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NOTE

Pathology

Oxazolone-induced gastrointestinal disorders enhance the oral transmission of AA amyloidosis in mice

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ABSTRACT. Amyloid A (AA) amyloidosis is a lethal disease characterized by systemic AA amyloid deposition, and is reported in many animal species. Despite experiments have shown that AA amyloidosis can be transmitted orally, horizontal transmission and cross-species transmission are concerns, the transmission mechanism has been unknown. In this study, we examined the oral transmission efficiency of AA amyloidosis using oxazolone-induced gastrointestinal disorder mice. As a result, the upper or lower gastrointestinal disorder groups developed more severe amyloid deposition in systemic tissues than the group without gastrointestinal disorders. The results of this study suggest that gastrointestinal damage promotes the oral transmission of AA amyloidosis.

KEY WORDS: AA amyloidosis, gastrointestinal disorder, mouse, oral transmission, oxazolone

Amyloidosis is a progressive and intractable disease caused by systemic or localized amyloid deposition. Amyloid A (AA) amyloidosis is a systemic amyloidosis that has been reported as a fatal disease in various animal species [7, 16]. AA amyloidosis is secondary to chronic inflammatory diseases, such as rheumatoid arthritis [15], but the detailed pathogenic mechanism has not yet been elucidated. Experimentally, AA amyloidosis can be induced by the long-term administration of inflammatory stimuli, such as silver nitrate and casein [19]. In addition, the inoculation of splenic extracts derived from AA amyloidosis-affected donor animals to other recipient animals with inflammation leads to the rapid development of AA amyloidosis. This phenomenon is known as "transmission of AA amyloidosis", and the inoculated extract is called "amyloid-enhancing factors" (AEF) [14]. Amyloid fibrils in the AEF are thought to act as seeds for amyloid deposition in the recipient animals [12]. Since the oral transmission of AA amyloidosis has been demonstrated experimentally [13], there are concerns about horizontal transmission among animals or cross-species transmission from animals to humans similar to prion diseases. Despite these concerns, the mechanism of the oral transmission of AA amyloidosis has not yet been elucidated due to the lack of appropriate animal models that are useful for pathological analyses. The transmission efficiency of amyloid by oral administration is much lower than that by intravenous or intraperitoneal administration. As such, an oral transmission model requires long-term administration or a large amount of AEF, leading to low reproducibility between experiments [10, 11].

In prion diseases, oral exposure is involved in the transmissible pathogenesis [5, 8]. During the transmission of prion diseases, abnormal prion proteins are absorbed and amplified in Peyer's patches, and this pathway is thought to play an important role in the pathogenesis of prion diseases [3]. Recently, it has been reported that inflammation in the intestinal tract enhances the absorption of abnormal prion proteins and exacerbates the disease pathology [18]. In this study, we hypothesized that AA amyloidosis involves an oral transmission mechanism similar to that of prion diseases. Therefore, oral transmission experiments were carried out using mice with experimentally induced upper or lower gastrointestinal disorders.

In all experiments, 6-week-old female C57BL/6J mice (Japan SLC, Hamamatsu, Japan) were used. All mice were bred under conventional conditions with *ad libitum* access to food and water. The research protocols were approved by the Animal Care and Use Committee at the Tokyo University of Agriculture and Technology (Approved No. 31-47), and the research was performed according to the guidelines for animal experiments at the university.

4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one (OXA; Sigma-Aldrich, St. Louis, MO, USA) was used to induce enteritis. As an AEF, amyloid fibrils were extracted from the spleen and liver of AA amyloidosis-affected mice using Pras' method [17]. Lipopolysaccharide (LPS; O111: B4, Sigma-Aldrich) was used as an inflammatory stimulus.

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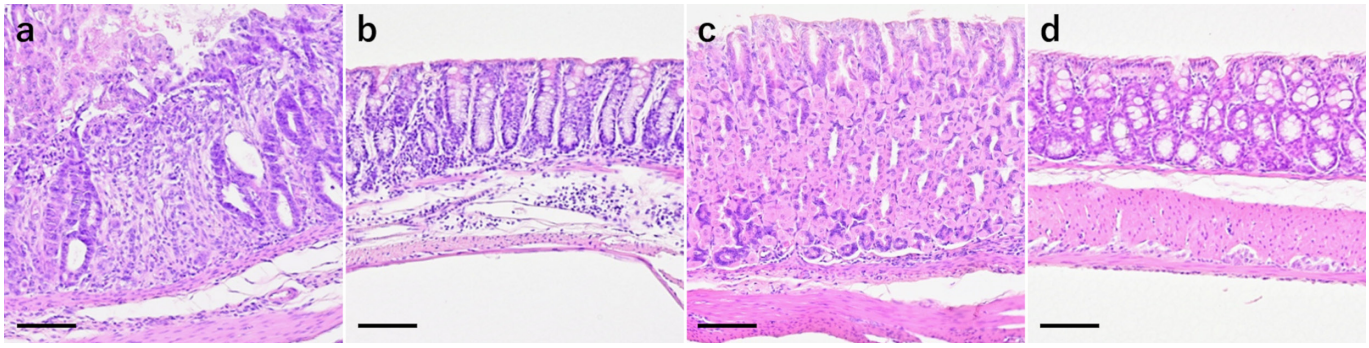

Fig. 1. Histological features of the gastrointestinal tract. a: Stomach tissue from the orally oxazolone (OXA)-treated group in experiment 1. Transmural inflammatory cell infiltration and erosion are observed. b: Colon tissue from the intracolonically OXA-treated group in experiment 1. Infiltration of inflammatory cells is observed in the lamina propria and submucosa. c: Stomach tissue from the orally OXA-treated group in experiment 2. d: Colon tissue from the intracolonically OXA-treated group in experiment 2. Minute inflammation was observed (c, d). Hematoxylin and eosin stain. Bars=100 µm.

At first, an OXA-induced upper or lower gastrointestinal disorder model was developed (Experiment 1). The following procedures were used with reference to previous research [21]. Three mice were allocated into each experimental group, i.e., the orally OXA-treated group and the intracolonically OXA-treated group. On day 0, the dorsal skin of the mice was shaved, and 150 µl of 3% (w/v) oxazolone diluted with an equal amount of acetone and olive oil was dropped onto the shaved area. On day 7, 100 µl of 1% (w/v) oxazolone diluted with 50% ethanol was administered orally or intracolonically to the mice under anesthesia. Necropsy was performed on day 10. From day 0 to day 7, there was no weight loss in either group after the initial exposure to OXA. Then, both groups showed a slight weight loss after the second exposure to OXA (day 7 to day 9), but the body weights had begun to recover at necropsy (day 10) in both groups. Throughout the experiment period, there was no significant change in body weight among the groups (Supplementary Fig. 1a). At necropsy, mice were euthanized by exsanguination under deep anesthesia with 4% isoflurane, and tissue samples of the stomach, duodenum, and colon were collected. Histologically, typical oxazolone-induced allergic inflammation was observed in both groups. In the oral administration group, erosion and severe transmural inflammatory cell infiltration with neutrophils were observed in the stomach (Fig. 1a). In the intracolonic administration group, mild erosion and moderate inflammatory cell infiltration was confirmed (Fig. 1b). For the quantification of gastrointestinal inflammation, hematoxylin and eosin-stained upper and lower gastrointestinal tissues were evaluated on a 7-point scale based on the scoring criterion of chemically induced enteritis proposed by Erben *et al.* [4]. The mean inflammation scores of the oral and intracolonic groups were 5 and 4, respectively (Supplementary Table 1). In the oral administration group, inflammation was limited to the stomach, and was not observed in the lower intestine.

Next, we examined the oral transmission of AA amyloidosis using the oxazolone-induced gastrointestinal disorder mice model developed above (Experiment 2). Thirty-seven mice were allocated into groups A to F (Supplementary Table 1). From day 0 to day 10, upper gastrointestinal disorders were induced in groups C and D, and lower gastrointestinal disorders were induced in groups E and F using the same procedures as in experiment 1. On day 10, mice in groups B, D, and F were orally inoculated with 30 µg/g body weight of amyloid fibrils. All mice were inoculated with 2 mg/kg body weight of LPS intraperitoneally twice per week from day 10 to day 38, and necropsied on day 41. In groups C to F, which were treated with oxazolone, the body weight change from day 0 to day 10 was similar to that in experiment 1 (Supplementary Fig. 1b). After the administration of LPS (Groups A, C, and E) or LPS and AEF (Groups B, D, and F) on day 10, rapid weight loss was observed in all groups. From day 12 to day 14, the body weight began to recover in all groups, and on day 17, it had recovered to the same level as on day 10. After day 10, there were no significant differences in body weight among the groups. At necropsy, mice were euthanized by exsanguination after deep anesthesia with 4% isoflurane, and the liver, spleen, kidney, heart, lung, stomach, duodenum, and colon were collected. Histologically, in the orally OXA-treated groups (groups C and D), inflammatory cell infiltration was very mild, and no mucosal damage was observed (Fig. 1c). In the intracolonically OXA-treated groups (groups E and F), mild inflammatory cell infiltration was observed in the lamina propria (Fig. 1d). The scores in both the orally OXA-treated groups (groups C and D) and the intracolonically OXA-treated groups (groups E and F) were significantly lower than those in the groups in experiment 1 (Fig. 2), and comparable to those in the OXA-untreated group (group B). These results indicate that the gastrointestinal inflammation in both groups had recovered to the normal level by the time of necropsy.

Amyloid deposition was determined by polarization microscopy of Congo red-stained specimens. The degree of amyloid deposition in each tissue was scored as follows: score 0, no deposition; 1, mild deposition only in the vessel walls; 2, mild deposition in the vessel walls and interstitial tissues; 3, moderate deposition in the vessel walls and interstitial tissues; and 4, severe deposition in the vessel walls and interstitial tissues. No amyloid depositions were observed in any tissues in groups A, C, and E, which did not receive AEF (Fig. 3). In group B, moderate amyloid deposition that was limited to the spleen was observed in one case. In contrast, in four cases in each of groups D and F, mild-to-severe amyloid deposits were observed in various organs, although mainly in the spleen (Fig. 4a). The average score of each tissue in individual mice was calculated as the

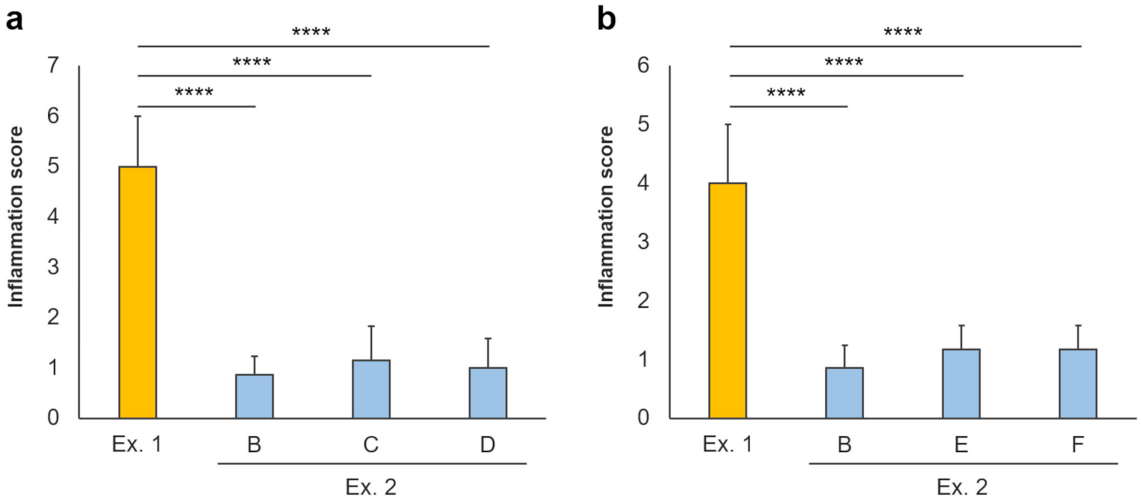

Fig. 2. Comparison of the mean inflammation scores for each group. A: Scores of the stomach in the orally OXA-treated groups in experiments 1 and 2. B: Scores of the colon in the intracolonically OXA-treated groups in experiments 1 and 2. In both comparisons, groups in experiment 2 showed a significant decrease in the inflammation score when compared to groups in experiment 1. Among the groups in experiment 2, there was no significant increase in the scores of the OXA-treated groups (C to F) when compared to the OXA-untreated group (B). Ex. 1, experiment 1; Ex. 2, experiment 2. The error bar indicates the standard deviation. ****P<0.0001 vs. Ex. 1; Tukey's test.

Group	A				B							C							D							E						F					
AEF injection	-				+							-							+							-						+					
Oxazolone injection	-				-							Oral							Oral							Intracolonic						Intracolonic					
No.	1	2	3	4	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	1	2	3	4	5	6
Liver																																					
Spleen																																					
Kidney																																					
Heart																																					
Lung																																					
Stomach																																					
Duodenum																																					
Colon																																					ND

Fig. 3. Distribution and degree of amyloid deposition in groups A to F. The severity of amyloid deposition is represented as: 0, white; 1, yellow; 2, orange-yellow; 3, orange-red; 4, red; and ND, no data.

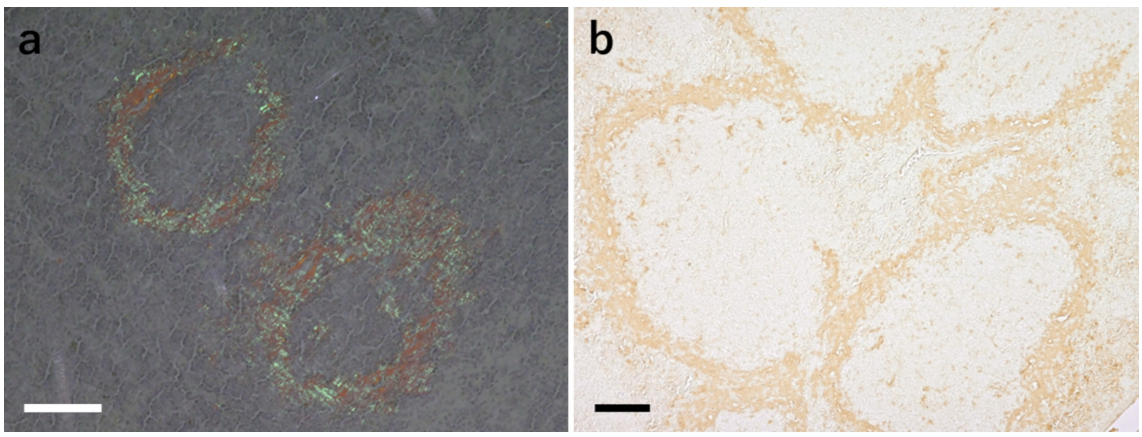

Fig. 4. Histological and immunohistochemical features of splenic amyloid deposition. a: In the spleen, amyloid deposition was observed around white palp. No. 1 in group D. Hematoxylin and eosin stain. b: Amyloid deposits were positive for AA. No. 1 in group D. Immunohistochemistry. Bars=100 µm.

amyloid-index (AI) score. The mean AI score of group D was significantly higher than that of group B (Supplementary Fig. 2). By immunohistochemistry (IHC) with anti-mouse serum AA polyclonal antibody (Cloud-Clone Corp., Houston, TX, USA) as the primary antibody, the amyloid deposits were positive for amyloid A, and they were diagnosed as AA amyloidosis (Fig. 4b).

In this study, the groups with OXA-induced gastrointestinal disorders developed a more severe AA amyloidosis pathology than the group without gastrointestinal disorders. In general, oxazolone-induced colitis is characterized by acute inflammation that occurs 3 to 4 days after OXA treatment and subsequent rapid recovery [6]. In this study as well, the body weight changes and pathological findings suggest that the inflammatory symptoms improved rapidly in both the orally and intracolonically OXA-treated groups. Therefore, it is unlikely that inflammatory stimulation in the intestinal tract is a direct etiology of amyloidosis. Regarding prion diseases, sensitivity to ingested abnormal prion proteins was increased by experimental bacterial enteritis in a mouse model [18]. In the mouse model, it was suggested that the enhancement of the antigen-uptake capacity of M cells and mononuclear phagocytes in Peyer's patches, which are associated with inflammatory reactions, affects the uptake and amplification of abnormal prion proteins [3]. Although further analyses will be required, this study also suggests that the activation of gastrointestinal immunity associated with damage to the mucosal barrier and inflammation may have increased the uptake of amyloid in the intestinal tract, leading to systemic amyloid deposition. However, it should also be noted that not only enteritis, but also the presence of minor oral wounds has been reported to be involved in prion disease pathology [2]. In this study, the OXA-treated mice had developed mucosal erosions in the early stages, so it is also necessary to consider the possibility that amyloid directly invaded the bloodstream.

In this study, there was no clear difference in tissue distribution of amyloid deposition between the orally and intracolonically OXA-treated mice. In the oral transmission of amyloidosis, the initial site of amyloid deposition is the spleen, which subsequently spreads throughout the body [11, 13]. In this study as well, the more severe deposition was observed in the spleen, supporting that amyloid absorbed in the gastrointestinal tract propagates to the spleen rather than being amplified at the absorption site.

Although a number of risk factors, such as enteritis or oral wounds described above, have been reported to be involved in prion disease pathology, little is understood about the factors that influence oral transmission of AA amyloidosis [12]. In the oral transmission of AA amyloidosis, large doses of AEF are required for inducing amyloid deposition [11], and even higher doses are required for cross-species transmission [1, 9]. While there have been several reports of AA deposition in foods [20], the amount of amyloid contained in these foods was very small, and it seems unlikely that the large amount required for cross-species transmission would be ingested by humans [12]. However, the results of this study suggest the possibility that gastrointestinal tract disorders may enhance the oral transmission of AA amyloidosis even at the low doses of amyloid fibrils found in foods.

In conclusion, this study revealed that experimental upper and lower gastrointestinal disorders enhanced the oral transmission of AA amyloidosis in mice. Further study is needed to elucidate the pathogenic mechanisms of oral transmission of AA amyloidosis.

CONFLICTS OF INTEREST. The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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