Influence of apoA-I and apoE on the formation of serum amyloid A-containing lipoproteins in vivo and in vitro

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Abstract **Serum amyloid A (SAA) circulates bound to HDL_3 during the acute-phase response (APR), and recent evidence suggests that elevated levels of SAA may be a risk factor for cardiovascular disease. In this study, SAA-HDL was produced in vivo during the APR and without the APR by injection of an adenoviral vector expressing human SAA-1. SAA-HDL was also produced in vitro by incubating mouse HDL with recombinant mouse SAA and by SAA-expressing cultured hepatoma cells. Whether produced in vivo or in vitro, SAA-HDL floated at a density corresponding to that of human HDL_3 (d 1.12 g/ml) separate from other apolipoproteins, including apolipoprotein A-I (apoA-I; d 1.10 g/ml) when either apoA-I or apolipoprotein E (apoE) was present. In the absence of both apoA-I and apoE, SAA was found in VLDL and LDL, with low levels in the HDL and the lipid-poor fractions suggesting that other HDL apolipoproteins are incapable of facilitating the formation of SAA-HDL. We conclude that SAA does not exist in plasma as a lipid-free protein. In the presence of HDL-associated apoA-I or apoE, SAA circulates as SAA-HDL with a density corresponding to that of human HDL_3. In the absence of both apoA-I and apoE, SAA-HDL is not formed and SAA associates with any available lipoprotein.**—Cabana, V. G., N. Feng, C. A. Reardon, J. Lukens, N. R. Webb, F. C. de Beer, and G. S. Getz. **Influence of apoA-I and apoE on the formation of serum amyloid A-containing lipoproteins in vivo and in vitro. *J. Lipid Res.* 2004.** 45: **317–325.**

Supplementary key words apolipoprotein A-I • high density lipoprotein • acute-phase response

Atherosclerosis is now recognized as a chronic inflammatory response in which a vast number of mediators of the inflammatory process are superimposed upon hyperlipidemia (1, 2). Two proteins of the innate acute inflammatory response are the focus of intensive research, C-reactive protein and serum amyloid A (SAA). These proteins have been shown to exhibit higher risk relationships with cardiovascular disease than serum cholesterol (3, 4). Whether these proteins have any causal relationship with cardiovascular diseases or are simply markers of the inflammatory process is not known.

This study focuses on SAA, a plasma protein that increases by greater than 1,000-fold within 24 to 48 h after the induction of the acute-phase response (APR) by infectious and noninfectious inflammatory processes. A highly conserved family of proteins, SAA is present in all vertebrates from fish to human (5–7). Despite years of study, the biological significance of its dramatic increase during the APR remains unclear. SAA may play a role in both amyloidogenesis and atherogenesis. Its role in amyloidogenesis is better defined, SAA being the precursor of the AA protein deposited in secondary amyloidosis. Its role in atherogenesis is circumstantial, although evidence is accumulating that it may be more than an innocent bystander and may affect processes and functions related to atherogenesis. For example, SAA circulates in plasma bound to HDL_3, and when incubated with HDL in vitro, can displace apolipoprotein A-I (apoA-I) (8, 9), which may result in decreased HDL levels. We have shown also that SAA can displace the antioxidant enzyme paraoxonase from HDL (10) and that this may contribute to the reduction in the antioxidant properties of HDL after the acute phase (11). Thus, although basal HDL may protect against atherosclerosis, this activity of HDL may be either attenuated or reversed when it contains SAA. SAA may function in cholesterol transport (12) in an isoform-specific manner (13). It apparently binds cholesterol and could alter HDL-mediated cholesterol efflux (14, 15), thereby affecting the transport of cholesterol from the tissues back to the liver for excretion. SAA mRNA has been detected in cells of the human vessel wall involved in atherogenesis, such as

Abbreviations: adv-huSAA1, recombinant adenovirus expressing human SAA1; APR, acute-phase response; $EA^{-/-}$, mice deficient in both the apoA-I and apoE genes; LPS, lipopolysaccharide; $RE^{-/-}$, mice deficient in both the apoE gene and the recombination activation gene 2; r-moSAA, recombinant mouse SAA; SAA, serum amyloid A.

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Manuscript received 30 September 2003 and in revised form 17 October 2003.

Published, JLR Papers in Press, November 1, 2003.
DOI 10.1194/jlr.M300414-JLR200

endothelial cells, smooth muscle cells, macrophage-derived foam cells, and adventitial macrophages (16). This expression pattern has been observed also in the aortic lesions of C57BL/6 and apoA-II transgenic mice fed an atherogenic diet and in apoE gene knockout mice fed a chow diet (17). Among endothelial cells, those lining the lumen of atherosclerotic vessels express the highest level of SAA mRNA, suggesting a role for SAA in the pathogenesis or regression of atherosclerotic plaques (16). SAA induces the migration, adhesion, and tissue infiltration of monocytes (18), cells strongly implicated in the pathogenesis of atherosclerosis. Atherosclerosis-susceptible strains of mice on an atherogenic diet accumulate lipid oxidation products, activate the nuclear factor-κB transcription factor involved in the inflammatory response, and activate inflammatory genes. The induction of inflammatory genes, including SAA, is associated with susceptibility to aortic lesion formation (19). A high-fat, high-cholesterol diet induces the synthesis of the acute phase (but not the constitutive) SAA isoforms (20). Its presence in cells of the atherosclerotic plaque and its several possible functions in the atherogenic process indicate that more extensive study of these functions is called for.

SAA exists in the plasma bound to HDL_3. We have previously shown that HDL particles containing only SAA exist in the plasma of normal and apoA-I-deficient mice after the induction of the APR by bacterial lipopolysaccharide (LPS) (21). In this report, we have further analyzed the formation of SAA-containing lipoproteins in the presence of either apoA-I or apoE in the following systems: *1*) in vivo during the APR induced by LPS injection; *2*) in vivo without the APR by injection of adenoviruses expressing human SAA1 (adv-huSAA1); *3*) in vitro by incubation of non-APR sera with recombinant SAA; and *4*) in cultured hepatoma-derived cells infected with adv-huSAA1. We have also analyzed the formation of SAA-containing lipoproteins in vivo and in vitro in the absence of both apoA-I and apoE. In all of the systems in which either apoA-I or apoE was present, the SAA was found mainly in particles with a density comparable to that of human HDL_3. However, in mice deficient in both apoE and apoA-I genes ($EA^{-/-}$) that lack HDL, SAA associated almost exclusively with VLDL and intermediate density lipoprotein (IDL) and/or LDL. We conclude that apoA-I and apoE play an important role in the formation of SAA-HDL.

MATERIALS AND METHODS

Materials

LPS *Escherichia coli* serotype 0127:B8, tribromoethyl alcohol, tertiary amyl alcohol, horseradish peroxidase-coupled antibodies, and protease inhibitors were purchased from Sigma Chemical (St. Louis, MO). Tris-glycine 4–20% polyacrylamide gels were from Invitrogen (Carlsbad, CA). Immobilon-P was purchased from Millipore (Bedford, MA), and broad-range nonstained (6,500–200,000 Da) or prestained Kaleidoscope (7,600–216,000 Da) standards were obtained from Bio-Rad (Richmond, CA). The enhanced chemiluminescence (ECL) kit was supplied by Amersham Corporation (Arlington Heights, IL). All cell culture media reagents were from Gibco (Gaithersburg, MD). Kits for enzymatic measurement of cholesterol and phospholipids were purchased from Roche Diagnostics (Indianapolis, IN) and Wako Chemicals (Osaka, Japan), respectively.

Production of SAA-HDL in vivo during the APR

Mice deficient in both the apoE gene ($apoE^{-/-}$) and the recombination activating gene 2 ($RAG2^{-/-}$) gene ($RE^{-/-}$) were produced by mating the respective strains at the animal facilities of the University of Chicago as described (22). C57BL/6 mice purchased from Jackson Laboratories (Bar Harbor, ME) were used as controls. Mice deficient in both the apoA-I and apoE genes ($EA^{-/-}$ mice) were bred and maintained in the animal facilities of the University of Chicago as described (10). The mice were housed in a specific-pathogen-free environment in a temperature-controlled room with a 12-h light-dark cycle. All of the mice were fed mouse chow diet, and food and water were available at all times. During injections and bleedings, the mice were anesthetized by intraperitoneal injection (17.5 μl/g body weight) of a sterile Avertin solution [2.5% in saline prepared from a stock solution of equal tribromoethyl alcohol and tertiary amyl alcohol (w/v)].

The APR was induced in vivo by intraperitoneal injection of 50 μg of bacterial LPS per mouse. After 24 h, the mice were killed by cardiac puncture under anesthesia. Blood was collected without EDTA, and the sera separated by centrifugation were stored at 4°C in the presence of 1 mM PMSF (in ethanol) and anti-bacterial agents (per milliliter of plasma: 0.5 μg of gentamycin sulfate, 50 μg of NaN_3, and 1 μg of chloramphenicol) and used within 2 weeks. Sera were used because some of the mice used in this study were also used for the study of paraoxonase (10), which is irreversibly inactivated by EDTA. All procedures performed on the mice were in accordance with National Institutes of Health and institutional guidelines.

Production of SAA-HDL in vitro without the APR

The construction of a replication-defective adv-huSAA1 was described previously (23). Infectious titers of adenovirus preparations were determined by plaque assay using HEK-293 cells as described (24). Mice were injected via the retro-orbital sinus with various amounts of the virus [expressed as plaque-forming units (pfu)]. Blood was collected at various time points by retro-orbital or cardiac puncture and processed as described above.

Production of SAA-HDL by in vitro incubation

Two hundred fifty microliters of sera from $RE^{-/-}$ mice was incubated with recombinant mouse SAA (r-moSAA) at a ratio of 600 μg of SAA per milliliter of serum, the ratio usually found during the APR. The r-moSAA was isolated from the pGEX bacterial system according to procedures detailed previously (21). We have shown that in vitro this recombinant SAA formed fibrils similar to prion proteins as analyzed by atomic force microscopy (25). After a 1 h incubation at room temperature, the incubation mixture was subjected to density gradient centrifugation as described below. The fractions were collected, and the distributions of apoA-I and SAA were analyzed as described below.

Cell culture

The rat hepatoma-derived McA RH7777 cells were obtained from the American Type Culture Collection (ATCC No. CRL 1601; Rockville, MD). The cells were maintained in high-glucose (4.5 g/l) DMEM with 10% FBS, 5% horse serum, and 1% each of glutamine, penicillin, and streptomycin (henceforth referred to as growth medium). Subconfluent cells were trypsinized, and 1.5×10^6 cells were replated in T-75 culture flasks and grown for 48 h in growth medium. After washing twice with growth me-

dium, adv-huSAA1 with predetermined multiplicity of infection in serum-free medium was added and incubated in serum-free medium for 1 h. The virus media were removed, and the cells were washed twice with serum-free medium and then grown for 48 h in DMEM with 1% lipoprotein-deficient serum isolated as described (26) and supplemented with 1% each of glutamine, penicillin, and streptomycin. The cells were separated by centrifugation, and the supernatant culture media were harvested in solution containing a protease cocktail (0.1% aprotinin, 1 mM PMSF, 2 mM EDTA, and 0.02% NaN_3), concentrated by centrifugal filtration (Centriprep; Amicon, Beverly, MA), and the lipoprotein fractions were isolated by density gradient centrifugation as described below.

Lipoprotein fractionation

The density distribution of apolipoproteins was analyzed by equilibrium density gradient centrifugation according to procedures described previously (27). In this procedure, 250 μl of serum or 2 ml of concentrated culture supernatant was fractionated on 3–20% NaBr gradients by centrifugation for 66 h at 38,000 rpm in a SW41 Ti rotor. After centrifugation, 30 0.4 ml fractions were collected using a gradient fractionator with UV monitor (ISCO, Lincoln, NE) and pump assembly (Brandel). The refractive index of each of the fractions was assessed as an indicator of the density based on the refractive index of salt solutions of known concentration, density, and refractive index. The fractions were dialyzed against Tris-buffered saline (10 mM Tris, 150 mM NaCl, 0.01% EDTA, and 20 mM NaN_3, pH 7.4) and used for analyses.

Apolipoprotein and lipid analysis

One microliter of plasma or 2–5 μl of fraction from the density gradient centrifugation was loaded per lane on a 4–20% polyacrylamide Tris-glycine gel in a sample buffer containing 5% β-mercaptoethanol. The gels were either stained with Coomassie blue or used for electrotransfer of the protein bands to Immobilon-P membrane for Western immunoblotting using polyclonal antibodies to the respective proteins as described (9).

The approximate proportion of apolipoproteins present in a given lipoprotein fraction was assessed by scanning densitometry of the protein bands developed on a photographic film and quantitated (Advanced Quantifier; BioImage) or quantitated using a chemiluminescence imager (Alpha Innotech, San Leandro, CA). Where indicated, apoA-I was quantitated by radial immunodiffusion (28) after denaturation of the protein with 1% Triton X-100, using antibody against mouse apoA-I.

Total cholesterol was quantified enzymatically (Roche Diagnostics). Phospholipids were quantified by an enzymatic-colorimetric assay of choline-containing phospholipids (Wako Chemicals). All lipoprotein analyses were performed by methods standardized against Centers for Disease Control and Prevention-furnished standards. Protein was quantified according to the procedure of Lowry et al. (29) with SDS to disrupt the lipid micelles (30) using BSA as a standard.

RESULTS

Production of SAA in vivo during the APR in $RE^{-/-}$ mice

In published studies, we have shown that the induction of the APR by LPS in wild-type C57BL/6 mice changes the HDL profile from a monodisperse peak (peak at 1.10 g/ml) on density gradients into two separate peaks (peaks at 1.09 and 1.12 g/ml) within 24 h of injection of LPS (9, 21). We also showed that SAA-HDL (d 1.12 g/ml) essentially devoid of other apolipoproteins can be produced in wild-type C57BL/6 mice and in the absence of apoA-I in apoA-I-deficient ($apoA\text{-}I^{-/-}$) mice after the induction of the APR (21). The production of SAA-HDL in $apoA\text{-}I^{-/-}$ mice showed that apoA-I is not required for the production of these particles. In the current study, we further examined the role of apoA-I and apoE in the production of SAA-HDL initially using immunoincompetent double gene knockout $RE^{-/-}$ mice. These mice are deficient in both apoE and RAG2. $ApoE^{-/-}$ mice develop atherosclerosis spontaneously on chow diet and are used as models for the development of atherosclerosis. $RAG2^{-/-}$ mice lack mature T and B cells. This immunodeficiency allows the sequential injections of replication-defective adenoviruses carrying the SAA gene to maintain a high SAA level without the complication of a host-adaptive immune response related to the APR or the viral vector.

Our results show that even in the absence of mature T and B cells, $RE^{-/-}$ mice were able to mount a characteristic rapid increase of SAA in plasma after the injection of LPS. This is expected, as the induction of SAA is a part of the innate acute inflammatory response. Before the injection of LPS, a single HDL peak was present at d 1.10 g/ml as detected by density gradient ultracentrifugal flotation of plasma (**Fig. 1A**, fraction 20). Twenty-four hours after LPS injection (Fig. 1B), two HDL peaks were present: one at d 1.09 g/ml (fraction 19) and a second at the region equivalent to human HDL_3 (fractions 22–23; d 1.12 g/ml). This is similar to the results obtained upon induction of the APR in C57BL/6 mice (21).

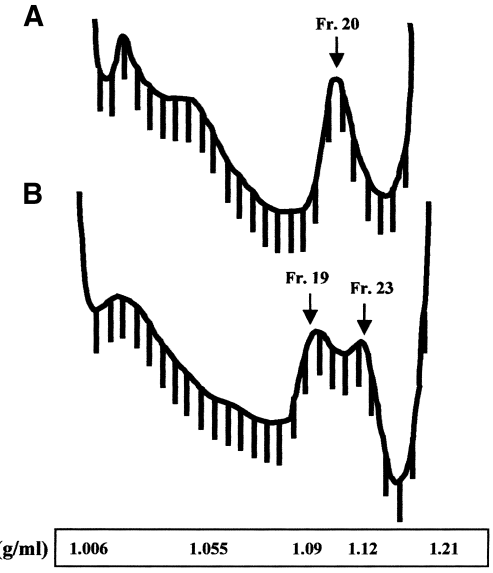

Fig. 1. Ultracentrifugal flotation profiles of acute-phase response plasma from mice deficient in both apolipoprotein E (apoE) and the recombination activation gene 2 ($RE^{-/-}$ mice). Plasma obtained before (A) and 24 h after (B) the injection of lipopolysaccharide (LPS) were subjected to equilibrium density gradient ultracentrifugal flotation as described in Materials and Methods. The gradient fractions were monitored at an optical density of 280 nm. The densities of the fractions are shown at the bottom. Representative profile of n = 10.

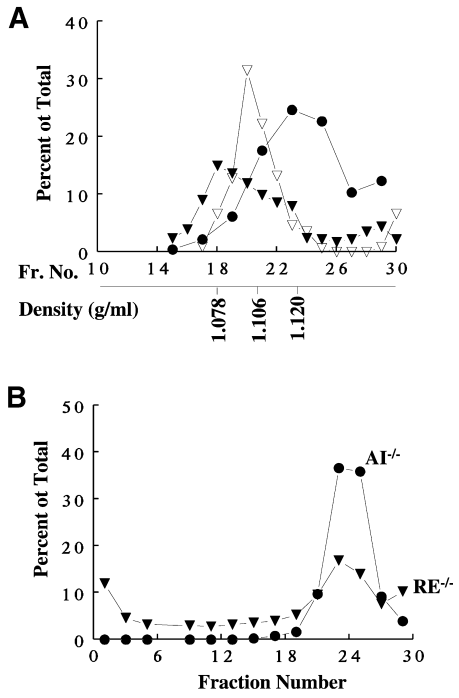

Fig. 2. Density distribution of serum amyloid A (SAA) and apolipoprotein A-I (apoA-I). Fractions obtained from density gradient centrifugation of sera from $RE^{-/-}$ mice and mice deficient in the apolipoprotein A-I gene ($AI^{-/-}$ mice) before and 24 h after injection of LPS were subjected to SDS-PAGE and blotted with antibody against the respective apolipoproteins. The SAA bands were quantitated by densitometric scanning using the BioImage Intelligent Quantifier program. The apoA-I was quantitated by radioimmunodiffusion as described in Materials and Methods. A: HDL gradient fractions (Fr) from $RE^{-/-}$ mice before injection (open symbols) and 24 h after injection (closed symbols) of LPS blotted for apoA-I (triangles) and SAA (circles). B: Density gradient fractions from LPS-injected $RE^{-/-}$ mice (triangles) and apoA-$I^{-/-}$ mice (circles) immunoblotted for SAA. Representative profiles of n = 6.

Western immunoblotting of the fractions for mouse apoA-I and SAA followed by densitometric scanning of the protein bands showed that before the injection of LPS, apoA-I had a monodisperse distribution that peaked at d 1.10 g/ml (**Fig. 2A**). Twenty-four hours after LPS injection, apoA-I had a broader distribution, with its peak shifted to lighter density at 1.08 g/ml. SAA floated separately from apoA-I, with a peak at d 1.12 g/ml, suggesting that they are on separate particles. We have previously shown that ~90% of the SAA is found in the density of HDL_3 in C57BL/6 and apoA-$I^{-/-}$ mice (9, 21) (Fig. 2B, **Table 1**). In contrast, in the $RE^{-/-}$ mice, which have high levels of VLDL and IDL/LDL (22), 14.1 ± 6.5% of SAA was in the VLDL/IDL/LDL fractions (Fig. 2B, Table 1). Most of the SAA (69.7 ± 1.5%) was still found in the fraction corresponding to human HDL_3 (d 1.12 g/ml), with less than 15% in the lipid-poor fractions (fractions 28–30).

Production of SAA in vivo in the absence of the APR

Production of SAA-HDL in the absence of the APR was induced in $RE^{-/-}$ mice by the injection of adenoviruses carrying a single copy of the adv-huSAA1 gene (5×10^8 pfu/mouse). Three days after injection, the level of adv-huSAA1 was ~50 μg/ml (**Fig. 3A**). This is substantially lower than the mouse SAA level achieved after the induction of the APR by LPS injection. The levels progressively decreased thereafter. Because the level of SAA decreased progressively after a single dose of adv-huSAA1, injections of 5×10^8 pfu of adv-huSAA1 per mouse were repeated every 21 days. After each injection of the adenovirus, huSAA levels in the plasma increased 3 days postinjection, and although the level decreased by 21 days after each injection, the levels were always higher than that at 21 days after the previous injection (Fig. 3B). Although endogenous mouse SAA increased modestly with each viral injection (Fig. 3C), these levels were low compared with the 24 h post-LPS injection level, showing that a full APR was not a significant complication of the adenoviral injections.

Analysis of the $RE^{-/-}$ plasma at 3 days after injection revealed huSAA and endogenous apoA-I floating at separate density peaks; the SAA floated at d 1.12 g/ml, and apoA-I peaked at d 1.10 g/ml (**Fig. 4A**). At this low level of SAA, greater than 90% of the SAA was found in HDL (Fig. 4B), even in the presence of high VLDL and LDL/IDL in these animals. Similar distributions of huSAA were obtained after adenoviral injections into apoA-$I^{-/-}$ and C57BL/6 mice (data not shown). With a 10-fold higher dose of adv-huSAA1 (50×10^8 pfu), higher levels of huSAA were produced, with ~15% of the SAA found in VLDL and <10% in the lipid-poor fraction (data not shown). This distribution is almost identical to that observed during the APR of these mice, indicating that SAA-HDL is capable of being formed in vivo in the absence of the full APR.

Production of SAA-HDL in vitro

Sera from control $RE^{-/-}$ mice were incubated with 600 μg of r-moSAA per milliliter (the level commonly found during the APR), and the lipoproteins were separated by density gradient centrifugation. Analysis of the fractions (**Fig. 5**, **Table 2**) showed a similar distribution of SAA and apoA-I to that observed during the APR in vivo (Fig. 2A). SAA formed a peak at d 1.12 g/ml separate from apoA-I, suggesting the formation of separate particles. In contrast to a single well-defined peak of apoA-I at d 1.10 g/ml in the serum incubated alone, the serum incubated with SAA

TABLE 1. Distribution of SAA in vivo in three mouse strains

Mouse Strain	SAA			
	VLDL	IDL/LDL	HDL	Lipid-Poor
	% of total			
apoA-$I^{-/-}$ [a]	0	0	86.9	13.1
$RE^{-/-}$ [b,c]	10.1 ± 4.0	4.4 ± 2.5	69.7 ± 1.5	13.0 ± 3.0
$EA^{-/-}$ [b]	25.0 ± 5.0	41.5 ± 10.9	19.0 ± 5.3	14.0 ± 5.2

apoA-I, apolipoprotein A-I; apoA-$I^{-/-}$, mice deficient in the apoA-I gene; $EA^{-/-}$, mice deficient in both the apoA-I and apolipoprotein E (apoE) genes; IDL, intermediate density lipoprotein; $RE^{-/-}$, mice deficient in both the apoE gene and the recombination activation gene 2; SAA, serum amyloid A.

[a] Average, n = 2.

[b] Mean ± SD, n = 6.

[c] Similar distributions were observed in apo$E^{-/-}$ mice.

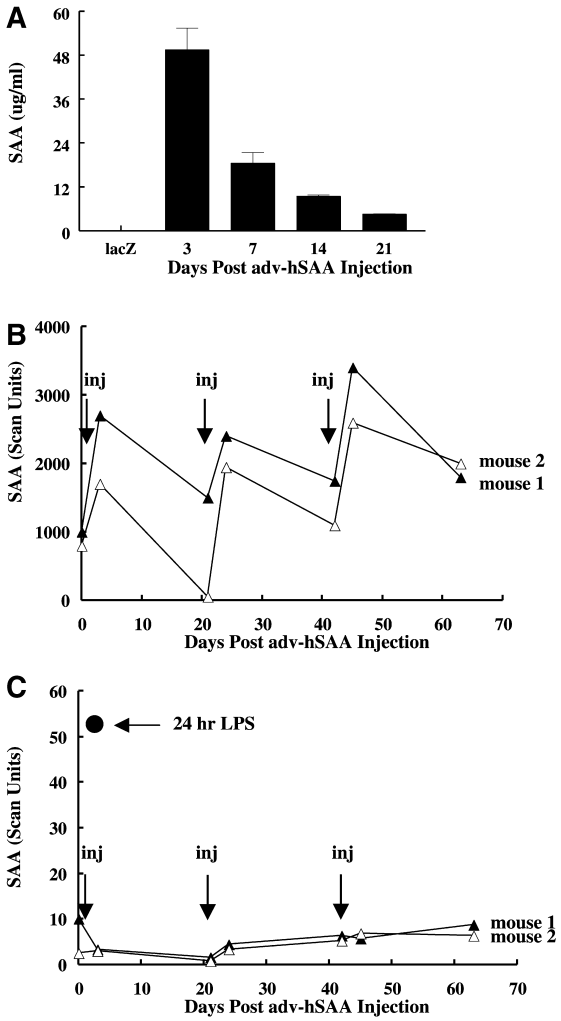

Fig. 3. Human SAA (huSAA) levels in RE$^{-/-}$ plasma after injection of adenovirus expressing huSAA (adv-huSAA1) or LacZ. The huSAA in the mouse plasma was detected by Western immunoblotting using a huSAA-specific antibody and quantitated (mean ± SD) by densitometric scanning of the SAA bands using lipid-free recombinant huSAA as a standard. A: Levels of huSAA in plasma up to 21 days after a single injection with 5×10^8 plaque-forming units (pfu) of adv-huSAA1 (n = 4). B: Levels of huSAA in mice injected (inj) with 5×10^8 pfu of adv-huSAA1 every 21 days (n = 2). SAA levels were quantitated at 3 and 21 days after injection. C: Levels of mouse SAA in the same mice as in B. (Note that the data in B and C represent scan units and are not comparable in the two graphs, because two different antibodies of different binding affinities were used in the immunoblotting.) For comparison, the levels of mouse SAA at 24 h after injection of LPS are shown in C.

showed a more polydisperse apoA-I pattern, suggesting remodeling of the particles after the addition of recombinant SAA. Furthermore, almost 20% of the SAA was found in the VLDL fraction. This was accompanied by a doubling of the amount of apoA-I in the VLDL as well (Table 2).

Formation of SAA-containing lipoproteins by hepatoma cells

McA RH7777 cells are a rat hepatoma cell line that secretes VLDL and HDL-like particles. McA RH7777 cells were infected with adv-huSAA1 at different multiplicity of

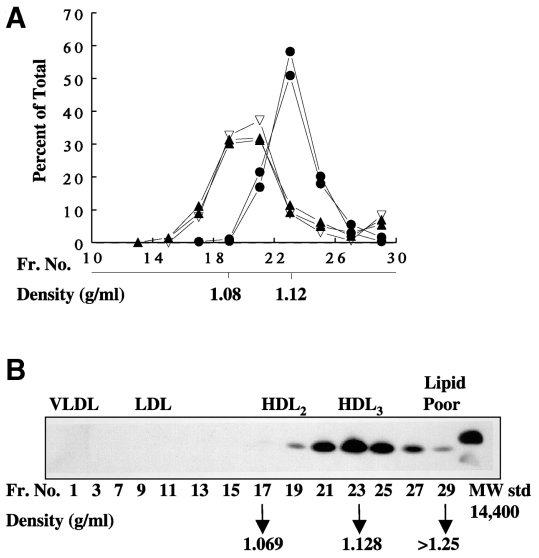

Fig. 4. Density distribution of SAA and apoA-I in RE$^{-/-}$ mouse plasma after the injection of adv-huSAA1. Sera obtained at 3 days after injection of 5×10^8 pfu of adv-huSAA1 or adv-LacZ into RE$^{-/-}$ mice were fractionated by density gradient ultracentrifugation. Fractions were subjected to SDS-PAGE and immunoblotting. A: The apolipoprotein bands in the HDL fractions were quantitated by densitometric scanning and plotted as percentages of total. Open triangles, apoA-I in adv-LacZ-infected mice; closed triangles, apoA-I in adv-huSAA1-infected mice (2 mice); and closed circles, huSAA in adv-huSAA1-infected mice (2 mice). B: Western immunoblot of fractions from the whole gradient showing that more than 90% of the SAA was associated with HDL_3. MW std, molecular weight standard.

infection levels. The apolipoproteins produced were analyzed by Western immunoblotting. The huSAA was produced in a dose-dependent manner (**Fig. 6A**). The production of huSAA had no effect on the production of the endogenous rat apoA-I (Fig. 6B). When SAA-containing lipoproteins were isolated from the culture media of these

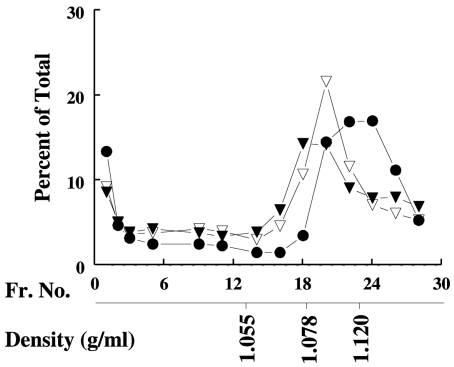

Fig. 5. Formation of SAA-containing particles in vitro. Serum from RE$^{-/-}$ mice was incubated with lipid-free recombinant mouse SAA (r-moSAA) at a ratio of 600 μg of SAA per milliliter of serum and separated by density gradient centrifugation. The apolipoproteins in the fractions were quantitated by densitometric scanning of the specific bands of immunoblots: apoA-I from serum incubated alone (open triangles) and apoA-I (closed triangles) and huSAA (closed circles) after incubation of the mouse serum with r-moSAA.

TABLE 2. Distribution of apoA-I and SAA in $RE^{-/-}$ and $EA^{-/-}$ sera after in vitro incubation with or without recombinant mouse SAA

	$RE^{-/-}$ Mice			$EA^{-/-}$ Mice
	apoA-I		SAA[b]	SAA[b]
Lipoprotein[a]	−r-moSAA	+r-moSAA	+r-moSAA	+r-moSAA
	% of total			
VLDL	9.8	22.8	19.3	26.2
IDL/LDL	9.7	8.5	12.3	32.4
HDL	67.1	54.2	50.1	16.3
Lipid-poor	11.2	14.2	18.2	25.1

r-moSAA, recombinant mouse SAA.
[a] Sera pooled from three mice were used in each incubation.
[b] SAA was below the limit of detection in the preincubation serum.

cells and subjected to density gradient centrifugation, analysis of the fractions showed that the particles containing huSAA and rat apoA-I were separately distributed. Fractions from the cells infected with adv-huSAA1 at a multiplicity of infection of 5 are shown in Fig. 6C, but each of the different doses of adv-huSAA1 produced a similar pattern. SAA-containing particles formed by the cells were distributed mainly in the HDL region, with a peak at d 1.111 g/ml, whereas the peak of the endogenous rat apoA-I-containing particles was at d 1.078 g/ml, thus showing a separate distribution than that observed in serum. However, although the particles formed peaks at separate densities, both the endogenous rat apoA-I and the huSAA floated at densities lighter than that of particles formed in vivo. No significant amount of SAA was found in other fractions.

Influence of apoA-I and apoE on the production of SAA-containing particles

In the murine models described in this and previously published reports (9), most of the SAA is associated with HDL in vivo. This is the case in a number of different mouse strains (C57BL/6, $RE^{-/-}$, apoA-$I^{-/-}$), whether SAA is induced as part of the APR or by adenoviral vector-mediated gene transfer. Similar results are also seen in studies in vitro, in which normal mouse HDL is incubated with either recombinant SAA or with hepatoma cells expressing SAA. To assess whether the production of SAA-HDL requires the presence of preexisting HDL particles, we performed studies using $EA^{-/-}$ mice. We have previously shown that $EA^{-/-}$ mice have no detectable HDL (10). Serum cholesterol and phospholipid are found almost exclusively in the VLDL fractions in these mice (**Fig. 7A**, B), and greater than 90% of the HDL-associated enzyme paraoxonase is lipid-poor (10).

The results depicted in **Fig. 8** clearly illustrate the differences in the lipoprotein distribution of SAA and other HDL apolipoproteins in normal mice, $RE^{-/-}$ mice, and $EA^{-/-}$ mice during the APR. Note that in C57BL/6 and $RE^{-/-}$ mice, SAA is detected primarily in HDL-containing fractions, confirming our previous results (9, 21, 28). In contrast, little SAA is detected in HDL-containing fractions in $EA^{-/-}$ mice that lack preexisting HDL.

Although the $EA^{-/-}$ mice can mount an APR after injection of LPS, SAA floated mostly in VLDL and IDL/LDL, with low levels in the HDL region and in the lipid-poor fraction (**Fig. 9A**, Table 1). In all three strains studied, the lipid-poor SAA in vivo was 13–14% of the total. The particles in the HDL region of the $EA^{-/-}$ mice may

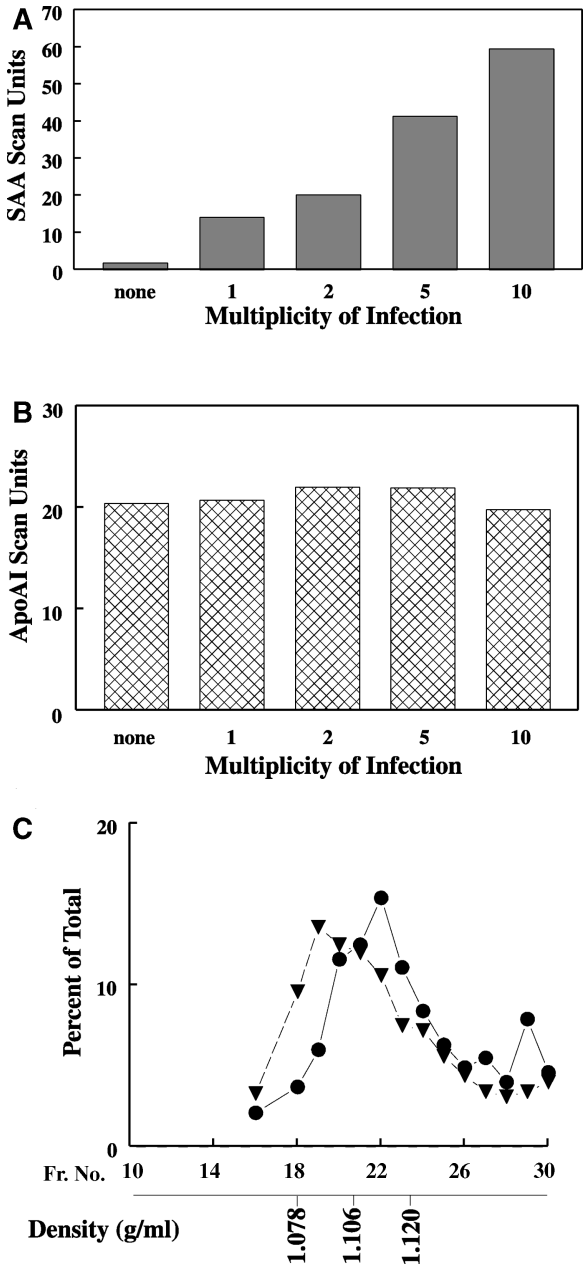

Fig. 6. Formation of SAA-HDL in hepatoma cells. McA RH7777 cells were infected with adv-huSAA1 as described in Materials and Methods. After harvest and separation of the cells, the lipoproteins in the media were isolated by density gradient centrifugation. HuSAA and endogenous rat apoA-I were analyzed on the Alpha Imager after Western immunoblotting with specific antibodies. A: HuSAA produced at different multiplicity of infections (moi). B: Endogenous rat apoA-I from the same culture. C: Distribution of huSAA (closed circles) and rat apoA-I (closed triangles) on the HDL particles secreted from McA RH7777 cells infected at an moi of 5. Similar patterns were observed in all of the cultures regardless of the moi. The densities of the fractions (Fr) are indicated at the bottom. Representative profile of n = 3.

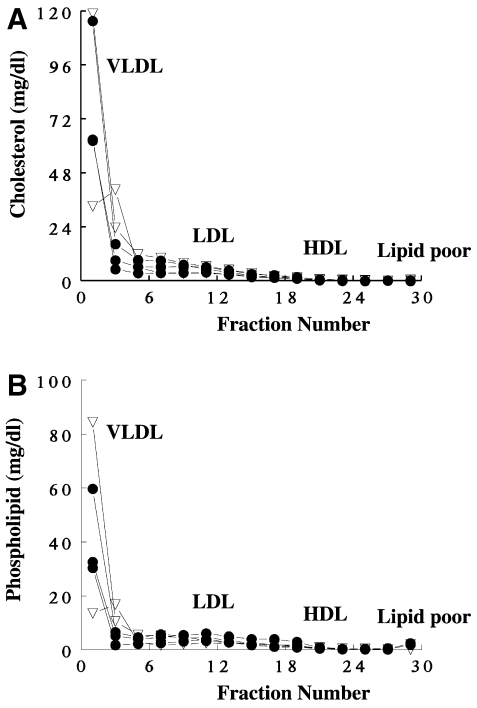

Fig. 7. Distribution of cholesterol and phospholipids in the sera from mice deficient in both the apoA-I and apoE genes ($EA^{-/-}$ mice). $EA^{-/-}$ mice were injected with LPS as described in Materials and Methods, and the sera of control mice (open symbols; n = 2) and LPS-injected mice (closed symbols; n = 3) obtained at 24 h after injection were separated on density gradients and the fractions analyzed for cholesterol (A) and phospholipid (B).

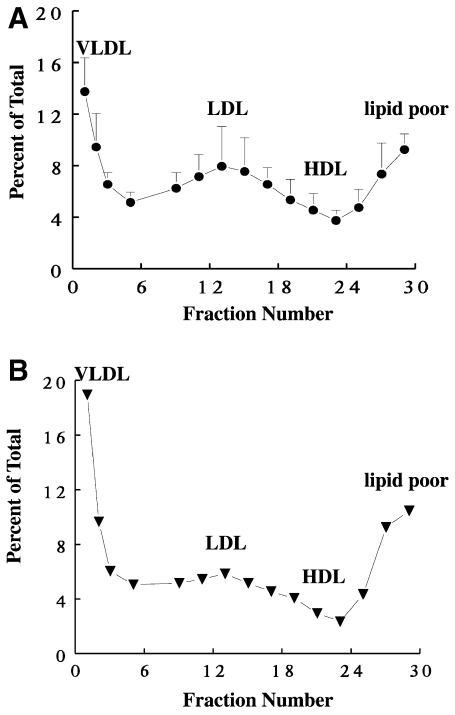

Fig. 9. Distribution of SAA in sera of $EA^{-/-}$ mice. A: Distribution of SAA in sera of LPS-injected $EA^{-/-}$ mice (mean ± SD, n = 6). B: Distribution of SAA after incubation of $EA^{-/-}$ sera with r-moSAA as described in the legend to Fig. 5.

represent the trailing edge of the LDL peak, because only 3.8 ± 0.8% of total SAA was in fraction 23 (d 1.12 g/ml), the usual peak of SAA in the other mice. In some $EA^{-/-}$ mice, only background levels of SAA were found in the HDL density region. Incubation of $EA^{-/-}$ serum with r-moSAA again showed a distribution of SAA similar to what occurred in vivo (Fig. 9B, Table 2). Here too, the majority of the SAA was found mainly in the VLDL and the IDL/LDL. The amount of SAA in the HDL and the lipid-poor fraction after in vitro incubation was slightly higher than what was found in vivo (Table 2).

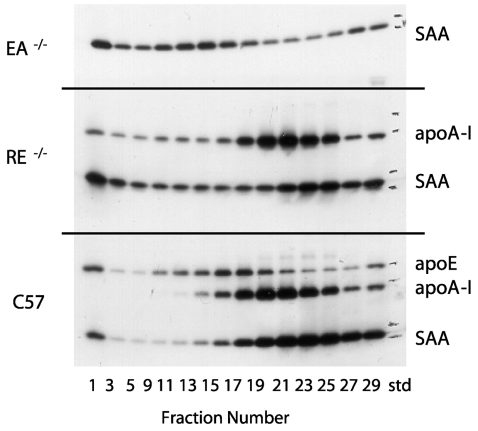

Fig. 8. Distribution of apoE, apoA-I, and SAA in $EA^{-/-}$, $RE^{-/-}$, and C57BL/6 mice. The acute phase was induced in the mice by injection of LPS, and the sera obtained at 24 h after injection were fractionated by density gradient centrifugation. The distribution of SAA, apoA-I, and apoE in the gradient fractions was examined by Western immunoblotting.

DISCUSSION

We have previously demonstrated in the plasma of wild-type and apoA-I-deficient mice the presence of HDL particles containing SAA but devoid of other apolipoproteins (21). The existence of SAA-HDL in apoA-$I^{-/-}$ mice that was similar in density and size to SAA-HDL from wild-type mice shows that apoA-I is not a prerequisite for the production of SAA-HDL. In this report, we describe the production of SAA-containing particles in the presence of HDL-associated apoA-I or apoE in the following systems: *1*) in vivo in $RE^{-/-}$ mice during the APR induced by LPS injection; *2*) in vivo in $RE^{-/-}$ mice without the APR by adv-huSAA1 injection; *3*) in vitro by incubation of non-APR HDL from $RE^{-/-}$ mice with recombinant SAA; and *4*) in cultured hepatoma cells infected with adv-huSAA1. However, SAA-HDL was not produced in vivo and in vitro in the absence of both apoA-I and apoE in $EA^{-/-}$ mice. These data suggest that SAA remodels HDL to generate a particle that contains predominantly SAA. In the absence of HDL, SAA-HDL is not formed and SAA associates with the other lipoproteins in the plasma.

SAA-HDL was produced in vivo in the double knockout

mice lacking both the apoE and the RAG2 genes. We have previously shown that the absence of mature T and B cells in these mice has a site-specific effect on the development of atherosclerosis (22). In this study, we demonstrate that these mice are able to mount an APR characterized by a rapid increase of SAA in the plasma. Because the production of SAA is induced by cytokines, particularly interleukin 1 and interleukin 6, the production of SAA by the $RE^{-/-}$ mice shows that cytokines produced by cells of the adaptive immune system are not required for the production of SAA. Because these mice are not able to mount an adaptive immune response, it is possible to repeatedly inject the adv-huSAA1, thereby maintaining an elevated level of SAA. This provides a valuable tool to study the role of SAA in atherosclerosis without the complications associated with the systemic alterations related to the APR. However, even in the absence of the adaptive immune system, there is a steady decline in the production of SAA over a 21 day period in these adenovirus-infected mice. Perhaps other viral vectors (e.g., adeno-associated virus) would be more effective at producing a sustained level of SAA over an extended period.

SAA has a very strong preference for HDL, with a bias toward small, dense HDLs. Because most control mouse sera contain very low levels of small, dense HDLs, it is likely that this pattern is elicited by the remodeling of HDL under the influence of SAA. That the SAA itself is responsible for the remodeling of the HDL is indicated by the in vitro incubation of sera with recombinant SAA, which generates a similar remodeling as long as HDL containing apoE or apoA-I is present.

The absence of SAA-HDL in the $EA^{-/-}$ mice suggests that other HDL apolipoproteins (i.e., apoA-II and apoA-IV) that are present in the $EA^{-/-}$ mice are incapable of supporting the formation of separate SAA-containing HDL particles. This result is somewhat unexpected in view of our observation that in other strains of mice, SAA is often found in HDL density fractions that lack significant quantities of either apoE or apoA-I (21). This raises the question of why such an SAA-enriched HDL-like particle is not detected in the double knockout mice. We favor the notion that the remodeling by SAA of preexisting or nascent HDL containing either apoA-I or apoE generates this unique SAA-HDL. The measurements reported here relate to the mass of apolipoproteins in the "mature" lipoprotein fractions. This does not necessarily indicate how SAA emerges from the cells that produce it (i.e., as a free protein or bound to lipid with or without apoA-I or apoE). Hoffman and Benditt (31) have suggested that SAA may be produced in the liver as free protein and becomes bound to lipoproteins in plasma. Neither apoE nor apoA-I is required for the production of SAA-HDL, but their combined absence does not allow for the formation of SAA-HDL.

Two possible explanations for this can be entertained. First, it is possible that either apoE or apoA-I may serve as a chaperone for the production of SAA-HDL. Alternatively, it is possible that SAA, being a lipid binding apolipoprotein, may first bind to HDL-like nascent particles, resulting in the reorganization of its lipid components to generate an SAA-HDL-type particle. According to this second scenario, SAA will have a preference for lipoproteins in the HDL size range, and only in the absence of such lipoproteins or when the capacity of the HDL to bind SAA is exceeded will it satisfy its lipid binding propensities by associating with VLDL and/or LDL. Relatively modest levels of SAA exist in plasma as free protein. SAA can be found in VLDL (27, 32), but as long as either apoA-I or apoE is present in the plasma, this never exceeds 15–20% of the total plasma SAA. It is not clear whether SAA and apoA-I are found in the same VLDL particles. The VLDL of $apoE^{-/-}$ mice contains measurable quantities of apoA-I, which could facilitate the transport of SAA in this lipoprotein fraction; however, this cannot account for SAA in the VLDL (or LDL) fraction of $EA^{-/-}$ mice. It is clear that neither apoE nor apoA-I is a requirement for the presence of SAA in VLDL. Further studies are required to resolve these issues.

The most puzzling observation in these $EA^{-/-}$ mice relates to the high proportion of SAA associated with fractions exhibiting the density of IDL/LDL despite the very small proportion of cholesterol or phospholipid in these fractions. We have not ruled out the presence of lipids other than the cholesterol and phospholipid (i.e., lecithin) assayed in these fractions. This is not unique to the in vivo situation, as SAA added to the $EA^{-/-}$ serum also partly targets the IDL/LDL fraction (20–50%). It is not clear whether this SAA associates with endogenous IDL/LDL or with a unique particle sedimenting in this density range. Analysis of these fractions by SDS-PAGE showed the presence of apolipoprotein B-48 and apolipoprotein B-100 (data not shown). It is notable that when LDL is the predominant lipoprotein, as in mice deficient for both the LDL receptor and the apolipoprotein B editing enzyme (apobec-1) (33), less than 20% of SAA associates with the endogenous LDL (data not shown).

SAA is found in atherosclerotic plaques, possibly produced locally by cells in the lesion (16, 17), or in association with HDL. Whether SAA plays a role in atherogenesis is not known. The double knockout $EA^{-/-}$ mice provide a valuable tool to study the role of SAA in atherogenesis. The mode of secretion of SAA could also be important in the context of the atherosclerotic plaque. An association of SAA with HDL could enable more SAA to gain access to tissue fluid, including the intimal space at the atherosclerotic plaque. Is the presence of HDL important for the secretion of SAA by the cells of the plaque? Is the production of apoE by these cells necessary for SAA secretion in the plaque? These are a few of the many questions that remain to be answered about the synthesis of SAA in the vessel wall. Further studies are required to elaborate on these questions.

This work was supported by American Heart Association grant 0050506N (V.G.C.) and by National Institutes of Health Grant HL-57334 (G.S.G.).

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