Some Effects of the Administration of Endotoxin in Mice

SPECIFIC CLEAVAGE OF SERUM ALBUMIN BY AN ACID PROTEASE AND THE GENERATION OF AMYLOID SERUM COMPONENT

PETER D. GOREVIC, YORAM LEVO, PREM C. CHATPAR, BLAS FRANGIONE, and E. C. FRANKLIN, *Departments of Medicine and Pathology, Rheumatic Diseases Study Group, Irvington House Institute, New York University Medical Center, New York 10016*

ABSTRACT Endotoxin has been shown to induce amyloidosis in mice and to result in the appearance in serum of large amounts of amyloid-related protein (SAA). After injection of 300 μg lipopolysaccharide *Escherichia coli*, SAA behaves as an acute phase reactant with levels reaching a peak of >600 μg/ml at 18–22 h and returning to base line (<50 μg/ml) by 48 h in each of four strains tested; only the endotoxin-resistant C3H/HeJ strain showed a smaller response. Lesser, though significant, elevations were also found after subcutaneous injection of 25 mg of casein, bovine serum albumin, ovalbumin, or monomeric immunoglobulin G, whereas pyrogen-free human serum albumin/U. S. Pharmacopeia failed to raise SAA levels. SAA generation may thus be a result of endotoxin contamination of these protein preparations.

Also present in equivalent amounts in acidified serum from endotoxin-treated mice, but barely detectable in control sera, was a 3,000-dalton molecule whose amino acid sequence is identical to the amino terminal 24 residues of mouse albumin. The appearance of SAA and the amino terminal albumin fragment after endotoxin were unaffected by pretreatment with cobra venom factor, and equivalent levels were found in C5-deficient mice. Pretreatment with pepstatin in vivo, or before acidification in vitro, prevented the appearance of the albumin fragment but had no effect on the appearance of SAA, whereas leupeptin and antipain did not affect the appearance of either SAA or the albumin fragment. These studies suggest that the generation of SAA after endotoxin administration does not involve complement activation or intravascular proteolytic activity, whereas the liberation of a specific peptic-like cleavage product of albumin appears to be the consequence of an acid protease.

Received for publication 29 June 1978 and in revised form 22 September 1978.

INTRODUCTION

Bacterial endotoxins are known to possess a variety of biological effects in vivo (1). Some of these, such as activation of the complement and coagulation cascades, represent, in part, direct effects of lipopolysaccharides (LPS)[1] on target cells (leukocytes, platelets, macrophages), possibly via adsorption to membrane receptors (2–4), whereas other hemodynamic and metabolic consequences of endotoxemia are presumed to be largely mediated by subsequent increased blood levels of proteolytic enzymes or vasoactive substances of lysosomal and extralysosomal origin (5–7).

Previous work in mice and mink has shown that single subcutaneous (SC) injections of *Escherichia coli* LPS causes >100-fold increases in the level of amyloid-related serum component (SAA) during the 24-h period after injection (8, 9). Human volunteers given etiocholanolone show a similar dramatic rise and comparable kinetics (10). In the course of studies designed to isolate and characterize murine SAA (11), we noted the appearance of a novel 3,000-dalton molecule in equivalent amounts in acidified endotoxemic mouse serum and identified it as the amino terminal 24 residue fragment of mouse albumin (12). The present report discusses its formation and the appearance of SAA in relation to the pathogenesis of secondary amyloidosis.

METHODS

Animals. C57BL/6, AKR, C3H/HeJ, and BALB/c mice were obtained from Jackson Laboratories, Bar Harbor, Maine. CF1 mice originated from Charles River Breeding Laboratories, Wilmington, Md. Each experiment involved 30–40 8-wk-old

[1] *Abbreviations used in this paper*: AA, tissue amyloid protein; BGG, bovine gamma globulin; BSA, bovine serum albumin; CVF, cobra venom factor; HSA/USP, human serum albumin, U. S. Pharmacopeia; LPS, lipopolysaccharide; OVA, ovalbumin; PEG, polyethylene glycol; RIA, radioimmunoassay; SAA, serum amyloid-related protein; SC, subcutaneous.

J. Clin. Invest. © *The American Society for Clinical Investigation, Inc.*, 0021-9738/79/02/0254/08 $1.00
Volume 63 February 1979 254–261

male mice, and in each case an equal number of uninjected control animals were studied in parallel to eliminate the possibility of intercurrent animal room infections.

Induction of SAA. Each animal was given one of the following SC injections: (*a*) 300 μg lipopolysaccharide W *E. coli* 055:B5 (Phenol-Westphal, Difco Laboratories, Detroit, Mich.); (*b*) 25 mg ovalbumin (OVA), 5 × recrystallized (Miles Laboratories, Inc., Kankakee, Ill.); (*c*) 25 mg bovine serum albumin (BSA) 5 × recrystallized, Fraction V (Miles Laboratories, Inc.); (*d*) 25 mg bovine gamma globulin (BGG), Fraction II (Miles Laboratories, Inc.) (Before injection, BGG solution was centrifuged at 105,000 *g* for 1 h at 4°C in a Spinco model L5-65 ultracentrifuge [Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.] to remove aggregated IgG; (*e*) 25 mg human serum albumin U. S. Pharmacopeia (HSA/ USP; (*a–e* were dissolved in 0.5 ml normal saline total volume per animal) (*f*) 25 mg casein (Fisher Scientific Co., Fairlawn, N. J.), dissolved in 0.3 N $NaHCO_3$ by heating to 60°C for 10–15 min; 0.5 ml total volume per animal. In each case, animals were sacrificed by exsanguination 18–24 h later. Each animal yielded ≅1 ml of blood. Blood from groups of mice was pooled, allowed to clot at room temperature for 3–4 h and sera were separated for further studies.

Fractionation of sera. Pooled sera were dialyzed (molecular weight cutoff of 12,000 daltons) overnight (18 h) against distilled water. Serum was applied to a Sephadex G-100 column (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) (83 × 4.5 cm; vol = 144 ml; elution rate, 19.2 ml/h; fraction vol, 4.8 ml; temperature, 23°C) equilibrated with 10% formic acid (pH 1.3). Unless otherwise stated, 7 ml of serum was applied to each column. For chromatography in 10% formic acid, 88% acid (Fisher Scientific Co.) was added to serum to bring the final concentration to 10% just before sample application. In experiments where the sera were brought to neutral pH after standing in 10% formic acid at room temperature for 6 h, 1 N NaOH was added in amounts sufficient to achieve pH 7.0 before application of Sephadex G-100 buffered with pH 7, phosphate-buffered saline (PBS). Both SAA and the low molecular weight albumin fragment were further purified by recycling on Sephadex G-75 (83 × 4.5 cm; flow rate, 13.8 ml/h) in 10% formic acid (13).

Isolation of albumin before and after cleavage in acidified endotoxemic serum. Albumin was isolated by starch zone electrophoresis (14) from normal mouse serum or the albumin fraction obtained from endotoxemic mouse serum after fractionation on Sephadex G-100 in 10% formic acid (peak 2, Fig. 1).

Molecular weights. Molecular weights and purity of protein samples were determined by sodium dodecyl sulfate polyacrylamide gradient gel electrophoresis (15). Standards included OVA (45,000 mol wt), light chain (22,500 mol wt), ribonuclease A (13,700 mol wt), and glucagon (3,485 mol wt).

Amino acid analyses. These were performed on a Durrum D-500 automatic amino acid analyzer (Durrum Instrument Corp., Palo Alto, Calif.). Proteins were hydrolyzed under vacuum at 110°C for 20 h in 6 N HCl containing 0.1% phenol to prevent destruction of tyrosine (16).

Carboxy terminal amino acids. Carboxy terminal amino acids were determined by ½ h and overnight digestions with Carboxypeptidase A (Worthington Biochemical Corp., Freehold, N. J.) (17).

Amino acid sequence. Determinations were performed by the method of Edman and Begg (18) with a Beckman model 890C sequencer (Beckman Instruments, Fullerton, Calif.) as previously described by using the accelerated dimethylallylamine-trifluoroacetic acid buffer program (19). Each recovered amino acid was identified by gas and thin layer chromatography and by back hydrolysis with hydriodic acid (20, 21).

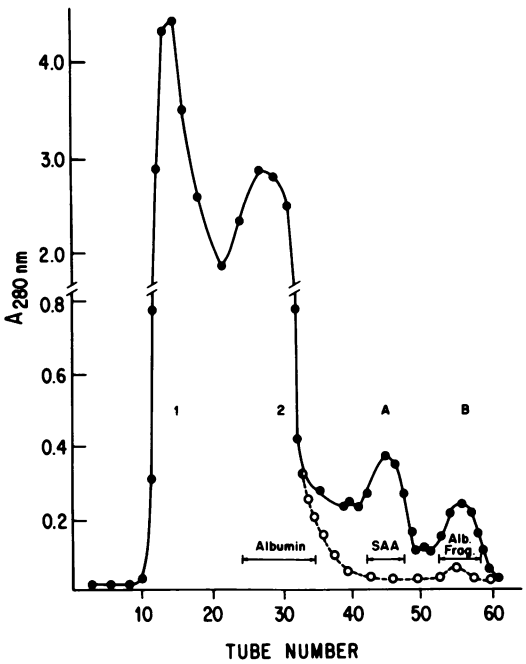

FIGURE 1 Gel filtration on Sephadex G-100 of 7 ml serum dialyzed overnight against distilled water and diluted to a concentration of 10% formic acid just before application. Closed circles indicate the elution pattern of serum from groups of mice injected SC with 300 μg LPS and exsanguinated 18–20 h later. Open circles show the elution pattern of an equal amount of serum from control, uninjected mice. Also indicated is the presence of immunoreactivity for native albumin or SAA determined by immunodiffusion or RIA. Peak B did not react with antialbumin antisera and did not elicit an antibody response in two rabbits.

Decomplementation. Mice were decomplemented by four intraperitoneal injections of cobra venom factor (CVF) (Cordis Laboratories, Miami, Fla.) given 12 h apart (first dose, 3 U/mouse; subsequent doses, 1 U/mouse) (22). Endotoxin was administered immediately after the last injection. Radial immunodiffusion against antimouse C3 (kindly supplied by Dr. Victor Nussenzweig, New York University Medical Center), showed levels to be <10% normal at the time of sacrifice.

Enzyme inhibitors. Enzyme inhibitors were kindly provided by Dr. Walter Troll, Department of Environmental Medicine, New York University School of Medicine. In vivo, the sodium salt of pepstatin was dissolved in water to a concentration of 1 mg/ml. Each animal received 50 μl i.p. and 0.4 ml SC 4 h before receiving endotoxin. Antipain and leupeptin were administered by a modification of a protocol developed by Dr. Mortimer Levitz, New York University School of Medicine. Each animal received 5 mg leupeptin and 6 mg antipain total in two divided doses in saline SC (0.2 ml/injection) given 16 and 4 h before receiving endotoxin. In vitro, 1 mg sodium pepstatin or 1 mg each of antipain and leupeptin was dissolved in 1 ml distilled water and added to 8 ml serum from endotoxemic mice after dialysis against water. The mixture was incubated ½ h at 37°C and then made up to 10% formic acid before gel filtration on a Sephadex G-100 column as described above.

Antisera. Antisera to the following were raised in rabbits: (*a*) C57BL/6 low molecular weight SAA, isolated as described above (13); (*b*) C57BL/6 tissue amyloid protein (AA), derived

from livers and spleens of casein-injected mice, extracted by water solubilization (23) and gel filtration (19); (*c*) C57BL/6 albumin, isolated by starch block electrophoresis (14). In each case, rabbits were immunized with 1 mg protein in complete Freund's adjuvant, injected into the footpads, in divided doses, followed by three weekly booster injections of 0.5 mg in complete Freund's adjuvant. Animals were bled 1–2 wk after the last booster, and antisera were tested by double diffusion.

Radioimmunoassay (RIA) for SAA. RIA for SAA was carried out as previously described (24), using chloramine T and sodium metabisulfite for iodination (25) and precipitation of antigen-antibody complexes with polyethylene glycol (PEG) (26). In this study, a monospecific antiserum to C57BL/6 low molecular weight SAA showing partial identity to tissue AA in double diffusion was used. Unlike antihuman SAA (27), this antiserum was not inhibited by albumin. A highly purified preparation of C57BL/6 low molecular weight SAA (0.5 mg/ml), previously shown to be homogeneous by polyacrylamide gel electrophoresis (15) and amino acid sequence analysis (11) was used for iodination and the preparation of standard curves. 100 μl of specific rabbit antiserum, diluted 1:2,000 (50% maximal binding) was added to 50 μl of unknown serum (diluted 1:100 and 1:500, each run in triplicate) and incubated 30 min at 37°C, followed by 50 μl of I^{125}-SAA having 16,000 cpm. All dilutions were done with 0.5% BGG (Fraction II) in 0.1 M barbital-buffered saline, pH 8.2. The mixture was incubated 90 min at 37°C, and the reaction stopped by addition of 100 μl chilled (4°C) 30% PEG (Matheson, Coleman, and Bell, East Rutherford, N. J.). Complexes insoluble in PEG were centrifuged at 2,500 rpm for 30 min at 4°C and the pellet counted in a gamma counter (Nuclear Chicago, Chicago, Ill.). Results are recorded as micrograms of SAA per milliliter. Standard curves were linear down to 5 ng/ml. Normal values for mouse sera of the various strains studied were consistently <50 μg/ml (mean, 18.8 μg/ml).

RESULTS

Identification and isolation of SAA and the amino-terminal fragment of albumin. As seen in Fig. 1, when fractionated on Sephadex G-100 columns in 10% formic acid, the elution pattern of pooled dialyzed serum from endotoxin-injected animals differed strikingly from that of control, uninjected mice. The serum of animals receiving 300 μg *E. coli* LPS 18–20 h before sacrifice was characterized by the appearance of two retarded peaks (A and B), both of which were not present in significant amounts in serum of control animals. Initially both retarded peaks were separately purified on Sephadex G-75 to remove minor contaminants before sequencing; in later experiments, this proved necessary only for peak A. Both peaks were homogeneous and had molecular weights of 12,000 and 3,000 daltons determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 2). Calculated yields were up to 1 mg/ml serum for each (mean, 615 μg/ml). Similar elution patterns were obtained on groups of C57BL/6, AKR, BALB/c, and CF1 mice receiving 300 μg *E. coli* LPS. Though some variation in the height of the retarded peaks was noted between runs, the size of peak A was consistently less for C3H/HeJ sera than for other strains studied. Serial studies on sera

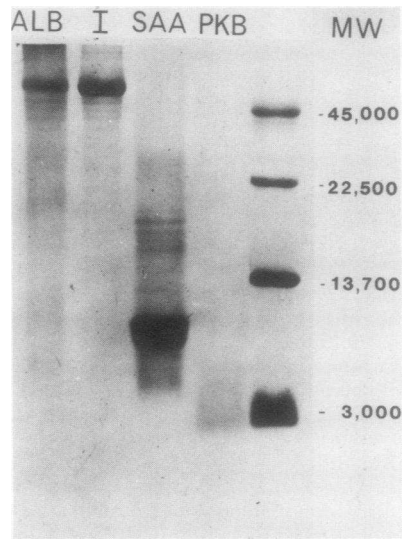

FIGURE 2 10–20% polyacrylamide gradient gel showing intact mouse albumin (ALB), albumin from acidified endotoxemic serum (I), SAA, and the 24 residue amino terminal albumin fragment (PKB). MW, molecular weight.

diluted to 10% formic acid and stored for 1–3 d at 23°C before gel filtration showed peak A to be particularly labile and to disappear with time under acid conditions, whereas peak B seemed to increase (Fig. 3). No change was noted in the elution profile of serum predialyzed against water for up to 5 d before acidification and gel filtration.

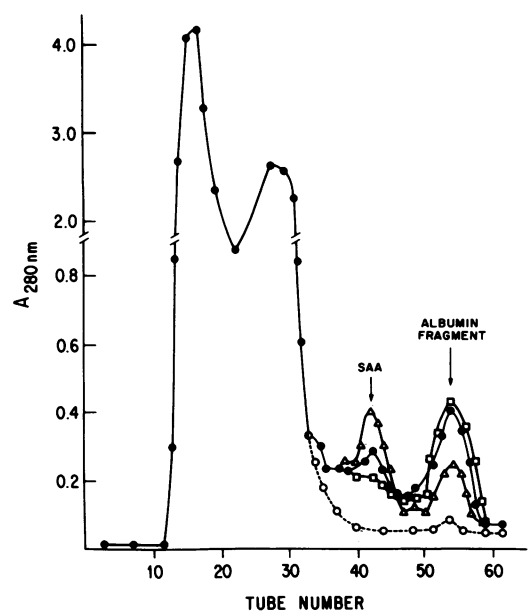

FIGURE 3 Elution pattern of serum from LPS-injected (△) and control (○) mice run under acid conditions. Endotoxemic serum was applied immediately (day 1) (△) or left in 10% formic acid at 23°C for (●) 2 or (□) 3 d before application.

TABLE I
Amino Acid Analyses of Mouse Albumin, Fragment I, and Fragment II

Amino acid	Albumin*	Fragment I*	Fragment II (peak B)‡
Asp	9.5	9.4	2.3
Thr	6.1	6.1	0.4
Ser	4.9	4.9	1.1
Glu	14.3	14.4	3.8
Pro	4.6	4.3	—
Gly	3.9	3.6	2.4
Ala	9.8	10.0	2.4
Cys	3.9	4.1	—
Val	5.5	5.6	1.3
Met	1.7	1.7	—
Ileu	3.7	3.4	1.1
Leu	10.6	10.9	2.8
Tyr	4.4	4.6	—
Phe	5.7	5.6	1.1
His	2.4	2.2	2.1
Lys	5.7	6.0	2.1
Trp	—	—	—
Arg	3.3	3.2	1.1
Total	100.0	100.0	24.0

Fragment I is albumin isolated from peak 2 of LPS-treated mice serum. Fragment II (peak B) is the amino terminal 24 residue fragment of albumin.
* Residues per 100.
‡ Residues per 24 (Fig. 1).

Peak A was identified as SAA, because the amino acid sequence to residue 10 was virtually identical to that of AA for each of the four strains of mice (11) and because each preparation gave a reaction of identity with SAA by double diffusion using a monospecific antisera.

The presence of small amounts of A and B in control serum similarly fractionated was shown by reacting a fraction with the same elution volume as peak A with anti-SAA and by noting a very small increase in OD 280 at the position of peak B. No SAA or AA immunoreactivity by RIA could be detected in the higher molecular weight peaks 1 and 2 (Fig. 1).

Peak B was identified as the amino terminal 24 residues of mouse serum albumin on the basis of amino acid analysis (Table I) and complete amino acid sequence determination to residue 24 (Fig. 4). Digestion with carboxy-peptidase A yielded Val-Leu thus establishing the sequence as complete. Mouse albumin was isolated by starch zone electrophoresis from control serum and from peak 2 (Fig. 1) of acidified serum from mice injected with LPS. Material isolated from both sources gave a reaction of identity with an antiserum to mouse albumin in double diffusion and appeared homogeneous and identical by cellulose acetate and gradient gel electrophoresis (Fig. 2). Peak B failed to react with the antiserum (Fig. 1) and failed to elicit an antibody response in two rabbits.

Albumin from control serum had an amino-terminal sequence identical to that of peak B (Fig. 4) and had an amino acid composition similar to human and rat albumin (Table I). Albumin isolated from peak 2 of LPS-treated mouse serum yielded a major sequence beginning with isoleucine, which was homologous to the sequence of human and bovine albumin starting at position 25 (28, 29) (Fig. 4). Because no sequence corresponding to that of intact albumin was detected, all albumin in this sample of acidified endotoxemic mouse serum was cleaved at leucine-isoleucine to release the amino terminal 24 residue fragment.

Role of acid pH in the generation of the albumin fragment. Sera from both control and LPS-injected

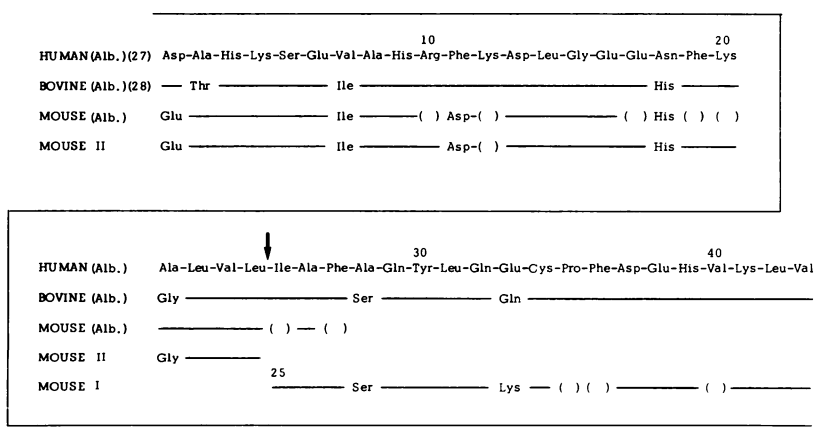

FIGURE 4 Amino terminal sequences of human (27), bovine (28), and C57BL/6 mouse (this study) albumins (ALB). These are compared to the amino terminal sequence of albumin isolated from acidified endotoxemic mouse serum by starch zone electrophoresis (mouse I) and Peak B (mouse II). ↓ denotes the presumed point of cleavage by acid protease generated during endotoxemia.

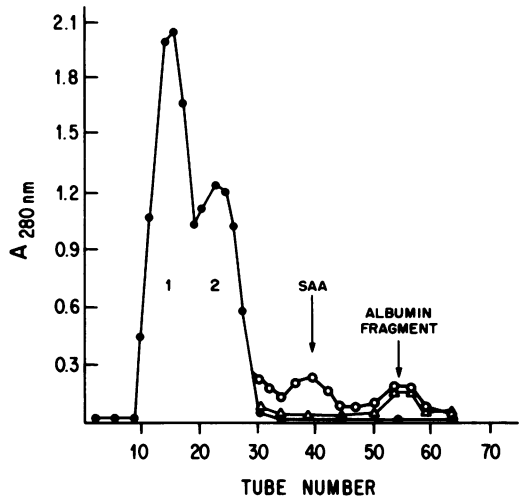

FIGURE 5 Gel filtration on Sephadex G-100 of 4.5 ml endotoxemic serum dialyzed overnight against distilled water and either (*a*) applied to a neutral (PBS) column (●) (*b*) applied to an acid (10% formic acid) column (○), or (*c*) acidified 6 h in 10% formic acid, neutralized with NaOH, and applied to a neutral column (△). When peaks 1 and 2 of (*a*) were pooled, lyophilized, and reapplied to an acid column, the elution profile of (*b*) was obtained.

mice, predialyzed to remove molecular species <12,000 daltons, were fractionated on Sephadex G-100 under neutral conditions. The elution profiles shown in Fig. 5 yielded the two high molecular weight peaks seen on the acid columns; however, peaks A and B were not apparent (Table II). When these high molecular weight peaks from the neutral column were pooled, dialyzed, lyophilized, and reapplied to Sephadex G-100 in 10% formic acid, the two retarded peaks corresponding to low molecular weight SAA and the albumin fragment were again seen (Fig. 5). To be certain that the albumin fragment once formed did not remain complexed to another protein, we examined the role of acid in releasing the albumin fragment. Serum from LPS-injected mice was dialyzed, and then acidified and allowed to stand at room temperature for 6 h; aliquots were then applied to an acid column or neutralized with NaOH and run under neutral conditions. Although low molecular weight SAA (peak A) was not seen in the elution profile of the neutral column, the size of peak B was similar on the neutral and the acid column (Fig. 5).

TABLE II
Effect of Various Factors on the Appearance of SAA and the Albumin Fragment in LPS-Treated Mice

	SAA		
	G-100	RIA	Albumin fragment
		μg/ml	
Control	0	10–50	±
LPS-injected	+	615	+
AKR (C5 deficient)	+	600	+
CVF	+	1,002	+
Leupeptin-antipain			
In vivo	+	660	+
In vitro	+	660	+
Pepstatin			
In vivo	+	660	0
In vitro	+	660	0

Presence (+) or absence (0) of SAA and the 24 residue amino terminal fragment of albumin (peak B), assessed by gel filtration (Sephadex G-100) after various conditions of pretreatment before administration of LPS. Also shown are peak SAA levels (microgram per milliliter) determined by RIA of unfractionated serum pooled from groups of 30–40 animals.

Induction with other agents. Increase in serum SAA level and the appearance of the albumin fragment were examined by RIA and gel filtration after injection of OVA, BSA, monomeric IgG, casein, and HSA/USP. After a single injection, all agents except HSA/USP caused significant elevations of SAA and the generation of the albumin fragment, the former effect peaking at 18–24 h and returning to base line by 48 h. Peak SAA levels after LPS injection for C57BL/6N, AKR, BALB/c, and CF1 strains were comparable and averaged 615 μg/ml, whereas those for C3H/HeJ were 196 μg/ml. These findings are in substantial agreement with data previously reported by McAdam and Sipe (8) and by Benson et al. (30). Marked differences of peak SAA levels were noted when the effect of the various protein preparations injected in equal milligram amounts were compared. Peak levels after injection of 25 mg casein were approximately one-third those found after injection of 300 μg LPS *E. coli*, but could be increased in a dose-dependent fashion twofold if up to 60 mg of casein was administered. Good general agreement was found between the area of peak A and immunoreactive SAA determined by RIA (Fig. 6). HSA/USP, 25 mg/animal, did not cause elevations of SAA levels above control values or the generation of detectable amounts of the amino terminal 24 residue albumin fragment.

Decomplementation. A role for the complement system in the generation of SAA monomer and the appearance of the amino-terminal albumin fragment was evaluated by testing the effect of endotoxin on C5-deficient AKR mice and mice rendered deficient in C3 by serial injections of CVF (Table II). Hypocomplementemia did not abrogate the development of peaks A or B after LPS. By RIA, SAA levels were 600 μg/ml for the AKR mice and 1,002 μg/ml for C3-depleted mice, and the size of peak B was comparable to sera derived from animals receiving only endotoxin.

Effect of enzyme inhibitors. Specific cleavage of mouse albumin at leucine-isoleucine under acid conditions suggested the presence in serum of an acid protease with peptic activity. This was examined

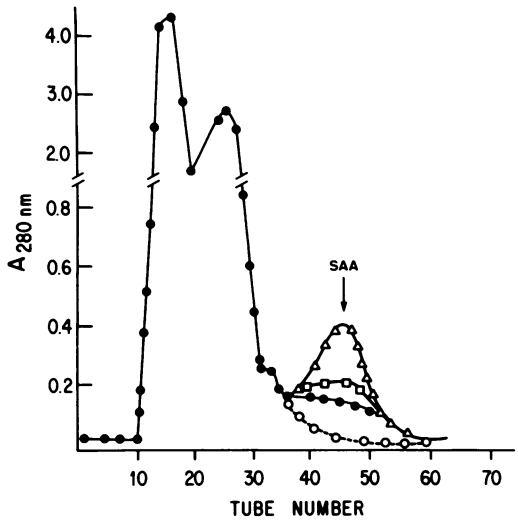

FIGURE 6 Elution patterns of acidified sera fractionated 18–24 h after injection of LPS *E. coli* (300 μg) (△) or casein, BSA (□), OVA, monomeric IgG (●), or HSA/USP (○) (25 mg each) per animal. Peak B was present in amounts roughly correlating to SAA level and is not shown in this figure. SAA levels (micrograms per milliliter) determined by RIA of sera pooled from groups of 30–40 animals averaged 615 (range, 400–1,000) for LPS, 292 for casein, 240 for BSA, 75 for OVA, 57 for monomeric IgG, and 21 for HSA/USP.

further by the use of specific acid protease inhibitors. In vivo pretreatment with pepstatin effectively suppressed the appearance of the albumin fragment, but had no effect on the generation of low molecular weight SAA. A direct effect of pepstatin on the intravascular generation of the albumin fragment was corroborated by the ability of the inhibitor to prevent cleavage when preincubated with endotoxemic mouse serum in vitro at an enzyme:substrate ratio of 0.5–1:1 (wt/wt) (Table II). By contrast, animals pretreated in vivo with antipain and leupeptin developed SAA and albumin fragment peaks comparable to controls receiving LPS without pretreatment. No in vivo effect was found with these inhibitors (Table II).

DISCUSSION

Injection of a single dose of endotoxin has been shown to cause striking elevations in the levels of SAA in mink and mice (8, 9) and to lead to the deposition of amyloid fibrils in tissues (9, 31). Endotoxin challenge is of further interest in that the rise in SAA levels is accompanied by the appearance of a specific peptic cleavage product of mouse albumin in acidified endotoxemic serum. After the injection of a single dose of LPS *E. coli*, there is a rise in SAA levels from microgram to milligram amounts, peaking within 24 h and returning to base line by 48 h (8, 30). The magnitude of the SAA response appears to be influenced by the nature and the amount of the inducing stimulus (8, 30) as well as by genetic factors (8, 32, 33); peak SAA levels in the endotoxin resistant C3H/HeJ strain mice are less than one-third those obtained in other strains tested (8). Serial studies have shown essentially identical kinetics of SAA generation in each of the experimental models studied to date; however, it is not yet known whether this reflects the release of a preformed precursor with rapid serum turnover, or de novo synthesis. Though many other substances have been shown to have a similar effect, it has been suggested that all may act by virtue of contamination with minute amounts of endotoxin (34). Such contamination has been shown for several commercially obtained proteins using either the limulus assay or intraocular inflammatory effects in rabbits. Of the proteins tested, only pyrogen-free pharmaceutical HSA/USP failed to show endotoxin activity (35), and it is of note that this was the only protein that failed to generate SAA or albumin fragments. It seems possible that the rate and degree of amyloid deposition may be directly related to the amount of SAA released as well as factors involved in its processing.

SAA circulates as a high molecular weight complex with lipoprotein (36, 37) and can interact with serum albumin (27, 38). Therefore, it is of interest that exposure of serum from endotoxin-treated animals to 10% formic acid results in the dissociation of the SAA complex and the cleavage of mouse serum albumin at position 24 to yield a second related peak. The 24 residue fragment corresponds to the "asp" fragment of albumin produced by mild digestion with pepsin at pH 3.0 (39, 40). Acid may be necessary for optimal activity of the protease, may cause its dissociation from an inhibitor, or may perhaps induce conformational changes in the albumin. Previous work of Foster has established that albumin undergoes major conformational changes in the pH range 2–5 (41), with progressive "acid expansion" of the molecule to globular parts linked by flexible segments (42). These changes are associated with great variations in susceptibility to pepsin digestion (43–45) and may explain why albumin is cleaved exclusively at residue 24 under these conditions. Lack of other proteolytic fragments of albumin in acidified serum is suggested by the almost identical molecular weights of control and endotoxemic mouse albumin, and the absence of intact albumin or other albumin fragments in acidified endotoxemic serum. Thus digestion of albumin at a single site previously shown to be susceptible to pepsin was demonstrated (39).

A specific role for an enzyme with pepsin-like activity is suggested by the cleavage point, Leu-Ileu, the pH requirement, and the ability of pepstatin pretreatment in vivo and in vitro to block its generation. The efficacy of pepstatin in vivo was first demonstrated by Aoyagi et al. (46) and is confirmed in the present study. Pepstatin is an acid protease inhibitor of broad

specificity with no activity for neutral or alkaline proteases; leupeptin and antipain inhibit trypsin, cathepsins A and B, plasmin and papain (47). In contrast, pepstatin has no effect on the release of low molecular weight SAA, and pretreatment with leupeptin and antipain was without effect on either process. The fact that acidification of normal mouse serum resulted in the expected release of SAA, but failed to yield an albumin fragment, makes cleavage by acid highly improbable. In addition, the finding that neutralization after acidification of endotoxemic serum had no effect on the appearance of peak B makes it unlikely that the role of acid pH is to dissociate the low molecular weight albumin fragment from a complex after proteolysis. However, the close association of certain acid proteases (e.g., cathepsins D and E) with lysosomes suggests the possibility of some function in the local pericellular inflammatory environment (48). Because no albumin fragment could be identified at neutral pH, it seems unlikely that digestion occurs under physiological conditions.

Although both the acute rise in SAA level and the appearance of acid protease activity directed against the amino terminal end of mouse albumin result from endotoxemia, a possible relationship between the two remains speculative. Interest in the effect of decomplementation resulted from consideration of whether either effect might relate to coagulopathy and(or) complement consumption secondary to the endotoxemic state (2, 3). However, generation of the albumin fragment was similar to normals in congenitally (C5) deficient mice or in mice made hypocomplementemic by prior administration of CVF.

Previous work has established amino and carboxy terminal heterogeneity of SAA, suggesting that it may originate by proteolysis of a larger precursor. Similarly, the conversion of SAA probably also involves proteolytic cleavage of a carboxy terminal segment (3). Because limited digestion by pepsin, trypsin, or lysosomal hydrolases can convert certain light chains in vitro to material with the tinctorial and ultrastructural properties of amyloid fibrils (49–51), it seems possible that proteolysis may play a role in the generation of several types of amyloid, perhaps by converting a soluble precursor into a fibrillar fragment. Recent work in man has suggested that the development of amyloid may relate to individual, possibly genetic, differences in the degradation of SAA to AA by specific monocyte-associated proteolytic enzymes (52). Such work emphasizes the importance of local tissue factors in the pathogenesis of this disease, and studies are currently in progress to define the physiological controls of these phenomena.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical assistance of Ms. Joan Zaretzky and Ellen Rosenwasser and the secretarial expertise of Ms. Marlene Chavis and Beverly Coopersmith.

This work was supported by U. S. Public Health Service research grants AM 01431, AM 02594, and AG 00458, the Irvington House Institute, the Michael and Helen Schaffer Fund, and the Arthritis Foundation.

REFERENCES

1. Elin, R. J., and S. M. Wolff. 1976. Biology of endotoxin. *Annu. Rev. Med.* **27**: 127–141.
2. Miller, R. L., M. J. Reichgott, and K. L. Melmon, 1973. Biochemical mechanisms of generation of bradykinin by endotoxin. *J. Infect. Dis.* **128**: 5144–5156.
3. Frank, M. M., J. E. May, and M. E. Kane. 1973. Contributions of the classical and alternative complement pathways to the biological effects of endotoxin. *J. Infect. Dis.* **128**: S 176–181.
4. Cline, M. J., K. L. Melmon, W. C. Davis, and H. E. Williams. 1968. Mechanism of endotoxin interaction with human leukocytes. *Br. J. Haematol.* **15**: 539–547.
5. Weissman, G., and L. Thomas. 1962. Studies on lysosomes. I. The effects of endotoxin, endotoxin tolerance and cortisone on the release of acid hydrolases from a granular fraction of rabbit liver. *J. Exp. Med.* **116**: 433–450.
6. Janoff, A., G. Weissmann, B. Zweifach, and L. Thomas. 1962. Pathogenesis of experimental shock. IV. Studies on lysosomes in normal and tolerant animals subjected to lethal trauma and endotoxemia. *J. Exp. Med.* **116**: 451–466.
7. Sleeman, H. K., P. B. Lamborn, J. W. Diggs, and C. E. Emery. 1971. Effects of endotoxin and histamine on serum enzyme activity. *Proc. Soc. Exp. Biol. Med.* **138**: 536–541.
8. McAdam, K. P. W. J., and J. D. Sipe. 1976. Murine model for human secondary amyloidosis: genetic variability of the acute phase serum SAA response to endotoxins and casein. *J. Exp. Med.* **144**: 1121–1127.
9. Anders, R. F., K. Nordstoga, J. B. Natvig, and G. Husby. 1976. Amyloid-related serum protein SAA in endotoxin-induced amyloidosis of the mink. *J. Exp. Med.* **143**: 678–683.
10. McAdam, K. P. W. J., R. J. Elin, J. D. Sipe, and J. M. Wolff. 1978. Changes in human serum amyloid A and Creactive protein after etiocholanolone-induced inflammation. *J. Clin. Invest.* **61**: 390–394.
11. Gorevic, P. D., Y. Levo, B. Frangione, and E. C. Franklin. 1978. Polymorphism of tissue and serum amyloid proteins in murine amyloidosis. *J. Immunol.* **121**: 138–140.
12. Gorevic, P. D., Y. Levo, E. C. Franklin, and B. Frangione. 1977. Increased proteolytic activity due to endotoxin in the mouse—its role in the generation of SAA and an albumin fragment. *Clin. Res.* **25**: 358A.
13. 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**: 1415–1418.
14. Kunkel, H. G. 1954. Zone electrophoresis. *In* Methods Biochemical Analysis. D. Glick, editor. Interscience Pubs., Inc., John Wiley & Sons, Inc. New York. **1**: 141.
15. Margolis, J., and K. G. Kendrick. 1968. Polyacrylamide gel electrophoresis in a continuous molecular sieve gradient. *Anal. Biochem.* **25**: 347–362.
16. Sanger, F., and E. O. P. Thompson. 1963. Halogenation of tyrosine during acid hydrolysis. *Biochim. Biophys. Acta.* **71**: 468–471.
17. Ambler, R. P. 1967. Enzymic hydrolysis with carboxypeptidases. *In* Methods Enzymology. C. H. W. Hirs, editor. Academic Press, Inc., New York. **11**: 155.

18. Edman, P., and G. Begg. 1967. A protein sequenator. *Eur. J. Biochem.* **1**: 80–91.
19. Levin, M., E. C. Franklin, B. Frangione, and M. Pras. 1972. The amino acid sequence of a major non-immunoglobulin component of some amyloid fibrils. *J. Clin. Invest.* **51**: 2773–2776.
20. Pisano, J. J., and T. J. Bronzert. 1969. Analysis of amino acid phenylthiohydantoins by gas chromatography. *J. Biol. Chem.* **244**: 5597–5607.
21. Summers, M. R., G. W. Smythers, and S. Oroszlan. 1973. Thin-layer chromatography of sub-nanomole amounts of phenylthiohydantoins (PTH) amino acids on polyamide sheets. *Anal. Biochem.* **53**: 624–628.
22. Cochrane, C. G., H. J. Muller-Eberhard, and B. J. Aikin. 1970. Depletion of plasma complement in vivo by a protein of cobra venom: its effect on various immunologic reactions. *J. Immunol.* **105**: 55–69.
23. Pras, M., M. Schubert, D. Zucker-Franklin, A. Rimon, and E. C. Franklin. 1968. The characterization of soluble amyloid prepared in water. *J. Clin. Invest.* **47**: 924–933.
24. Rosenthal, C. J., and E. C. Franklin. 1975. Variation with age and disease of an amyloid protein-related serum component. *J. Clin. Invest.* **55**: 746–753.
25. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of I^{131}-labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89**: 114–123.
26. Desbuquois, B., and G. D. Aurbach. 1971. Use of polyethylene glycol to separate free and antibody-bound peptide hormones in radio-immunoassay. *J. Clin. Endocrinol. Metab.* **33**: 732–738.
27. Franklin, E. C. 1976. Some properties of serum amyloid A protein. Inhibition of precipitation by complexing to albumin. *J. Exp. Med.* **144**: 1679–1682.
28. Behrens, P. Q., A. M. Spiekerman, and J. R. Brown. 1975. Sequence of human serum albumin. *Fed. Proc.* **34**: 591.
29. Brown, J. R. 1976. Structural origins of mammalian albumin. *Fed. Proc.* **35**: 2141.
30. Benson, M. D., M. A. Scheinberg, T. Shirahama, E. S. Cathcart, and M. Skinner. 1977. Kinetics of amyloid protein A in casein-induced murine amyloidosis. *J. Clin. Invest.* **59**: 412–417.
31. 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**: 615–626.
32. McAdam, K. P. W. J., and J. L. Ryan. 1978. C57BL/10/CR mice: nonresponding to activation by the lipid A moiety of bacterial lipopolysaccharide. *J. Immunol.* **120**: 249–254.
33. Watson, J., M. Largen, and K. P. W. J. McAdam. 1978. Genetic control of endotoxic responses in mice. *J. Exp. Med.* **147**: 39–49.
34. Cohen, A. S., and E. S. Cathcart. 1972. Casein-induced experimental amyloidosis. *Methods Achiev. Exp. Pathol.* **6**: 207–242.
35. Bito, L. Z. 1977. Inflammatory effects of endotoxin-like contaminants in commonly used protein preparations. *Science (Wash. D. C.).* **196**: 83–85.
36. Benditt, E. P., and N. Eriksen. 1977. Amyloid protein SAA is associated with high density lipoprotein from human serum. *Proc. Natl. Acad. Sci. U. S. A.* **74**: 4025–4028.
37. Skogen, G., J. B. Natvig, T. Michaelsen, and R. F. Anders. 1977. A high molecular weight serum protein is the carrier for amyloid-related protein, SAA. *Scand. J. Immunol.* **6**: 1363–1368.
38. Rosenthal, C. J., and E. C. Franklin. 1977. Serum amyloid (SAA) protein-interaction with itself and serum albumin. *J. Immunol.* **119**: 630–634.
39. Peters, T., and C. Hawn. 1967. Isolation of two large peptide fragments from the amino and carboxyl-terminal positions of bovine serum albumin. *J. Biol. Chem.* **242**: 1566–1573.
40. Peters, J., Jr., and F. A. Blumenstock. 1967. Copper-binding properties of BSA and its amino-terminal peptide fragment. *J. Biol. Chem.* **242**: 1574–1578.
41. Foster, J. F. 1960. Serum Albumin. *In* The Plasma Proteins. F. W. Putnam, editor. Academic Press, Inc., New York. **1**: 179.
42. Slayter, E. M. 1965. An electron microscopic study of the conformational change in BSA at low pH. *J. Mol. Biol.* **14**: 443–452.
43. Weber, G., and L. B. Young. 1964. Fragmentation of BSA by pepsin. I. The origin of the acid expansion of the albumin molecule. *J. Biol. Chem.* **239**: 1415–1423.
44. King, T. P. 1973. Limited pepsin digestion of BSA. *Arch. Biochem. Biophys.* **156**: 509–520.
45. Hilak, M. D., J. M. Harmsen, W. C. M. Braan, J. J. M. Joordens, and G. A. J. Van Os. 1974. Conformational studies of large fragments of BSA in relation to the structure of the molecule. *Int. J. Pept. Protein. Res.* **6**: 95–101.
46. Aoyagi, T., S. Kunimoto, H. Morishima, T. Takeuchi, and H. Umezawa. 1971. Effect of pepstatin on acid proteases. *J. Antibiotics.* **24**: 687–691.
47. Umezawa, H. 1976. Structure and activities of protease inhibitors of microbial origin. *In* Methods Enzymology. **45**: 678–695.
48. Dingle, J. T. 1976. The role of neutral and acid proteinases in connective tissue turnover. *In* Proteolysis and Physiological Regulation, D. W. Ribbons and K. Brew, editors. Academic Press, Inc., New York. 339–355.
49. Linke, R. P., D. Zucker-Franklin, and E. C. Franklin. 1973. Morphologic, chemical and immunologic studies of amyloid-like fibrils formed from Bence-Jones proteins by proteolysis. *J. Immunol.* **111**: 10–23.
50. Tan, M., and W. Epstein, 1972. Polymer formation during degradation of human light chain and Bence Jones proteins by an extract of the lysosomal fraction of normal human kidney. *Immunochemistry.* **9**: 9–16.
51. Glenner, G. G., D. Ein, E. D. Eanes, H. A. Bladen, W. Terry, and D. Page. 1971. The creation of "amyloid" fibrils from Bence-Jones proteins in vitro. *Science (Wash. D. C.).* **174**: 712–714.
52. Lavie, G., D. Zucker-Franklin, and E. C. Franklin. 1978. Degradation of serum amyloid A protein by surface-associated enzymes of human blood monocytes. *J. Exp. Med.* **148**: 1020–1031.