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Pathology

Species-barrier on the cross-species oral transmission of bovine AA amyloidosis in mice

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ABSTRACT. In AA amyloidosis, cross-species oral transmission has been demonstrated in several animal models. While it is known that the transmission efficiency of AA amyloidosis between different species is lower than that among the same species, the mechanism of this species-barrier is unclear. In this study, we found at first that mice orally given a large amount of bovine AA simultaneously with inflammatory stimulation did not develop AA amyloidosis. Therefore, we hypothesized that the low efficiency of the cross-species oral transmission of AA amyloidosis might be due to the low absorption rate in Peyer's patches. To evaluate the hypothesis, we next investigated whether bovine AA was taken up by Peyer's patches and translocated to other organs *in vivo* and *ex vivo* models. The direct absorption of bovine AA by Peyer's patches was not observed. Besides, translocation of bovine AA to the mesenteric lymph nodes, spleen, liver, or kidney was not observed except the mesenteric lymph node of a single mouse. Thus, absorption of bovine AA by Peyer's patches occurred much less efficiently in mouse models of cross-species oral transmission of AA amyloidosis. The present study suggests that the less efficient amyloid uptake by Peyer's patches may be involved in the species-barrier of oral transmission of AA amyloidosis.

KEY WORDS: AA amyloidosis, cross-species transmission, oral transmission, Peyer's patches

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Amyloidosis is a progressive disease caused by systemic or localized deposition of a misfolded fibrillary protein (amyloid) which is derived from a physiologically functional precursor protein. Human amyloidosis is classified into 36 types according to the type of precursor protein [1].

Amyloid A (AA) amyloidosis is a type of systemic amyloidosis found in a variety of animals including humans [10, 17]. Serum AA (SAA), the precursor protein of AA, is an acute phase protein synthesized in the liver in response to cytokines such as interleukin 6 [31]. AA fibril formation is triggered by supersaturation of SAA in serum associated with persistent inflammation. When chronic inflammation occurs due to inflammatory diseases such as rheumatoid arthritis, an accumulation of AA deposits occurs in tissues and causes AA amyloidosis. AA amyloidosis can be induced in a mouse model by continuous inflammatory stimulation [22]. In addition, the administration of a homologous or heterologous amyloid to mice under inflammatory conditions results in the development of amyloidosis in a dramatically shortened period. This phenomenon is termed the "transmission of AA amyloidosis". The administered amyloid functions as a fibril nucleus for amyloid formation/elongation and is described as an amyloid enhancing factor (AEF) [12]. AEFs induce AA amyloidosis following intravenous or intraperitoneal administration, as well as oral administration [20].

In recent years, various studies have revealed amyloid deposition in foods such as beef [35, 37] and *foie gras* [32]. In addition, it has been reported that the AEF activity is demonstrated in mice following oral administration of bovine AA (bAA) fibrils [7, 18, 37]. Therefore, cross-species transmission of AA amyloidosis via amyloid-contaminated commercial meat is a concern as a human health risk factor, so elucidation of the oral transmission mechanism is required for accurate risk analysis. However, there are few reports examining the mechanisms for the absorption of orally ingested AA amyloid or its dynamics in the body. Furthermore, while it is known that the transmission efficiency of AA amyloidosis between different species is lower than that among the same species [18, 28], the mechanism of this species-barrier is unclear.

Peyer's patches (PP) are lymphoid follicles located under the intestinal mucosa and contain specialized M cells responsible

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for transcytosis of antigens in the intestinal lumen to the subepithelial lymphoid tissue [16, 26]. Since PP takes up antigens non-specifically, they represent a major infection site for various pathogens including prions [13]. Orally administered prions are captured by M cells in the follicle-associated epithelium and taken up into the PP. Prions are then thought to initially accumulate in follicular dendritic cells and subsequently in the spleen and secondary lymphoid tissues, and ultimately migrating to the central nervous system [27, 34]. Our previous research has demonstrated that amyloid is initially observed in the spleen of animals that develop AA amyloidosis following oral amyloid administration [25]. Therefore, a lymphatic-mediated absorption mechanism similar to prion disease is suggested for AA amyloidosis; however, the details are currently unknown.

In this study, absorption of bAA through PP and subsequent pathological transmission were not observed in mouse models of cross-species oral transmission of AA amyloidosis. This study proposes that the cause of low efficiency in the cross-species oral transmission of AA amyloidosis may be due to the low absorption rate in PP.

MATERIALS AND METHODS

Animals

A total of six ddY mice (obtained from Tokyo Laboratory Animals Science Co., Ltd., Tokyo, Japan), twelve ICR mice (obtained from Japan SLC, Hamamatsu, Japan), and sixty C3H/HeN mice (Japan SLC) were used in the present study. Thirty 6-week-old female C3H/HeN mice were used in both Experiments 1 and 2. In Experiment 3, six retired female ddY mice and three 6-week-old female ICR mice were used. In Experiment 4, nine 6-week-old female ICR mice were used.

All mice were sorted into groups of 3 to 6 and acclimatized for several days in a conventional environment. Tap water in a bottle and solid feed (Lab Diet 5001, Japan SLC) were supplied without restriction. At the termination of the experiments, the mice were euthanized by exsanguination after deep anesthesia with 4% isoflurane (DS Pharma Animal Health Co., Ltd., Osaka, Japan). The research described herein was approved (31-45, R02-76, R02-77) by the Animal Care and Use Committee at Tokyo University of Agriculture and Technology (TUAT) and the research was performed according to the guidelines for animal experiments at TUAT.

Preparation of materials

bAA fibrils were extracted from the liver of a cow with AA amyloidosis using a water extraction method [29]. The extracted fibrils were positive for Congo Red (CR) staining (data not shown). The extracted fibrils were suspended in distilled water at 2.72 mg/ml, stored at −20°C until use, and sonicated just before administration. To prepare amyloid-laden liver homogenates, the same liver as used for amyloid extraction was diluted 5-fold with saline and homogenized. Similarly, amyloid-non-laden liver homogenates were prepared from the liver of an amyloidosis-free cattle. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis using the extracted bAA as a standard indicated that the amyloid-laden liver homogenates contained approximately 14 mg/g of bAA (data not shown). Each sample was stored at 4°C until use and sonicated just before administration.

Human-derived recombinant α-synuclein (α-syn) monomer solution was provided by Dr. Tsukakoshi (TUAT). The α-syn monomer solution was diluted to 2 mg/ml with PBS and the fibrils were prepared according to the method described previously [36]. Fibril formation was confirmed using the Thioflavin T assay and CR staining [6] (Supplementary Fig. 1). The fibrils were stored at 4°C until use and sonicated just before administration.

Experiment 1: Evaluation of AEF activity of bAA using an in vivo model

AEF activity of orally inoculated bAA was initially evaluated using an *in vivo* model. Thirty mice were sorted into three groups (A–C; n=10) and 100 mg of liver homogenate, with or without amyloid deposition, were administered to groups A and B, or group C, respectively. All mice received liver homogenates a total of 6 times (on every other day for 12 days). To examine the effect of the presence of lipopolysaccharide (LPS) in the intestinal tract on amyloid absorption [5], groups A and C were orally administered 10 mg/kg body weight (BW) LPS (O111: B4, Sigma-Aldrich Japan, Tokyo, Japan) with a feeding needle 30 min before each liver homogenate administration. After the last liver homogenate administration, LPS was subcutaneously administered at 5 mg/kg BW twice a week for 4 weeks. Mice were necropsied 4 days after the final LPS administration. At necropsy, PP, mesenteric lymph nodes (MLN), spleen, liver, and kidney were collected.

Experiment 2: Verification of bAA absorption using an in vivo model

A short-term *in vivo* experiment was conducted to evaluate bAA absorption in PP. Thirty mice were sorted into 10 groups (A-J; n=3) and treated with a single administration of 100 mg of liver homogenate with amyloid deposition. The effect of LPS on PP absorption [5] was determined by orally administering 10 mg/kg BW of LPS to the mice in groups A-E 30 min before administration of the liver homogenates. Mice were maintained for up to 24 hr after oral administration of LPS and were necropsied at various time points to collect PP tissues.

Experiment 3: Verification of amyloid absorption in PP using an ex vivo model

An *ex vivo* experiment was performed to determine the effects of exposing PP to high concentrations of bAA, and to compare the absorption of bAA with other materials. All mice were euthanized by deep anesthesia and the intestinal tracts were collected. Both ends of the PP-containing intestine were ligated approximately 5 mm before and after each PP. bAA fibrils (54 μg/20 μl) or α-syn fibrils (40 μg/20 μl) were inoculated into each intestinal lumen, with PBS used as an inoculation control. After inoculation, each intestine fragment was cultured in D-MEM / Ham's F-12 with L-Glutamine, Phenol Red, HEPES, and Sodium Pyruvate

(Wako, Osaka, Japan) at 37°C and sampled overtime for up to 6 hr.

Experiment 4: Evaluation of dynamics of bAA inoculated into PP

In vivo experiments were performed to examine the translocation of bAA after absorption by PP. Nine mice were assigned to three groups (A–C; n=3). A 1% solution of tissue marking dye (Thermo Fisher Scientific K.K., Tokyo, Japan) was added to the bAA fibrils solution to label the administration site. Laparotomy was performed on each mouse under inhalation anesthesia with isoflurane. The fibril solution was inoculated into one PP, and the peritoneum and epidermis were sutured. The control group was inoculated with PBS. Mice were bred for 1 day to 3 weeks and necropsied at various post-treatment time points. At necropsy, the PP, MLN, spleen, liver, and kidney tissues were collected.

Histological and immunohistochemical analysis

All collected tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. For histological examination, the tissue samples were cut into 3 µm and stained with hematoxylin and eosin (H&E) or CR. Rabbit anti-human α-syn polyclonal antibody (Bioss Antibodies, Woburn, MA, USA) or mouse anti-bovine SAA1 monoclonal antibody (Clone: 25BF12) was used for immunohistochemistry (IHC) [33]. Horseradish peroxidase-labeled anti-rabbit IgG antibody (Dako, Santa Clara, CA, USA) or horseradish peroxidase-labeled anti-mouse IgG antibody (Dako) was used as the secondary antibody. A diaminobenzidine-4HCl substrate kit (Liquid DAB + Substrate Chromogen System, Dako) was used to detect immunoreactions.

RESULTS

Experiment 1: Evaluation of AEF activity of bAA using an in vivo model

Histological analysis showed that amyloid deposition was observed in each one animal of groups A and group C (Table 1), suggesting that oral inoculation of amyloid-laden liver homogenate did not enhance the amyloid deposition. In a mouse of group A, amyloid deposits were observed only around the white pulp of the spleen, whereas in a mouse of group C, moderate to severe amyloid deposits were observed around the white pulp of the spleen, in the perisinusoidal space (space of Disse) of the liver, and the tubular stroma of the kidney. By immunohistochemistry, amyloid deposits were negative for bovine SAA1.

Experiment 2: Verification of bAA absorption using an in vivo model

Table 2 shows the results for each treatment group in Experiment 2. IHC analysis of bAA localization indicated that bAA was not detected in PP up to 24 hr post-administration, regardless of LPS pre-treatment. Also, no bAA localization was observed in the villi.

Experiment 3: Verification of amyloid absorption in PP using an ex vivo model

Table 3 shows the results for each treatment group in Experiment 3. IHC analysis of bAA localization indicated that bAA was not detected in PP at any of the time points examined (Fig. 1a). In contrast, α-syn localization was observed mainly in the PP follicles at 1, 2, and 4 hr post-inoculation (Fig. 1b). At 2 hr post-inoculation, α-syn localization in PP was also observed in the subepithelial dome. Non-specific reactions were not observed in PBS-treated negative control tissue. In all groups, necrosis, autolysis, and detachment of the follicle-associated epithelium were observed in the tissue at 4 to 6 hr post-inoculation.

Experiment 4: Evaluation of dynamics of bAA inoculated into PP

Table 4 shows the results for each treatment group in Experiment 4. IHC analysis showed that bAA localization was observed in the MLN of only a single animal in group A. Within the MLN, macrophages in the subcapsular sinus showed positive reactions for bAA (Fig. 2). In the other animals, bAA localization was not detected in any of the organs, including PP, over the study period (1 day to 3 weeks). Besides, the dye inoculated simultaneously with bAA had disappeared at the time of necropsy in some cases, and the inoculation site could not be confirmed macroscopically.

DISCUSSION

Oral transmission of bovine AA amyloidosis to mice has been demonstrated in several previous studies [7, 18, 37]. However, in this study, oral administration of bAA did not cause significant induction of AA amyloidosis with or without LPS-pretreatment. In Experiment 1, a total of 600 mg of liver homogenate was orally administered over a 12-day period, which contained at least 8.4 mg of bAA fibrils. In a previous report, Cui *et al*. induced amyloidosis by orally administering a total of 700 mg bAA-laden liver homogenate to mice for 28 days [7]. Although Cui *et al*. did not specify the amount of amyloid fibrils present in their homogenates, it is possible that an equivalent or a higher dose of amyloid fibrils was administered for a longer period of time than in the current study. It is thought that the induction of amyloidosis was not observed in the present study because the accumulation of bAA was insufficient to acquire or maintain AEF activity due to the low efficiency of oral absorption bAA in mice (between species).

Since oral transmission of AA amyloidosis could not be confirmed, the bAA absorption in PP was evaluated using *in vivo* and *ex vivo* experiments. In the *ex vivo* experiment using mouse intestine, bAA fibrils were exposed to follicle-associated epithelia of PP for up to 6 hr, however no fibril absorption was observed in the PP. In contrast, tissues exposed to α-syn resulted in positive reactions in dendritic cells in the subepithelial dome and follicular dendritic cells in the germinal center. These results indicate that M cells do not take up amyloid non-specifically, and it is possible that M cell uptake is a selective process. It is thought that AA amyloidosis and

Table 1. Amyloid enhancing factor activity of bovine amyloid A *in vivo* (Experiment 1)

Group	LPS-Pretreatment	Bovine liver homogenate	Mice with AA deposition after 6 weeks	Distribution of AA
A	+	AA laden liver	1/10	Spleen
B	-		0/10	-
C	+	AA non-laden liver	1/10	Spleen, Liver, Kidney

LPS: lipopolysaccharide, AA: amyloid A.

Table 2. Bovine amyloid A absorption in Peyer's patches using an *in vivo* model (Experiment 2)

Group	LPS-pretreatment	Time between pretreatment and sacrifice (hr)	PP with bAA in each group
A	+	2	0/3
B	+	4	0/3
C	+	8	0/3
D	+	12	0/3
E	+	24	0/3
F	-	2	0/3
G	-	4	0/3
H	-	8	0/3
I	-	12	0/3
J	-	24	0/3

LPS: lipopolysaccharide, PP: Peyer's patches, bAA: bovine AA.

Table 3. Amyloid absorption in Peyer's patches using an *ex vivo* model (Experiment 3)

Injection material	Given dose	Incubation period (hr)	PP with injection material in each group	Localization
bAA fibrils	54 µg/20 µl	0.5	0/3	-
		1	0/3	-
		2	0/3	-
		4	0/2	-
		6	0/2	-
Recombinant α-syn fibrils	40 µg/20 µl	0.5	0/3	-
		1	1/2	Follicle
		2	3/3	Follicle, SED
		4	3/3	Follicle
		6	0/1	-
PBS (negative control)	20 µl	6	NA	-

PP: Peyer's patches, bAA: bovine AA, α-syn: α-synuclein, SED: subepithelial dome, PBS: phosphate buffered saline.

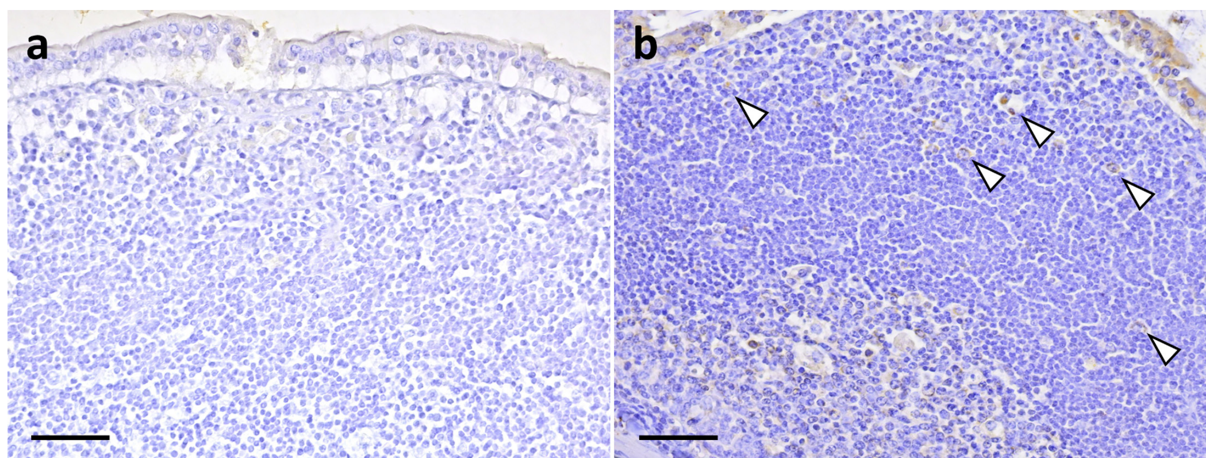

Fig. 1. Immunohistochemistry of Peyer's patches in Experiment 3. The images show immunohistochemistry performed with anti-bovine AA (bAA) antibody in group D (a), and anti-human α-synuclein (α-syn) antibody in group H (b). Cells in the subepithelial dome are positive for α-syn (arrowheads). Scale bars=50 µm.

Table 4. Dynamics of bovine amyloid A following inoculation of Peyer's patches (Experiment 4)

Group	Injection material	Given dose	Time point	Distribution of injected bAA				
				PP	MLN	Spleen	Liver	Kidney
A	bAA fibrils	27 µg	1 day	0/3	1/3	0/3	0/3	0/3
B	bAA fibrils	27 µg	3 days	0/3	0/3	0/3	0/3	0/3
C	bAA fibrils	27 µg	1 week	0/3	0/2	0/3	0/3	0/3

PP: Peyer's patches, MLN: mesenteric lymph nodes, bAA: bovine AA.

prion disease require recipient-derived amyloid precursor protein for replication of amyloid fibrils [20, 23]. During the replication of amyloid in cross-species transmission, difference in amino acid sequences between the donor and recipient constitutes a barrier to transmission [21]. As a result, the transmission efficiency of heterologous amyloid is lower than that of homologous amyloid, suggesting the existence of a so-called species-barrier [9, 24]. Moreover, no absorption of bAA was observed in PP in an *in vivo* experiment, indicating that the species-barrier may exist not only in the replication mechanism but also at the absorption stage.

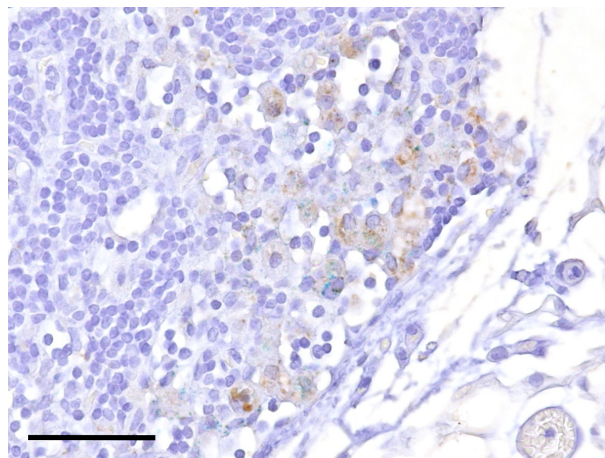

Fig. 2. Immunohistochemistry of mesenteric lymph nodes from a mouse in group A of Experiment 4. Several macrophages in the subcapsular sinus are positively stained for bAA. Scale bar=50 µm.

α-syn is a protein that forms abnormal aggregates in the neuronal cytoplasm and causes neurodegenerative diseases such as Parkinson's disease [14]. Recently, Braak proposed a hypothesis regarding the pathogenesis of sporadic Parkinson's disease. The hypothesis states that a non-specified neurotropic pathogen triggers the induction of a conformational change in α-syn. Moreover, the resultant pathology might initially occur in the enteric nervous system and subsequently retrogradely ascend to the central nervous system along neuronal pathways, leading to the Parkinson's disease pathology [2, 3]. More recently, it has been reported that inoculation of recombinant α-syn fibrils into the striatum [19], olfactory bulb [30], or gut [15] causes amplification of α-syn fibrils in the brain and induces α-synucleinopathy-like pathology in the mice, suggesting the occurrence of oral transmission of α-synucleinopathy. However, intestinal absorption of α-syn has not been demonstrated to date. In this study, we demonstrated α-syn absorption in PP using an *ex vivo* model. This result further supports the previous hypothesis that the onset of α-synucleinopathy can be initiated by oral exposure to α-syn.

In PP, particle size and polarity are involved in absorption capacity. For example, it is reported that India ink particles with a diameter of about 2 µm are easily absorbed in mouse PP and subsequently reach the spleen, while particles with a diameter of about 9 µm remain in the PP [8]. This study showed that α-syn, a misfolded protein similar to bAA, may be absorbed in the epithelium of PP and is retained by dendritic cells in follicles, similar to what is observed with prions. α-syn is a protein composed of 140 amino acids that normally exists in an unfolded state and consists of an N-terminal amphipathic region, a C-terminal acidic region, and an intermediate hydrophobic region [4]. Previously, it was reported that hydrophobic particles are more easily absorbed in PP than hydrophilic particles [11]. Since bAA fibrils were not absorbed in PP, it is suggested that α-syn possess structural advantages that facilitate absorption in PP relative to bAA. Further research is required to elucidate the PP absorption mechanism.

It is reported that exposure of PP to LPS increases the absorption capacity of PP in the intestinal lumen [5]. However, absorption of bAA was not observed in this study either with or without LPS-pretreatment. As mentioned above, it is possible that bAA is not easily absorbed. To verify the effect of LPS on oral transmission of AA amyloidosis, it is likely to be better to use homologous amyloids.

In the last experiment, the post-absorption migration pathway of bAA fibrils to various organs over time was evaluated following direct inoculation of bAA fibrils to the PP of mice. It was found that localization of bAA in the subcapsular sinus was observed one day after inoculation. The PP contains efferent lymph vessels that carry lymphocytes to the mesenteric lymph nodes [13]. As mentioned above, macrophages that have migrated from the parenchyma to the lymph nodes show phagocytic activity against foreign substances that have invaded the lymphatic sinus via the lymph vessels. In this study, the transfer of bAA from PP to MLN was confirmed. Therefore, it is suggested that if bAA is absorbed by PP, it may translocate to other tissues via the lymphatic tract.

Elucidation of the absorption mechanism is indispensable for assessing the risk of dietary amyloid to human health. In this study, we provide evidence to support the hypothesis that cross-species transmission of amyloidosis, other than prion diseases, occurs through the lymphatic system by demonstrating the absorption of α-syn and the translocation of bAA from PP to MLN. However, it should be noted that this study did not demonstrate direct absorption of bAA in PP with subsequent the pathological transmission, indicating the potential presence of a species-barrier that mediates selective absorption in PP. Various previous studies on the cross-species transmission of bovine AA amyloidosis have shown that bAA in beef can be a causative agent of human disease [35, 37]. Given that the amount of amyloid contained in diet is negligible, and that very large doses are required for cross-species transmission due to the species-barrier, the risk of AA amyloid in commercial meat does not appear to be highly problematic. However, further analysis of the mechanism of oral amyloid transmission is required to accurately evaluate risk.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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