Addition of gangliosides to an adapted milk formula modifies levels of fecal *Escherichia coli* in preterm newborn infants

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Because some gangliosides bind bacteria, we tested the influence of supplementing an adapted milk formula with gangliosides, at a total concentration of 1.43 mg/100 kcal, on the fecal microflora of preterm infants. At all sampling times, feces from infants fed with ganglioside-supplemented formula had significantly lower relative content of *Escherichia coli* than feces from infants fed with control milk formula; the difference was especially significant at age 7 days postnatal ($P < .001$). At age 30 days postnatal, fecal bifidobacterial counts were higher in infants fed with ganglioside-supplemented formula ($P < .05$). We conclude that gangliosides at concentrations present in human milk significantly modify the fecal flora. (J Pediatr 1998;133:90-4)

The neonatal intestine is colonized in a stepwise process that depends on environmental factors, bacterial interactions, and the host itself.[1] The colonization process in breast-fed infants differs from that in formula-fed ones.[2,3] Human milk feeding has been related to a lower incidence of diarrheal morbidity.[4,5] The lower pH, which favors the predominance of bifidobacteria over acid-sensitive gram-negative bacilli,[6] specific antibodies,[7] lactoferrin,[7] glycolipids,[8,9] glycoproteins,[10,11] and oligosaccharides,[12-15] and the free fatty acids released by human milk during partial digestion[16,17] may all contribute to the antiinfectious properties of human milk.

Gangliosides, acidic glycosphingolipids, are present in human milk but present in lower quantities and with a different pattern of distribution in milk formula[18]; they appear to inhibit the action of *Vibrio cholerae* and *Escherichia coli* enterotoxins.[19] Because human milk gangliosides bind to surface receptors in *E. coli*,[20] which may play a role in bacterial attachment, this might decrease the adhesion of that bacterium to the intestinal epithelium, leading to a lower rate of colonization of the neonatal intestine by this potential pathogen.

In this study we determined the effect of supplementing an adapted milk formula with gangliosides, at a total concentration similar to that found in human milk, on the fecal microflora of preterm newborn infants, a group highly susceptible to neonatal infections, including those of enteral origin.

GMF	Ganglioside-supplemented milk formula
MF	Milk formula

Methods

Subjects and Experimental Design

A total of 43 healthy preterm infants born between 32 and 36 weeks of gestational age (estimated by the last menstrual period and confirmed by the Dubowitz method[21]) were initially included in the study. All were born at the University of Granada Hospital and were free of major neonatal disease. They were able to tolerate enteral feeding within 48 hours after birth. Infants who had significant causes of neonatal morbidity were excluded from the study. The use of antibiotics was also considered as an exclusion criterion. The study protocol was approved by the ethics committee of the University of Granada Hospital, and informed consent was obtained from all parents.

Infants were paired for birth weight, length, and gestational age, and randomly assigned to 2 groups. One group (the MF group) consisted of 22 infants who received an adapted low birth weight infant formula based on the latest recommendations of the European Society for Paediatric Gastroenterology and Nutrition.[22] One of the infants was excluded from the study because he did not follow the feeding regimen correctly, and anoth-

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Supported by PULEVA (currently Abbott Laboratories) and by grant 900020 from the Centro para el Desarrollo Tecnológico e Industrial and from the Ministerio de Industria, Comercio y Turismo.

Submitted for publication Jan. 30, 1997; revisions received July 14, 1997, Oct. 30, 1997, and Mar. 11, 1998; accepted Mar. 24, 1998.

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0022-3476/98/$5.00 + 0 **9/22/90623**

er one because gastroenteritis developed during the period of study. The second group (the GMF group) consisted of 21 infants who received the same formula supplemented with 1.43 mg gangliosides per 100 kcal. One of the infants was excluded because of an alteration in the feeding regimen. The total number of infants who completed the study, and those whose fecal samples were considered, was 20 for the MF group (weight, 1946.25 ± 79.60 g; length, 43.40 ± 2.58 cm; gestational age, 33.76 ± 0.24 weeks) (mean ± SEM) and 20 for the GMF group (weight, 2004.45 ± 41.15 g; length, 44.15 ± 1.98 cm; gestational age, 33.87 ± 0.20 weeks).

The sample size was estimated according to previous studies performed in our department, in which the main outcome variable was also the bacterial counts in the feces of the neonates.[23] To detect 0.8 SD, with a β power of 80% and an α of 0.05, we needed at least 20 infants per group. There were no evident clinical or anthropometric differences between the two groups at entry or during the study. All the infants included in the study had an adequate nutritional status for their gestational age at birth, according to the standard curves for children born in the south of Spain[24] and according to international standard curves.[25] Postnatal growth was evaluated by weight (daily), height (weekly), and cephalic perimeter (weekly). Growth in the two groups was compared with reference standards for Spanish children.[26] At the end of the study, the weight and length averages in the dietary groups were 2233 ± 132 g and 45.6 ± 1.12 cm for the MF group, and 2260 ± 130 g and 45.7 ± 1.30 cm for the GMF group. Fecal samples were obtained in both infant groups at 3, 7, and 30 days after birth.

Table I. Culture media, times, and conditions of incubation for the groups of microorganisms

Microorganisms	Culture media	Atmosphere	Time (h)
Total aerobes	Plate count agar	O_2	72
Total anaerobes	Eugon agar + 5% defibrinated horse blood	CO_2+H_2	72
Coliforms	Violet red bile agar	O_2	24
Enterobacteria	Violet red bile agar + glucose	O_2	24
E. coli	MacConkey + MUG	O_2	24
Enterococci	Slanetz and Bartley	O_2	48
Bifidobacteria	Modified Petuely*	CO_2+H_2	120

MUG, 4-Methylumbelliferyl-β-D-glucuronide.
*Modified Petuely medium was of the following composition per liter: 35 g lactose, 10 g ascorbic acid, 5 g dipotassium phosphate, 2.5 g ammonium sulfate, 10 g sodium acetate trihydrate, 0.45 g cysteine chlorhydrate, 5 ml Tween 80, 0.2 g *N*-acetylglucosamine, 18 g agar, 5 μg biotine, 500 μg calcium pantothenate, 0.5 g magnesium sulfate heptahydrate, 10 mg ferric sulfate heptahydrate, 7 mg manganous sulfate monohydrate, 10 mg sodium chloride, 10 mg cytidine-5′-monophosphate, 10 mg adenosine-5′-monophosphate, 10 mg guanosine-5′-monophosphate, 10 mg uridine-5′-monophosphate, 10 mg inosine-5′-monophosphate, and distilled water up to 1000 mL.

Table II. Mean logarithmic microbial counts per gram of dry feces of preterm newborn infants fed on milk formula and ganglioside-supplemented milk formula

Microorganisms	Diet	3 d	7 d	30 d
Coliforms	MF	9.40 ± 0.40	9.99 ± 0.16	9.93 ± 0.14
	GMF	9.81 ± 0.36	9.95 ± 0.10	9.97 ± 0.08
Enterobacteria	MF	9.43 ± 0.40	10.03 ± 0.16	9.97 ± 0.14
	GMF	9.87 ± 0.36	10.00 ± 0.10	10.05 ± 0.08
E. coli	MF	6.02 ± 0.78	8.62 ± 0.64	7.04 ± 0.75
	GMF	3.51 ± 0.55*	4.79 ± 0.71†	4.25 ± 0.64
Enterococci	MF	9.06 ± 0.44	9.53 ± 0.26	9.81 ± 0.76
	GMF	9.79 ± 0.18	10.04 ± 0.14	9.79 ± 0.10
Total aerobes	MF	10.28 ± 0.23	10.52 ± 0.14	10.44 ± 0.09
	GMF	10.62 ± 0.11	10.65 ± 0.08	10.42 ± 0.08
Total anaerobes	MF	10.58 ± 0.24	10.87 ± 0.09	10.55 ± 0.08
	GMF	10.63 ± 0.12	10.79 ± 0.09	10.59 ± 0.08
Bifidobacteria	MF	8.86 ± 0.63	10.20 ± 0.22	9.43 ± 0.27
	GMF	10.11 ± 0.26	10.16 ± 0.17	10.16 ± 0.13‡

Results are means ± SEM. Twenty samples were analyzed for each feeding group. Samples were collected at 3, 7, and 30 days of life. Kruskal-Wallis and Friedman nonparametric tests were used to determine the effects of diet and postnatal age as sources of variation.
*$P < .01$ (versus MF group).
†$P < .001$ (versus MF group).
‡$P < .05$.

Feeding Regimens

All infants were fed according to their appetite and tolerance to ensure an intake of at least 100 kcal/kg/d by the 10th day of life. Both milk formulas (supplemented or not supplemented with gangliosides) provided 80 kcal/dL, 2 g protein per 100 kcal (60% whey, 40% casein protein), 5.4 g fat per 100 kcal, 9.9 g carbohydrate per 100 kcal, and 0.6 g minerals and vitamins per 100 kcal.[22] The oral intake was calculated as previously described.[27] The average caloric intake during the study was 126 ± 12 kcal/kg/d in the MF group and 123 ± 11 kcal/kg/d in the GMF group. GMF was supplemented with 1.43 mg gangliosides/100 kcal, so the average dose of gangliosides per child was 1.80 ± 0.16 mg/kg/d.

Gangliosides

We chose gangliosides from porcine brain to supplement our formulas, because the distribution was closer than others to that found in human milk.[28] Gangliosides were purified and quantified by us as previously described,[29,30] adapted for large-scale ganglioside purification. Gangliosides were identified according to the nomenclature of Svennerholm.[31] The

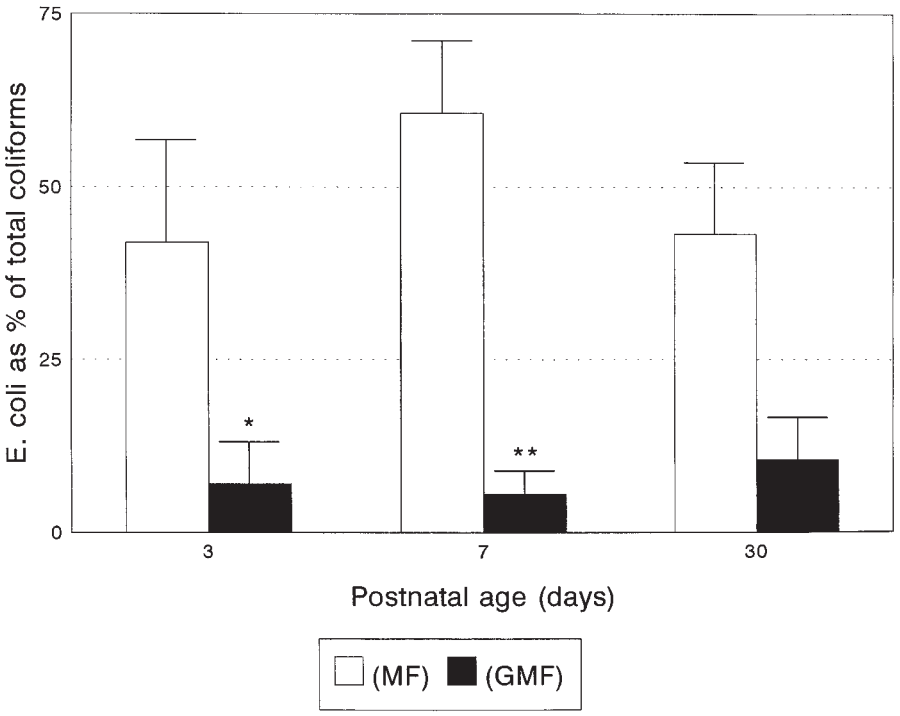

Fig. 1. Content of *E. coli* expressed as mean percentage of total coliforms. Values were calculated for fecal samples obtained on postnatal days 3, 7, and 30 from infants fed milk formula (*MF*) or ganglioside-supplemented milk formula (*GMF*). Results are means ± SEM. Kruskal-Wallis nonparametric test was used to determine the effects of diet as source of variation. Versus MF group: *$P < .01$; **$P < .001$.

distribution of the main individual gangliosides in preterm human milk as well as in pig brain was previously reported by our group.[32]

In preliminary toxicologic studies, 16 neonatal pigs received a formula supplemented with gangliosides from the fifth day of life at concentrations nearly 20 times higher (145 mg/100 g dry product) than those used in this study. Another 16 neonatal pigs received the same formula without supplemented gangliosides. At 21 and 45 days postnatal age, 8 pigs from each group were killed. No differences in weight gain and clinical course were observed between the experimental and control groups, and no toxic effects were observed.

Analytic Procedures

Feces were collected at defecation and immediately placed in sterile plastic bags under anaerobic conditions (CO_2 + H_2 atmosphere). Feces were always collected early in the morning. Nurses from the hospital undertook the collection immediately after defecation, and they had been previously trained to place feces immediately in sterile bags under anaerobic conditions. Occasionally the fecal specimens were obtained at defecation induced with a cotton swab inserted into the anus. Samples were transported and processed within 1 to 3 hours after collection. The fecal samples were analyzed as previously described.[23] The culture media, microorganisms investigated, atmosphere, and time of incubation are detailed in Table I. Results were expressed as the number of microorganisms per gram of dry feces. The results of bacterial counts were log transformed, and the means for each group of bacteria were calculated according to Best.[33]

Statistical Analysis

The data were processed with BMDP statistical software.[34] To determine the effects of diet and postnatal age as sources of variation, we used the Kruskal-Wallis and Friedman nonparametric tests because the data did not show a normal distribution according to Shapiro and Wilks' W statistic. The significance of the differences between the percentages of fecal samples positive for *E. coli* or bifidobacteria in the two groups was assessed with a chi-square test.

RESULTS

The logarithmic counts of *E. coli* were significantly lower in the feces of infants fed ganglioside-supplemented milk formula (GMF group) than in the feces of those receiving the standard milk formula (MF group) (Table II) on days 3 ($P < .01$) and 7 ($P < .001$), and there was a trend toward significance on day 30 ($P = .0515$).

In infants receiving GMF, the levels of bifidobacteria remained fairly stable throughout the period of sample collection, whereas in the MF group the microbial counts had increased on day 7 and had decreased on day 30. There were no significant differences between log bifidobacteria counts in the two groups on postpartum day 3, although the counts for this group of bacteria tended to be higher in the GMF group than in MF-fed infants. However, by day 30, bifidobacteria counts were significantly higher in the GMF than in the MF group ($P < .05$). In the group fed unsupplemented formula, the logarithmic counts of bifidobacteria tended to be lower ($P = .0578$) on day 30 than on day 7 of life.

We found no significant effects of diet or postnatal age on logarithmic counts of coliforms, enterobacteria, enterococci, aerobes, and anaerobes.

The mean percentages of *E. coli* were lower in the GMF than in the MF group at all postnatal ages (Fig. 1); the differences between the groups were especially significant on day 7 post partum ($P < .001$) but were also evident at 3 days ($P < .01$), and at 30 days the P value was close to significance ($P = .0548$).

The percentage of fecal samples in which we found *E. coli* and bifidobacteria was calculated for both groups. For *E. coli*, we considered the bacterium present when we counted more than 1000 colonies in fecal samples obtained on day 3 of age and more than 10,000 colonies in fecal samples at 7 and 30 days. For bifidobacteria, we considered the bacterium present when we counted more than

10,000 colonies in fecal samples at 3 days and more than 100,000 colonies in fecal samples at 7 and 30 days.

We found that the percentage of fecal samples that met the criteria for the presence of *E. coli* (Fig. 2) was significantly higher in the MF than in the GMF group at all postnatal ages. This difference was significant on day 7 ($P < .01$) and day 30 ($P < .05$). There was no difference between the groups in the percentage of fecal samples that contained bifidobacteria on postpartum days 7 and 30, but on day 3 the percentage was significantly lower ($P < .05$) in the MF than in the GMF group.

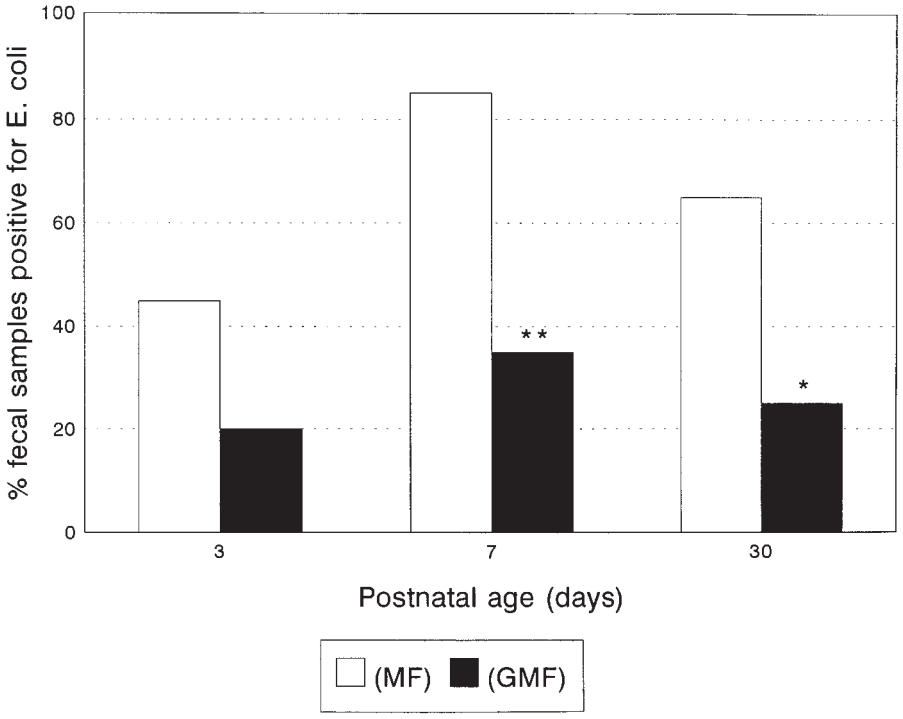

Fig. 2. Percentage of fecal samples that satisfied criteria for presence of *E. coli*. Values were calculated for fecal samples obtained on postnatal days 3, 7, and 30 from infants fed milk formula (*MF*) or ganglioside-supplemented milk formula (*GMF*). *E. coli* organisms were considered present when there were more than 1000 colonies on day 3 and more than 10,000 colonies on days 7 and 30. Differences between feeding groups were assessed with a chi-square test. Versus MF group: *$P < .05$; **$P < .01$.

DISCUSSION

The exact role of gangliosides in human milk is not well understood. Although Carlson and House[35] found a significant increase of brain ganglioside total content after oral administration of *N*-acetylneuraminic acid to rats, other experiments using radioactive G_{M1} monosialoganglioside contradict this finding.[36] The latter results suggest that only a small proportion of gangliosides is absorbed after oral ingestion, and therefore their presence in human milk is likely to correlate with a biologic role in the neonatal gastrointestinal tract.

The lack of a breast-fed infant control group is also a limitation of our study. The original study design considered that group, but because of the hospital routine, which usually includes a low birth weight infant formula to feed preterm infants, it was impossible to complete such group. A future study comparing breast-fed infants and infants fed ganglioside-supplemented formula should be undertaken.

Although we do not know the exact mechanism by which dietary gangliosides reduce the fecal levels of *E. coli*, in vitro experiments suggest that gangliosides interact with specific *E. coli* strains.[37] The inhibitory effect of gangliosides on *E. coli* enterotoxin is well known,[19] but there is also some evidence to suggest that gangliosides inhibit the adherence of this bacterium to intestinal epithelial cells.[20] Likewise, the meconium and feces of breast-fed newborn infants have been reported to inhibit the adhesion of S-fimbriated *E. coli* to epithelial cells.[38] The stronger inhibitory capacity found in meconium has been linked to the concentration of sialic acid. It was recently shown that meconium gangliosides are oncofetal gangliosides, and their structure resembles that of some human milk oligosaccharides and some buttermilk gangliosides.[39] These data suggest that sialylated oligosaccharides and other compounds with conjugated sialylated carbohydrates (glycoproteins and glycolipids) could function as receptor-analogous structures for bacterial adhesins. Such compounds could modify the intestinal microflora in the neonate and reduce the infectious capacity of these bacteria.

We also found a difference between the two groups in fecal levels of bifidobacteria, which suggests that the colonization of bifidobacterial flora is faster in infants fed with a milk formula supplemented with gangliosides. It has been reported recently that fortification of infant formula with *N*-acetylneuraminic acid–containing substances may promote the growth of bifidobacteria.[40] Other milk compounds, namely nucleotides[23] and sialylated oligosaccharides,[41] have also been implicated as bifidobacterial growth promoters.

We conclude that ganglioside-supplemented milk formulas can promote the growth of bifidobacteria and suppress the growth of *E. coli* and possibly other potentially pathogenic microorganisms in the intestine of preterm newborn infants. However, further studies are required to clarify the mechanisms implicated in this action and the relevance of this finding to clinical outcomes in the human neonate, especially in undeveloped countries where gastroenteritis caused by *E. coli* are particularly abundant.

We thank Juan Miguel Fernández, María Luisa Jiménez, and Antonio José Gómez for their technical assistance, and Dr María Ramírez for her help with statistical processing of the data. We also thank Karen Shashok for improving the English, and Drs William MacLean, Stephan Ladisch, and Ricardo Uauy for their critical reading of the manuscript.

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