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Absorption of Horseradish Peroxidase (HRP) In Vitro Across Bovine Jejunal and Ileal Epithelia Around the Time of Weaning

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Summary

Using the everted sac methodology as well as an Ussing chamber, we investigated changes in the absorption of horseradish peroxidase (EC 1.11.1.7, HRP (40 kDa)) in jejunum and ileum segments isolated from male Holstein cattle around the time of weaning (6 to 15 wks old). By the everted sac method, HRP transport (HRP concentration on the serosal side sampled after a 60-min-incubation) at 15 wks of age was significantly greater than that at 6 wks of age, in both segments of the intestine. Absorption was not significantly different between the jejunum and ileum. Addition of Na^+/K^+ATPase inhibitor (ouabain, 1 mM) did not cause any significant change in HRP absorption, whereas Na^+/H^+anti-transporter inhibitor (amiloride, 1 μM) significantly increased the absorption in both sacs at 8 wks of age. By the Ussing chamber method, there were no significant differences between the values for Jsm and Jms, while the Jnet value was nearly zero for both epithelia. In addition, the flux (Jms) of Lucifer yellow, a cell-membrane-impermeable fluorescence dye, was significantly greater at 6 than at 13 wks of age in the ileal epithelia, although the flux was significantly greater in the jejunal than the ileal epithelia at both ages. From these findings, we conclude that: 1) bovine jejunal and ileal epithelia are able to absorb a large molecule such as the HRP protein; 2) HRP transport occurs in a concentration-dependent manner and may in part be via a paracellular pathway; 3) the increased HRP transport shown at 15 wks of age may not be caused by an increased use of the paracellular pathway.

Key words: horseradish peroxidase, absorption, transport, calf

During the first weeks of life after birth, neonates are obliged to take colostrum and milk from their mothers for several weeks until weaning, and then their intake changes to solid materials such as roughage and cereal. We recently reported changes in the expression of nutrient transporters and related enzymes around weaning time in the ruminant alimentary tract. We demonstrated that

weaning reduced the expression of sodium-dependent glucose transporter (SGLT1) and CD36 (a fatty acid transporter) (Hayashi et al., 2005) as well as leptin (Yonekura et al., 2002) in the gastrointestinal tract, but increased the activity of carbonic anhydrase (Kitade et al., 2002) in the parotid gland of calves.

Neonatal disease resistance in farm animals is greatly influenced by a passive immunisation just after birth : the intestinal absorption of immunoglobulins from their mother's colostra. Although the intestinal capacity to absorb macromolecules is influenced by both diet- and animal-related factors, the details of the absorption process and the mechanisms regulating its cessation are poorly understood (Sangild, 2003).

There is a report to show that intestinal transport of a macromolecule substance (horseradish peroxidase : HRP) across the descending colon is much greater in cattle than in sheep (McKie et al., 1999). When macromolecules such as proteins and polymer antigens are transported from the mucosal to the serosal side of the gut, the cellular pathway is thought to be via a paracellular rather than via a transcelluler route. This is because it is generally believed that the transport of macromolecules via the paracellular route can avoid proteolytic digestion by intracellular protease activity. However, this assumption has been put under suspicion because HRP and some peptides remain intact after transport across a monolayer of a human colonic epithelial cell line (HT29-19A)(Terpend et al., 1998). On the other hand, as the colon has been shown to be relatively impermeable to macromolecules, polyethylene glycols of various sizes have been used as an indicator of paracellular flow in the intestine (Hollander et al., 1986, 1989 ; Krugliak et al., 1989 ; Krugliak et al., 1990 ; Seidman et al., 1986). Stress and inflammatory conditions significantly increase this permeability in all parts of the intestine including the colon (Hollander et al., 1989 ; Seidman et al., 1986 ; Bijlsma et al., 2001).

On the other hand, horseradish peroxidase (HRP) is a macromolecule protein (molecular weight 40 kD), and is partially absorbed across the intestinal mucosa without digestion by proteases (Terpend et al., 1998), indicating the possibility that as this feature resembles that of an abnormal prion protein (PrP^{sc}), HRP is an appropriate substitute for the study of the transport of PrP^{sc}.

In the present study, in order to determine the mechanism for the intestinal transport of macromolecular proteins, we investigated : 1) the effects of aging around the time of weaning on HRP transport ; 2) the effects of ouabain and amiloride on HRP absorption ; and 3) the transport mechanisms of HRP. Each of these processes was investigated using the everted sac and Ussing chamber methods in isolated segments of the bovine jejunum and ileum.

Materials and Methods

Intestinal tissue samples

Holstein male cattle at 6～8 wks ($n=32$), and 13～15 wks ($n=5$) were used. The cattle were anaesthetized by an overdose injection of thiopental sodium (Labonal, Tanabe, Osaka, Japan) and killed by exsanguination. Then, intestinal segments of the jejunum (10～50 cm descendant from the pylorus) and the ileum (10～50 cm ascendant from the cecum) were immediately removed and washed with physiological saline. For experiments with everted sacs, sacs (10 cm length, two sacs for each segment) were made, which were then filled with HEPES-buffered saline (HBS) inside, and conserved in HBS bubbled with 100% O_2 before use in the incubation. The composition of HBS was as follows (g/l): NaCl: 6.716, KCl: 0.402, $MgCl_2$: 0.204, $NaHCO_3$: 4.766, D-glucose: 1.350, HEPES: 4.766 (pH 7.4 adjusted with NaOH). For experiments using the Ussing chamber, washed intestinal segments were dissected open in a vertical direction, and epithelial tissue sheets were separated from the muscle layer under a stereomicroscope. These were mounted on an Ussing chamber (tissue exposure area, 1 cm^2).

Everted sac method

Everted sacs were pre-incubated in HBS (150 ml) for 30 min at 37°C. Following this, HRP (Sigma) was added to the mucosal-side HBS (final concentration 2,500 nM), and incubated for a further 60 min. After 60 min of incubation, one ml of serosal-side HBS was taken and stored in a plastic tube (O.D. 12 mm, Length 70 mm).

In the experiment with ouabain, everted sacs were made by filling the serosal side with HBS (serosal-side) containing 1 mM ouabain (Sigma, MO). In the experiment with amiloride, amiloride (final concentration 1 mM) was added together with HRP to the mucosal-side HBS. An additional experiment was performed where the pH of the HBS was reduced to 5.4. In this experiment, everted sacs were incubated in HBS for which the mucosal-side pH was adjusted 5.4, while the pH of the HBS injected into the sacs was 7.4.

Ussing chamber method

Both the mucosal- and serosal-sides of the epithelial tissues were filled with 15 ml of HBS, and were maintained at 37°C for 30 min under a continuous stream of bubbling 100% O_2. Then, HBS on both sides was changed with 15 ml of fresh HBS, in which either the mucosal- or the serosal-side solution contained HRP (final concentration 100 μM), and the incubation was continued for a further 60 min under 100% O_2. After the incubation, 500 μl of the solution from the side that did not contain HRP was sampled.

Lucifer yellow (LY), a cell membrane-impermeable fluorescence dye, was

added separately to both sides of the incubation after a preincubation of 30 min (final concentration 100 μg/ml)(Clark et al., 2003). The incubation period and sampling of the solution were same as that described for the HRP experiments.

HRP assay

A portion of each sampled solution was freeze-dried over-night, and stored at -30°C. After the addition of 500 μl of distilled water, freeze-dried samples were centrifuged at 2,100 rpm for 10 min, and the supernatant was diluted with HBS at a ratio of 1 : 3. The assay procedure was based on a published method (McKie et al., 1999) : Briefly, 60 μl of the diluted sample solution was mixed with 540 μl of reactive solution (10 ml of 0.2 M NaH_2PO_4, 200 μl of 0.2 M Na_2HPO_4, 1,020 μl of 0.3% O-dianisidine, 4.08 μl of 30% H_2O_2, made up to 40.8 ml with distilled water) for 10 min at 25°C, and then the reaction was terminated by the addition of 24 μl of 4% NaN_3. Standard concentrations were used at 0$\sim$40 nM. The absorbance was photometrically measured at 450 nm (AA-610, Shimazu, Kyoto, Japan).

Statistical analysis

The results are presented as the mean$\pm$S.D. The data were analyzed using Student's t-test and Duncan's multiple comparison test.

Results

Everted sac method

HRP transport, as measured by the everted sac method in segments of the

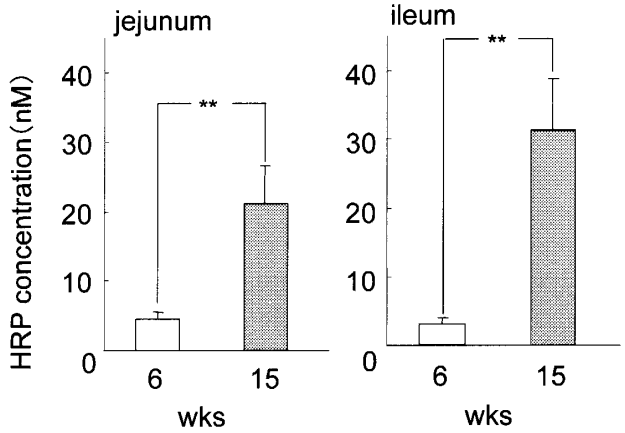

FIG. 1. Everted sac method : Serosal HRP concentration of the intestinal sacs after incubation for 60 min in HBS containing HRP (2,500 nM). Intestinal segments were isolated from the proximal jejunum and distal ileum at 6 ($n=8$) and 15 ($n=15$) wks of age. There is a significant difference between characters with different letters ($p<0.05$, Duncan's test).

jejunum and ileum from cattle at 6 and 15 wks of age is shown in Fig. 1. In both segments, HRP transport at 15 wks of age was significantly greater than that at 6 wks of age. However, at all ages, there was no significant difference between the jejunal and ileal segments.

We investigated the effect of the addition of Na^+/K^+ATPase inhibitor

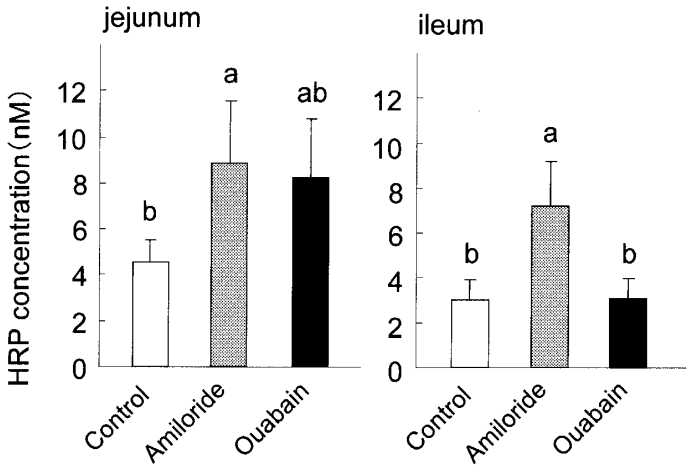

FIG. 2. Everted sac method: Serosal HRP concentration of the intestinal sacs after incubation without or with ouabain (1 mM) or amiloride (1 μM) for 60 min in HBS containing HRP (2,500 nM). Intestinal segments were isolated from the proximal jejunum and distal ileum at 8 ($n=6$) wks of age. There is a significant difference between characters with different letters ($p<0.05$, Duncan's test).

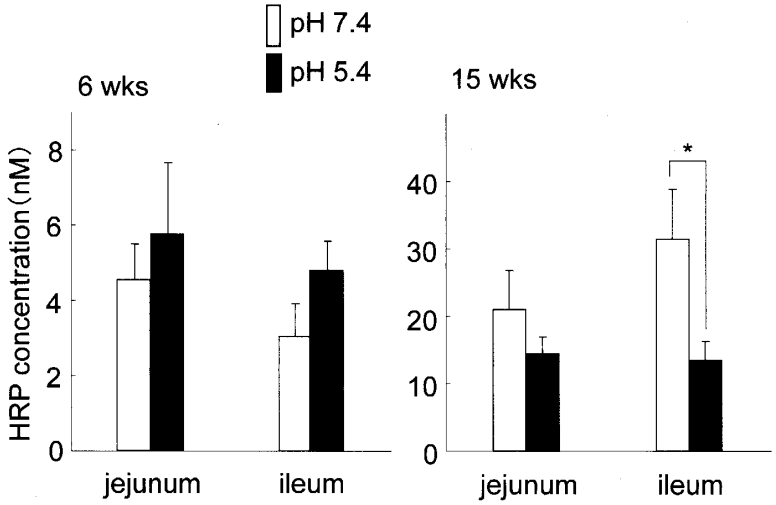

FIG. 3. Everted sac method: Serosal HRP concentration of the intestinal sacs after incubation in HBS with normal pH (7.4) or low pH (5.4) for 60 min in HBS containing HRP (2,500 nM). Intestinal segments were isolated from the proximal jejunum and distal ileum at 6 ($n=8$) and 15 ($n=5$) wks of age. $^*p<0.05$ (Student's t-test).

(ouabain, 1 mM) and Na^+/H^+transporter inhibitor (amiloride, 1 nM) on HRP transport, using the segments at 8 wks of age (Fig. 2). In both intestinal segments, HRP transport was significantly increased by the addition of amiloride, whilst the addition of ouabain did not cause any significant effect on HRP transport.

In order to investigate the effects of enhanced H^+concentration in HBS on HRP transport, we measured HRP transport at an external pH of 5.4 at 6 and 15 wks of age (Fig. 3). HRP transport was only significantly decreased in the ileal

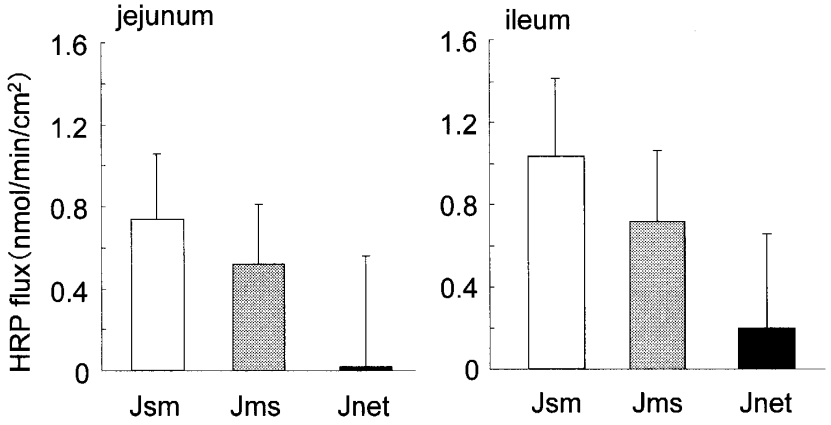

FIG. 4. Ussing chamber method : Unidirectional HRP transport was measured with stripped epithelia attached to Ussing chambers after incubation in HBS for 60 min. Intestinal segments were isolated from the proximal jejunum and distal ileum at 8 ($n=6$) wks of age. HBS on both sides contained HRP (100 μM).

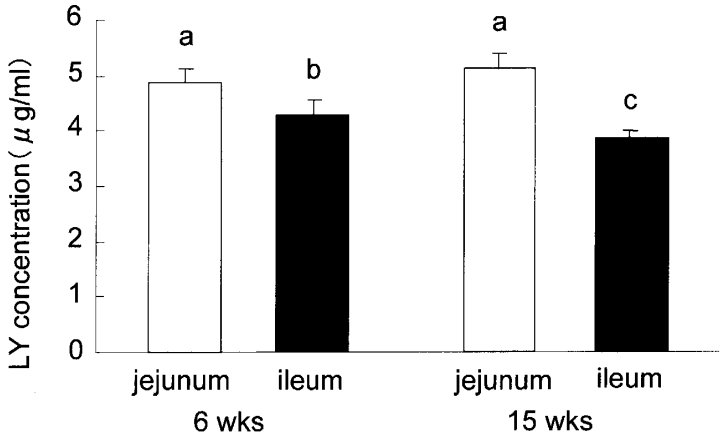

FIG. 5. Ussing chamber method : The Lucifer yellow concentration on both sides was measured using stripped epithelia attached to a Ussing chamber after incubation in HBS for 60 min. Intestinal segments were isolated from the proximal jejunum and distal ileum at 6 ($n=6$) and 15 ($n=7$) wks of age. HBS containing Lucifer Yellow (100 μg/ml) was used separately on both sides of the epithelia.

segments isolated at 15 wks of age.

Ussing chamber method

We also measured HRP transport fluxes using the Ussing chamber method, across jejunal and ileal epithelia at 8 wks of age (Fig. 4). In both intestinal epithelia, no significant difference was found between Jsm and Jms, while Jnet was nearly 0. In addition, there was no significant difference in fluxes between either of the intestinal epithelia.

Finally, we measured LY flux (Jms) in order to investigate whether or not the intercellular pathway of the epithelia changes between 6 and 15 wks of age (Fig. 5). For the jejunal epithelium, there was no significant difference in LY permeability between either age. However, LY permeability in the ileal epithelium at both ages was significantly lower than that of 6 wks of age, and LY permeability at 15 wks of age was significantly lower than that at 6 wks of age.

Discussion

From the findings using the everted sac method, HRP transport was significantly increased between the ages of 6 and 15 wks (Fig. 1). This result suggests that the physiological and morphological characteristics of the intestinal transport system may change around 15 wks of age in order to increase the rate of macromolecular transport. It is reported that HRP permeability was significantly decreased in the intestine of mice and rats when treated with GLP-2 for 10 days (Benjamin et al., 2000 ; Cameron et al., 2003). The authors of these reports suggest that intestinal cell proliferation is stimulated by GLP-2 treatment, resulting in a decreased HRP permeability because the degradation of HRP was increased due to a lengthened transit time for the transcytosis of large molecules. Another possibility may be that the integrity of the tight junctions was altered (Cameron et al., 2003), due to morphological changes in the intestinal epithelium during this period of ageing.

There were no significant differences in the HRP transport between the jejunal and ileal intestinal segments at any age (Fig. 1). From this finding, it seems likely that there are no cells specific for the HRP transport of HRP in one of the intestinal segments only, and that the ability of both parts of the intestine is approximately the same. However, it is reported that HRP is transported in the bovine and ovine colon (McKie et al., 1999), rat jejunum (Ducroc et al., 1983 ; Heyman et al., 1982 ; Kiliaan et al., 1998), and porcine jejunum, ileum and colon (Boudry et al., 2004). Therefore, it remains to be established whether the ability of HRP transport occurs at the same level in the other segments of the gastrointestinal tract, as we used only the segments isolated from the proximal jejunum and the distal ileum in this study. In our preliminary experiment, however, HRP

transport in the proximal jejunum and the distal ileum were greatest relative to the other segments of the gastrointestinal tract.

We investigated the effects of ouabain (1 mM) (a Na^+/K^+ ATPase inhibitor), amiloride (a Na^+/H^+ exchange system inhibitor) and increased H^+ gradient on HRP transport. This is because some peptides and amino acids are known to be transported through the mucosal cell membrane and into the cell because of the H^+gradient or secondary active transport system, established by the activation of a Na^+/K^+ pump located on the serosal cell membrane (D'Mello, 2003). However, HRP transport was not significantly changed by the addition of ouabain to incubations of both intestinal segments. This finding suggests that HRP transport does not depend on a Na^+/K^+ pump, or HRP transport is via the paracellular pathway. Interestingly, HRP transport was significantly increased by the addition of amiloride. We do not know the precise mechanism for this, but a plausible explanation may be that a decreased intracellular Na^+ concentration induced by amiloride spared ATP consumption by the Na^+/K^+ pump, resulting in the acceleration of HRP transport via an unknown pathway. Additionally, HRP transport was significantly decreased by the reduction in pH outside of the mucosal membrane (increased H^+ gradient) at 15 wks of age, but not at any other age. This finding suggests that HRP transport may not be dependent on the H^+ gradient, and agrees with the previous finding that immunoglobulin absorption was not affected by pH in neonatal calves (Quigley III et al., 2000).

Using the Ussing chamber method, we found that HRP transport is passive because there was no significant difference between Jsm and Jms. From the use of a cell-membrane-impermeable fluorescence dye, it seems likely that paracellular permeability was significantly decreased in the ileum rather than in the jejunum, both at 6 and 15 wks weeks of age. This result coincides with the result for the increased H^+gradient, and suggests that significantly increased HRP transport at the age of 15 wks is not due to an increased transport via the paracellular pathway. In other words, the increased HRP transport at 15 wks of age is caused via the intracellular pathway.

As mentioned in Introduction, HRP was used as an appropriate substitute for the study of the transport of PrP^{sc} (Terpend et al., 1998). However, little is known of the mechanisms for the absorption of orally ingested PrP^{sc}, or for its transport to and storage in the brain and spinal cord. The causative agent for BSE is suspected to be an abnormal prion protein (PrP^{sc}) (Jhon and Mark, 1997 ; Yamauchi, 2001 ; Yamauchi and Ono, 1996). The prion protein (PrP) gene exists on the 20th chromosome in the human and produces the normal prion protein (PrP^{c})(Yamauchi and Ono, 1996). PrP^{c} is synthesized in a variety of tissues, although PrP^{sc} accumulates particularly in the brain of animals suffering from a prion disease.

In conclusion, we found that HRP is absorbed from both the jejunum and the

ileum at the same level, and that transport is increased at approximately 15 wks of age. It is also suggested that HRP transport is via a passive transport system, and that the increased HRP transport at the age of 15 wks is not brought about by the paracellular route. In the future, it will be necessary to investigate how HRP is transported at the other ages and when HRP is consumed orally.

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References

Benjamin, M.A., McKay, D.M., Yang, P.-C., Cameron, H., and Pedue, M.H., Glucagon-like peptide-2 enhances intestinal epithelial barrier function of both transcelluler and paracellular pathways in the mouse. *Gut*, **47**, 112–119 (2000)

Bijlsma, P.B., van Raaiiji, M.T., Dobbe, C.J.G., Timmerman, A., Kiliaan, A.J., Taminiau, J.A.J., and Groot, J.A., Subchronic mild noise stress increases HRP permeability in rat small intestine in vitro. *Physiol. Behav.*, **73**, 43–49 (2001)

Boudry, G., Peron, V., Le Huerou-Luron, I., Lalles, J.P., and Save, B., Weaning induces both transient and long-lasting modifications of absorptive, secretory, and barrier properties of piglet intestine. *J. Nutr.*, **134**, 2256–2262 (2004)

Cameron, H.L., Yang, P.C., and Perdue, M.H., Glucagon-like peptide-2-enhanced barrier function reduces pathophysiology in a model of food allergy. *Am. J. Physiol.*, **284**, G905–912 (2003)

Clark, E.C., Patel, S.D., Chadwick, P.R., Warhurst, G., Curry, A., and Carlson, G.L., Glutamine deprivation facilitates tumour necrosis factor induced bacterial translocation in Caco-2 cells by depletion of enterocyte fuel substrate. *Gut*, **52**, 224–230 (2003)

D'Mello, J.P.F., pp. 41-70. "Amino Acids in Animal Nutrition". 2nd Ed., CABI Publishing, Cambridge (2003)

Ducroc, R., Heyman, M., and Beaufrere, B., Horseradish peroxidase transport across rabbit jejunum and Peyer's patches in vitro. *Am. J. Physiol.*, **245**, G54–58 (1983)

Hayashi, H., Yonezawa, T., Kanetani, T., Terada, F., Katoh, K., and Obara, Y., Expression of mRNA for Na^+/glucose transporter 1 (SGLT1) and fatty acid translocase (CD36) in the ruminant gastrointestinal tract before and after weaning. *Anim. Sci. J.*, **76**, 339–344 (2005)

Heyman, M., Ducroc, R., and Desjeux, J.F., Horseradish peroxidase transport across adult rabbit jejunum in vitro. *Am. J. Physiol.*, **242**, G558–564 (1982)

Hollander, D., Koyama, S., and Dadufalza, V., Polyethylene glycol 900 permea-

bility of rat intestinal and colonic segments in vivo and brush border membrane vesicles in vitro. *J. Lab. Clin. Med.*, **113**, 505-515 (1989)

Hollander, D., Vadhein, C.M., and Brettholz, E., Increased intestinal permeability in patients with Crohn's disease and their relatives. A possible etiologic factor. *Ann. Int. Med.*, **105**, 883-885 (1986)

Jhon, C., and Mark, S.P., Prion diseases. Oxford University Press, Oxford (1997)

Kiliaan, A.J., Saunders, P.R., Bijlsma, P.B., Berin, M.C., Taminiau, J.A., Groot, J.A., and Perdue, M.H., Stress stimulates transepithelial macromolecular uptake in rat jejunum. *Am. J. Physiol.*, **275**, G1037-44 (1998)

Kitade, K., Takahashi, K., Yonekura, S., Katsumata, N., Furukawa, G., Osuga, S., Nishita, T., Katoh, K., and Obara, Y., Effects of nutritional conditions around weaning on carbonic anhydrase activity of the parotid gland and ruminal and abomasal epithelia of Holstein calves. *J. Comp. Physiol.*, **B172**, 379-385 (2002)

Krugliak, P., Hollander, D., and Ma, T.Y., Mechanisms of polyethylene glycol 400 permeability of perfused rat intestine. *Gastroent.*, **97**, 1164-1170 (1989)

Krugliak, P., Hollander, D., and Schlaepfer, C., Polyethylene glycol 400 penetration of the colonic epithelial barrier of the rat. *Gastroent.*, **99**, 1001-1007 (1990)

McKie, A.T., Zammit, P.S., and Naftalin, R.J., Comparision of cattle and sheep colonic permeabilities to horseradish peroxidase and hamster scrapie prion protein in vitro. *Gut*, **45**, 879-888 (1999)

Quigley III, J.D., French, P., and James, R.E., Effect of pH on absorption of immunoglobulin G in neonatal calves. *J. Dairy Sci.*, **83**, 1853-1855 (2000)

Sangild, P.T., Uptake of colostral immunoglobulins by the compromised newborn farm animal. *Acta Vet. Scand., Suppl.*, **98**, 105-22 (2003)

Seidman, E.G., Hanson, D.G., and Walker, W.A., Increased permeability to polyethylene glycol 4000 in rabbits with experimental colitis. *Gastroent.*, **90**, 120-126 (1986)

Terpend, K., Boisgerault, F., Blaton, M.A., Desjeux, J.F., and Heyman, M., Protein transport and processing by human HT29-19A intestinal cells effect of interferon gamma. *Gut*, **42**, 538-545 (1998)

Yamauchi, K., *Kyou-gyu Byou*, Kawade Shobo Shinsha, Tokyo (2001) (in Japanese, with English summary)

Yamauchi, K., and Ono, T., *Purion Byou*, Kindai Shuppan, Tokyo (1996) (in Japanese, with English summary)

Yonekura, S., Kitade, K., Furukawa, G., Takahashi, K., Katsumata, K., Katoh, K., and Obara, Y., Effects of aging and weaning on RNA expression of leptin and CCK receptors in the calf rumen and abomasums. *Domest. Anim. Endocrinol.*, **22**, 25-35 (2002)