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Original Article

Acceleration of murine AA amyloidosis by oral administration of amyloid fibrils extracted from different species

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We herein report that experimental murine amyloid A (AA) deposition is accelerated by oral administration of semipurified amyloid fibrils extracted from different species. Three groups of mice were treated with semipurified murine AA amyloid fibrils, semipurified bovine AA amyloid fibrils or semipurified human light chain-derived (Aλ) amyloid fibrils for 10 days. After 3 weeks, each mouse was subjected to inflammatory stimulation by subcutaneous injection with a mixture of complete Freund's adjuvant supplemented with *Mycobacterium butyricum*. The mice were killed on the third day after the inflammatory stimulation, and the spleen, liver, kidney and gastrointestinal tract were examined for amyloid deposits. Amyloid deposits were detected in 14 out of 15 mice treated with murine AA amyloid fibrils, 12 out of 15 mice treated with bovine AA amyloid fibrils and 11 out of 15 mice treated with human Aλ amyloid fibrils. No amyloid deposits were detected in control mice receiving the inflammatory stimulant alone or in amyloid fibril-treated mice without inflammatory stimulation. Our results suggest that AA amyloid deposition is accelerated by oral administration of semipurified amyloid fibrils when there is a concurrent inflammatory stimulation.

Key words: amyloid enhancing factor, murine AA amyloidosis, oral administration, semipurified amyloid fibrils

Amyloidosis is a disease condition characterized by deposition of amyloid in various tissues and organs. In most types of amyloidosis, the proteins or protein precursors that form the amyloid fibrils have been identified.[1] However, the mechanism leading to the β-pleated sheet conformation of the protein and the formation of amyloid fibrils from the precursor proteins remain to be elucidated.[2]

It is known that the amyloid fibril protein in experimentally induced amyloidosis is amyloid A (AA), of which the precursor protein is serum amyloid A protein (SAA), an acute phase apolipoprotein.[3] AA amyloid fibrillogenesis is a necessary step in a two-stage or biphasic process.[4] The first phase involves synthesis of a precursor protein in sufficient quantities to initiate amyloid deposition. This is known as the pre-amyloid phase, which can endure for several days to years without actual amyloid deposition, and the duration of the pre-amyloid phase depends on inflammatory stimuli and the levels of SAA. The second phase, the amyloid phase, is thought to result from the generation of a nidus or fibrillar network onto which amyloid can be deposited. This step is dependent on the metabolism and structural properties of the SAA molecule, and on the presence of serum amyloid P component, apolipoprotein E and proteoglycans.[5–7] The pre-amyloid phase of experimentally induced AA amyloidosis can be greatly shortened in recipient animals when amyloid enhancing factor (AEF) is administered by intravenous or intraperitoneal injection, or concomitant ingestion with an inflammatory episode.[8,9] AEF is poorly defined, but AA amyloid fibrils,[10] amyloid-like synthetic fibrils[11,12] and denatured silk[13] have AEF-like effects.

Herein, we report that AA amyloidosis is rapidly formed in mice following oral administration of amyloid fibrils extracted from different species. This finding indicates that, under certain inflammatory conditions, oral administration of exogenous amyloid fibrils increases the onset of murine AA amyloidosis.

MATERIALS AND METHODS

Animals

Experiments were performed in 7-week-old female ICR mice (Japan SLC, Shizuoka, Japan) weighing 25–28 g. The protocol was approved by the Committee for Ethics of Animal Experiments of Yamaguchi University School of Medicine

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and carried out under the Guidelines for Animal Experiments of Yamaguchi University School of Medicine, and relevant laws (No. 105) and notifications (No. 6) of Japan.

Preparation of amyloid fibrils

Semipurified murine AA amyloid fibrils were extracted from the amyloid-laden liver of AKR mice, which had been subcutaneously injected with 0.5 mL of 10% casein each day for 42 days prior to death.[14] Semipurified bovine AA amyloid fibrils were extracted from the amyloidotic liver of a bovine with AA amyloidosis.[15] Semipurified human λ light chain-derived (Aλ) amyloid fibrils were extracted from an amyloidotic liver obtained at autopsy of an individual with Aλ amyloidosis.[16] Briefly, amyloid fibrils were extracted with distilled water, according to the method of Pras *et al*.[17] Amyloid deposits were dissected from 50 g of liver for 30 min at 4°C. The pellet was then resuspended, homogenized and centrifuged; this step was repeated 10 times. The supernatant from the last centrifugation had an absorbance at 280 nm of less than 0.05. The pellet was homogenized with cold distilled water and centrifuged as described above; this step was repeated five times. Supernatants from centrifugations 2–5 contained amyloid fibrils. To recover amyloid fibrils, 0.15 mol/L NaCl was added to the supernatants and the mixture was centrifuged at 15 000 *g* for 1 h. Smears of the pellets were stained with alkaline Congo red and examined under polarized light to confirm the presence of amyloid fibrils. The semipurified amyloid fibrils were diluted with distilled water to 10 mg wet weight/mL, and immediately stored at 4°C until use.

Induction of experimental AA amyloidosis

Experimental protocols are shown in Table 1. Mice were divided into six groups (A–F), with 15 animals in each group. Three groups of mice were treated each day for 10 days by oral administration of 10 mg/day of either semipurified murine AA amyloid fibrils (group A), semipurified bovine AA amyloid fibrils (group B) or semipurified human Aλ amyloid fibrils (group C), by an animal feeding needle (Fuchigami Company, Kyoto, Japan). After 3 weeks, mice were injected with an inflammatory stimulant consisting of complete Freund's adjuvant supplemented with *Mycobacterium butyricum* to induce amyloidosis.[18] Mice in group D were treated with semipurified murine AA amyloid fibrils for 10 days and injected with the inflammatory stimulant the day after the last amyloid fibril feeding. Mice in group E were treated with distilled water instead of the semipurified amyloid fibrils for 10 days and injected with the inflammatory stimulant 3 weeks after the last amyloid fibril feeding. All mice in groups A–E were killed on the third day after inflammatory stimulation. Mice in group F were treated with the semipurified murine AA amyloid fibrils for 10 days, received no inflammatory stimulant and were killed after 5 weeks.

Histological and immunohistochemical studies

Specimens from the liver, spleen, kidney and gastrointestinal tract were fixed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE) and alkaline Congo red. Sections stained with alkaline Congo red were observed under polarized light. The amount of amyloid deposited in each spleen was graded as follows: 0, no amyloid; 1 +, ⩽10%; 2 +, 10–25%; 3 +, 25–50%; and 4 +, > 50% replacement with amyloid. For identification of amyloid protein in recipient mice, an immunohistochemical study using the labeled streptavidin–biotin method[19] was performed with rabbit antimouse AA antiserum[20] as a primary antibody. Immunohistochemical analysis was performed on sections from mice exposed to the semipurified human Aλ amyloid fibrils, with rabbit antihuman Aλ antiserum (provided by Dr

Table 1 Experimental protocols and results of murine amyloidosis induced by exposure to semipurified amyloid fibrils

Group	Treatment (10 times)	Lag period after treatment	Inflammatory stimulation	No. mice with amyloidosis (n = 15)	Amount of amyloid
A	Semipurified murine AA amyloid fibrils	3 weeks	+	14	2 +
B	Semipurified bovine AA amyloid fibrils	3 weeks	+	12	1 + to 2 +
C	Semipurified human Aλ amyloid fibrils	3 weeks	+	11	1 + to 2 +
D	Semipurified murine AA amyloid fibrils	none	+	2	2 +
E	Distilled water	3 weeks	+	0	0
F	Semipurified murine AA amyloid fibrils	none	–	0	0

AA, amyloid A; Aλ, human light chain-derived. The amount of amyloid deposited in each spleen was graded as follows: 0, no amyloid; 1 +, ⩽10%; 2 +, 10–25%; 3 +, 25–50%; and 4 +, > 50% replacement with amyloid.

George G Glenner, University of California, San Diego, USA). As a negative control, the primary antibody was replaced by non-immune rabbit serum (Dako A/S, Glostrup, Denmark).

RESULTS

Semipurified amyloid fibrils stained with Congo red showed green birefringence under polarized light (Fig. 1), indicating that the extracted materials contained amyloid fibrils. A summary of the results is given in Table 1. Briefly, amyloid deposits were found in 14 out of 15 mice in group A, 12 out of 15 mice in group B and 11 out of 15 mice in group C. In group D mice, amyloid deposits were found in two out of 15. These amyloid deposits stained positively with Congo red (Fig. 2a) and showed birefringence under polarized light (Fig. 2b). In groups E and F, amyloid deposits were not found in any of the mice examined. In tissue sections from mice in groups A, B and C, amyloid deposits were seen in the perifollicular area of the spleen (Fig. 3a), the space of Disse (Fig. 3b), vessel walls in the liver and kidney, including the glomerular tufts (Fig. 3c), around the papillae and along the tubules. The amount of amyloid in the spleen was scored as 2 + in group A mice (Fig. 4a), 1 + to 2 + in group B mice (Fig. 4b), 1 + to 2 + in group C mice (Fig. 4c), and 2 + in group D mice. Amyloid deposits were not detected in the gastrointestinal tract of any of the mice examined. Amyloid deposits in mice of all groups immunohistochemically reacted with antimouse AA antiserum (Fig. 3a–c), regardless of the type of semipurified amyloid fibrils administered, indicating that the amyloid protein was derived from AA protein. In the negative controls performed with non-immune rabbit serum as the primary antibody, no amyloid deposits reacted with the serum. Amyloid deposits in tissue sections from mice exposed to semipurified human Aλ amyloid fibrils did not react with antihuman Aλ antiserum.

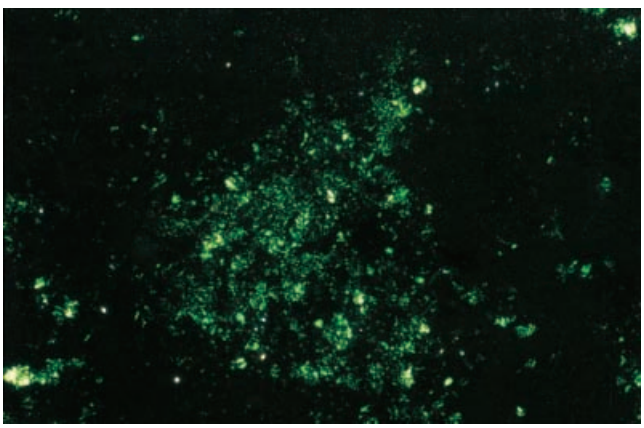

Figure 1 Smear of semipurified murine AA amyloid fibrils shows green birefringence under polarized light (alkaline Congo red).

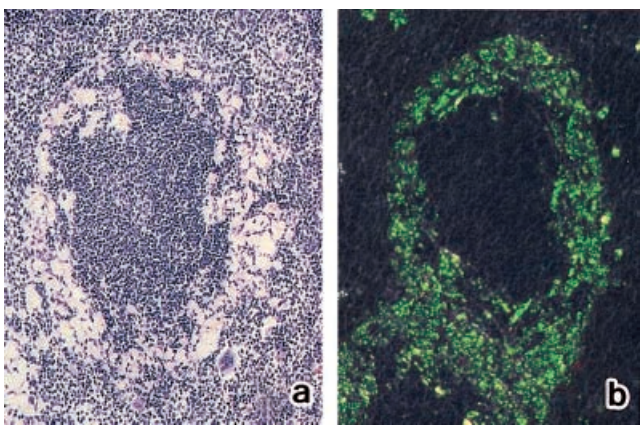

Figure 2 Splenic tissue from a group A mouse. (**a**) Amyloid deposits stain positively (Congo red). (**b**) Apple-green birefringence under polarized light.

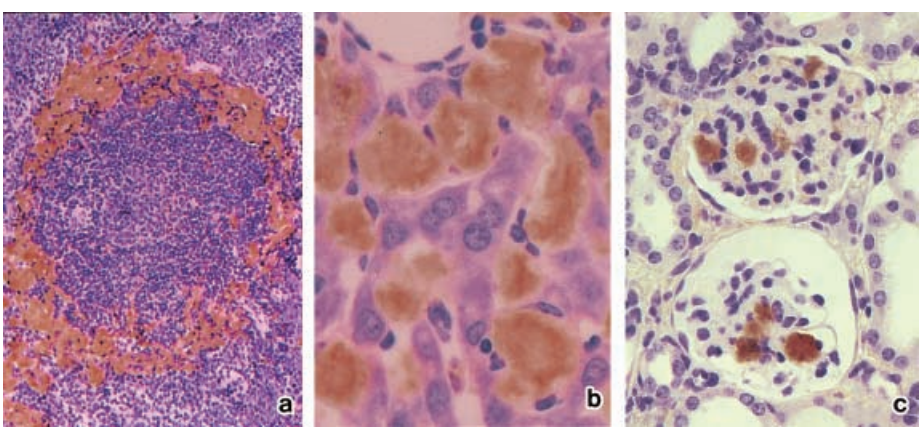

Figure 3 (**a**) Splenic tissue from a group C mouse. Amyloid deposits reactive with antimouse AA antiserum in the perifollicular area (staining was scored 2 +). (**b**) Liver specimen from a group A mouse. Amyloid deposits are seen along the space of Disse (HE). (**c**) Kidney section from a group A mouse. Nodular amyloid deposits are seen in the glomerular tufts, and the deposits react with antimouse AA antiserum.

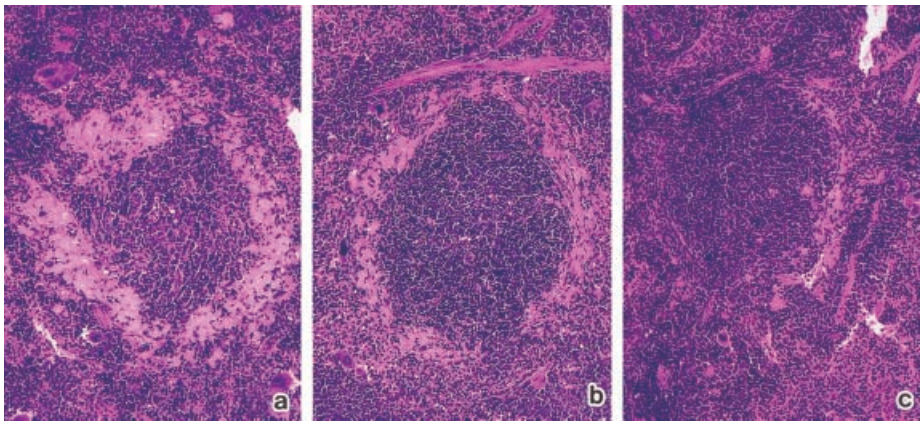

Figure 4 Amount of amyloid deposit in the spleen. (**a**) Group A mouse, 2 +; (**b**) group B mouse, 1 + to 2 +; (**c**) group C mouse, 1 + to 2 + (HE).

DISCUSSION

Secondary or amyloid protein A amyloidosis occurs in individuals with long-standing inflammatory diseases. An essential factor for the development of AA amyloidosis is a continual high plasma concentration of SAA. However, it is still unclear why only a subset of such individuals develops AA amyloidosis. Therefore, in addition to high concentrations of an amyloidogenic protein, other factors are thought to be necessary in the pathogenesis of AA amyloidosis. In this context, one such factor is AEF.[8] Experimentally induced AA amyloidosis can be greatly accelerated in recipient animals when AEF is administered concomitantly with inflammatory stimuli.[8,9] Although AEF was discovered several decades ago,[21,22] the nature of the active component in AEF continues to receive much attention. Despite intensive efforts during the past few decades, the active component of AEF has not been defined. Amyloid fibrils extracted from different types of amyloidoses from a wide variety of species display biologically similar AEF activity in experimental animals. In fact, Niewold *et al.*[10] showed that intravenous and intraperitoneal injection of hamster AA amyloid fibrils, bovine AA amyloid fibrils and human light chain-derived (Aλ) amyloid fibrils markedly accelerated hamster amyloidosis. Furthermore, intravenous injection of amyloid-like fibrils made from synthetic peptides of transthyretin[11] or denatured silk[13] accelerates murine AA amyloidosis. By double immunogold labeling and microautoradiographic methods, Johan *et al.*[12] reported that intravenously administered, radiolabeled, heterologous, amyloid-like, synthetic fibrils reached to the lung and spleen, and were associated with topographical deposition of murine protein AA fibrils in the recipient mouse. They suggested that synthetic amyloid-like fibrils have an *in vivo* nidus activity and that amyloid-enhancing activity may occur through this mechanism.

Presently, there is convincing evidence that exposure, most probably dietary, to abnormal prions causes not only endemic bovine spongiform encephalopathy (BSE),[23] but also a new variant of Creutzfeldt–Jakob disease in humans.[24] Prion diseases are associated with the accumulation of a conformational isomer (PrP^{Sc}) of host-derived prion protein (PrP^{C}), and PrP^{Sc} forms amyloid fibrils. The exogenous abnormal form of the prion protein is generally regarded as a seed that promotes the association of cellular proteins after invasion. Although the mechanism of amyloidogenesis might not be identical to that of orally transmissible prion diseases, it prompted us to examine whether amyloid fibrils extracted from different species enhance amyloid deposition in recipient animals by oral administration.

In the present study, we postulate that murine AA amyloidosis occurs over a relatively short period with oral administration of semipurified murine AA amyloid fibrils, semipurified bovine AA amyloid fibrils or semipurified human Aλ amyloid fibrils, and inflammatory stimuli. Amyloid deposits were seen in the spleen, liver and kidney of recipient mice, but no amyloid deposits were found in the gastrointestinal tract. The amyloid protein found in recipient mice was immunohistochemically identified as AA type, despite the administration of non-AA amyloid fibrils. On the basis of these results, we speculate that semipurified amyloid fibrils are transported through the digestive tract to organs where amyloid deposits commonly occur; however, the reason why no amyloid deposits were found in the gastrointestinal tract is not clear. A few recent studies have shown that oral administration of AEF accelerates amyloid deposition in recipient animals.[25,26] Elliott-Bryant and Cathcart reported that AA amyloidosis was rapidly formed in CBA/J mice following oral administration of an AEF extracted from the liver of CE/J mice that were resistant to AA amyloid induction and had no detectable amyloid fibrils in the liver.[25] They suggested that a transmissible agent

present in the diet may be a contributory factor in amyloid fibrillogenesis. More recently, in a mouse model of senile systemic amyloidosis, Xing *et al*. reported that an orally administered series of AApo AII amyloid fibrils isolated from old R1.P1-*Apoa2*^{c} mice induced amyloidosis in young R1.P1-*Apoa2*^{c} mice.[26] Previously described results, in which AEF is transmissible by oral administration, are in accordance with those in the current study. However, previous studies[25,26] used donor and recipient animals of the same species (i.e. AEF was extracted from mice and administered to mice) whereas in the current study, amyloid fibrils were extracted from mouse, bovine or human tissues and administered to mice. Thus, the results of our present study, in which oral ingestion of amyloid fibrils extracted from different species caused amyloid deposition, may be important in understanding the etiology of AA amyloidogenesis in humans.

Since the incidence of chronic inflammatory diseases such as tuberculosis and leprosy have decreased in recent years, rheumatoid arthritis (RA) is now the most common disease involving secondary AA amyloidosis, especially in elderly patients with a long history of RA. Autopsy studies indicate that the incidence of secondary amyloidosis in RA patients may be between 20 and 25%.[27] A major unresolved question is why only some individuals with long-standing inflammatory conditions and a high plasma SAA concentration develop amyloid deposits. Therefore, other factors in addition to the high level of SAA are likely to be important in human amyloidosis. In lepromatous leprosy, secondary amyloidosis was remarkably higher in patients from Carville, Louisiana (24% incidence), whose diet consisted of large amounts of animal fat, than in patients from Guadalajara, Mexico (6% incidence), whose diet is much lower in animal fat.[28] Although it is unknown whether such animal fat contains AEF amyloid or an AEF-like substance, several reports have described secondary AA amyloidosis developing spontaneously in various domestic animals, including cow, horse, dog and fowl.[29] Indeed, an autopsy survey by Fujinaga showed that incidental amyloidosis was found in 22 (1.2%) out of 1800 senile cows.[15] In conjunction with the results of the current study, these data raise the possibility that intake of foods containing animal tissues causes secondary AA amyloidosis in certain patients with long-standing inflammatory diseases. Further studies are needed to clarify the relationship between AA amyloidosis and dietary habits in patients with long-standing inflammatory diseases.

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REFERENCES

1 Husby G, Araki S *et al.* The 1990 guidelines for nomenclature and classification of amyloid and amyloidosis. In: Natvig JB, Førre Ø, Husby G *et al.,* eds. *Amyloid and Amyloidosis.* Dordrecht*:* Kluwer Academic Publishers, 1991; 7–11.
2 Falk RH, Comenzo RL, Skinner M. The systemic amyloidoses. *N. Engl. J. Med.* 1997; **25**: 898–909.
3 Skinner M, Shirahama T, Benson MD, Cohen AS. Murine amyloid protein AA in casein-induced experimental amyloidosis. *Lab. Invest.* 1977; **36**: 420–427.
4 Sipe JD, McAdam KP, Uchino F. Biochemical evidence for the biphasic development of experimental amyloidosis. *Lab. Invest.* 1978; **38**: 110–114.
5 Emsley J, White HE, O'Hara BP *et al.* Structure of pentameric human serum amyloid P component. *Nature* 1994; **367**: 338–345.
6 Wisniewski T, Frangione B. Apolipoprotein E: A pathological chaperone protein in patients with cerebral and systemic amyloid. *Neurosci. Lett.* 1992; **135**: 235–238.
7 Snow AD, Willmer J, Kisilevsky R. Sulfated glycosaminoglycans: a common constituent of all amyloids? *Lab. Invest.* 1987; **56**: 120–124.
8 Axelrad MA, Kisilevsky R, Willmer J, Chen SJ, Skinner M. Further characterization of amyloid-enhancing factor. *Lab. Invest.* 1982; **47**: 139–146.
9 Yokota T, Ishihara T, Kawano H, Takahashi M, Fujinaga Y, Uchino F. Amyloid enhancing factor (AEF) in the aging mouse. *Virchows Arch. A* 1989; **414**: 511–514.
10 Niewold TA, Gruys E, Kisilevsky R, Shirahama TS. Fibril amyloid enhancing factor (FAEF)-accelerated amyloidosis in the hamster is not dependent on serine esterase activity and mononuclear phagocytosis. *Scand. J. Immunol.* 1991; **34**: 101–107.
11 Ganowiak K, Hultman P, Engström U, Gustavsson Å, Westermark P. Fibrils from synthetic amyloid-related peptides enhance development of experimental AA-amyloidosis in mice. *Biochem. Biophys. Res. Commun.* 1994; **199**: 306–312.
12 Johan K, Westermark G, Engström U, Gustavsson Å, Hultman P, Westermark P. Acceleration of amyloid protein A amyloidosis by amyloid-like synthetic fibrils. *Proc. Natl Acad. Sci. USA* 1998; **95**: 2558–2563.
13 Kisilevsky R, Lemieux L, Boudreau L, Sheng YD, Fraser P. New clothes for amyloid enhancing factor (AEF): silk as AEF. *Amyloid* 1999; **6**: 98–106.
14 Cui D, Hoshii Y, Takahashi M, Kawano H, Iwata T, Ishihara T. An immunohistochemical study of amyloid P component, apolipoprotein E and ubiquitin in human and murine amyloidoses. *Pathol. Int.* 1998; **48**: 362–367.
15 Fujinaga Y. Incidence of amyloid deposits in various organs in senile cows and their pathological characteristics. *Yamaguchi Med. J.* 1990; **39**: 293–303 (in Japanese with English abstract).
16 Hoshii Y, Takahashi M, Ishihara T, Uchino F. Immunohistochemical classification of 140 autopsy cases with systemic amyloidosis. *Pathol. Int.* 1994; **44**: 352–358.
17 Pras M, Schubert M, Zucker-Franklin D, Rimon A, Franklin EC. The characterization of soluble amyloid prepared in water. *J. Clin. Invest.* 1968; **47**: 924–933.
18 Ram JS, Delellis RA, Glenner GG. Amyloid. III. A method for rapid induction of amyloidosis in mice. *Int. Arch. Allergy Appl. Immunol.* 1968; **34**: 201–204.
19 Shi ZR, Itzkowitz SH, Kim YS. A comparison of three immunoperoxidase techniques for antigen detection in colorectal carcinoma tissues. *J. Histochem. Cytochem.* 1988; **36**: 317–322.

20 Imada N. Pathological study on amyloidosis: Isolation and purification of amyloid fibril protein and biochemical and immunological analysis. *Yamaguchi Med. J.* 1981; **30**: 149–162 (in Japanese with English abstract).
21 Willerson JT, Gondon JK, Talal N, Barth WF. Murine amyloid transfer of an amyloid-accelerating substance. *Arthritis Rheum.* 1969; **12**: 232–240.
22 Shirahama T, Lawless OJ, Cohen AS. Heterologous transfer of amyloid- -human to mouse. *Exp. Biol. Med.* 1969; **130**: 516–519.
23 Bruce ME, Will RG, Ironside JW *et al.* Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature* 1997; **389**: 498–501.
24 Collinge J, Sidle KC, Meads J, Ironside J, Hill AF. Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. *Nature* 1996; **383**: 685–690.
25 Elliott-Bryant R, Cathcart ES. Amyloid enhancing factor and dietary transmission in accelerated amyloid A amyloidosis. *Clin. Immunol. Immunopathol.* 1998; **88**: 65–69.
26 Xing Y, Nakamura A, Chiba T *et al.* Transmission of mouse senile amyloidosis. *Lab. Invest.* 2001; **81**: 493–499.
27 Suzuki A, Ohosone Y, Obana M *et al.* Cause of death in 81 autopsied patients with rheumatoid arthritis. *J. Rheumatol.* 1994; **21**: 33–36.
28 Williams RC, Cathcart ES, Calkins E, Fite GL, Rubio JB, Cohen AS. Secondary amyloidosis in lepromatous leprosy: possible relations of diet and environment. *Ann. Intern. Med.* 1965; **62**: 1000–1007.
29 Zschiesche W, Jakob W. Pathology of animal amyloidoses. *Pharmacol. Ther.* 1989, 1990; **41**: 49–83.