

MURINE TISSUE AMYLOID PROTEIN AA  
NH<sub>2</sub>-Terminal Sequence Identity with Only One of Two Serum  
Amyloid Protein (ApoSAA) Gene Products

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In amyloidosis associated with chronic inflammatory disease processes, the characteristic extracellular protein fibrils that form deposits in various tissues are largely composed of an 8.5-kd polypeptide called protein AA (1, 2). Amino acid sequence analysis reveals that protein AA is homologous to the NH<sub>2</sub>-terminal portion of a 12-kd high density lipoprotein (HDL)<sup>1</sup> apoprotein, apoSAA (2-5). ApoSAA is normally a trace plasma constituent but is elevated many fold in response to a variety of disease states and pathologic conditions (6, 7). Since chronically elevated plasma apoSAA levels are predisposing to protein AA-type amyloidosis, it is currently believed that in amyloidosis a precursor-product relationship exists in which elevated apoSAA levels lead to the accumulation of the partial breakdown product, protein AA, found in amyloid fibril deposits.

Recent studies of apoSAA structure have revealed that in several mammalian species more than one form of apoSAA exists. In humans, two major (8) and four minor (9) apoSAA isotypes have been identified. Similarly, in the rabbit (10), monkey (11), mink (12), and mouse (12, 13) two electrophoretically distinct apoSAA isotypes have been described. The two murine apoSAA isotypes, apoSAA<sub>1</sub> (12.6 kd, pI = 6.35) and apoSAA<sub>2</sub> (11.8 kd, pI = 6.20), are present in nearly equal amounts and constitute as much as 20% of the total mouse HDL protein content during acute inflammation (13). With regard to amyloidosis, a question of interest is whether one or both apoSAA isotypes contribute to the formation of amyloid protein AA deposits. To address this question, we have purified the two major murine apoSAA isotypes, characterized them by NH<sub>2</sub>-terminal amino acid analysis, and compared the sequences obtained with that of murine amyloid protein AA. Our results indicate that the two major murine apoSAA isotypes are separate gene products and that amyloid protein AA has NH<sub>2</sub>-terminal identity with only one of these, namely apoSAA<sub>2</sub>.

**Materials and Methods**

*Sources and Purification of ApoSAA and Protein AA.* ApoSAA was obtained from the pooled serum of mice 20 h after intraperitoneal administration of 50 μg of bacterial

This work was supported in part by grants HL-03174, HL-07312, and GM-15731 from the National Institutes of Health.

<sup>1</sup> *Abbreviations used in this paper:* HDL, high density lipoprotein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate.

J. EXP. MED. © The Rockefeller University Press · 0022-1007/84/02/0641/06 \$1.00 641  
Volume 159 February 1984 641-646

lipopolysaccharide (*Salmonella typhi* lipopolysaccharide W, Difco Laboratories, Detroit, MI), as described previously (13). BALB/c mice were obtained from a stock maintained in the Department of Microbiology and Immunology at the University of Washington. C57BL/KsJ and CBA/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

HDL ( $1.063 < d < 1.21$  g/ml) was prepared by sequential ultracentrifugation and the delipidated apoproteins were separated by analytical isoelectric focusing using pH 3.5–10.0 ampholytes (400  $\mu$ g protein applied per gel), as described previously (13). Bands containing apoSAA<sub>1</sub> and apoSAA<sub>2</sub> were located by reference to opaque areas of companion gels, fixed in a solution of 14.7% trichloroacetic acid and 4% sulfosalicylic acid. The apoproteins were eluted from excised and homogenized gel slices by two successive extractions in ~10 vol of 8 M urea/formic acid, pH 3.0, with overnight mixing by vortex at room temperature. The extracts were then dialyzed in Spectrapor 3 tubing against 0.5% acetic acid and lyophilized. Residual ampholytes were removed by two successive extractions with 0.25 ml of 10% trichloroacetic acid, in which the protein was precipitated by a 10-min centrifugation at ~13,800 g, with a final precipitation in 0.2 ml of -10°C acetone.

Amyloid protein AA was extracted from amyloidotic livers of C57BL/Ks mice injected with *Candida albicans* (14), from amyloidotic spleens of CBA/J mice subjected to 2–3 wk of daily casein administration (15), and from mixed amyloidotic tissue (liver, spleen, kidney) of casein-treated C57BL/6J mice (the latter provided by Dr. Z. Ali-Khan, Department of Microbiology and Immunology, McGill University). Samples of tissue were homogenized, extracted, and centrifuged (finally in 6 M urea/0.01 M HCOONa/HCOOH, pH 3.0) essentially as previously described (14). The centrifuged urea/formic acid extracts were lyophilized after dialysis in Spectrapor 3 tubing vs. 0.5% CH<sub>3</sub>COOH. Protein AA was isolated from the lyophilized products by Sephadex G-100 chromatography in the urea/formic acid buffer (14). In another approach, crude protein AA was extracted from the washed and homogenized tissues by sonication and boiling in 7.5% sodium dodecyl sulfate (SDS); protein AA was purified by elution (16) following SDS/urea polyacrylamide gel electrophoresis (PAGE) (16) of the crude extract.

*Analysis.* SDS/urea PAGE was performed as described elsewhere (13).

NH<sub>2</sub>-terminal amino acid sequences were determined with a Beckman sequencer (Model 890C) as described previously (17).

## Results and Discussion

Fig. 1 shows SDS/urea PAGE patterns of mouse HDL apoproteins (*a* and *b*) and purified amyloid proteins (*c–e*). By comparison with the apoprotein content of control mouse HDL (*a*), the HDL obtained from mice 20 h following endotoxin administration is enriched in two polypeptides, apoSAA<sub>1</sub> (12.6 kd) and apoSAA<sub>2</sub> (11.8 kd). Lanes *c* and *d* show purified apoSAA<sub>1</sub> and apoSAA<sub>2</sub>, respectively, prepared by isoelectric focusing. Lane *e* shows chromatographically purified murine amyloid protein AA (8.6 kd).

Fig. 2 shows the NH<sub>2</sub>-terminal amino acid sequences of purified murine apoSAA<sub>1</sub>, apoSAA<sub>2</sub>, and amyloid protein AA obtained from several mouse strains. Each purified apoSAA isotype was observed to be a single polypeptide through 26 NH<sub>2</sub>-terminal residues, differing from the other isotype in two positions, namely residues 6 and 7. Whereas apoSAA<sub>1</sub> contained valine and histidine, respectively, in these positions, the corresponding sequence in apoSAA<sub>2</sub> was isoleucine-glycine. This difference was a consistent finding within the three mouse strains (BALB/c, C57BL/KsJ, and CBA/J) examined. A comparison with two previous reports (12, 18) reveals that our findings are consistent with those of Anders et al. (12), who found both valine and isoleucine at position 6, the

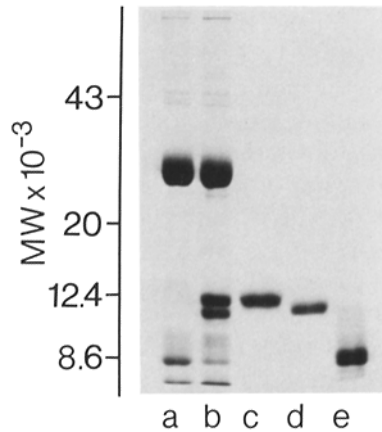


FIGURE 1. SDS/urea polyacrylamide gel electrophoretic patterns of (a) normal mouse (BALB/c) HDL apoproteins, (b) HDL apoproteins obtained 20 h following bacterial endotoxin administration, (c) apoSAA<sub>1</sub> and (d) apoSAA<sub>2</sub>, purified by isoelectric focusing, and (e) murine (C57BL/6J) amyloid protein AA. Molecular weight (MW) markers are ovalbumin (43 kd), soybean trypsin inhibitor (20 kd), cytochrome c (12.4 kd), and monkey amyloid protein AA (8.6 kd).

Protein	Mouse Strain	Observed Sequence																																				
		1	5	10	15	20	25	30	35																													
SAA <sub>1</sub>	CBA/J	G	F	F	S	F	V	H	E	A	F	Q	G	G	D	M	R	A	Y	T	D	M	K	E	A	N	W	K	N	S	D	K	Y	F	H	A	R	
SAA <sub>1</sub>	BALB/c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SAA <sub>1</sub>	C57BL/ksJ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
SAA <sub>2</sub>	CBA/J*	-	-	-	-	?	I	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
SAA <sub>2</sub>	BALB/c	-	-	-	-	-	I	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
SAA <sub>2</sub>	C57BL/ksJ	-	-	-	-	-	I	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
AA	CBA/J	-	-	-	-	-	I	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AA	C57BL/ks**	-	-	-	-	-	I	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AA	C57BL/6J	-	-	-	-	-	I	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

\*Mixture of 40% as written and 60% starting at residue 6.

\*\*From Eriksen *et al.* (14).

FIGURE 2. Amino-terminal sequences of apoSAA isotypes and tissue protein AA from various strains of mice. A hyphen indicates identity with apoSAA<sub>1</sub> (top line). A question mark indicates that the identification was not accomplished. Single letter code: alanine, A; arginine, R; asparagine, N; aspartic acid, D; glutamine, Q; glutamic acid, E; glycine, G; histidine, H; isoleucine, I; lysine, K; methionine, M; phenylalanine, F; serine, S; threonine, T; tryptophan, W; tyrosine, Y; valine, V.

presumptive result of analyzing a mixture of apoSAA<sub>1</sub> and apoSAA<sub>2</sub>; at position 7 they found only glycine. Anders *et al.* (12) also reported that ~35% of their sample, lacking the NH<sub>2</sub>-terminal pentapeptide, began at isoleucine, a phenomenon that we also encountered in one of three samples of apoSAA<sub>2</sub> (from CBA/J mice). In another study, Gorevic *et al.* (18) found a mixture of glutamic acid and valine at position 6 in sequences of apoSAA from several mouse strains (including BALB/c), isoleucine with valine at this position in only one instance,

and glycine in position 7 in all cases; at other positions they found mixtures of amino acids not observed either by us or by Anders et al. (12). The failure of previous workers to detect histidine in addition to glycine at position 7 (as expected from samples containing both apoSAA<sub>1</sub> and apoSAA<sub>2</sub>) might be explained if, in the earlier studies, only the organic-soluble PTH-amino acid derivatives were examined at this position, leaving the aqueous-soluble histidine-PTH derivative undetected. The unambiguous assignment of a single amino acid sequence to each of the two apoSAA isotypes in the present study clearly identifies them as distinct polypeptides translated from different genes rather than as posttranslationally modified forms of a single precursor.

We next examined the amino terminal sequence of protein AA found in amyloid deposits to determine whether it corresponded to one or both serum apoSAA isotypes. The NH<sub>2</sub>-terminal segments of protein AA from several strains of mice were examined for isotype-specific sequences in positions 6 and 7. The sequences obtained for protein AA isolated from the mixed tissues (liver, spleen, and kidney) of CBA/J mice and C57BL/6J mice, and the previously published sequence of liver protein AA from C57BL/Ks mice (14) are shown in Fig. 2. In all cases, amyloid protein AA showed NH<sub>2</sub>-terminal amino acid sequence identity with murine apoSAA<sub>2</sub>. Sequences corresponding to apoSAA<sub>1</sub>, i.e., containing the valine-histidine pair in positions 6 and 7, were not detected under conditions that would have identified 5%. Furthermore, we observed differences between amyloid protein AA and apoSAA<sub>1</sub> at residues 27, 30, and 31 (as shown, Fig. 2), although a comparison with both serum isotypes was not possible in this region.

In one experiment, protein AA was purified by elution from gel slices after SDS PAGE (16) of proteins obtained by extraction of amyloidotic C57BL/6J tissues in 7.5% SDS, a method that solubilizes >90% of the tissue protein content (J. Hoffman, unpublished observations). Again, the protein AA obtained by this method exclusively contained apoSAA<sub>2</sub>-type sequences, indicating that these observations do not result from an artifact of our preparative methods.

Gorevic et al. (18) originally suggested the possibility that murine amyloid protein AA might be derived from only one of several forms of apoSAA polypeptides. Our present findings show, first, that the two major murine apoSAA isotypes are separate gene products and second, that amyloid protein AA could be derived from only one of these, namely apoSAA<sub>2</sub>. Several mechanisms could explain these observations. Differences in the structure of murine apoSAA<sub>1</sub> and apoSAA<sub>2</sub> may play a role in the selective deposition of apoSAA<sub>2</sub>-derived protein AA during the pathogenesis of amyloidosis, or both proteins may be initially deposited but broken down at different rates, leading to the selective retention of apoSAA<sub>2</sub>-derived protein AA. Alternatively, differential gene expression during chronic inflammation could result in the preferential synthesis of apoSAA<sub>2</sub> during amyloidogenesis. We note that in addition to the two major isotypes sequenced here we observe trace bands upon isoelectric focusing that may correspond to minor apoSAA isotypes (13), consistent with the recent report of an mRNA-derived plasmid that encodes a polypeptide having only 71% identity with the NH<sub>2</sub>-terminal region (residues 8–28) of murine amyloid protein AA (19).

We recently reported the complete 104-amino acid sequence of one fraction

of human apoSAA (13) containing two distinct proteins that differ from each other only at positions 52 and 57. The first 20 residues of both are identical with those reported for human protein AA from several laboratories (20–22), but amino acids reported for the tissue amyloid proteins at residues 23, 53, 60, 66, 69, 71, and 75 are not in accord with our identifications in the two apoSAA subspecies found (13). Thus, there appear to be several human allotypes and/or gene loci coding for apoSAA and protein AA. By analogy with our findings using the animal model, the intriguing possibility is suggested that in humans protein AA is derived from a subset of the total complement of apoSAA isotypes.

### Summary

Amyloid protein AA is the presumptive fragment of an acute phase serum apolipoprotein, apoSAA. Two major murine apoSAA isotypes (apoSAA<sub>1</sub> and apoSAA<sub>2</sub>) have been identified. The NH<sub>2</sub>-terminal amino acid sequences of purified murine apoSAA<sub>1</sub> and apoSAA<sub>2</sub> have been examined and compared with that of murine amyloid protein AA. Our results indicate that apoSAA<sub>1</sub> and apoSAA<sub>2</sub> are separate gene products and that amyloid protein AA has NH<sub>2</sub>-terminal amino acid sequence identity with only one of these isotypes, namely apoSAA<sub>2</sub>.

We thank Virginia Wejak for her help in preparing the manuscript.

### References

1. Benditt, E. P., and N. Eriksen. 1971. Chemical classes of amyloid substance. *Am. J. Pathol.* 65:231.
2. Rosenthal, C. J., E. C. Franklin, B. Frangione, and J. Greenspan. 1976. Isolation and partial characterization of SAA—an amyloid-related protein from human serum. *J. Immunol.* 116:1415.
3. Benditt, E. P., and N. Eriksen. 1977. Amyloid protein SAA is associated with high density lipoprotein from human serum. *Proc. Natl. Acad. Sci. USA.* 74:4025.
4. Benditt, E. P., N. Eriksen, and R. H. Hanson. 1979. Amyloid protein SAA is an apoprotein of mouse plasma high density lipoprotein. *Proc. Natl. Acad. Sci. USA.* 76:4092.
5. Skogen, B., A. L. Børresen, J. B. Natvig, K. Berg, and T. E. Michaelsen. 1979. High density lipoprotein as carrier for amyloid-related protein SAA in rabbit serum. *Scand. J. Immunol.* 10:39.
6. Levin, M., M. Pras, and E. C. Franklin. 1973. Immunologic studies of a major nonimmunoglobulin protein of amyloid. I. Identification and partial characterization of a related serum component. *J. Exp. Med.* 138:373.
7. 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.
8. Eriksen, N., and E. P. Benditt. 1980. Isolation and characterization of the amyloid related apoprotein (SAA) from human high density lipoprotein. *Proc. Natl. Acad. Sci. USA.* 77:6860.
9. Bausserman, L. L., P. N. Herbert, and K. P. W. J. McAdam. 1980. Heterogeneity of human serum amyloid A proteins. *J. Exp. Med.* 152:641.
10. Tobias, P. S., K. P. W. J. McAdam, and R. J. Ulevitch. 1982. Interactions of bacterial lipopolysaccharide with acute-phase rabbit serum and isolation of two forms of rabbit serum amyloid A. *J. Immunol.* 128:1420.

11. Parks, J. S., and L. L. Rudel. 1979. Isolation and characterization of high density lipoproteins in the non-human primate (Vervet). *J. Biol. Chem.* 254:6716.
12. Anders, R. F., J. B. Natvig, K. Sletten, G. Husby, and K. Nordstoga. 1977. Amyloid-related serum protein SAA from three animal species: comparison with human SAA. *J. Immunol.* 118:229.
13. Hoffman, J. S., and E. P. Benditt. 1982. Changes in high density lipoprotein content following endotoxin administration in the mouse: formation of serum amyloid protein-rich subfractions. *J. Biol. Chem.* 257:10510.
14. Eriksen, N., L. H. Ericsson, N. Pearsall, D. Lagunoff, and E. P. Benditt. 1976. Mouse amyloid protein-AA: homology with nonimmunoglobulin protein of human and monkey amyloid substance. *Proc. Natl. Acad. Sci. USA.* 73:964.
15. Benson, M. D., M. A. Scheinberg, T. Shirahama, E. S. Cathcart, and M. Skinner. 1977. Kinetics of serum amyloid protein A in casein-induced murine amyloidosis. *J. Clin. Invest.* 59:412.
16. Hager, D. A., and R. R. Burgess. 1980. Elution of proteins from sodium dodecyl sulfate-polyacrylamide gels, removal of sodium dodecyl sulfate, and renaturation of enzymatic activity: results with sigma subunit of *Escherichia coli* RNA polymerase, wheat germ topoisomerase, and other enzymes. *Anal. Biochem.* 109:76.
17. Parmelee, D. C., K. Titani, L. H. Ericsson, N. Eriksen, E. P. Benditt, and K. A. Walsh. 1982. Amino acid sequence of amyloid-related apoprotein (apoSAA<sub>1</sub>) from human high-density lipoprotein. *Biochemistry.* 21:3298.
18. Gorevic, P. D., Y. Levo, B. Frangione, and E. C. Franklin. 1978. Polymorphism of serum amyloid A (AA and SAA) proteins in the mouse. *J. Immunol.* 121:138.
19. Stearman, R. S., C. A. Lowell, W. R. Pearson, and J. F. Morrow. 1982. Regulation of synthesis of amyloid A-related protein. *Ann. N.Y. Acad. Sci.* 389:106.
20. 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.
21. Sletten, K., and G. Husby. 1974. The complete amino acid sequence of non-immunoglobulin amyloid fibril protein AS in rheumatoid arthritis. *Eur. J. Biochem.* 41:117.
22. Møyner, K., K. Sletten, G. Husby, and J. B. Natvig. 1980. An unusually large (83 amino acid residues) amyloid fibril protein AA from a patient with Waldenström's macroglobulinaemia and amyloidosis. *Scand. J. Immunol.* 11:549.