

AMYLOID-RELATED SERUM PROTEIN SAA IN  
ENDOTOXIN-INDUCED AMYLOIDOSIS OF THE MINK

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Amyloid fibrils deposited in secondary amyloidosis contain a unique protein (AA) as the major constituent (1-3). An antigenically related serum protein (SAA) (4, 5), present in normal sera in low concentration (6), is elevated in amyloidosis and in diseases which predispose to amyloidosis, such as rheumatoid arthritis, leprosy, and tuberculosis (4-8).

The finding that SAA has a monomeric form about 50% larger than AA (9, 10) is consistent with the suggestion that SAA represents a circulating precursor of the fibril protein (4, 5). However, direct evidence that SAA is converted into AA is lacking. To elucidate further the relationship between SAA and AA, we have studied the serum level of SAA during the development of amyloidosis. For this, we have used an animal model in which amyloidosis was induced in mink by repeated endotoxin injections (11). Endotoxin-induced amyloid fibrils in mink contain a protein equivalent to human AA, and the sera of some mink contain a related protein, mink SAA (12).

A dramatic increase in the serum level of SAA occurred within 24 h of the first endotoxin injection. Subsequently, the concentration of SAA decreased to relatively low levels, but increased again during the early amyloidotic phase.

### Materials and Methods

*Animals.* 19 mink (*Mustela vison*) of the standard type were used, 6 males and 13 females. All mink were less than 20-mo old at the beginning of the study.

*Antiamyloid Antiserum.* Rabbits were immunized with alkali-degraded amyloid fibrils (DAM) isolated from the liver of a mink with endotoxin-induced amyloidosis (12). Before use, the antiserum was absorbed with pooled normal mink serum.

*Induction of Amyloidosis.* *Escherichia coli* (O26:B6) endotoxin (Difco Laboratories, Detroit, Mich.) was dissolved in sterile saline (2 mg/ml), stored at -20°C, and thawed immediately before use. 13 mink received subcutaneous injections of endotoxin three times each week (2-6 mg according to body weight). Four control mink were similarly injected with sterile saline. Two additional mink received a single endotoxin injection (5 mg).

*Assessment of Amyloidosis.* Liver, spleen, and kidney sections, were examined for amyloid after staining with hematoxylin and eosin, Congo red, and thioflavine T. For immunochemical analyses 1 g of tissue was homogenized for 5 min in 20 ml phosphate-buffered saline using a VirTis "45" homogenizer (VirTis Co., Inc., Gardiner, N. Y.) and then centrifuged at 27,000 g for 30 min. The supernate was discarded and the washing procedure repeated three times. The final tissue sediment was dispersed in 4 ml 0.1 M NaOH, allowed to stand at room temperature overnight, and adjusted to pH 8.5 with 1 M HCl (liver) or dialysed for 16 h against water (spleen). The preparations were centrifuged at 27,000 g for 30 min and the supernates tested for protein AA by double diffusion against rabbit antimink DAM. The degree of amyloidosis was graded +, ++, or

+++ if the maximum dilution at which protein AA could be detected was 1:1, 1:10, or 1:100, respectively.

*Protein SAA Concentration.* Serum samples, from blood collected from the ventral tail artery, were stored at  $-20^{\circ}\text{C}$ . A semiquantitative estimation of the concentration of protein SAA was performed by double-diffusion analysis of twofold dilutions of the sera, using antimink DAM. There is good agreement between the concentration of SAA determined in this way and the concentration determined by radial diffusion (5).

*Antiendotoxin Antibodies.* Antibodies to endotoxin were determined by hemagglutination of sheep red blood cells coated with endotoxin (13), employing U-shaped microtiter plates (Cooke Laboratory Products Div., Dynatech Laboratories Inc., Alexandria, Va.). Hemagglutination titers were read after the plates were incubated at  $37^{\circ}\text{C}$  for 1 h and stored overnight at  $4^{\circ}\text{C}$ .

## Results

*Development of Amyloidosis.* Amyloid was first detected in a mink (mink 9) killed 28 days after the commencement of the course of endotoxin (Table I). In this mink amyloid was detected in the liver immunochemically but not histologically, whereas neither method detected amyloid in the spleen. All mink which received a longer course of endotoxin had marked amyloid deposition in both liver and spleen, whereas no evidence of amyloidosis was found in any mink receiving endotoxin for less than 28 days. No amyloid was found in the four control mink, which received saline injections for 7 wk.

*Protein SAA Concentration.* There was a sharp increase in the concentration of SAA after the first endotoxin injection (Fig. 1). SAA was detectable in only four mink before receiving any endotoxin (titers of 1, 4, 4, and 8) but 24 h after the first endotoxin injection all mink had a titer of either 64 or 128. During the subsequent 3-wk period, and before the deposition of detectable amyloid, the concentration of SAA in the sera decreased considerably, returning to near control levels at day 21 (Fig. 1). During the amyloidotic phase there was again a rise in the concentration of SAA with another subsequent decrease. The concentration of SAA during the amyloidotic phase never reached that which persisted

TABLE I  
*Induction of Amyloidosis in Endotoxin-Treated Mink*

Mink	Length of endotoxin treatment	Evidence of amyloidosis				
		Histology			Immunochemistry	
		Liver	Spleen	Kidney	Liver	Spleen
	<i>days</i>					
1	1	-	-	-	-	-
2	1	-	-	-	-	-
3	7	-	-	-	-	-
4	7	-	-	-	-	-
5	13	-	-	-	ND*	ND
6	13	-	-	-	ND	ND
7	21	-	-	-	-	-
8	21	-	-	-	-	-
9	28	-	-	-	+	-
10	53	+++	+++	-	+++	++
11	53	++	+++	-	+++	+++
12	60	++	+++	-	+++	++
13	62	+	++	-	+++	++

No evidence of amyloidosis was found in the control mink, all four of which received three saline injections each week for 7 wk.

\* ND, not determined.

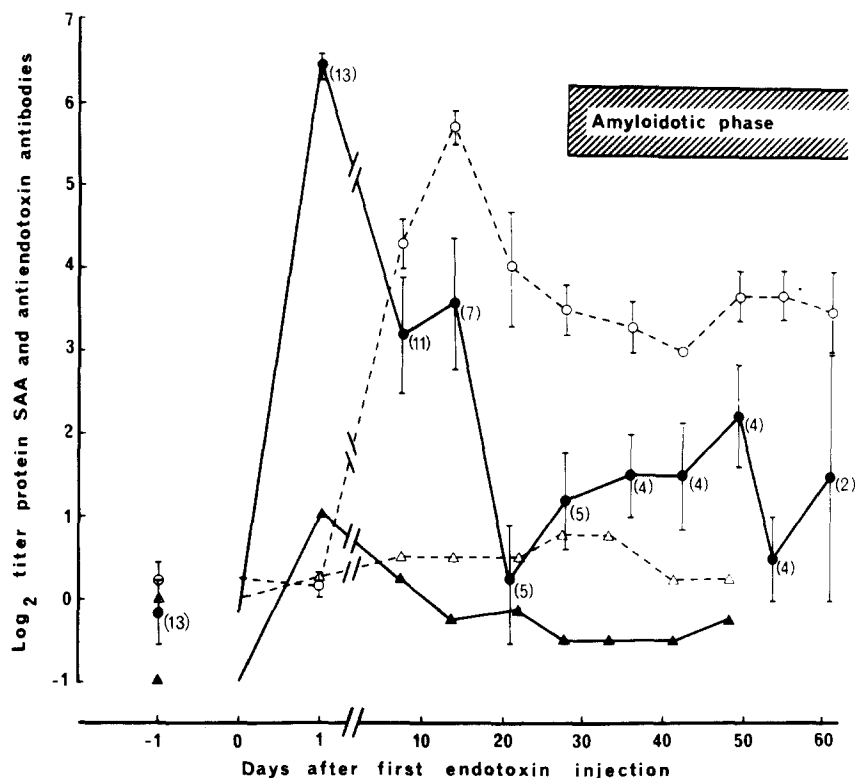


FIG. 1. Concentration of protein SAA: (●), treated and (▲), control; and antiendotoxin antibodies: (○), treated and (△), control; during the induction of amyloidosis by repeated endotoxin injections. The numbers in parentheses indicate the number of treated animals from which the geometric means and standard error of the means were calculated. All the control values were calculated from four mink.

for the first 14 days after the commencement of endotoxin treatment (Fig. 1).

All four control mink were negative for SAA when tested before receiving the first saline injection. At day 1 two of these mink had detectable SAA (a titer of 8 in each case). One of these mink maintained an elevated level of SAA throughout the 7-wk period of the experiment (titers ranged from 2 to 8), whereas the other was still positive at day 8 (titer of 4) but was negative when bled subsequently. Of the other two control mink, one was negative for SAA throughout the experiment and the other was negative at all bleeds except day 22 (titer of 2). Thus the SAA concentration did rise in some control mink but was consistently much lower than in the mink receiving endotoxin (Fig. 1). Either low amounts of endotoxin in the saline solution or inflammation resulting from the repeated bleeding could have caused the elevation of SAA in the control mink.

As SAA had already risen to high levels when blood was first taken 24 h after the first endotoxin injection, two additional mink were given a single subcutaneous injection of endotoxin (5 mg) and blood samples were collected at intervals during the subsequent 24-h period. To facilitate repeated bleeding the mink were anesthetized with nembutal. SAA was first detected at 3 h in one mink and

at 6 h in the other. One mink died at 6 h, presumably because of a synergistic effect between anesthetic and endotoxin. In the surviving mink the concentration of SAA increased progressively throughout the 24-h period. At 24 h the titer of SAA was 64, consistent with the results of the other experiment.

*Antibodies to Endotoxin.* Hemagglutination assays first detected antibodies 8 days after the first endotoxin injection at a time when the concentration of SAA was decreasing (Fig. 1). Thus, SAA was elevated before a detectable antibody response to the endotoxin. The highest antibody levels were detected at day 15, about 2 wk before the onset of amyloidosis, but significant levels of antibodies were detectable throughout the amyloidotic phase.

*Additional Experiments in Other Species.* The generation of SAA after endotoxin injection has also been studied in rabbit, mouse, and man. In all these species a marked increase in the concentration of SAA was observed within the same short period of time, similar to that in the mink.

### Discussion

Endotoxin, an effective amyloidogenic substance in various animal species including mink (11, 14), is reported here to be a potent stimulus for the generation of SAA, the putative precursor of the amyloid fibril protein AA. Whereas amyloidosis was first detected after 4 wk of endotoxin treatment, the concentration of SAA in the serum increased rapidly during the 24 h after the first endotoxin injection. Thus, in this animal model, elevation of SAA concentration occurs before the deposition of detectable amyloid, as in secondary amyloidosis in man, where high concentrations of SAA are detected in diseases such as rheumatoid arthritis (5), leprosy (8), and tuberculosis (6), before the development of amyloidosis.

The rise in SAA concentration before the formation of amyloid is consistent with the hypothesis that SAA is a precursor of AA, and the relatively lower levels of SAA found during the amyloidotic phase may be due in part to the conversion of SAA to AA with subsequent deposition in amyloid fibrils. However, the concentration of SAA fell sharply from the early high level well before the deposition of detectable amyloid. This suggests that mechanisms other than the formation of amyloid fibrils, for example, tolerance to endotoxin, may operate to limit the SAA response to later endotoxin injections.

The onset of amyloidosis 4 wk after the peak SAA concentration suggests that an elevated concentration of SAA per se is not a sufficient condition for the deposition of AA in amyloid fibrils. Formation of fibrils most likely also depends upon other events triggered by recurrent stimulation of SAA production. The tendency for the concentration of SAA to increase again during the early amyloidotic phase suggests that the onset of amyloidosis may depend on an induced defect of SAA catabolism.

We have recently established that the 20 amino acid N-terminal sequence of human SAA is identical to that of AA<sup>1</sup> and, therefore, conversion of SAA into AA would involve cleavage of SAA towards the C-terminus. However, an alternative possibility is that both SAA and AA are derived by proteolysis from

<sup>1</sup> Anders, R. F., K. Sletten, and J. B. Natvig. Manuscript in preparation.

a common, larger precursor. The N-terminal heterogeneity in SAA<sup>1</sup> as in some preparations of AA, the rapid appearance of SAA in serum after treatment with endotoxin, and the finding of an amyloid fibril protein AA larger than the SAA subunit (15) support this suggestion.

A more common experimental model of amyloidosis is casein-induced amyloidosis in mice. Investigations with this model to determine whether an antibody response to casein is important in the pathogenesis of amyloidosis have given conflicting results (16). Our results allow no conclusions as to the importance of the observed antiendotoxin response for the genesis of amyloid fibrils but provide clear evidence that such a response is not necessary for the generation of SAA.

Observations in man have indicated that SAA is an acute-phase reactant (6) and endotoxin is known to stimulate the synthesis of other acute-phase reactants. However, unlike the majority of acute phase reactants, SAA is not a glycoprotein (R. F. Anders, unpublished observation), and in one study the correlation between levels of SAA and C-reactive protein was poor (6). Whether SAA is synthesized in the liver as are other acute-phase reactants or is generated elsewhere, possibly by proteolytic degradation of a precursor molecule, is currently under investigation.

### Summary

The concentration of the amyloid AA-related serum protein (SAA) was markedly increased after endotoxin injections in mink, mouse, rabbit, and man. It was particularly studied during the development of endotoxin-induced amyloidosis of the mink. Protein SAA was markedly elevated in all mink 24 h after the first endotoxin injection but had fallen to relatively low levels before the onset of amyloidosis at about 4 wk. These results are consistent with SAA being a circulating precursor of the amyloid fibril protein, AA. However, both proteins may be derived by proteolysis from a common precursor.

*Received for publication 25 November 1975.*

### References

1. Benditt, E. P., N. Eriksen, M. A. Hermodson, and L. H. Ericsson. 1971. The major proteins of human and monkey amyloid substance: common properties including unusual N-terminal amino acid sequences. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 19:169.
2. Husby, G., K. Sletten, T. E. Michaelsen, and J. B. Natvig. 1972. Antigenic and chemical characterization of non-immunoglobulin amyloid proteins. *Scand. J. Immunol.* 1:393.
3. Levin, M., E. C. Franklin, B. Frangione, and M. Pras. 1972. The amino acid sequence of a major nonimmunoglobulin component of some amyloid fibrils. *J. Clin. Invest.* 51:2773.
4. Levin, M., M. Pras, and E. C. Franklin. 1973. Immunologic studies of the major nonimmunoglobulin protein of amyloid. *J. Exp. Med.* 138:373.
5. Husby, G., and J. B. Natvig. 1974. A serum component related to nonimmunoglobulin amyloid protein AS, a possible precursor of the fibrils. *J. Clin. Invest.* 53:1054.

6. Rosenthal, C. J., and E. C. Franklin. 1975. Variation with age and disease of an amyloid A protein-related serum component. *J. Clin. Invest.* 55:746.
7. Kronvall, G., G. Husby, D. Samuel, G. Bjune, and H. Wheate. 1975. Amyloid-related serum component (protein ASC) in leprosy patients. *Infect. Immun.* 11:969.
8. McAdam, K. P. W. J., R. F. Anders, S. R. Smith, D. A. Russell, and M. A. Price. 1975. Association of amyloidosis with erythema nodosum leprosum reactions and recurrent neutrophil leucocytosis in leprosy. *Lancet.* 2:572.
9. Anders, R. F., J. B. Natvig, T. E. Michaelsen, and G. Husby. 1975. Isolation and characterization of amyloid-related serum protein SAA as a low molecular weight protein. *Scand. J. Immunol.* 4:397.
10. Linke, R. P., J. D. Sipe, P. S. Pollock, T. F. Ignaczak, and G. G. Glenner. 1975. Isolation of a low-molecular-weight serum component antigenically related to an amyloid fibril protein of unknown origin. *Proc. Natl. Acad. Sci. U. S. A.* 72:1473.
11. Nordstoga, K. 1972. Amyloidosis in mink induced by repeated injections of endotoxin. *Acta Pathol. Microbiol. Scand. Sect. A Pathol.* 80:159.
12. Husby, G., J. B. Natvig, K. Sletten, K. Nordstoga, and R. F. Anders. 1975. An experimental model in mink for studying the relation between amyloid fibril protein AA and the related serum protein SAA. *Scand. J. Immunol.* 4:811.
13. Andersson, B., and H. Blomgren. 1971. Evidence for thymus-independent humoral antibody production in mice against polyvinylpyrrolidone and *E. coli* lipopolysaccharide. *Cell. Immunol.* 2:411.
14. Barth, W. F., J. T. Willerson, R. Asofsky, J. N. Sheagren, and S. M. Wolff. 1969. Experimental murine amyloid. III. Amyloidosis induced with endotoxins. *Arthritis Rheum.* 12:615.
15. Lian, J. B., M. D. Benson, M. Skinner, and A. S. Cohen. 1975. A 25,000 dalton protein constituent of human amyloid fibrils related to protein AA. *Arch. Biochem. Biophys.* 171:197.
16. Cohen, A. S., and E. S. Cathcart. 1972. Casein-induced experimental amyloidosis. I. Review of cellular and immunologic aspects. *Methods Achiev. Exp. Pathol.* 6:207.