Lactoferrin and Iron Absorption in Newborn Infants

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ABSTRACT. Results from experiments in this laboratory using ^{59}Fe suggest that bovine lactoferrin (Lf) has no effect on iron absorption in rats. A study was therefore carried out in newborn infants to measure the effects of Lf on iron retention. Bovine Lf was labeled with the stable isotope ^{58}Fe and fed to 7-day-old infants in a standard milk formula. Iron retention was estimated by measuring the unabsorbed ^{58}Fe excreted in the feces during the following 3 days using neutron activation analysis. The results were compared with those obtained from a group of infants fed a similar level of iron as ferric chloride, labeled with ^{58}Fe, together with 30 mg ascorbic acid. There was a very wide variation in percent iron retention amongst the infants but no overall difference between the Lf and ferric chloride groups. This confirms the previous findings in rats that Lf does not influence the availability of nonheme iron. (Pediatr Res 22: 651–654, 1987)

Abbreviations

Lf, lactoferrin

AAS, atomic absorption spectrometry

NAA, neutron activation analysis

The effect of Lf on iron absorption in infants is not clear. Many studies have been performed in animals using ^{59}Fe but such high energy radioisotopes cannot be used in infants for ethical reasons. However, recent developments in stable isotope methodology using ^{58}Fe and neutron activation analysis (1) have made labeled studies possible in human subjects for whom radioisotope administration would not be acceptable. The most recent evidence in rats (2) suggest that Lf has no effect on iron absorption, but it is uncertain as to whether or not these findings can be extrapolated to man. A study was therefore carried out in newborn infants, already participating in a study on Lf and fecal flora, in which the effect of Lf on iron absorption was determined by comparing iron retention from ^{58}Fe-labeled bovine Lf with that from ferric chloride plus ascorbic acid. In addition, the level of bovine Lf in the stools of 11-day-old infants fed Lf-fortified or unfortified formulae was measured to discover to what extent the Lf was broken down by digestive enzymes and microorganisms.

MATERIALS AND METHODS

Thirty-six babies (16 boys and 20 girls) were recruited at Sorrento Maternity Hospital, Birmingham, as part of a larger study on Lf and fecal flora. The babies were full term and healthy, weighing 2060–3800 g (mean 3140 ± 490 g), with a gestational age of 36–41 wk (mean 39.5 ± 1.0 wk).

There were 25 Caucasians, nine Asians, and two West Indian babies. Their mothers had opted to bottle feed and gave written informed consent to the investigation. Seven babies were subsequently excluded from the study: one on the basis of noncompliance (incomplete fecal collection), one was given an incorrectly prepared labeled feed, the feces from three others were inadvertently pooled following freeze-drying and two babies had chronic constipation. Results from 29 babies are presented. The babies were fed from birth on ad libitum quantities of one or two formulas specially prepared by Nestlé for this study. Each formula was manufactured from demineralized whey, skimmed milk, a butter and vegetable fat mixture, minerals, and vitamins. One formula was used without modification (Basic formula) and the other consisted of the basic formula plus bovine Lf (Lf formula). The composition of these two formulas is shown in Table 1. They differed only in the amounts of added Lf and since this carried a small amount of iron they also have a slightly different iron content.

On the 7th day after birth half of the babies in each group were given a single dose of ^{58}Fe-labeled bovine Lf and the rest were given ^{58}Fe-labeled ferric chloride plus ascorbic acid (for method of preparation see below). All subsequent diapers were collected for 3 days. During this period the mothers were instructed by the nurses to use two liners in each diaper. The uncontaminated part of each diaper was discarded and the remainder frozen and the fecal material removed and freeze-dried. Any stained diaper material was soaked for at least 12 h in 0.1 M HCl, the dilute acid extract freeze-dried, and the solid residue added to the freeze-dried stools.

Iron retention was measured by subtracting the fecal ^{58}Fe (unabsorbed label), with due allowance for naturally occurring ^{58}Fe (0.33%), from the administered dose after adjusting for leftover feed, vomit, etc, as described previously (3). Fecal bovine Lf was estimated in a 24-h stool collection 11 days after birth (see below for details). For comparison, stools were also collected from 28 (11 boys, 17 girls) exclusively breast-fed babies (mean birth weight 3260 ± 400 g) 11 days after birth and fecal human Lf was measured using antihuman Lf antibody (Serotec, Kidlington, Oxfordshire, England).

Preparation and administration of ^{58}Fe-labeled Lf and ferric chloride. Freeze-dried bovine Lf, partially saturated with iron, was supplied by Nestlé Research Department (Nestec, Vevey, Switzerland) and dissolved in 0.1 M KHCO₃ before use.

Elemental iron, containing 71.5% ^{58}Fe (mass atom percent), used for labeling the ferric chloride, or 65.8%, used for labeling the Lf, obtained from AERE, Harwell, Oxfordshire, England, was dissolved in 0.1 M HCl. The solution was filtered and the iron concentration measured by atomic absorption spectrometry (see below). Half of the infants recruited to the study were given 3.032 g of the ferric chloride solution (285 μg ^{58}Fe), together with 30 mg L-ascorbic acid, in a small volume of milk formula containing no added Lf or iron. The solutions and dry ascorbic acid were stored separately in small plastic vials at −18° C until use. The remaining infants were given ^{58}Fe-labeled Lf, fully saturated with iron; although the Lf in human milk is only partially saturated this was the only means of ensuring sufficient ^{58}Fe in a physiological dose of Lf for subsequent detection of unabsorbed isotope in the feces.

The labeled Lf solution was prepared by adding citric acid (10 mol/mol iron) to the ^{58}Fe-ferric chloride and neutralizing to pH 7.0 with KHCO$_3$. Volumes containing 147 μg ^{58}Fe and 420 mg Lf (4.54 ml) were dispensed into plastic containers and stored at −18° C until required.

The labeled Lf or ferric chloride plus ascorbic acid were administered to the infants at approximately 1000 h as follows: 8 g of basic formula was mixed into 30 ml cooled boiled water in a plastic jug together with the ^{58}Fe solution. The empty ^{58}Fe vial was rinsed three times with a total volume of 25 ml water, dispensed from a syringe, the washings added to the feed, and the mixture transferred to a bottle. Any leftover feed was placed in a small plastic bottle, together with washings (using 20 ml water) from the jug and bottle, and freeze-dried.

Fecal Lf. Twenty-four-h stool collections made on day 11 were analyzed for bovine lactoferrin. Feces were homogenized in phosphate-buffered saline, centrifuged, and filtered through Whatman glass microfiber filters. Measurement of bovine lactoferrin was by immunoturbidimetry using partially purified bovine lactoferrin antibody raised in a rabbit. The assay was

calibrated using bovine lactoferrin supplied by Nestlé. The bovine lactoferrin gave a 5% cross-reactivity with human Lf. Results were expressed as mg fecal Lf per day.

AAS. Fecal samples, milk formulae, ^{58}Fe-labeled Lf, and reference materials (National Bureau of Standards, Office of Standard Reference Materials, Washington, D.C.) were analyzed for total iron by ashing in silica crucibles at 480° C for 48 h, taking the ash up in hot concentrated HCl, and diluting to an appropriate volume with distilled water; iron solutions were measured directly after dilution. The iron content was determined using a PU9000 AAS (Pye Unicam, Cambridge, England).

NAA. Samples were usually ashed to reduce bulk by removing organic material. Accurately weighed portions were placed in small plastic capsules and irradiated together with standards for 70 h in the Consort reactor (100 kW) at Imperial College, Ascot, at a flux of approximately 1.2×10^{16} neutrons/m^2/s. They were allowed to decay for 2 wk to reduce the activity from short-lived radionuclides and were then analyzed by γ-ray spectrometry using a Ge(Li) semiconductor detector (43 ml volume; resolution 1.81 keV at 1332 keV and 8.1% efficiency) and a Nuclear Data (ND 6600) multichannel analyzer (Nuclear Data, Bourne End, Bucks, England) with dedicated computer and Fortran programs for spectral analysis. The counting time was 2 h/sample to give a counting error of <1%.

Statistical analysis. The percentage iron retention results were not normally distributed, therefore differences between the two groups were analyzed using the Mann-Whitney test (4). Differences in fecal weights and iron content were analyzed by one-way analysis of variance and where this showed a treatment effect, differences between groups were tested using Student's *t* test (5).

Ethical consideration. The study was approved by South Birmingham Health Authority's Committee for the Supervision of Clinical Ethics, and the Ethical Committee of the AFRC Institute of Food Research, Norwich Laboratory.

Table 1. *Composition (wt/100 ml) of reconstituted formulas fed to infants**

Formula	Basic	L
Added bovine Lf (mg)	0	285
Iron (μg)	40	86
Protein (g)		1.7
Fat (g)		3.4
Carbohydrate (g)		7.4
Energy (kcal)		67
(KJ)		280
Ca (mg)		53
P (mg)		30
Zn (mg)		0.5
Cu (μg)		40
Ascorbic acid (mg)		5.4

* The composition of other nutrients are not shown but are similar to those for NAN, a formula manufactured by Nestlé.

RESULTS

There were no differences in mean gestational age between the groups, but there was a difference in birth weight ($p < 0.05$), probably explained by differences in the boy:girl ratio (Table 2).

The mean weights of feces collected during the 3 days following the ^{58}Fe-labeled feed are shown in Table 3, together with total iron content and iron concentration (μg/g dry weight). The infants were subdivided into four groups for statistical analysis: group 1, previously fed basic formula, given ^{58}Fe ferric chloride; group 2, fed basic formula, given ^{58}Fe Lf; group 3, fed L formula, given ^{58}Fe ferric chloride; group 4, fed L formula, given ^{58}Fe Lf. There were significant differences between the four groups in fecal dry weights ($p < 0.05$), but these differences were apparently unrelated to either the formula fed or the source of labeled iron. There were no differences in fecal iron concentration or total iron excreted during the 3-day period.

The individual figures for percent iron retention from the ^{58}Fe labeled ferric chloride and Lf are illustrated in Figure 1. Mean percent Fe retention from ferric chloride was 44.4 (SD 25.8) and

Table 2. *Details of babies studied (mean ± SD)*

Groups	1	2	3	4
n	8	8	8	5
Caucasian	5	5	6	4
Asian	1	3	2	1
West Indies	2	0	0	0
Boys:girls	2:6	6:2	3:5	1:4
Birth wt (g)	2980	3620	3120	3100
	±500	±280	±330	±410
Wt range (g)	2060–3480	3080–4000	2600–3500	2660–3740
Gestational age (wk)	39–40	38–41	39–40	38–40

Table 3. Wt (mean ± SD) and iron content of feces collected from 7 to 10 days after birth

Group	1	2	3	4
Formula fed on days 1–14 (iron content per 100 ml)	Basic (40 µg)	Basic (40 µg)	L (86 µg)	L (86 µg)
[58]Fe labeled iron source (given at day 7)	FeCl₃	Lf	FeCl₃	Lf
Dry wt feces per 3 days (g)	16.37[a*] ±4.39	21.49[b] ±3.96	16.68[a,b] ±6.97	13.24[a] ±1.95
Fe concentration (µg/g)	72.4 ±42.6	88.2 ±29.1	108.6 ±48.4	101.9 ±14.2
Total Fe/3 days (µg)	1120 ±480	1900 ±830	1640 ±510	1350 ±320

[*a,b] Values in a row with different superscripts are significantly different (group $2 > 1$, $p < 0.05$; group $2 > 4$, $p < 0.01$).
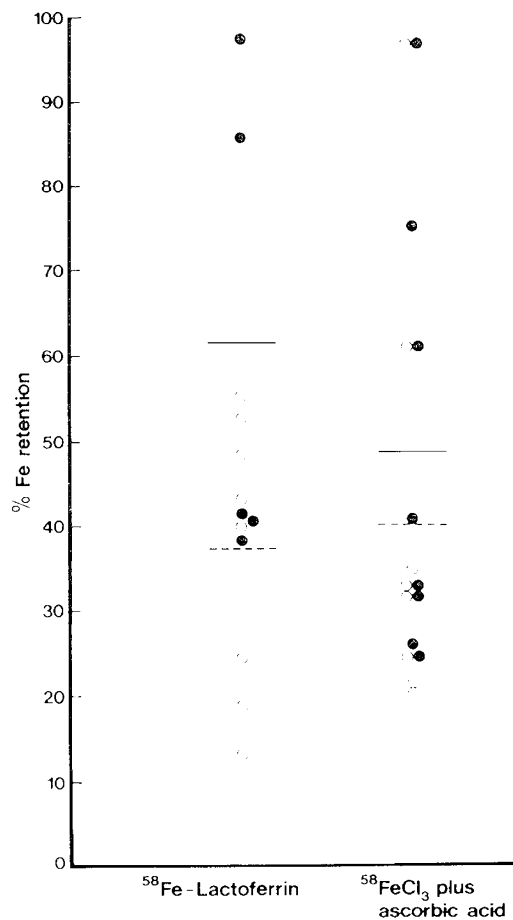

Fig. 1. Percent Fe retention from [58]Fe labeled-bovine Lf or [58]FeCl₃ in 7-day-old infants previously fed basic (○) or L formula (●). Horizontal bars represent group means for infants fed basic (–) or L formula (——).

with a range of 2.10–15.80, which was significantly higher than that from the babies receiving the L formula ($p < 0.001$).

DISCUSSION

Breast-fed infants consume much higher levels of Lf than bottle-fed infants. The normal concentration of human milk ranges from about 7 mg/ml in colostrum to not less than 1 mg/ml in mature milk (6), whereas mature bovine milk only contains 20–200 µg Lf/ml (7). Lf is very resistant to proteolytic degradation and when recovered from the feces of newborn infants retains its ability to bind iron (8). However, the recovery of Lf in the stools of infants fed a formula containing bovine Lf was low, which indicates that much of it had been degraded in the gut.

The iron retention results shown in Figure 1 demonstrate the very wide variation in absorption from both Lf and ferric chloride between individual infants. This was unrelated to gestational age.

Unfortunately, it was not possible to give fecal markers to infants to check for completeness of fecal collection, as is customary in studies of this kind in adults (3). The collection period of 3 days was a convenient length of time; it would not have been possible to carry it out for any longer in this study, and 3 days was considered to be sufficient time for any unabsorbed isotope to be excreted. However, some of the infants were suffering from constipation, which would have affected transit time. If the percent iron retention data in Figure 1 are examined in greater detail, it can be seen that five infants appeared to retain more than 70% of the [58]Fe labeled iron. These high figures may be correct, but, more likely, they are an overestimate resulting from incomplete collection of unabsorbed isotope.

The iron retention results indicate that there was no significant difference in iron absorption from ferric chloride or iron bound to Lf. Furthermore, the addition of bovine Lf to milk formula given to infants from birth onward (formula L), as part of a larger study on fecal flora, did not appear to affect the way in which the infants responded to different sources of iron. One explanation for the lack of difference between Lf-bound and inorganic iron, as also demonstrated in rats (1), would be that the iron is dissociated from the Lf in the acid environment of the stomach, and that with the rise in pH along the duodenum there is no appreciable rebinding of iron to Lf, as already demonstrated in vitro in rats (9). The iron can then enter the nonheme pool in the gut. Unfortunately because the recovery of bovine Lf in the feces of 11-day-old infants was low, the possibility that the Lf-Fe complex was absorbed cannot be excluded. The results from this study indicate that Lf-bound iron is handled by the body in exactly the same way as other nonheme dietary iron. Further studies are required to confirm this hypothesis.

from Lf 46.2 (SD 23.9). There was no significant difference between the two iron sources, nor did the previous formula (basic or L) have any effect on iron retention from either ferric chloride or Lf.

The mean bovine Lf concentration in the 24-h stool collected on day 11 after birth was 0.55 ± 0.65 mg (range 0–2.2) in infants fed the basic formula and 4.50 ± 1.52 mg (range 2.4–7.1) in those fed the Lf-fortified formula from birth. As expected, the babies fed the formula containing added bovine Lf excreted significantly more Lf in their stools than babies fed the basic formula ($p < 0.001$). However, most of the Lf from the L formula (285 mg/100 ml) appeared to have been broken down.

When a similar assay was performed on stools from 11-day-old breast-fed babies, the Lf levels were 8.32 ± 4.28 mg/24 h,

formulae, and C. Boyle and N. Burton for their invaluable assistance with the infants.

REFERENCES

1. Fairweather-Tait SJ, Minski MJ, Richardson DP 1983 Iron absorption from a malted cocoa drink fortified with ferric orthophosphate using the stable isotope ^{58}Fe as an extrinsic label. Br J Nutr 50:51–60
2. Fairweather-Tait SJ, Wright AJA, Piper Z 1986 The influence of human and bovine lactoferrin on iron absorption from ferrous sulphate in rats. Proc Nutr Soc 45:40A
3. Fairweather-Tait SJ, Minski MJ 1986 Studies on iron availability in man, using stable isotope techniques. Br J Nutr 55:279–285
4. Siegel S 1956 Nonparametric Statistics for the Behavioural Sciences. McGraw-Hill, London, pp 116–127
5. Snedecor GW, Cochran WG 1967 Statistical Methods, 6th ed. Iowa State University Press, Ames, IA, pp 100–106
6. Masson PL, Heremans JF 1971 Lactoferrin in milk from different species. Comp Biochem Physiol [B] 39:119–129
7. Brock JH 1980 Lactoferrin in human milk: its role in iron absorption and protection against enteric infection in the newborn infant. Arch Dis Child 55:417–421
8. Spik G, Brunet B, Mazurier-Dehaine C, Fontaine G, Montreuil J 1982 Characterisation and properties of the human and bovine lactoferrins extracted from the faeces of newborn infants. Acta Paediatr Scand 71:979–985
9. Huebers HA, Huebers E, Csiba ER, Finch CA 1983 The significance of transferrin for intestinal iron absorption. Blood 283–290

Errata

The article by Walterspiel JN, Kaplan SL, and Mason EO, Jr. 1986 Pediatr Res 20:237–241 titled "Protective effect of subinhibitory polymyxin B alone and in combination with ampicillin for overwhelming *Haemophilus influenzae* type B infection in the infant rat: Evidence for *in vivo* and *in vitro* release of free endotoxin after ampicillin treatment" was published with an incorrect middle initial for Dr. Walterspiel. The correct spelling is JN Walterspiel—**not** JW Walterspiel. The author regrets the error.

Errors have been found in the article by Jain SK, titled "Prematurity and lecithin-cholesterol acyltransferase deficiency in newborn infants," Pediatr Res 19:58–60, 1985.

Figures 1 and 2, on Y-axis, LCAT parentheses should read "cholesterol esterified, nmol/ml/h" instead of "^{14}C-cholesterol esterified, nmol/ml/h," because ^{14}C-cholesterol was used as a marker but the LCAT activity was expressed in terms of both labeled and nonlabeled cholesterol esterified by the LCAT. Also in "Materials and methods" it should have been stated that blood was collected into EDTA tubes instead of heparinized tubes. The author regrets the errors.