Differential plasma clearance of murine acute-phase serum amyloid A proteins SAA1 and SAA2

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Serum amyloid A (SAA) proteins SAA1 and SAA2 are prominent acute-phase reactants which circulate in association with the high-density-lipoprotein (HDL) fraction of plasma. Plasma levels of SAA1 and SAA2 increase dramatically, by as much as 1000-fold, within 24 h of tissue injury and then rapidly decrease with cessation of the inflammatory stimulus, suggesting that SAA clearance and/or catabolism is important to the re-establishment of homeostasis. In this context, aberrant SAA catabolism has long been considered a potential factor in the pathogenesis of reactive amyloidosis. To initiate studies aimed at understanding the differential regulation of SAA metabolism, we have produced ³⁵S-labelled murine SAA1 and SAA2 in *Escherichia coli*, bound them individually to HDL, and then compared the plasma-

clearance characteristics of SAA1 and SAA2 under normal and acute-phase conditions. When bound to normal HDL, SAA2 [half-life ($t_{1/2}$) = 30 min] was cleared significantly faster than SAA1 ($t_{1/2}$ = 75 min). Clearance of SAA1 and SAA2 was significantly slower when each was bound to acute-phase HDL as opposed to normal HDL, when clearance rates were determined in acute-phase mice versus normal mice, and when normal HDL was remodelled to contain both recombinant isotypes rather than just one of the isotypes. Thus it appears that an increased amount of SAA on HDL, or possibly the combined presence of both isotypes on HDL, is associated with a prolongation in the plasma half-life of SAA.

INTRODUCTION

The serum amyloid A (SAA) family of proteins in both humans and mice of the type A phenotype (e.g., BALB/c and CBA/J) is encoded by four genes. SAA1 and SAA2, the products of two nearly identical genes, are extremely sensitive acute-phase reactants; SAA3 in mice is a quantitatively minor constituent of acute-phase plasma, whereas in humans the gene for SAA3 is not expressed; SAA4, also termed 'C-SAA' and originally referred to as 'SAA5' in mice, is relatively dissimilar to SAA1 and SAA2 and is not hyperinducible [1,2]. Triggered by inflammation, the macrophage-derived cytokines interleukin-1, interleukin-6, and tumour-necrosis factor act synergistically on hepatocytes to induce synthesis of SAA1 and SAA2 [3–5]. SAA1 and SAA2 levels in plasma, in turn, are elevated as much as 1000-fold. The physiological impact of this dramatic increase has yet to be defined. SAA1 and SAA2 circulate exclusively with the high-density-lipoprotein (HDL) component of plasma [6,7], hence roles in reverse cholesterol transport and/or cholesterol metabolism are under investigation [8–10]. In other studies, SAA has been shown to exhibit chemoattractant activity for monocytes, neutrophils and T-lymphocytes, suggesting a proinflammatory function in the recruitment of phagocytic cells [11,12].

Fibrillar deposits of SAA-derived amyloid A (AA) protein characterize reactive amyloidosis, an infrequent, but often fatal, disease affecting patients who have chronic inflammatory conditions such as rheumatoid arthritis [13]. While prolonged elevation of SAA seems to be a necessary condition for AA amyloid pathogenesis, it certainly is not the only determinant. Many patients have chronic inflammation, but only a small

percentage develop amyloidosis. Additional factors, including aberrant SAA metabolism and interaction with such macromolecules as glycosaminoglycans, amyloid P component and the poorly defined amyloid enhancing factor, may be involved in pathogenesis.

Amyloidosis-associated mutations, analogous to those found in the genes for a number of other amyloid-forming proteins, have not been identified in SAA genes.

Mice have long served as a model for both the acute-phase response and the development of reactive amyloidosis. The structure and induction characteristics of murine SAA genes and proteins closely parallel those in humans. In addition, AA amyloid formation can be experimentally induced in mice by repeated injections of an inflammatory stimulus. Although levels of both acute-phase SAA isotypes are elevated in the blood, only murine SAA2 has been detected in amyloid deposits [14–16], and in humans a preponderance of SAA1-derived AA protein has been noted [17]. The basis for selective deposition is not apparent, especially in the light of the fact that the two isotypes share approx. 95 % structural identity [18].

Since an understanding of SAA metabolism may provide clues about SAA function as well as amyloidogenesis, we have undertaken a characterization of SAA metabolism in the murine model. Our initial study has focused on the kinetics of SAA plasma clearance. Previous studies by others have shown SAA to be cleared more rapidly than, and hence independently of, HDL apolipoproteins AI and AII [19–22]. Using biosynthetically ³⁵S-labelled recombinant SAA1 and SAA2, we have now differentiated the clearance properties of the two acute-phase SAA isotypes in mice.

Abbreviations used: (S)AA, (serum) amyloid A; apoAI, apolipoprotein AI; HDL, high-density lipoprotein; SSCP, single-strand conformation polymorphism.

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MATERIALS AND METHODS

Expression of recombinant SAA

Murine SAA1 and SAA2 cDNAs were amplified by the PCR from cloned plasmids pbSAA1.14 and pbSAA2.63 [18] kindly provided by Dr. K.-I. Yamamoto and Dr. S. Migita (Department of Molecular Immunology, Kanazawa University, Kanazawa, Japan). Amplified fragments were inserted into pET-21a(+) (Novagen, Madison, WI, U.S.A.) between *Nde*I and *Bam*HI cloning sites. Recombinant pET-SAA plasmids were transformed into *Escherichia coli* strain B834(DE3)pLys (Novagen). Plasmid DNA isolated from large-scale cultures of an SAA1 clone and an SAA2 clone was sequenced to verify identity to SAA1 and SAA2 cDNA sequences respectively.

SAA expression was induced by culturing *E. coli* under the conditions previously described for expression of human SAA [23], except when SAA was biosynthetically radiolabelled. Expression in the presence of [³⁵S]methionine was carried out as follows. Mid-exponential-phase cultures (8 ml) were used to inoculate 100 ml of Luria-Bertani medium containing 500 µg/ml carbenicillin and 34 µg/ml chloramphenicol; these cultures were grown for 3 h at 37 °C. Cells were collected by centrifugation, rinsed once in 100 ml of minimal medium (M9 salts) and then resuspended in 100 ml of minimal medium supplemented with 500 µl/ml carbenicillin, 34 µg/ml chloramphenicol, 1 mM isopropyl β-D-thiogalactoside to induce expression, and 5 mCi of [³⁵S]methionine (³⁵S-EXPRESS, Amersham International; 3000 Ci/mmol). After 30 min, 200 µg/ml rifampicin was added to inhibit *E. coli* RNA polymerase. After incubation for an additional 2 h, cells were pelleted by centrifugation.

Purification of recombinant SAA

Recombinant murine SAA proteins were purified from *E. coli* extracts according to the scheme used to purify recombinant human SAA [23]. Purification involved Sepharose CL-6B gel-filtration chromatography (Pharmacia Biotech Inc., Piscataway, NJ, U.S.A.), followed by chromatofocusing in Polybuffer exchanger PBE 94 (Pharmacia) between pH 8.0 and pH 5.0. SAA-containing column fractions were identified by immunoreactivity with anti-(murine SAA) antiserum produced in this laboratory. SAA in chromatofocusing pools was precipitated with 80 % satd. (NH₄)₂SO₄ to remove non-dialysable Polybuffer components. Final preparations were dialysed against water, freeze-dried, and then analysed by SDS/PAGE. N-terminal sequences of purified SAA proteins were determined on an Applied Biosystems model 473A protein sequencer using the manufacturer's standard cycles. Cleavage with CNBr was accomplished by overnight treatment with 1 % CNBr in 70 % formic acid at room temperature.

Single-strand conformation polymorphism (SSCP) analysis

SAA mRNA sequences in ICR and BALB/c mice were compared by SSCP analysis. Hepatic RNA isolated from mice experiencing an acute-phase response was reverse-transcribed and then enzymically amplified using the Perkin-Elmer GeneAmp RNA PCR Kit protocol and reagents (Perkin-Elmer/Cetus, Norwalk, CT, U.S.A.). PCR reaction mixtures contained 30 nM ³²P-labelled dCTP and either primers A and B or primers C and B (A: 5'-CTCCTGGGAGTCTGCCATG-3', B: 5'-AGTCCAGGAGGTCTGTAGTAA-3', C: 5'-AGGAAGCTA-ACTGGAAAACTC-3'). Primers A and B match both SAA1 and SAA2 perfectly, while primer C anneals specifically to SAA1. Amplification was accomplished by 30 cycles of de-

naturing at 94 °C for 30 s, annealing at 63 °C for 1 min, and extension at 72 °C for 1 min. The 317 bp fragment amplified by primers A and B was digested with *Sma*I prior to SSCP analysis; the 226 bp fragment amplified by primers C and B was analysed directly. SSCP analysis in polyacrylamide gels was carried out as previously described [24].

PAGE and immunoblot analysis

Tricine/SDS/PAGE was carried out as described by Schagger and von Jagow [25]. Separating, spacing and stacking layers contained 16.5, 10 and 4 % polyacrylamide respectively. Electrophoresis was conducted at 25 mA for 16–18 h using a cathode buffer consisting of 0.1 M Tris, 0.1 M Tricine and 0.1 % SDS, pH 8.2, and an anode buffer of 0.2 M Tris/HCl, pH 8.9. Urea/SDS/PAGE was performed in 11.5 % polyacrylamide gels containing 6.4 M urea as previously described [26]. Coomassie Blue-stained proteins were quantified in wet gels using a Molecular Dynamics laser-based Personal Densitometer SI. Immunoblot analyses were performed as described previously [27]. Rat anti-(mouse AA protein) monoclonal antibody produced in this laboratory or rabbit anti-(recombinant mouse SAA2) antiserum was used for detection.

Isolation of HDL

Blood was collected in 0.1 % EDTA from NIH Swiss ICR mice which had been either maintained in a healthy state or injected subcutaneously with azocasein, 0.5 ml of a 10 % (w/v) solution in 0.01 M sodium bicarbonate, pH 8.0, 20 h prior to bleeding to induce an acute-phase response. HDL were isolated from plasma by sequential flotation ultracentrifugation. The plasma fraction floating at a density between 1.06 and 1.21 g/ml was collected and dialysed against PBS using Centricon-3 microfiltration units (Amicon Corp., Danvers, MA, U.S.A.). All HDL preparations were examined for the presence of endogenous SAA by SDS/PAGE/immunoblot analysis. HDL prepared from healthy mice contained no immunodetectable SAA. HDL from casein-injected mice contained SAA easily revealed by Coomassie Blue staining with apoAI/(SAA1 + SAA2) ratios ranging between 3.5 and 5.0; in these preparations SAA represented approx. 15 % of the total apolipoprotein. The SAA1/SAA2 ratio on acute-phase HDL particles ranged between 1.2 and 1.6.

Binding of recombinant SAA to HDL

Pelleted *E. coli* cells from 100 ml cultures grown in the presence of [³⁵S]methionine were resuspended in 8 ml of PBS, lysed by freezing-thawing, and sonicated on ice to shear DNA. The lysate was centrifuged at 10000 g for 10 min to prepare a soluble extract. Plasma or isolated HDL in PBS equivalent to the amount of HDL in 4 ml of plasma was added to the soluble extract. The *E. coli* extract/HDL mixture was incubated at 37 °C for 15 min and its density then adjusted to 1.21 g/ml with solid KBr. SAA-HDL were isolated by flotation ultracentrifugation at 541000 g for 6 h and applied to a column of Sephacryl S-200 equilibrated and eluted with PBS. Fractions were monitored for absorbance at 280 nm and for radioactivity and were analysed by Tricine/SDS/PAGE.

Using this binding protocol, various combinations of SAA-HDL were achieved. These included: [³⁵S]SAA1 on normal HDL (HDL from healthy mice), [³⁵S]SAA2 on normal HDL, [³⁵S]SAA1 on acute-phase HDL (HDL from mice experiencing acute inflammation) and [³⁵S]SAA2 on acute-phase HDL. The plasma clearance kinetics of each of these SAAs were studied in

healthy mice and in mice with acute inflammation induced by a subcutaneous injection of casein. A final set of [35 S]SAA–HDL samples consisted of normal HDL remodelled to contain both recombinant SAA1 and recombinant SAA2, only one of which was radiolabelled.

Plasma clearance of SAA

Plasma clearance was monitored in NIH Swiss ICR (outbred) female mice; in most instances, the mice were retired breeders. Young ICR or BALB/c female mice, 8–12 weeks old, were used in some experiments to investigate whether age or strain influenced the plasma clearance rate. Each mouse was injected via the tail vein with [35 S]SAA–HDL in PBS (50–100 μ l containing 25–50 μ g of [35 S]SAA; $(2-3) \times 10^6$ c.p.m.). After anesthetizing mice with metaflane, blood samples (~ 50 μ l) were collected retro-orbitally into heparin-coated capillary tubes at 4 min, 30 min, 1 h, 2 h, 3 h, and 4 h post-injection. Radioactivity (c.p.m.) in a 10 μ l aliquot of plasma was measured by liquid-scintillation counting and expressed as a fraction of that in the aliquot collected at 4 min. The fractional values used to construct plasma clearance curves were the mean $\pm$ S.E.M. of fractional values determined for all mice in each of the experimental groups. Data from all mice were included, since no statistical differences were found between the values obtained in young-versus-old or in ICR-versus-BALB/c mice. Curves were generated using Cricket Graph Software, Version 1.3.2. SAA levels in plasma collected before and 4 min after injection of [35 S]SAA–HDL were measured by a direct binding ELISA similar to that used to determine human SAA concentrations [28].

RESULTS

Expression and purification of SAA1 and SAA2

Expression of murine SAA1 and SAA2 cDNAs in *E. coli* strain B834(DE3)pLys was accomplished using the pET-21a(+) vector. Cultures were maintained in high concentrations of carbenicillin (500 μ g/ml) before induction as well as during expression of SAA. Highly purified SAA1 and SAA2 were obtained from *E. coli* extracts by Sepharose CL-6B gel-filtration chromatography followed by chromatofocusing between pH 8.0 and pH 5.0. SAA1 was eluted from the chromatofocusing column in two peaks, at pH 7.2 and pH 6.9. SAA2 was also eluted in two peaks, at pH 7.0 and pH 6.7. The protein in each of the peaks generated a single Coomassie Blue band when examined by SDS/PAGE (Figure 1) and was immunoreactive with anti-SAA antiserum. Typical yields from 1-litre cultures were 2 mg of SAA1 in the pH 7.2 peak, 2 mg of SAA1 in the pH 6.9 peak, 4 mg of SAA2 in the pH 7.0 peak and 1 mg of SAA2 in the pH 6.7 peak.

The SAA identity of purified proteins was confirmed by N-terminal sequencing for 15–20 residues. All of the SAA proteins for which sequence data were obtained began with glycine, indicating cleavage of the N-terminal formylmethionine. Residue yields from sequencing were lower than expected for the SAA1-pH 6.9 and SAA2-pH 6.7 preparations. When these samples were cleaved by CNBr and the resulting peptides sequenced, residue yields from internal peptides were 2–3 times higher than yields from the N-terminal peptide, implying that one-half to two-thirds of the protein in the SAA1-pH 6.9 and SAA2-pH 6.7 preparations was N-terminally blocked. The fact that the yield of residues in the N-terminal peptide was not increased after CNBr treatment suggested that the recombinant proteins did not contain formyl-methionine at the N-terminus. It seems more likely that the blocking group was bound directly to glycine, the first residue of murine SAA. Sequence analysis of

Figure 1 Tricine/SDS/PAGE analysis of purified recombinant SAA

SAA proteins were purified from *E. coli* lysates by Sepharose CL-6B chromatography and chromatofocusing (CF). M, molecular-mass markers. Lanes: 1, SAA1 (CF pH 7.2 peak); 2, SAA1 (CF pH 6.9 peak); 3, SAA2 (CF pH 7.0 peak); 4, SAA2 (CF pH 6.7 peak).

SAA1-pH 7.2 and SAA2-pH 7.0 before and after CNBr cleavage gave no indication of blocked protein. The elution of both blocked and unblocked SAA in SAA1-pH 6.9 and SAA2-pH 6.7 samples may indicate the presence of multimeric molecules comprised of both blocked and unblocked SAA.

Comparison of SAA sequences in ICR and BALB/c mice

SSCP analysis revealed no differences between the SAA mRNA sequences in ICR mice and the SAA mRNA sequences in BALB/c mice, i.e., the electrophoretic migration pattern of single-stranded SAA1 and SAA2 cDNA fragments amplified from ICR liver RNA was identical with the migration pattern of SAA fragments amplified from BALB/c liver RNA (results not shown). In addition, the pattern of bands in these samples matched the pattern generated from cloned BALB/c SAA1 and SAA2 cDNAs. On the basis of this finding, ICR mice were used throughout these studies as a source of plasma and HDL and as the primary strain in which SAA plasma clearance was monitored.

Binding of SAA to HDL

As part of our overall goal to compare the plasma clearance kinetics of murine SAA1 and SAA2, methodology was developed for obtaining HDL remodelled to contain a defined, radiolabelled SAA isotype. According to this protocol, SAA proteins were expressed in *E. coli* under conditions allowing for biosynthetic incorporation of [35 S]methionine (Figure 2). A soluble extract prepared from lysed *E. coli* was incubated with murine plasma or isolated HDL; [35 S]SAA–HDL complexes formed during the incubation were isolated by flotation ultracentrifugation. The SAA which became associated with HDL in this manner had been neither denatured nor freeze-dried. In contrast, binding purified SAA to HDL requires that the SAA first be solubilized by treatment with denaturing agents, e.g. 6 M urea. The presence of urea limits the amount of SAA which can be added to plasma or HDL. Therefore it is not surprising that much higher levels of SAA on HDL were achieved using SAA-containing *E. coli* extracts rather than soluble purified SAA (results not shown).

SDS/PAGE analysis of normal HDL before and after in-

Figure 2 Urea/SDS/PAGE analysis of lysates of *E. coli* induced to express SAA

Gels containing 11% polyacrylamide and 6.4 M urea were stained with Coomassie Blue (lanes 1–4) and then autoradiographed (lanes 5 and 6). M, molecular-mass markers. Lanes: 1, SAA1 clone grown in rich medium; 2, SAA2 clone grown in rich medium; 3, SAA1 clone grown in minimal medium plus [^{35}S]methionine; 4, SAA2 clone grown in minimal medium plus [^{35}S]methionine; 5, autoradiograph of lane 3; 6, autoradiograph of lane 4. Upper arrow, SAA2; lower arrow, SAA1.

Figure 3 Tricine/SDS/PAGE analysis of [^{35}S]SAA–HDL formed by incubating *E. coli* lysates with HDL

Gels were stained with Coomassie Blue (lanes 1, 2 and 4) and autoradiographed (lanes 3, 5, 6 and 7). M, molecular-mass markers. Lanes: 1, normal HDL prior to incubation with lysates; 2, normal HDL after incubation with SAA1 lysate; 3, autoradiograph of lane 2; 4, normal HDL after incubation with SAA2 lysate; 5, autoradiograph of lane 4; 6, acute-phase HDL after incubation with SAA1 lysate; 7, autoradiograph of acute-phase HDL after incubation with SAA2 lysate. The faint band in normal HDL (lane 1) at the position of SAA was not immunoreactive with anti-SAA antibody.

incubation with SAA-containing *E. coli* extracts showed SAA1 or SAA2 to be the only *E. coli*-derived protein in the post-incubation HDL preparation (Figure 3, lanes 1, 2 and 4). Autoradiography confirmed [^{35}S]SAA as the sole radiolabelled protein in both [^{35}S]SAA–normal HDL and [^{35}S]SAA–acute-phase HDL preparations (Figure 3, lanes 3, 5, 6 and 7). As shown in Figure 2, lanes 5 and 6, many proteins in addition to SAA had been biosynthetically radiolabelled; the fact that none of these other proteins bound to HDL underscores the specificity of the SAA–HDL interaction.

To ensure that final [^{35}S]SAA–HDL preparations were free of non-bound, aggregated or denatured SAA, HDL isolated by flotation ultracentrifugation were subjected to gel-filtration chromatography through Sephacryl S-200 (which also served to remove the KBr). The SAA elution profile was compared with the elution profile of other HDL components by SDS/PAGE

Table 1 ApoAI/SAA ratios in final preparations of [^{35}S]SAA–HDL

Ratios were calculated from densitometric image of Coomassie Blue-stained bands after SDS/PAGE.

Preparation	ApoAI/SAA
[^{35}S]SAA1 on normal HDL	15–20*
[^{35}S]SAA2 on normal HDL	6*
[^{35}S]SAA1 on acute-phase HDL	2.2†
[^{35}S]SAA2 on acute-phase HDL	2.7†

* HDL contained only recombinant SAA.

† HDL contained endogenous SAA1 and SAA2, as well as recombinant SAA.

analysis of column fractions. In most cases, a few of the leading fractions were significantly enriched in SAA relative to apolipoprotein AI (apoAI). Since it was possible that these fractions contained aggregated SAA or SAA-phospholipid vesicles formed by sonication of *E. coli*, they were excluded from the [^{35}S]SAA–HDL pool used for plasma clearance studies.

The relative amount of recombinant SAA bound to HDL was evaluated by comparing apoAI/SAA ratios in final [^{35}S]SAA–HDL preparations as determined by laser-based densitometric quantification of Coomassie Blue bands after SDS/PAGE (Table 1). Under identical incubation conditions employing the same HDL preparation, higher levels of SAA2 than SAA1 were achieved on normal HDL. On the basis of Coomassie Blue staining intensities, the relative amount of [^{35}S]SAA2 in *E. coli* extracts was 3.7 times higher than the amount of [^{35}S]SAA1 (Figure 2, lanes 3 and 4), suggesting that the higher apoAI/SAA ratio in final [^{35}S]SAA1–HDL preparations was due to lower expression of SAA1 rather than reduced affinity for HDL. Expression of SAA1 appeared to be more sensitive than SAA2 to the minimal culture conditions employed during induction in the presence of [^{35}S]methionine. SAA1 production in minimal medium was approximately one-half of what it was in rich medium, whereas SAA2 production decreased by less than 10% (Figure 2, lanes 1–4).

Clearance of SAA from plasma

The presence of either [^{35}S]SAA1 or [^{35}S]SAA2 as the only radiolabelled component on HDL made it possible to independently monitor the plasma clearance of each SAA protein. Mice of the ICR and BALB/c strains were injected intravenously with [^{35}S]SAA–HDL, blood samples were taken at various times post-injection, and aliquots of plasma were analysed by liquid-scintillation counting to determine radioactivity remaining in blood at each of the time points. As determined by ELISA, [^{35}S]SAA–HDL injections did not result in measurable increases in plasma levels of SAA in either healthy or acute-phase mice.

Plasma clearance rates were different for SAA1 and SAA2 in three of the first four situations studied (Figure 4; Table 2). [^{35}S]SAA2 bound to normal HDL (containing no immunodetectable endogenous SAA) was cleared significantly faster than [^{35}S]SAA1 in both healthy and acute-phase mice (Figures 4A and 4B). In healthy mice, a 50% decrease in the plasma level of ^{35}S was seen 30 min after injection of SAA2–normal HDL and 75 min after injection of SAA1–normal HDL. In acute-phase mice, 50% levels were reached at 55 min (SAA2) and > 120 min

Figure 4 SAA plasma-clearance curves

^{35}S SAA–HDL were injected intravenously into mice, blood samples were collected at the indicated time points, and radioactivity was determined in 10 μl aliquots of plasma. Data points were generated by expressing the radioactivity in each of the samples as a fraction of the radioactivity in the 4 min sample. Each point represents the mean $\pm$ S.E.M. for n mice. In some cases S.E.M. bars are not clearly visible because they are very short. (A) ^{35}S SAA on normal HDL injected into healthy mice: ○, SAA1, $n = 6$; ●, SAA2, $n = 10$. (B) ^{35}S SAA on normal HDL injected into acute-phase mice: ○, SAA1, $n = 5$; ●, SAA2, $n = 4$. (C) ^{35}S SAA on acute-phase HDL injected into healthy mice: ○, SAA1, $n = 3$; ●, SAA2, $n = 6$. (D) ^{35}S SAA on acute-phase HDL injected into acute-phase mice: ○, SAA1, $n = 2$; ●, SAA2, $n = 4$.

Table 2 Comparison of plasma clearance of SAA1 and SAA2

Sample	Condition of injected mouse	Time post-injection at which ^{35}S SAA level was decreased by 50% (min)	
		SAA1	SAA2
^{35}S SAA on normal HDL*	Healthy	75	30
	Acute-phase	> 120	55
^{35}S SAA on acute-phase HDL*	Healthy	~ 120	65
	Acute-phase	~ 120	~ 120
^{35}S SAA + SAA on normal HDL†	Healthy	~ 120	60

* HDL contained recombinant SAA1 or SAA2.

† HDL contained recombinant SAA1 and SAA2, only one of which was radiolabelled.

(SAA1) post-injection. ^{35}S SAA2 was also cleared faster than ^{35}S SAA1 in healthy mice when each of the isotopes was bound to acute-phase HDL (Figure 4C). Under these conditions, the time required to reduce plasma levels by 50% was approx. 65 min for SAA2 and 125 min for SAA1. Only in acute-phase mice with acute-phase HDL serving as the ^{35}S SAA carrier were the time courses of SAA1 and SAA2 clearance the same; 50% levels were reached approx. 120 min following injection (Figure 4D).

Figure 5 SAA plasma-clearance curves generated using normal HDL which had been remodelled to contain both recombinant isotopes

HDL containing ^{35}S SAA1 plus unlabelled SAA2 or ^{35}S SAA2 plus unlabelled SAA1 were injected into healthy mice. Clearance of ^{35}S SAA from plasma was then monitored as described in the legend to Figure 4. SAA was not detectable on the HDL prior to remodelling or in the plasma of mice prior to injection. ^{35}S SAA1 plus unlabelled SAA2: ○, $n = 2$; ^{35}S SAA2 plus unlabelled SAA1, ●, $n = 4$.

The kinetics of SAA plasma clearance were influenced by the presence or absence of endogenous SAA on the injected SAA–HDL and by the health status (healthy or acute-phase) of the mice in which clearance was monitored (Figure 4; Table 2).

Clearance in healthy mice was slower when SAA (either SAA1 or SAA2) was bound to acute-phase HDL as opposed to normal HDL (Figure 4C versus Figure 4A). In addition, clearance of either isotype bound to normal HDL was slower in acute-phase mice than in healthy mice (Figure 4B versus Figure 4A). Clearly the presence of high levels of endogenous SAA either on HDL carrying the [35 S]SAA or in the circulation of the mouse injected with [35 S]SAA resulted in slower clearance of the radiolabelled isotype.

To investigate whether the slower clearance depicted in Figure 4(B), 4(C) and 4(D) relative to that seen in Figure 4(A) simply reflected preferential clearance of endogenous SAA or was in fact related to the combined presence of SAA1 and SAA2 or to the increased amount of total SAA, normal HDL were remodelled to contain both recombinant SAA isotypes, only one of which was radiolabelled. Clearance of each was then monitored in healthy mice and the time course compared with that determined when only a single isotype was present on HDL. Interestingly, the addition of even small amounts of unlabelled recombinant SAA1 caused a significant prolongation in the apparent half-life of [35 S]SAA2 (from 30 to 60 min) (compare Figure 4A and Figure 5). Similarly, the apparent half-life of [35 S]SAA1 increased from 75 min to > 120 min when unlabelled recombinant SAA2 was present.

DISCUSSION

Efficient production of murine SAA1 and SAA2 has been achieved in *E. coli* using pET-21a(+). The induction protocol, designed to maximize retention of the plasmid, is the same as that used successfully in this laboratory for expression of human SAA proteins [23]. High concentrations of carbenicillin are included in the culture medium, and the medium is replaced twice prior to induction to disfavor overgrowth of cultures by cells lacking plasmid. Analysis of pET-SAA plasmid DNA verified the presence of correct SAA1 and SAA2 sequences. The *E. coli*-expressed SAAs were purified by gel-filtration chromatography and chromatofocusing and subjected to N-terminal protein sequencing for further confirmation of SAA identity. Sequencing revealed that recombinant murine SAA1 and SAA2, like the naturally occurring murine SAA proteins, undergo excision of formylmethionine from the N-terminus, resulting in glycine as the first residue. In contrast, recombinant human SAA proteins produced using the same pET expression system retain methionine at the N-terminus and have arginine in the second position [23]. It has been reported that aminopeptidase cleavage of formylmethionine is determined by the residue in the penultimate position: the extent of cleavage decreases as the size of the penultimate amino acid side chain increases [29]. Our finding that cleavage occurs between methionine and glycine in mouse SAA, but not between methionine and arginine in human SAA, is consistent with this relationship.

While harsh chemical conditions were required for purification and characterization of SAA, physiological conditions were maintained throughout the course of metabolic studies. The SAA presented to HDL and subsequently injected into mice had been neither denatured nor freeze-dried. Furthermore, incorporation of [35 S]methionine during biosynthesis allowed SAA to be radiolabelled without chemical modification, an inevitable consequence of radiolabelling by other methods, e.g. iodination or methylation. The independent expression and radiolabelling of SAA1 and SAA2 provided a means by which to individually determine and compare the plasma half-lives of the two isotypes. Using this approach it was also possible to remodel either SAA-

rich or SAA-poor HDL. In contrast, previous studies of SAA plasma clearance utilized HDL isolated from acute-phase animals as the substrate for radiolabelling such that all protein constituents, including SAA1 and SAA2, became labelled [19,20,22]. The methodology adopted for this study obviated the need for SDS/PAGE resolution of proteins in order to monitor SAA plasma clearance.

The validity of directly counting 35 S in plasma as a measure of SAA depended on two conditions: (1) that SAA be the only radiolabelled entity in the injected sample; and (2) that all of the SAA be in a physiologically native form, i.e., non-denatured, non-aggregated. Satisfying the first condition, a single radiolabelled protein was revealed when [35 S]SAA-HDL preparations were subjected to SDS/PAGE and autoradiography (Figure 3). It should be noted that the SAA-normal HDL preparations represented in Figure 3, lanes 3 and 5, were formed by incubating *E. coli* extracts with plasma rather than with isolated HDL, as was done for the SAA-acute-phase HDL preparations shown in lanes 6 and 7. Plasma-clearance data generated from the former preparations were no different from those obtained in later experiments in which HDL were used. With regard to the second condition, several steps were taken to safeguard against the inclusion of non-physiological [35 S]SAA in the HDL preparations used for injection. First, insoluble SAA was removed from *E. coli* lysates by centrifugation and, secondly, [35 S]SAA-HDL preparations were isolated by flotation ultracentrifugation and finally by Sephacryl S-200 gel-filtration chromatography.

The SAA-plasma-clearance data presented here expand upon those obtained in previous studies in which SAA was radiolabelled as part of acute-phase HDL [20,22]. In these previous experiments, although individual protein constituents were resolved by SDS/PAGE, separate clearance curves were not generated for SAA1 and SAA2. By injecting 125 I-labelled acute-phase HDL into healthy mice, Hoffman and Benditt [20] determined an SAA plasma half-life of 75–80 min, while Tape and Kisilevsky [22], using 3 H-labelled HDL, determined a half-life of 1.4 h (84 min) in healthy mice. Presumably both of these values represent a composite of SAA1 and SAA2 half-lives. Under comparable conditions ([35 S]SAA on acute-phase HDL injected into healthy mice), our data indicated half-lives of 125 min for SAA1 and 65 min for SAA2.

The study by Tape and Kisilevsky [22] also included a comparison of SAA plasma clearance in healthy mice ($t_{1/2} = 1.4$ h) and mice with either an acute inflammatory condition ($t_{1/2} = 2.4 \pm 0.9$ h) or inflammation leading to AA amyloidosis ($t_{1/2} = 1.4 \pm 0.4$ h). They interpreted these values to mean that the apparent half-life of SAA was fixed regardless of the physiological state and that clearance took place through an unsaturable mechanism. In partial agreement, we found the apparent half-life of SAA1 bound to acute-phase HDL to be the same in healthy and acute-phase mice ($t_{1/2}$ values of ~ 120 min). The apparent half-life of SAA2 bound to acute-phase HDL, however, was much longer in acute-phase mice than in healthy mice ($t_{1/2} \sim 120$ min versus $t_{1/2} = 65$ min).

The general trend seen in Figures 4(A)–4(D) is a prolongation in the plasma half-life of SAA as the total amount of SAA in the system increases. From the most SAA-poor (Figure 4A) to the most SAA-rich (Figure 4D) condition, apparent half-lives of SAA1 and SAA2 increased 2- and 4-fold respectively. This trend appears to suggest that sites for SAA uptake from plasma may be rate-limiting. The results presented in Figure 5, however, cast doubt on this possibility. Even a small amount of recombinant SAA1, not equal to the amount of endogenous SAA present on acute-phase HDL, caused [35 S]SAA2 to lose the rapid-clearance characteristics it exhibited when present alone on HDL ($t_{1/2} =$

60 min versus 30 min) and to have a clearance time course mimicking that of [35 S]SAA2 bound to acute-phase HDL ($t_{1/2}$ = 65 min). A similar prolongation effect was observed for SAA1 in the presence of recombinant SAA2 ($t_{1/2}$ increased from 75 min to ~ 120 min). To explain this effect, it could be postulated that one isotype sterically hinders clearance of the other; perhaps the topography of HDL is such that the two isotypes restrict the accessibility of each other to molecules involved in clearance, e.g. receptors.

The most prolonged plasma half-life, ~ 2 h, was observed for both isotypes under acute-phase conditions. It is tempting to speculate that this slower clearance reflects a physiological 'need' for SAA during the acute-phase response. In the healthy animal, in contrast, there would be no function to perform; in fact, the presence of SAA potentially could be detrimental and therefore trigger aggressive clearance mechanisms.

In the simplest of all situations examined in the present study, either [35 S]SAA1 or [35 S]SAA2 was bound to normal HDL and then injected into normal mice, i.e. the radiolabelled recombinant isotype was the only detectable SAA in the system. The observation that the clearance rates of SAA1 and SAA2 were significantly different under these conditions suggests inherent differences in the clearance properties of the two isotypes. Such differences could derive from differences in the relative strength of interaction with HDL, affinity for receptors involved in clearance, and/or susceptibility to degradative processes active in the circulation. Just as it is difficult to envision significant differences in the clearance mechanism of two proteins having 95 % amino acid identity, it has also been difficult to explain why only one of these isotypes, namely SAA2, is found in amyloid deposits. In this context, one could propose that, since SAA2 is normally cleared more rapidly than SAA1, more severe consequences may result from perturbation or retardation of its clearance, potentially giving rise to a situation favourable to SAA2 fibrillogenesis. A mechanistic understanding of SAA plasma clearance under homeostatic conditions (in the non-amyloidotic mouse) will possibly provide clues about the basis of selective SAA2 deposition.

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