Effect of the Intestinal Flora on Amyloid Deposition in a Transgenic Mouse Model of Familial Amyloidotic Polyneuropathy

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Abstract: Familial amyloidotic polyneuropathy (FAP) is a hereditary disease characterized by the systemic accumulation of amyloid fibrils. A mutant transthyretin (TTR) gene is mainly responsible for the disease. However, the variable age of onset and low penetrance might be due to environmental factors, one of which is the intestinal flora. Three types of intestinal flora were introduced into a transgenic (Tg) mouse FAP model, 6.0-hMet30. The CV1 and CV2 group transgenic mice were transferred with the intestinal flora from two different mouse facilities housed under conventional conditions, and the SPF group transgenic mice were kept under specific pathogen free conditions in our facility. All the mice were maintained under controlled temperature, humidity and bacterial conditions. Over a period of 28 months, amyloid was not deposited in the SPF and CV1 groups. In contrast, amyloid was deposited in the esophagus and small intestine of two of the three CV2 mice at 18 months. Many neutrophils infiltrated the lesions. The numbers of tissue neutrophils were higher in the CV2 group than in the SPF and CV1 groups at 18 months. The CV2 flora included fewer gram-positive anaerobic cocci as well as higher proportions of yeasts, staphylococci and enterobacteriaceae compared with the SPF and CV1 flora. These findings suggest that the intestinal flora plays an important role in amyloid deposition.

Key words: Familial amyloidotic polyneuropathy, amyloid, intestinal flora

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Introduction

It is a well-known fact that both genetic and environmental factors are involved in the development of most human diseases. However, analyses of environmental factors are not easy to carry out. One approach is to establish a transgenic mouse model and to use this model for the analysis of environmental factors. We previously produced transgenic mouse models for a human disease, familial amyloidotic polyneuropathy (FAP) by microinjecting a mutant human transthyretin gene (hTTR Val30Met) carrying three different promoters into fertilized eggs of C57BL/6J mice [1–3]. FAP is an autosomal dominant disorder characterized by the systemic accumulation of amyloid fibrils in the peripheral nerves and other organs. All FAP amyloid is composed of variant TTR molecules with single amino acid substitutions. Although over 80 mutations have been reported, the most common FAP is type I (FAP aTTR Val30Met), in which the atypical TTR has methionine substituted for valine at amino acid position 30 [4]. Most FAP patients are heterozygous for the mutated TTR gene. The symptoms of homozygous individuals are less severe and onset is later than in heterozygotes [5].

Type I FAP is widespread in Japan, Portugal and Sweden. The average ages of onset in Japanese and Portuguese patients are 40.1 and 33.5 years, respectively, whereas northern Swedish patients with the same mutation generally develop the condition at 56 years of age. The penetrance in the Portuguese FAP population is 48.4%, whereas in northern Sweden it is only 2% and most carriers are asymptomatic [6–8]. Discordant symptoms in monozygotic twins with atypical TTR have been reported [9]. One of a monozygotic twin developed typical FAP symptoms seven years ago, i.e. at the age of 55 years, while the other remains absolutely healthy. The variable age of onset and low penetrance in Swedish FAP patients, and the discordance in one pair of monozygotic twins might be explained by environmental factors.

In our transgenic mouse models of FAP housed under conventional (CV) conditions at the Institute of Molecular Embryology and Genetics, amyloid deposition occurred in the gastrointestinal tract, kidneys, heart, thyroid and skin [10]. However, under specific pathogen-free (SPF) conditions, amyloid was never deposited

(manuscript in preparation). These mice were maintained under controlled temperature, humidity and bacterial population conditions, whereas these conditions were not well controlled for the CV group. One of the key differences between CV and SPF mice was the intestinal flora and amyloid deposits were mainly attached to the gastrointestinal tract in our CV mice. Therefore, this factor may be relevant to amyloid deposition.

In this experiment, we used a transgenic mouse line carrying the entire coding region for a mutant hTTR gene with an approximately 6-kb upstream coding region, termed 6.0-hMet30 mice. In this line the expression pattern of the 6.0-hMet30 transgene is similar to that in normal humans in terms of developmental, tissue-specific, and quantitative expression [3]. Amyloid deposition initially appeared at 9 months of age in this line [10]. The 6.0-hMet30 mice were kept under the same environmental conditions, such as temperature and humidity, but the type of intestinal flora differed. We compared amyloid deposition among the mice with three types of intestinal flora and evaluated their effects on amyloidogenesis.

Materials and Methods

Animals: Two-cell embryos obtained by *in vitro* fertilization of superovulated eggs from C57BL/6J females (CLEA Inc., Tokyo, Japan) with sperm from 6.0-hMet30 Tg males were cryopreserved in liquid nitrogen. Frozen embryos were thawed and transferred to the oviducts of pseudopregnant ICR (CLEA) female mice [11, 12]. The CV1 group was transferred with the intestinal flora from their foster-parents housed under the CV condition at the Inoue Experimental Animal Center after raising for two months after birth. The CV2 group was originally kept under SPF conditions until 3 months of age. Then, this group was transferred with the intestinal flora from the CV mice maintained at the Institute of Molecular Embryology and Genetics by maintaining them together for two months. The SPF group was transferred with the intestinal flora by raising with mice housed under SPF conditions at the Center for Animal Resources and Development. Thereafter the three groups of mice were housed in separate vinyl isolators equipped with a sterilizing air filter (ICM Co., Ltd., Ibaraki, Japan). As both transgenic (Tg) and

non-transgenic (nonTg) mice died of unknown causes after 16 months, the experiment of the CV2 group was terminated at 18 months. Transgenic mice were identified by polymerase chain reaction (PCR) of tail DNA with two primer sets that detect the second and fourth exons, and nonTg littermates were kept as controls. The serum concentration of hTTR was determined by immuno-blot assay with rabbit anti-human TTR antibodies (MBL Co., Ltd., Nagoya, Japan) and calibrated with purified hTTR (CALBIOCHEM Japan Ltd., Tokyo, Japan).

Housing conditions: The vinyl isolators were placed in a room with a controlled 12-h light-dark cycle, at $22 \pm 2^\circ\text{C}$ and a relative humidity level of 50–70%. The TPX cages (CLEA) contained autoclaved wood chips. The mice were fed with a commercial pelleted diet (Funabashi Farm Co., Chiba, Japan) sterilized by ^{60}Co γ -irradiation at 50 kGy and given access to autoclaved water *ad libitum*.

Monitoring of microbes: All animals were tested for the following microbes at each sampling point: *Mycoplasma pulmonis*, *Corynebacterium kutscheri*, *Salmonella* spp., *Pasteurella pneumotropica*, *Streptococcus pneumoniae*, *Bordetella bronchiseptica*, *Citrobacter rodentium*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Clostridium piliforme*, mouse hepatitis virus, Sendai virus, Ectromelia virus, mouse adenovirus, *Syphacia* spp., and ectoparasites. In the SPF group, the monitored microbes were totally absent. *Pseudomonas aeruginosa* and *Aspicularis tetraptera* (nematodes) in the CV1 group and *Mycopetes musculus* (mites) in the CV2 group were detected.

Histopathological examination: Tissues, comprising heart, kidneys, spleen, liver, lungs, pancreas, esophagus, stomach, small and large intestines, mesenteric lymph nodes, urinary bladder, thyroid gland, bone marrow, sciatic nerves, eyes and brain, were fixed in 4% paraformaldehyde in phosphate-buffered saline, and embedded in paraffin blocks. Sections cut from the blocks were stained with hematoxylin and eosin for light microscopy. Five fields (25 mm²) in sections of esophagus and small and large intestine tissues were examined at a magnification of 400x, the numbers of neutrophils being determined. Paraffin sections were also stained with Congo red for examination under a polarization microscope to detect amyloid. The amyloid deposits were immunostained with anti-human TTR

antibodies by the avidin-biotin complex method.

Analysis of the intestinal flora: Fecal bacteria were cultured according to Mitsuoka *et al.* [13–15], except that an anaerobic chamber (85% N₂, 5% CO₂, and 10% H₂; ICM Co., Ltd., Osaka, Japan) replaced the 'plate-in-bottle' method [13]. Mouse fecal samples were collected before sacrifice, immediately weighed and then transferred to the anaerobic chamber. The samples were homogenized with a 50-fold volume of anaerobic diluent, serially diluted (10^{-1} to 10^{-8}), and then spread on 16 plates containing non-selective and selective agar media. M10 and EG were used in the anaerobic chamber at 37°C for 4 days. Eight agar plates (EG, BL, NBGT, BS, ES, VS, NN and LBS agar) were incubated 37°C for 3 days in an anaerobic steel wool jar filled with oxygen-free CO₂, and 6 (TS, DHL, TATAC, PEES, PD and NAC) agars were incubated at 37°C for 24–48 h aerobically. Isolated bacteria were identified by Gram staining, colony morphology and aerobic growth. The numbers of bacteria are expressed as the log₁₀ count of viable bacteria per gram wet weight of feces.

Statistical procedures: The groups were statistically compared using the unpaired *t*-test.

Results

Transgene expression and amyloid depositions: 6.0-hMet30 mice were simultaneously produced by *in vitro* fertilization and embryo transfer. In total, 179 transgenic mice (96 male, 83 female) were identified by PCR of tail DNA and immunoblotting. The average serum hTTR concentrations at 6, 18, 24, and 28 months were 10.12 ± 4.24 (SD) mg/dl (n=10), 11.73 ± 7.721 mg/dl (n=10), 9.83 ± 5.28 mg/dl (n=10) and 9.14 ± 3.64 mg/dl (n=21) in the CV1 group, and 8.92 ± 3.90 mg/dl (n=16), 7.88 ± 3.98 mg/dl (n=14), 11.91 ± 4.61 mg/dl (n=11) and 12.68 ± 2.17 mg/dl (n=5) in the SPF group, respectively. The serum hTTR concentration did not significantly differ between the CV1 and SPF groups ($P < 0.05$). Despite constant expression of the mutant hTTR, amyloid was not deposited in the CV1 or SPF group of Tg mice over a period of 28 months (Table 1). Amyloid was deposited in the esophagus, stomach (squamous epithelium) and small intestine of two of three CV2 mice at 18 months (Fig. 1A-D). The amyloid deposits were stained with anti-human TTR antibodies (data not shown). Amyloid deposition in

Table 1. Incidence of amyloid deposition

Group	Mice	Age (months)			
		6	18	24	28
SPF	Tg	0/16 ^{a)}	0/14	0/11	0/5
	nonTg	0/19	0/14	0/9	0/5
CV1	Tg	0/10	0/10	0/10	0/21
	nonTg	0/10	0/10	0/10	0/4
CV2	Tg	0/2	2/3		
	nonTg	0/1	0/3		

a) Number positive/Number examined

Fig. 1. Histopathological analysis of amyloid deposition. Amyloid deposited in the esophagus and small intestine was stained with Congo red and hematoxylin (A and C, respectively) and exhibited characteristic yellow-green birefringence under polarizing light (B and D, respectively); bar = 50 μ m. Many neutrophils (arrow head) can be seen around the amyloid deposits in the esophagus and small intestine, respectively. (E and F). H&E stain; bar=10 μ m.

Fig. 2. The numbers of neutrophils in the lamina propria. The numbers of tissue neutrophils in the esophagus, small intestine and large intestine were higher in the CV2 group than in the CV1 and SPF groups at 18 months. Significant differences ** ($p < 0.01$) vs. SPF group.

other tissues was not evident.

Neutrophil infiltration of Tg mice: Many neutrophils infiltrated the lesions (Fig. 1E, F). The numbers of neutrophils in the lamina propria of the digestive tract were examined in the mice at 18 months (Fig. 2). The average numbers of neutrophils in the esophagus, small intestine, and large intestine were 0.36 ± 0.41 , 3.10 ± 2.10 and 1.86 ± 1.83 cells/mm² in the SPF group ($n=28$), 0.91 ± 1.26 , 5.11 ± 2.68 and 7.91 ± 5.07 cells/mm² in the CV1 group ($n=20$), and 1.63 ± 0.91 , 8.50 ± 3.42 and 13.26 ± 9.22 cells/mm² in the CV2 group ($n=6$), respectively. The numbers of tissue neutrophils in the esophagus, small intestine and large intestine were higher in the CV2 than in SPF group ($p < 0.01$).

Intestinal flora of Tg mice: The differences in intestinal flora among these three groups of Tg mice were compared to evaluate the effect of the intestinal flora on amyloid deposition (Table 2). All mice examined were female except for the case of the SPF group at 28 months, as no difference in amyloid deposition was observed between males and females [2, 10]. NonTg mice examined simultaneously had almost the same intestinal flora composition as Tg mice. The intestinal flora in the CV2 group, in which amyloid was deposited, was compared with those in the SPF and CV1 groups. Yeasts were identified in the CV2 group in all samples at a level of 10^7 , but never in the SPF and CV1 groups. The numbers of staphylococci and

enterobacteriaceae were higher in the CV2 group than in the CV1 and SPF groups. Gram-positive anaerobic cocci (GPAC) were undetectable in the CV2 group at 18 months, yet the levels remained above 10^9 in the CV1 and SPF groups for over 28 months. *Pseudomonas* was detected in the CV1 and CV2 groups, but not at all in the SPF group. There were no significant differences in other microorganisms.

Discussion

The presence of a mutant TTR gene and its product is essential for the development of FAP. TTR is a 5.5-KDa protein, consisting of four identical polypeptide chains of 127 amino acids [16, 17], and plays an important functional role in the plasma transport of both thyroid hormone and retinol (vitamin A). Most FAP patients are heterozygous for the mutant TTR gene. Tetramers consisting of both normal and atypical TTR may be less stable than wild-type TTR [18], implying that hetero-tetramers dissociate into monomeric amyloidogenic intermediates [19]. The atypical monomeric structure self-assembles, resulting in the formation of amyloid fibrils [20]. We previously reported that the serum level of hTTR in 6.0-hMet30 mice reached the human level of 10 mg/dl at 1 month of age. However, amyloid was first deposited in the small intestine at 9 months, and then in other tissues including the gastrointestinal tract, salivary glands, heart, lungs, liver, spleen, kidneys, testes and skin at 24 months [10].

Although the CV1 group exposed to the CV flora from the Inoue Experimental Animal Center did not develop amyloid deposition, the CV2 group exposed to the CV flora from the Institute of Molecular Embryology and Genetics developed amyloid deposition in the gastrointestinal tract. This suggests that the CV2 flora affected amyloid deposition. Thus, the amyloid depositions might be the result of the change of flora. If the intestinal flora affects the amyloid deposition, it is important to examine the differences among floras in SPF, CV1 and CV2. There were, in fact, several differences among SPF, CV1 and CV2 as described in the results.

The findings as to the intestinal flora in the CV2 group are as follows. The number of GPAC, one of the dominant anaerobes, was significantly reduced. On the other hand, the amount of aerobes, such as yeasts, staphylococci and enterobacteriaceae, was increased. This

Table 2. Intestinal flora of Tg mice

Age of mice (months)	6			18		
	SPF (n=6)	CV1 (n=4)	CV2 (n=2)	SPF (n=9)	CV1 (n=5)	CV2 (n=3)
Enterobacteriaceae	6.6 ± 0.8	6.8 ± 0.6	7.5 ± 0.6	6.5 ± 0.6	6.4 ± 0.7	7.8 ± 0.2 ^{b)}
Pseudomonas	n.d.	6.9 ± 0.4	3.8 ± 1.1 ^{a)}	n.d.	5.2 ± 0.4	7.0 ± 0.4(2)
Gram-negative rods	n.d.	n.d.	n.d.	n.d.	5.8 ± 0.8	n.d.
Streptococci	7.3 ± 0.6	7.4 ± 0.4	7.6 ± 0.4	8.3 ± 0.8(6)	7.6 ± 0.4	8.5 ± 1.1
Staphylococci	4.3 ± 1.4	4.6 ± 1.4	6.2 ± 0.0	4.6 ± 0.9	3.7 ± 0.7	7.7 ± 0.6 ^{b)}
Corynebacterium	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Yeast	n.d.	n.d.	7.2 ± 0.2	n.d.	n.d.	7.3 ± 1.0
Lactobacilli	9.4 ± 0.3	9.7 ± 0.1	9.2 ± 0.4	9.3 ± 0.3	9.2 ± 0.2	9.1 ± 0.4
Bacteroidaceae	9.5 ± 0.9	9.6 ± 0.3	10.4 ± 0.2	10.2 ± 0.2	9.8 ± 0.3 ^{a)}	10.4 ± 0.3
Veionellaceae	n.d.	n.d.	n.d.	8.3(1)	n.d.	n.d.
Gram-positive anaerobic cocci	9.5 ± 0.7(5)	9.9 ± 0.3	9.2 ± 0.2	9.2 ± 1.0	10.1 ± 0.2	n.d.
Gram-positive anaerobic rods	8.7 ± 0.8	9.0 ± 0.3	9.1 ± 0.0	8.9 ± 0.7	8.8 ± 0.6	8.9 ± 0.2(2)
Clostridia	n.d.	n.d.	n.d.	9.0 ± 0.1(2)	n.d.	n.d.
Total organism	10.3 ± 0.3	10.2 ± 0.2	10.5 ± 0.0	10.4 ± 0.2	10.3 ± 0.2	10.5 ± 0.2

Age of mice (months)	24		28	
	SPF (n=5)	CV1 (n=5)	SPF (n=5)	CV1 (n=5)
Enterobacteriaceae	6.1 ± 0.2	6.1 ± 0.6	5.5 ± 0.5	6.4 ± 0.8
Pseudomonas	n.d.	5.8 ± 0.7	n.d.	5.6 ± 1.0
Gram-negative rods	6.4 (1)	6.6 ± 1.6(2)	nd.	n.d.
Streptococci	7.1 ± 0.3	8.4 ± 0.2 ^{b)}	6.3 ± 0.7	8.0 ± 0.3 ^{b)}
Staphylococci	4.5 ± 0.7	5.4 ± 0.2	5.3 ± 0.4	5.0 ± 2.0(4)
Corynebacterium	n.d.	n.d.	n.d.	n.d.
Yeast	n.d.	n.d.	n.d.	n.d.
Lactobacilli	9.5 ± 0.2	9.2 ± 0.2	9.4 ± 0.3	9.4 ± 0.3
Bacteroidaceae	9.8 ± 0.2	9.8 ± 0.1	9.7 ± 0.2	9.4 ± 0.4
Veionellaceae	n.d.	n.d.	n.d.	n.d.
Gram-positive anaerobic cocci	9.5 ± 0.4	9.3 ± 0.9	9.8 ± 0.3	9.3 ± 0.9
Gram-positive anaerobic rods	9.6 ± 0.3	8.6 ± 0.5 ^{a)}	9.1 ± 0.5	9.1 ± 0.4
Clostridia	n.d.	n.d.	n.d.	n.d.
Total organism	10.3 ± 0.2	10.4 ± 0.1	10.3 ± 0.2	10.1 ± 0.4

n.d.: not detected, (No. of mice), Statistically significant at the a) $p < 0.05$ and b) $p < 0.01$ levels when compared with the SPF group.

is consistent with the notion that anaerobes are more effective at controlling the multiplication of aerobes such as enterobacteriaceae, *Pseudomonas aeruginosa* and staphylococci [15, 21]. This microbial environment could be regarded as uncontrollable aerobe growth. Since the pathogenic aerobe, *Pseudomonas aeruginosa*, was detected in both the CV1 and CV2 groups, it may not have any effect on amyloid deposition.

The numbers of tissue neutrophils in the esophagus and small intestine were higher in the CV2 group than in the CV1 and SPF groups at 18 months. However, neutrophilic infiltration in other tissues was unremarkable. Concerning the number of neutrophils in the CV2

group, both Tg and nonTg mice had an increased number of neutrophils and there was no difference between Tg and nonTg mice (data not shown). Since the nonTg mice do not have a mutant TTR gene and never develop amyloid deposition, CV2 flora rather than amyloid deposition may be the cause for the neutrophilic infiltration. This poses the question as to whether neutrophilic infiltration is associated with amyloid deposition or not. In this experiment, only two out of three transgenic mice developed amyloid deposition, and the number of mice examined was too few to draw a conclusion. In addition, there seemed to be no difference in the number of neutrophils between Tg and nonTg

mice. Thus, further analysis will be needed to answer this question.

In the CV2 group, the number of aerobes, such as yeasts and staphylococci, were increased. As neutrophils play an essential protective role against such aerobes by oxidant-mediated fungicidal activity [22] or by absorbing pathogens into phagosomes [23], it is possible that neutrophil chemotaxis was facilitated by the intestinal flora of the CV2 group. This is consistent with the data that the chemotactic activity of neutrophils and macrophages was higher in SPF than germ-free mice [24]. Stone *et al.* detected human neutrophil protease, elastase and cathepsin G in amyloid fibrils obtained from patients with five types of systemic amyloidosis including FAP [25]. They suggested that these proteases in the azurophilic granules become associated with amyloid precursor proteins in an extracellular inflammatory microenvironment or in phagolysosomes, and thus play a role in amyloid deposition. Although FAP is a hereditary disorder caused by a mutant hTTR gene, our results suggest that the intestinal flora plays an important role in amyloidogenesis, and that control of the microbial environment may prevent the onset of the disease.

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References

1. Yamamura, K., Wakasugi, S., Maeda, S., Inomoto, T., Iwanaga, T., Uehira, M., Araki, K., Miyazaki, J., and Shimada, K. 1987. Tissue-specific and developmental expression of human transthyretin gene in transgenic mice. *Dev. Genet.* 8: 195–205.
2. Wakasugi, S., Inomoto, T., Yi, S., Naito, M., Uehira, M., Iwanaga, T., Maeda, S., Araki, K., Miyazaki, J., Takahashi, K., Shimada, K., and Yamamura, K. 1987. A transgenic mouse model of familial amyloidotic polyneuropathy. *Proc. Japan. Acad.* 63: 344–347.
3. Nagata, Y., Tashiro, F., Yi, S., Murakami, T., Maeda, S., Takahashi, K., Shimada, K., Okamura, H., and Yamamura, K. 1995. A 6-Kb upstream region of the human transthyretin gene can direct developmental, tissue-specific, and quantitatively normal expression in transgenic mouse. *J. Biochem.* 117: 169–175.
4. Connors, L.H., Richardson, A.M., Thébrege, R., and Costello, C.E. 2000. Sequence communication; tabulation of transthyretin (TTR) variants as of 1/1/2000. *Amyloid: Int. J. Exp. Clin. Invest.* 7: 54–69.
5. Holmgren, G., Bergstöm, S., Drugge, U., Lundgren, E., Nording-Sikstöm, C., Sandgren, O., and Steen, L. 1992. Homozygosity for the transthyretin-Met30-gene in seven individuals with familial amyloidosis with polyneuropathy detected by restriction enzyme analysis of amplified genomic DNA sequences. *Clin. Genet.* 41: 39–41.
6. Nakazato, M., Shiomi, K., Miyazato, M., and Matsukura, S. 1992. Type I familial amyloidotic polyneuropathy in Japan. *Internal. Med.* 31: 1335–1338.
7. Sousa, A., Coelho, T., Barros, J., and Sequeiros, J. 1995. Genetic epidemiology of familial amyloidotic polyneuropathy (FAP)-type I in Póvoa do Varzim and Vila do Conde (North of Portugal). *Am. J. Hum. Genet.* 60: 512–521.
8. Holmgren, G., Costa, P.M.P., Andersson, C., Asplund, K., Steen, L., Beckman, L., Nylander, P.O., Teixeira, A., and Saraiva, M.J.M. 1994. Geographical distribution of TTR met30 carriers in northern Sweden: discrepancy between carrier frequency and prevalence rate. *J. Med. Genet.* 31: 351–354.
9. Holmgren, G., Ando, Y., Wikstöm, L., Rydh, A., and Suhr, O. 1997. Discordant symptoms in monozygotic twins with familial amyloidotic polyneuropathy (FAP) (TTR Met30). *Amyloid* 4: 178–180.
10. Takaoka, Y., Tashiro, F., Yi, S., Maeda, S., Takahashi, K., Sasaki, Y., and Yamamura, K. 1997. Comparison of amyloid deposition in two lines of transgenic mouse that model familial amyloidotic polyneuropathy, type I. *Transgenic Res.* 6: 261–269.
11. Nakagata, N. 1995. Studies on cryopreservation of embryos and gametes in mice. *Exp. Anim.* 44: 1–8.
12. Nakagata, N. 1996. Use of cryopreservation techniques of embryos and spermatozoa for production of transgenic (Tg) mice and for maintenance of Tg mouse lines. *Lab. Anim. Sci.* 46: 236–238.
13. Mitsuoka, T., Ohno, K., Benno, Y., Suzuki, K., and Namba, K. 1976. Die Faekalflora bei Menschen. IV. Mitteilung: Vergleich des neu entwickelten Verfahrens mit dem bisherigen üblichen Verfahren zur Darmfloraanalyse. *Zbl. Bakt. Hyg., I. Abt. Orig. A* 234: 219–233.
14. Mitsuoka, T., Sega, T., and Yamamoto, S. 1965. Eine verbesserte Methodik der qualitativen und quantitativen Analyse der Darmflora von Menschen und Tieren. *Zbl. Bakt., I. Abt. Orig.* 195: 455–469.
15. Itoh, K., Mitsuoka, T., Sudo, K., and Suzuki, K. 1983. Comparison of fecal flora of mice based upon different strains and different housing. *Z. Versuchstierk.* 25: 135–146.

16. Kanda, Y., Goodman, D.S., Canfield, R.E., and Morgan, F.J. 1974. The amino acid sequence of human plasma prealbumin. *J. Biol. Chem.* 249: 6796–6805.
17. Blake, C.C.F., Geisow, M.J., Oatley, S.J., Rerat, B., and Rerat, C. 1978. Structure of prealbumin: secondary, tertiary and quaternary interactions determined by Fourier refinement. *J. Mol. Biol.* 121: 339–356.
18. Colon, W., Lai, Z., McCutchen, S.L., Miroy, G.J., Strang, C., and Kelly, J.W. 1996. FAP mutations destabilize transthyretin facilitating conformational changes required for amyloid formation. *Ciba. Found. Symp.* 199: 228–238.
19. Colon, W. and Kelly, J.W. 1992. Partial denaturation of transthyretin is sufficient for amyloid fibril formation *in vitro*. *Biochem.* 31: 8654–8660.
20. Kelly, J.W., Colon, W., Lai, Z., Lashuel, H.A., McCulloch, J., McCutchen, S.L., Miroy, G.J., and Peterson, S.A. 1997. Transthyretin quaternary and tertiary structural changes facilitate misassembly into amyloid. *Adv. Protein Chem.* 50: 161–181.
21. Itoh, K. and Freter, R. 1989. Control of *Escherichia coli* populations by a combination of indigenous clostridia and lactobacilli in gnotobiotic mice and continuous-flow cultures. *Infect. Immun.* 57: 559–565.
22. Diamond, R.D. 1995. Mechanisms involved in neutrophil-mediated killing of *Candida albicans* hyphae. *Jpn. J. Med. Mycol.* 36: 1–6.
23. Gresham, H.D., Lowrance, J.H., Caver, T.E., Wilson, B.S., Cheung, A.L., and Lindberg, F.P. 2000. Survival of *Staphylococcus aureus* inside neutrophils contributes to infection. *J. Immunol.* 164: 3713–3722.
24. Yamazaki, S., Ueda, K., and Someya, S. 1981. Non-immunological resistance of mice to infection with a virulent strain of *Mycobacterium bovis*. S. Sasaki (ed.), *Advances in Germfree Research*. pp. 419–423. Tokai Univ. Press, Tokyo.
25. Stone, P.J., Campistol, J.M., Abraham, C.R., Rodgers, O., Shirahama, T., and Skinner, M. 1993. Neutrophil proteases associated with amyloid fibrils. *Biochem. Biophys. Res. Commun.* 197: 130–136.