## *Brief Definitive Report*

# MURINE MODEL FOR HUMAN SECONDARY AMYLOIDOSIS: GENETIC VARIABILITY OF THE ACUTE-PHASE SERUM PROTEIN SAA RESPONSE TO ENDOTOXINS AND CASEIN

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Amyloidosis is the generic name for conditions characterized by the tissue deposition of protein fibrils having a β-pleated sheet conformation. In man, at least three major varieties of amyloid fibril proteins have been identified: amyloid of immunoglobulin origin, amyloid of neurohormonal origin, and the nonimmunoglobulin amyloid of unknown origin which consists of protein AA. Immunoglobulin amyloid occurs in patients with myeloma-associated amyloidosis, and in some primary amyloid syndromes; neuroendocrine amyloid is usually localized to tissues of neuroectodermal origin where the major fibril protein is a hormone or prohormone; and amyloid protein AA occurs in patients with amyloidosis secondary to chronic inflammatory diseases, such as rheumatoid arthritis, familial Mediteranean fever, and leprosy. Immunoglobulin and neuroendocrine amyloid have not been described in the mouse. To date, all murine amyloid proteins studied are similar (1) and show amino acid sequence homology with AA proteins from man, monkey, mink, guinea pig, and duck (2).

Antisera to denatured AA fibrils cross-react with a serum protein designated SAA, which is thought to be a precursor of the AA fibril protein, as its $NH_2$-terminal sequence is identical to that of AA (3, 4). Murine SAA is similar in structure to human SAA in that it has a native mol wt of 160,000 and it can be dissociated to a more stable species of mol wt 12,500 (5–7). Recent studies suggest that human SAA is an acute-phase protein (8), which is elevated in a variety of acute and chronic conditions (9). Elevated SAA levels have been detected by double immunodiffusion, within 24 h of endotoxin administration in mink and mice (10).

In this study we utilize a solid-phase radioimmunoassay for AA protein (6) to characterize the acute-phase nature of murine SAA in response to bacterial lipopolysaccharide (LPS) and casein, which are known to induce amyloidosis in mice when given by repeated daily injections. Several studies have suggested that immune dysfunction is important in amyloidogenesis and in order to evaluate this, a comparison was made of the ability of LPS, a known B-cell mitogen, to stimulate SAA elevation in 12 strains of mice. These included T-cell-deficient nude mice, two strains (C3H/HeJ and CBA/N) known to be defective in a range of B-cell activities, including stimulation by LPS, as well as strains that have been commonly used for amyloid induction in the past. The SAA response of two syngeneic strains, C3H/HeN and C3H/HeJ, has been compared using LPS extracted in phenol from *Salmonella typhosa* or *Escherichia coli* K235. The

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amyloid resistance of AJ mice and colchicine-treated mice has been assessed to examine whether this resistance is a result of decreased acute-phase SAA production. Finally, preliminary studies on the site of origin of SAA are reported.

## Materials and Methods

*Animals.* 6- to 8-wk-old male mice were obtained from either The Jackson Laboratory, Bar Harbor, Maine or from Animal Production, National Institutes of Health, Bethesda, Md. Strains tested were Swiss Webster GP, C57BL6, C57BL6Be, CDF, DBA/2FD, CBA/N, CBA/J, BALB/c, nu/nu(BALB), AJ, C3H/HeN, and C3H/HeJ.

*SAA Inducers.* LPS extracted by the Westphal method (W) from *S. typhosa* (Difco Laboratories, Detroit, Mich.) was given at the sublethal dose of 50 $\mu$g intraperitoneally (i.p.). Phenol-extracted LPS from *E. coli* K235 was kindly donated by Doctors J. Ryan and L. M. Glode, NIH. Casein Hammersten and azocasein were injected in saline i.p. at a dose of 14 mg in 0.2 ml.

*Colchicine.* Colchicine was given 1–4 h before injection of LPS at a dosage of up to 0.1 mg i.p., which had previously been shown to prevent the formation of amyloidosis in mice (11, 12).

*Tissue Origin of SAA.* Mice were sacrificed at the height of an acute SAA response to LPS and homogenates of lung, heart, thymus, lymph node, spleen, kidney, and liver were incubated with 10% (vol/vol) formic acid for 24 h at 37°C, before being assayed by radioimmunoassay for AA cross-reacting material. In order to minimize the amount of SAA-rich blood remaining in the organs, anesthetized animals were exchange transfused with saline before death, using about four times total blood volume by cardiac perfusion. All organs except spleen looked pale after this procedure.

*Radioimmunoassay for SAA.* A solid-phase radioimmunoassay for murine AA was developed using the method recently described for the human AA protein (1, 6). Rabbit anti-mouse AA (kindly made available by Dr. Scott Pollock, NIH) was purified by affinity chromatography (6, 13). Serum samples were incubated at 37°C for 24 h in 10% formic acid before assay on plastic microtitration plates coated with anti-AA immunoglobulin.

## Results

*Strain Differences.* An initial screening experiment of SAA response to LPS *S. typhosa* in 12 strains of mice showed marked strain variation (Fig. 1). The SAA concentration for all strains except C3H/HeJ was elevated by 8 h and rose from a baseline of less than 200 ng/ml to more than 200 $\mu$g/ml by 24 h. At this stage a precise comparison cannot be made among the other 11 strains, except to note that the thymus-deficient nude mice had high baseline SAA levels, which increased after LPS administration, and no abnormality in the response of CBA/N mice was noted.

*The Acute-Phase SAA Response.* A more detailed time-response experiment in C3H/HeN mice (Fig. 2*a*) showed a dramatic increase in SAA levels, beginning 4–6 h after LPS and rising to a maximum at 18–22 h. Control animals given saline showed no rise in SAA. In contrast to C3H/HeN in which amyloid is easily induced, AJ mice are amyloid resistant (14). However, their time-SAA response curves are very similar (Figs. 2*a* and 2*b*). Casein and azocasein administration also caused elevation in SAA levels to 150 $\mu$g/ml by 8 h in C3H/HeN and AJ mice. C3H/HeJ mice produced similar increases in SAA concentration after casein, in contrast to their diminished SAA response to LPS.

Tissues harvested at the height of the SAA response to LPS showed that liver homogenates had 20 times and kidney homogenates 10 times the concentration per gram wet weight of tissue of AA cross-reacting material found in organs from a control animal, which had received saline. Spleen, lung, and heart homogenates had low SAA concentrations in both control and test animals.

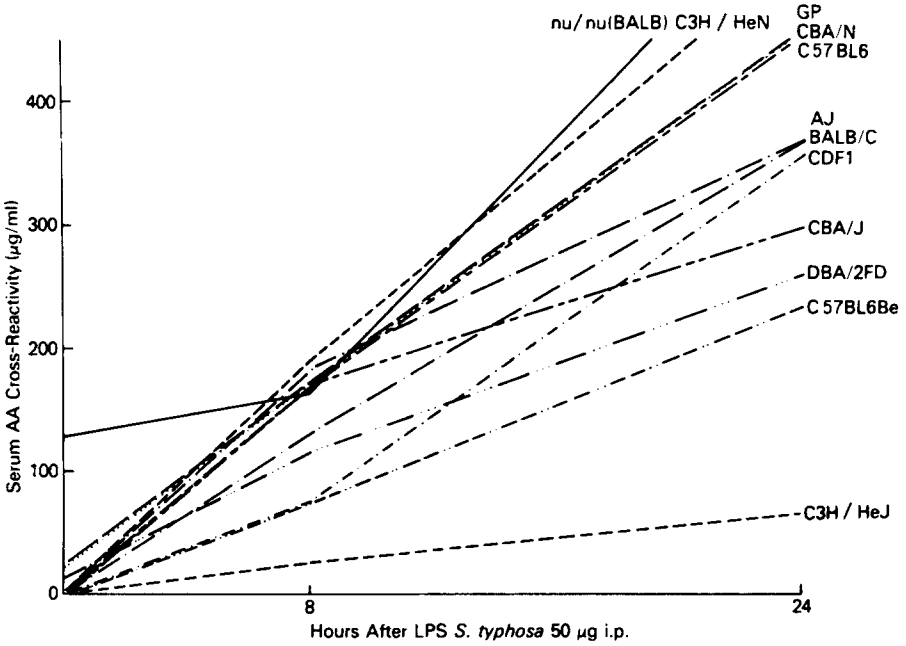


FIG. 1. SAA response of 12 strains of mice to i.p. 50 μg *S. typhosa* (W). Two mice of each species were bled from the orbital sinus at time points 0, 8 h, and 24 h after LPS. Control mice of each strain bled at 24 h after saline showed no rise in SAA level.

Experiments with colchicine were designed to assess whether its documented activity of preventing amyloid induction in mice (11, 12) could be attributed to blocking the acute elevations of SAA observed after LPS or casein injections. However, pretreatment of mice with colchicine, in dosages which block amyloid induction, did not cause any decrease in SAA levels observed at 8 and 24 h, and colchicine alone caused no rise in SAA levels.

*Dose-Response in C3H/HeJ Mice.* The low SAA response of C3H/HeJ mice to LPS administration was investigated by comparison with the syngeneic strain C3H/HeN using two different phenol-extracted LPS preparations from *S. typhosa* and *E. coli* K235 (Fig. 3). C3H/HeJ animals were less sensitive than C3H/HeN mice to both LPS preparations, in terms of lethality and their SAA response. A maximal SAA concentration of 750 μg/ml was obtained with 5 μg of either LPS preparation in C3H/HeN mice; the $LD_{50}$ was 100 μg with *S. typhosa* LPS and between 100–250 μg with *E. coli* K235 LPS. But in the C3H/HeJ animals 1,000 μg LPS *S. typhosa* and more than 3,000 μg of the *E. coli* K235 material were required to stimulate levels of SAA comparable to that induced in C3H/HeN mice and none of the animals died at those dosages. Individual variability was particularly noted in C3H/HeN animals.

## Discussion

For over 100 yr, secondary amyloidosis has largely remained an abstruse interest of pathologists as an untreatable, progressive condition which complicates chronic, longstanding infectious, or inflammatory diseases. It therefore

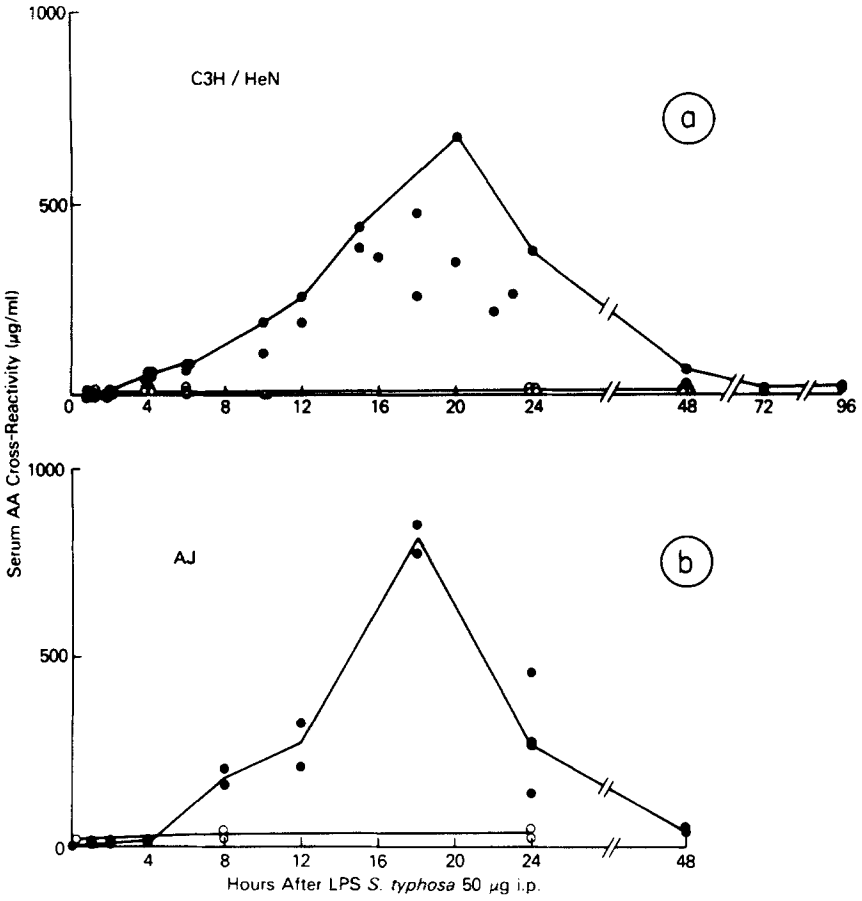


FIG. 2. (*a*) SAA response of C3H/HeN and (*b*) amyloid-resistant AJ mice after i.p. 50 µg LPS *S. typhosa* (W); (●—●), test; (○—○), control animals (i.p. saline).

comes as a surprise to find that the precursor protein of the amyloid AA fibril is a normal serum protein which fluctuates many hundred-fold within hours of an acute stimulus. A report that SAA acts as an immunosuppressant in in vitro assays (15) indicates the possible functional significance of this acute-phase protein.

In this study two polyclonal B-lymphocyte mitogens, LPS and casein (16), have been shown to cause acute elevation of SAA concentration in 12 strains of mice. These included the T-cell-deficient nude mice and the CBA/N strain which are thought to have immature B cells. However, C3H/HeJ animals, which are insensitive to many of the actions of LPS (17), produced low levels of SAA in response to two preparations of LPS (*S. typhosa* and *E. coli* K235). The SAA response at high LPS dosages might be explained by contamination of the phenol-extracted LPS with a cell wall peptide mitogen (18).

In contrast to the low SAA response of C3H/HeJ mice to LPS, they resembled C3H/HeN in SAA response to casein derivatives which, like LPS, are thought to be B-cell mitogens. This finding supports the concept that the defect in C3H/HeJ is limited to a range of B-cell responses to LPS. Mice treated with colchicine and

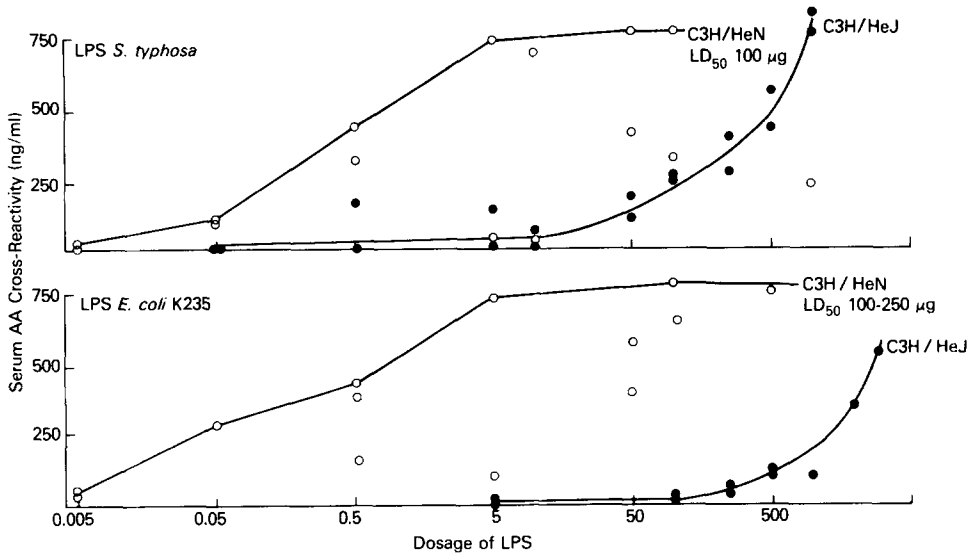


FIG. 3. LPS dose-SAA response curves of C3H/HeN and C3H/HeJ animals after two types of LPS: *S. typhosa* (W) and *E. coli* K235 (phenol extracted). Clinical manifestations of diarrhea and conjunctivitis were noted at dosages of LPS over 5 μg in C3H/HeN mice ($LD_{50}$, 100–250 μg) but were not seen in C3H/HeJ mice, none of which died after LPS.

AJ mice have been shown to be resistant to amyloid induction. Since they produce normal quantities of SAA in response to LPS, it appears that the amyloid resistance is related, not to SAA synthesis or release, but to the processing and catabolism of SAA at the lysosomal level, where fibril formation is thought to occur.

Although the SAA concentration is highest at 24 h, when liver, and to a lesser extent, kidney were the organs containing most AA cross-reactivity, amyloid fibrils are not observed in tissues until 7–10 days later. Interaction with antibodies and overloading of the lysosomal enzyme systems which normally process SAA may be reasons why amyloid deposition does not occur immediately and requires prolonged antigenic stimulation.

## Summary

The serum precursor SAA of the secondary amyloid protein AA has been detected by solid-phase radioimmunoassay as a normal serum α-globulin of mol wt 160,000, which dissociates to a more stable 12,500 dalton moiety on treatment with formic acid. In 12 strains of mice, including T-cell-deficient nude mice, treated with the amyloid-inducing agents lipopolysaccharide (LPS) or casein, SAA behaved as an acute-phase reactant. SAA concentration rose to about 750 μg/ml by 24 h and returned to less than 1 μg/ml by 48 h. Since the amyloid-resistant colchicine-treated mice and AJ mice had a normal SAA response to LPS, it appears that their resistance to amyloid induction is due to the nature of their SAA processing rather than decreased SAA production. C3H/HeJ mice, which have defective B-lymphocyte responses to LPS, required extremely high dosages of LPS to cause SAA elevation, although their SAA response to casein was normal. This suggests that SAA is an acute-phase protein produced as a

result of B-lymphocyte stimulation. Preliminary evidence suggests that at the height of an acute SAA response, liver homogenates are particularly rich in protein AA cross-reacting material.

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