intake from milk of the infants in the BF group was generally similar until 11 months of life, so that these infants served as a control group (ie, continuous high-cholesterol intake) for the 12-month test period. Infants randomized to the RF+cholesterol group were fed RF+cholesterol until 6 months of age and then were fed RF until 11 months of age (ie, high-cholesterol intake crossed over to low-cholesterol intake). Conversely, infants randomized to the RF group were fed RF until 6 months of age and then fed RF+cholesterol until 12 months of age (ie, low-cholesterol intake crossed over to a high-cholesterol intake).

Infants enrolled in arm 2 underwent testing for lipid profiles and FSR determination at 11 months of age and 12 months of age after the cholesterol challenge period. At 11 months of age, infants in all 3 diet groups (BF, RF+cholesterol, and RF) received an additional 250 mg of cholesterol powder (similar to the concentration of cholesterol found in 1 egg) per day for 1 month. The cholesterol challenge, in arm 2 of the study during the 12th month, was used to magnify any existing, persistent differences between the groups to determine whether such effects on FSRs were imprinted, ie, when uniformly challenged with increased cholesterol intake, infants who were formerly on high-cholesterol intakes would be more able to inhibit endogenous FSRs compared with infants who were formerly on low-cholesterol intake.

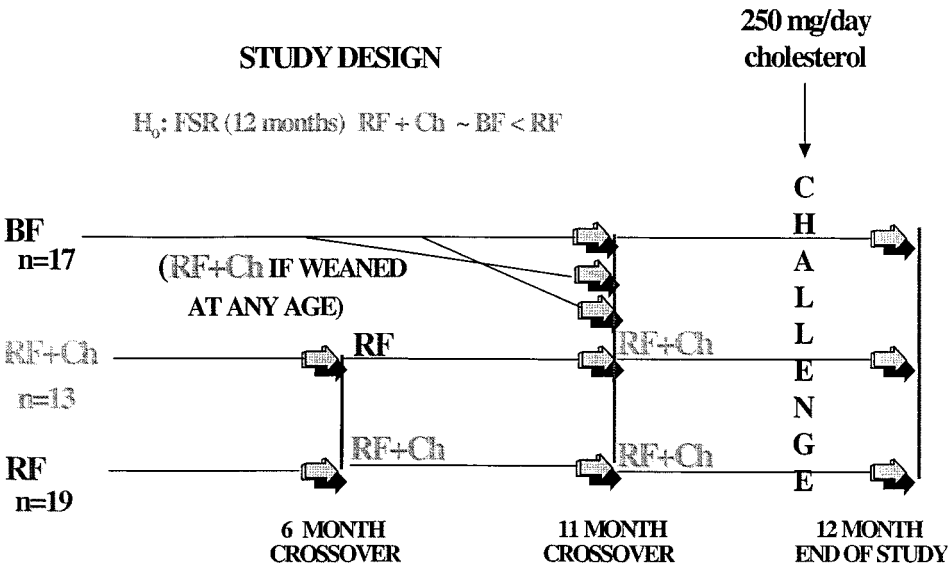

Fig 1. Arm 2: Long-term effect of cholesterol supplementation. H_o, hypothesis; BF, breast-fed; RF+cholesterol, regular formula+cholesterol; regular formula, RF.

Protocol

Random assignments for formula-fed infants were generated using computerized random number tables. BF infants were not randomized. Mothers who had already decided to breast feed their babies were recruited for the BF group. With the obvious exception of the BF group, physicians, nurses, and families were blinded to the diet assignment. The double-blind was maintained until the 12-month assessment was complete.

Formula was provided as ready to feed cans. The cholesterol content was the only difference in composition between the RF and the RF+cholesterol. Crystalline-free cholesterol powder was thoroughly dissolved in component oils, forming an emulsion, prior to combining macronutrient ingredients by the manufacturer. Analysis of a random batch of RF+cholesterol was made prior to distribution to the infants by the manufacturer to insure that the cholesterol contents were within 0.85 ± 0.05 mmol/L (33 ± 2 mg/L) or 3.44 ± 0.05 mmol/L (133 ± 2 mg/L), respectively. The nutrient composition of formula is summarized in Table 1. All infants began receiving formula within the first 3 to 7 days of life. Formula was provided free to the formula-fed infants for the entire duration of the study to improve compliance with dietary assignments. At 11 months of age, all infants underwent a 4-week period of additional cholesterol intake, as 250 mg of free crystalline food grade cholesterol powder (Wyeth-Ayerst Laboratories, Philadelphia, PA) per day. The study dietician instructed the mothers on how to give the cholesterol powder mixed with baby food to their infants. To minimize the variation in solid food intake among the infants, diets other than milk intake were controlled for by supplying coupons for designated commercial processed baby foods in the same dietary sequence for all 3 groups. During the cholesterol challenge period, all formula-fed infants received RF+cholesterol formula to ensure cholesterol intakes were similar between all the infants during the challenge period.

Mothers from the 3 groups were required to keep a monthly 3-day diet diary until the testing period at 4 months. Mothers recorded information regarding the frequency of breast-feeding, the volume of supplemental RF+cholesterol per day (BF group), and the volume of formula (RF or RF+cholesterol) per day in the formula groups. Upon introduction of solids, the diary also included recording of all types of solid food intake to determine cholesterol, fat, and fatty acid intake. In the general population, milk and baby food intake together account for approximately 50% to 70% of total caloric intake of infants from 6 to 12 months of age.[21] Three-day diaries were the most time-consuming to administer (including the time necessary for instruction for their use), but they offered the highest degree of accuracy with the lowest proportion of missing foods. The diets were analyzed by an experienced registered dietician to determine whether any significant disparity occurred among the diet groups that may confound the outcome variable of FSR. All blood samples were drawn by the hospital phlebotimist, placed on ice, and centrifuged within 30 minutes. Fractionated blood samples were used to determine plasma lipid concentrations, as well as cholesterol biosynthesis measurement. The red blood cell fraction was frozen at -80°C in Cincinnati and sent to McGill University for FSR determination. Cholesterol synthesis rates were determined at 4 or 11 and 12 months of age. Measurements were performed over a 48-hour period. On day 1, 8 mL blood was obtained to determine baseline body water deuterium enrichment and baseline erythrocyte membrane cholesterol deuterium enrichment. Infants were then orally given 500 mg/kg body weight of deuterium oxide (D_2O, 99.96% deuterium; Isotech, Miamisburg, OH). On day 2, 8 mL blood for deuterium excess enrichment was obtained, after which 50 mg/kg body weight of D_2O was orally given to maintain constant body water deuterium enrichment. On day 3, a final blood sample was obtained. All blood samples (8 mL each day on days 1 to 3) were drawn between 9:00 AM and 11:00 AM. Sixty-nine infants completed the study. Adequate blood samples and/or volume for FSR determination at both 11- and 12-month time points could not be obtained from 20 infants; BF (n = 8), RF (n = 4), and RF+cholesterol (n = 8). The study protocol and use of experimental infant formula were reviewed and approved by the Institutional Review Boards of all the involved hospitals, and informed consent was obtained from families before enrollment of the infants.

Table 1. Composition of Types of Milk and Formula Used in Study

	Breast Milk*	Regular Formula†	Regular Formula + Cholesterol‡
Calories	680	676	676
Protein (g)	10.5	15.0	15.0
Carbohydrates (g)	72.0	72.3	72.3
Fat (g)	39.0	36.3	36.3
Polyunsaturated/ saturated ratio	0.3	0.8	0.8
Cholesterol (mg)	120.0	35.0	135.0
Linoleic acid (mg)	3,971	8,790	8,790

* Composition of human breast milk varies with stage of lactation and among mothers. Composition of formulas based on manufacturer's estimates. Values are per liter.

† Regular formula (SMA, 20 calories/ounce, ready to serve iron fortified, 33 mg/L cholesterol; Wyeth-Ayerst Laboratories, Philadelphia, PA).

‡ Regular formula + cholesterol (SMA, 20 calories/ounce ready to serve iron fortified, 133 mg/L cholesterol; Wyeth-Ayerst Laboratories, Philadelphia, PA).

Laboratory Methods

Centrifuged and fractionated blood samples were used to determine cholesterol biosynthesis measurement, as well as plasma lipid concentrations. Plasma cholesterol, triglyceride, high-density lipoprotein-cholesterol (HDL-C), and LDL-C levels were determined using an automated analyzer (Hitachi 747, San Jose, CA) using methods validated by Medical Research Laboratories (Dr Evan Stein) in Cincinnati, OH, with enzymatic techniques validated by the National Institutes of Health Lipid Research Clinics (Iowa City, IA). This laboratory participated in and remained certified by the Part III Program of the National Heart, Lung, and Blood Institute (Bethesda, MD) and the Centers for Disease Control and Prevention (Atlanta, GA). Internal quality assurance was performed every 20 samples by means of frozen material with target values having been assigned by the Centers for Disease Control and Prevention. The HDL was isolated with heparin and 2 mol/L manganese chloride. The precipitated lipoproteins were sedimented by centrifugation, leaving the clear supernate, containing HDL-C that was then analyzed enzymatically. Triglycerides were also determined enzymatically. Plasma LDL-C concentrations were calculated from plasma total-cholesterol concentrations using the Friedwald equation[22] used previously with infants.[9-11] Interassay coefficients of variability for LDL-C and HDL-C were less than 5% and 3%, respectively.

Determinations of cholesterol FSR were performed using procedures described by Jones et al[17] as the rate of incorporation of deuterium into red blood cell membrane cholesterol. Erythrocyte lipids were extracted by organic solvent and dried under nitrogen. Duplicates of 1.5 mL red blood samples were used. A total of 6 mL of methanol was added, and samples were heated in a 55°C water bath for 15 minutes. Hexane/chloroform (4:1 vol/vol) was added and the mixture shaken for 15 minutes, upon which 0.75 mL distilled water was added and shaken again for another 10 to 15 minutes. This was followed by centrifugation for 15 minutes at 1,500*g*. The upper hexane-chloroform layer containing the lipid was removed. The lipid was re-extracted using hexane-chloroform, upon which the upper layer was again removed and com-

bined with the first. The solvent was then evaporated by drying under nitrogen, leaving the lipid extract.

The extract was redissolved in a small amount of chloroform and streaked onto thin-layer silica (TLC) gel plates, which were prepared in the lab. Plates were developed in petroleum ether/ethyl ether/acetic acid (135:15:1.5 vol/vol/vol) for approximately 1 hour and air dried. Lipid bands were visualized in iodine vapor against a cholesterol standard. The free cholesterol band presented itself as the first band from the bottom of the TLC plate. This free cholesterol band was scraped from the TLC plate and extracted 3 times using 6 mL, 4 mL, and 3 mL, respectively, of hexane/chloroform/ether (5:2:1 vol/vol/vol). The pool solvent was dried under nitrogen, leaving the purified lipid extract.

The extract containing the free cholesterol was redissolved in a small amount of chloroform and transferred into a Pyrex combustion tube, 18 cm in length (Corning Glass Works, Corning NY). Ground copper oxide (0.6 g) (BDH, Toronto, Ontario) and a 2 to 2.5 cm piece of silver wire (1 mm diameter) were added to each tube. The solvent was then removed by gradual heating under vacuum until it was boiled off, leaving the cholesterol. Pressure was allowed to return to baseline, upon which tubes were sealed under vacuum using a hydrogen torch. Tubes then underwent combustion at 540°C for 4 hours to produce water and carbon dioxide, upon which they were allowed to slowly cool to room temperature.

Combustion water produced previously by combustion was cryogenically separated from CO_2 by vacuum distillation into 10-cm Pyrex tubes containing 60 mg zinc shavings (Biogeochemical Labs, Indiana University, Bloomington, IN). Tubes were sealed under vacuum using a hydrogen torch. Water in tubes then underwent reduction at 540°C for 30 minutes to produce hydrogen-deuterium gas.

Samples of plasma (100 μL) were diluted with Montreal tap water (of known isotopic abundance) 2-fold for baseline and 6-fold for the 24- and 48-hour blood draws. These 200-μL and 600-μL samples were placed under nitrogen into 10-cm Pyrex tubes containing 60 mg zinc. Tubes were sealed under vacuum and water was reduced in a 540°C oven for 30 minutes to produce hydrogen-deuterium gas. Plasma-water enrichment was measured after dilution of 24- and 48-hour plasma samples with water of known isotopic abundance to bring the enrichment into the working range of the International Atomic Energy Agency (Vienna, Austria) mass spectrometer calibration standards.

Deuterium enrichment of the resultant gas was measured by dual inlet isotope ratio mass spectrometry (VG Isogas 903D, Micromass, Cheshire, UK). Reduction gas was loaded directly into the instrument. Samples were compared with 2 gas standards with electrical H_3^+ compensation to determine ratios. Values were expressed relative to the enrichment of standard mean ocean water (SMOW) in parts per million.

Erythrocyte cholesterol deuterium enrichment values at 24 and 48 hours were expressed relative to the corresponding mean plasma water sample enrichment at each time point, after correction for the deuterium-protium ratio in cholesterol, to yield FSRs (pool/day) for the free cholesterol pool. The FSR index represents that fraction of the free portion of the rapidly turning over central cholesterol pool that is synthesized in 24 hours as per the formula[17]:

$$\mathrm{FSR}(\%/\mathrm{day}) = (\delta_{\mathrm{cholesterol}}/\delta_{\mathrm{plasma}}) \times 0.478$$

in which δ refers to deuterium enrichment above baseline over 24 hours. The factor of 0.5 is used to yield a daily FSR from each respective 48-hour time period.

Statistical Analysis

The main outcome variables were FSR and plasma lipid concentrations. The statistical package of StatView (Abacus Concepts, Berkeley, CA) was used for the statistical analysis. Population normality was tested by Kolmogorov-Smirnov goodness of fit testing. One-way analysis of variance was used to test differences between groups, and the Tukey-Kramer method for multiple comparisons was used to determine differences between pairs of groups.[23] Using a standard deviation of 1.39% for FSR observed in regular cow milk-based formula-fed infants at 4 months of age from previous studies,[9-11] a sample size of 10 in each group gives a power of 80% in detecting a 20% difference in FSR between groups at an alpha value of 0.05 for a 2-sided test. Statistical significance was considered for P values less than .05. Results are presented as mean ± SEM.

RESULTS

Sixty infants were recruited for arm 1, as previously described,[9] of whom 37 completed the study. In arm 2, a total of 108 infants were recruited, of whom 69 completed the study. Mothers of these infants were approached by study personnel within 24 to 36 hours after delivery. All mothers who had chosen *a priori* to formula feed their infants were approached for the formula group. Reasons for dropping out included: infants weaned from breast feeding before 4 months (n = 3), failure to thrive (n = 1), noncompliance with study protocol (n = 1), formula intolerance and change by pediatricians (n = 10), parent withdrawal from the study (n = 3), and lost to follow-up (n = 7). An additional 9 infants dropped out of the study, and 6 were transferred to arm 1 of the study during the first 6 months of the study when formula was not available due to manufacturing difficulties.

In arm 2, 25 infants exclusively BF for the first 12 months of life comprised the BF group (13 males, 12 females). Forty-four infants were randomized according to a computer-generated random numbers table to receive regular cow milk-based formula (RF) (SMA, ready to serve iron fortified, 0.85 mmol cholesterol/L, 33 mg cholesterol/L; Wyeth-Ayerst Laboratories, Philadelphia, PA) (n = 23) or regular cow milk-based formula + cholesterol (RF+cholesterol) (SMA, ready to serve iron fortified, 3.44 mmol cholesterol/L, 133 mg cholesterol/L; Wyeth-Ayerst Laboratories) (n = 21). Some of the data for infants at 4 months of age have been previously reported.[9]

Growth in weight, length, and ponderal index occurred at the expected normal progression and were equivalent for each group (data not shown). An experienced dietician, skilled in the analysis of food diaries, determined that there was no significant disparity in food intake, other than milk, among the 3 groups that may have confounded the outcome variable of FSR.

Analysis of variance indicated that at 4 months of age plasma total-cholesterol concentrations were higher ($P < .02$) in BF (4.31 ± 0.21 mmol/L; 166.7 ± 8.1 mg/dL) compared with RF (3.37 ± 0.23 mmol/L; 131.2 ± 9.15 mg/dL) as shown in Table 2. There was an intermediate response in plasma total-cholesterol concentrations (3.81 ± 0.18 mmol/L; 148.2 ± 7.31 mg/dL) for infants fed RF+cholesterol. The difference between the plasma total-cholesterol concentrations for RF and RF+cholesterol groups at 4 months did not reach significance. Total-cholesterol concentration was higher in the RF groups at 11 months (4.20 ± 0.17 mmol/L, 162.9 ± 6.5 mg/dL; $P < .02$) and 12 months (4.06 ± 0.20 mmol/L, 157.9 ± 7.5 mg/dL; $P < .05$) compared with the RF group at 4 months (3.37 ± 0.23 mmol/L, 131.22 ± 9.2 mg/dL), as seen in Table 2. Total-cholesterol concentration for the RF+cholesterol infants was higher ($P < .04$) at 12 months (4.30 ± 0.13 mmol/L, 166.9 ±

Table 2. Plasma Concentrations (mmol/L [mg/dL]) as a Function of Diet and Time (total-cholesterol and LDL-C)

	Total Cholesterol			LDL-C		
Age (mo)	RF* (n)	RF + Ch† (n)	BF‡ (n)	RF* (n)	RF + Ch† (n)	BF‡ (n)
4	3.37 ± 0.23[a,d,e]	3.81 ± 0.18[b,c]	4.31 ± 0.21[a,b]	1.21 ± 0.18[f,j,k]	1.77 ± 0.22[g,h,i]	2.27 ± 0.23[f,g]
	131 ± 9 (9)	148 ± 7 (10)	167 ± 8 (13)	49 ± 10 (9)	68 ± 8 (10)	88 ± 9 (13)
11	4.20 ± 0.17[d]	3.86 ± 0.11	3.92 ± 0.19	2.17 ± 0.14[j]	1.97 ± 0.10[f,h]	2.10 ± 0.17
	163 ± 7 (19)	150 ± 4 (13)	152 ± 7 (17)	84 ± 5 (19)	77 ± 4 (13)	82 ± 7 (17)
12	4.06 ± 0.20[e]	4.30 ± 0.13[c]	4.16 ± 0.14	2.18 ± 0.15[k]	2.51 ± 0.22[h,i]	2.36 ± 0.12
	158 ± 8 (19)	167 ± 5 (13)	162 ± 6 (17)	85 ± 6 (19)	97 ± 9 (9)	92 ± 5 (17)

NOTE. Mean ± SEM. Groups with the same superscripts are significantly different ($P < .05$ by repeated measures analysis and Tukey-Kramer method). Plasma cholesterol 5.17 mmol/L = 200 mg/dL.

* RF, regular cow milk protein-based formula (SMA, 33 mg cholesterol/L).

† RF + Ch, regular cow milk protein-based formula + cholesterol (SMA, 133 mg cholesterol/L).

‡ BF, breast milk.

5.1 mg/dL) compared with 4 months (3.81 ± 0.18 mmol/L, 148.2 ± 7.3 mg/dL) of age.

Similar to plasma total cholesterol concentrations, plasma LDL-C concentrations were assessed as higher ($P < .04$) in BF (2.27 ± 0.23 mmol/L, 87.7 ± 9.3 mg/dL) compared with RF (1.21 ± 0.18 mmol/L, 47.1 ± 7.0 mg/dL) groups at 4 months of age, presented in Table 2. Similar to total-cholesterol, there was an intermediate response in plasma LDL-C concentrations (1.77 ± 0.22 mmol/L, 68.8 ± 8.4 mg/dL) for infants fed RF+cholesterol at 4 months. The difference between the plasma LDL-C concentrations for RF and RF+cholesterol groups at 4 months did not reach significance. Similar to total-cholesterol concentrations, plasma LDL-C concentrations were higher ($P < .05$) in the RF+cholesterol group (2.51 ± 0.22 mmol/L, 97.5 ± 8.7 mg/dL) at 12 months compared with the RF+cholesterol group (1.97 ± 0.10 mmol/L, 76.5 ± 3.9 mg/dL) at 11 months, presented in Table 2. LDL-C concentration was higher in the RF groups at 11 months (2.17 ± 0.14 mmol/L, 84.1 ± 4.9 mg/dL; $P < .002$) and 12 months (2.18 ± 0.15 mmol/L, 85 ± 5.7 mg/dL; $P < .002$) compared with the RF group (1.21 ± 0.18 mmol/L, 49.3 ± 10.4 mg/dL) at 4 months, as seen in Table 2. No other significant differences were found for total-cholesterol, LDL-C (Table 2), plasma triglycerides, or HDL-C between the groups at 4, 11, and 12 months of age as shown in Table 3. Deuterium enrichments of body water for infants at 4 or 11 and 12 months of age were not different among treatment groups as shown in Fig 2. At 11 months, cumulative deuterium erythrocyte cholesterol enrichment was higher ($P < .01$) in the BF group compared with the formula-fed groups as seen in Fig 3. There was no significant difference in cumulative deuterium erythrocyte cholesterol enrichment between RF+cholesterol- and RF-fed infants at 11 months (Fig 3) or among the groups at 12 months of age Fig 4.

Fractional synthetic rate of RF+cholesterol-fed infants at 11 months (2.71% ± 0.56%/day) and 12 months (3.79% ± 0.48%/day) was lower ($P < .0001$) than at 4 months of age (8.29% ± 0.37%/day) as shown in Fig 5. Similarly, mean FSR of RF-fed infants at 11 months (3.58% ± 0.57%/day) and 12 months (3.15% ± 0.48%/day) was lower ($P < .0001$) than at 4 months of age (8.58% ± 0.27%/day). Conversely, FSR of BF infants was higher ($P < .03$) at 11 months (6.16% ± 1.05%/day) and higher ($P < .007$) at 12 months (4.34% ± 0.66%/day) of age compared with BF infants at 4 months (2.19% ± 0.29%/day) as shown in Fig 5. FSR of BF infants at 11 months (6.16% ± 1.05%/day) was higher ($P < .02$) than both RF-fed (3.58% ± 0.57%/day) or RF+cholesterol-fed (2.71% ± 0.56%/day) infants. No significant differences in FSR were observed across dietary groups at 12 months of age.

DISCUSSION

The essential question addressed here was whether changes in intake of dietary cholesterol used in otherwise identical infant formula affects endogenous cholesterol synthesis and

Table 3. Plasma Concentrations (mmol/L [mg/dL]) as a Function of Diet and Time (HDL-C and triglycerides)

	HDL Cholesterol			Triglyceride		
Age (mo)	RF* (n)	RF + Ch† (n)	BF‡ (n)	RF* (n)	RF + Ch† (n)	BF‡ (n)
4	1.19 ± 0.05	1.08 ± 0.06	1.13 ± 0.06	2.28 ± 0.39	2.10 ± 0.18	1.97 ± 0.17
	46 ± 2 (9)	42 ± 2 (10)	44 ± 3 (13)	203 ± 35 (9)	186 ± 16 (10)	175 ± 16 (13)
11	1.06 ± 0.06	1.15 ± 0.06	1.08 ± 0.07	2.08 ± 0.19	1.63 ± 0.21	1.62 ± 0.16
	41 ± 2 (19)	45 ± 3 (13)	42 ± 3 (17)	185 ± 17 (19)	145 ± 18 (13)	144 ± 14 (17)
12	1.09 ± 0.05	1.16 ± 0.05	1.12 ± 0.07	1.70 ± 0.19	1.65 ± 0.22	1.62 ± 0.30
	43 ± 2 (19)	45 ± 2 (13)	44 ± 3 (17)	151 ± 17 (19)	146 ± 20 (9)	144 ± 27 (17)

NOTE. Mean ± SEM. Plasma triglycerides 2.3 mmol/L = 200 mg/dL; plasma cholesterol 5.17 mmol/L = 200 mg/dL.

* RF, regular cow milk protein-based formula (SMA, 33 mg cholesterol/L).

† RF + Ch, regular cow milk protein-based formula + cholesterol (SMA, 133 mg cholesterol/L).

‡ BF, breast milk.

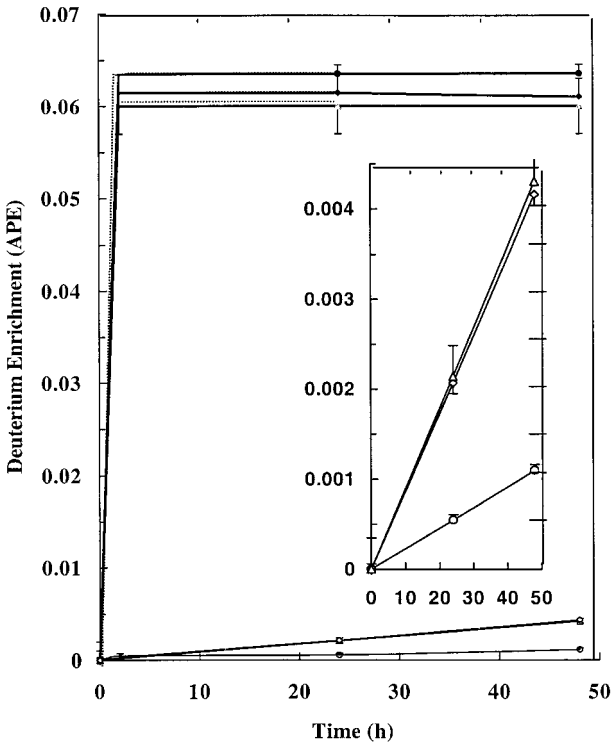

Fig 2. Deuterium enrichments (mean ± SEM) of plasma water (BF, ●; RF-fed, ◀; RF+cholesterol-fed, ◆) and erythrocyte cholesterol (BF, ○; RF-fed, ◇; RF+cholesterol-fed, ◁) infants at 4 months of age.

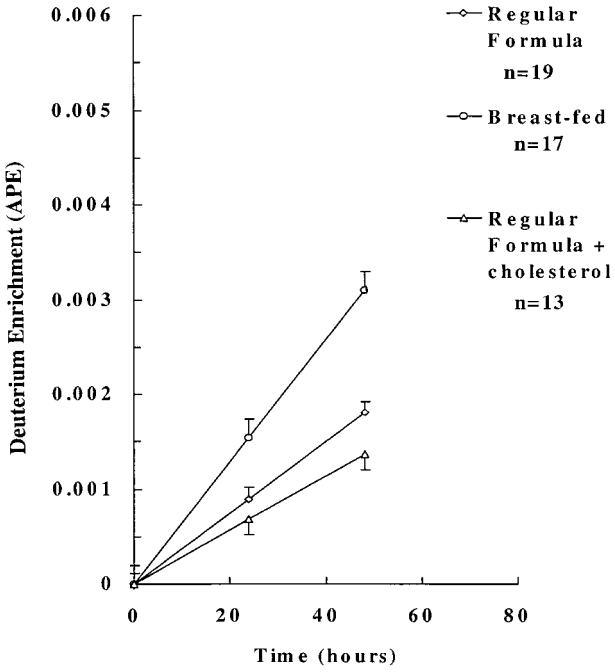

Fig 3. Deuterium enrichment of erythrocyte cholesterol for RF-, BF, and RF+cholesterol-fed infants at 11 months of age. *Deuterium enrichment was higher ($P < .01$) in BF compared with both formula-fed groups. There was no significant difference between the formula groups. Mean ± SEM.

serum lipoprotein-lipid concentrations. A test of the null hypothesis showed that there were significant differences at 4, 11, and 12 months for the major outcome measure, FSR, but that there were variable differences for serum lipoprotein concentrations following 4 months or a full year of feeding the defined diets. The data indicate the relative insensitivity of cholesterol synthesis and circulatory levels to changes in dietary cholesterol infants at 1 year of age, regardless of early cholesterol intakes.

Cholesterol synthesis is especially difficult to measure in humans for several reasons. Its synthesis rate is low (less than 5%/day), it is distributed into several kinetically distinct compartments, it has a complex transport system, and its synthesis is regulated by feedback inhibition of a single rate-limiting enzyme, which has a circadian oscillation.[24,25] Although radioactive tracer kinetic measurements have been made in adults, similar studies have not been undertaken in infants because of radiation exposure, frequent blood collections, and the likelihood that growing infants might not be in a steady-state over the duration of the study. Four-month-old infants, such as in arm 1 of the present study, grow very slowly (approximately 0.4% day), and 11- and 12-month-old infants in arm 2 are undergoing slightly more variable growth rates; they are assumed to be in a de facto steady-state during the 3-day study period. Relating the actual state of growth of each infant with their respective FSR data would have been possible if individual pool sizes had been determined in the current study. However, limited blood volume availability precluded determina-

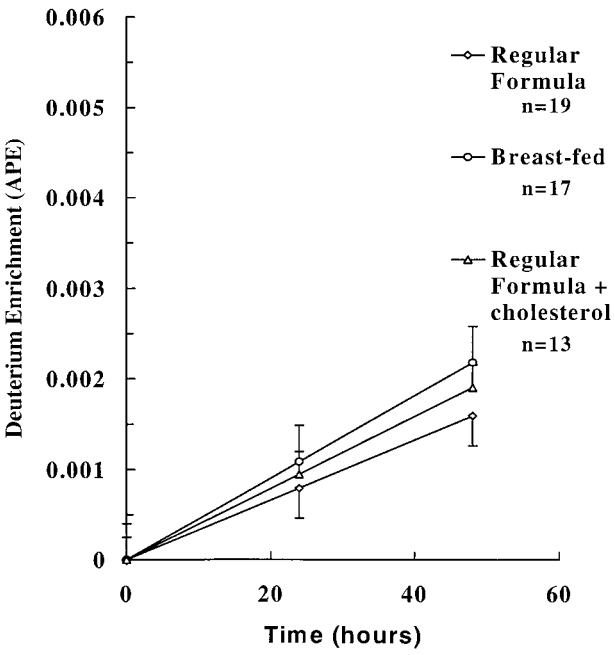

Fig 4. Deuterium enrichment of erythrocyte membrane cholesterol for RF-, BF, and RF+cholesterol-fed infants at 12 months of age. All diet groups received a 1 month, 250 mg/day cholesterol challenge. There were no significant differences among the diet groups. Mean ± SEM.

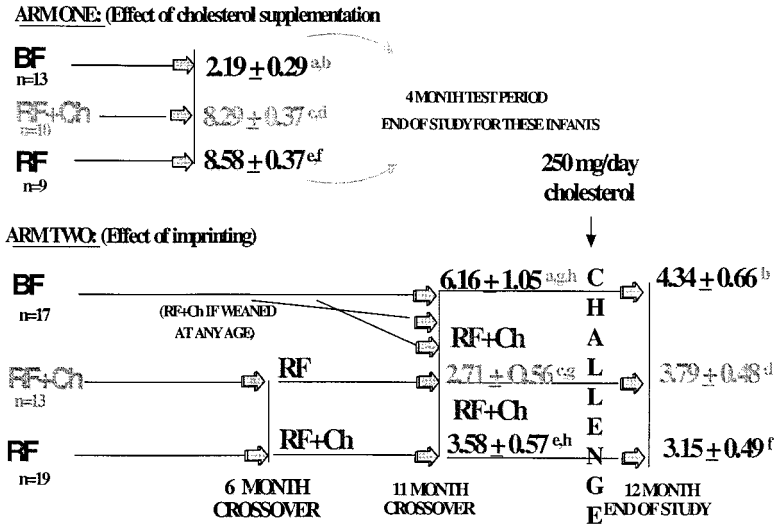

Fig 5. FSR (mean ± SEM) of BF, RF+ cholesterol-fed, and RF-fed infants at 4, 11, and 12 months of age. Values with the same superscripts are significantly different. FSR of BF higher (P < .03) at 11 and higher (P < .007) at 12 compared with 4 months. FSR of RF+cholesterol lower (P < .0001) at 11 and 12 months compared with 4 months. FSR of RF lower (P <0 0001) at 11 and 12 months compared with 4 months. FSR of BF higher (P < .02) than RF and RF+cholesterol at 11 months.

tion of cholesterol absolute synthetic rate (ASR) for each infant.

Clinical studies in human infants have previously suggested that serum cholesterol levels of BF infants are higher than those of formula-fed infants.[7,9-11,26-30] In the present report, the significant differences in plasma total-cholesterol and LDL-C between feeding groups present at 4 months of age failed to persist at 11 or 12 months. With one notable exception, plasma LDL-C concentration was significantly higher in the RF+cholesterol-fed group at 12 months, after the cholesterol challenge, compared with plasma LDL-C concentration at 11 months, while FSRs remained relatively constant. In contrast, previous work has demonstrated no significant relationship between the intake of neonatal dietary cholesterol and serum cholesterol concentrations during the first 6 months of life and the serum cholesterol concentrations on a normal diet at 12 months,[6,31] 18 to 24 months,[7] 3 to 4 years,[32] 5 years,[33] or 15 to 19 years[7] of age.

Our results demonstrate profoundly reduced cholesterogenesis, irrespective of early dietary cholesterol intake in formula-fed infants at 11 and 12 months of age compared with a similar group of neonates at 4 months of age, as assessed by deuterium enrichment of erythrocyte membrane cholesterol. Long-term metabolic responses to increased neonatal dietary cholesterol in human infants potentially include higher cholesterol absorption, elevated serum total cholesterol, diminished endogenous synthesis, and increased fecal cholesterol excretion.[9,29] Unfortunately, cholesterol absorption was not determined in the present study; it would have been valuable to have assessed whether the changes in cholesterol synthesis rates were offset by reciprocal shifts in cholesterol absorption, as seen in adults after perturbation of cholesterol metabolism.[34] However, results of specific effects of addition of dietary cholesterol on cholesterol metabolism in humans have been somewhat ambiguous, with no effect reported in some,[6,9,35] but not all studies.[29] In contrast to our findings in human infants, some human studies show a possible long-term effect of early dietary cholesterol intake. Adult men and women who were BF in infancy had lower serum cholesterol concentrations at 30 to 50 years of age compared with adults who were previously formula-fed.[13] Conversely, serum cholesterol concentrations were significantly lower in 7- to 12-year-old school children who received low cholesterol formula in infancy compared with those who were formerly BF.[14] A study of 465 subjects demonstrated that the most important predictor of high serum cholesterol concentrations in men at 18 years of age was duration of breast-feeding during early infancy.[15]

Understandably, studies examining effects of early dietary cholesterol on cholesterol metabolism in human infants have focused on differences between breast and commercial formula-fed infants. Attributing the observed differences solely to the cholesterol component of the milk may be misleading, as other factors, such as the fatty acid composition of formula, may be equally as important in regulation of circulating lipid levels.[30] As such, because the fatty acid composition of formula was not identical to that of human milk, a potential confounding effect of fatty acid composition cannot be ruled out in the present study. Alternatively, the form of dietary cholesterol may have been responsible for the dissimilarity between supplemented formula and breast milk. Breast milk contains about two thirds of its cholesterol in esterified form,[36] a form that may undergo absorption at a different efficiency compared with free unesterified cholesterol found in the formula.

The dietary crossover at 6 months from low to high, versus high to low, cholesterol intake did not appear to affect subsequent cholesterol synthesis rates in the present study. Present data suggest the absence of dietary cholesterol transition effects on cholesterol synthesis rates or serum lipids during infancy, when dietary cholesterol intake is altered at 6 months of age. Dietary cholesterol transition effects have been demonstrated to influence cholesterogenesis in adulthood. When deuterium incorporation and urinary mevalonate were used to measure the response of cholesterol biosynthesis to 50, 350, or 650 mg cholesterol/2,800 kcal,[17] modest reductions in synthesis rates were observed in individuals with low, normal, or elevated plasma cholesterol concentrations when making transitions from low- to medium and low- to high-cholesterol diets. Down-regulation of synthesis in response to dietary cholesterol was

related to the rate of synthesis while on the low-cholesterol diet, but independent of plasma cholesterol levels.[17] In the present study, FSR values of the RF+cholesterol-fed infants at 11 and 12 months of age were independent of dietary cholesterol intake; conversely plasma LDL-C concentration exhibited greater dependency. The elevated standard error measure for FSR in the BF infants at 12 months could reflect 2 populations, however; those exclusively BF and those whose intake included some proportion of formula. As such, there is a risk that the study possessed insufficient power to detect differences in FSR at 12 months due to type II error.

The diet-related changes in infant plasma cholesterol concentrations reported in this study at 4 months and by others,[9-11] appear, on the basis of our current findings, to be transient in nature. Moreover, the differences in FSR observed between BF and formula-fed infants at 4 months of age were not present at 11 or 12 months. From these results, we suggest that cholesterol synthesis may play a dominant role in early infancy, but more of a passive role by weaning age in regulating cholesterol homeostasis. The resistance of synthesis rates to changes in dietary cholesterol level observed at weaning age in our study has been demonstrated in adults. Sterol-balance methodology used by Grundy et al[37] to measure body cholesterol synthesis demonstrated that the amount of cholesterol did not significantly reduce synthesis in normolipidemic subjects. Similarly, the addition of 250 mg dietary cholesterol to self-selected low-cholesterol diets resulted in no changes in synthesis rates, as measured by deuterium incorporation.[38]

In summary, to our knowledge, this is the first study in infants evaluating the possible imprinting effect of early dietary cholesterol intake on endogenous cholesterol synthesis later in life. Results indicate that although substantial intersubject variation can be observed, early exposure to infant formula supplemented with cholesterol, within the physiologic range found in human milk, does not induce differences in central pool synthesis rates, in response to later cholesterol challenge, in normal infants during the first year of life. It is, however, likely that infants at 12 months of age are undergoing significant developmental changes and major changes in diet. These processes may be exerting larger effects on cholesterol synthesis, underneath which smaller differences cannot be identified. Notwithstanding, the present findings support the view that dietary cholesterol causes minimal changes in cholesterol metabolism about 6 months after dietary exposure in young infants.

ACKNOWLEDGMENT

The excellent technical assistance of Janny Brons is gratefully acknowledged. We would especially like to thank Kathryn Pramuk, MS, RD, at Wyeth-Ayerst.

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