FULL PAPER *Anatomy*

Persorption of Bovine Lactoferrin from the Intestinal Lumen into the Systemic Circulation via the Portal Vein and the Mesenteric Lymphatics in Growing Pigs

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ABSTRACT. The absorption and the transportation of intestinally administrated bovine lactoferrin (LF) were immunohistochemically and physiochemically investigated in the small intestine of growing pigs. At the apical halves of the small intestinal villi, bovine LF was absorbed by transcytosis as small vesicles through villous columnar epithelial cells. The presence of bovine LF-positive membranes of transcytotic vesicles suggests that the absorption was mediated by LF-binding factors on the epithelial cell membranes. Almost all of the absorbed bovine LF was demonstrated to be transported via the lymphatics and the portal vein into the systemic circulation. The LF-concentration in systemic circulation was significantly higher at 1 hr following intestinal administration of bovine LF. Bovine LF-positive lymphocytes also were transferred into the systemic circulation from intestine via the lymphatics and the portal vein.
KEY WORDS: bovine lactoferrin, enzyme-linked immunosorbent assay, intestine, persorption, swine.

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Lactoferrin (LF) belongs to the transferrin family that transports Fe^{3+} irons [15]. LF is present in the milk of all mammalian species investigated to date with the exception of the dog and the rat [19]. The concentration of LF is quite high in milk [15], and markedly higher levels exist in the cord blood, tears, and vaginal mucus of humans [17]. Sequential studies have clarified the physiological nature of LF. Briefly, milk LF plays a central role in intestinal Fe-absorption in the small intestine [8]. LF also expresses some active biological effects on host defense mechanisms: regulation of cytokine secretion from the immunocompetent cells, regulation of cellular differentiation and proliferation of various leucocytes [3, 6], and antibacterial activity [30, 31]. The effect of LF on cellular proliferation [16, 24, 25] and the stimulation of protein synthesis [7] have also been reported in hepatocytes. Thus, LF is a very advantageous physiological activator for humans and animals [For more details, see the reviews of 5 and 17].

Neonatal pigs absorb a wide variety of macromolecules such as immunoglobulins from the intestine to the blood for about the first 36 hr of life [27]. In neonatal pigs, LF is also persorbed into the portal vein by enterocytes [5]. Orally administrated bovine LF in piglets is absorbed in the small intestine and is resecreted into bile via hepatocytes [12]. The absorbed bovine colostral components including LF reach into the cerebrospinal fluid in neonatal pigs [13] and calves [30]. On the other hand, LF is resistant to the digestive enzymes of 3-weeks-old piglets, but is sensitive to the digestive enzymes of adult pigs [28]. In addition, after gut closure, orally administrated bovine LF is not detected in the systemic circulation of 8- and 14-day-old piglets, using immuno-electrophoresis technique [9]. At 6 weeks, however, bovine LF is transferred from the intestinal lumen into the blood circulation, as shown by using double antibody ELISA technique with higher sensitivity [12].

Thus, the intestinal absorption of bovine LF in pigs after the weaning period has remained controversial. The pig is one of the useful models for the study of nutritional issues in humans, because of the similarity of pig's digestive function similar to that of humans [23]. In addition, the characteristics of the LF-receptor in porcine intestines are very similar to the bovine one [10]. Therefore, we investigated the absorption and the transportation route of intestinally administrated bovine LF in growing pigs.

MATERIALS AND METHODS

Animals: Eight pigs (Duroc x Large White; 10–12 weeks old) were used for this study. Their body weights were 19–22 kg. The care and use of these animals were approved by the Animal Research Committee of Tottori University.

LF administration for immunohistochemistry: Four pigs were used in this experiment. Surgery was performed under halothane anesthesia. After laparotomy, intestinal loops of 10 cm length were separated by ligation at duodenum, jejunum, and ileum. The ligation was carefully performed avoiding both the vascular and the nervous system in the cranial mesentery. Five m*l* of 5% bovine LF in saline warmed to 37°C was injected into each loop by means of a plastic syringe equipped with a 24-gauge needle. In one animal 5 m*l* of saline was injected as an experimental control, and the other one had no operation as an intact control. The pigs were let lie quietly under anesthesia.

Tissue processing: At 30 min after bovine LF-infusion, an overdose of pentbarbital sodium (Abbott Lab., U.S.A.) was intravenously injected in order to cause death. Immediately after euthanasia, parts of the loops, liver and spleen were extracted. The tissues were sliced, immersion-fixed in

PLP (periodate-lysine-paraformaldehyde) for 24 hr at 4°C, and were snap frozen in liquid nitrogen with reference to an embedding method described by Barthel and Raymond [1]. Sections of 4 μm in thickness were cut using a Coldtome HM505E (Carl Zeiss, Germany) and were placed on slide glasses precoated with 0.2% 3-aminopropyltriethoxysilane (Shinetsu, Japan).

Immunohistochemistry: The sections were rinsed with 0.05%-Tween-added phosphate buffered saline (pH7.4, PBS), followed by treatment with absolute methanol and 0.5% H_2O_2 for 30 min, respectively. Following blocking with 1% non-immunized chicken serum, the sections were reacted with horseradish peroxidase (HRP)-conjugated anti bovine LF goat IgG (diluted at 1:100; Bethyl Lab., U.S.A.) for 20 hr at 4°C. After rinsing with PBS, the sections were incubated with 3,3'-diaminobenzidine (DAB) containing 0.03% H_2O_2, and were counterstained with Mayer's hematoxylin.

Analysis of LF concentration in blood plasma: Four pigs were used for the measurement of bovine LF concentration in blood plasma.

The pigs were anesthetized with 3% halothane in air during the operation. A cannula for blood collection was inserted into the left external jugular vein. After the collection of blood (1 m*l*) as a pre-administration control, bovine LF (1 g/kg, body weight) maintained at 37°C was infused into the duodenum via a sharp end of a 24-gauge needle over 2 min. Under conscious conditions, peripheral blood (1 m*l*) was collected into heparinized tubes at 1, 4 and 12 hr after the bovine LF infusion. The samples were centrifuged at 1,100 g at 4°C for 15 min, the plasma was separated and stored at –80°C until analysis.

Quantitative analysis of bovine LF was carried out according to the previous report [13]. In brief, a flat-bottomed microwell plate with 96 wells was incubated with anti-bovine LF goat IgG (20 *n*g/100 μl; Bethyl Lab., U.S.A.) at 4°C overnight. After washing, a 200 μl aliquot of 1% gelatin in PBS was added to each well and incubated at 37°C for 30 min. After washing, we added samples to the wells. Plates were incubated at 37°C for 2 hr, then the wells were washed. Then 100 μl of anti-bovine LF rabbit IgG (Yagai Inc., Tokyo), which was diluted to 2,000-fold with egg albumin PBS, were added to each well. After 2 hr of incubation at 37°C, the plates were washed. Then 100 μl of HRP-conjugated anti-rabbit IgG (H+L) goat IgG, (American Qualex International Inc., U.S.A.), which was diluted to 2,000-fold with egg albumin PBS, were added to each well. After 2 hr of incubation at 37°C, the plates were washed and 100 μl of the reaction mixture [0.1 M citric acid-PBS containing 5 mM H_2O_2 and 2 mM 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] were added. After 1 hr of incubation at 37°C, the optical density of the reaction mixture was measured at 405 nm with a multiscan plate reading spectrophotometer (Sunrise Classic, Tecan Japan, Japan). The measureable range of bovine LF was 1–300 *n*g/m*l*.

Statistical analysis: Quantitative data were expressed as means ± SD. Unpaired Student's *t*-test was employed for the detection of statistical significances with a *P* value of less than 0.05.

RESULTS

Immunohistochemistry: Administrated bovine LF was detected as a liquid or in granulated form in both the luminal contents and the mucous layer on the intestinal villi.

Cytoplasms of villous columnar epithelial cells contained small amount of bovine LF in the apical intestinal villi throughout the small intestine. Bovine LF was scarcely contained in the lower halves of the intestinal villi. The striated borders and the lateral cell membranes of the epithelial cells strongly bound bovine LF in the apical intestinal villi, and the immuno-reaction intensities gradually decreased toward the middle portion of the intestinal villi (Fig. 1). The cytoplasm also contained numerous bovine LF-positive small vesicles, and their membranes also apparently bound bovine LF (Fig. 2).

The exfoliation of apoptotic villous epithelial cells was accelerated along the entire intestines. The cytoplasms of exfoliating epithelial cells were also shown to be intensely bovine LF-positive (Fig. 3). Both the exfoliated cells and apoptotic bodies were also strongly bovine LF-positive in the intestinal lumen (Figs. 1 and 2).

The lamina propria in the apical villi contained a high concentration of bovine LF. The concentration gradually decreased toward the middle portion of the intestinal villi (Fig. 1).

The cell surface membranes of many intraepithelial lymphocytes apparently possessed bovine LF. In addition, bovine LF-positive lymphocytes were also found in the villous lamina propria. Moreover, a few macrophages in the lamina propria were laden with bovine LF-positive substances in their cytoplasmic vacuoles (Fig. 4).

Bovine LF could be scarcely detected in the blood plasma of subepithelial capillaries, whereas it was contained in the central lymphatics in the apical villi. The submucosal lymphatics contained moderately bovine LF-positive substances, but the submucosal venules showed weakly positive ones. The blood plasma of submucosal arteries scarcely contained bovine LF. The positive intensities in the lumen of the vasculature were the strongest in the ileum. Predominant bovine LF-positive lymphocytes were transferred from the intestine through the lymphatics. A few LF-positive lymphocytes were also detected in the submucosal venules and the submucosal arteries (Fig. 5).

No bovine LF-positive substances were found in livers or spleens of bovine LF-administered animals. In addition, bovine LF was detected neither in any tissues of the experimental control nor in the intact control.

LF concentration in blood plasma: Bovine LF was detected in the peripheral blood at different time points. The bovine LF concentration was steeply and significantly increased ($P<0.05$), and reached a peak value of 318.3 ± 62.2 *n*g/m*l*, at 1 hr after intraduodenal bovine LF infusion. It was noted that bovine LF concentration gradually declined after 1 hr (Fig. 6).

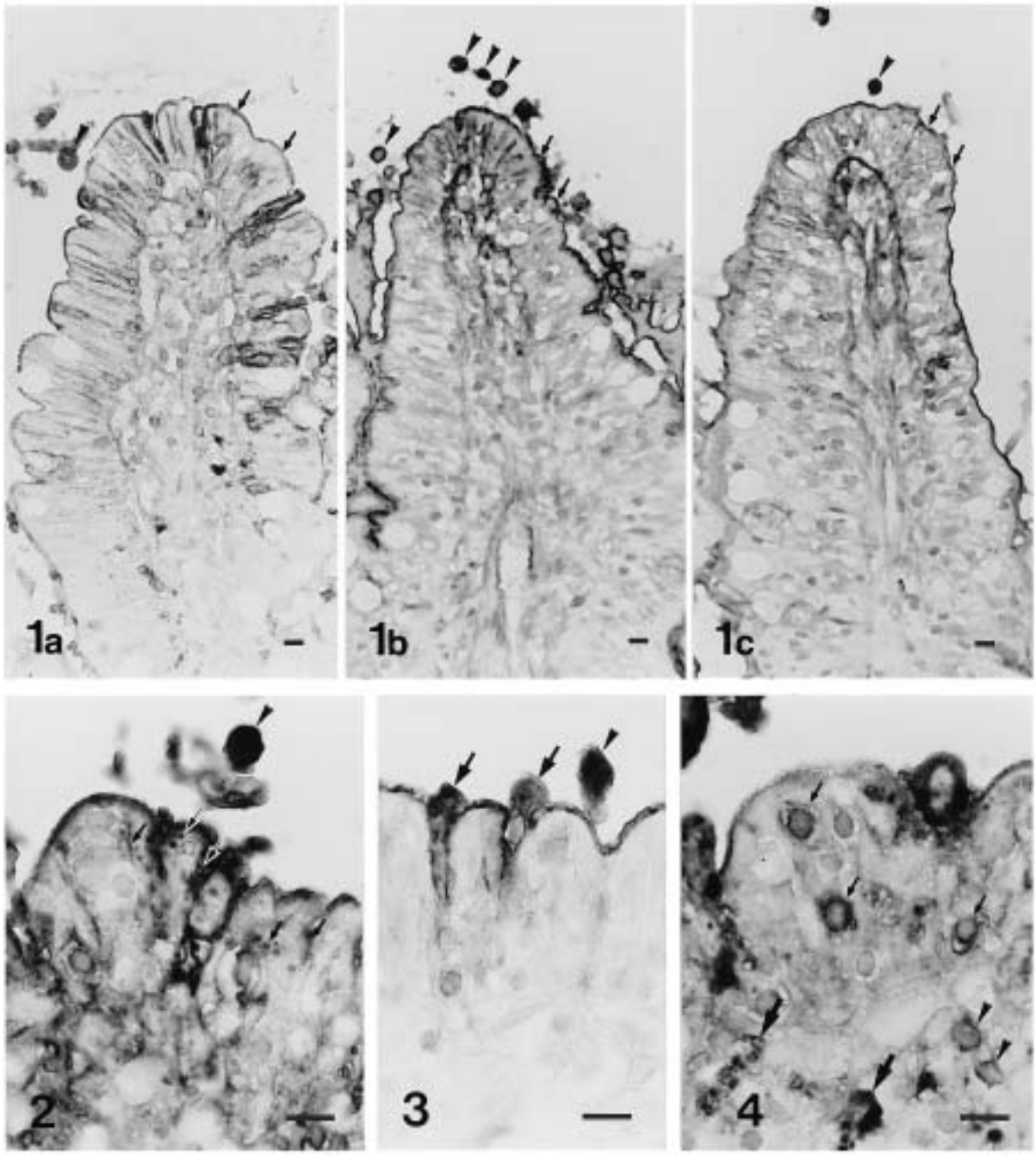

Fig. 1. Enzyme-immunohistochemistry on the duodenum (a), jejunum (b), and ileum (c) of bovine LF-treated pigs. Weak LF-positivity is seen in the epithelial cytoplasm at the apical halves of intestinal villi. Both the striated borders (small arrows) and the lateral membranes of epithelial cells are apparently bovine LF-positive. The reaction of the lamina propria was strongly positive in apical villi and gradually decreased toward the lower portion. Arrowheads, exfoliated apoptotic cells. Bar = 10 μm.

Fig. 2. Epithelium of apical intestinal villi of the duodenum. The cytoplasm of villous epithelial cells contains bovine LF-positive small vesicles (small arrows). Arrowheads, exfoliated apoptotic cells. Bar = 10 μm.

Fig. 3. Exfoliating apoptotic epithelial cells (arrows) and an exfoliated cell (arrowhead) which are strongly bovine LF-positive. Bar = 10 μm.

Fig. 4. Bovine LF-positive lymphocytes. Positive cell surfaces are seen in intraepithelial lymphocytes (small arrows) and those in the lamina propria (arrowheads). Macrophages also possess bovine LF-positive vacuoles in the lamina propria (arrows). Bar = 10 μm.

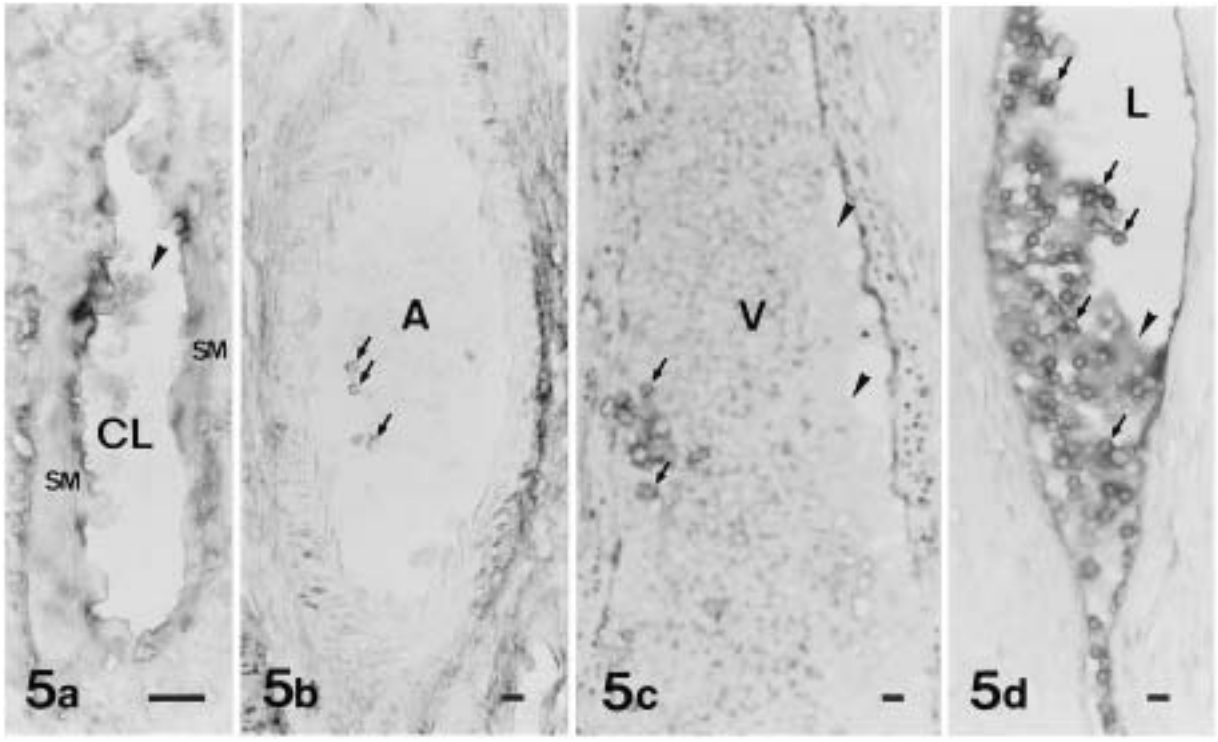

Fig. 5. Bovine LF in the intestinal vascular system. a) Lumen of a central lymphatic (CL) contains weakly bovine LF-positive substance (arrowhead). b) Bovine LF is scarcely visible in the lumen of a submucosal artery (A). c) A submucosal venule (V) contains weakly bovine LF-positive plasma (arrowheads). d) A submucosal lymphatic (L) contains apparently bovine LF-positive substance (arrowhead). SM, villous smooth muscle fiber; Small arrows, lymphocytes with bovine LF-positive cell surfaces. Bar = 10 μm.

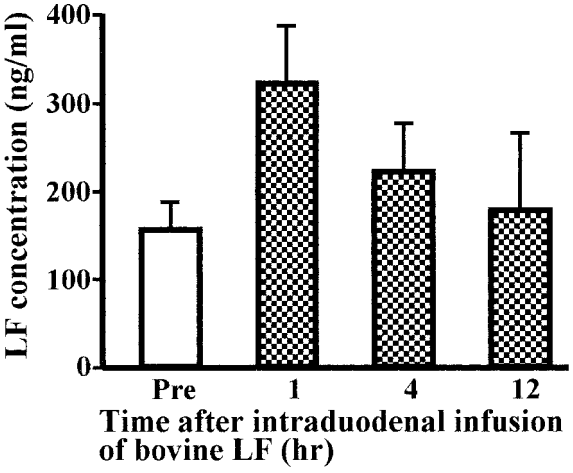

Fig. 6. LF concentration in the blood plasma after intraduodenal infusion of bovine LF (1 g/m*l*) at 10-weeks-old pigs. The concentration at 1 hr is significantly increased over that prior to infusion (Pre) ($P<0.05$). Each value represents mean ± S.D. of four animals.

DISCUSSION

Before gut closure, orally-administrated bovine LF is persorbed from the small intestine to the systemic blood circulation via the lymphatics and the portal vein in neonatal pigs that have not been provided colostrum [12]. After the gut closure, the orally ingested bovine LF is not detected in systemic circulation of 8–14-days-old piglets, whereas the orally administrated bovine LF is detected in the systemic circulation of 6-weeks-old pigs [9]. In this study, the intestinally injected bovine LF was demonstrated in villous columnar epithelial cells of apical intestinal villi throughout the small intestine in 10–12-weeks-old pigs. This finding suggests that the absorption ability of bovine LF is retained for a long time following the weaning period in growing pigs.

LF has several important biological functions, such as antibacterial and immunoprotective effects [4, 18]. The entero-hepatic circulation of heterogeneous LF [12] might be more effective on intestinal host defense mechanisms in both neonatal and weaner piglets. LF is also present in the secondary granules of human neutrophils [17, 20]. The concentration of serum LF varies with the neutrophil count in human cyclic neutropenia [26]. Thus, the endogenous LF derived from neutrophils might be also excreted into bile, suppress bacterial growth and translocation, and be reabsorbed via the lymphatics and portal vein into the systemic circulation of growing pigs.

LF receptor for human LF has been detected in the microvillous membrane of enterocytes of human and rabbit

small intestine [22]. In suckling piglets the enterocytes have LF-receptor for porcine LF, and its density is homogeneous throughout the small intestine [11]. The porcine LF receptors of enterocytes in the piglet small intestine, however, do not bind human and bovine LF unlike porcine LF [11], whereas the binding of porcine LF was apparently inhibited by the co-culture of bovine LF [10]. Thus, the affinity of bovine LF to porcine intestinal LF receptor is inconsistent. On the other hand, bovine LF is transported into the mucosal lamina propria via endocytotic vesicles with LF-binding factors on the cell membrane of villous columnar epithelial cells in neonatal pigs [12]. In the present study, bovine LF bound the cell membrane of both microvilli and the transcytotic vesicles. This finding demonstrates that LF receptor has the ability to bind bovine LF on enterocytes in growing pigs as well as in suckling piglets.

Human peripheral lymphocytes do not possess detectable intracellular and surface LF receptor in the resting phase [21]. In the activated peripheral lymphocytes, however, LF receptors for human LF appear on their cell surfaces [21]. LF is effective in the proliferation and differentiation of lymphocytes [2, 14]. In the present study, intraepithelial and subepithelial lymphocytes appeared LF-positive. Furthermore, these bovine LF-positive and possibly activated intraepithelial lymphocytes were transferred via both the lymphatics and the portal vein to the systemic circulation. This phenomenon might constitute an evidence of the effective regulation of heterogeneous LF on the systemic host defense mechanisms.

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REFERENCES

1. Barthel, L. K. and Raymond, P. A. 1990. Improved method for obtaining 3-microns cryosections for immunocytochemistry. *J. Histochem. Cytochem.* **38**: 1383–1388.
2. Bennett, R. M. and Davis, J. 1981. Lactoferrin binding to human peripheral blood cells: An interaction with B-enriched population of lymphocytes and subpopulation of adherent mononuclear cells. *J. Immunol.* **127**: 1211–1216.
3. Birgens, H. S. 1991. The interaction of lactoferrin with human monocytes. *Dan. Med. Bull.* **38**: 244–252.
4. Brock, J. 1995. Lactoferrin: a multifunctional immunoregulatory protein? *Immunol. Today* **16**: 417–419.
5. Brock, J. H. 2002. The physiology of lactoferrin. *Biochem. Cell Biol.* **80**: 1–6.
6. Broxmeyer, H, E., Gentile, P., Cooper, S., Lu, L., Juliano, L., Piacibello, W., Meyers, P. A. and Cavanna, F. 1984. Functional activities of acidic isoferritins and lactoferrin *in vitro* and *in vivo*. *Blood Cells* **10**: 397–426.
7. Burrin, D. G., Wang, H., Heath, J. and Dudley, M. A. 1996. Orally administered lactoferrin increases hepatic protein synthesis in formula-fed newborn pigs. *Pediatr. Res.* **40**: 72–76.
8. Cox, T. M., Mazurier, J., Spik, G., Montreuil, J. and Peters, T. J. 1979. Iron binding proteins and influx of iron across the duodenal brush border. Evidence for specific lactotransferrin receptors in the human intestine. *Biochim. Biophys. Acta* **588**: 120–128.
9. Fransson, G. B., Thoren-Tolling, K., Jones, B., Hambraeus, L. and Loennerdal, B. 1983. Absorption of lactoferrin-iron in suckling pigs. *Nutr. Res.* **3**: 373–384.
10. Gislason, J., Douglas, G. C., Hutchens, T. W. and Lonnerdal, B. 1995. Receptor-mediated binding of milk lactoferrin to nursing piglet enterocytes: A model studies for absorption of lactoferrin-bound iron. *J. Pediatr. Gastroenterol. Nutr.* **21**: 37–43.
11. Gislason, J., Iyer, S., Hutchens, T. W. and Loennerdal, B. 1993. Lactoferrin receptors in piglet small intestine: Lactoferrin binding properties, ontogeny, and regional distribution in the gastrointestinal tract. *J. Nutr. Biochem.* **4**: 528–533.
12. Harada, E., Itoh, Y., Sitizyo, K., Takeuchi, T., Araki, Y. and Kitagawa, H. 1999. Characteristic transport of lactoferrin from the intestinal lumen into the bile via the blood in piglets. *Comp. Biochem. Physiol. Part A.* **124**: 321–327.
13. Harada, E., Sugiyama, A., Takeuchi, T., Sitizyo, K., Syuto, B., Yajima, T. and Kuwata, T. 1999. Characteristic transfer of colostral components into cerebrospinal fluid via serum in neonatal pigs. *Biol. Neonate* **76**: 33–43.
14. Hashizume, S., Kuroda, K. and Murakami, H. 1983. Identification of lactoferrin as an essential growth factor for human lymphocytic cell lines in serum-free medium. *Biochim. Biophys. Acta* **763**: 377–382.
15. Kanyshkova, T. G., Buneva, V. N. and Nevinsky, G. A. 2001. Lactoferrin and its biological functions. *Biochemistry (Moskow)* **66**: 1–7.
16. Kohno, Y., Shiraki, K., Mura, T. and Ikawa, S. 1993. Iron-saturated lactoferrin as a co-mitogenic substance for neonatal rat hepatocytes in primary culture. *Acta Paediatr.* **82**: 650–655.
17. Levay, P. F. and Viljoen, M. 1995. Lactoferrin: A general review. *Haematologica* **80**: 252–267.
18. Lonnerdal, B. and Iyer, S. 1995. Lactoferrin: molecular structure and biological function. *Ann. Rev. Nutr.* **15**: 93–110.
19. Masson, P. L. and Heremans, J. F. 1971. Lactoferrin in milk from different species. *Comp. Biochem. Physiol. B* **39**: 119–129.
20. Masson, P. L. and Heremans, J. F. and Dive, C. H. 1966. An iron-binding protein common to many external secretions. *Clin. Chim. Acta* **14**: 735–739.
21. Mazurier, J., Legrand, D., Hu, W. L., Montreuil, J. and Spik, G. 1989. Expression of human lactotransferrin receptors in phytohemagglutinin -stimulated human peripheral blood lymphocyte. Isolation of the receptors by antiligand affinity chromatography. *Eur. J. Biochem.* **179**: 481–487.
22. Mazurier, J., Montreuil, J. and Spik, G. 1985. Visualization of lactotransferrin brush-border receptors by ligand blotting. *Biochem. Biophys. Acta* **821**: 453–460.
23. Miller, E. R. and Ullrey, D. E. 1987. The pig as a model for human nutrition. *Annu. Rev. Nutr.* **7**: 361–382.
24. Nichols, B. L., Mckee, K. F., Henry, J. F. and Putman, M. 1987. Human lactoferrin stimulates thymidine incorporation into DNA of rat crypt cells. *Pediatr. Res.* **21**: 563–567.
25. Nichols, B. L., Mckee, K. F. and Huebers, H. A. 1990. Iron is not required in the lactoferrin stimulation of thymidine incorporation into the DNA of rat crypt enterocytes. *Pediatr. Res.* **27**: 525–528.

26. Olofsson, T., Olsson, I., Venge, P. and Elgefors, B. 1977. Serum myeloperoxidase and lactoferrin in neutropenia. *Scand. J. Haematol.* **18**: 73–80.
27. Payne, L. C. and Marsh, C. L. 1962. Gamma globulin absorption in the baby pig: The nonselective absorption of heterologous globulins and factors influencing absorption time. *J. Nutr.* **76**: 151–158.
28. Schmitz, M., Hagemeister, H., Goertler, I., Bindels, J. G. and Barth, C. A. 1988. Does bovine lactoferrin resist absorption in the small intestine of neonatal and adult pigs? pp. 103–104. *In*: Milk Proteins, Nutritional, Clinical, Functional and Technological Aspects (Barth, C. A. and Schlimme, E. eds.), Steinkopff Verlag, Darmstadt.
29. Talukder, M. J. R., Takeuchi, T. and Harada, E. 2002. Transport of colostral macromolecules into the cerebrospinal fluid via plasma in newborn calves. *J. Dairy Sci.* **85**: 514–524.
30. Tomita, M., Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H. and Kawase, K. 1991. Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. *J. Dairy. Sci.* **74**: 4137–4142.
31. Visca, P., Dalmastri, C., Verzili, D., Antonini, G., Chiancone, E. and Valenti, P. 1990. Interaction of lactoferrin with *Escherichia coli* cells and correlation with antibacterial activity. *Med. Microbiol. Immunol.* **179**: 323–333.