Preparation and Evaluation of Fortified Human Milk for Very-Low-Birth-Weight Infants

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Efforts to develop experimentally based feeding protocols using human milk for very-low-birth-weight (VLBW) infant reveal two major problems. First, there is little information on the nutrient requirements of these infants (1) and second, the description or understanding of the changes undergone by banked human milk between collection and feeding is limited (2).

We have approached the first problem by making pragmatic decisions. The range of protein and energy requirements chosen was based on the factorial calculations of Fomon et al. (3), who used intrauterine growth rates as the principal factor in their computations. Additionally, assumptions were made regarding the efficiency of nutrient utilization in the maintenance range and interindividual variation in nutrient requirements. Although such an approach does not address directly the uncertainties regarding optimal extrauterine growth rates, maturation of body composition, and development of mature metabolic abilities (4), it does provide reference points for experimental trials.

The second problem requires the determination of the composition of human milk in selected circumstances. Investigators of the Children's Nutrition Research Center, Baylor College of Medicine, Houston; in the Immunology Division of the Department of Pedi-
atrics of the University of Texas Medical Branch, Galveston; and
of the Department of Animal Science, Texas A&M University,
College Station, have collaborated for 4 years in studies of the
nutritional and immunologic composition of milk produced by women
who delivered term (5,6) or preterm infants (7,8) and who weaned
their infants gradually from the breast (9,10). We also have exam-
ined the effects of specific collection (11), storage (11,12), and
processing conditions on the composition of mature human milk.
In these studies, variables such as the duration of pregnancy and
lactation, completeness of emptying of the breast, time of day, diet,
maternal age, and parity have been controlled. Diet and nutrient
stores were controlled by qualitatively screening maternal intakes
and restricting subject recruitment to middle- and upper-income
groups for whom the availability of food was not a limiting factor.
Central to these studies were analyses of milk of women delivering
prematurely.
We have found, as have other investigators, that women delivering
prematurely had levels of protein N in their milk (PM) ~21% higher
than those of women delivering term infants (7). The difference in
protein N between PM and milk from women delivering at term
(TM) persisted for only 8 weeks and decreased with time. By the
fourth week of lactation, the concentration of protein N was equal
to that observed in TM at 2 weeks.
We found no differences in the caloric concentrations of these
milks (7), which is in agreement with reports by Gross et al. (13),
but at variance with observations made by Anderson et al. (14),
who reported a 30% higher energy density in PM than TM. The
gestational ages of the infants included in the Anderson study (26–
33 weeks), however, were younger than in ours (30–36 weeks).
Samples in the Anderson study were obtained over 24 hr; in con-
trast, samples in our study represented the entire content of a single
breast emptied once between 8 a.m. and 12 noon. Despite these
methodological differences, it appears that the disparity between
our findings and those of Anderson and co-workers is caused not
by higher energy concentrations in the PM assayed but by relatively
lower energy values in the TM they analyzed. Our values for PM,
those obtained by Gross et al., and those of Anderson et al. were 66.4, 65.4, and 68.0 kcal/dl, respectively. Analogous values from each of these laboratories for TM were 67.7, 62.3, and 58.5 kcal/dl, respectively.

From a practical standpoint, it is not uncommon for mothers to produce more milk than their VLBW infant can consume. If it were possible to identify diurnal patterns in the fat content of human milk, then one could select the most calorically concentrated milk as food for such infants. Moreover, we have found that certain key nutrients may be present in lower concentrations in PM than in TM. Mean Ca and P concentrations in PM were consistently 84 and 81%, respectively, of values obtained in samples of TM collected at 2-week intervals through the first 3 months of lactation.

In studies of milk composition during gradual weaning, we have observed that protein and Na concentrations rise as milk production falls, but other key nutrients such as Ca and energy show less consistent changes (9).

We were unable to identify a source of milk with nutrient concentrations high enough to meet the estimated needs of VLBW infants. The nutrient concentrations of the milks studied would have necessitated the feeding of volumes >175 ml/kg in order to meet our estimates of requirements. This has been a source of concern, because it is our view that a conservative regimen for the volume of fluid administered is generally appropriate for VLBW infants. Many of these infants suffer from chronic respiratory disorders, which are thought to benefit from fluid restriction. Bell et al. (15) recently reported observing a greater incidence of patent ductus arteriosus and congestive heart failure in infants managed with liberal fluid regimens. Formulas with caloric densities greater than 0.8 kcal/ml can be administered to the infant by a continuous nasogastric infusion. This is the principal feeding method used at Texas Children's Hospital and allows the delivery of higher energy and protein levels without the risks that may be entailed by high-volume feedings.

The immunologic composition of milk has been more difficult to determine. We have measured the concentrations of lysozyme, lac-
toferrin, secretory IgA (sIgA), total IgA, sIgA antibodies to a pool
of *E. coli* antigens, and leukocytes in TM through 2 years of
unrestricted lactation (5,6). Lysozyme levels fell transiently during
the first 4 weeks and then rose dramatically over the next 20 weeks.
Lysozyme levels remained stable thereafter for 2 years. Lactoferrin
levels fell over the first 12 weeks and then remained stable for the
remainder of the 2-year period we have studied. The IgA, both
total and secretory, fell over the first 12 weeks and then rose over
the next few weeks to levels observed at 4 weeks and remained at
this level thereafter. Leukocyte levels fell over the initial 4 weeks
and remained low for the duration of the period studied. During
weaning, levels of these components either rose modestly or re-
mained stable.

The pattern of compositional changes for certain immunologic
factors differed substantially between PM and TM (8). Lysozyme
and lactoferrin levels were greater in PM during each 2-week in-
terval of the 3 months of observation. As in TM, sIgA is the
predominant form of IgA in PM. Both total IgA and sIgA were
observed to rise linearly between the sixth and 12th week of lac-
tation. Leukocyte concentrations were of particular interest, and
we found that viable leukocyte numbers in PM were lower at 2
weeks and higher at 12 weeks than in TM. Whether or not these
differences are beneficial to the premature infant is not clear, nor
are the mechanisms responsible for these differences apparent.
Nevertheless, there are differences between PM and TM, and there
is an obvious need to establish the *in vivo* functions of these com-
ponents as well as the intakes necessary to ensure that they are
effective.

Evaluations of collection, storage, and processing protocols were
conducted either before or simultaneously with the studies that have
been described. These were performed to define banking proce-
dures to be used in the feeding protocol. Initial studies compared
the composition of milks obtained by hand expression or gentle
suction applied by an electric pump (11). Differences in volume
and concentrations of fat suggested that the electric pump provided
more representative samples, especially from mothers inexperienced with hand expression.

The effects of storage in Pyrex®, polyethylene, and polypropylene containers at 4°C also were evaluated (11). The concentrations of immunologic factors listed above and of Cu, Zn, Na, Fe, vitamin A, and protein N were measured at 4 and 24 hr of storage. Nutrient concentrations were not affected to a measurable extent by these treatments. The concentrations of several immunologic factors, however, were affected, but the changes were not related to the container used for storage with two exceptions (11). Concentrations of sIgA antibodies to E. coli antigens fell much more in milks stored in polyethylene containers. Also, after 24 hr, more macrophages, neutrophils, and lymphocytes were measured in the fluid phase of milks stored in Pyrex than in samples stored in polypropylene, although neither container was clearly better. Polypropylene containers, however, were chosen for all subsequent studies because there were significant losses of sIgA antibodies in polyethylene bags, there was difficulty in handling the flexible bags, and Pyrex containers were easily broken.

The effects of storage at 37, 4, and −72°C in polypropylene containers have also been evaluated (11). Ascorbic acid showed the most marked changes with storage of the nutrients studied. Its concentration in milk stored at 4°C for 24 hr was 60% of the value observed after 4 hr storage at the same temperature. At 48 hr, the concentration fell to ~40% of the 4-hr value. Values were comparable in milks stored at 4 hr at 4 and 37°C and for 24 hr at −72°C. The transient maintenance of ascorbic acid concentrations suggests that it is protected, at least temporarily, by other factors that are oxidized more easily or that it is compartmentalized in such a way that it is protected. The decrease in ascorbic acid levels with prolonged storage suggests that other labile components might be oxidized too.

Generally, noncellular immunologic factors were highest in samples stored at 37°C regardless of storage time. Notable exceptions were total IgA and sIgA, for which similar values were observed regardless of the storage temperature. Lymphocytes, on the other
hand, were present in highest numbers in samples stored at 4°C, but \( ^3 \)H-thymidine incorporation following PHA stimulation decreased progressively with time at all storage temperatures.

These studies indicate that many constituents are preserved when banked human milk is stored at 4°C for periods of 24 hr or less. Although some immunologic components appeared to be preserved better at 37°C, concomitant studies of bacterial growth indicated that there were many problems in preventing bacterial overgrowth when milk was maintained at this temperature.

**THE PREPARATION OF HUMAN MILK FRACTIONS**

The next series of experiments focused on the preparation of skim and cream fractions that could be added easily to PM and thereby increase its protein and energy densities. Levels were achieved that would permit the use of preparations for feeding VLBW infants containing \( \sim 450 \) mg N/kg and \( \sim 130 \) kcal/kg while the intake volume was maintained at \( \sim 130 \) ml/kg for the first 3 to 4 weeks and 160 ml/kg for the remaining 3 to 4 weeks of study. Our first choice was to use human milk components (Fig. 1) to obtain the skim and cream fractions. A short-time/high-temperature protocol was evaluated to process donor milk (DM) for this purpose. Heating DM at 72°C for 15 sec had only slight adverse effects on potentially heat-labile nutrients and preserved the immunologic identity of the proteins studied (IgA, lactoferrin, and lysozyme) at acceptable levels. This treatment also had adequate bactericidal effects and could be shown to destroy added cytomegalic virus.

For the preparation of skim and cream fractions, donor milk was screened to minimize the use of milk possibly contaminated with pathogens and pollutants. Acceptable milk was heated as described above in an APV heat exchanger with heating and cooling times of less than 5 sec. Cream and skim fractions were prepared from the heat processed DM by using a DeLaval cream separator. The cream fraction was lyophilized and stored at \(-20°C\) in foil-laminated pouches. The skim fraction was dialyzed to reduce the lactose
FORTIFIED HUMAN MILK FOR VLBW INFANTS

Donor Milk

- Frozen
- Thawed
- Pooled

Pasteurization
72°C
15 sec.

Lactose (50%)
Small MW Compounds

Dialysis

Skim Fraction

Skim Fraction
Protein, minerals, lactose

lyophilization

5 gram packets

Cream Fraction

Cream Fraction

lyophilization

5 gram packets

Fresh
Mother's Milk

A B

A B

A

Nursery

Fortified
Mother's Milk

content by ~50% against a solution containing Ca, K, Na, P, Mg, Cu, Mn, and Fe salts that was made up to maintain the mineral-to-N ratio in the skim milk. The end product of this process was lyophilized and stored, as was the cream fraction. These fractions are used to fortify PM to levels (~346 mg N/dl and 100 kcal/dl) that meet conservative fluid requirements or to lower levels when more liberal fluid intakes are indicated.

Milk from mothers of premature infants was analyzed periodically to determine its total N and caloric density. Analyzed fractions of skim and cream were added to this milk in the proportions necessary to provide the infant with the desired levels of N and energy. Eventually, our goal was to compare clinical outcomes between infants fed this type of human milk preparation and those fed synthetic formula in isonitrogenous and isocaloric amounts. The design should permit us to test the in vivo significance of the functional components of human milk. Before this comparison

FIG. 1. Outline of protocol followed in the preparation of skim and cream fractions from donor milk: (A) laboratory analysis; (B) bacteriology.
TABLE 1. Description of VLBW infants fed fortified banked human milk

<table>
<thead>
<tr>
<th>Parameter</th>
<th>First period</th>
<th>Second period</th>
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<tbody>
<tr>
<td>Birth weight (g)</td>
<td>1,191 ± 183</td>
<td>1,207 ± 114</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>29.0 ± 0.7</td>
<td>30.0 ± 0.5</td>
</tr>
<tr>
<td>Age during balance (days)</td>
<td>17.0 ± 4.0</td>
<td>33.0 ± 3.0</td>
</tr>
<tr>
<td>Weight gain (g/kg/day)</td>
<td>16.0 ± 5.0</td>
<td>18.0 ± 5.0</td>
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could be carried out, however, it was important to assess the utilization of N and fat from the human milk preparation described. These assessments were conducted in a series of metabolic studies. Very-low-birth-weight infants of mothers who were planning to breast feed were enrolled from the neonatal nurseries of Texas Children's Hospital, Houston. The infants had to be 28 to 30 weeks gestation, approximate weight for gestational age at birth, and free of prolonged respiratory difficulties and major congenital anomalies. It also was required that they be on full enteral feeds by 15 days of life. The breast-feeding consultant met with the mother and family of the infant to review the protocol, which described the initiation, establishment, and maintenance of lactation without a suckling infant. Instructions were given for the collection and daily delivery of the mother's milk to the hospital. There, the milk from each mother was fortified with individually determined amounts of lyophilized fractions of skim and cream. The milk was usually fed within 24 hr of collection and seldom after 36 hr. Two feeding periods were evaluated. Human milk was fortified to a caloric density of 1.0 kcal/ml and was fed at ~130 ml/kg per day via a continuous nasogastric infusion for the first month. The second 4-week period entailed the use of milk fortified to ~0.8 kcal/ml and fed at 160 ml/kg per day. Ninety-six-hour balance studies were conducted during each period as part of the total assessment of the feeding protocol. Table 1 summarizes selected subject parameters,
and Table 2 outlines their intakes. For both periods, N from processed DM accounted for \( \sim 28\% \) of the total N fed. This was not the case for fat. In the first period, \( \sim 37\% \) of the total fat fed came from processed DM, and 14% in the second period.

The utilization of N from PM and processed DM was compared by regression of the amount of N absorbed versus the amounts of N from PM and DM that were fed. This calculation was performed to assess the apparent digestibility of N from these two sources. In both balance periods, N from DM and PM was highly digestible. The regression coefficients calculated from the data of the first balance were 0.99 for DM and 0.90 for PM. In the second balance period, the analogous values were 0.95 and 1.00, respectively. Each of these coefficients was significant at \( p<0.05 \). The utilization of proteins was also evaluated by regression of the amount of N retained versus the amounts of N from DM and PM that were fed. The regression coefficients for DM and PM N in the first balance period were 0.80 and 0.80, respectively \( (p<0.05) \). In the second period, the values were considerably lower, 0.43 and 0.61, respectively. However, only the coefficient for PM was significant.

We have speculated about causes of possible differences in the coefficients by estimating the efficiency with which N was retained in both periods. The lower values in the second balance period may reflect a trend toward efficiencies seen in older children and adults who are consuming maintenance levels of protein (16). Efficiencies close to 65% are reported for these groups. Conversely, the high

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\text{TABLE 2. Intake of VLBW fed fortified banked human milk}
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<thead>
<tr>
<th></th>
<th>First period</th>
<th>Second period</th>
</tr>
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<tbody>
<tr>
<td>N (mg/kg)</td>
<td>469.0 ± 51.0</td>
<td>468.0 ± 46.0</td>
</tr>
<tr>
<td>N from PM (mg/kg)</td>
<td>338.0 ± 58.0</td>
<td>340.0 ± 55.0</td>
</tr>
<tr>
<td>N from DM (mg/kg)</td>
<td>131.0 ± 32.0</td>
<td>128.0 ± 48.0</td>
</tr>
<tr>
<td>Fat intake (g/kg)</td>
<td>7.3 ± 0.7</td>
<td>6.8 ± 0.8</td>
</tr>
<tr>
<td>Fat from PM (g/kg)</td>
<td>4.6 ± 0.7</td>
<td>5.8 ± 0.5</td>
</tr>
<tr>
<td>Fat from DM (g/kg)</td>
<td>2.7 ± 0.6</td>
<td>1.0 ± 0.6</td>
</tr>
<tr>
<td>Gross energy (kcal/kg)</td>
<td>136.0 ± 5.0</td>
<td>136.0 ± 5.0</td>
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values observed in the first balance period may reflect a failure to meet the requirement for protein; in that case, a higher efficiency might be expected. Alternatively, the lower values in the second balance period may reflect a progressive deficit of a nutrient or nutrients that limited the efficiency of N utilization. The results of one additional analysis should be noted here. The regression of nitrogen retention versus the amounts of nitrogen absorbed and metabolizable energy was also calculated. In the first balance, we observed a mean increase of 2.1 mg N per added kilocalorie; in the second balance, this value dropped to 1.0 mg N per added kilocalorie. Although it is too early to comment on the statistical significance of these differences, it is of interest to make comparisons with analogous values obtained from studies in adults. Earlier work done in adults indicates an improvement in nitrogen retention of 2 to 4 mg per added kilocalorie when N and energy are provided at the maintenance range and no other nutrients are limiting (17). The apparent decrease from 2 to 1 mg N per added kilocalorie suggests that energy or another nutrient may limit the efficient utilization of N. These are speculations that require completion of the study before more definite statements can be made. However, the available data suggest that N from both sources is used equally.

We assessed the bioavailability of fat in a similar manner. The quantity of fat that was absorbed was regressed against the quantities of fat from PM and DM that were fed. In the first balance, fat from both sources was absorbed to a similar extent. The coefficients relating fat absorption to the levels of fat intake from DM and PM were 1 for both sources. These results suggest that fat from both sources was absorbed very efficiently.

We anticipate completion of these studies in the next few months. If preliminary findings are confirmed, that nitrogen and fat from processed and fresh milk are absorbed and retained with similar efficiencies, comparisons with data from similar experiments in infants fed synthetic formulas will be particularly useful. The use of fresh PM fortified with skim and cream fractions permits iso-nitrogenous and isocaloric feeding studies that evaluate the efficacy
of human milk in promoting optimal nutrient utilization and immune function.

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