Fecal transmission of AA amyloidosis in the cheetah contributes to high incidence of disease

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AA amyloidosis is one of the principal causes of morbidity and mortality in captive cheetahs (Acinonyx jubatus), which are in danger of extinction, but little is known about the underlying mechanisms. Given the transmissible characteristics of AA amyloidosis, transmission between captive cheetahs may be a possible mechanism involved in the high incidence of AA amyloidosis. In this study of animals with AA amyloidosis, we found that cheetah feces contained AA amyloid fibrils that were different from those of the liver with regard to molecular weight and shape and had greater transmissibility. The infectious activity of fecal AA amyloid fibrils was reduced or abolished by the protein denaturants 6 M guanidine-HCl and formic acid or by AA immunodepletion. Thus, we propose that feces are a vehicle of transmission that may accelerate AA amyloidosis in captive cheetah populations. These results provide a pathogenesis for AA amyloidosis and suggest possible measures for rescuing cheetahs from extinction.

Results

Cheetah AA Amyloid Fibril Proteins and Specific Antiserum Against Them. Until now, specific anti-cheetah AA antiserum has not been produced. To produce anti-cheetah AA antiserum, we isolated AA amyloid fibril fraction from the liver of Cheetah 68 (10, 11) or synthetic amyloid-like fibrils (12, 13), providing further evidence for transmissibility.

The cheetah species (Acinonyx jubatus) is in danger of extinction and is included on The World Conservation Union list of vulnerable species. Although efforts have been made in wildlife sanctuary parks and zoos worldwide to prevent extinction, a steady increase in the size of the cheetah population is hampered by the high prevalence of certain diseases in captive cheetahs. In particular, systemic AA amyloidosis is regarded as an increasingly important cause of morbidity and mortality in captive cheetahs as prevalence increased from 20% in pre-1990 reported necropsies to an unusual 70% of necropsied cheetahs in 1995 (14). Despite much effort, the pathogenesis for AA amyloidosis in cheetahs is still only partially understood. Inflammatory diseases, especially chronic lymphoplasmacytic gastritis, found in 100% of cheetahs with AA amyloidosis (14), and genetic homogeneity have been considered as causes for the increased incidence of AA amyloidosis (15). However, environmental epidemiological studies indicate that breeding conditions have a prominent effect on the incidence of AA amyloidosis. A high rearing density is always associated with early age of onset, and with the high incidence and severity of AA amyloidosis, findings similar to sheep scrapie and cervid CWD. Thus, sustained epidemics of sheep scrapie and cervid CWD appear to be principally due to horizontal (animal to animal) transmission, although the routes of natural transmission remain to be clarified (16, 17). The propagation of AA amyloidosis among captive cheetah populations may also depend on a horizontal transmission pathway. Identification of the mode of transmission is a prerequisite for disease control.

In this study, we show that the feces from a cheetah with AA amyloidosis can act as a possible transmission origin to accelerate the transmission of AA amyloidosis.
Fig. 1. Cheetah AA amyloid fibril proteins and specific antiserum. (a) The isolated AA amyloid fibril fractions from the livers of C68 and C66 (20 μg) were applied to 16.5% Tris glycine SDS/PAGE gel followed by CBB staining. Lane 1, murine AA amyloid fibril fractions from the liver of C68 (lane 1), human (lane 2), mouse (lane 3), cheetah, and murine AApoAII(C) amyloid fibril fraction (lane 5) were used as molecular weight markers. Molecular masses of two main and two faint bands corresponded to the AA monomer. (b) Amino acid sequencing from four bands in C68 sample. AA amyloid fibril fragments with different lengths were obtained. The amyloid fibril fraction from C68 was used as antigen to produce specific anti-cheetah AA antiserum. (c) Amyloid deposition in the liver of C68 was detected in Congo red-stained sections by standard microscopy (Left), by green birefringence under polarized microscopy (Center), and by immunohistochemical staining (Right). The anti-cheetah AA antiserum was used as primary antibody (dilution 1:1,000). (Magnification, ×200.) (d) AA amyloid fibril fractions from the liver of C68 (lane 1), human (lane 2), mouse (lane 3), cow (lane 4), and murine AApoAII(C) amyloid fibril fraction (lane 5) were used (12 μg of protein per well) to perform Western blot analysis with anti-cheetah AA antiserum as primary antibody (dilution 1:3,000).

(C68) with severe AA amyloidosis. When the isolated fibril fraction was separated with 16.5% SDS/PAGE, two major bands with molecular masses of ~9.0 kDa and two faint bands were seen by Coomassie Brilliant Blue (CBB) staining (Fig. 1a). Based on their molecular masses, the four bands were determined most likely to be the monomeric forms of AA amyloid fibril protein. The four bands were cut and subjected to amino acid sequencing (Fig. 1b). This revealed that the sequences of the four bands were consistent with cheetah AA sequence determined in ref. 18. It also revealed that the isolated amyloid fibril fraction contained several cheetah AA amyloid fragments with different lengths. The longest fragment was the 93-amino acid-long fragment, and N-terminal and/or C-terminal deleted fragments also existed (Fig. 1b). In addition to C68, we also isolated the AA amyloid fibril fraction from the liver of C66 with severe AA amyloidosis by sequencing. The result was identical to the result from C68 (Fig. 1a; sequence data not shown).

We used the isolated fibril fraction from C68 to produce antiserum against cheetah AA protein. To evaluate the specificity of anti-cheetah AA antiserum, we performed immunohistochemical staining of liver specimens that had been identified with severe amyloid deposition by Congo red staining. The amyloid deposition was stained positively with anti- cheetah AA antiserum (Fig. 1c). In addition, Western blot analysis was also performed by using the de novo antiserum as primary antibody (Fig. 1d). The anti- cheetah AA antiserum recognized all forms of cheetah AA proteins, including the monomer, dimer, and oligomer. It also reacted with human AA protein, because three dominant bands corresponding to the monomer, dimer, and trimer of human AA protein were recognized. All forms of cheetah AA protein (monomer, dimer, and oligomer) had relatively larger molecular mass compared to human AA protein. This was due to an eight-amino acid insertion in the C-terminal part (from amino acids 69–76) of cheetah AA protein (18). In contrast, the antiserum did not recognize murine or bovine AA proteins [Fig. 1d and supporting information (SI) Fig. S1]. There was also no immunoreaction with AApoAII protein, the amyloid protein of AApooAII amyloidosis that is another systemic amyloidosis occurring in mice.

Existence of Distinctive AA Amyloid Fibrils in the Feces of a Cheetah with Amyloidosis. We isolated the amyloid fibril fraction from the feces of C68 and performed Western blot analysis with anti- cheetah AA antiserum. A clear single immunoreactive band (presumptive monomer) at a 7.0 kDa molecular mass was seen in fecal samples (Fig. 2a). As a control, we also isolated the amyloid fibril fraction from the liver of C68, and several bands corresponding to the monomer, dimer, and oligomers were seen by Western blot analysis. Unexpectedly, we found that the molecular mass of the fecal AA amyloid protein monomer was ~2.0 kDa smaller than that from the liver (Fig. 2a). In addition to C68, we also examined three fecal samples from C67, C78, and C90. The same results were obtained; a single band with a molecular mass of 7.0 kDa was present in all fecal samples (data not shown). The difference in molecular mass was confirmed by amino acid sequencing, indicating the deletion of both N- and C-terminal sequences from the fecal AA amyloid protein compared to the liver AA amyloid protein (Fig. 2b). Transmission electron microscopy of the negatively stained liver amyloid sample revealed amyloid-characteristic straight and unbranched fibril images. However, the fibril image from feces was different from the liver exhibiting much thinner and smaller fibrils, implicating a divergent ultrastructure between the two kinds of fibrils (Fig. 2c). To exclude the possibility that soluble serum precursor SAA was excreted into the feces and then formed amyloid fibrils during the isolation process, we also examined the fecal sample from TT-253, a cheetah that had very minor AA...
microscopy (detected by green birefringence in Congo red-stained sections under polarized fibrils (C and D) (grade 1) or liver fibrils (I and J) (grade 3) and to formic acid treated fecal fibrils (A and B) (grade 4) or untreated liver fibrils (G and H) (grade 3).

antiserum (to induce AA amyloidosis. Equal quantities of amyloid fractions (100 µg) and liver (C68) AA amyloid fibril fractions were untreated (N), or mouse experimental AA amyloidosis.

AA amyloid proteins might be involved in the propagation of AA amyloidosis and further characterized its transmissibility with AA amyloid fibrils in feces had a higher transmissibility than that in liver. No amyloid deposition in spleens of mice induced by fecal amyloid fibril fraction (100 µg) was determined in Congo red-stained tissue sections (four mice per group). (b) The degree of AA amyloid deposition in induced mice was determined by isolation of AA amyloid fibril fractions from the spleens of mice in each group (filled squares, fecal; open diamonds, liver) followed by Western blot analysis (20 µg of protein per well) and quantification using National Institutes of Health Images. The means and SE were determined by the relative ratios of AA amyloid protein levels in each group versus the group receiving 10 µg of amyloid fibrils fraction from the feces (*, P < 0.05; **, P < 0.01).

Fig. 3. High transmissibility of feces from the cheetah with amyloidosis. (a) Fecal (C68 and C67) and liver (C68) AA amyloid fibril fractions were untreated (N), or treated by guanidine-hydrochloride (G) or formic acid (F) and injected into mice to induce AA amyloidosis. Equal quantities of amyloid fractions (100 µg) were used in each experiment. The degree of amyloidosis was determined by the amyloid deposition observed in Congo red-stained sections of the spleen (*, P < 0.05; **, P < 0.01). (b) AA deposition in the spleens of mice exposed to untreated fecal fibrils (A and B) (grade 4) or untreated liver fibrils (G and H) (grade 3). Deposition observed in mice exposed to guanidine-hydrochloride treated fecal fibrils (C and D) (grade 1) or liver fibrils (I and J) (grade 3) and to formic acid treated fecal fibrils (E and F) (grade 0) or liver fibrils (K and L) (grade 1). Deposition was detected by green birefringence in Congo red-stained sections under polarized microcopy (upper) and immunohistochemical staining with anti-mouse AA antiserum (lower). (Magnification, × 200.)

The amyloid-inducing activity of the fecal fraction was disrupted by both 6 M guanidine-HCl and formic acid denaturation as most injected mice did not develop amyloidosis, and only a few had very minor degrees of deposition. With regard to the liver fraction, formic acid treatment caused a nearly complete loss of amyloid-inducing activity, whereas the guanidine-HCl treated fraction retained high amyloid-inducing activity (Fig. 3a). The amyloid depositions that stained positively with Congo red were identified as AA amyloid deposition by immunohistochemical staining with anti-mouse AA antiserum (Fig. 3b). These results suggested that the amyloid fraction isolated from the feces might possess greater amyloid-inducing activity.

Quantitation of the Transmissibility of AA Amyloid Fibrils from the Feces. The amyloid fibril fraction from the feces was serially diluted in distilled water (DW) and injected into mice at doses of 1 µg, 10 µg, and 100 µg to induce AA amyloidosis. The degree of amyloid deposition, graded with Congo red staining, increased in a dose-dependent manner (Fig. 4a). We also induced the disease in mice by injection of 10 µg of the liver amyloid fibril fraction. We found that these mice had lower amyloid deposition compared to those receiving 10 µg of fecal fibrils.

In addition to Congo red staining, the degree of amyloid deposition was evaluated by Western blot analysis, using equal quantities of the AA amyloid fibril fractions isolated from spleens of induced mice in each group, followed by AA protein quantification, using National Institutes of Health image software (Fig. 4b). This showed that the intensity of the bands stained positively with anti-mouse AA antiserum increased proportionately to the logarithm of the AA amyloid fibril fraction dosage (1–100 µg). In mice injected with 10 µg of liver amyloid fibril fraction, the intensity of bands was appreciably lower than from mice injected with 10 µg of fecal amyloid fibril fraction and was only similar in degree to mice injected with 1 µg of fecal matter. These results confirmed that AA amyloid fibrils in feces had a higher transmissibility than that in liver. No samples isolated from mouse spleens reacted with anti-cheetah AA antiserum, which demonstrated that the amyloid deposition observed in the mouse tissue was derived from mouse AA fibrils but not the cheetah fibrils in the inocula (Fig. S2).

The Requisite of Fecal AA Amyloid Protein for Transmission. To establish whether AA amyloid protein contained in the fecal amyloid fibril fraction was a requisite for the amyloid-inducing activity, the amyloid fraction was immunodepleted with anti-
group induced by the untreated sample (National Institutes of Health Image. The means and SE were determined by the analyzed (20 exposed to UT, ID-IgG, or ID-AA for five cycles of immunodepletion. AA red-stained sections. (AA amyloid protein levels in the spleens from mice (Fig. 5 amyloid-inducing activity because of the presence of AA amyloid fibril protein. The reliability of the amyloid grade was confirmed by stained sections. (c) AA amyloid protein levels in the spleens from mice exposed to UT, ID-IgG, or ID-AA for five cycles of immunodepletion. AA amyloid proteins were isolated from each group mice, and Western blots were analyzed (20 μg of protein per well) followed by quantitative analysis using National Institutes of Health Image. The means and SE were determined by the relative ratios of AA amyloid protein levels for each induced group versus the group induced by the untreated sample (*, P < 0.05; **, P < 0.01). cheetah-AA antiserum. Control experiments showed that five cycles of immunodepletion were required to remove AA proteins from the fecal fraction (Fig. 5a). Thus, fecal amyloid proteins were depleted five times with either anti-heetah AA antiserum or normal rabbit IgG and were subsequently used to induce AA amyloidosis in mice. As shown in Fig. 5b, immunodepletion with anti-heetah-AA antiserum completely prevented the amyloid-inducing activity of the fecal AA amyloid fraction, because no induced mice suffered from amyloidosis. In contrast, five cycles of immunodepletion with normal rabbit IgG antibody only partially decreased the amyloid-inducing activity, which was associated with only a partial loss of AA amyloid protein. The reliability of the amyloid grade was confirmed by Western blot analysis of the amyloid fractions isolated from the spleens of mice (Fig. 5c). These results show that cheetahs suffering from AA amyloidosis pass fecal matter that possesses amyloid-inducing activity because of the presence of AA amyloid fibril protein.

Discussion
It is currently accepted that systemic AA amyloidosis is an increasingly important cause of morbidity and mortality in captive cheetah populations (14). For conservation of this species, therefore, it is critical to elucidate the etiology of AA amyloidosis. As with sheep scrapie and cervid CWD, the routes of transmission are among the most debated and intriguing issues. Infectious CWD prions in saliva have been identified to be involved in transmission in high-density captive situations (19, 20). Recently, available evidence indicates that an environmental reservoir of infectivity contributes to the continuation of these diseases in affected populations. These infectious agents can be transmitted by flesh flies (21) or hay mites (22) and can directly enter the environment from decomposing carcasses of infected animals (23). Environmental contamination by excreta from infected cervids has also seemed the most plausible explanation for the dissemination of CWD (24). Scrapie-infected hamsters and Creutzfeldt-Jakob disease (CJD) patients were reported to excrete urinary protease-resistant PrP isoform (25), indicating that urinary excretion from infected animals may provide a vector for horizontal transmission. However, there are studies that are not consistent with these findings (26, 27). Perhaps unrecognized nephritic conditions may underlie these discrepant observations, because it has been reported that urinary prion excretion is found only in scrapie-infected mice with lymphocytic nephritis (28). In this study, we observed several bands with high molecular weights that reacted with anti-cheetah AA antiserum in the whole urine sample, but not in the urine pellet in which AA amyloid fibrils should be recovered. We thought that the possibility for a transmission pathway through urine might be low, but it could not be ruled out.

In addition to urine, the alimentary shedding route has been considered as a possible transmission pathway (29). Abnormal prion protein is present in gut-associated lymphoid tissues of mule deer infected with CWD, consistent with an alimentary shedding route (30). In this study, we showed that the fecal fraction from a cheetah with amyloidosis had AA amyloid fibrils and possessed high transmissibility. In mouse AAp0AII amyloidosis, regarded recently as another transmissible amyloidosis (5–7), we also demonstrated that the feces could serve as an agent to induce amyloidosis in recipient mice (31). These results shed new light on the etiology involved in the high incidence of AA amyloidosis in cheetahs.

In this study, we unexpectedly found that the amyloid fibril fraction from feces had smaller amyloid fibrils and higher sensitivity to denaturation treatment than the liver amyloid fibril fraction. In mammalian prion, it has been demonstrated that there is a very strong correlation between seeding capability and amyloid fibril conformation (32, 33). Similarly, in yeast prion, it also has been indicated that [PSI+] with stronger infectivity typically have less stable fibrils in vivo than strains with weaker infectivity (34), and the prion strain with relatively smaller prion particles is always associated with greater frangibility and increased sensitivity to denaturants (35). The enhanced frangibility is presumably involved in the increase in seeding efficiency and prion infectivity, while the high sensitivity probably results from structural differences in inter-molecular contacts and a shorter, less stable amyloid core. The divergent ultrastructure between the fecal and the liver fibrils identified by transmission electron microscopy may be responsible for the different characteristics of transmissibility and sensitivity to denaturation treatment, analogous to prion protein.

It has been reported that AA amyloidosis can be experimentally induced by i.v. or i.p. administration of AA amyloid fibrillar extracts in recipient mice (10). A few recent studies have shown that AA-containing extracts also had amyloid-inducing activity when administered orally to mice (36, 37). In AAp0AII amyloidosis, we reported that an oral administration of AAp0AII amyloid fibrils induced amyloidosis in recipient mice (38). Thus, it is plausible that oral ingestion of AA-containing fecal matter caused amyloid deposition in the cheetah population. At this juncture, the manner in which fecal matter is initially absorbed by the cheetahs is not clear. This may occur during mutual grooming (licking of the fur contaminated by fecal matter). Recently it was shown that a prion agent could bind to whole soil and common soil minerals and retain infectivity for a prolonged period (23, 39). Thus, soil may act as a reservoir capable of contaminating both food and fur. It is also unknown how AA fibril proteins enter the feces. Because AA amyloidosis was also in the small intestines of AA amyloidosis cheetahs, it is possible that AA proteins enter the feces through exfoliated mucosa.

In conclusion, we found that cheetahs with amyloidosis pass fecal matter that had strong seeding efficiency and should be regarded as a transmission medium. To control the incidence of AA amyloidosis and reduce the likelihood of the animal’s extinction, prevention of the transmission with excretion from
chvee...s with amyloidosis should be considered along with reduction of precursor SAA levels.

**Materials and Methods**

**Animals.** C68 was a domestic male cheetah that died of AA amyloidosis at 4 years of age. AA amyloidosis was systemic and severe. C66 (died at 8 years of age) and C90 (died at 11 years of age) were female cheetahs that had systemic AA amyloidosis similar to C68. C67 was a female cheetah that died at age 13 years with minor AA amyloidosis seen in the kidney. C78 was a male cheetah that died at 1 year of age and had a minor systemic AA amyloidosis. TT253 was a female cheetah that died at 17 years of age with very minor AA amyloidosis localized only in the esophagus. Because the amount of fecal amyloid fibril fraction obtained from a single cheetah was not sufficient, we used fecal fractions from C68 and C67 for induction of experimental AA amyloidosis, fecal fraction from C90 in immunodepletion experiments, and fecal amyloid fibril fraction from C78 for amino acid sequencing.

Eight-week-old male C57BL/6 mice (Japan SLC) were used for the induction of experimental AA amyloidosis. Mice were raised in the Division of Laboratory Animal Research, Research Center for Human and Environmental Sciences, Shinshu University, under clean conventional conditions at 24°C with a 12-h/12-h light/dark cycle. A commercial diet (MF; Oriental Yeast) and water were provided ad libitum. A female JW/C57K mouse purchased from Japan SLC was used for the production of antiserum against AA amyloid fibril proteins. All experiments were performed with the consent of the Animal Care and Use Committee of Shinshu University School of Medicine.

**Production of Antiserum for Cheetah AA Amyloid Fibril Proteins.** Crude amyloid fibrils were isolated in a water suspension fraction from the liver of C68 and further purified by ultracentrifugation (40). The amyloid fibrils were partially degraded in 0.1 N of NaOH at 37°C for 1 h and used for immunization. Antiserum was produced in a rabbit by s.c. injection of the partially degraded AA amyloid fibrils suspended in DW with TiterMax (CytRx Norcross) adjuvant. The rabbit was given booster doses after 2 and 4 weeks with incomplete Freund’s adjuvant and bled 2 weeks after the last dose.

**Isolation of Amyloid Fibrils and Western Blot Analysis.** AA amyloid fibril fractions were isolated from feces of cheetahs and from human (an autopsied patient) tissue. Isolation of amyloid fibrils was performed with rabbit anti-cheetah AA antiserum (1:1,000 dilution) or anti-mouse AA antiserum (1:5,000 dilution). Protein contents in the fibril preparations were determined with a protein assay kit (Bio-Rad). Proteins in the amyloid fibril fractions were separated by 16.5% SDS/PAGE with a Tris-tricine buffer system and electro-transferred to a polyvinyliden difluoride (PVDF) membrane (Bio-Rad). AA on the blots was detected with either anti-cheetah AA antiserum (13,000 dilution) or anti-mouse AA antiserum (1,500,000 dilution).

**Histological and Immunohistochemical Studies.** Specimens from the livers of cheetahs or spleens of mice induced by various fibrils were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were stained with Congo red and observed by using polarizing microscopy. For identification of amyloid protein, an immunohistochemical study, using labeled streptavidin-biotin, was performed with rabbit anti-cheetah AA antiserum (1:3,000 dilution) or anti-mouse AA antiserum (1:5,000 dilution).

**Amino Acid Sequence Analysis of Amyloid Fibril Proteins.** The isolated liver amyloid protein from C68 was separated with 16.5% SDS/PAGE followed by CBB staining, and the AA protein bands were excised for amino acid sequence analysis described in SI Text. The fecal AA amyloid fibril fractions were also isolated followed by CBB staining. Because the fecal amyloid fibril sample did not show a clean detectable band with CBB, the gel region corresponding to the band immunodetected by anti-cheetah AA antiserum was excised for sequence analysis.
