ABSTRACT Why breastfed infants absorb extrinsic iron (EFe) exceptionally well is an unexplained phenomenon. Our objective was to identify effects of human milk fractions (HMF) on bioavailability of EFe. HMF were prepared by centrifugation followed by successive ultrafiltration using 10-, 3- and 1-kDa molecular weight cutoff membranes. EFe was added to HMF before and after treatment with digestive enzymes. Solubilization of EFe by HMF was characterized by scintillation counting of radioiron and by size exclusion chromatography/inductively coupled plasma mass spectrometry (SEC/ICPMS) of stable iron. Effects of HMF on EFe uptake and basolateral transfer were assessed by using confluent Caco-2 cells in bicameral chambers. Whey fractions of low molecular weight (MW) derived from 10-kDa filtrate, except the 1-kDa filtrate, were as effective as ascorbate and nitrilotriacetate in solubilizing EFe at intestinal pH. Basolateral radioiron transfer from Caco-2 cell monolayers was greater in the presence of low MW whey fractions than in the presence of ferrous ascorbate. The 3-kDa filtrate and 3-kDa retentate fractions promoted basolateral transfer of cellular radioiron taken up previously. SEC/ICPMS of the 1-kDa retentate fraction revealed a UV-absorbing peak of MW ∼4.2 kDa that contained iron and that solubilized added ferric iron both before and after in vitro digestion with pepsin, pancreatin and bile extract. Our results suggested that a low MW component of breast milk whey enhances iron bioavailability. Because the iron solubilization activity is resistant to in vitro digestion, it is plausible that the component is active in vitro and may explain the excellent absorption of EFe by breastfed infants. J. Nutr. 133: 449–455, 2003.

KEY WORDS: • Caco-2 cells • iron absorption • breast milk
• inductively coupled plasma mass spectrometry

Breastfed infants absorb extrinsic (nonmilk) iron exceptionally well (1). Absorption of iron and its incorporation into hemoglobin measured by the extrinsic tag method are greater in breastfed than in formula-fed term infants (2) and have been correlated inversely to plasma ferritin concentration in breastfed term infants (2,3). Absorption of extrinsic iron by fasted adults is greater in the presence of human milk than in the presence of cow’s milk (4). The higher bioavailability of iron when breast milk is given is not fully explained by the factors known to affect iron absorption (5). Once it was thought that lactoferrin, abundant in human but not in cow’s milk, might facilitate iron absorption (6). However, absorption of extrinsic iron has been shown to be slightly greater when lactoferrin is absent than when it is present (7).

Our objective was to identify components in human milk that enhance extrinsic iron absorption. Sequential processes obligatoly to absorption of extrinsic iron include iron solubilization, iron uptake into and passage through the enterocyte and iron transfer across the basolateral membrane to the circulation. Monolayers of Caco-2 cells properly prepared on porous supports facilitate precise comparisons of the effects of treatment variables on these mechanistic processes (8–15). Caco-2 cells modify their ferrokinetics with changes in cellular iron status, possess the proper morphology and cellular constituents and exhibit characteristics similar to those observed in human studies (8,14,16,17). Moreover, they are useful for quantification of food iron bioavailability (17).

In this research, we used monolayers of Caco-2 cells to screen fractions of breast milk for their ability to enhance transepithelial flux of extrinsic iron. Then some of the most stimulatory fractions were studied for their influence on iron solubilization. Finally, the most stimulatory of these fractions was characterized as to the molecular weight of the active component, its iron binding capacity and its resistance to in vitro digestion. For clarity and brevity, our findings are reported in the sequence that these physiologic processes normally occur rather than in the order the experiments were conducted.

The amount of iron intrinsic to breast milk is insufficient to meet the iron needs of breastfed infants for longer than a few
months even if one assumes the intrinsic iron is absorbed completely. Extrinsic, nonmilk iron is present in the gastrointestinal tract of infants from endogenous secretions and from exogenous sources such as supplements and other foods. We hypothesized that persistence of specific peptides from breast milk in the proximal small intestine facilitates absorption of extrinsic iron and contributes beneficially to the iron status of the breastfed infant.

We report here the existence of several breast milk fractions that promote solubilization of extrinsic iron at intestinal pH and enhance flux of extrinsic iron across Caco-2 cell monolayers. At least one of these fractions has substantial iron content, binds an appreciable amount of extrinsic iron and is resistant to digestion by pepsin, pancreatin and bile extract.

MATERIALS AND METHODS

Materials and reagents. Antibiotic-antimycotic solution, nonessential amino acids and trypsin-EDTA (4) were purchased from Gibco (Grand Island, NY). Earle’s balanced salt solution without bicarbonate (EBSS), fetal bovine serum, HEPES, 2-(N-morpholino)ethanesulfonic acid; NTA, nitrilotriacetic acid; OFGM, outer-fat-globular membrane; PEEK, polyethylene ketone; SEC, size exclusion chromatography.

Fractionation of human milk. Aliquots of surplus human milk from samples collected under approved research protocols were combined by personnel of the Pediatric Metabolism Unit of the University of Iowa and were stored frozen at −80°C before use. Aliquots of each reconstituted fraction usually were brought to room temperature and digested in the vials within closed vessels in a microwave digestion apparatus (Milestone 1200 Mega; Milestone, Monroe, CT). Iron in fractions was determined by using flame atomic absorption spectrophotometry versus working standard solutions prepared from spectrometric standard iron from the National Institute of Standards and Technology (Gaithersburg, MD). Iron in uptake buffer and in chromatographic eluates of some milk fractions before and after incubations was determined by using inductively coupled plasma mass spectrometry (ICPMS). Protein was determined by the Lowry method (19).

Preparation of fractions for studies of iron solubilization and transmonolayer flux. Apical (uptake) buffer was freshly prepared by addition of 10 mmol of MES/L to EBSS to make EBSS/MES at pH 6.0 with or without the addition of phenol red (100 μmol/L) as desired. Iron concentration of freshly prepared uptake buffer was 0.10 ± 0.05 (mean ± se) μmol/L as determined by ICPMS. Lyophilized aliquots of milk fractions typically were reconstituted to a protein concentration of 2 g/L in EBSS/MES. To simulate exposure to infant gastric fluid, aliquots of each reconstituted fraction usually were brought to pH 3.0 by dropwise addition of 5 mol of HCl/L and warmed for 15 min at 37°C. Then all aliquots usually were brought to pH 6.0 by dropwise addition of 1 mol of NaHCO3/L and maintained at 37°C for 12 min before use in experiments of solubilization and transmonolayer flux of iron. For characterization of solubilization by using ICPMS, the solute limitations of ICPMS were met by omission of EBSS, diminution of the MES concentration from 10 mmol/L to 0.1–0.3 mmol/L and increase in the pH of MES from 6.0 to 6.5–7.0 by the addition of ammonium hydroxide.

Stock solutions contained ascorbic acid (20 mmol/L in H2O), nitrilotriacetic acid (5 mmol/L in H2O), ferrous sulfate (1 mmol/L in 0.1 mmol of HCl/L), ferric chloride (1 mmol/L in 10 mmol of HCl/L) and 59FeCl3 (specific radioactivity, 32.3 GBq/mmol; 0.573 μmol of...
Fe/L in 10 mmol of HCl/L). For studies of transmonolayer iron flux, ferrous/ascorbate control solutions (10 and 15 µmol of iron/L, ferrous/ascorbate molar ratio 1:2) were prepared freshly by the addition of 1 or 1.5% vol/vol ascorbic acid and ferrous sulfate stock solutions to EBSS/MES, pH 6.0.

**Iron solubilization.** For iron solubilization studies by measurement of soluble radioiron equilibrated with extrinsic stable iron, prepared aliquots of 3KR, 3KF, 1KR and 1KF in EBSS/MES were brought to iron concentration of 20 µmol/L by addition of ferric chloride stock solution. For comparison purposes, iron solubilization was carried out by adding desferrioxamine mesylate (D-9533; Sigma Chemical), crystalline cobalamin (V-2876; Sigma Chemical) and oxidized insulin chain B (I-6383; Sigma Chemical). Human apotransferrin (T-2252; Sigma Chemical), human milk lactalbumin (L-7269; Sigma Chemical), bovine lung aprotinin (H-5748; Sigma Chemical), hen egg lysozyme (A-4529; Sigma Chemical), oxidized insulin chain B (I-6383; Sigma Chemical), crystalline cobalamin (V-2876; Sigma Chemical) and desferrioxamine mesylate (D-9533; Sigma Chemical) were used for molecular weight calibration curve by identifying the absorbance peak of the 1KR fraction was estimated graphically from the molecular weight calibration curve by identifying the absorbance peak of the 1KR fraction.

**Transmonolayer flux of iron.** Relationships between components of the Caco-2/transwell system were illustrated in Figure 2. Procedures and conditions were as described previously (17) except that apical buffer was EBSS/MES, pH 6.0, and basolateral buffer was EBSS/Hepes, pH 7.4. In all experiments, 59FeCl3 (833 Bq/4.5 mL preincubation volume) was added to apical buffer (blank), ferrous ascorbate control solution and prepared milk fractions 10 min before incubation with cell monolayers. In some experiments, extrinsic stable iron as ferric chloride stock solution was added (final extrinsic iron concentration, 15 µmol/L) concomitantly with the radioiron. Radioactivity in cell lysates was considered to represent radioiron cellular content plus binding, and radioactivity in basolateral buffer was considered to represent net basolateral transfer.

**Loading of Caco-2 cells with stable and radioiron.** In a manner identical to the control incubations of the studies of transmonolayer
flux, Caco-2 cell monolayers were exposed apically to ferrous/ascorbate control solution and $^{59}$Fe for 1 h at 37°C. Then, the apical and basolateral solutions were removed, cells were rinsed with 1 mL of EBSS and the solutions were saved for measurement of radioactivity. Monolayers were then incubated for 1 h with apical 3KF or 3KR but without added $^{59}$Fe. Control for these incubations was EBSS/MES without added $^{59}$Fe. Fresh (i.e., nonradioactive), EBSS/HEPES, pH 7.4, was placed in the basal chambers. After incubation, apical and basolateral solutions and cell monolayers were counted for radioactivity.

**Statistical Analysis.** Analysis of the data were performed by using one-way ANOVA. Tukey’s multiple comparison test (Prism; GraphPad, San Diego, CA) was used to compare group means. Treatment comparisons were considered statistically significant when probabilities were <0.05.

**RESULTS**

**Iron and protein in milk fractions.** The 10KF fraction of mature human whey possessed 39% of the iron (iron concentration: 2.68 ± 0.18 μmol/L, mean ± SEM, n = 3) native to human milk (iron concentration: 5.75 ± 0.14 μmol/L) and the highest iron/protein ratio (67 ± 1.8 μmol/L) of the fractions analyzed (OGFM, 10KR, 10KF, casein). The OGF M fraction of the milk was second highest in intrinsic iron content (31% of milk iron) and highest in iron concentration (28.6 ± 1.2 μmol/L), followed by the 10KR fraction of whey at 26% of milk iron (17.9 ± 1.8 μmol/L). The casein fraction contained 2% of the protein and 1.4% of the iron (9.8 ± 1.8 μmol/L).

**Radioiron solubility.** The solubility of radioiron in the presence of whey fractions in EBSS/MES and with stable iron concentration brought to 20 μmol/L by the addition of ferric chloride is illustrated in **Figure 3**. Results are expressed as percentage of the radioactivity in reference ferric chloride (20 μmol/L) in 10 mmol of HCl/L, pH 2.0. Radioiron solubility in ferric chloride in EBSS, pH 6.0, without whey fractions was minimal (22.0 ± 0.2%). Solubility with 1KF added to the EBSS was only slightly, but significantly, greater (30.0 ± 0.3%). Radioiron solubilities in the presence of ascorbate or 1KR in EBSS were ~80% of the reference value in dilute HCl and solubilities in the presence of NTA, 3KF or 3KR were ~90–94% of reference.

**Results of size exclusion chromatography of 1KR are illustrated in Figures 4 and 5.** Integration of ultraviolet absorbance versus time at wavelength of 280 nm revealed that lyophilized, resuspended 1KR contained two broad peaks of 77 and 14 area%, respectively (chromatogram C, Fig. 4). When fit to the molecular weight calibration curve, the partition coefficient for the larger peak corresponded to an apparent molecular weight of 4.2 kDa. After in vitro enzymatic digestion of 1KR, the total chromatogram area (chromatogram D, Fig. 4) decreased to 50% of the chromatogram area of native 1KR (chromatogram C, Fig. 4), and the larger peak decreased to 52 area%, whereas a second peak at longer elution time comprised 39 area%. Size exclusion chromatography of native human α-lactalbumin (chromatogram A, Fig. 4) revealed a single, sharp peak of 100 area% with an elution time shorter than those of the broad peaks from 1KR. In vitro enzymatic digestion of α-lactalbumin decreased total chromatogram area (chromatogram B, Fig. 4) to 27% of the area of chromatogram A, and the products of proteolysis were distributed over a wide range of elution times.

**Selected ion monitoring by ICPMS of chromatographic effluents versus time revealed that lyophilized, redissolved 1KR exhibits a substantial peak of ionic intensity at m/z = 54 (chromatogram E, Fig. 5) at an elution time that corresponded to the maximum for ultraviolet absorbance of the larger 1KR
peak and a similarly shaped intensity versus time curve at m/z = 57 (data not shown). The ratios of the intensities for m/z = 57 versus m/z = 54 at corresponding time points are similar to the natural abundance ratio for \(^{57}\text{Fe}/^{54}\text{Fe}\). The 1KR in solution also exhibits a small peak of intensity at m/z = 57 at an elution time similar to that observed for the cobalamin standard, as well as two very small peaks at m/z = 50, at different elution times from the aforementioned peaks, that may be ascribable to the presence of chromium complexes of low molecular weight (data not shown). The addition of FeCl\(_3\) to 1KR solution results in very intense, similarly-shaped peaks at m/z = 54 (chromatogram D, Fig. 5) and m/z = 57 (data not shown) with ratios of intensities similar to the natural abundance ratio for the isotopes of iron observed at these nominal m/z values, and the plots for m/z = 50 and m/z = 59 are unaffected by addition of FeCl\(_3\) (data not shown).

Effects of digestive treatment on the iron content and iron-binding capacity of 1KR are illustrated (chromatograms A, B and C, Fig. 5). After change in pH and incubation with pancreatin/bile extract, peak intensities at m/z = 54 (chromatogram B, Fig. 5) and at m/z = 57 are only slightly decreased and peak shapes are skewed to longer elution times relative to pretreatment chromatograms (e.g., chromatogram E, Fig. 5). The addition of incubation with pepsin to the treatment procedure before incubation with pancreatin/bile extract caused a further decrease in peak intensities, but peak heights and areas remained substantial (chromatogram C, Fig. 5). Chromatogram A (Fig. 5) illustrates that the addition of ferric chloride after change in pH and treatment with pepsin, pancreatin and bile extract resulted in almost as much bound iron with the same peak shape and elution time as does addition of FeCl\(_3\) to untreated 1KR (chromatogram D, Fig. 5). ICPMS chromatograms of human \(\alpha\)-lactalbumin at m/z = 50, 54 (left inset, Fig. 5), 57 and 59 establish that lactalbumin contains very little iron and confirm that elution time for lactalbumin-associated transition metals differs from that of 1KR-associated transition metals.

Radioiron flux through Caco-2 cell monolayers. Integrity of the Caco-2 cell monolayers during incubation with milk fractions was studied by inclusion of phenol red (100 \(\mu\)mol/L) in apical chambers of some of the replicates of all the treatments shown in Figures 6 and 7. Less than 2% of the dye appeared in the basolateral chambers after 1-h incubation at 37\(^\circ\)C. Results for radioiron with and without phenol red were consistent with each other. Also, the integrity of tight junctions after incubation was confirmed for selected monolayers by electron microscopy and by measurements of transepithelial electrical resistance.

![Figure 5](image-url) Size exclusion chromatography: Ion intensity by inductively coupled plasma mass spectrometry at m/z = 54 versus elution time for 1-kDa retentate (1KR) fraction of breast milk whey before and after digestion with pepsin, pancreatin and bile extract. Conditions as in Figure 4, except flow rate = 0.5 mL/min. (Left inset) Human \(\alpha\)-lactalbumin as in Figure 4, chromatogram A. (Right inset, E) 1KR (24.5 mg) was dissolved in mobile phase (1 mL). (Right inset, D) Half of 1KR dissolved as in E was treated by exposure to 50 \(\mu\)L of freshly prepared aqueous FeCl\(_3\) (1 mmol/L) followed by removal of insoluble iron (see text). (Center, B) 1KR (26.2 mg) was dissolved (1.1 mL) and half was brought to pH 6.6 as in Figure 4, chromatograms A and C, followed by digestion only with pancreatin/bile extract and centrifugation as in Figure 4, chromatograms B and D before injection of supernatant. (Center, C) 1KR was dissolved as in center, B and half was prepared, digested with pepsin, pancreatin and bile extract, centrifuged, and injected as in Figure 4, chromatograms B and D. (Center, A) The remainder (0.45 mL from center, C) of 1KR digested with pepsin, pancreatin and bile extract was treated with FeCl\(_3\) as in right inset, D.
Effects of milk and milk fractions after pH change on cellular content plus binding and on basolateral transfer of extrinsic radioiron are illustrated (Fig. 6). The percentage radioiron content plus binding (means ± SEM) of Caco-2 cell monolayers postincubation was highest in the presence of ferrous ascorbate (FeASC). Of all the milk fractions, OFGM and 3KR elicited the highest radioiron content plus binding. Radioiron content plus binding in the presence of 10KF was slightly greater than that of the blank and 3KF. Means for radioiron content plus binding in the presence of milk, casein, 10KR, 1KR and 1KF were lower than that of the blank. In this set of experiments, the extrinsic apical iron contributed by the 59Fe was 5.73 nmol/L. The apical intrinsic iron concentrations of the various treatments were in the range of 1–10 μmol/L, which is commonly used in Caco-2 cell studies of iron flux (in μmol/L): milk, 1.2; OFGM, 1.1; 10KR, 1.1; 10KF, 2.4; 3KR, 1.4; 3KF, 4.9; 1KR, 5.4; 1KF, 2.5; casein, 0.84; blank, 0; FeASC, 10.0.

Basolateral radioiron transfer (Fig. 6) in the presence of 10KF, 3KF and 1KR was about fivefold, fourfold and fourfold, respectively, that of the FeASC control when reported as percentage of total radioactivity. If equilibration of the radiolabel with intrinsic iron in each fraction is assumed, these mean values (in pmol of iron/well) are for 10KF, 270; 3KF, 448; 1KR, 446; and FeASC, 219. Therefore, iron transfer is apparently greater for these fractions than for ascorbate, even though the apical iron concentrations in the presence of these fractions are substantially lower than the apical iron concentration in the presence of ascorbate in this experiment. Values for radioiron transfer in the presence of milk and 3KR were not significantly different from that of FeASC, and OFGM and 1KF elicited slightly greater transfer. Transfer in the presence of casein and 10KR was significantly less than that of all foregoing treatments except 3KR. Casein, 10KR and 3KR elicited transfer not significantly different from that of the blank.

Radioiron flux in monolayers preincubated with stable and radioactive iron. Preincubation of Caco-2 cells with ascorbate, radioiron and iron concentration at 10 μmol/L in apical medium (Fig. 7) resulted in radioiron content plus binding of 12–14% of total radioactivity as in Figure 6. Basolateral radioiron transfer after preincubation with ferrous ascorbate and 59Fe was <1% of total radioactivity, as in Figure 6. After radioiron-free 3KF and 3KR were incubated with these Caco-2 cell monolayers that already bound radioiron, values for mean monolayer radioiron content plus binding in the presence of 3KF and 3KR were 5.05 ± 0.05% and 6.08 ± 0.04%, respectively, of total radioactivity compared with 10.8 ± 0.1% for EBSS/MES (Fig. 7). The means for basolateral radioiron transfer after incubation with 3KF (7.49 ± 0.05%) and 3KR (5.67 ± 0.13%) were significantly greater than that after incubation with EBSS/MES alone (3.22 ± 0.10%).

**DISCUSSION**

Fe(III) at 20 μmol/L is effectively solubilized at intestinal pH by 3KF, 3KR and 1KR but is poorly solubilized by 1KF (Fig. 3). Ultrafiltration membranes do not have extremely sharp molecular weight cutoffs; they will pass appreciable amounts of molecules with weights up to two times the nominal cutoff value of the membrane but do not normally retain substantial amounts of molecules with weights below the nominal cutoff value. Therefore, the results illustrated in Figure 3 support solubilization of ferric iron by a human whey component of 1–6 kDa. The results with 1KF in Figures 3 and 6 are consistent and support those of a previous report that free citrate can decrease iron uptake by Caco-2 monolayers when the concentration of citrate is greater than that of ascorbate or cysteine, as is the situation in human milk (23).

Ferric iron is almost as well solubilized by 1KR after as before pH change and in vitro enzymatic digestion (Fig. 5). Size exclusion chromatography of 1KR before and after in vitro enzymatic digestion (Figs. 4 and 5) shows a broad ultraviolet absorbance peak with an elution time that corresponds to a molecular weight of ~4.2 kDa and that co-chromatographs with iron content and iron-binding activity but not with human α-lactalbumin. The observations that the ultraviolet absorbance of this broad peak is only moderately decreased (while that of the α-lactalbumin positive control is severely reduced) on enzymatic treatment and that the iron content and iron-binding capacity are not proportionately diminished suggest that an iron-binding component is even more resistant to digestion than are other components of 1KR. Taken together, Figures 3–6 strongly suggest that a digestion-resistant whey component of ~4.2 kDa enhances solubilization of nonheme, ferric iron at intestinal pH in support of increased iron absorption.

The basolateral radioiron transfer by Caco-2 cell monolayers in the presence of unfraccionated breast milk is similar to that for ferrous ascorbate (Fig. 6), which is the customary positive control for extrinsic iron bioavailability experiments in Caco-2 cells. Relative to ferrous ascorbate, some milk frac-
tions adversely affect, and others promote, radioiron flux through Caco-2 monolayers (Fig. 6–7). For examples, the casein and 10KR fractions resulted in basolateral transfer not significantly greater than blank and significantly less than all treatments except 3KR (Fig. 6). Our interpretation is that these unhydrolyzed casein- and lactoferrin-containing fractions of human milk can interact with extrinsic iron to minimize its uptake from apical medium. Whether this holds true for enzymatic digests of these fractions is not yet known.

In contrast to the casein and 10KR fractions, the 10KF and the low molecular weight whey fractions derived therefrom (except for 3KR) elicit several-fold more radioiron transfer than do ferrous ascorbate and unfraccionated breast milk (Fig. 6). These observations, combined with the property of ultrafiltration membranes to pass molecules of mass up to twice the nominal molecular weight cutoff value, suggest that a whey component of <6 kDa facilitates basolateral transfer of iron.

The interpretation that a whey component of <6 kDa facilitates basolateral transfer of iron is reinforced by the results of the iron-loading experiments (Fig. 7). More than half of the radioiron in monolayers preincubated with ferrous ascorbate is transferred to basolateral medium when the monolayers subsequently are incubated with 3KF in the apical medium, but less than half of the radioiron is transferred during incubation with an equal amount of protein from 3KR. Kinetic studies in isolated duodenal segments and in Caco-2 cell monolayers have shown that basolateral transfer of iron is increased by subsequently absorbed chelators of iron such as EDTA and citrate anions (10,24). We suggest that iron-binding peptides from breast milk can be taken up by enterocytes and can mobilize iron from the intracellular lable pool for basolateral transfer.

The possibility must be considered that milks from other species possess low molecular weight components that enhance basolateral iron transfer. The iron distribution in bovine whey is similar to that in human milk whey in that about one half is bound to whey proteins and the other half is associated with low molecular weight compounds (6). However, there are more differences than similarities between protein composition of milks of these species (25). In bovine whey, β-lactoglobulin predominates over α-lactalbumin. Human milk contains no β-lactoglobulin, and α-lactalbumin is the predominant whey protein. Nonetheless, bovine skim milk exhibits protein bands on urea-PAGE that are of lower molecular weight than α-lactalbumin and are not observed in the whole casein fraction (26).

We conclude that low molecular weight whey fractions of breast milk enhance flux of extrinsic ferric iron across Caco-2 cells. We speculate that one or more peptides from these fractions resistant to digestion may enhance absorption of nonmilk iron in the infant proximal small intestine. Increased understanding of this phenomenon is expected to lead to development of ferric compounds that are more effective as iron fortificants for populations at risk and are more stable than commonly used ferrous forms that can be readily oxidized.

LITERATURE CITED


