Lactoferrin receptors in piglet small intestine: Lactoferrin binding properties, ontogeny, and regional distribution in the gastrointestinal tract

Jóhannes Gíslason, Suhasini Iyer, T. William Hutchens, and Bo Lönnerdal

Department of Nutrition, University of Uppsala, Sweden; Department of Nutrition, University of California, Davis, CA; and United States Department of Agriculture/Agriculture Research Station Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, TX USA

Bioavailability of iron from human milk is known to be high. This may be due to a receptor-mediated mechanism in the small intestine that facilitates the absorption of iron from lactoferrin. Our aim in this study was to evaluate the ontogeny and localization of lactoferrin receptors within the small intestine. The piglet was used as an animal model because lactoferrin is a major iron-binding protein in both human and porcine milk. Kinetics of lactoferrin interaction with its receptor and receptor density were determined in relation to the age of the piglet (day 0–21 after birth) and the location (duodenum, jejunum, and ileum) within the small intestine. Specific and saturable binding of ^{59}Fe-labelled pig lactoferrin by brush border membranes purified from piglet intestine was observed. Pig transferrin, human, and bovine lactoferrin did not bind to the porcine lactoferrin receptor. Lactoferrin binding occurred throughout the intestine independent of age of the piglet; receptor number (15×10^{14}/mg protein) and affinity ($K_d = 3 \times 10^{-7}$ M) were relatively constant from birth until weaning. Thus, it is possible that lactoferrin receptors throughout the intestine may play a role in iron absorption throughout infancy.

Keywords: iron; iron-binding protein; lactoferrin; lactoferrin receptor; milk; piglets

Introduction

The iron concentration of human milk is low compared with infant formula or weaning foods. Nevertheless, several investigators[1–3] found that a majority of infants exclusively breast fed for 6–9 months could maintain their iron status at the same level as formula-fed infants up to 6 months of age. Isotope studies[4–6] and iron balance studies[7] have indicated a higher bioavailability of iron from human milk than from infant formula and cow's milk. Lactoferrin is a major iron-binding protein in milk from many species. Genetically and structurally related to transferrin,[8] lactoferrin is a single chain (80 kDa) glycoprotein, possessing one iron-binding site in each of its two main structural domains.[9] Although related to transferrin, human lactoferrin binds iron approximately 200–300 times stronger than transferrin. Lactoferrin also maintains a higher affinity for iron at decreased pH; complete dissociation is only observed at pH 2.[10] The concentration of lactoferrin varies in milk from different species and there seems to be an inverse relationship between the lactoferrin and transferrin concentrations in milk.[11,12]

The primary physiological role of lactoferrin is still debated. It has been reported to have a bacteriostatic effect in the small intestine by withholding iron from iron-demanding pathogens.[13–15] Lactoferrin has also been suggested to play a role in iron absorption during infancy.[16] Cox et al.[17] showed that human lactoferrin has the ability to deliver iron to human intestinal mucosa biopsies. Evidence for the presence of specific lactoferrin receptors in brush border membranes from the small intestine has been provided in the case of rhesus mon-

This work was supported by NIH grant DK-47850, Swedish Council for Forestry and Agricultural Research, Swedish Nutrition Foundation, and the Medical Faculty of the University of Uppsala.
Address reprint requests to Dr. Bo Lönnerdal at the Department of Nutrition, University of California, Davis, CA 95616-8669 USA.
Received August 21, 1992; accepted February 1, 1993.

keys,[18,19] mice,[20] and humans.[21] It should be noted that all these species have a relatively high lactoferrin concentration in their milk. This may suggest that lactoferrin plays a role in receptor-mediated iron absorption in these species. In rat milk, however, transferrin is the major iron-binding protein. Consistent with this observation, Kawakami et al.[22] reported a receptor in the rat brush border membrane that bound rat transferrin. This receptor exhibited less specificity than lactoferrin receptors previously described in other species, because it also bound bovine lactoferrin, although with lower affinity.

An animal model that is frequently used in nutrition research is the pig. During the first week of life, the piglet may increase its birth weight by 100%. During this period of high growth rate, increased requirements for iron call for efficient intestinal iron absorption. Porcine milk contains lactoferrin and its concentration is relatively high during the first week after delivery (1.3 mg/mL), then declines during lactation.[23] In addition, the gastrointestinal development of piglets is similar to that of human infants.[24]

In the present study, we explored whether porcine milk lactoferrin would bind to a specific receptor in the piglet small intestine. One objective was to identify such a receptor and evaluate the lactoferrin-receptor interaction, binding kinetics, receptor number, and specificity. Our second aim was to evaluate the above parameters with regard to age of the piglet and location within the intestine. Because porcine milk also contains transferrin, we also evaluated the binding of transferrin to the receptor.

Methods and materials

Animals

Four sows (Swedish land race) were maintained on a regular pig diet (Faculty of Veterinary Medicine, University of Agricultural Science, Uppsala, Sweden) and delivered healthy litters of 10–15 piglets. At regular intervals (0–2, 5, 8–9, 14–16, and 21–22 days of age) milk was sampled and two piglets from each sow were killed. Piglets were fasted for 2–3 hr before they were killed. The youngest piglets (0–1 days) were fed a synthetic diet (consisting of 10 g glucose, 6 g sodium chloride, and 6 g sodium bicarbonate, dissolved in 1 L water) during the night for 5–10 hr to prevent dehydration before being killed.

Tissue preparation

Piglets were killed by intraperitoneal injection of pentobarbital. The small intestines were immediately dissected and flushed with ice-cold saline and cut into three equal segments. Each part was slit open and the mucosa was scraped with a glass slide. All steps were performed on ice. Consecutive samples of 2–3 mL were immediately flash frozen in liquid nitrogen and stored frozen at −70° C until use. Samples of small intestine used in the lactoferrin-binding studies were obtained from the proximal part of each of the three segments.

Preparation of brush border membrane vesicles (BBMV)

BBMVs were prepared from 1.5–2.5 g of mucosa by differential centrifugation and magnesium precipitation techniques as described by Muir et al.[25] and modified by Davidson and Lönnerdal.[18] Recovery and purity of the vesicle preparation were determined by measuring the increase in sucrase activity[26] over that of the initial mucosa homogenate. Na-K-ATPase[27] was also assayed as a basolateral membrane marker. Protein content was determined by the modified Lowry method.[28] The final vesicle suspension was used in binding studies immediately after preparation.

Isolation of porcine lactoferrin

Skimmed sow's milk (2–7 days of lactation) was used for isolation of porcine lactoferrin on a Sephadex CM-50 ion exchange column (Pharmacia, Uppsala, Sweden) in 0.02 M Na-phosphate buffer pH 7.0.[23] The isolated protein was found to be pure as assessed by polyacrylamide gel electrophoresis according to Laemmli.[29] The purified lactoferrin was desaturated by dialysis against 0.1 M citric acid for 24 hr followed by a second dialysis against deionized water.

Isolation of porcine transferrin

Transferrin was isolated from pig serum by a modification of the methods of Sawatzki et al.[30] and Baumstark.[31] Serum was dialyzed overnight against 50 volumes of 10 mmol/L $CaCl_2$ in 1.14 M NaCl. The dialysate was centrifuged (10,000*g*, 10 min) to remove fibrin. The supernatant was brought to 50% saturation with solid $(NH_4)_2SO_4$, stirred gently for 1 hr at 4° C and centrifuged (10,000*g* for 10 min). The particle-free supernatant was brought to 70% saturation with solid $(NH_4)_2SO_4$ and gently stirred for 1 hr at 4° C. The precipitate was centrifuged (10,000*g* for 10 min) and the pellet was dissolved in 25 mmol/L Tris-HCl buffer pH 7.8 and dialyzed against the same buffer overnight.

The dialyzed crude transferrin fraction was applied to a DEAE-Sepharose (Pharmacia, Piscataway, NY USA) column (5 × 12.4 cm) with 50 mmol/L Tris-HCl, pH 7.8. Transferrin was eluted ~90% pure with a linear gradient from 50 mmol/L to 500 mmol/L Tris-HCl buffer at pH 7.8. The isolated protein was found to be pure as assessed by polyacrylamide gel electrophoresis.[30]

Labeling of the proteins with ^{59}Fe

^{59}Fe-citrate was prepared by adding a 1000-fold molar excess of citrate to $^{59}FeCl_3$ in 1 M HCl. After 10 min, the pH was adjusted to 7 with 1 M NaOH. Iron-free lactoferrin or transferrin was dissolved in 50 mmol/L Tris buffer (pH 7.5) containing 0.1% sodium bicarbonate. ^{59}Fe-citrate was added in an amount sufficient to saturate the protein and the solution was left at room temperature overnight to ensure maximum binding of iron. Unbound ^{59}Fe was removed by passing the solution through an Excellulose GF-5 desalting column (Pierce, Rockford, IL USA).

Lactoferrin binding assay

BBMVs were suspended at a concentration of 1 mg protein/mL. Twenty μL of this suspension were incubated at 37° C with varying concentrations (0.1–1.25 μmmol/L) of labeled lactoferrin or transferrin for various times (15 sec to 10 min). The reaction was stopped by the addition of ice-cold saline, followed by immediate vacuum-filtration on Millipore filters (prewetted 0.22 μm). Filters were rinsed three times with 1 mL saline and then counted in a gamma counter (Gamma 8500, Beckman Instruments, Fullerton, CA USA) to determine the amount of ^{59}Fe-lactoferrin (or ^{59}Fe-transferrin) associated with the BBMVs. Non-specific binding of ^{59}Fe-

lactoferrin to BBMVs was determined in each experiment by addition of a 100-fold molar excess of unlabeled ligand to parallel incubations. Non-specific binding of ^{59}Fe to the filter membranes was monitored by incubation of ^{59}Fe-labeled protein in the absence of BBMVs. All incubations were performed in triplicate. Binding experiments were performed using ^{59}Fe-labeled porcine lactoferrin and transferrin (Tf), bovine lactoferrin (Lf), and human lactoferrin (Lf). Bovine and human lactoferrin were prepared by affinity chromatography using immobilized monoclonal antibodies against these proteins.[21]

Results

The isolated BBMVs prepared for these investigations were characterized by an enrichment of sucrase activity 17–20 fold relative to the original homogenate; there was no detectable Na-K-ATPase activity in the BBM suspension. Enzyme recoveries were generally 20–30%.

The time course for binding of porcine Lf to the BBMVs is shown in *Figure 1*. Each data point represents the average of three experiments done in triplicate. Initial binding of lactoferrin was rapid and reached its maximum at about 5 min. Addition of a 100-fold molar excess of unlabeled lactoferrin resulted in complete displacement of bound ligand. Saturation of the lactoferrin receptor was observed at a lactoferrin concentration of 1 μmol/L (*Figure 2*).

Equilibrium binding analyses were performed with BBMVs prepared from piglets that were 0–22 days old. Lactoferrin receptor number and affinity constants were calculated from the intercept and the slope of the Scatchard plot, respectively. *Figure 3* is representative of the several Scatchard plots generated from our equilibrium binding data. There were no significant differences in receptor affinity or receptor number observed with BBMVs from three different locations within the intestine (*Table 1*). The lactoferrin binding affinity (K_d) was about 0.3 μmol/L. The number of binding sites ranged from 12–16 × 10^{14}/mg BBMV protein. When comparing lactoferrin binding data from piglets of different ages, we found no significant differences for either dissociation constants (K_d) or the number of binding sites (*Table 2*).

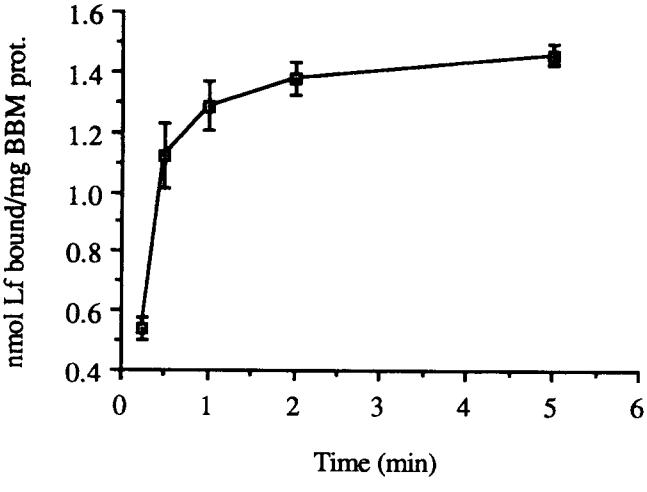

Figure 1 Time course of porcine lactoferrin binding to brush-border membrane vesicles (37° C, pH 7). Values represent average data from three separate experiments performed in triplicate. Bars indicate the standard deviation.

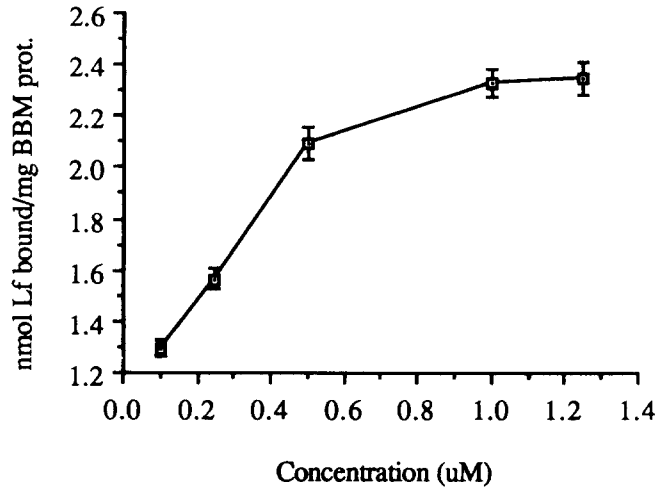

Figure 2 Saturation of porcine lactoferrin receptors on brush-border membrane vesicles. Lactoferrin (0.1–1.25 μmol/L) was incubated with 20 μg of BBMV protein for 5 min at 37° C.

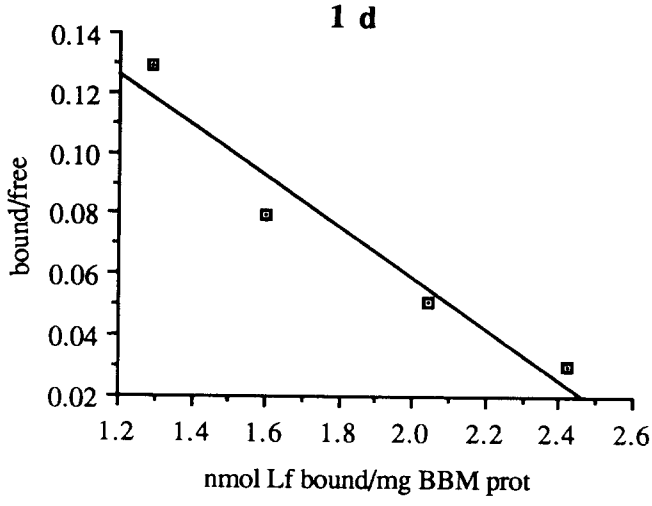

Figure 3 Scatchard analyses of BBMV lactoferrin receptor binding data obtained from piglets of different ages. These are representative of numerous plots generated from the data obtained in the study. Values are expressed as means of triplicates.

Table 1 Kinetics of porcine lactoferrin binding to BBMVs prepared from different locations within the 10-day old piglet small intestine

Location (cm from pylorus)	Dissociation constant (1 μmol/L)	Number of binding sites (× 10^{14}/mg protein)
0–120	3.2 ± 0.8	15 ± 1.1
240–360	2.8 ± 0.6	16 ± 1.1
480–600	3.1 ± 0.4	15 ± 1.1

Table 2 Kinetics of porcine lactoferrin binding to BBMVs prepared from the upper part (duodenum) of the small intestine of piglets of different age groups

Age of piglet (days)	Dissociation constant (1/μmol/L)	Number of binding sites ($\times 10^{14}$/mg protein)
0	2.8 ± 0.6	15 ± 1.1
10	3.1 ± 0.7	12 ± 1.0
21	2.4 ± 0.6	15 ± 1.2

Bovine Lf, human Lf, and pig Tf did not bind to the porcine lactoferrin receptor, demonstrating a high degree of specificity for the receptor (*Figure 4*).

Discussion

Several characteristics of the lactoferrin binding to isolated BBMVs (e.g., affinity and specificity) clearly suggest the presence of specific lactoferrin receptors in piglet small intestine. The affinity constant for porcine lactoferrin (3 μmol/L) was found to be similar to that previously reported for lactoferrin receptors in human[21] and rhesus monkey[18,19] intestine. In addition, the number of binding sites in the piglet was similar to what was found in the primate species. Thus, the piglet appears to be a reasonable animal model for studies of lactoferrin interactions with its receptor.

Masson and Heremans[11] found by employing a semiquantitative technique that the concentration of lactoferrin and transferrin in porcine milk is similar. We found, however, that porcine BBMVs did not bind transferrin. Kawakami et al.[22] found that BBMVs isolated from rat intestine did bind transferrin but not lactoferrin. However, lactoferrin has been reported to be present only in trace concentrations in rat milk, whereas transferrin occurs in high concentrations.[11] Thus, transferrin in rat milk might play the same role in that species as lactoferrin does in milk from other species. The presence of transferrin in porcine milk with no detectable transferrin receptors in the intestine presents an apparent anomaly. It should be recognized, however, that transferrin is involved in the delivery of iron to the mammary gland.[32] Thus, the presence of transferrin in pig milk might be a consequence of the transfer of transferrin into the mammary cell, with subsequent export into milk.

For a receptor-ligand interaction to have a biological relevance, the concentration of the ligand at the site of the receptor has to be comparable to the binding constant of the receptor for its ligand. Our results show that the intestinal lactoferrin receptor in nursing piglets has a relatively low affinity (K_d = 0.3 μmol/L). This affinity is in general agreement with the affinity of lactoferrin for receptors in brush-border membranes from other species.[18–21] In porcine milk, lactoferrin concentrations range from 15 μmol/L (1.2 mg/mL) in colostrum to 3.8 μmol/L (0.3 mg/mL) in mature milk.[24] If 50% of the ingested lactoferrin resists proteolysis in the stomach and the small intestine, and if the volume is not significantly changed, the luminal concentration of lactoferrin could reach 1.5–2 μmol/L in a 1-week-old piglet. These

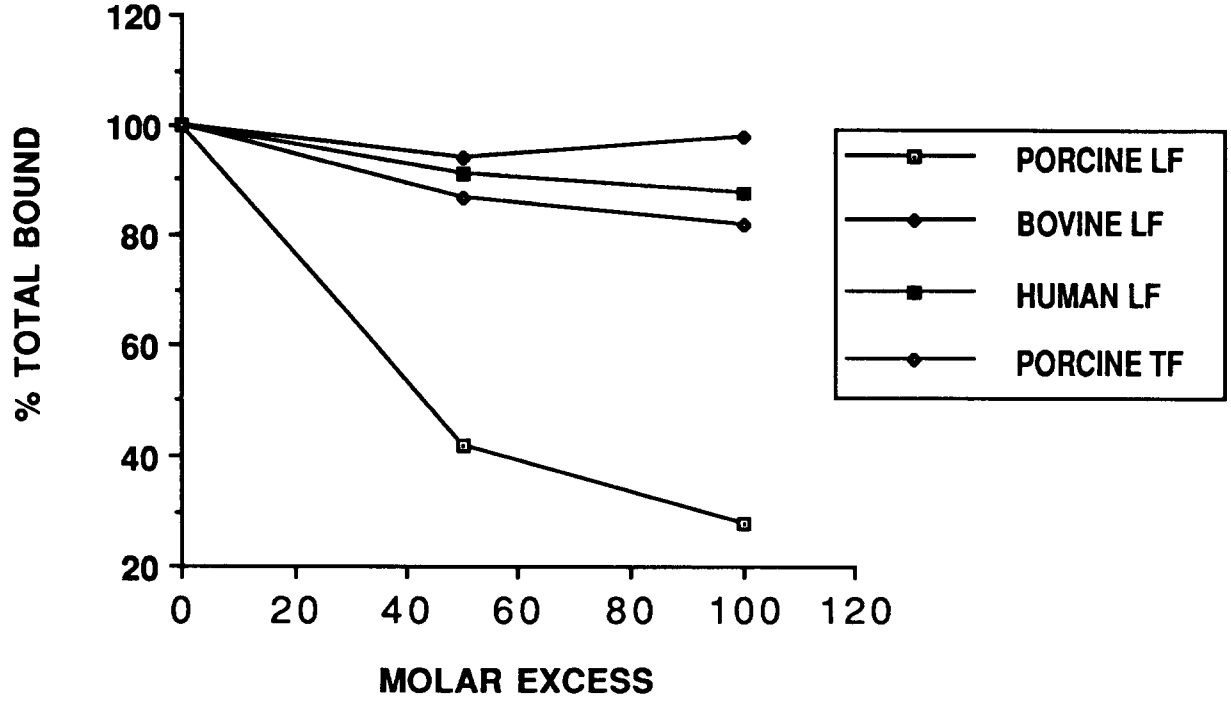

Figure 4 Binding of bovine lactoferrin, human lactoferrin, and porcine transferrin to the porcine lactoferrin receptor.

assumptions are far from reliable, but they do indicate that a significant fraction of the lactoferrin present in sow's milk has to be protected against proteolysis to occupy the lactoferrin receptor to any significant extent. Schmitz et al.[33] showed that bovine lactoferrin could resist proteolytic degradation through the piglet small intestine. We have also shown that a significant proportion (2–6% of intake; 10–150 mg/d) of human milk lactoferrin can be found intact in the stool of breast fed infants.[34] Thus, although a major proportion of lactoferrin may bind to the receptor and become internalized, the occurrence of some intact lactoferrin in the stool demonstrates that digestion of lactoferrin is limited.

We did not find any age-related differences in receptor number (per mg protein) or affinity. In a previous study in rhesus monkeys, we found a higher number of receptors during infancy than in tissue from fetuses or older monkeys. The binding affinity, however, was similar at all ages examined.[18] In this study we only investigated the nursing period, i.e., from birth to 22 days of age, and did not find any difference in receptor number. It is possible that receptor number is higher during infancy and equally high during the entire nursing period.

The uniform presence of the receptor in all parts of the small intestine increases the potential for receptor-mediated processes to be effective in iron transport relative to other known mechanisms of iron absorption, which are known to occur mainly in the proximal part of the small intestine (duodenum). There are reasons to suggest that, because of high gastric pH and therefore low pepsin activity, only a small amount of milk casein is digested in the stomach.[35] In species with milk high in casein, this will result in a relatively high concentration of incompletely digested casein. Casein may therefore bind iron in the duodenum, resulting in decreased availability of iron at the site where it is most efficiently absorbed. The presence of intact lactoferrin and lactoferrin receptors in the jejunum and ileum may then facilitate absorption of iron gradually being released from partly digested casein in the distal parts of the small intestine. This scenario requires lactoferrin intact enough to bind iron and to its receptor.

Acknowledgments

The authors thank Dr. Bernt Jones for his valuable guidance and practical help with housing of the animals and tissue collection.

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