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Alymphoplasia mice are resistant to prion infection via oral route.

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Abstract

The major cause of infection in animal prion diseases is thought to be consumption of prion-contaminated stuff. There is evidence that the enteric nerve system (ENS) and gut-associated lymphoid tissues (GALT) are involved in the establishment of prion infection through alimentary tract. To elucidate the initial entry port for prion, we inoculated prion to alymphoplasia (*aly*) mice showing a deficiency in systemic lymph nodes and Peyer's patches. The *aly/aly* mice were susceptible to prion infection by intra-cranial inoculation and there were no differences in incubation periods between *aly/aly* mice and wild-type C57BL/6J mice. Incubation periods in *aly/aly* mice were about 20 days longer than those in C57BL/6J mice with the intra-peritoneal inoculation. The *aly/aly* mice were completely resistant to prion infection by per os administration, while C57BL/6J mice were sensitive as they entered the terminal stage of disease around 300 days post inoculation. PrP^{Sc} were detected in the intestine and spleen of C57BL/6J mice inoculated with prion intra-peritoneally or orally ; however PrP^{Sc} was not detected in the spleen and intestine of *aly/aly* mice. Prion infectivity was detected in the intestines and spleens of prion-inoculated C57BL/6J mice, even after the early stages of exposure, while no infectivity was detected in these tissues of prion-inoculated *aly/aly* mice. No apparent differences were observed in the organization of the enteric nerve system between wild-type and *aly/aly* mice. These results indicate that GALT rather than ENS acts as the primary entry port for prion after oral exposure.

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Introduction

Transmissible spongiform encephalopathies (TSEs or prion diseases) are a group of fatal neurodegenerative diseases that include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting diseases (CWD) in deer and elk, and Creutzfeldt-Jakob disease in humans. Prion diseases have a long asymptomatic incubation period followed by a relatively short clinical phase, and they are characterized by the accumulation of disease-specific, protease-resistant isoforms of prion protein (PrP), designated PrP^{res} or PrP^{Sc}, in the central nervous system (CNS). PrP^{Sc} is post-translationally generated from the normal, protease-sensitive isoform of PrP, designated PrP^{sen} or PrP^C, which is expressed in many tissues and is particularly strongly expressed in the CNS. Although PrP^{Sc} is derived from host gene-encoded normal host protein, PrP^C, a line of evidences suggests that PrP^{Sc} is a major component of the TSE agent.

Although the CNS is the only site of histopathologically discernible damage, the port of entry for exogenous prion in animal prion diseases such as scrapie, BSE, and CWD is thought to be an alimentary tract. The route of neuroinvasion of prion has been well documented using prion-infected rodent models; there are at least two pathways for neuroinvasion, one is retrograde along the parasympathetic fibers of the vagus nerve to the medulla oblongata, and the other is along the sympathetic fibers of the splanchnic nerve to the thoracic/lumbar spinal cord.¹⁻⁴⁾ The existence of the two pathways to CNS is also confirmed by the extensive immunohistochemical analysis of naturally occurring sheep scrapie.⁵⁾ In the early stages of oral ex-

posure to prion, PrP^{Sc} can be detected in the nerve cells of enteric nerve system (ENS) and follicular dendritic cells (FDC) of tonsil and other submucosal lymphoid follicles in the alimentary tract.⁶⁻⁸⁾ These data indicate that ENS and Gut-associated lymphoid tissues (GALT) are the initial entry port for prion infection. However, it is unclear which of these is primarily important for the establishment of prion infection in the alimentary tract and subsequent neuroinvasion.

The alymphoplasia (*aly*) mutation in mice is autosomal recessive and is characterized by a deficiency in systemic lymph nodes and Peyer's patches.⁹⁾ Recently, the *aly* allele was found to carry a point mutation causing in amino acid substitution in the carboxy-terminal of NF- κ B inducing kinase (NIK).¹⁰⁾ Due to the lack of Peyer's patches, *aly* mice provide a suitable model for analyzing the involvement of GALT in the initial entry of prion in the alimentary tract. To elucidate the involvement of ENS and GALT in the establishment of prion infection, we analyzed the prion susceptibility of *aly* mice with various routes of infection. The results indicate GALT is a key tissue for the establishment of prion infection through the oral route.

Materials and Methods

Mice

ALY/NscJcl-*aly* (*aly/aly*) mice and their wild-type, C57BL/6J mice, were purchased from CLEA Japan Inc. ICR mice for bioassay were also purchased from CLEA Japan Inc.

Experimental inoculation

Mouse-adapted scrapie Obihiro strain was propagated in ICR mice. After entering the terminal stage of the disease, mice were sacrificed under anesthesia and brains were

collected. The brains were used as a source of brain homogenate for experimental inoculation. For intra-cranial (i.c.) inoculation, 20 μ l of 1 % brain homogenate was injected into the left hemisphere. Mice assigned to the intraperitoneal (i.p.) inoculation group received 100 μ l of 0.1% brain homogenate. Oral administration (p.o.) was carried out as described by Maignien et al.¹¹⁾ Animals were placed in individual cages equipped with a liquid delivery system consisting of a 1.5-ml Eppendorf tube with a 3-mm hole at the bottom. Tubes were filled with 100 μ l of a mixture of Endolipid (20% soya oil, 1.2% egg lecithin, 2.5% glycerol in water) and 20% brain homogenate (Endolipid : brain homogenate =1:1). Consumption of the infectious preparations was individually monitored.

Bioassay

During the course of experimental infection, 2 mice were sacrificed at each time point, and their brains, spleens and intestines were collected. Fifty milligrams of each tissue from the two mice was pooled and homogenized in PBS (10% w/w) a Multi-beads shocker (Yasuikiki, Japan) at 2,000 rpm for 1 min, followed by sonication for 30 sec. ICR mice were inoculated by i.c. route with 20 μ l of the tissue homogenates and were observed until they exhibited the clinical symptoms of the terminal stage.

Detection of PrP^{Sc}

Samples were prepared as described previously,¹²⁾ with minor modifications described below. Minced tissues were homogenized in about eight volumes of buffer consisting of 2 % (v/v) Zwittergent 3-12, 0.5% sodium deoxycholate, 100 mM NaCl, and 50 mM Tris-HCl (pH7.5). Homogenates were digested with collagenase (0.5mg/100mg tissue) and DNase I (40 μ g/100mg tissue) with constant

rotation at 37°C for 6 to 12 hr until lumps of tissue were dispersed. Proteinase K (50 μ g/100 mg tissue) was then added, and the homogenates were further incubated for 1 hr. Pefabloc was added at a final concentration of 2 mM to stop proteinase K digestion. Samples were then centrifuged at 68,000 g for 40 min at 20°C. The pellets were then dissolved in eight volumes (relative to the starting tissue sample) of 6.25% Sarkosyl in Tris-HCl, pH 8.0. After incubation at 37°C for 30 min, the samples were centrifuged at 12,000g for 5 min at 20°C. Supernatants were brought to 12% (w/v) NaCl, and were centrifuged at 100,000g for 40 min at 4 °C. The resulting pellets were dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (5% SDS, 4M Urea, 5% 2-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue, 62.5 mM Tris-HCl, pH 6.8). Immunoblot was performed as described elsewhere with some modifications.¹³⁾ SDS-PAGE was carried out using 14% Bis-Tris gels (Invitrogen) according to the supplier's instruction. Western transfer to Immobilon-P transfer membranes (Millipore) was carried out using the Trans-blot mini cell (Bio-Rad) at 60 V for 2 hr. After transfer, membranes were blocked for 1 hr at room temperature with 5% skim milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST) and were then incubated for 1 hr with primary antibodies diluted in the 1% skim milk in PBST. Blots were then washed with PBST and incubated with secondary antibody for 1 hr. Blots were visualized using an ECL Western blot detection kit (Amersham).

Immunohistochemical (IHC) detection of PrP^{Sc} was carried out as described elsewhere.¹⁴⁾ B103 polyclonal antibodies against bovine PrP synthetic peptide were used for detection.¹⁵⁾

Staining of ENS

Tissues were fixed with 10% formalin, 0.2% picric acid in PBS, and wholemount specimens or cryosections (20 μm) were stained with antibodies against Protein Gene Product 9.5. Antibody reactivity of the antibody was visualized using the avidin-biotin complex method as described elsewhere.¹⁶⁾

Results

Susceptibility of *aly/aly* mice to prion via various route of inoculation

In order to examine the susceptibility of *aly/aly* mice to prion infection, we inoculated *aly/aly* and C57BL/6J mice with brain homogenates of scrapie-infected mice via i.c., i.p. or p.o. route. Table 1 shows the incubation periods to reach the terminal stage of disease. No significant differences were observed in the incubation periods between *aly/aly* mice (159 days) and C57BL/6J mice (165 days) when they were inoculated with prion via i.c. route, and no apparent differences in clinical manifestation were seen between *aly/aly* and C57BL/6J mice. Furthermore, no differences were observed in the accumulation of PrP^{Sc} in the mouse brains (Fig. 1) or in neuro-histopathological findings (data not shown), indicating that the *aly* phenotype, which is caused by a point mutation in NIK, does not influence the neuropathogenesis of prion diseases nor prion replication in the CNS.

In i.p. inoculation, the incubation period in *aly/aly* mice was prolonged by 28 days, as

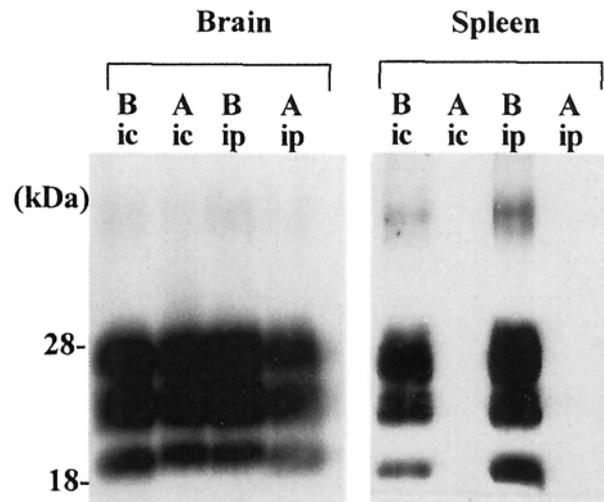


Fig. 1. Detection of PrP^{Sc} in brain and spleen. Mice exhibiting symptoms of the terminal stage of disease were sacrificed and examined for PrP^{Sc}. B, C57BL/6J mice; A, *aly/aly* mice. i.c., inoculated by intra-cranial route; i.p., inoculated by intra-peritoneal route. Molecular mass markers are in kilo Daltons.

compared with C57BL/6J mice, but all mice developed the typical clinical symptoms of scrapie. Although PrP^{Sc} levels in the brain were the same, there was a striking difference in the accumulation of PrP^{Sc} in spleen; PrP^{Sc} was not detected in the spleens of scrapie-affected *aly/aly* mice (Fig. 1). The severe combined immunodeficiency (SCID) mouse spleen did not support prion replication due to the lack of mature FDC, and SCID mice were found to be resistant to prion when low doses of prion were administered i.p. In contrast, SCID mice developed clinical symptoms with-

Table 1. Susceptibility of *aly/aly* mice to prion exposure via various routes.

Route	Concentration, amount of homogenate	Infectious dose ¹⁾ (LD ₅₀)	Period to terminal stage (days, mean \pm SD)	
			C57BL/6J (Attack rate)	<i>aly/aly</i> (Attack rate)
i.c.	10%, 20 μl	10 ⁶	165 \pm 5 (4/4)	159 \pm 8 (4/4)
i.p.	0.1%, 200 μl	10 ⁵	251 \pm 9 (6/6)	279 \pm 6 (4/4)
p.o.	10%, 100 μl	5 x 10 ⁶	307 \pm 7 (7/7)	>700 (0/5)

¹⁾Infectious doses were expressed as 50% lethal dose (LD₅₀).

out any PrP accumulation in the spleen when moderate doses of prion were administered i. p., although the incubation periods were longer than in wild-type mice.^{17,18)} This difference can be explained by direct spreading to the CNS from peripheral nerves. The prolonged incubation periods in *aly/aly* mice inoculated with prion via i.p route, without accumulation of PrP^{Sc} in spleen, could thus be explained by the same mechanism.

Obvious differences in susceptibility were observed when mice were challenged by p. o. route ; all of wild-type mice entered the terminal stage within 307 ± 7 days post infection (dpi) ; however, none of the *aly/aly* mice showed clinical symptoms and remained healthy throughout the experimental period (700days) . IHC analysis showed that PrP^{Sc} was present in the GATL of C57BL/6J mice at the terminal stage of the disease but was not present in that of *aly/aly* mice at the end of the experiment (Fig. 2). These results indicate that *aly/aly* mice are susceptible to prion replication in the CNS, but that neuroinvasion did not take place via oral consumption.

Prion infectivity in alimentary tract and spleen

In an effort to determine whether uptake and replication of prion occur in the alimentary tract of *aly/aly* mice, prion infectivity in the intestine was analyzed by bioassay. In the p.o. group, two mice from each mouse strain were sacrificed at 19, 43, and 83 dpi and tissue homogenates were inoculated i.c. into ICR mice for bioassay. Infectivity was detected in the intestines of C57BL/6J mice at each time point, however, no infectivity was detected in the intestines of *aly/aly* mice (Table 2) , suggesting that prion did not replicate in the intestine of *aly/aly* mice. Infectivity was not detected in the spleens of *aly/aly* mice inoculated p.o., while considerable amounts of in-

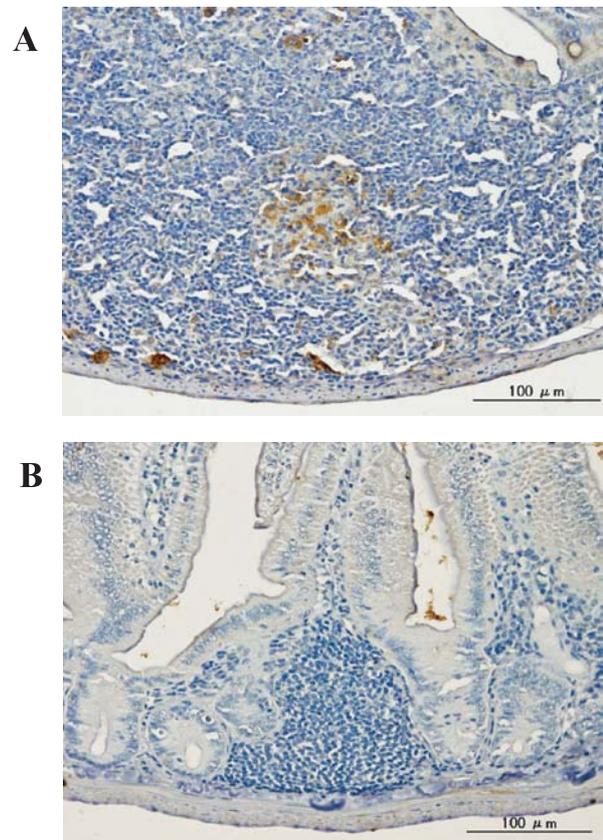


Fig. 2 . Immunohistochemical detection of PrP^{Sc} in the ileum.

The ilea of mice inoculated p.o. were examined for PrP^{Sc}. (A) C57BL/6J mice at 309 dpi. (B) *aly/aly* mice at 700 dpi.

fectivity were detected in the spleens of the corresponding C57BL/6J mice at 80 dpi. Furthermore, traces of infectivity were detected in the spleens of *aly/aly* mice, even after i.p. inoculation ; one of five mice manifested the terminal stage of the disease at 381 dpi (Table 2) and the brain of this mouse was positive for PrP^{Sc} (data not shown) , indicating that the lymphoid tissues of *aly/aly* mice did not support prion propagation.

Organization of ENS in aly/aly mice

Data described above suggested that GALT is essential as an initial entry port for prion infection, however, no information was available regarding the influence of NIK mu-

Table 2 . Prion infectivity in spleen and ileum.

Group	dpi ¹⁾	C57BL/6J		<i>aly/aly</i>	
		Spleen	Ileum	Spleen	Ileum
p.o.	19	NT ²⁾	246 ± 36 (6/6) ³⁾ 6.3 × 10 ³	NT	>300 (0/5)
	43	NT	220 ± 18 (4/4) 3.7 × 10 ⁴	NT	>425 (0/5)
	83	176 ± 4 (5/5) 8.9 × 10 ⁶	307 ± 21 (5/5) ⁴⁾ 6.2 × 10 ²	>450 (0/5)	>450 (0/5) ⁴⁾
i.p.	80	188 ± 31 (4/4) 2.0 × 10 ⁶	NT	>450 (0/4) 381 (1/5)	NT

¹⁾Spleens and/or ilea were collected from C57BL/6J or *aly/aly* mice at indicated days post infection.

²⁾Not tested.

³⁾Upper column shows incubation periods (days, mean ± SD) and attack rates (in parenthesis) of mice used for bioassay, while lower column shows estimated infectivity (LD₅₀/g tissue) from the incubation periods.

⁴⁾Ileum homogenates of the group were treated at 60°C for 30 min before inoculation to mice for bioassay.

tation on the organization of ENS. Therefore, we analyzed the organization of ENS by immunohistochemistry with protein gene product 9.5 as an ENS marker (Fig. 3). No obvious deficiency in organization of nerve fibers, or submucosal and intramuscular plexus was observed in *aly/aly* mice, supporting the idea that GALT is a primary target for prion entry via oral consumption.

Discussion

Although the pathway of invasion into CNS is well characterized,^{1,3-5)} it remains unclear how prion enter the host, particularly under natural circumstance. The major route of infection in naturally occurring prion diseases in animals is believed to be oral consumption of the infectious agent. Accumulating evidence suggests that the ENS and GALT are the primary target sites for prion entry and replication in peripheral tissues.^{1,3,4)} We therefore attempted to address the question of whether the ENS or GALT is important in the establishment of prion infection via the oral route. Our results demonstrated that GALT is essential for initial uptake of prion from the gut lumen to the alimentary tract. No differences were observed

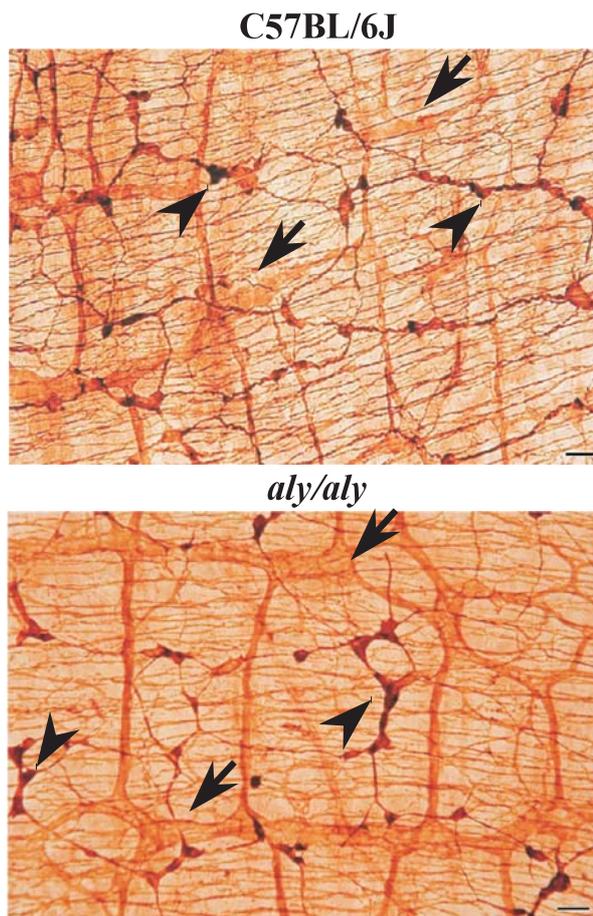


Fig. 3 . Organization of enteric nerve system. Extended intestine specimens were stained with anti-protein gene product 9.5 antibodies. Arrowheads indicate submucosal plexus, whereas arrows indicate intra-muscular plexus. Bar : 50 μm

in the organization of ENS between *aly/aly* and C57BL/6J mice, suggesting that prion adsorption via the epithelial cells of digestive tract and subsequent entrance into peripheral nerve fibers or blood stream is unlikely.

The follicle-associated epithelium (FAE) that covers the dome of Peyer's patches or submucosal lymphoid follicle contains villus and M cells. M cells act as major ports of entry for enteric pathogens via trans epithelial transport¹⁹⁾, and Heppner *et al.* reported that prion could be transported from the apical to the basolateral compartment in an *in vitro* model comprising epithelial cells morphologically and functionally resembling M cells.²⁰⁾ It was reported that functional B cells are required for the development of the FAE, Payer's patches and M cells.²¹⁾ B cells of *aly/aly* mice are functionally impaired so that *aly/aly* mice may have reduced M cell numbers and/or functionally impaired M cells in FAE. Mice deficient in B lymphocytes (μ MT mice), both B and T lymphocytes (RAG-1^{-/-} mice), or in tumor necrosis factor and lymphotoxin- α , in which the number of Peyer's patches is reduced, were resistant to oral prion challenge.²²⁾ In contrast, β 7 integrin-deficient mice, in which B cells in Peyer's patches are severely reduced, which also possess normal numbers of Peyer's patches, were sensitive to oral prion infection.²²⁾ The difference in prion susceptibility among those B cell-deficient mice following oral inoculation may be explained by the numbers of Peyer's patches and M cells.²²⁾ The *aly/aly* mice were highly resistant to oral prion infection, similar to RAG-1^{-/-} and μ MT mice, and thus it is of interest to determine whether the presence of M cells and M cell function in *aly/aly* mice are involved in the initial entry of prion into the gastro-intestinal tract.

One of the interesting questions is why ruminants appear sensitive to prion via oral

exposure. Anatomical and histological characteristics might explain the susceptibility of ruminants to prion. The ileal Peyer's patches are large organs in young lambs, extending for up to 2.5 cm, are estimated to contain over 100,000 follicles,²³⁾ and develop well in the distal ileum. FEA of cattle was reported to contain higher M cell or M cell-like populations than that of rodents.^{23,24)} These features suggest that the intestines of ruminant possess more ports of entry for prion than those of rodents. The scrapie susceptibility of sheep is thought to decline with growth.²⁵⁾ The involution of the ileal Peyer's patch at puberty and the accompanying drastic reduction in the number of follicles and FAE may contribute to the reduced susceptibility to prion that is observed in older animals.

PrP^{Sc} and prion infectivity can be readily detected in the lymphoreticular tissues of scrapie-infected sheep, in contrast, PrP^{Sc} and prion infectivity were scarcely detected in the lymphoreticular tissues of BSE-affected cattle.²⁶⁾ However, PrP^{Sc} has been detected in the ENS of BSE-affected cattle (Iwata *et al.*, submitted for publication). Thus efficient replication of BSE agent in peripheral lymphoreticular tissues may not be essential for the neuroinvasion of BSE agent after oral exposure. The *aly/aly* mice possess normal ENS organization but are resistant to prion infection via the p.o. route, suggesting that the ENS does not act as a port of entry for prion after oral exposure.

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