Nervonic Acid in Red Blood Cell Sphingomyelin in Premature Infants: An Index of Myelin Maturation?

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The present study addresses the question whether nervonic acid (24:1n-9) accumulation in sphingomyelin (SM) of red blood cells (RBC) could yield information on cerebrum maturation in premature infants. The study included 28 premature eutrophic infants of 31.5 wk gestational age. Eleven were fed with human milk, nine with a regular formula and eight with an α-linolenate-enriched formula. The fatty acid composition of the SM fraction was determined by gas–liquid chromatography on a 50-m fused silica capillary column. At 32 wk gestational age, the main fatty acids in SM were 16:0, 18:0, 20:0, 22:0, 24:0 and 24:1n-9. After five weeks of feeding, at week 37 of postconceptional age, the most striking variation was a rise in 24:1n-9, from 9.9 ± 0.7 to 12.8 ± 0.9 ($P < 0.02$), regardless of regimen in all three feeding groups. The rise in 24:1n-9 after birth in premature eutrophic infants is the beginning of a trend toward the higher levels in 24:1n-9 observed in mature newborns and older infants. The 24:1n-9 level in SM of RBC from premature infants may reflect 24:1n-9 levels in SM of brain and could thus reflect brain maturity.
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In recent years, the need for n-3 polyunsaturated fatty acids (PUFA) in early brain development has been pointed out by many investigators (1–6). However, few studies have been devoted to the metabolism and the role of nervonic acid (24:1n-9) which, together with lignoceric acid, constitutes the major long chain fatty acid of myelin. Accumulation of these two acids may reflect the increase in myelin during gestation in animals (7) and in humans (8). Nervonic acid, which is synthesized by elongation of oleic acid (9), is also characteristic of the sphingomyelin (SM) of red blood cells (RBC) and is one of the four major fatty acids of this lipid fraction in adults (10,11) and in infants (12).

The only data on nervonic acid distribution in infant RBC had previously been obtained on full-term newborns (12) during the first few hours after birth (13) and during the first month of life (14). The present work was undertaken to investigate whether, in premature infants, nervonic acid might accumulate in SM of RBC as in the cerebrum and thus reflect maturation. It compared three different types of milk-feeding (human, regular formula and α-linolenate-enriched formula) on PUFA metabolism in premature infants. The results suggest that nervonic acid in SM of RBC could indeed be used as an index of maturation in premature newborns.

MATERIALS AND METHODS

The study included 28 premature eutrophic infants (11 males and 17 females) admitted to the Neonatal Department of the Regional Hospital Center of Montpellier for nutritional assessment and treatment. Mean gestational age was 31.5 wk (29 to 33) and mean birthweight 1630 g (1270–1980 g). Eleven infants were fed human milk, nine received regular formula feeding (Pregallia), and eight an α-linolenate-enriched formula (enriched Pregallia). Both formulas were from Nutripharm (Levallois-Perret, France). The α-linolenic acid-enriched formula was obtained by isocaloric partial substitution of fats with colza oil [low-erucic acid rapeseed (LEAR) oil; Codex Alimentarius, Canadian General Standard Board, 1987] from Nutripharm. The fatty acid distribution in percent weight of total diet fatty acids for stearic, oleic, linoleic and α-linolenic acids was 5.05, 25.50, 12.50 and 0.55, respectively, in the regular formula and 5.25, 30.25, 12.50 and 1.95, respectively, in the enriched formula. The percent composition in stearic and oleic acids (the main precursors of nervonic acid) was not very different from that in human milk from the Montpellier lactarium (8.10, 34.90, 12.65, 1.10 for stearic, oleic, linoleic and α-linolenic acids, respectively). Informed consent was obtained from parents and the protocol of the study was approved by the Regional Hospital Center Ethics Committee. Three blood samples were obtained from each of 25 infants at 2 (D_2) and 15 d (D_{15}) of milk-feeding, and the third one (Dterm, the equivalent of full-term) at 37 wk postconceptional age. Two infants discontinued participation in the study before Dterm and one was enrolled after D_2.

Lipid analyses. Blood was collected in Na-heparinized glass tubes. An aliquot was taken for hematocrit measurement in order to determine the precise volume of blood corresponding to 200 μL of RBC pelleted under these conditions. The fatty acid composition of SM could thus be expressed as μg of each fatty acid per mL of RBC.

After centrifugation (2000 rpm for 10 min) the RBC pellet was washed by resuspending and centrifugation in isotonic saline solution containing 1 mM ethylenediaminetetraacetic acid (EDTA). The RBC lipids were extracted according to the method of Folch *et al.* (15) using a chloroform/methanol mixture containing 1 mM butylated hydroxytoluene (BHT) (Sigma Chimie, St-Quentin-Fallavier, France) as an antioxidant. Chloroform, pure analytical grade, was from SDS (Peypin France) and methanol from Prolabo (Paris, France). The extract was left to evaporate until dry under a nitrogen stream and the residue was redissolved in a minimal quantity of chloroform/methanol mixture (1:1, vol/vol) for thin-layer chromatography on Silica gel 60 F_{254} (Merck, Darmstadt, Germany) using chloroform/methanol/H_2O (65:25:4, by vol) as developing solvent (16), to which BHT (1 mM) was added.

The phospholipid fractions were visualized at 250 nm after spraying with 0.2% 2′,7′-dichlorofluorescein in ethanol (Merck). SM was identified by comparison to known standard (Sigma Chimie).

The silica gel bands containing SM were scraped off and 7.5 μg (125 μL of a methanol solution) of free heneicosanoic acid 21:0 (Sigma Chimie) was added as an internal

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Abbreviations: BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; LEAR oil, low-erucic acid rapeseed oil; PUFA, polyunsaturated fatty acids; RBC, red blood cells; SM, sphingomyelin.

standard. The fatty acids were transesterified with sulfuric acid in methanol (1:19, vol/vol) at reflux. The methyl esters were extracted with hexane and analyzed by gas-liquid chromatography (17) with the following modification: a fused silica capillary column (50 m × 0.32 mm) CP Sil 88 from Chrompack (Les Ullis, France) was used on a Fractovap 2900 Chromatograph (Erba Science, Massy, France). The temperatures were: ionization detector, 250°C; inlet, 230°C; oven program, 10°C/min from 100 to 170°C and 2°C/min from 170 to 200°C.

The amount of each fatty acid was calculated by reference to the internal standard of 21:0 using an ENICA-21 integrator (Delsi France, Argenteuil, France). Results are expressed ± SEM, and Student's paired *t*-test was used for statistical analysis.

RESULTS

The fatty acid composition of SM of premature infant RBCs is reported in Table 1. Results are expressed both as a percent distribution of individual fatty acids and as μg per mL of RBC. The main variation over time concerns nervonic acid (24:1n-9)—a significant rise is observed from 9.9 ± 0.7 to 12.8 ± 0.9 percent between D_2 and Dterm ($P < 0.02$), which is compensated for by small declines in percentages of 16:0 and 18:0. Similar observations can be made when the results are expressed as μg of each fatty acid per mL of RBC. Whereas the total amount of SM fatty acid decreased between D_2 and Dterm, the mean concentration of nervonic acid rose from 29.8 ± 3.6 to 34.4 ± 3.4, reflecting an increased contribution of this acid to the total fatty acid content of SM in RBC during this period.

Figure 1 shows the time course of the variations in percent nervonic acid in the SM of the RBC of each infant. There is an overlap zone ranging from 5 to 15% between values at D_2 and Dterm, but about 80% of the infants

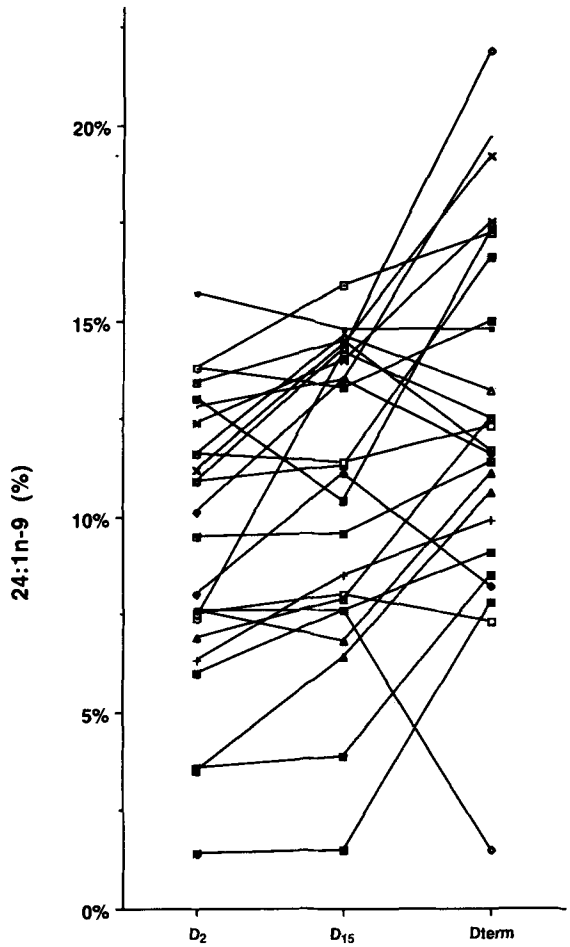

FIG. 1. Time course of the variation in percent nervonic acid in sphingomyelin of red blood cells from 28 premature infants between birth (wk 32 of gestation) and the wk 37 postconceptional age. Abbreviations as in Table 1.

TABLE 1

Variation in the Fatty Acid Composition of Red Blood Cell Sphingomyelins in Premature Infants Between Birth (wk 32 of gestation) and Theoretical Full-Term[a]

	Fatty acid composition (%)			Fatty acid composition (μg/mL of RBC)		
	D_2[b] n = 27[c]	D_{15} n = 28	Dterm[b] n = 26	D_2[b] n = 27	D_{15} n = 28	Dterm[b] n = 26
16:0	37.3 ± 1.3	36.7 ± 1.2	35.3 ± 1.4	97.4 ± 5.1	90.4 ± 5.1	88.8 ± 5.0
18:0	17.8 ± 0.6	16.6 ± 0.6	15.7 ± 0.7[d]	49.0 ± 3.9	41.9 ± 3.0	41.6 ± 4.1
20:0	2.5 ± 0.1	2.7 ± 0.1	2.7 ± 0.1	6.7 ± 0.4	6.7 ± 0.4	6.7 ± 0.4
22:0	6.0 ± 0.2	6.5 ± 0.2	6.6 ± 0.2	16.6 ± 1.2	16.3 ± 1.1	17.0 ± 1.1
24:0	15.3 ± 0.9	15.6 ± 0.8	15.2 ± 1.0	44.8 ± 4.6	40.6 ± 3.3	39.5 ± 3.5
24:1n-9	9.9 ± 0.7	11.0 ± 0.7	12.8 ± 0.9[e]	29.8 ± 3.6	29.0 ± 2.7	34.4 ± 3.4
18:1n-9	2.0 ± 0.3	1.8 ± 0.2	2.0 ± 0.3	6.1 ± 1.0	4.8 ± 0.7	5.7 ± 1.1
18:2n-6	0.4 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	1.4 ± 0.3	1.1 ± 0.2	1.3 ± 0.3
18:3n-3	trace	trace	trace	trace	0.1 ± 0.0	0.1 ± 0.0
20:4n-6	0.4 ± 0.1	0.3 ± 0.0	0.5 ± 0.1	1.1 ± 0.2	0.7 ± 0.1	1.7 ± 0.6
20:5n-3	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
22:6n-3	trace	trace	trace	trace	trace	trace
Total	91.8	91.7	91.4	253.1	232.0	237.0

[a]Mean ± SEM of sphingomyelin fatty acids. RBC, red blood cells; D_2, day 2 of milk feeding; D_{15}, day 15 of milk feeding; Dterm, week 37 of postconceptional age.
[b]Student's *t*-test (D_2 *vs.* Dterm).
[c]n = Number of patients.
[d]$P < 0.05$.
[e]$P < 0.02$.

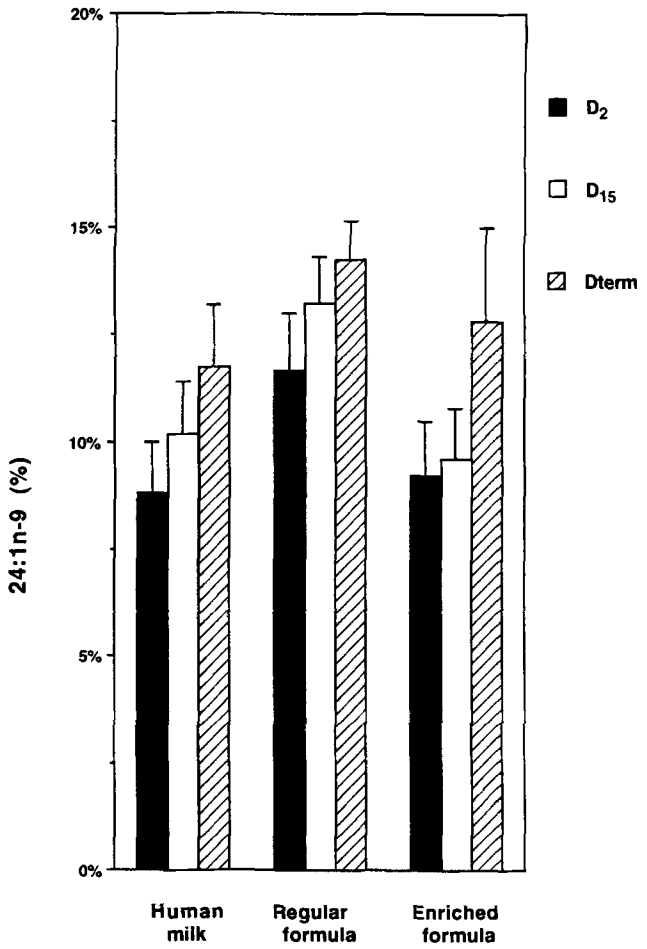

FIG. 2. Time course of the variation in percent nervonic acid in sphingomyelin of red blood cells from three groups of premature infants fed human milk, regular formula and α-linolenate-enriched formula. D_2 and D_{15} correspond to 2 and 15 d of feeding and Dterm to 37 wk postconceptional age.

experienced an increase. In two patients, the pattern was atypical. In the first case, the level, which was 7% on D_2 and D_{15}, drastically declined to 1% at Dterm. In the second case, where the mother had undergone treatment for a pre-eclamptic syndrome, the level rose from 1% (D_2-D_{15}) to 7% (Dterm).

In Figure 2, the infants are considered as a function of their feeding (human milk, regular formula feeding or α-linolenic colza oil-enriched formula feeding). Mean percent nervonic acid increased progressively between D_2 and Dterm in the three groups, suggesting that this increase is independent of the type of feeding (milk or formula feeding).

DISCUSSION

A comparison of the fatty acid composition of the brain of precocial and nonprecocial species has revealed considerable variations in the proportions of nervonic acid around full-term (18). In the guinea pig (precocial species), the main rise (fivefold) in nervonic acid is observed during the 25 d before birth, whereas in the rat (nonprecocial species) it occurs between birth and adulthood (fourfold). In humans, born at term, brain development is accompanied by an approximately 10% increase (15.1-26.4%) in total brain cerebrosides nervonic acid levels and a 15% increase (13.0-28.0%) in sulfatides that occur between birth and two months. Maximum levels are being reached at four years (about 43%) and then remain almost constant until and throughout adulthood (19). This accumulation in brain nervonic acid is related both to an increase in the relative abundance of myelin *vs.* gray matter and to a specific increase in the actual percentage of nervonic acid in myelin SM (10-32% between 1 and 6 yr) (20). The relationships and exchanges between nervonic acid pools in RBC and the central nervous system are not well understood, but published data indicate a rise in nervonic acid in the SM of RBC between birth at term (11.2% at 7 d) (14) and the fifth year of life (25.4%) (21).

The results of the above study indicate that nervonic acid accumulation in SM of RBC starts earlier and that the rise we observe between a postconceptional age of 32-37 wk (from 9.9 to 12.8%) is part of a process extending over a longer period of time and beginning much earlier than full-term. It is to be compared to the accumulation in the cerebrosides observed in the cerebrum of human fetuses obtained after medical termination of pregnancy at this time (1.9-10% of fatty acids between 30-32 wk and full-term) (8). Apparently, nervonic acid levels increase simultaneously in the SM of RBC and in the central nervous system from at least six weeks before full term until the fourth year. This parallelism is surprising as very long chain fatty acids are derived from two different pools—from the circulation and hepatic synthesis for RBC and mainly from *"in situ"* synthesis in the brain. Simultaneous accumulation in two different compartments could imply, amongst other factors, exchanges between the pools or simultaneous maturation of the biosynthetic systems. This needs to be investigated further.

With regard to nervonic acid, the SM of RBC could be an indication of the fatty acid composition of tissue membranes, and especially of myelin cerebrum membranes, and thus nervonic acid could be considered as an index of myelin maturation. Maternal dietary restriction was recently shown to result in a drastic decrease of 24:0 and 24:1n-9 in cerebrosides and sulfatides of suckling rats (7). Whether the levels of 24:1n-9 in SM of RBC could provide information on the consequences of undernourishment in the myelination process of the central nervous system remains to be established. We have recently observed that the levels of stearic and oleic acids are significantly higher in fetal than in maternal plasma lipids between week 18 and week 37 of gestation (22), suggesting that these two acids are of special importance to the fetus, possibly in supplying C_{24} fatty acids. Moreover, in the same study, the oleic acid level decreased by about 25-30% in fetal plasma. Such a decrease at a time of high energy requirements when nervonic acid biosynthesis is particularly active could increase the demand for oleic acid.

In the present study, the premature infants received three different types of feeding (human milk, regular formula and α-linolenic acid-enriched formula). An increase in nervonic acid was observed in all three groups, indicating that endogenous synthesis together with milk or formula feeding provided enough precursors for the

normal evolution of nervonic acid in the SM of RBC and probably in the central nervous system; the percentages of oleic and stearic acids in formula and milk feeding were about 25-35 and 5-8%, respectively. As expected, linolenate supplementation introduced as LEAR oil did not interfere with oleate elongation and conversion to nervonic acid.

A rise in nervonic acid did not occur in a very small number of the infants, probably as a result of individual variations rather than clinical abnormalities as postconceptional ages and birthweights were within the same limited range. One infant, whose mother had pre-eclamptic syndrome and was being treated with glucocorticoids, antimineralocorticoids and a β-blocker, had very low levels of nervonic acid (about 1%) at birth. This might have been due to the medical treatment of the mother, to an alteration in the hemodynamic conditions of the feto-maternal unit or to other factors, such as the specific gestational conditions, because a tendency to recovery occurred several weeks after birth, although this recovery only reached to the lower end of the overall range for the group.

Overall, our results suggest that the incorporation of nervonic acid into membranes in humans, as reflected by its level in RBC, increases between 32 and 37 wk. Results from the literature show that this evolution proceeds during the first years of development, as values of 25.4% have been reported for five-year-old children (21). A steady state is likely to be reached because the percentage of nervonic acid in SM of RBC remains stable thereafter with values ranging from 21 to 25% having been reported for adults (10,11).

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