Adenoviral expression of murine serum amyloid A proteins to study amyloid fibrillogenesis

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Serum amyloid A (SAA) proteins are one of the most inducible acute-phase reactants and are precursors of secondary amyloidosis. In the mouse, SAA₁ and SAA₂ are induced in approximately equal quantities in response to amyloid induction models. These two isotypes differ in only 9 of 103 amino acid residues; however, only SAA₂ is selectively deposited into amyloid fibrils. SAA expression in the CE/J mouse species is an exception in that gene duplication did not occur and the CE/J variant is a hybrid molecule sharing features of SAA₁ and SAA₂. However, even though it is more closely related to SAA₂ it is not deposited as amyloid fibrils. We have developed an adenoviral vector system to overexpress SAA proteins in cell culture to determine the ability of these proteins to form amyloid fibrils, and to study the structural features in relation to amyloid formation. Both the SAA₂ and CE/J SAA proteins were synthesized in large quantities and purified to homogeneity. Electron microscopic analysis of the SAA proteins revealed that

the SAA₂ protein was capable of forming amyloid fibrils, whereas the CE/J SAA was incapable. Radiolabelled SAAs were associated with normal or acute-phase high-density lipoproteins (HDLs); we examined them for their clearance from the circulation. In normal mice, SAA₂ had a half-life of 70 min and CE/J SAA had a half-life of 120 min; however, in amyloid mice 50% of the SAA₂ cleared in 55 min, compared with 135 min for the CE/J protein. When the SAA proteins were associated with acute-phase HDLs, SAA₂ clearance was decreased to 60 min in normal mice compared with 30 min in amyloidogenic mice. Both normal and acute-phase HDLs were capable of depositing SAA₂ into preformed amyloid fibrils, whereas the CE/J protein did not become associated with amyloid fibrils. This established approach opens the doors for large-scale SAA production and for the examination of specific amino acids involved in the fibrillogenic capability of the SAA₂ molecule *in vitro* and *in vivo*.

INTRODUCTION

Serum amyloid A (SAA) proteins are normally found in low concentrations (1–5 µg/ml) but can increase 500–1000-fold within 24 h of an acute-phase response [1–3]. The function of the SAA proteins is currently unknown but they are thought to have a role in altered lipid flow during inflammation [4]. During the acute-phase response the increased SAA proteins associate with high-density lipoprotein (HDL) particles, becoming the most abundant lipoprotein present, and displace apolipoprotein A-I (apo-AI) from the HDLs [5]. The acute-phase SAA proteins are found associated principally with the HDL₃ subfraction [6]. The SAA proteins are predominantly produced in the liver and are induced by a variety of cytokines including interleukin 1, interleukin 6 and tumour necrosis factor [7,8]. Previous studies have shown that SAA proteins are the precursors to reactive amyloid [amyloid A protein (AA)], both in man and mouse [9]. The AA fibrils consist mainly of residues 1–76 (the N-terminus) of the SAA proteins, although the length of the fibrillar peptide varies depending on the tissue location [10]. In most mouse strains, SAA₁ and SAA₂ are the acute-phase SAA proteins; during inflammation-associated amyloid formation, only SAA₂ is selectively cleared into amyloid fibrils [11]. Because of the similarities between the SAA proteins, the SAA₂ protein must

have a unique secondary and tertiary structure that allows its fibrillogenic properties.

We have identified a strain of mice (CE/J) that is totally resistant to amyloid formation [12]. The CE/J mouse contains a unique SAA isoform that is a composite molecule of the SAA₁ and SAA₂ proteins. The CE/J and SAA₁ proteins differ from the SAA₂ protein in 5 and 9 of their 103 amino acids respectively, suggesting that the amyloidogenicity is defined by these differences [13]. The N-terminal region of the CE/J protein is similar to the non-fibrillogenic SAA₁ protein, with the sixth and seventh residues being valine and histidine respectively [13], whereas in the SAA₂ molecule the residues at positions 6 and 7 are isoleucine and glycine. This region of the SAA₂ protein has been shown to be important for amyloid fibrillogenesis by using synthetic peptides to mimic fibril formation *in vitro* [14]. We and others have shown that the CE/J and SAA₁ proteins lack the ability to undergo a conformational change to a β -pleated sheet in the presence of heparan sulphate proteoglycans and Ca²⁺, whereas SAA₂ can easily form β -pleated structures [15]. These amino acid differences have been suggested to result in structural changes that enable the SAA₂ protein to form β -pleated sheets. Arrangement of the structures allows the SAA₂ protein to form amyloid fibrils.

Several studies have attempted to express recombinant SAA

Abbreviations used: AA, amyloid A protein; AEF, amyloid-enhancing factor; apo-AI, apolipoprotein A-I; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HDL, high-density lipoprotein; IEF, isoelectric focusing; LPS, lipopolysaccharide; MOI, multiplicity of infection; SAA, serum amyloid A.

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proteins *in vitro*; however, production of the mature protein and production of protein in sufficient quantities for structural determination has been difficult [16,17]. To understand the structural properties and how this selective incorporation of SAA₂ into amyloid occurs, we have developed adenoviral vectors to overexpress the murine SAA (CE/J and SAA₂) proteins. These recombinant proteins were purified and analysed for their fibrillogenic properties. In addition, both the CE/J and SAA₂ proteins were radiolabelled *in vitro* and examined for clearance *in vivo*. Finally, the radiolabelled proteins were studied for their ability to be incorporated into murine amyloid. The results show that SAA₂ is more rapidly cleared from the circulation than the CE/J SAA protein and that this rapid clearance of the SAA₂ protein is enhanced when injected into amyloidotic mice or in the presence of acute-phase HDLs. In addition, the recombinant radiolabelled SAA₂ protein is found associated with amyloid but the CE/J SAA is not incorporated into these fibrils. The methodology described allows the synthesis and purification of large quantities of SAA proteins for structural analysis to determine the fibrillogenic nature of the SAA₂ protein. In addition, the utility of the adenoviral vectors allows the analysis of SAA proteins *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

All reagents were of the highest available grade and, unless indicated otherwise, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Animals

Male BALB/c mice (Jackson Laboratory, Bar Harbor, ME, U.S.A. and Harlan Sprague-Dawley, Indianapolis, IN, U.S.A.) 8–12 weeks of age were used in the experiments. Mice were maintained in rodent cages as outlined by the University Institutional Animal Care and Use Committee.

Isolation of lipoproteins

HDLs were isolated from normal animals and animals subjected to inflammatory response. Inflammation was induced in mice by subcutaneous injection of 0.5 ml of 2% (w/v) aqueous AgNO₃. For collection of normal HDL, untreated animals were anaesthetized with Metofane and exsanguinated via heart puncture into 7% (w/v) EDTA-coated syringes. For acute-phase HDLs, animals were prepared as described above and after 24 hours were subjected to anaesthesia and heart puncture. Plasma from both preparations was subjected to HDL purification by sequential ultracentrifugation as described previously [13]. The samples were recentrifuged for 20 h at 242 000 *g* and the upper layer was saved for HDL. Samples were dialysed extensively against saline/EDTA and sterile-filtered.

Adenoviral preparation

Replication-defective adenoviral vectors were prepared with a cytomegaloviral promoter and SV40 poly(A)⁺ site as described previously [18]. Vectors contained 0–1 and 9–16 map units of the DNA sequence from adenovirus type 5. The constructs were sequenced and tested by PCR for correct orientation. The transfer vector containing the CE/J or SAA₂ cDNA and the adenovirus 5 DNA were transfected (or infected) into HEK-293 (human embryonic kidney) cells, which contain a portion of the adenoviral genome needed for replication. After 6 h the medium was

changed; the cells were then incubated overnight. On the following day the monolayers were covered with Noble agar and incubated for 10 days to allow plaques to develop. The plaques were analysed for recombinant virus, tested for the production of protein and propagated for the studies outlined. Plaque lysate [multiplicity of infection (MOI) of 5–10] was added to 50–150 mm plates of 293 cells and harvested after 90% of the cells had been infected (2–3 days). Viral lysates were collected and centrifuged through CsCl solution to purify the virus, then passed through a Sepharose CL-4B column to remove salts. This protocol resulted in purified virus that was ready for use in infection.

Tissue culture and radiolabelling

CV-1 monkey kidney cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS). Confluent 10 cm dishes of CV-1 cells were infected with adenoviral vectors at an MOI of 100 [viruses were used at 10⁸ plaque-forming units (pfu) per 10⁷ cells in 1 ml of DMEM] for 1 h followed by incubation in DMEM/10% (v/v) FCS for 24 h. Infected cells were washed with sterile PBS and incubated with either normal or acute-phase mouse HDL at a concentration of 200 µg/ml in DMEM plus 200 µCi/ml (4.44 × 10⁻¹⁰ mBq/ml) of [³⁵S]methionine (Trans³⁵S-label; ICN). Cells were incubated for 4 h, then medium was collected and subjected to HDL purification as described above. HDL was dialysed against PBS and sterile-filtered before injection into mice.

HDL samples were quantified by standard techniques described previously [19]. In addition, samples were counted to determine the specific radioactivity of each HDL preparation. The specific radioactivity (trichloroacetic acid-precipitable d.p.m./ng of HDL protein) was calculated by counting the labelled sample and quantifying the amount of HDL protein in each sample (average specific radioactivity was 50 d.p.m./ng of protein). The SAA in each sample was also quantified and was identical for each preparation (specific radioactivity was 167 d.p.m./ng of SAA protein). The specific radioactivities were the same for all preparations.

Protein purification and analysis

HDL isolated from normal and acute-phase mice was subjected to Western blot analysis before incubation, to verify the nature of the HDL particle. After incubation of HDL with CV-1 cells, HDL (5 µg) was subjected to electrophoresis on 4–20% (w/v) polyacrylamide gels and stained with Coomassie Blue to identify protein bands [13]. Recombinant protein was subjected to isoelectric focusing (IEF) to verify the SAA isotype [13]. In addition, recombinant protein was N-terminally sequenced as described previously [20]. HDL-SAA was delipidated and SAA proteins were subjected to gel-filtration chromatography for purification and PAGE analysis as described previously [21]. For radiolabelled SAA, gels were dried and autoradiography was performed to determine the relative labelling of the SAA protein compared with other HDL proteins.

Fibril formation *in vitro*

Each purified recombinant protein was dissolved in 10% (v/v) acetic acid at a concentration of 10 mg/ml, incubated for 24 h at room temperature, then neutralized with concentrated NH₄OH [14]. Samples were dried on glass slides, stained with Congo Red and examined under polarized light for fibril formation [14]. Samples were diluted 1:5 or 1:10 with distilled water, placed on

Formvar grids, then stained with 1 % (w/v) uranyl acetate [14] and examined by electron microscopy.

Amyloid induction

AA amyloidosis was induced in mice by injecting 200 μ g of amyloid-enhancing factor (AEF) into the lateral tail vein followed by injection of 0.5 ml of 2 % (w/v) AgNO_3 . AEF was prepared as described previously [22,23]. Animals developed amyloid within 48–72 h. For the studies outlined, animals were injected with AEF and AgNO_3 and were maintained for 1 week before the injection of radiolabelled SAA–HDL. Amyloid-containing mice were injected with 5×10^6 d.p.m. per mouse (SAA_2 or CE/J) HDL (100 μ g of HDL protein, 30 μ g of SAA protein) and killed after 24 h. Mice were perfused with cold PBS followed by 4 % (w/v) paraformaldehyde. Tissues were excised and embedded in OCT medium and frozen on dry ice. Sections (30 μ m) were cut on a cryostat and mounted on slides. The slides were exposed to X-ray film (Kodak, Rochester, NY, U.S.A.) for 12–24 h.

Half-life determination

Mice were injected with radiolabelled HDL as follows: (1) normal mice injected with normal HDL; (2) normal mice injected with acute-phase HDL; (3) amyloidotic mice injected with normal HDL; and (4) amyloidotic mice injected with acute-phase HDL. These groups were further separated into CE/J-labelled protein and SAA_2 -labelled protein on the HDLs. Mice were injected, and samples were collected at the indicated times. Animals were injected with 5×10^6 d.p.m. per mouse (100 μ g of HDL protein, 30 μ g of SAA protein). Blood samples (50 μ l) were collected in heparin-coated capillary tubes by retro-orbital venous plexus under anaesthesia at time points up to 24 h after injection. Liquid-scintillation counting was used to determine the amount of radioactivity remaining after injection. Plasma samples were prepared by centrifugation of blood and duplicate counting (10 μ l) of the trichloroacetic acid-precipitable radioactivity. In addition, the amount of SAA remaining was determined by Western blot analysis. The amount of radioactivity remaining at the indicated time was expressed as a percentage (percentage of ^{125}I remaining) of the original injected radiolabelled HDL (at 10 min after injection). This percentage was plotted against time to determine the half-life of the SAA proteins.

Statistical analysis

All results are presented as means $\pm$ S.E.M. Clearance and half-life determinations were analysed by analysis of variance with Fisher's protected least significant difference. $P < 0.05$ was considered indicative of a significant difference.

RESULTS

Adenoviral vector expression of SAA proteins

To develop an expression system for the generation of large amounts of SAA proteins and to examine the clearance of SAA proteins from the plasma, we constructed adenoviral vectors that synthesized the CE/J and SAA_2 proteins *in vitro*. The cDNAs for the CE/J and SAA_2 proteins were cloned into the adenoviral vector system as described previously [18]. The vector utilizes the cytomegaloviral promoter and the SV40 polyadenylation signal. The cDNA species were amplified by PCR to remove any 5' and 3' regions that might interfere with expression, then cloned into the adenoviral transfer vector and then sequenced to verify the

Figure 1 Protein analysis of normal HDL with SAA

Upper panel: SDS/PAGE of normal mouse HDL (lane 1), acute-phase mouse HDL (lane 2), control CV-1 cell HDL (lane 3) and CV-1 cells expressing recombinant SAA_2 on bovine HDL (lane 4). Total protein (5 μ g) from each sample was subjected to electrophoresis. Abbreviation: AI, apo-AI (bovine and mouse respectively). Bottom panel: IEF and immunoblotting of adenoviral vector-expressed SAA proteins: lane 1, HDL from CV-1 cells expressing recombinant SAA_2 ; lanes 2 and 3, HDL from CV-1 cells expressing the recombinant CE/J SAA; lane M (markers), mouse SAA_1 and SAA_2 (acute-phase HDL).

clone (results not shown). The transfer vector was transfected into 293 cells with adenoviral DNA, and examined for plaque formation. Positive plaques were expanded, purified and examined for protein expression in 293 cells (results not shown). Adenoviral vectors were used to infect CV-1 monkey kidney cells at an MOI of 1:100 and incubated in the presence of FCS HDL. Supernatants were collected from the infected CV-1 cells and HDL containing the recombinant SAA proteins was isolated by density gradient ultracentrifugation. The isolated HDLs were subjected to SDS/PAGE for protein analysis. Figure 1 (upper panel) shows the SAA proteins found associated with normal mouse HDL (lane 1), acute-phase mouse HDL (lane 2), fetal calf HDL isolated from control CV-1 cells (lane 3) and the SAA_2 protein expressed in CV-1 cells associated with fetal calf HDL (lane 4): the presence of a protein band in the SAA size range associated with the virus-infected cells is evident. Western blot analysis indicated that this band was SAA immunoreactive. On the basis of quantitative immunoblotting, approx. 50 μ g/ml SAA protein was found on the fetal calf HDL (400 μ g/ml); up to 80 % of the secreted SAA protein was incorporated into the HDL, and 20 % was free or VLDL/LDL-associated (results not shown). The SAA–HDL was subjected to IEF for isoform analysis and compared with SAA proteins from a lipopolysaccharide (LPS)-injected BALB/c mouse (Figure 1, lower panel). It is apparent from the IEF gel that the CV-1 cells were

Table 1 N-terminal sequence analysis of recombinant proteins

Protein	Sequence
SAA ₂	GFFSFIGEAFQG
CE/J	GFFSFVHEAFLG

Figure 2 Electron microscopy of the recombinant SAA proteins

Purified recombinant proteins were prepared as described in the Materials and methods section and subjected to electron microscopy: (A) recombinant SAA₂ protein; (B) recombinant CE/J SAA; (C) control protein (apo AI); (D) native fibrils extracted from splenic tissue of a mouse treated with casein for 21 days. Magnification used, × 84 000.

capable of synthesizing and secreting both the SAA₂ (lane 1, pI 6.30) and CE/J (lanes 2 and 3, pI 6.15) proteins.

Amyloid fibril formation

To determine whether the SAA proteins retained predicted fibrillogenic properties after purification, samples were examined by electron microscopy and Congo Red staining. CV-1 cells were infected with either the CE/J or SAA₂ adenoviral vector. Fetal calf HDL was ultracentrifuged from CV-1 cell medium and subjected to Western blot analysis (results not shown). Western blot analysis showed that the recombinant SAA proteins were associated with the fetal calf HDL. The ultracentrifuged HDL from infected CV-1 cells was subjected to delipidation and ion-exchange chromatography; fractions were collected as described previously [13]. Apo-AI and SAA (CE/J and SAA₂) fractions

Figure 3 Recombinant radiolabelled SAA proteins

CV-1 cells were infected with recombinant SAA adenoviruses for 24 h and then incubated in DMEM plus methionine-free medium for 30 min. Cells were then incubated with [³⁵S]methionine and normal mouse HDL for 4 h. Samples were isolated and ultracentrifuged as described in the Materials and methods section. Radiolabelled SAA/HDL was subjected to SDS/PAGE and autoradiography. Lane 1, CE/J SAA HDL; lane 2, SAA₂ HDL.

were pooled separately and the protein concentration was determined. N-terminal sequence analysis confirmed the identity of the SAA proteins (Table 1). CV-1 cells produced approx. 50 mg of SAA protein per litre of medium; on purification, approx. 15 mg of purified recombinant protein was obtained from each litre. Samples were concentrated by filtration (Centricon10 concentrators; Amicon, Beverly, MA, U.S.A.); purified recombinant SAA and apo-AI proteins were dissolved in 10% (v/v) acetic acid at a concentration of 10 mg/ml and incubated at room temperature for 24 h. The SAA₂ protein formed a precipitate after the addition of acetic acid that showed apple-green birefringent material after staining with Congo Red (results not shown). In addition, electron microscopic analysis demonstrated fibrillar components with a diameter of approx. 10 nm (Figure 2A). The structure of the fibrillar material was similar to that seen in native fibrils isolated from amyloidotic mouse spleens (Figure 2D). The CE/J protein did not show any significant fibrillar material; however, the protein did show aggregation in solution (Figure 2B). As a control, purified apo-AI was treated in the same manner and did not show fibril-like material (Figure 2C).

SAA clearance in normal and amyloidotic mice

We examined the incorporation of SAA into amyloid fibrils by performing experiments designed to determine the clearance rate of the respective SAAs. Radiolabelled SAA was synthesized by CV-1 cells as described in the Materials and methods section. Cells were incubated in the presence of normal mouse HDL or acute-phase mouse HDL. HDL was floated ultracentrifugally from culture media and analysed by SDS/PAGE to determine the specificity of incorporation of the radiolabel into SAA. Representative samples of CE/J SAA HDL (Figure 3, lane 1) and SAA₂ HDL (lane 2) on normal mouse HDL are shown. The infected CV-1 cells produced only labelled SAA proteins; the other lightly labelled bands were aggregates of the SAA proteins

Figure 4 Clearance of SAA proteins in normal and amyloidotic mice

Radiolabelled recombinant proteins (HDL, 100 μ g per mouse), prepared as described in the legend to Figure 3, were injected into normal or amyloidotic mice and examined for clearance. Animals were bled at the given times and radioactivity was analysed. Triplicate samples (10 μ l) were counted in a scintillation counter and plotted against time (trichloroacetic acid-precipitable counts). Six animals were used per time point. (A) Normal SAA/HDL injected into a normal mouse; (B) normal SAA/HDL injected into an amyloidotic mouse; (C) acute-phase SAA/HDL injected into a normal mouse; (D) acute-phase SAA/HDL injected into an amyloidotic mouse.

that are always detected on HDL and identified by Western blots (results not shown). Radiolabelled SAA HDL (100 μ g of protein per 5×10^6 d.p.m. for each mouse) was injected into normal and amyloidotic mice to examine the relative clearance of SAA proteins (Figure 4 and Table 2). As can be seen in Figure 4, CE/J SAA was cleared more slowly under all conditions than was the SAA₂ protein. In addition, the SAA₂ protein was cleared faster than the CE/J protein in the amyloidotic mice than in the normal mice [Figure 4B (2.5-fold) compared with Figure 4A (1.7-fold) respectively; $P < 0.05$]. When clearance of SAA₂ protein is compared with that of the CE/J on acute-phase HDL in amyloidotic mice, the SAA₂ was cleared even faster (Figure 4D) (4-fold; $P < 0.01$). The SAA₂ protein was shown to clear with a half-life of 30 min under conditions of acute-phase HDL in amyloidotic mice. It was expected that the radiolabelled SAA₂ protein would clear at a slower rate on acute-phase HDL owing to the presence of unlabelled SAA present on the particle. However, the results here suggest that the alterations in the

Table 2 Half-life determination of apo-SAA

Results are means $\pm$ S.D. for eight animals per group.

Mouse group	HDL	Calculated half-life (min)
Normal	Normal CE/J	120 $\pm$ 10
	Normal SAA ₂	70 $\pm$ 11
Amyloidotic	Normal CE/J	135 $\pm$ 14
	Normal SAA ₂	55 $\pm$ 8
Normal	Acute-phase CE/J	115 $\pm$ 16
	Acute-phase SAA ₂	60 $\pm$ 10
Amyloidotic	Acute-phase CE/J	120 $\pm$ 18
	Acute-phase SAA ₂	30 $\pm$ 12

structure of the acute-phase HDL and the structure of the SAA protein are important in determining the clearance rate of the SAA₂.

Figure 5 Incorporation of radiolabelled SAA₂ into amyloid tissue

Radiolabelled SAA–HDLs were injected into amyloidotic mice and examined for the incorporation of radioactivity into amyloid. Mice ($n = 6$) were injected with 100 μ g of SAA–HDL and spleens were removed 24 h later. Top panel: mice injected with SAA₂/HDL. Bottom panel: mice injected with CE/J SAA/HDL. Spleens were examined histologically for the incorporation of radioactivity into amyloid.

Table 3 Scintillation counting of splenic tissue after injection of radio-labelled SAA

Results are means $\pm$ S.D. for six animals per group.

Mouse group	HDL	Radioactivity (d.p.m.)
Normal	CE/J	1250 $\pm$ 427
	SAA ₂	3780 $\pm$ 398
Amyloidotic	CE/J	1830 $\pm$ 576
	SAA ₂	26 400 $\pm$ 2340

Association of recombinant SAA₂ with amyloid

The amyloid association of the SAA proteins *in vivo* was examined by injection of radiolabelled recombinant SAA proteins into normal and amyloidotic mice (Figure 5). Amyloidotic mice were prepared by the injection of AEF and AgNO₃ and injected with radiolabelled SAA–HDL after 7 days to minimize the effects of acute-phase HDL on fibril deposition. HDL analysis on amyloid mice at 7 days showed that SAA returned to approximately normal levels (results not shown). Radiolabelled CE/J and SAA₂ HDL was prepared by incubation of CV-1 cells with normal mouse HDL. Samples were ultracentrifuged and HDL was purified. Both the CE/J and SAA₂ HDL were labelled to equal efficiencies, then equivalent radioactive counts and equivalent amounts of protein were injected into mice. Mice were injected with radiolabelled CE/J–SAA and SAA₂–SAA and spleens were collected after 24 h. As shown in Figure 5, CE/J–SAA displayed some association with the amyloid present in the spleen (bottom panel), whereas SAA₂–SAA showed significant amounts of radiolabelled protein incorporated into the splenic amyloid (top panel). These results suggest that the CE/J–SAA proteins might

Figure 6 Adenoviral expression of SAA *in vivo*

Mice were injected with LPS (10 μ g) and blood was collected after 24 h (lane 1); other mice were not injected (lane 2) or injected with CE/J adenovirus (10⁹ pfu) (lane 3) and blood was collected after 3 days. LPS was injected intraperitoneally in 100 μ l, adenoviruses were injected in 100 μ l intravenously. Isolated HDLs (5 μ g) were subjected to SDS/PAGE and staining with Coomassie Blue. The SAA band is indicated.

be able to associate to some degree with the established amyloid, but they are still incapable of initiating amyloid formation. Normal spleens showed background levels of radioactivity and degradation of SAA proteins (results not shown). In addition, scintillation counting of the control and amyloidotic spleens after injection of radiolabelled protein gave results that agreed with the autoradiographic results (Table 3).

Adenoviral-directed SAA expression *in vivo*

The versatility of the adenoviral vector system was shown by injection of the CE/J adenoviral vectors into mice and examination of protein expression. Figure 6 shows the level of expression of the adenoviral vectors in the mouse. Mice were injected with 10 μ g of LPS for 24 h (lane 1), not injected (lane 2) or injected with 10⁹ pfu per mouse for 3 days (lane 3); blood was collected and examined for SAA on HDL particles. As seen in Figure 6, the SAA adenovirus-injected mice had SAA levels comparable to those in acute-phase mice. IEF analysis demonstrated a single species of SAA that migrated at the same pI as the CE/J protein (results not shown). The SAA in the virus-injected mice represents a single SAA (CE/J) species, whereas in the acute-phase mice this is a composite of both SAA₁ and SAA₂. The SAA levels reached approx. 800 μ g/ml in the adenovirus-injected mice. These results indicate that the adenoviral system can be used *in vitro* and *in vivo* to analyse the structural features necessary for amyloid fibrillogenesis.

DISCUSSION

Previous studies have demonstrated the expression of SAA proteins *in vitro* by using *Escherichia coli* and baculovirus expression systems [16,17]. The use of these two systems has proved to be a problem owing to the toxic effects of the recombinant protein in bacterial cells and the inefficient processing of the SAA molecules into mature protein. Both the baculoviral and bacterial systems resulted in the production of 2 mg of protein per 2.5 $\times$ 10⁸ cells and up to 3 mg/l protein respectively, but the proteins were not secreted by either type of

cell. To overcome some of these problems, a methionine residue has been added to the N-terminus of the mature SAA molecule to permit the synthesis and secretion of the mature protein [16]. However, the addition of a methionine to the very hydrophobic N-terminal region could create problems for HDL association and amyloid formation. Recent studies have shown some success in the generation of mature SAA proteins both in mammalian cells and *E. coli* [24,25]. In the bacterial system the synthesis of sufficient quantities of protein for structural studies and the generation of mature protein remain problematic [25]. In the mammalian expression system used by Patel et al. [24], the cells generated 30 mg of protein per litre of medium for the wild-type protein, but only 3 mg/l protein for the mutants. In addition there are still problems of loss of the plasmid in cells under selection and of maintaining expression in the mammalian cells. We have developed an adenoviral vector system for the efficient expression of mature SAA protein *in vitro*, in which our yield was approx. 50 mg/l of medium. Moreover it is highly reproducible and has the convenience of secretion of the SAA proteins and association on HDL.

In this study we have demonstrated the expression of the mouse CE/J and SAA₂ recombinant proteins by using adenoviral vector-infected CV-1 cells. The CV-1 cells produce and secrete SAA protein at 50 mg/l; more than 80% of this protein was associated with HDL. These cells maintained a continuous production of SAA protein if the medium was changed on a regular basis (results not shown). Protein samples subjected to IEF indicated that the proteins were of the same pI as the endogenous mouse proteins, i.e. recombinant CE/J protein had a pI of 6.15 and recombinant SAA₂ had a pI of 6.3. This suggests that the SAA proteins are accurately processed in the CV-1 cells and therefore provide a source of material for structural studies *in vitro*. The novelty of the adenoviral vector system permits the large-scale production of the SAA proteins, and reproducibility and flexible expression of the SAA proteins either *in vitro* or *in vivo*.

As stated above, the SAA₂ was cleared selectively from the circulation and deposited into amyloid fibrils [11]. CE/J mice are resistant to amyloid deposition and this resistance is thought to be due to amino acid differences in the N-terminus of the SAA protein [13]. We and others have shown that synthetic peptides against the amino terminal portion of the SAA CE/J protein (residues 1–15) do not form amyloid fibrils *in vitro*, whereas the N-terminus of the SAA₂ peptides are capable of forming fibrillar material ([14] and M. S. Kindy and J. Yu, unpublished work). The recombinant SAA₂ protein in the present study formed amyloid fibrils as determined by electron microscopy and Congo Red staining. The CE/J protein showed aggregation of the protein but did not have an amyloid structure when stained with Congo Red. These results confirm previous work that recombinant protein can form amyloid and that the N-terminus is important for the fibrillogenic property of the molecule [16,24]. Turnell et al. [26] predicted that alteration of the glycine residue in position 8 of the human SAA_{1,2} protein would disrupt fibril formation; Patel et al. [24] have shown that mutation in this glycine residue resulted in a protein that was incapable of forming fibrils. In the mouse, the SAA₂ protein has a glycine residue at position 7 of the mature protein, whereas SAA₁ and the CE/J protein have a histidine residue. The N-terminus is thus probably important for the fibrillogenic property of the molecule [16,24]. These studies will lead to the development of adenoviral vectors that contain mutations in the positions 6 and 7 of the SAA₂ protein to determine which amino acid or amino acids are important for amyloid fibrillogenesis.

To determine the clearance of SAA in the mouse, we used

radiolabelled SAA proteins isolated from adenovirus-infected cells. Our results were similar to those published previously; however, the conditions used here to generate SAA–HDL do not represent the harsh conditions used to isolate or radiolabel the SAA molecules, as described previously [9,19,25]. As noted by Kluge-Beckerman et al. [25], the previous studies focused on the clearance of SAA from the circulation in normal mice and showed that the SAA half-life *in vivo* was approx. 80 min. However, this half-life was not capable of distinguishing between the different clearance rates of the individual SAA isotypes. Kluge-Beckerman et al. [25], using an *E. coli* system, labelled SAA with [³⁵S]methionine and isolated the radiolabelled SAA by incubation of the bacterial lysates with purified mouse HDL after lysis by freeze–thawing and sonication. Under the conditions used in this study, the SAA could associate with the HDL as it was presumably synthesized in a fashion similar to that synthesized from the mouse liver without subjecting the SAA to freeze–thawing or lysis. Our results are similar to those seen previously in that the SAA₁ had a half-life of 75 min, in comparison with 30 min for SAA₂. Our results indicate that the CE/J protein in normal mice had a calculated half-life of 120 min, in comparison with 70 min for the SAA₂ protein.

Several investigators have examined the clearance of SAA proteins during the acute phase to determine if there are differences in SAA₁ and SAA₂ [19,25]. Mice were injected with LPS or AgNO₃ and the clearance of SAA protein was determined. One study found that there was no difference in the half-life of the SAA proteins when comparing normal and acute-phase conditions [19]. In contrast, a recent study showed that there were differences between the clearances of SAA₁ and SAA₂ depending on the type of HDL used and/or the state of the animal (i.e. normal or acute phase) [25]. Under most conditions the SAA₁ was cleared much more slowly than SAA₂, whereas when acute-phase animals were injected with radiolabelled SAA, on acute-phase HDL, the half-life of SAA₁ was the same as that of SAA₂. The use of acute-phase conditions to study the clearance of SAA might result in difficulty in interpreting the results owing to the multitude of factors that are in process during an acute-phase response. In this regard we decided to use mice that had established amyloid deposits, where the acute-phase response had subsided, to study the clearances of CE/J and SAA₂ isotypes. Mice were devoid of an acute-phase response as determined by the lack of SAA proteins in the plasma. Animals were injected with normal or acute-phase HDL containing radiolabelled SAA proteins. Under these conditions, the CE/J SAA protein had a half-life of approx. 120 min, whereas the SAA₂ protein was cleared faster when associated with normal HDL (55 min) and even faster when associated with acute-phase HDL (30 min). This is consistent with the hypothesis of selective rapid clearance of SAA₂ into amyloid deposits. This may be due partly to some displacement of unlabelled SAA from the HDL during the synthesis of radiolabelled SAA in the CV-1 cells, or a change in the structure of the acute-phase HDL particle to allow more rapid dissociation of the SAA.

A number of studies have demonstrated that the HDL particle undergoes changes during the induction of an acute-phase response. HDL cholesterol and apo-AI levels decrease markedly during acute inflammation and are also lower than normal in chronic inflammatory conditions [27]. The HDL particle becomes smaller and more dense, which could affect lipoprotein metabolism. It has been shown that the group II non-pancreatic phospholipases are classical acute-phase reactants and are elevated in inflammatory states [28]. In addition, group II non-pancreatic phospholipases can act on lipoproteins as substrates [29]. In transgenic mice expressing sPLA₂, HDL levels were

decreased, HDLs were smaller and phospholipid content was decreased [30]. These studies indicate the altered nature of the HDL particle during inflammation, which might affect the metabolism of the HDL particle as well as the clearance of lipoproteins associated with these particles. The altered structure of the HDL particle and the structure of the SAA₂ protein might contribute to the selective and rapid clearance of SAA₂ into amyloid fibrils.

The development of an adenoviral vector system for expression of recombinant SAA proteins has several advantages over the bacterial and other mammalian protein expression systems. First, the adenoviral system permits the generation of large quantities of SAA proteins that will be needed for structural analysis. Secondly, the ability to infect a variety of different cells to determine the optimal cell type for expression gives a unique flexibility. Thirdly, the adenoviral vectors can be used *in vivo* to study the effects of expression of altered SAA molecules on amyloid formation. This is important in defining how specific structural alterations in SAA would influence fibrillogenesis.

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