Macrophage cholesterol efflux and the active domains of serum amyloid A 2.1

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Abstract Serum amyloid A 2.1 (SAA2.1) suppresses ACAT and stimulates cholesteryl ester hydrolase (CEH) activities in cholesterol-laden macrophages, and in the presence of a cholesterol transporter and an extracellular acceptor, there is a marked increase in the rate of cholesterol export in culture and in vivo. The stimulation of CEH activity by SAA2.1 is not affected by chloroquine, suggesting that it operates on neutral CEH rather than the lysosomal form. With liposomes containing individual peptides of SAA2.1, residues 1–20 inhibit ACAT activity, residues 74–103 stimulate CEH activity, and each of residues 1–20 and 74–103 promotes macrophage cholesterol efflux to HDL in culture media. In combination, these peptides exhibit a profound effect, so that 55–70% of cholesterol is exported to media HDL in 24 h. The effect is also demonstrable in vivo. [^{3}H]cholesterol-laden macrophages injected intravenously into mice were allowed to establish themselves for 24 h. Thereafter, the mice received a single intravenous injection of liposomes containing intact SAA1.1, SAA2.1, peptides composed of SAA2.1 residues 1–20, 21–50, 51–80, 74–103, or SAA1.1 residues 1–20. Only liposomes containing intact SAA2.1 or its residues 1–20 or 74–103 promoted the efflux of cholesterol in vivo. A single injection of each of the active peptides is effective in promoting cholesterol efflux in vivo for at least 4 days.—Kisilevsky, R., and S. P. Tam. **Macrophage cholesterol efflux and the active domains of serum amyloid A 2.1. *J. Lipid Res.* 2003.** 44: **2257–2269.**

Supplementary key words acyl-CoA:cholesterol acyltransferase • macrophages • in vivo

Atherogenesis is characterized by the accumulation of cholesterol in aortic and arterial walls in cells such as macrophages. This accumulation is likely due to an imbalance between the biosynthesis, uptake, and esterification of cholesterol on the one hand, and, on the other hand, the deesterification and export of unesterified macrophage cholesterol. Attempts to limit macrophage cholesterol accumulation have therefore focused on enzymatic or receptor activities concerned with *a*) cholesterol biosynthesis [3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase)] (1, 2), *b*) cholesterol influx (LDL and scavenger receptors) (3, 4), and *c*) the storage of cholesterol through its esterification (ACAT) (5, 6), all of which control the cholesterol input side of this equation. The output side of the equation is controlled by *d*) the mobilization of stored cholesteryl esters through their deesterification (cholesterol esterase) (7), *e*) the transport of unesterified cholesterol through the cell membrane (e.g., the ABCA1 transporter) (8, 9), and *f*) the presence of an adequate supply of an extracellular plasma cholesterol acceptor (e.g., HDL).

An analogous set of events concerning macrophage cholesterol uptake and export occurs at sites of acute tissue injury. Such injuries result in local cell death and the release of cellular membrane fragments. These are ingested by macrophages arriving at the site of injury as part of the local acute inflammatory reaction. Because these membrane fragments are composed of up to 50% cholesterol (10, 11), the macrophages acquire a considerable cholesterol load and become foam cells. A mechanism to mobilize this cholesterol for excretion or reuse is therefore required. Our past work has suggested that this role is exercised by a major acute-phase protein, serum amyloid A 2.1 (SAA2.1) (12).

SAA represents a family of proteins whose evolutionary history is at least 600×10^6 years (13, 14). Four functional SAA genes have been described in the mouse and three in humans (15–18). SAA4 is expressed constitutively in both humans and mice, and its protein product is a constant but minor apolipoprotein of HDL (19). It is the only form of SAA that is glycosylated (20, 21). SAA3 is a pseudogene not expressed in humans (22). In mice, SAA3 is produced primarily in extrahepatic tissues such as adipocytes and macrophages (16, 17). During inflammation, SAA3 can be

Abbreviations: ApoA-I, apolipoprotein A-I; CEH, cholesteryl ester hydrolase; CQ, chloroquine; DIDS, 4,4-diisothiocyanotostilbene-2,2′-disulfonic acid; LPDS, lipoprotein-depleted serum; MCD, methyl-β-cyclodextrin; RBC, red blood cell; SAA2.1, serum amyloid A 2.1.

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found in small quantities associated with HDL (23). SAA1.1 and -2.1 are major acute-phase proteins present in human and mouse plasma and are produced primarily in the liver. Their plasma concentration may increase two to three orders of magnitude (0.001–1 mg/ml) following tissue injury, regardless of the cause (24). During such injury, 30–80% of the apolipoprotein composition of HDL is composed of SAA1.1 and -2.1 in roughly equal proportions (25). In the mouse, each of these acute-phase isoforms is 103 residues long, differing from each other by only nine amino acids (18). In humans, these isoforms are 104 amino acids in length, differing from each other by only seven amino acids (18). A comparison of the complete human and mouse sequences of isoforms 1.1 and 2.1 may be found in Sipe and Westermark (18).

As shown previously, SAA2.1, but not SAA1.1, inhibits ACAT activity and enhances cholesterol esterase activity in intact cholesterol-laden macrophages (12), in postnuclear macrophage homogenates (26), and with purified pancreatic neutral cholesteryl ester hydrolase (CEH) (26). In doing so, it drives stored cholesteryl esters into their unesterified form, which, in the presence of a functional cholesterol transporter and an extracellular cholesterol acceptor, is rapidly exported from the macrophage (12). This effect is observable in culture and in vivo (12).

To further characterize and define the effects of SAA2.1, we have, in our present work, attempted to optimize the culture conditions under which it exerts its effect on macrophage cholesterol efflux. We have also determined the domains of SAA2.1 that are responsible for its inhibitory effect on macrophage ACAT activity, its promotion of cholesterol esterase activity, and its enhancement of cholesterol efflux from cholesterol-laden macrophages in culture and in vivo.

MATERIALS AND METHODS

Animals

Swiss white CD1 6–8-week-old female mice were obtained from Charles Rivers, Montreal, Quebec. Mice were kept in a temperature-controlled room on a 12 h light/dark cycle. They were fed with Purina Lab Chow pellets and water ad libitum.

Chemicals

All chemicals were reagent grade and purchased from Fisher Scientific (Nepean, Ontario), Sigma (St. Louis, MO), ICN (Aurora, OH), or BioRad (Hercules, CA). Dulbecco's modified Eagle's medium (DMEM) and FBS were purchased from Life Technologies (Burlington, Ontario). Radiolabeled [1-^{14}C]oleic acid (52 mCi/mmol), [1,2,6,7-^{3}H(N)]cholesterol (82 Ci/mmol), and [cholesteryl-1,2,6,7-^{3}H(N)]oleate (84 Ci/mmol) were obtained from DuPont NEN (Boston, MA).

Peptides

Peptides corresponding to amino acid residues 1–20, 21–50, 51–80, and 74–103 of the murine SAA2.1 protein sequence were synthesized by solid-phase peptide synthesis using 9-fluorenylmethoxycarbonyl as an α-amino protecting group in a PE Applied Biosystems 433A peptide synthesizer (Protein Synthesis Laboratory at Queen's University). A peptide corresponding to amino acid residues 1–20 of murine SAA1.1 protein sequence was also synthesized. The purity of the synthetic peptides was established by analytical high-performance liquid chromatography (HPLC) and ion-spray mass spectrometry. The peptides were dialyzed against distilled water and lyophilized before use.

Preparation of red blood cell membranes as a source of cholesterol

To mimic the ingestion of cell membrane fragments by macrophages at sites of tissue injury, red blood cell (RBC) membrane fragments were prepared and used as a source of cholesterol as described previously (26). Similar quantities of cholesterol (as RBC membrane fragments) were used in all experiments. The concentration of cholesterol in the RBC membrane preparations was determined using the method of Allain and coworkers (27) with the aid of a Sigma cholesterol 20 reagent kit.

Preparation of HDL and acute-phase HDL and purification of apolipoprotein A-I and SAA isoforms

HDL and acute-phase HDL were isolated from normal and inflamed mice, respectively, using sequential density flotation as described previously (28, 29). Briefly, inflammation was induced by subcutaneous injection of 0.5 ml of 2% $AgNO_3$ under the loose skin of the upper back of the mice. Twenty-four hours later, after CO_2 narcosis, the animals were exsanguinated by cardiac puncture, and the blood was collected into 0.5% EDTA (final concentration). The plasma was then separated from the RBCs by centrifugation. The induction of inflammation and SAA synthesis and the isolation of apolipoprotein A-I (apoA-I), SAA1.1, and SAA2.1 from acute-phase murine plasma was performed as described previously (28). Separation and purification of these proteins was accomplished by reverse-phase HPLC as described previously (29). The purity of the isolated proteins was established by mass spectrometry and N-terminal sequence analysis as published previously (28, 29).

Preparation and characterization of apo-lipid complexes

ApoA-I, SAA1.1, SAA2.1, synthetic peptides corresponding to amino acid residues 1–20 of murine SAA1.1 and -2.1, respectively, and synthetic peptides corresponding to amino acid residues 21–50, 51–80, and 74–103 of murine SAA2.1 were reconstituted with lipids to form liposomes. These liposomes were prepared by the cholate dialysis procedure described by Jonas, Kezdy, and Wald (30), using 1-palmitoyl-2-oleoyl-phosphatidylcholine-cholesterol-apolipoprotein-sodium cholate in the molar ratio 100:25:1:250. Cholesterol was included to stabilize the liposomes and to give them a composition more similar to that of HDL. All preparations were performed in 0.5 ml of buffer containing 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 0.005% EDTA. The reaction mixture was stirred thoroughly and incubated for 12 to 16 h at 4°C. At the end of the equilibration period, the sample was dialyzed extensively in PBS at 4°C. After removal of any unreacted or precipitated lipid by centrifugation at 15,000 *g*, 15°C, for 1 h, the liposomes were filtered on a 1.5 × 50 cm Sepharose CL-4B column. Following concentration, the liposomes were sterilized by filtration through a 0.45 μm Millipore filter and mixed at various concentrations with tissue culture medium. The chemical compositions of various protein-containing liposomes have been reported previously (12) and were obtained from protein determinations using the method of Lowry and coworkers (31), phospholipid determinations using a colorimetric kit (Wako Chemicals, Richmond, VA), and enzymatic analyses of free cholesterol (Sigma cholesterol reagent kit).

Cell culture

J774 macrophages (from American Type Tissue Collection, Manassas, VA) were cultured in 6-well tissue culture plates at one million cells per well and grown to 90% confluence in 2 ml DMEM supplemented with 10% FBS. The medium was changed three times a week. In some experiments, nearly confluent monolayers were cultured in the presence of chloroquine (CQ) (100 μM), 8-bromo-cAMP (0.3 mM), or methyl-β-cyclodextrin (MCD) (0.1 mM).

Cholesterol loading and determination of cell cholesterol esterification

To load the cells with cholesterol, nearly confluent monolayers were washed three times with PBS containing 2 mg/ml fatty acid-free BSA (PBS-BSA) and incubated for 5 h in DMEM supplemented with 5% lipoprotein-depleted serum (LPDS) (d > 1.25 g/ml) and 175 μg of RBC membrane cholesterol. For the purpose of pool equilibration of the added cholesterol, cell cultures were rinsed twice with PBS-BSA and incubated overnight in DMEM containing 5% LPDS. The relative activity of ACAT was determined in cholesterol-laden cells that had been cultured in medium containing no liposomes, protein-free liposomes, or liposomes containing 0.5 μM synthetic peptides (final culture concentration) corresponding to amino acid residues 1–20, 21–50, 51–80, and 74–103 of murine SAA2.1, respectively. Following 3 h incubations with the above media, the cells were incubated for another 3 h period after addition of [^{14}C]oleate (32, 33). Cells were chilled on ice and washed twice with PBS-BSA and twice with PBS. After addition of [^{3}H]cholesteryl oleate (6,000 dpm/well) as an internal standard, the lipids were extracted from the cells and analyzed by thin-layer chromatography as described previously (32, 33). The radioactivity in appropriate spots was measured to determine the incorporation of radioactivity into cholesteryl esters as a measure of ACAT activity.

Rates of hydrolysis of cholesteryl ester in J774 cells

Nearly confluent J774 cells were labeled with [^{14}C]oleate during cholesterol loading with RBC membranes as described above. Cells were then incubated for up to 24 h with 2 ml DMEM containing 5% LPDS and 50 μg/ml of either native HDL, SAA-HDL, liposomes containing 2 μM (final culture concentration) of apoA-I, SAA1.1, or -2.1, or liposomes containing 0.5 μM (final culture concentration) synthetic peptides corresponding to amino acid residues 1–20, 21–50, 51–80, and 74–103 of murine SAA2.1, respectively. To determine the rate of cholesteryl ester hydrolysis, 2 μg/ml of Sandoz 58-035 (an ACAT inhibitor) was added during lipoprotein or liposome incubation to prevent re-esterification of liberated [^{14}C]oleate and free cholesterol. To examine whether the rate of cholesteryl ester hydrolysis involved the lysosomal CEH, the cells were cultured in the presence of 50 μg/ml of either native HDL or SAA-HDL together with CQ (100 μM), an agent that neutralizes the lysosomal proton gradient (34, 35). At various time points, cellular lipids were extracted and analyzed for cholesteryl ester radioactivity as described above.

Cholesterol efflux in tissue culture and in vivo

J774 cells were enriched with cholesterol by incubating with RBC membrane fragments (175 μg as cholesterol) that had been previously labeled with 0.5 μCi/ml [^{3}H]cholesterol at 37°C for 24 h in 0.2% BSA. After loading with the labeled RBC membrane for 6 h, cells were washed three times with PBS-BSA followed by an 18 h equilibration period during which monolayers were exposed to DMEM-BSA. Cells were washed four times with PBS-BSA prior to the efflux studies. Cells were then incubated at 37°C with 2 ml DMEM-BSA and the indicated additions plus 2 μg/ml of Sandoz 58-035. At the indicated time points, 100 μl aliquots of the efflux media were collected and centrifuged to remove cell debris, and 75 μl aliquots were then used to measure the exported counts. The cell layers were then washed twice with ice-cold PBS-BSA and twice with PBS. A portion of the cells were lysed in 0.1 N NaOH to estimate both the remaining radioactivity and the cellular protein content. Cellular lipids were extracted from the remaining portion of the cells and analyzed by thin-layer chromatography as described previously (32, 33). Briefly, the percentage of the radiolabeled cholesterol that was released was calculated by dividing the total amount of [^{3}H]cholesterol in the incubation medium at each sampling time by the total [^{3}H]cholesterol in the cells at time zero. An extra set of cells was harvested at the beginning of the efflux period to determine initial cellular [^{3}H]cholesterol and cholesterol mass. Efflux was calculated from the fraction of initial [^{3}H]cholesterol, or cholesterol mass, exported or remaining in the cells, respectively, at each time point. The effects of various concentrations (0–100 μg/ml) of HDL or SAA-HDL were then determined. For this dose-response study, the fractional efflux of labeled cholesterol or cholesterol mass was determined after an 8 h incubation of the monolayers with various concentrations of either HDL or SAA-HDL as indicated. To examine whether cholesterol export from J774 cells to medium containing liposomes with 2 μM (final culture concentration) of apoA-I, SAA1.1, or SAA2.1 is a cAMP-dependent process, the radiolabeled cholesterol-laden cells were incubated overnight with 8-Br-cAMP (0.3 mM) prior to the addition of the aforementioned liposomes. This concentration of cAMP was maintained during the liposome incubation period.

To map the domains in SAA2.1 that are responsible for the increase in cholesterol export, J774 macrophages were loaded with cholesterol and labeled as described above. They were then incubated with either HDL (10 μg/ml) or liposomes containing 0.5 μM synthetic peptides corresponding to amino acid residues 1–20, 21–50, 51–80, or 74–103 of murine SAA2.1 for 4 h. In some experiments, the above-noted cells were exposed simultaneously to 0.5 μM (final culture concentration) synthetic peptides corresponding to amino acid residues 1–20 and 74–103 of murine SAA2.1 for 4 h. After the incubation period, the cells were washed three times with PBS-BSA to remove all the liposomes, and the amount of [^{3}H]cholesterol radioactivity efflux to the medium containing 100 μg of HDL was measured at various time points as described above. To test the "shuttle-sink" for cholesterol efflux, we carried out studies in which cell cholesterol efflux to medium was determined when the medium was supplemented with protein-free liposomes or liposomes containing 2 μM (final culture concentration) of SAA1.1 or SAA2.1 together with MCD (0.1 mM), a nonlipoprotein particle that can function as a cholesterol shuttle.

To determine cholesterol export, in vivo experiments were conducted as described and validated previously (12). Briefly, J774 macrophages were cholesterol loaded with RBC membranes and [^{3}H]cholesterol as described previously (12). Cells were washed four times with PBS-BSA and then detached from the culture dishes. Five million cells in 200 μl DMEM were injected into mice through the tail vein. At various time points thereafter, ~25 μl of blood was collected from the tail vein of each animal into heparinized capillary tubes and then centrifuged for 5 min in an Adams Autocrit Centrifuge to separate RBCs from plasma. Cholesterol efflux was determined by measuring the appearance of [^{3}H]cholesterol in plasma by scintillation spectrometry. To study whether export of cholesterol from J774 cells to plasma is influenced by SAA2.1 or its domains, 24 h after injection of cholesterol-loaded and labeled J774 macrophages into mice, the same animals received, by intravenous injection, 200 μl of protein-free liposomes, liposomes containing SAA1.1 or -2.1, or lipo-

somes containing synthetic peptides corresponding to amino acid residues 1–20, 21–50, 51–80, and 74–103 of murine SAA2.1, respectively. The concentration of the injected SAA1.1 and -2.1 liposomes was 20 μM. Assuming a mouse total blood volume of 2 ml, the final blood concentration was ~2 μM. The liposomes containing the synthetic peptides were injected at a concentration of 5 μM, resulting in a final blood concentration of 0.5 μM. At various time points following the second injection, ~25 μl of blood was collected from the tail vein of each animal and analyzed as described above.

Protein determinations

Protein concentration was determined using the method of Lowry and coworkers (31), with the aid of a BioRad protein assay kit.

Statistical analysis

Unpaired Student's *t*-tests were used to compare group means. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Effect of varying concentrations of HDL or SAA-HDL on macrophage cholesterol efflux

A comparison of the influence of varying concentrations of HDL versus SAA-HDL on macrophage cholesterol efflux in culture is illustrated in **Fig. 1A**, **B**. Figure 1A demonstrates the efflux data over an 8 h period using [^{3}H]cholesterol. The percent of total cellular cholesterol emerging during the 8 h period increases in a nonlinear fashion with increasing concentrations of HDL or SAA-HDL, reaching a value of 33.4 ± 2.1% and 40.5 ± 2.6%, respectively, at 100 μg/ml. Similarly, in parallel experiments, when the residual mass of free cholesterol is determined after 8 h of incubation with varying concentrations of HDL or SAA-HDL, 24.8 ± 2.3% and 35.5 ± 2.2% is exported, respectively.

Effect of CQ on CEH activity in J774 macrophages

Previously (12), using macrophages preloaded with radiolabeled cholesteryl esters, we demonstrated that SAA-HDL caused a 3-fold increase in CEH activity. The de-esterification of cellular cholesteryl esters involves two types of hydrolases: neutral (cytoplasmic) or acid (lysosomal) hydrolases. To examine whether the increase in CEH activity was due to an enhancement of the lysosomal pathway, cholesterol-laden and labeled macrophages were incubated in the presence of SAA-HDL and 100 μM CQ, a lysosomal enzyme inhibitor. This concentration of CQ has been shown to completely inhibit lysosomal hydrolases in cultured cells (36). Hence, a shallower decline in labeled esterified cholesterol would indicate involvement of the acid hydrolase in this process. The results presented in **Fig. 2** show that the rate of loss of labeled cholesteryl ester was not affected by the presence of 100 μM CQ in cells treated with SAA-HDL. Furthermore, incubation with HDL (50 μg/ml) and CQ had similar rates of degradation of labeled cholesteryl ester when compared with HDL treatment alone (Fig. 2). These findings indicate that nonlysosomal hydrolases are responsible for the hydrolysis

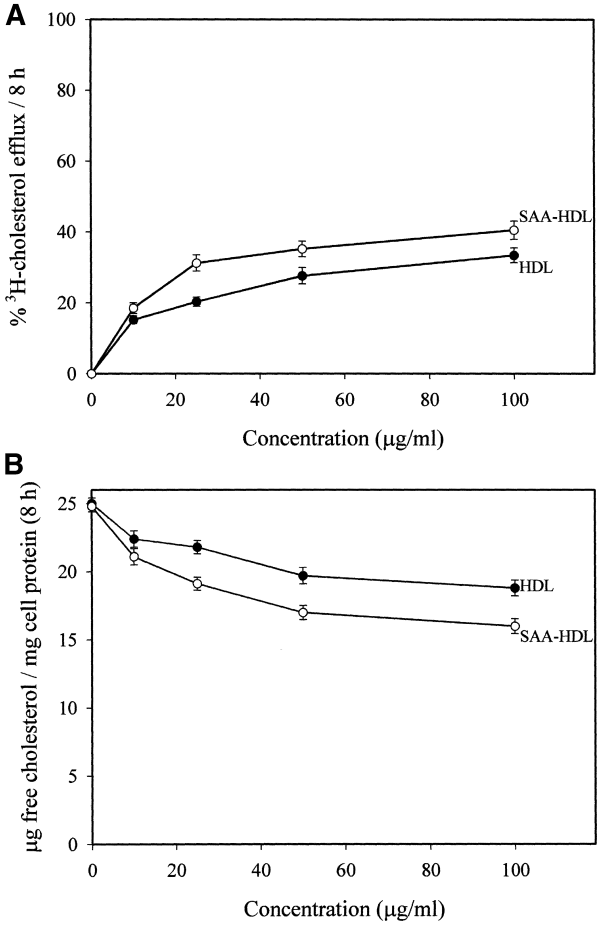

Fig. 1. Effects of increasing concentration of HDL and serum amyloid A (SAA)-HDL on the efflux of cholesterol from J774 cells. Monolayers of J774 cells grown in 6-well tissue culture plates were enriched with cholesterol by incubating with red blood cell (RBC) membrane fragments (175 μg as cholesterol) that had been previously labeled with 0.5 μCi/ml [^{3}H]cholesterol at 37°C for 24 h in 0.2% BSA. After loading with the labeled RBC membranes for 6 h, cells were washed with PBS-BSA three times followed by an 18 h equilibration period during which monolayers were exposed to DMEM-BSA. Cells were washed four times with PBS-BSA prior to the efflux studies. Cells were then incubated at 37°C with 2 ml DMEM-BSA plus 2 μg/ml of Sandoz 58-035 and increasing concentrations (0–100 μg/ml) of either HDL (closed circles) or SAA-HDL (open circles). A: The fractional efflux of labeled cholesterol was determined after an 8 h incubation as described in Materials and Methods. B: The mass of free cholesterol at time zero or remaining in the cells after an 8 h incubation with either HDL or SAA-HDL. Results are the mean ± SEM of three determinations.

of labeled cholesteryl esters in macrophages exposed to native HDL or SAA-HDL.

Effects of cAMP and MCD on cholesterol efflux from J774 cells

We have previously demonstrated that liposomes containing SAA2.1, but not SAA1.1, are more effective than apoA-I-containing liposomes in mobilizing free cholesterol from cholesterol-loaded macrophages (12). This SAA2.1-promoted export process was shown to be coupled to the ATP binding cassette transporter (ABCA1) pathway

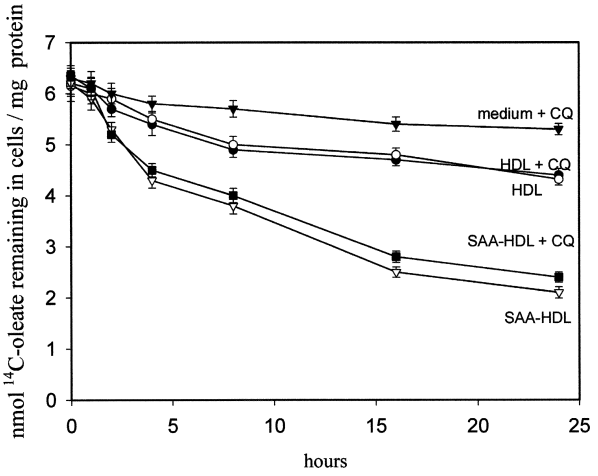

Fig. 2. Cholesterol esterase activity in cholesterol-loaded macrophages exposed to HDL or SAA-HDL in the absence or presence of chloroquine (CQ). Nearly confluent J774 cells were cholesterol loaded with RBC membrane fragments and labeled with [^{14}C]oleate as described in Materials and Methods. The cells were then incubated for up to 24 h in the presence of 2 μg/ml of the ACAT inhibitor Sandoz 58-035 with 2 ml DMEM containing 5% lipoprotein-depleted serum medium supplemented with 50 μg/ml HDL (open circles), 50 μg/ml HDL + 100 μM CQ (closed circles), 50 μg/ml SAA-HDL (inverted open triangles), 50 μg/ml SAA-HDL + 100 μM CQ (closed squares) or medium alone + 100 μM CQ (inverted closed triangles). After the indicated intervals, cellular lipids were extracted and analyzed for cholesteryl ester radioactivity. Results are the mean ± SEM of four determinations.

(12). In addition, other investigators have demonstrated that lipid efflux to apolipoproteins can be stimulated by treatment of murine macrophages with cAMP analogs (37, 38). Also, the expression of ABCA1 is induced by cAMP treatment (37, 38). We wished, therefore, to examine the effect of 8-bromo-cAMP (0.3 mM) on cholesterol efflux to liposomes containing various apolipoproteins when incubated with cholesterol-laden J774 macrophages. Such cells were prelabeled with [^{3}H] cholesterol in the presence of Sandoz 58-035, an ACAT inhibitor, to ensure that all of the radiolabeled cholesterol released from the cells was derived from the unesterified cholesterol pool, and the cells were treated overnight with 8-bromo-cAMP. This was followed by incubation with various acceptors in the presence of cAMP. At the indicated time points, the fractional release of cellular labeled cholesterol was determined as described in Materials and Methods. When compared with untreated cells, cAMP treatment over the first 8 h resulted in the efflux of 55.9% and 35.8% of cellular cholesterol to liposomes containing SAA2.1 and apoA-I, respectively (**Fig. 3A**). No stimulation of efflux was observed when cells were exposed to SAA1.1 liposomes with or without cAMP treatment. Furthermore, we have demonstrated previously that SAA1.1 liposomes are not any more effective than protein-free liposomes in promoting cholesterol efflux from cholesterol-laden macrophages (12). Moreover, cAMP treatment did not stimulate cholesterol export to culture medium not containing liposomes (data not shown).

To determine whether an apolipoprotein-free acceptor such as cyclodextrin can catalyze the removal of cholesterol from macrophages, cholesterol-loaded and labeled J774 cells were incubated with various liposomes and MCD (0.1 mM). As shown in Fig. 3B, no stimulation of cholesterol efflux to medium without a cholesterol acceptor was observed at this concentration of MCD. As shown in Fig. 3A, treatment of cells with liposomes containing SAA2.1, but not SAA1.1 or protein-free liposomes, resulted in a 2.3-fold increase in both the initial rate (0–8 h), and final percent (51.2%) of cholesterol efflux. Using SAA2.1 liposomes, MCD treatment over the first h enhanced the quantity of free cholesterol efflux 2- to 2.5-fold, (compare Fig. 3A, B). Furthermore, the combined presence of SAA2.1, MCD, and cAMP over a 24 h period prompted the release of 72.5% of radiolabeled cellular cholesterol (Fig. 3B). Parallel experiments examining the mass of residual free cholesterol during identical incubations are illustrated in Fig. 3C, D. Using this parameter, when compared with untreated cells, cAMP treatment (0–8 h) resulted in a 49.8% and 32.2% efflux of cholesterol to liposomes containing SAA2.1 and apoA-I, respectively (Fig. 3C). By 24 h, this had increased to 52.4% and 34.9%, respectively (Fig. 3C). As shown in Fig. 3D, no stimulation of cholesterol efflux to medium containing no liposomes was observed in the presence of MCD. Using SAA2.1 liposomes, MCD treatment over the first 2 h enhanced the quantity of free cholesterol efflux 2-fold (compare Fig. 3C, D). MCD had no effect on cholesterol efflux with SAA1.1 or protein-free liposomes (Fig. 3D). Furthermore, cAMP treatment caused a further 11% increase (47.6% to 58.1%) in cholesterol efflux in cells exposed to MCD and liposomes containing SAA2.1 (Fig. 3D).

Identifying domains of SAA2.1 responsible for inhibiting ACAT and enhancing CEH activities

Previously, we demonstrated that exposure of cholesterol-loaded macrophages to SAA2.1 resulted in a marked reduction of ACAT and enhancement of CEH activities (12). To map the domains in SAA2.1 that are responsible for modulating these two enzyme activities, we used four synthetic peptides corresponding to amino acid residues 1–20, 21–50, 51–80, and 74–103 of the murine SAA2.1 protein sequence, which span the entire sequence of this isoform. Residues 1–20 correspond closely to residues 1–16 generated previously from the native protein by CNBr cleavage (28) and shown to profoundly inhibit macrophage ACAT activity in postnuclear homogenates (26). Residues 21–50, 51–80, and 74–103 span an 80 residue peptide (residues 24–103) generated previously from the native protein by CNBr cleavage, which possessed the enhancing property for purified pancreatic neutral CEH (26). The incorporation of [^{14}C]oleate into cholesteryl ester was used as a measure of ACAT activity as described in Materials and Methods. The relative ACAT activity was determined in cholesterol-laden cells that had been cultured in medium in the absence of liposomes or in the presence of protein-free liposomes or liposomes at 0.5 μM synthetic peptides corresponding to amino acid residues 1–20, 21–

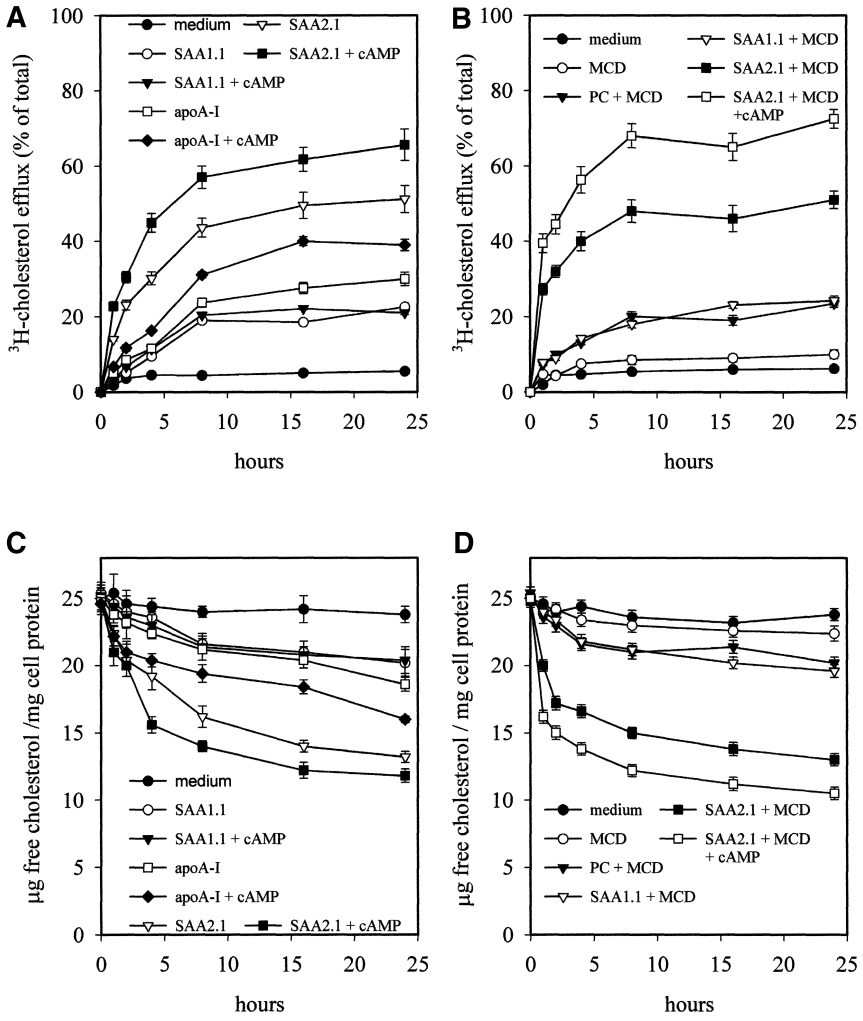

Fig. 3. Time course of cholesterol efflux from cholesterol-laden macrophages exposed to various liposomes in the absence or presence of cAMP or methyl-β-cylcodextrin (MCD). J774 cells were cholesterol loaded and labeled as described in Fig 1. A: Cells were then incubated in medium containing 2 μg/ml Sandoz 58-035 (closed circles) or the same medium plus liposomes containing 2 μM of either apolipoprotein A-I (apoA-I) (open squares), SAA1 (open circles), or SAA2.1 (inverted open triangles). Similar experiments were also carried out, except that the cells were preincubated with 8-bromo-cAMP (0.3 mM) overnight prior to the incubation and, during the incubation period, with liposomes containing 2 μM of either apoA-I (closed diamonds), SAA1.1 (inverted closed triangles), or SAA2.1 (closed squares). The fractional efflux of labeled cholesterol was determined as described in Materials and Methods. B: Cholesterol-loaded and labeled J774 cells were incubated with medium containing 0.1 mM MCD alone (open circles), or the same medium plus protein-free liposomes (inverted closed triangles), or liposomes containing 2 μM of either SAA1.1 (inverted open triangles) or SAA2.1 (closed squares). In some experiments, the cells were preincubated with cAMP (0.3 mM) for 12 h prior to incubation and, during the incubation period, with liposomes containing 2 μM of SAA2.1 plus MCD (open squares). After the indicated time intervals, the medium was collected and centrifuged at 10,000 *g* for 10 min, and radiolabeled cholesterol in the supernatant was measured as described in Materials and Methods. Cellular lipids were analyzed for remaining free and esterified [^{3}H]cholesterol. Results are expressed as percent of total (cell plus medium) radioactivity in each well. Total [^{3}H]cholesterol was 2.5–2.8 × 10^5 dpm/mg cell protein (A) and 2.6–2.9 × 10^5 dpm/mg cell protein (B). C: In parallel experiments as described in Fig. 3A, the mass of residual cell free cholesterol was determined at the end of the designated incubations. D: In parallel experiments as described in Fig. 3B, the mass of residual cell free cholesterol was determined at the end of the designated incubations. Values are the mean ± SEM of four determinations.

50, 51–80, and 74–103 of murine SAA2.1. Following 6 h incubations, only the cells that had been exposed to liposomes containing synthetic peptides corresponding to amino acid residues 1–20 of SAA2.1 showed a 2.5-fold decrease in ACAT activity (**Fig. 4A**), while other liposome treatments had no significant effect on ACAT.

Experiments were also carried out to identify the domains in SAA2.1 that are responsible for enhancing CEH activity in J774 cells that had been preloaded and radiolabeled with cholesteryl esters (Fig. 4B). This was performed using liposomes containing one of each of the four synthetic peptides of SAA2.1 noted above. These studies were

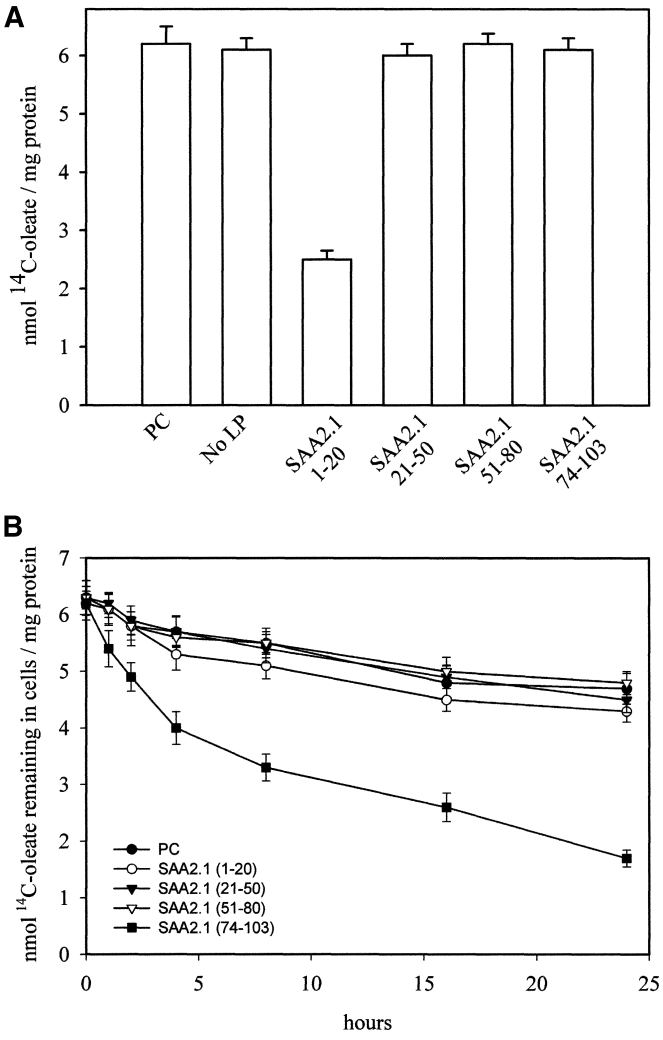

Fig. 4. Mapping the active domains in SAA2.1 that are responsible for inhibiting ACAT and enhancing cholesteryl ester hydrolase activities. Nearly confluent monolayers of J774 cells were incubated in the presence of RBC membrane fragments and labeled with [^{14}C]oleate. A: The ACAT activity was determined as described in Materials and Methods using cholesterol-laden cells that had been cultured with DMEM-BSA alone (no LP); medium plus protein-free liposomes (PC), or liposomes containing 0.5 μM synthetic peptides corresponding to amino acid residues 1–20, 21–50, 51–80, and 74–103 of murine SAA2.1. B: Time course of cholesterol esterase activity in cholesterol-loaded macrophages exposed to protein-free liposomes (PC, closed circles) or liposomes containing 0.5 μM synthetic peptides corresponding to amino acid residues 1–20 (open circles), 21–50 (inverted closed triangles), 51–80 (inverted open triangles), and 74–103 (closed squares) of murine SAA2.1 was determined as described in Fig. 1. After the indicated intervals, cellular lipids were extracted and analyzed for cholesteryl ester radioactivity. Results are the mean ± SEM of four determinations.

done in the presence of Sandoz 58-035, an ACAT inhibitor, to prevent the reesterification of liberated cholesterol and [^{14}C]oleate. Incubations proceeded for various time periods, as indicated, following which the remaining quantities of ^{14}C-labeled cholesteryl oleate in cells were measured to determine the rate of hydrolysis of cholesteryl ester. With re-esterification blocked, there were no significant differences in the rate of hydrolysis of ^{14}C-labeled cholesteryl oleate in cells cultured in the presence of protein-free liposomes or liposomes at 0.5 μM synthetic peptides corresponding to amino acid residues 1–20, 21–50, and 51–80 from the N-terminal of murine SAA2.1, respectively. However, an equivalent amount of liposomes containing the synthetic peptide corresponding to residues 74–103 of SAA2.1 caused a 3-fold increase in CEH activity.

Cholesterol efflux from cholesterol-loaded J774 cells that had been preincubated for 4 h with HDL or liposomes containing various synthetic SAA2.1 peptides, followed by 24 h with HDL

Cholesterol-loaded macrophages labeled with [^{3}H]cholesterol were preincubated for 4 h in the absence or presence of either HDL (10 μg/ml) or liposomes at 0.5 μM synthetic peptides corresponding to amino acid residues 1–20, 21–50, 51–80, or 74–103 of murine SAA2.1. In some experiments, the combination of two synthetic peptides (0.5 μM each) corresponding to amino acid residues 1–20 and 74–103 of SAA2.1 was also used. Following incubation, the cells were washed extensively with DMEM-BSA to remove all radioactivity and the various liposomes in the preincubation medium. So that there would be a common cholesterol acceptor after the preincubation with the various liposomes, the chase efflux media consisted of DMEM-BSA alone or medium containing HDL (50 μg/ml). At various time points, the efflux media were collected, analyzed for [^{3}H]cholesterol, and the results expressed as a percent of the total counts present in the cells at the beginning of the HDL incubation. Also determined (**Table 1**) was the percent efflux of the radiolabeled cellular cholesterol relative to the cellular [^{3}H]cholesterol content before the preincubation step (i.e, at the start of the experiment). In identical parallel experiments, the percent mass of free cholesterol exported during the 4 h preincubation with various liposomes (**Table 2**) and that remaining in the cells at varying time points during the 24 h incubations were determined. The results shown in **Fig. 5A**, **B** indicate that [^{3}H]cholesterol efflux to medium containing 0.2% BSA was 5.5 ± 0.9% of total counts and 5.2 ± 1.0% by mass, respectively. Cells cultured in the presence of HDL (50 μg/ml) exported 22.5 ± 1.9% of total cellular [^{3}H]sterol and 17.1 ± 1.8% by mass, respectively, to the medium. Preincubation of cells with liposomes at 0.5 μM synthetic peptides corresponding to amino acid residues 21–50 or 51–80 of SAA2.1 did not cause any significant changes in the rate of [^{3}H]cholesterol efflux into the medium containing the HDL. However, when cholesterol-laden J774 cells labeled with [^{3}H]cholesterol were preincubated with liposomes at 0.5 μM synthetic peptides corresponding to amino acid residues 1–20 or 74–103 of SAA2.1, it was observed that 45.3 ± 2.9% and 33.6 ± 3.0% of total cellular [^{3}H]cholesterol (41.3 ± 1.7% and 34.9 ± 1.9% by mass) were released into the medium when the cells were subsequently cultured in the presence of HDL. Under similar culturing conditions, preincubation with the combination of these two synthetic peptides of SAA2.1 resulted in the export of 71.5 ± 3.2% of total cellular [^{3}H]sterol (54.5 ± 1.7% by mass) to HDL. In addition, in

TABLE 1. Efflux of [^{3}H]cholesterol from cholesterol-loaded J774 cells that had been preincubated for 4 h with HDL or liposomes containing various synthetic SAA2.1 peptides, followed by 24 h with HDL

Addition	Total Cell [^{3}H]Cholesterol at t = 0	[^{3}H]Cholesterol in Medium after 4 h Preincubation	Total Cell [^{3}H]Cholesterol 4 h Postincubation	[^{3}H]Cholesterol Efflux after 24 h HDL Incubation Relative to	
				t = 0	t = Post 4 h
	dpm/mg protein	%	*dpm/mg protein*	%	
Medium	248,010	2.0	243,040	5.4	5.5
HDL	258,606	7.8	238,480	20.7	22.5
$SAA2.1_{(1–20)}$	249,000	12.0	219,120	39.9	45.3
$SAA2.1_{(21–50)}$	259,458	7.6	239,700	21.2	22.9
$SAA2.1_{(51–80)}$	265,001	8.0	243,681	21.1	23.0
$SAA2.1_{(74–103)}$	256,000	11.2	227,240	29.8	33.6
$SAA2.1_{(1–20)}$ + $SAA2.1_{(74–103)}$	275,000	13.0	239,122	62.2	71.5

J774 cells were cholesterol loaded and labeled with [^{3}H]cholesterol as described in Fig. 5. The radioactivity determined at this time (t = 0) is shown in column 2. Cells were then preincubated for 4 h in the absence or presence of either HDL (10 μg/ml) or liposomes containing 0.5 μM synthetic peptides corresponding to amino acid residues 1–20, 21–50, 51–80, and 74–103 of murine serum amyloid A 2.1. (SAA2.1). In some experiments, a combination of two synthetic peptides (0.5 μM each) corresponding to amino acid residues 1–20 and 74–103 of SAA2.1 was also used. Following this 4 h incubation, the radioactivity emerging in the medium was determined as described in Materials and Methods and expressed as percent of that originally in the cells (column 3). The residual radioactivity associated with the cells after the 4 h preincubation was calculated by subtracting the value in the medium from the radioactivity originally in the cells (column 4). The radioactivity emerging during the 24 h chase incubation (HDL 50 μg/ml) was determined and expressed either as percent of the total cell radioactivity at time zero (column 5) or as percent of the total cell radioactivity following the 4 h preincubation (column 6). Results are the average of four determinations, and the maximum range was ±6% of the mean values.

the presence of both active peptides, the initial rate of cholesterol efflux to HDL during the first 2 h was twice as fast when compared with the results with liposomes containing either synthetic peptide alone (Fig. 5A).

Cholesterol efflux studies in vivo

To examine cholesterol export in vivo, experiments were conducted as described previously (12), in which we confirmed that cholesterol release from intravenously injected cholesterol-laden macrophages was not a function of cell death but rather was dependent on a functional cholesterol transporter. Five million [^{3}H]cholesterol-loaded J774 cells were injected intravenously into noninflamed mice. After 24 h of equilibration, the animals were injected intravenously with medium alone (200 μl) or 200 μl medium containing protein-free liposomes or liposomes to a final concentration of 2 μM of SAA1.1 or -2.1 (assuming a total mouse blood volume of 2 ml). Cholesterol export was determined over a 96 h period by measuring the appearance of [^{3}H]cholesterol in plasma. Among

TABLE 2. Efflux of cholesterol mass from cholesterol-loaded J774 cells that had been preincubated for 4 h with HDL or liposomes containing various synthetic SAA2.1 peptides, followed by 24 h with HDL

Addition	Free Cholesterol Mass at t = 0	Cholesterol in Medium after 4 h Preincubation	Free Cholesterol 4 h Postincubation	Cholesterol Efflux after 24 h HDL Incubation Relative to	
				t = 0	t = post 4 h
	μg/mg protein	%	*μg/mg protein*	%	
Medium	26.8	6.3	25.1	4.9	5.2
HDL	26.5	7.2	24.6	15.8	17.1
$SAA2.1_{(1–20)}$	27.8	10.1	25.2	37.4	41.3
$SAA2.1_{(21–50)}$	26.6	6.8	24.8	19.5	21.0
$SAA2.1_{(51–80)}$	26.4	5.3	25.0	14.8	15.6
$SAA2.1_{(74–103)}$	25.6	2.7	24.9	34.0	34.9
$SAA2.1_{(1–20)}$ + $SAA2.1_{(74–103)}$	28.5	13.7	24.6	47.0	54.5

J774 cells were cholesterol loaded as described in Fig. 5. The free cholesterol mass determined at this time (t = 0) is shown in column 2. Cells were then preincubated for 4 h in the absence or presence of either HDL (10 μg/ml) or liposomes containing 0.5 μM synthetic peptides corresponding to amino acid residues 1–20, 21–50, 51–80, and 74–103 of murine SAA2.1. In some experiments, a combination of two synthetic peptides (0.5 μM each) corresponding to amino acid residues 1–20 and 74–103 of SAA2.1 was also used. Following this 4 h incubation, the free cholesterol emerging in the medium was determined as described in Materials and Methods and expressed as percent of that originally in the cells (column 3). The residual free cholesterol associated with the cells after the 4 h preincubation was calculated by subtracting the value in the medium from the free cholesterol originally in the cells (column 4). The cholesterol emerging during the 24 h chase incubation (HDL 50 μg/ml) was determined and expressed either as percent of the cell free cholesterol at time zero (column 5) or as percent of the cell free cholesterol following the 4 h preincubation (column 6). Results are the average of four determinations, and the maximum range was ±10% of the mean values.

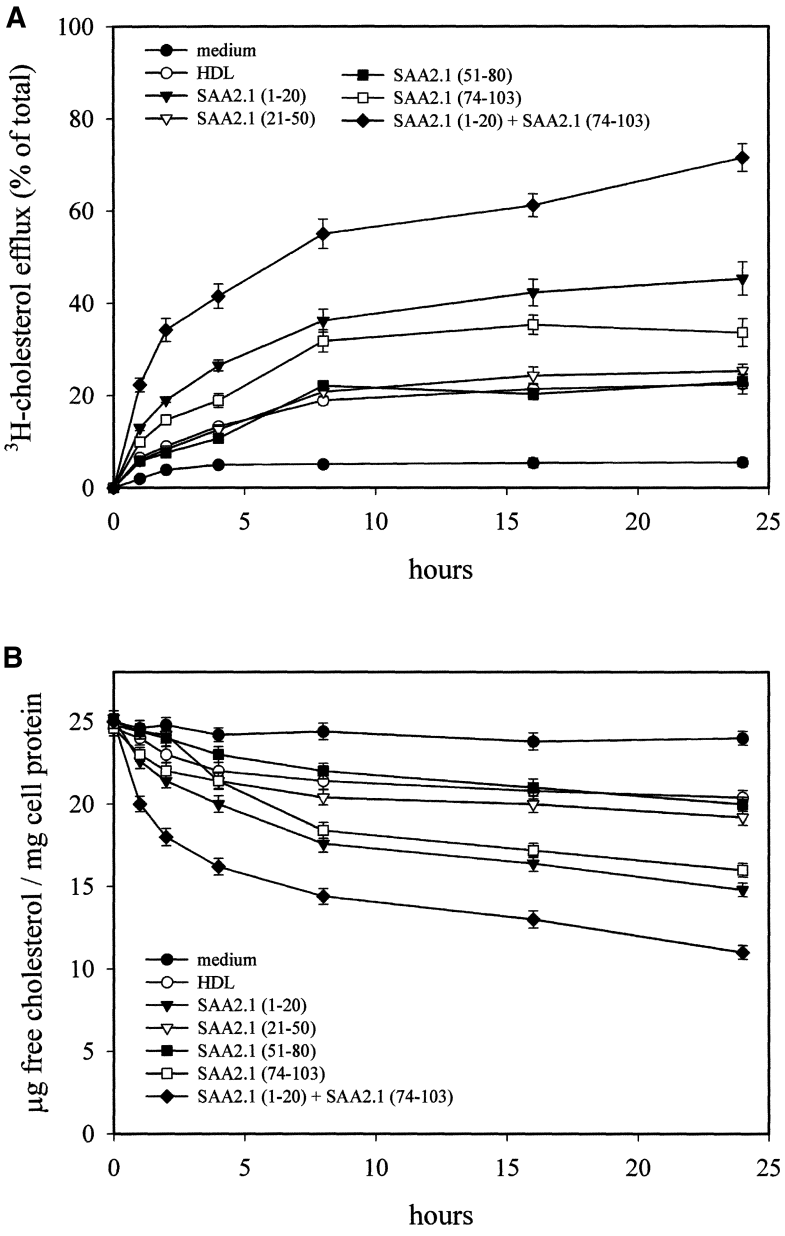

Fig. 5. Cholesterol export from cholesterol-loaded J774 cells that had been preincubated for 4 h with HDL or liposomes containing various synthetic SAA2.1 peptides, followed by 24 h with HDL. Cholesterol-loaded macrophages were labeled with [^{3}H]cholesterol as described in Fig. 1. Cells were preincubated in the absence or presence of either HDL (10 μg/ml) or liposomes containing 0.5 μM synthetic peptides corresponding to amino acid residues 1–20, 21–50, 51–80, and 74–103 of murine SAA2.1. In some experiments, the combination of two synthetic peptides (0.5 μM each) corresponding to amino acid residues 1–20 and 74–103 of SAA2.1 (closed diamonds) was also used. Following incubation, the cells were washed extensively with DMEM-BSA to remove all radioactivity and various liposomes in the preincubation medium. The chase efflux media consisted of DMEM-BSA alone (closed circles) or medium containing HDL (50 μg/ml). The results indicated by the open circles represent cholesterol efflux to the acceptor, HDL, in the medium from cells without any liposome pretreatment. At various time points, the efflux media were collected and analyzed for [^{3}H]cholesterol (A) and free cholesterol mass remaining in the cells (B) as described in Fig. 3. Total [^{3}H]cholesterol was 2.5–2.9 × 10^5 dpm/mg cell protein. Results are the mean ± SEM of four determinations.

these liposomes, only the liposomes containing SAA2.1 resulted in a 3-fold increase in cholesterol efflux to plasma of uninflamed animals (**Fig. 6**). The radioactivity released to plasma peaked 12 h after injection of liposomes containing SAA2.1 and remained elevated for at least the next 60 h when compared with the other liposomes injected. To confirm that the active domains of SAA2.1 demonstrated in tissue culture are responsible for the increase in cholesterol efflux in vivo, liposomes to a final concentration of 0.5 μM (assuming a mouse total blood volume of 2 ml) of synthetic peptides corresponding to amino acid residues 1–20, 21–50, 51–80, or 74–103 of murine SAA2.1 were each injected into uninflamed mice 24 h after the injection of five million [^{3}H]cholesterol-loaded J774 cells. As shown in **Fig. 7**, only liposomes containing synthetic peptides corresponding to residues 1–20 and 74–103 of SAA2.1 caused significant increases (3- to 4-fold) of [^{3}H]cholesterol efflux to plasma. In each case, the radioactivity peaked 16 to 20 h after injection of the liposomes and remained elevated for at least an additional 72 h (i.e., 96 h total).

DISCUSSION

SAA was discovered in the mid-1970s as a circulating plasma protein that cross-reacted with antibodies raised against the inflammation-associated amyloid peptide (amyloid A) (39). Over the next 20 to 25 years, it was shown that there was a family of evolutionary conserved genes that coded for several SAA isoforms (13), two of which (SAA 1.1 and SAA2.1) were major acute-phase proteins synthesized primarily in the liver in response to any insult that resulted in tissue injury and a reactive inflammatory response (40). Following synthesis and secretion, these isoforms associate primarily with HDL (>90%), dis-

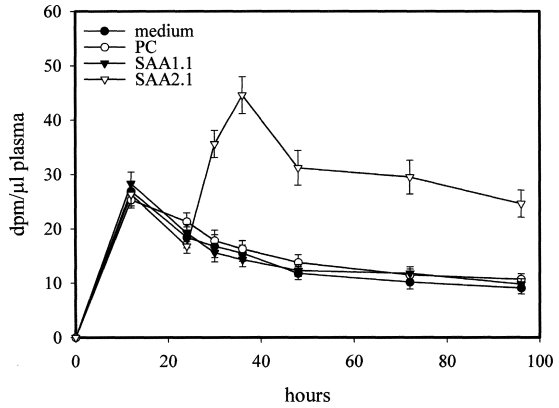

Fig. 6. Cholesterol export studies in vivo. J774 cells were cholesterol loaded and labeled with [^{3}H]cholesterol as described in Materials and Methods. Cells were then washed extensively and detached from the culture dishes. Five million cells in 200 μl DMEM were injected intravenously into mice through the tail vein. After 24 h of equilibration, the animals were injected with 200 μl medium alone (closed circles), medium containing protein-free liposomes (PC, open circles), or liposomes containing 2 μM of SAA1.1 (inverted closed triangles) or SAA2.1 (inverted open triangles). Cholesterol export was determined over a 96 h period by measuring the appearance of [^{3}H]cholesterol in plasma. At the indicated time points, ~25 μl of blood was collected from the tail vein of each animal. The blood samples were centrifuged to separate the RBCs from plasma, and the [^{3}H]cholesterol in plasma was then determined by scintillation spectrometry. Results are the mean ± SEM of five animals and are representative of three independent experiments. Error bars not shown are within the symbol dimensions.

placing apoA-I, apoA-II, and apoE (41, 42). During acute tissue injury, 30–80% of the apolipoprotein of HDL is composed of the acute-phase SAA isoforms (25), and it is this pool of SAA that serves as the precursor to AA amyloid peptides deposited as tissue AA amyloid (43–45). This role of SAA in the pathogenesis of inflammation-associated amyloidosis is clearly not its primary physiological function. The nature of the primary physiological function of SAA has been a subject of considerable interest for almost three decades and has generated a wide variety of ideas that have been reviewed recently (46). Among these is the concept that SAA influences cholesterol mobilization from macrophages at sites of tissue injury (47, 48). The rationale for this working hypothesis stems from several observations.

Tissue injuries, regardless of cause, result in local cell death and the release of cellular membrane fragments. These are ingested by macrophages arriving at the site of injury as part of the local acute inflammatory reaction. Because these membrane fragments are composed of up to 50% cholesterol (10, 11), the macrophages acquire a considerable cholesterol load and become foam cells. A mechanism to mobilize this cholesterol for excretion or reuse is therefore required. The long history of evolutionary conservation of the amino acid sequence of SAA, the profound response of hepatic SAA synthesis to acute tissue injury, and the predominant association of SAA with plasma HDL, a reverse cholesterol transporter, suggest that SAA has some beneficial role to play in cholesterol metabolism during the systemic response to acute tissue injury.

Our previous studies (12, 26, 49) are consistent with a role for SAA2.1 in the mobilization of stored macrophage cholesterol at sites of acute tissue injury, promoting its export to an extracellular cholesterol acceptor (12). This effect was shown to occur through a dual action on the enzyme activities (ACAT and CEH) that regulate the balance between cellular esterified and nonesterified cholesterol. The inhibition of ACAT and the enhancement of CEH activities by SAA2.1 shift the balance in favor of unesterified cholesterol, and in the presence of a functional transporter and extracellular acceptor, SAA2.1 promotes cholesterol efflux very effectively (12). These effects on ACAT and CEH are not secondary to the removal of the cholesterol from the cells by SAA2.1 or its peptides, because we have previously demonstrated identical effects on ACAT and CEH in cells in which macrophage cholesterol efflux is blocked by 4,4-diisothiocyanotostilbene-2,2′-disulfonic

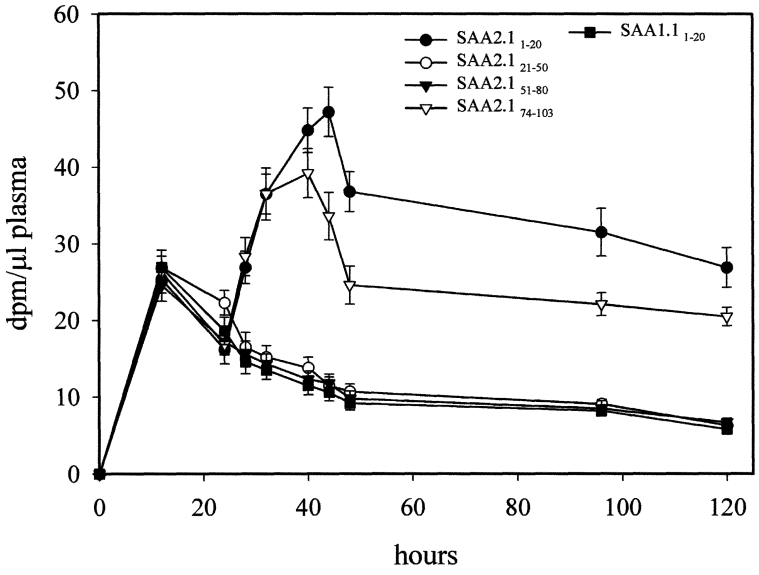

Fig. 7. Effects of liposomes containing various synthetic peptides of SAA2.1 on cholesterol efflux in vivo. Five million J774 cells were cholesterol loaded and labeled with [^{3}H]cholesterol and injected intravenously into mice as described in Fig. 6. After 24 h of equilibration, the animals were injected with 200 μl of liposomes containing 0.5 μM synthetic peptides corresponding to amino acid residues 1–20 (closed circles), 21–50 (open circles), 51–80 (inverted closed triangles), and 74–103 (inverted open triangles) of murine SAA2.1. In addition, liposomes containing 0.5 μM synthetic peptides corresponding to amino acid residues 1–20 (closed squares) of SAA1.1 were also used. Cholesterol export was determined over a 120 h period by measuring the appearance of [^{3}H]cholesterol in plasma. At the indicated time points, ~25 μl of blood was collected from the tail vein of each animal. [^{3}H]cholesterol in plasma was then determined by scintillation counting as described in Fig. 6. Results are the mean ± SEM of five animals and are representative of three independent experiments. Error bars not shown are within the symbol dimensions.

acid (DIDS), an inhibitor of the anion transport activities of the ABCA1 transporter (12). Furthermore, we have previously shown that native SAA2.1 and a CNBr-derived peptide from this isoform (specifically, residues 1–16) have a direct effect on ACAT activity in macrophage postnuclear homogenates (26). Moreover, native SAA2.1 and a CNBr-derived peptide from this isoform (specifically residues 24–103) have a direct enhancing effect on purified pancreatic CEH (26). Such observations are not consistent with secondary effects on ACAT and CEH through removal of cellular cholesterol, which would change the substrate pool for these enzymes. The observations with the postnuclear homogenates and the purified pancreatic form of CEH are also inconsistent with SAA2.1 affecting ACAT and CEH through a receptor-mediated second-messenger mechanism.

We have also previously demonstrated that the in vivo release of radiolabeled cholesterol from intravenously injected cholesterol-laden J774 macrophages is not a function of cell death or of the destruction of these cells by an immune or ongoing inflammatory reaction but rather is dependent on a functional transporter [see Fig. 6 in (12)]. Such cells, if injected intravenously into normal animals, release small quantities of labeled cholesterol over a 96 h period. If injected into mice that have a preexisting acute inflammatory reaction, such cells release large quantities of cholesterol over the same time period. Most importantly, such cells, if treated with DIDS, washed free of excess DIDS, and then injected into normal or inflamed mice, fail to release radiolabeled cholesterol to plasma to any significant degree in either group. If the large release of cholesterol from the injected cells were due to immune destruction of the J774 cells by the strain of mice used in the present studies, this should occur in both normal and inflamed mice. If the release of cholesterol from the injected cells were the result of their destruction by the provoked tissue injury in the inflamed mice, then it would be independent of DIDS. Furthermore, one can recreate the rapid in vivo release of labeled cholesterol in inflamed mice by pretreating the cholesterol-laden J774 cells with acute-phase HDL (i.e., SAA-HDL) or liposomes containing SAA2.1 prior to their intravenous injection into normal mice. This effect is not observed when such J774 cells are pretreated with HDL or liposomes containing SAA1.1 or apo A-I [see Figs. 7 and 8 in (12)]. These data point to SAA2.1 as the effector of cholesterol release from such macrophages.

In our present work, we have extended the above in vivo studies by injecting radiolabeled cholesterol-laden J774 cells into normal mice and allowing 24 h for these cells to establish themselves and for the equilibration of their cholesterol pools. Protein-free liposomes or those containing SAA1.1, SAA2.1, or short peptides spanning the entire length of isoform 2.1 were then injected intravenously. Radiolabeled cholesterol release was provoked only by liposomes containing SAA2.1 (see Fig. 6) and SAA2.1 peptides 1–20 and 74–103 (see Fig. 7). The cholesterol release was apparent within 6 h of the administration of isoform 2.1 or its relevant peptides and peaked ~16 h after the injection of these specific liposomes (i.e., ~40 h after the commencement of the experiment). These data are consistent with our previously published results (12) and the argument for the role of SAA2.1 in macrophage cholesterol mobilization in vivo.

Our present cell culture experiments demonstrate that in the presence of cAMP and MCD, liposomes containing SAA2.1 (but not SAA1.1) are able, within 24 h, to stimulate the export of 55–70% of the cholesterol from cholesterol-laden macrophages, as measured both by the mass of free cholesterol and the efflux of radiolabeled cholesterol. This effect of SAA2.1 resides in two domains, the N-terminal domain, which inhibits ACAT, and the C-terminal domain, which enhances CEH (Fig. 4A, B). The effect of MCD is most apparent in the initial few hours of the incubations but does not influence the degree of total cholesterol efflux, a parameter affected predominantly by SAA2.1. This effect of MCD is consistent with facilitation of passive transport as described in the literature. Acting in concert (Fig. 5A, B and Tables 1 and 2), these two peptides, as liposomes, even in the absence of cAMP and MCD, also stimulate the export of 55–70% of the cholesterol from cholesterol-laden macrophages, as measured by both the mass of free cholesterol released to the media and the efflux of radiolabeled cholesterol.

Our previous culture experiments demonstrated that SAA2.1 exerts an inhibitory effect on macrophage ACAT activity, even in the presence of the ABCA1 inhibitor of cholesterol transport, DIDS (12). This effect of SAA2.1 and its active peptides is therefore not likely due to a secondary removal of cholesterol from such cells, which changes the substrate pool for these enzymes. As demonstrated in Fig. 4A, the domain of SAA2.1 responsible for the ACAT inhibition resides in an N-terminal 20 residue peptide, likely the first 16 residues, as shown previously (26). Furthermore, this conclusion is supported by the observation that residues 1–16 have a direct effect on macrophage ACAT activity in postnuclear preparations, in which removal of cholesterol from the incubation cannot take place (26). As reported previously (12), to the best of our knowledge, this is the first report of a natural ACAT inhibitor. As suggested previously (12), the failure of SAA1.1 (residues 1–20) to exert an influence on macrophage ACAT activity likely resides in two amino acids that differ from those in SAA2.1 (residues 1–20). There is an IG-for-VH substitution at positions 6 and 7 in isoform 1.1 with respect to 2.1. Studies are now in progress to determine the conformational differences of these two peptides and whether this may explain their differing effects on ACAT.

The SAA2.1 domain responsible for the enhancement of CEH activity resides in the carboxy-terminal 30 residues (residues 74–103), and the CEH affected is likely to be the nonlysosomal form (Fig. 2). Our previous work has shown that the enhancing effect of SAA2.1 on macrophage CEH activity is not affected by the presence of the ABCA1 inhibitor of cholesterol transport, DIDS (12). Furthermore, a mixture of SAA1.1 and SAA2.1 has a direct enhancing effect on postnuclear macrophage and liver CEH activity (49), and an 80 residue peptide derived from native

SAA2.1 by CNBr cleavage, which contains residues 74–103, has a direct enhancing effect on purified pancreatic neutral CEH (26). These data cannot be explained by the removal of cholesterol from such incubations. To the best of our knowledge, this is the first reported natural or synthetic CEH enhancer. Furthermore, as discussed above, these peptides are functional in whole cells and in vivo, as shown by their effect on cholesterol efflux in cell culture and in cholesterol-laden macrophages previously established in noninflamed mice. A single injection, in the form of a liposome, is effective in vivo for at least 96 h.

The above observations with SAA2.1 do not appear to be consistent with recent proposals that SAA may in some way be a negative factor in the pathogenesis of atherosclerosis and cardiovascular disease. This view has become of interest to researchers investigating the pathogenesis of cardiovascular disease for several reasons.

Because SAA is an acute-phase protein, it is not surprising that its plasma concentration increases shortly after a myocardial infarction (50, 51). However, elevated levels of SAA have also been shown to be a poor prognostic indicator in patients with unstable angina prior to objective evidence of myocardial damage (52, 53). Furthermore, epidemiological data have indicated that SAA levels increase with age and that the segment of the population with the highest levels of SAA has the greatest liklihood of cardiovascular disease (54, 55). Such data, as well as evidence that SAA interferes with the protective effect of HDL against LDL oxidation (56), may cause a reduction in HDL LCAT activity (57, 58), and may reduce the cholesterol carrying capacity of HDL(48, 58), leave the impression that SAA may in some way be a causative factor in the pathogenesis of atherosclerosis.

In this regard, it should be pointed out that SAA plasma concentrations that correlate with age and clinical outcomes are elevated to only a fraction of those seen following acute tissue injury (54, 55). Furthermore, the clinical manifestation of vascular disease due to cholesterol deposition is a very late phenomenon in the course of this lipid accumulation. When examined histologically, these vessels exhibit not only lipid deposition but also a concomitant, ongoing, inflammatory process (59). It is therefore not surprising that SAA is modestly elevated in these silent clinical periods during which atherogenesis progresses. The plasma level of SAA may be a reflection of the degree and severity of the ongoing, clinically silent, atherogenic process, for which reason it serves as a satisfactory prognostic indicator or marker but not necessarily a negative pathogenetic indicator. Moreover, the potential influence of SAA on HDL LCAT activity and on the cholesterol-carrying capacity of HDL may well be a positive influence, as discussed previously (48, 49).

Although the observations we have made to date relate to cholesterol-laden macrophages at sites of acute tissue injury and the consequent inflammation, this information applied to cholesterol accumulation in macrophages at sites of atherogenesis may have profound implications. We are now in the process of assessing the effects of the active peptides of SAA2.1 in mouse models of atherogenesis. The questions of both the inhibition and the regression of atherosclerosis are being addressed. The identified active domains are small enough that positive antiatherosclerosis results can be followed by molecular modeling and the preparation of novel ACAT inhibitors and CEH enhancers, combinations of which may be potent mobilizers of cholesterol from atherosclerotic plaques.

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REFERENCES

1. Ness, G. C., and C. M. Chambers. 2000. Feedback and hormonal regulation of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase: the concept of cholesterol buffering capacity. *Proc. Soc. Exp. Biol. Med.* **224:** 8–19.
2. Blumenthal, R. S. 2000. Statins: effective antiatherosclerotic therapy. *Am. Heart J.* **139:** 577–583.
3. Linton, M. F., and S. Fazio. 2001. Class A scavenger receptors, macrophages, and atherosclerosis. *Curr. Opin. Lipidol.* **12:** 489–495.
4. Krieger, M., and J. Herz. 1994. Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Annu. Rev. Biochem.* **63:** 601–637.
5. Chang, T. Y., C. C. Chang, S. Lin, C. Yu, B. L. Li, and A. Miyazaki. 2001. Roles of acyl-coenzyme A:cholesterol acyltransferase-1 and -2. *Curr. Opin. Lipidol.* **12:** 289–296.
6. Rudel, L. L., R. G. Lee, and T. L. Cockman. 2001. Acyl-coenzyme A:cholesterol acyltransferase types 1 and 2: structure and function in atherosclerosis. *Curr. Opin. Lipidol.* **12:** 121–127.
7. St. Clair, R. W., P. G. Yancey, and M. A. Leight. 1995. Macrophage cholesterol balance: a potential site of genetic control of susceptibility to atherosclerosis. *Ann. N. Y. Acad. Sci.* **748:** 264–275.
8. Oram, J. F. 2002. ATP-binding cassette transporter A1 and cholesterol trafficking. *Curr. Opin. Lipidol.* **13:** 373–381.
9. Schmitz, G., and W. Drobnik. 2002. ATP-binding cassette transporters in macrophages: promising drug targets for treatment of cardiovascular disease. *Curr. Opin. Investig. Drugs.* **3:** 853–858.
10. Macchia, T., R. Mancinelli, D. A. Barbini, F. Taggi, U. Avico, and A. Cantafora. 1991. Determination of membrane cholesterol in normal and pathological red blood cells. *Clin. Chim. Acta.* **199:** 59–67.
11. Cooper, R. A. 1978. Influence of increased membrane cholesterol on membrane fluidity and cell function in human red blood cells. *J. Supramol. Struct.* **8:** 413–430.
12. Tam, S. P., A. Flexman, J. Hulme, and R. Kisilevsky. 2002. Promoting export of macrophage cholesterol: the physiological role of a major acute-phase protein, serum amyloid A 2.1. *J. Lipid Res.* **43:** 1410–1420.
13. Jensen, L. E., M. P. Hiney, D. C. Shields, C. M. Uhlar, A. J. Lindsay, and A. S. Whitehead. 1997. Acute phase proteins in salmonids: evolutionary analyses and acute phase response. *J. Immunol.* **158:** 384–392.
14. Santiago, P., J. L. Roig-Lopez, C. Santiago, and J. E. Garcia-Arraras. 2000. Serum amyloid A protein in an echinoderm: its primary structure and expression during intestinal regeneration in the sea cucumber Holothuria glaberrima. *J. Exp. Zool.* **288:** 335–344.
15. Lowell, C. A., D. A. Potter, R. S. Stearman, and J. F. Morrow. 1986. Structure of the murine serum amyloid A gene family. Gene conversion. *J. Biol. Chem.* **261:** 8442–8452.
16. Rokita, H., T. Shirahama, A. S. Cohen, R. L. Meek, E. P. Benditt, and J. D. Sipe. 1987. Differential expression of the amyloid SAA gene in liver and peritoneal macrophages of mice undergoing dissimilar inflammatory episodes. *J. Immunol.* **139:** 3849–3853.

17. Benditt, E. P., and R. L. Meek. 1989. Expression of the third member of the serum amyloid A gene family in mouse adipocytes. *J. Exp. Med.* **169:** 1841–1846.
18. Sipe, J. D., and P. Westermark. 1999. Revised nomenclature for serum amyloid A (SAA). *Amyloid.* **6:** 67–70.
19. Whitehead, A. S., M. C. Debeer, D. M. Steel, M. Rits, J. M. Lelias, W. S. Lane, and F. C. deBeer. 1992. Identification of novel members of the serum amyloid-A protein superfamily as constitutive apolipoproteins of high density lipoprotein. *J. Biol. Chem.* **267:** 3862–3867.
20. Debeer, M. C., M. S. Kindy, W. S. Lane, and F. C. deBeer. 1994. Mouse serum amyloid A protein (SAA(5)) structure and expression. *J. Biol. Chem.* **269:** 4661–4667.
21. Debeer, M. C., T. Yuan, M. S. Kindy, B. F. Asztalos, P. S. Roheim, and F. C. deBeer. 1995. Characterization of constitutive human serum amyloid A protein (SAA(4)) as an apolipoprotein. *J. Lipid Res.* **36:** 526–534.
22. Kluve-Beckerman, B., M. L. Drumm, and M. D. Benson. 1991. Nonexpression of the human serum amyloid-A three (SAA3) gene. *DNA Cell Biol.* **10:** 651–661.
23. Meek, R. L., N. Eriksen, and E. P. Benditt. 1992. Murine serum amyloid-A(3) is a high density apolipoprotein and is secreted by macrophages. *Proc. Natl. Acad. Sci. USA.* **89:** 7949–7952.
24. Morrow, J. F., R. S. Stearman, C. G. Peltzman, and D. A. Potter. 1981. Induction of hepatic synthesis of serum amyloid A protein and actin. *Proc. Natl. Acad. Sci. USA.* **78:** 4718–4722.
25. van der Westhuyzen, D. R., G. A. Coetzee, and F. C. de Beer. 1986. Serum amyloid A protein in plasma: characteristics of acute phase HDL. *In* Amyloidosis. J. Marrink and M. H. van Rijswijk, editors. Martinus Nijhoff, Dordrecht. 115–125.
26. Ely, S., R. Bonatesta, J. B. Ancsin, M. Kindy, and R. Kisilevsky. 2001. The in-vitro influence of serum amyloid A isoforms on enzymes that regulate the balance between esterified and un-esterified cholesterol. *Amyloid.* **8:** 169–181.
27. Allain, C. C., L. S. Poon, C. S. G. Chan, W. Richmond, and P. C. Fu. 1974. Enzymatic determination of total serum cholesterol. *Clin. Chem.* **20:** 470–475.
28. Ancsin, J. B., and R. Kisilevsky. 1999. The heparin/heparan sulfate-binding site on apo-serum amyloid A: implications for the therapeutic intervention of amyloidosis. *J. Biol. Chem.* **274:** 7172–7181.
29. Ancsin, J. B., and R. Kisilevsky. 1999. Laminin interactions with the apoproteins of acute-phase HDL: preliminary mapping of the laminin binding site on serum amyloid A. *Amyloid.* **6:** 37–47.
30. Jonas, A., E. Kezdy, and J. H. Wald. 1989. Defined apolipoprotein A-I conformations in reconstituted high density lipoprotein discs. *J. Biol. Chem.* **264:** 4818–4825.
31. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
32. Oram, J. F., A. J. Mendez, J. P. Slotte, and T. F. Johnson. 1991. High density lipoprotein apolipoproteins mediate removal of sterol from intracellular pools but not from plasma membranes of cholesterol-loaded fibroblasts. *Arterioscler. Thromb.* **11:** 403–414.
33. Mendez, A. J., G. M. Anantharamaiah, J. P. Segrest, and J. F. Oram. 1994. Synthetic amphipathic helical peptides that mimic apolipoprotein A-I in clearing cellular cholesterol. *J. Clin. Invest.* **94:** 1698–1705.
34. Myer, B. M., P. S. Tietz, T. E. Tarara, and N. F. LaRusso. 1995. Dynamic measurements of the acute and chronic effects of lysosomotropic agents on hepatocyte lysosomal pH using flow cytometry. *Hepatology.* **22:** 1519–1526.
35. Klempner, M. S., and B. Styrt. 1983. Alkalinizing thee intralysosomal pH inhibits degranulation of human neutrophils. *J. Clin. Invest.* **72:** 1793–1800.
36. Goldstein, J. L., S. E. Dana, J. R. Faust, A. L. Beaudet, and M. S. Brown. 1975. Role of lysosomal acid lipase in the metabolism of plasma low density lipoprotein. Observations in cultured fibroblasts from a patient with cholesteryl ester storage disease. *J. Biol. Chem.* **250:** 8487–8495.
37. Lin, G., and K. E. Bornfeldt. 2002. Cyclic AMP-specific phosphodiesterase 4 inhibitors promote ABCA1 expression and cholesterol efflux. *Biochem. Biophys. Res. Commun.* **290:** 663–669.
38. Oram, J. F., R. M. Lawn, M. R. Garvin, and D. P. Wade. 2000. ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages. *J. Biol. Chem.* **275:** 34508–34511.
39. Isersky, C., D. L. Page, P. Cuatrecasas, R. A. DeLillis, and G. G. Glenner. 1971. Murine amyloidosis: immunologic characterization of amyloid fibril protein. *J. Immunol.* **107:** 1690–1698.
40. Husby, G., G. Marhaug, B. Dowton, K. Sletten, and J. D. Sipe. 1994. Serum amyloid A (SAA): biochemistry, genetics and the pathogenesis of AA amyloidosis. *Amyloid.* **1:** 118–137.
41. Husebekk, A., B. Skogen, and G. Husby. 1987. Characterization of amyloid proteins AA and SAA as apolipoproteins of high density lipoproteins (HDL). Displacement of SAA from the HDL-SAA complex by apoA-I and apoA-II. *Scand. J. Immunol.* **25:** 375–382.
42. Hoffman, J. S., and E. P. Benditt. 1982. Secretion of serum amyloid protein and assembly of serum amyloid protein-rich high density lipoprotein in primary mouse hepatocyte culture. *J. Biol. Chem.* **257:** 10518–10522.
43. Meek, R. L., J. S. Hoffman, and E. P. Benditt. 1986. Amyloidogenesis. One serum amyloid A isotype is selectively removed from the circulation. *J. Exp. Med.* **163:** 499–510.
44. Husebekk, A., B. Skogen, G. Husby, and G. Marhaug. 1985. Transformation of amyloid precurser SAA to protein AA and incorporation in amyloid fibrils in vivo. *Scand. J. Immunol.* **21:** 283–287.
45. Tape, C., R. Tan, M. Nesheim, and R. Kisilevsky. 1988. Direct evidence for circulating apoSAA as the precursor of tissue AA amyloid deposits. *Scand. J. Immunol.* **28:** 317–324.
46. Kisilevsky, R., and S. P. Tam. 2002. Serum amyloid A, cholesterol metabolism, and cardiovascular disease. *Pediatr. Pathol. Mol. Med.* **21:** 291–305.
47. Kisilevsky, R. 1991. Serum amyloid-A (SAA), a protein without a function: some suggestions with reference to cholesterol metabolism. *Med. Hypotheses.* **35:** 337–341.
48. Kisilevsky, R., E. Lindhorst, J. B. Ancsin, D. Young, and W. Bagshaw. 1996. Acute phase serum amyloid A (SAA) and cholesterol transport during acute inflammation: a hypothesis. *Amyloid.* **3:** 252–260.
49. Lindhorst, E., D. Young, W. Bagshaw, M. Hyland, and R. Kisilevsky. 1997. Acute inflammation, acute phase serum amyloid A and cholesterol metabolism in the mouse. *Biochim. Biophys. Acta.* **1339:** 143–154.
50. Clifton, P. M., A. M. Mackinnon, and P. J. Barter. 1985. Effects of serum amyloid A protein (SAA) on composition, size, and density of high density lipoproteins in subjects with myocardial infarction. *J. Lipid Res.* **26:** 1389–1398.
51. Bausserman, L. L., A. Sadaniantz, A. L. Saritelli, V. L. Martin, A. M. Nugent, S. P. Sady, and P. N. Herbert. 1989. Time course of serum amyloid-A response in myocardial infarction. *Clin. Chim. Acta.* **184:** 297–306.
52. Liuzzo, G., L. M. Biasucci, J. R. Gallimore, R. L. Grillo, A. G. Rebuzzi, M. B. Pepys, and A. Maseri. 1994. Prognostic value of C-reactive protein and serum amyloid A protein in severe unstable angina. *N. Engl. J. Med.* **331:** 417–424.
53. Ridker, P. M., N. Rifai, M. A. Pfeffer, F. M. Sacks, L. A. Moye, S. Goldman, G. C. Flaker, and E. Braunwald. 1998. Inflammation, pravastatin, and the risk of coronary events after myocardial infarction in patients with average cholesterol levels. *Circulation.* **98:** 839–844.
54. Rosenthal, C. J., and E. C. Franklin. 1975. Variation with age and disease of amyloid A protein-related serum component. *J. Clin. Invest.* **55:** 746–753.
55. Jousilahti, P., V. Salomaa, V. Rasi, E. Vahtera, and T. Palosuo. 2001. The association of C-reactive protein, serum amyloid A and fibrinogen with prevalent coronary heart disease—baseline findings of the PAIS project. *Atherosclerosis.* **156:** 451–456.
56. Van Lenten, B. J., S. Y. Hama, F. C. de Beer, D. M. Stafforini, T. M. McIntyre, S. M. Prescott, B. N. La Du, A. M. Fogelman, and M. Navab. 1995. Anti-inflammatory HDL becomes pro-inflammatory during the acute phase response. Loss of protective effect of HDL against LDL oxidation in aortic wall cell cocultures. *J. Clin. Invest.* **96:** 2758–2767.
57. Steinmetz, A., G. Hocke, R. Saile, P. Puchois, and J. C. Fruchart. 1989. Influence of serum amyloid-A on cholesterol esterification in human plasma. *Biochim. Biophys. Acta.* **1006:** 173–178.
58. Kumon, Y., Y. Nakauchi, K. Kidawara, M. Fukushima, S. Kobayashi, Y. Ikeda, T. Suehiro, K. Hashimoto, and J. D. Sipe. 1998. A longitudinal analysis of alteration in lecithin-cholesterol acyltransferase and paraoxonase activities following laparoscopic cholecystectomy relative to other parameters of HDL function and the acute phase response. *Scand. J. Immunol.* **48:** 419–424.
59. Gotlieb, A. I., E. P. Benditt, and S. M. Schwartz. 1999. Blood vessels. *In* Pathology. E. Rubin and J. L. Farber, editors. Lippincott-Raven, Philadelphia, New York. 481–530.