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Trans-Endothelial and Trans-Epithelial Transfer of Lactoferrin into the Brain through BBB and BCSFB in Adult Rats

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ABSTRACT. The transportation of intravenously administered bovine lactoferrin (bLF) into the cerebrospinal fluid (CSF) was immunohistochemically investigated in adult rats. Administered bLF was detected in the vesicular membranes of endothelial cells in cerebral blood vessels 10 min after the infusion. Numerous immunoreactive small vesicles were also detected at the ependymal cells in the choroid plexus. Moreover, the bLF concentration in the CSF was significantly increased at 1–2 hr after the intravenous infusion of bLF (10 or 30 mg/kg). These findings clearly demonstrate that LF is possibly transported into the brain matter even in adult animals.

KEY WORDS: blood-cerebrospinal fluid barrier, lactoferrin, transcytosis.

Lactoferrin (LF) is known as a bioactive protein contained in blood, saliva, tears, hepatic bile, pancreatic juice and cerebrospinal fluid (CSF). Harada et al. [5] demonstrated that the LF contained in milk was transported into circulation from the intestinal lumen and excreted into bile, suggesting the possibility of the entero-hepatic circulation of LF in neonatal pigs. As well, orally administered LF was reported to be absorbed into systemic circulation not only in neonates [6, 14], but also in weaned pigs [5] and young cattle [16]. Talukder et al. [15] reported the detection of a specific LF-receptor in adult cow intestines, including the duodenum, jejunum, ileum, and colon, and Peyer’s patches in the jejunum and ileum. Interestingly, the density of LF-receptor was higher in Peyer’s patches than in other regions of the intestine. Thus, it is speculated that the LF transport mechanism is closely related to lymphatic organs in the weaned animal. Recently, we reported that intra-duodenally administered bovine LF (bLF) was successfully absorbed from the intestine via the lymphatic pathway in weaned pigs [10] and adult rats [13].

Hayashida et al. [7] reported novel functions of bLF, such as anti-nociception within the central nervous system of rats. This anti-nociceptive effect was induced by not only intra-peritoneal injection but also by oral administration. However, the passage of macromolecules from plasma into the brain is limited by the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB). These barriers to protein appear to be well formed in fetal and newborn animals, since tight junctions have been observed between cerebral endothelial cells and in the choroid plexus. Their report [7] suggested that orally administered LF was possibly transported into the brain by passing through the BBB and/or BCSFB. Using co-culture of bovine brain capillary endothelial cells and astrocytes as an in vitro model of the BBB, researchers demonstrated that LF crossed the BBB via receptor-mediated transcytosis [1]. However, the transported LF was not detected immunohistochemically. The present study investigated whether intravenously administered bLF was transported to the brain matter through the BBB and/or BCSFB in adult rats.

The bLF was provided from Tatua Biologics (Tatua, New Zealand). Its purity was 90% with an iron saturation of 15%. Coliforms, E. coli, S. aureus, Salmonella, and Listeria were not detected, and yeasts and moulds were less than one per gram.

Wistar strain male rats (220–280 g) were obtained from the Institute of Animal Reproduction (Ibaraki, Japan). The animals were maintained at a controlled temperature of 22±2°C with a 12:12-hr light/dark cycle (light cycle, 07:00–19:00), and were given standard chow (CE-2, Nihon Clea, Tokyo, Japan). The use of these animals, and the procedures performed on them, was approved by the Animal Research Committee at Tottori University.

Five rats were used for an immunohistochemical analysis. Under 25% urethane (4 g/kg, sc) anesthesia, bLF solution (10% in saline) was infused into the tail vein via a 26-gauge needle at a dose of 10 mg/kg body weight. At 10 min after the bLF infusion, periodate-lysine-paraformaldehyde fixative (PLP) was perfused intracardially for 20 min. The brain was frontally sliced and immersed in the same fixative overnight at 4°C. Tissue blocks were snap-frozen in liquid nitrogen, followed by cryosectioning with Coldtome HM505E (Carl Zeiss, Germany). Immunohistochemical analysis was performed according to the previous report [13]. In brief, the sections were rinsed with 0.05% Tween-20 or Tween-40 added phosphate buffered saline (pH 7.4, PBS), followed by treatment with absolute methanol and 0.5% H2O2 for 30 min, respectively. Following blocking with 1% wild bull-
frog serum, the sections were reacted with horseradish peroxide (HRP)-conjugated anti-bLF goat IgG (Bethyl Lab., U.S.A.) for 20 hr at 4°C. Finally, the sections were colored with 3, 3'-diaminobenzidine-HCl containing 0.03% H₂O₂, and were counter-stained with hematoxylin.

Sixty rats were used for measuring transported bLF concentrations in the CSF. We put a rat in a retaining box for intravenous injections and were given bLF solution in the tail vein at doses of 10 mg/kg or 30 mg/kg body weight in a conscious condition. The animal did not act violently even if we put the rat in the retaining box without pre-adaptation. We returned the rat to their home cage until sampling.

Before the infusion and at 30 min, 1 hr and 2 hr after the bLF infusion, CSF (0.2 ml) was collected by puncturing into the cisterna magna via a 26-gage needle under urethane (1 g/kg, sc) anesthesia. Physiological saline was infused into the tail vain in control rats to check a cross reactivity with endogenous LF in the CSF. Each rat was studied once only for the CSF sampling to prevent a contamination of blood. The CSF samples were centrifuged at 1,500 g at 4°C for 15 min and the supernatants were stored at −80°C until analysis. The bLF transported into the CSF was assayed quantitatively by double-antibody enzyme-linked immunosorbent assays (ELISA) described by Harada et al. [5]. The minimum detectable level for bLF was 2 ng/ml.

Data were expressed as mean ± SE. Unpaired Student's t-test was employed for the detection of statistical significances with a P value of less than 0.05.

At 10 min after the infusion, some administered bLF remained in the lumina of blood capillaries; it was also detected in the endothelial cells of capillary vessels in the brain white matter. The bLF-positive immunoreactions were found mainly on the membranes of vesicles in the endothelial cells (Fig. 1a).

Relatively strong immunoreactivity was detected in the vascular lumina and some ependymal cells in the choroid plexus in rats (Fig. 1b). Numerous vesicles in the ependymal cells of choroids plexus had positively stained membranes (Fig 1c). In any control rats, non-specific immunoreactivity was never detected (data are not shown), moreover the specificity of immunoreaction in the present protocol has been published in the previous report [13].

The bLF concentration in the CSF increased following intravenous infusion of bLF. In the high dose (30 mg/kg) group, transported bLF was detected in CSF at 30 min after the infusion. Thereafter, bLF concentrations in CSF were increased, and reached peak values at 1 hr in high dose group (621.8 ± 261.7 ng/ml) and at 2 hr in low dose group (388.1 ± 109.1 ng/ml), respectively (Fig. 2). bLF was never detected in the control group (data are not shown).

These findings suggest that the choroid plexus is one of the main routes for the transportation of LF into brain tissues in rats. Endogenous LF in the brain has been reported in mice [2]. The in situ synthesis of LF is up-regulated in cases of oxidative stress, as well as in the structural changes of the mouse brain that accompany aging [2].

It has also been reported that increased BBB permeability to macromolecules occurred under a variety of experimental conditions such as acute hypertension [18], ischemia [17], and administration of histamine [4] and cyclic nucleotide [9]. Plasma LF concentrations were markedly increased in inflammatory processes [3]. This suggests that in some specific pathological conditions, LF accumulation may occur in particular areas of the brain [3]. On the other hand, BBB or BCSFB permeability differs physiologically according to age. In our previous study, we reported bLF concentrations in plasma and CSF after infusion of bLF into the jejunum of newborn or weaned calves [14, 16]. Interestingly, the bLF concentration in the CSF was higher in weaned calves (800 ng/ml) than in newborn calves (180 ng/ml), while the bLF
concentration in plasma was higher in newborns (2,500 ng/ml vs. 2,000 ng/ml). In the present study, bLF concentration in the CSF reached 621 ng/ml after the intravenous infusion of bLF (30 mg/kg). This peak level was less than that in calves, while the bLF concentration in plasma was much higher in this study. The permeability of BCSFB to macromolecules may be affected by species specificity. It is thought that the LF is transported into the CSF via a receptor-mediated endocytotic mechanism [16]. This is a quite similar mechanism to that of the microvessels in the brain [1].

Moreover, Fillebeen et al. [1] have reported that the LF remained intact as a protein after its transport through the brain capillary epithelial cells by using co-culture of bovine brain capillary endothelial cells and astrocytes. LF is transported unidirectionally from the luminal to the abluminal side of the cells, and is not degraded during passage through the interendothelial pathway [1]. We also reported an intact size of LF transported into the CSF in the neonatal pigs [5]. Recently, Ji et al. [8] reported that brain uptake of LF in adult rats by using 125I-labeled LF. They reported that the brain uptake of 125I-LF represented approximately 0.016% of intravenously injected 125I-LF at 60 min, while there was no detectable amount of 125I-transferrin (TF) entering the brain after intravenous injection [8]. Strahan et al. [11] mentioned that although some TF molecules do enter the endothelia, most of them are recycled to the blood. These differences between LF and TF transport may be related with their receptor-mediated transport systems. However, the trans-endothelial transfer of TF into the brain through the BBB in vivo is still controversial.

In the present study, any immunoreactivity was not detected in astroglia nor microglia cells in cerebral white matter, although the bLF-positive immunoreactions were found on the membranes of vesicles in the endothelial cells (Fig. 1a). We think it is the reason for these phenomena that the bLF was concentrated within the vesicles in the cytoplasm, however, the bLF would be diffused to perivascular brain matter following a pass through the BBB. As a consequence concentrations of diffused bLF in the brain matter might be too low to detect microscopically.

By the way, the transported LF into the brain has various functions such as anti-inflammation and anti-oxidation action, etc. Recently, we reported novel functions of LF within the central nervous system, including anti-nociception [7] and anti-psychological stress actions [12]. In these cases, LF may allow the modification of neuronal reactions so as to prevent excess influences throughout the whole body. However, further experiments are necessary to clarify these points.

In this study, we detected immunoreactive bLF particles in the endocytes of microvessels and epithelial cells of the choroid plexus in the adult rats. This is one of the important information for understanding the transporting mechanisms of LF through the BBB and/or BCSFB.

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