

## **Linkage of Protection against Amyloid Fibril Formation in the Mouse to a Single, Autosomal Dominant Gene**

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### **Summary**

Inbred strains of mice provide a model for studies of the pathogenesis of amyloid A (AA) amyloidosis. All susceptible strains of mice described to date codominantly express two serum amyloid A (apoSAA) isoforms, apoSAA<sub>1</sub> and apoSAA<sub>2</sub>, of which only apoSAA<sub>2</sub> serves as a precursor for amyloid fibrils. In previous studies, we have shown that the CE/J strain, which produces a single, novel apoSAA isoform, apoSAA<sub>CE/J</sub>, is amyloid resistant. In the present study amyloid-resistant CE/J females were mated with amyloid-susceptible CBA/J males to produce F1 hybrid offspring which were then backcrossed to the parental CBA/J mouse strain. Amyloid susceptibility was determined in 30 backcrossed mice 72 h after injection of murine amyloid enhancing factor and silver nitrate. ApoSAA isoforms in plasma were separated by isoelectric focusing gel electrophoresis and visualized after immunoblotting with anti-AA antiserum. Amyloid A fibrils in spleen homogenates were denatured by formic acid and AA protein was quantified by ELISA using anti-mouse apoSAA antibodies. Values <5 apoSAA equivalent units were considered negative. 13 mice expressed an apoSAA<sub>1</sub> and apoSAA<sub>2</sub> doublet characteristic of CBA/J mice, whereas 17 mice, expressed the apoSAA<sub>CE/J</sub> isoform codominantly with apoSAA<sub>1</sub> and apoSAA<sub>2</sub>. The correlation of amyloid resistance to expression of the apoSAA<sub>CE/J</sub> isoform was absolute (17/17 were negative; mean score  $2.6 \pm 0.17$  [standard error of the mean] apoSAA equivalent units) and the correlation between amyloid susceptibility and the expression of apoSAA<sub>2</sub>/apoSAA<sub>1</sub> was also striking (12/13 were amyloid positive; mean score  $47.9 \pm 9.0$  [standard error of the mean] apoSAA equivalent units ( $P < 0.001$ )). This is not significantly different from the 50% segregation of apoSAA phenotypes expected for linkage to a single gene. These results indicate that a single gene governs apoSAA<sub>CE/J</sub> expression and thus confers protection against amyloid deposition even in the presence of apoSAA<sub>1</sub> and apoSAA<sub>2</sub> isoforms and show for the first time that resistance to AA amyloidosis is a dominant trait governed by a single gene.

**A**myloidosis is a disease characterized by progressive fibrillar protein deposits in various organs. To date more than 16 proteins have been shown to be amyloidogenic, including the  $\beta$ -amyloid protein characteristic of Alzheimer's disease (1). A common form of systemic amyloidosis is that which may develop secondary to chronic inflammatory diseases such as rheumatoid arthritis or leprosy (amyloid A [AA] amyloidosis) or as a result of a genetic abnormality as in familial Mediterranean fever (2). In the latter two syndromes, fibrils are composed of, or derived from, an acute phase protein, serum amyloid A (apoSAA).

AA amyloidosis has been described as a biphasic process (3, 4) that includes a latent, preamyloid phase that may last for decades before amyloid fibril formation takes place. The pathogenesis of AA amyloidosis is not fully understood, but

is known to involve a spectrum of factors including synthesis, catabolism, and structure of the precursor apoSAA, the pentaxin, serum amyloid P component, and other extracellular matrix constituents such as proteoglycans. The most commonly used experimental model of AA amyloidosis is one in which inbred strains of mice are subjected to multiple subcutaneous injections of casein or azocasein at intervals of 24 h or greater (5). In response to inflammatory stimulation, CBA/J and other amyloid-susceptible strains of mice, including A/J, codominantly express two major apoSAA isoforms, apoSAA<sub>1</sub> and apoSAA<sub>2</sub> (6-8). Only apoSAA<sub>2</sub> serves as a precursor for AA protein, which is comprised of the first 76 NH<sub>2</sub>-terminal amino acids and which is the predominant component of amyloid fibrils extracted from tissues (9-11). ApoSAA<sub>2</sub> is selectively and more rapidly cleared from

serum, although the reason for its exclusive deposition into amyloid fibrils in amyloid susceptible strains of mice is not clear. In our hands, most mouse strains tested to date vary with respect to the duration of the preamyloid phase with an induction time ranging between 5 d in CBA/J mice to >40 d in A/J mice (12). We have postulated that, in strains producing the amyloidogenic apoSAA<sub>2</sub> isoform, variability depends on the rate of production during the preamyloid phase of a poorly characterized substance called amyloid enhancing factor (AEF). AEF was first described as a "transfer factor" produced in donor mice that accelerated the induction of amyloid in recipient animals (13). Using a modification of the transfer model we have subsequently shown that the preamyloid phase in A/J mice can be reduced to <48 h by injecting them with AEF plus a single inflammatory stimulus (14).

We have recently discovered that inbred CE/J mice, which do not express the amyloidogenic apoSAA<sub>2</sub>, but rather a single, novel isotype, apoSAA<sub>CE/J</sub> (15) are completely resistant to amyloidosis (16). In pilot studies, F<sub>1</sub> hybrid mice were produced by mating CE/J females with CBA/J males: F<sub>1</sub> hybrid offspring were then backcrossed to the parental CE/J mouse strain. When challenged with azocasein, CBA/J mice developed amyloidosis within 10 d. Conversely, none of 20 CE/J mice, none of 10 F<sub>1</sub> hybrid CE/J × CBA/J mice, and none of 10 backcross F<sub>1</sub> × CE/J mice developed amyloidosis after 60 d (17). In the present study, we have used CBA/J × CE/J F<sub>1</sub> animals to complete the reciprocal backcross into the susceptible CBA/J parental strain.

## Materials and Methods

**Mice.** CBA/J mice (6 wk of age) were obtained through the Animal Procurement Program of the National Cancer Institute. CE/J mice (6–8 wk of age) were obtained from Jackson Laboratories, Bar Harbor, ME. All animals were housed in the Animal Research Facility at the E. N. Rogers Memorial VA Medical Center, Bedford, MA, under standard conditions. These facilities are AAALAC accredited and all experimental protocols were approved by the VA Animal Research Subcommittee and by the IACUC of Boston University School of Medicine.

**Amyloid Induction and Quantification.** 30 (CBA/J × CE/J) F<sub>1</sub> × CBA/J backcrossed mice were given a single intraperitoneal injection (200 µg total protein) of murine AEF (18) and a single, concomitant subcutaneous injection of 0.5 ml of 2% AgNO<sub>3</sub> as an inflammatory stimulus. 72 h later blood samples were taken by cardiac puncture after induction of CO<sub>2</sub> narcosis. The spleens were removed and representative samples were weighed, homogenized in normal saline, and AA fibrils denatured by incubation in 10% formic acid for 20 h at 37°C. AA protein was quantified by ELISA using anti-mouse apoSAA antibodies (15, 19). By this technique scores <5 apoSAA equivalent units are classified as negative.

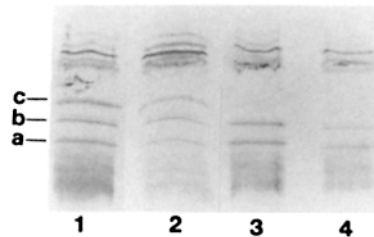
**Analysis of apoSAA Isoforms by IEF Gel Electrophoresis.** apoSAA proteins were separated by IEF and visualized after immunoblotting with anti-AA antiserum as described previously (16). Briefly, an ultrathin acrylamide gel was cast containing an ampholine gradient of 20% ampholines pH 3–10, 40% ampholines pH 4–6.5, and 40% ampholines pH 7–9 (Pharmacia LKB Biotechnology Inc.,

Piscataway, NJ) (20, 21). 20 µl of mouse plasma was lyophilized and delipidated with a 2:1 mixture of chloroform:methanol (22). The protein was then resuspended in 7 M urea, 1% decyl sodium sulfate, and 5% 2-mercaptoethanol. After IEF, proteins were transferred to nitrocellulose membranes. ApoSAA isoforms were detected with rabbit anti-mouse AA as primary antibody and an alkaline phosphatase-conjugated goat anti-rabbit IgG as secondary antibody.

## Results and Discussion

After treatment with AgNO<sub>3</sub> and AEF, spleens of the 17 backcrossed mice expressing the apoSAA<sub>CE/J</sub> isoform were all negative for amyloid (mean score 2.6 ± 0.17 (SEM)). In contrast, 12 of 13 mice expressing only apoSAA<sub>1</sub>/apoSAA<sub>2</sub> were positive (mean score 47.9 ± 9.0 (SEM)) ( $P < 0.001$ ). By IEF analysis, 13 of 30 mice expressed a characteristic apoSAA<sub>1</sub> and apoSAA<sub>2</sub> doublet, whereas 17 of 30 mice expressed the apoSAA<sub>CE/J</sub> isoform codominantly with apoSAA<sub>1</sub> and apoSAA<sub>2</sub> (Fig. 1, lane 1). This is not significantly different from the 50% segregation of apoSAA phenotypes expected if it were linked to a single gene. The correlation of amyloid resistance to expression of the apoSAA<sub>CE/J</sub> isoform was absolute (17/17 were negative) and the correlation between amyloid susceptibility and the expression of apoSAA<sub>2</sub> paired with apoSAA<sub>1</sub> was also striking (12/13 were amyloid positive). These results indicate that a single gene governs apoSAA<sub>CE/J</sub> expression and thus confers protection against amyloid deposition even in the presence of apoSAA<sub>1</sub> and apoSAA<sub>2</sub> isoforms. In combination with the results of our pilot study in which 100% of CBA/J × CE/J F<sub>1</sub> hybrids were negative for amyloid deposits (Table 1), these data support the hypothesis that the unique amyloidogenic molecular motif ascribed to apoSAA<sub>2</sub> protein (15) is incapable of overriding the structural properties of the apoSAA<sub>CE/J</sub> isoform in determining whether amyloid fibril formation occurs *in vivo*.

The novel apoSAA isoform, apoSAA<sub>CE/J</sub>, in CE/J strain mice has amino acid sequences characteristic of both apoSAA<sub>1</sub> and apoSAA<sub>2</sub>. It is identical to apoSAA<sub>1</sub> in the first 10 NH<sub>2</sub>-terminal amino acids, a region in which apoSAA<sub>2</sub> has sub-



**Figure 1.** IEF gel displaying apoSAA proteins after immunoblotting with anti-AA antiserum as described in Materials and Methods. Lane 1 shows the apoSAA<sub>1</sub> (a), apoSAA<sub>2</sub> (b), and apoSAA<sub>CE/J</sub> (c) isoform pattern always found in each CBA/J × F<sub>1</sub> backcrossed mouse ( $n = 17$ ). Lane 2 shows the similar isoform pattern found in parental F<sub>1</sub> CBA/J × CE/J hybrids. Lane 3 shows the isoform pattern previously described in CBA/J mice. Lane 4 shows the isoform pattern that is characteristic of CBA/J × F<sub>1</sub> backcrossed mice of which 12 developed amyloidosis.

**Table 1.** Summary of Studies Showing the Association of Amyloid Resistance with the apoSAA<sub>CE/J</sub> Isoform

Strain	Amyloid positive	apoSAA Profile			Reference
		apoSAA <sub>CE/J</sub>	apoSAA <sub>2</sub>	apoSAA <sub>1</sub>	
CBA/J	20/20	–	+	+	16
CE/J	0/20	+	–	–	16
F <sub>1</sub> (CBA × CE/J)	0/10	+	+	+	17
F <sub>1</sub> (CBA × CE/J) × CE/J	0/6	+	–	–	17
F <sub>1</sub> (CBA × CE/J) × CE/J	0/4	+	+	+	17
F <sub>1</sub> (CBA × CE/J) × CBA	12/13	–	+	+	*
F <sub>1</sub> (CBA × CE/J) × CBA	0/17	+	+	+	*

stitutions at positions 6 and 7 (Fig. 2). It differs from the amyloidogenic apoSAA<sub>2</sub> isoform in 6 amino acid residues although it is identical to apoSAA<sub>2</sub> at positions 75–77, the region at which serum amyloid precursor protein is cleaved to yield the AA fibril protein. It has been suggested that the amyloidogenic capacity of apoSAA resides in the amino acid residues of the NH<sub>2</sub>-terminus of the molecule (23). Peptides corresponding to the first 11 amino acids of apoSAA<sub>2</sub> but not apoSAA<sub>1</sub> were capable of forming fibrils in vitro. This implies that the amino acid differences at positions 6 and 7 (Val-His in apoSAA<sub>1</sub> compared with Ile-Gly in apoSAA<sub>2</sub>) might be the critical residues in the determination of amyloid fibril formation. In this regard, apoSAA<sub>CE/J</sub> is identical to apoSAA<sub>1</sub> in the first 10 amino acids but differs from both apoSAA<sub>1</sub> and apoSAA<sub>2</sub> at position 11 (Fig. 2).

There are several other possibilities for mechanisms by which the presence of apoSAA<sub>CE/J</sub> could confer resistance or protection. First, it might interfere with the process of fibrillogenesis itself, by binding a necessary cofactor or chaperone. AEF (14), proteoglycans (24), fibronectin (25), α 1-antichymotrypsin, and apolipoprotein E (26) have all been sug-

gested as possible modulators of fibril formation. By demonstrating that CE/J mice produce AEF in response to chronic inflammation (27), we have ruled out the possibility that an inherent lack of transfer factor is responsible for amyloid resistance in the CE/J mouse. A second possibility is that apoSAA<sub>CE/J</sub> could alter catabolism of other acute phase apoSAA proteins, possibly by interactions with cell-surface receptors on mononuclear phagocytic cells, to prevent cleavage of apoSAA<sub>2</sub> to the AA fibril. Third, it is conceivable that protection does not depend on apoSAA<sub>CE/J</sub> protein per se, and that a “resistance” factor is closely linked to the CE/J type apoSAA locus on chromosome 7 and presumably in a cluster of 79 kb or less (7, 28). A similar configuration was recently shown not to be the case in the A/J strain where the prolonged preamyloid phase was not determined by alleles at or close to the SAA locus (29).

The availability of murine strains that appear to be completely resistant to amyloid induction, even in the presence of amyloidogenic apoSAA precursor proteins, will be invaluable in answering a number of questions concerning structural specificity of interactions of apoSAA proteins with cholesterol and/or mechanisms of cell-apoSAA recognition. The discovery of a single gene which is inherited as an autosomal codominant trait for amyloid susceptibility in the murine model may have important implications with respect to the pathogenesis of amyloidosis secondary to familial Mediterranean fever, which is also governed by a single gene (2). Furthermore, the protective effect that is associated with the mouse apoSAA<sub>CE/J</sub> isoform may have relevance in certain forms of A-β amyloidosis, e.g., Alzheimer’s disease, where polymorphism in one or more gene loci may accelerate or retard amyloid fibril deposition (30).

	1	2	3	4	5	6	7	8	9	10	11
apoSAA <sub>1</sub>	Gly	Phe	Phe	Ser	Phe	Val	His	Glu	Ala	Phe	Gln
apoSAA <sub>2</sub>	Gly	Phe	Phe	Ser	Phe	Ile	Gly	Glu	Ala	Phe	Gln
apoSAA <sub>CE/J</sub>	Gly	Phe	Phe	Ser	Phe	Val	His	Glu	Ala	Phe	Leu

**Figure 2.** Comparison of NH<sub>2</sub>-terminal amino acid sequences (1–11) of apoSAA isoforms.

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