Promoting export of macrophage cholesterol: the physiological role of a major acute-phase protein, serum amyloid A 2.1

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Abstract **We show that murine macrophages that have ingested cell membranes as a source of cholesterol exhibit a marked increase in acyl-CoA:cholesterol acyl tranferase (ACAT) activity. Exposure of these macrophages to acute-phase high-density lipoprotein (HDL) results in a marked reduction of ACAT and enhancement of cholesteryl ester hydrolase (CEH) activities, phenomena not seen with native HDL. These complementary but opposite effects of acute-phase HDL on the two enzyme systems that regulate the balance between esterified (storage) cholesterol and unesterified (transportable) cholesterol are shown to reside with serum amyloid A (SAA) 2.1, an acute-phase apolipoprotein of HDL whose plasma concentration increases 500- to 1,000-fold within 24 h of acute tissue injury. Mild trypsin treatment of acute-phase HDL almost completely abolishes the apoliporotein-mediated effects on the cholesteryl ester cycle in cholesterol-laden macrophages. The physiological effect of SAA2.1 on macrophage cholesterol is to shift it into a transportable state enhancing its rate of export, which we confirm in tissue culture and in vivo. The export process is shown to be coupled to the ATP binding cassette transport system. Our findings integrate previous isolated observations about SAA into the sphere of cholesterol transport, establish a function for a major acute-phase protein, and offer a novel approach to mobilizing macrophage cholesterol at sites of atherogenesis.**—Shui Pang Tam, Alana Flexman, Jennifer Hulme, and Robert Kisilevsky. **Promoting export of macrophage cholesterol: the physiological role of a major acute-phase protein, serum amyloid A 2.1.** ***J. Lipid Res.*** **2002.** 43: **1410–1420.**

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The accumulation of lipids, especially cholesterol, in several vascular cell types such as macrophages and smooth muscle cells, is a defining pathologic feature of atherosclerosis (1). Two central issues are related to this problem. First is the mechanism by which cholesterol is synthesized/delivered and taken up by these cells, and second is the process by which these cells export cholesterol. A major aim in the treatment and prevention of atherosclerosis is to regulate the balance between cellular cholesterol uptake/synthesis and export to limit the intracellular cholesterol load. Such loads adversely influence the affected cells and eventually alter the structural integrity of the blood vessels.

An analogous, and perhaps instructive, set of events concerning cellular cholesterol uptake and export occurs at sites of acute tissue injury, one of the most common of pathologic processes. Such injuries result in local cell death and generation of large quantities of plasma membrane fragments rich in cholesterol (2). As part of the reactive acute inflammatory process macrophages arriving at such sites of injury ingest these fragments and thereby acquire a considerable cholesterol load, becoming foam cells (2). A cholesterol removal mechanism is therefore required to mobilize this cholesterol either for excretion or re-use. Our results suggest that the physiological role of one of the major acute-phase proteins synthesized by the liver in response to tissue injury, serum amyloid A (SAA) 2.1, is directed at the issue of macrophage cholesterol export.

SAA is a family of proteins encoded by four related genes whose evolutionary history is at least 600×10^6 years (3, 4). Of the four, isoforms 1.1 and 2.1 are major acute-phase proteins synthesized by the liver (5), and their plasma concentration may increase two to three orders of magnitude following tissue injury, regardless of cause. Within 15–20 h of an inflammatory stimulus, 2.5% of total liver protein synthesis can be devoted to the production of

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Abbreviations: AP-HDL, acute-phase high density lipoprotein; apoA-I, apolipoprotein A-I; CEH, cholesterol ester hydrolase; LPDS, lipoprotein depleted serum; RBC, red blood cell; T-AP-HDL, trypsin-modified acute-phase high density lipoprotein.

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these proteins (6), resulting in a dramatic increase in SAA's plasma concentration [0.001–1 mg/ml (a 500- to 1,000-fold increase)]. In plasma, 90% of SAA is found in the HDL fraction (7). In tissue culture, the acute-phase forms of SAA associate with HDL displace apoA-I and significantly remodel HDL's apolipoprotein composition (8). During tissue injury, 30–80% of the apolipoprotein composition of acute-phase HDL (AP-HDL) is made up of the acute-phase isoforms of SAA in roughly equal proportion (9). In the mouse, the acute-phase isoforms of SAA are 103 residues long and are remarkably similar in sequence, being 91% homologous (5) and differing from each other by only nine amino acids. Most past functional studies have examined mixtures of these two isoforms, and there are relatively few studies (10) comparing the functional properties of these isoforms individually.

Our working hypothesis regarding the physiological function of the acute-phase isoforms of SAA is that one or the other, and perhaps both, are involved in mediating removal of cholesterol from macrophages at sites of tissue injury (11, 12). To test this hypothesis and to mimic plasma membrane ingestion at sites of tissue injury, macrophages in tissue culture were exposed to red blood cell (RBC) membrane fragments as a source of cholesterol and their baseline acylCoA:cholesterol acyl transferase (ACAT), cholesterol ester hydrolase (CEH), and cholesterol export activities determined. Such cholesterol-laden macrophages were then examined for the effects of HDL, AP-HDL, trypsin-modified AP-HDL (T-AP-HDL), and liposomes containing apoA-I, SAA1.1, or SAA2.1 on macrophage ACAT, CEH, and cholesterol export activities. The in vivo release of cholesterol from such cholesterol-laden macrophages was then compared in the setting of acutely inflamed versus noninflamed mice.

MATERIALS AND METHODS

Animals

Swiss-white CD1 6- to 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, ON, Canada), Sigma (St. Louis, MO), ICN (Aurora, OH), or BioRad (Hercules, CA). DMEM and FBS were purchased from Life Technologies (Burlington, ON). 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).

Preparation of RBC membranes as a source of cholesterol

To mimic the ingestion of cell membrane fragments by macrophages at sites of tissue injury, RBC membrane fragments were prepared and used as a source of cholesterol as described previously (13). 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 (14), with the aid of a Sigma cholesterol 20 reagent kit.

Preparation of HDL, AP-HDL, and purification of apoA-I and SAA isoforms

HDL and AP-HDL were isolated from normal and inflamed mice respectively using sequential density flotation as described previously (15, 16). 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 collected into 0.5% EDTA (final concentration). The plasma was then separated from the red blood cells by centrifugation. The induction of inflammation and SAA synthesis and the isolation of apoA-I, SAA1.1, and SAA2.1 from acute-phase murine plasma was performed as described previously (15). Separation and purification of these proteins was accomplished by reverse phase high-pressure liquid chromatography as described previously (16). The purity of the isolated proteins was established by mass spectrometry and N-terminal sequence analysis as published previously (15, 16).

Trypsin modification of acute-phase HDL

Trypsin modification of AP-HDL was carried out as described by Oram and coworkers (17, 18). Briefly, AP-HDL (5 mg) was incubated with trypsin (Life Technologies, Burlington, ON) at a protein ratio of 40:1, respectively. The mixture was incubated at 37°C for 5 min. After incubation, the reaction tube was chilled on ice, and trypsin activity was inhibited by addition of phenylmethylsulfonylfluoride (1 mM). AP-HDL was separated from trypsin and degraded peptides by chromatography on a 1.5 × 50 cm Sepharose CL-4B column. When necessary, the T-AP-HDL was concentrated using Aquacide II (Calbiochem, La Jolla, CA). Apolipoprotein degradation by trypsin was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7–20% gradient gels, and proteins were stained with Commassie Blue. Lipoprotein size of native and trypsin-modified particles was determined by electrophoresis on 4–30% nondenaturing polyacrylamide gels (19).

Preparation and characterization of apolipoprotein-lipid complexes

Reconstitution of apoA-I-, SAA1.1-, and SAA2.1-containing liposomes was made by the cholate dialysis procedure as described by Jonas and coworkers (20), using 1-palmitoyl-2-oleoylphosphatidylcholine-cholesterol-apolipoprotein-sodium cholate in the molar ratio 100:25:1:250. Cholesterol was included to stabilize the liposome and give them a composition more similar to that of HDL. All preparations were done 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 12–16 h at 4°C. At the end of the equilibration period, the sample was dialyzed extensively in phosphate buffered saline at 4°C. After removing any unreacted or precipitated lipid by centrifugation at 15,000 *g* for 1 h at 15°C, 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 were obtained from protein determinations using the method of Lowry and coworkers (21), phospholipid determinations using a colorimetric kit (Wako Chemicals, Richmond, VA), and enzymatic analyses of free cholesterol (Sigma cholesterol reagent kit). The sizes of various liposomes were determined by electrophoresis on 4–30% nondenaturing polyacrylamide gels using reference protein standards.

Cell culture

J774 macrophages (American Type Tissue Collection, Manassas, VA) were maintained at 1 million cells per well and grown in 2 ml of DMEM supplemented with 10% FBS to 90% confluence. The medium was changed three times per week. Mouse peritoneal macrophages were isolated by injecting approximately 8 ml of cold (4°C) RPMI 1640 cell culture medium into the peritoneal cavity, massaging the abdomen, and then withdrawing the medium as described previously (22).

Cholesterol loading and determination of cell cholesterol esterification

To load the cells with cholesterol, nearly confluent mono-layers were washed three times with phosphate-buffered saline 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 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 cells without cholesterol loading and in cholesterol-laden cells that had been cultured in medium supplemented with native HDL, AP-HDL, or liposomes containing equimolar amounts of apoA-I, SAA1.1, or 2.1. Following 3 h incubation with the above media, the cells were incubated for another 3 h period after addition of [^{14}C]oleate (17, 23). Cells were chilled on ice and washed twice with PBS-BSA and twice with PBS. After addition of [^{3}H]cholesteryl oleate (6000 dpm/well) as an internal standard, the lipids were extracted from the labeled cells and analyzed by thin-layer chromatography as described previously (17, 23). 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

Newly 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 of DMEM containing 5% LPDS and 50 μg/ml of native HDL, AP-HDL, or liposomes containing 2 μmol of apoA-I, SAA1.1, or 2.1. To determine the rate of cholesteryl ester hydrolysis, 2 μg/ml of Sandoz 58-035 (an ACAT inhibitor) was added during lipoproteins or liposomes incubation to prevent re-esterification of liberated [^{14}C]oleate and free cholesterol. 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 loaded with cholesterol and simultaneously incubated for 3 h with 0.5 μCi/ml [^{3}H]cholesterol followed by an overnight equilibration period. Cells were washed four times with PBS/BSA prior to the efflux studies. Cells were then incubated at 37°C with DMEM/BSA and the indicated additions plus 2 μg/ml of Sandoz 58-035. At the indicated time points, the efflux media were collected and centrifuged to remove cell debris then used to measure the exported counts. The cell layers were then washed twice with ice-cold PBS/BSA and twice with PBS. Portions 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 (17, 23). The radioactivity in appropriate spots was measured to determine total cellular cholesterol counts. Efflux of radioactive label to the medium was calculated as the percentage of total counts (cell + medium counts) in each well. To examine whether cholesterol export from J774 cells to medium containing either HDL or AP-HDL involves the ABCA1 transporter process, the radio-labeled cholesterol-laden cells were incubated overnight with 400 μM (final concentration) of 4,4-disothiocyanostilbene-2,2′-disulfonic acid (DIDS), an inhibitor of the anion transport activities of ABCA1 (24), prior to the addition of either HDL or AP-HDL (50 μg/ml) to the culture medium. Cholesterol efflux to the medium was then determined at the indicated time points as described above. Efflux of radioactive label to the medium was calculated as the percentage of total counts in each well.

To determine cholesterol export in vivo, J774 macrophages were cholesterol loaded with RBC membranes and [^{3}H]cholesterol as described above. Cells were washed four times with PBS/BSA and then detached from the culture dishes. Five millions cells in 200 μl DMEM were injected into control mice or inflamed mice through the tail vein. At various time points, approximately 25 μl of blood were 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 red blood cells 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 mediated by the ABCA1 transporter pathway, or due to the endogenous destruction of the injected cells, the radio-labeled cholesterol-laden cells were incubated overnight with 400 μM (final concentration) of DIDS, and washed free of DIDS prior to their injection into uninflamed and inflamed mice. Inflammation, in the form of a small sterile abscess, was induced in the back by the subcutaneous injection of 0.5 ml of a 2% solution of $AgNO_3$ as described previously (25).

Protein determinations

Protein concentration was determined using the method of Lowry and coworkers (21), 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

Effects of red blood cell cholesterol loading on macrophage ACAT activity and the subsequent effect of HDL, AP-HDL, T-AP-HDL, and various protein-containing liposomes on this activity

Table 1 shows that RBC cholesterol loading resulted in a significant increase in both unesterified (33%), and par-

TABLE 1. Effect of cholesterol loading on cellular lipid from J774 macrophages

	Control	Cholesterol Loaded
	cellular lipid (μg/mg cell protein)[a]	
Free cholesterol	19.76 ± 0.52	25.68 ± 1.32
Cholesteryl ester	0.68 ± 0.23	8.57 ± 1.25

[a] Results shown are mean ± SEM of three independent experiments of triplicate cultures. As described in Materials and Methods, J774 macrophages were grown in 6-well plates; cells were loaded with 175 μg/ml cholesterol as red blood cell (RBC) membrane fragments; and the cells were then washed three times with PBS and cellular lipid was extracted and assayed.

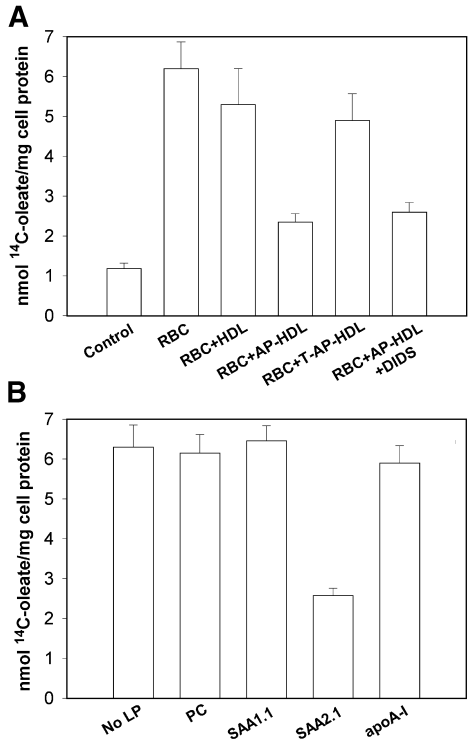

Fig. 1. Effects of red blood cell cholesterol loading on macrophage ACAT activity and the subsequent effect of HDL, acute-phase high density lipoprotein (AP-HDL), trypsin-modified acute-phase high density lipoprotein (T-AP-HDL), or various protein-containing liposomes on this activity. Nearly confluent monolayers of J774 cells were incubated in the absence (control) or presence of RBC membrane fragments (RBC) and labeled with [^{14}C]oleate. The ACAT activity was determined in cells without cholesterol loading and in cholesterol-laden cells that had been cultured in medium plus HDL, AP-HDL, T-AP-HDL, or AP-HDL + 400 μM DIDS as described in Materials and Methods (A), or with DMEM/BSA alone (no LP); medium plus protein-free liposomes (PC), or liposomes containing 2 μmol of apoA-I, SAA1.1 or 2.1 (B). In (A) differences in cellular ACAT activity between control cells and cells incubated with RBC are significant, with $P < 0.001$. No significant difference is found when comparing RBC versus either RBC + HDL or RBC + T-AP-HDL treatment. Differences between RBC and RBC + AP-HDL or RBC + AP-HDL + DIDS are significant, with $P < 0.005$. In (b) significant differences in cellular cholesteryl oleate radioactivity were observed when comparing any of no LP, PC, SAA1.1, or apoA-I to liposomes containing SAA2.1, with $P < 0.005$. Values are the mean ± SEM of four determinations.

ticularly, esterified cholesterol (12-fold) content in J774 macrophages. The incorporation of [^{14}C]oleate into cholesteryl esters in RBC cholesterol-loaded macrophages was five times higher than in control cells (**Fig. 1A**). Following 6 h incubations native HDL (50 μg/ml) had no significant effect on ACAT activity. In contrast, AP-HDL (50 μg/ml) decreased ACAT activity 2.5-fold (Fig. 1A). Similar results were seen when using mouse peritoneal macrophages (**Table 2**), the initial cells used when conducting these experiments. Since it proved tedious and time consuming to collect relatively few cells from the mice for each experiment, all subsequent experiments were done with the J774 cell line.

TABLE 2. Effect of RBC membrane fragments, and the subsequent effect of HDL or AP-HDL, on peritoneal macrophage ACAT activity

Treatment	Cholesterol Oleate	fmol per 2×10^6 Cells/h	Inhibition Relative to RBC	Inhibition Relative to HDL
	dpm		%	
None	463 ± 87	1,368 ± 256	—	—
RBC	5,132 ± 1435	15,143 ± 4242	—	—
HDL	3,502 ± 779	10,352 ± 2303	32	—
AP-HDL	1,082 ± 126	3,198 ± 361	79	69

Results are the mean ± SEM of three independent experiments of duplicate cultures. Mouse peritoneal macrophages were plated at 2×10^6 cells per well and allowed to adjust to culture conditions for 48 h. Media were then changed to contain 5% mouse lipoprotein-deficient serum with or without 175 μg/ml of cholesterol as RBC membrane fragments and incubated for 5 h. The media were replaced with media containing either, media alone, HDL, or acute-phase high-density lipoprotein (AP-HDL) all at 167 μg/ml for 3 h, following which [^{14}C]oleate, as an albumin complex, was added and incubated for 3 h. Total lipids were then extracted from each culture and separated by thin layer chromatography. The dpm in cholesterol oleate, as a measure of net cholesterol oleate formed, were determined and corrected for extraction efficiency.

When AP-HDL was treated with trypsin for 5 min and then incubated with cholesterol-loaded macrophages, this treatment almost completely abolished the ability of AP-HDL to inhibit ACAT activity (Fig. 1A). Trypsin-modified AP-HDL had no effect on its size distribution or electrophoretic mobility when compared with the parent particle as assessed by nondenaturing gradient gel electrophoresis (**Fig. 2A**). However, both apoA-I and SAA in AP-HDL were degraded by trypsin as determined by SDS-PAGE analysis (Fig. 2B). Furthermore, the inhibitory effect of AP-HDL on macrophage ACAT activity was not influenced by DIDS

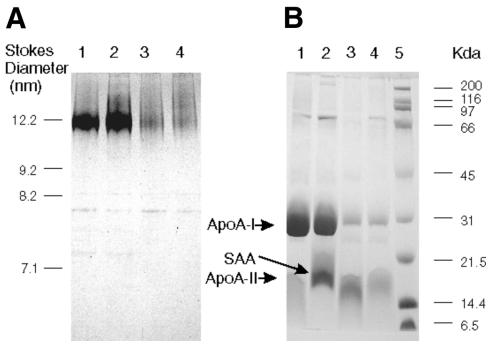

Fig. 2. Characterization of trypsin-modified AP-HDL. A: Native HDL (lane1), AP-HDL (lane2), and AP-HDL digested with trypsin for 5 min (lane 3) or 10 min (lane 4) were analyzed by nondenaturing gradient gel electrophoresis on 4–30% gradient gels. Trypsin-modified AP-HDL was prepared as described in the Materials and Methods section. Stokes diameters of native HDL, AP-HDL, and trypsin-modified AP-HDL were calculated from their mobility relative to protein standards supplied by Pharmacia. B: SDS-PAGE (7–20% gel) analysis of apolipoproteins derived from native HDL (lane 1), AP-HDL (lane 2), and AP-HDL digested with trypsin for 5 min (lane 3) or 10 min (lane 4). Molecular weights of various apolipoproteins were determined from their mobility relative to protein standards obtained from Bio-Rad. Twenty micrograms of protein were applied to each sample lane in both (A) and (B).

TABLE 3. Compositions and sizes of various apolipoprotein-containing liposomes

Liposomes	Composition	Size[a]
	PC-C-apolipoprotein (mol/mol)	*nm*
Apo A-I	82:18:1	10.8 ± 0.2
SAA1.1	72:16:1	11.2 ± 0.3
SAA2.1	75:17:1	10.5 ± 0.4

Results are mean ± SEM of four independent experiments.
[a] The diameter measurements were obtained from gradient gel electrophoresis on nondenaturing gels by reference to standard proteins.

(an inhibitor of ABCA1 transport pathway) treatment of cells (Fig. 1a). These results indicated that intact AP-HDL apolipoproteins and not lipids are responsible for its inhibiting effect of ACAT activity in cholesterol-laden macrophages, and that the effect of AP-HDL on ACAT is not influenced by an inhibition of cholesterol efflux.

To determine which of the major apolipoproteins of HDL, or AP-HDL, possessed the ACAT inhibitory properties, protein-free liposomes and liposomes containing apoA-I, SAA1.1, or SAA2.1 were examined for their effects on ACAT activity in cholesterol-loaded cells. The composition and sizes of various protein-containing liposomes did not differ much from one another and are shown in **Table 3**. Protein-free liposomes had no effect in the formation of [^{14}C]cholesteryl oleate when compared with cells cultured in the absence of liposomes (Fig. 1B). Similarly, liposomes containing SAA1.1 (2 μmol) did not affect the conversion of oleate into cholesteryl oleate. However, liposomes containing SAA2.1 (2 μmol) caused a 60% reduction in ACAT activity (Fig. 1B). Incubation of liposomes containing apoA-I (2 μmol) with cholesterol-laden J774 cells had no significant effect on cholesterol esterification.

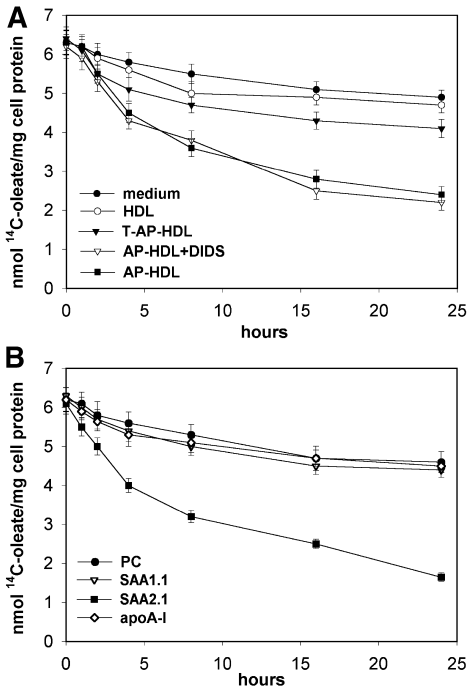

Fig. 3. Time course of cholesterol esterase activity in cholesterol-loaded macrophages exposed to HDL, AP-HDL, T-AP-HDL, or various liposomes. Nearly confluent J774 cells were cholesterol loaded with RBC membrane fragments and labeled with [^{14}C]oleate. The cells were then incubated for up to 24 h in the presence of 2 μg/ml of the ACAT inhibitor Sandoz 58-035 with 2 ml of DMEM containing 5% LPDS medium (open boxes), 50 μg/ml of HDL (open circles), 50 μg/ml AP-HDL (closed boxes), 50 μg/ml T- AP-HDL (closed down triangles), or 50 μg/ml AP-HDL + 400 μM DIDS (open down triangles) (A); or 2 ml of DMEM/LPDS supplemented with protein-free liposomes (closed circles), liposomes containing 2 μmol of either apoA-I (open diamond), SAA1.1 (open down triangles) or SAA2.1 (closed squares) (B). After the indicated intervals, cellular lipids were extracted and analyzed for cholesteryl ester radioactivity. Results are the mean ± SEM of four determinations.

Determination of cholesteryl ester hydrolase activity in J774 macrophages

Using macrophages preloaded with radio-labeled cholesteryl esters, we next examined the effect of native HDL, AP-HDL, and T-AP-HDL on CEH activity. This was done in the presence of Sandoz 58-035, an ACAT inhibitor, to prevent the re-esterification of liberated cholesterol and [^{14}C]oleate. Incubations proceeded for different times, 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 rates of hydrolysis of ^{14}C-labeled cholesteryl oleate in cells incubated without or with 50 μg/ml HDL (**Fig. 3A**). However, an equivalent amount of AP-HDL caused an approximately 3-fold increase in CEH activity. This enhancement of CEH activity by AP-HDL was not affected by DIDS (Fig. 3A), indicating that the effect was not dependent on cholesterol efflux. Mild trypsin treatment of AP-HDL reduced its ability to enhance CEH activity (Fig. 3A). The foregoing experiment was repeated with protein-free liposomes or with liposomes containing 2 μmol of apoA-I, SAA1.1, or SAA2.1 to determine which apolipoprotein was responsible for the increase in CEH activity. Among these various liposomes, only those containing SAA2.1 resulted in a 3-fold increase in the rate of cholesteryl ester hydrolysis (Fig. 3B).

Cholesterol export studies in tissue culture

The effects of AP-HDL and SAA2.1 liposomes on ACAT and CEH activities in cholesterol-loaded macrophages suggested that the balance between esterified and unesterified cholesterol in such treated cells would be shifted in favor of the latter, the transportable form of cholesterol. We therefore compared native HDL and AP-HDL for their abilities to mobilize cholesterol from RBC cholesterol-laden J774 cells that had been prelabeled with [^{3}H]cholesterol. [^{3}H]cholesterol efflux to medium containing 0.2% BSA was less than 6.0 ± 1.3% of total counts (**Fig. 4A**). Cells cultured in the presence of equivalent

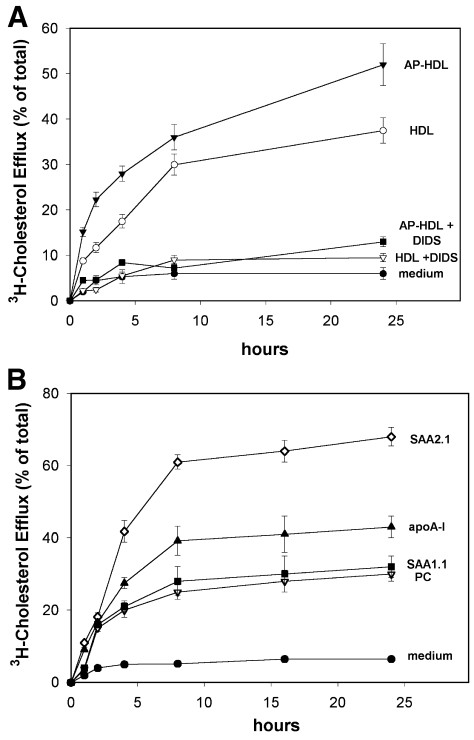

Fig. 4. Time course of cholesterol efflux from cholesterol-laden macrophages exposed to HDL, AP-HDL, or various liposomes. J774 cells were cholesterol loaded and simultaneously incubated for 3 h with DMEM/BSA containing 0.5 μCi/ml [^{3}H]cholesterol followed by an overnight equilibration period. Cells were then incubated in medium containing 2 μg/ml Sandoz 58-035 (closed circles) or the same medium plus 50 μg/ml of HDL (open circles), or AP-HDL (closed down triangles) (A); or the same medium plus liposomes containing no protein (open down triangles), liposomes containing equimolar 2 μmol of either apoA-I (closed up triangle), apoSAA1.1 (closed box), or apoSAA2.1 (open diamond) (B). To examine whether cholesterol export from J774 cells to medium containing either HDL or AP-HDL involves the ABC1 transporter process, the radio-labeled cholesterol-laden cells were incubated overnight with 400 μM DIDS (final concentration) prior to the addition of either 50 μg/ml of HDL (open down triangle), or AP-HDL (closed box) to the culture medium (A). After the indicated time intervals, the medium was collected and centrifuged at 10,000 *g* for 10 min, and radio-labeled cholesterol in the supernatant was determined. 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 (1.5–1.7) × 10^6 dpm/mg cell protein (A) and (1.8–2.2) × 10^6 dpm/mg cell protein (B). Values are the mean ± SEM of four determinations.

amounts of HDL or AP-HDL, exported 37.5 ± 2.8% and 52.0 ± 4.6% of total cellular [^{3}H]sterol to the medium, respectively (Fig. 4A). In addition, during the first 4 h of incubation with AP-HDL, the rate of cholesterol efflux was 2-fold faster than with HDL treatment. To determine whether export of cholesterol from J774 cells to the medium containing either HDL or AP-HDL involves the ATP binding cassette (ABCA1) transport pathway, we repeated the above experiment, but prior to the efflux studies, we incubated the cells overnight with 400 μM DIDS, an inhibitor of the transport activities of ABCA1 and blocker of acceptor-mediated cholesterol efflux in human fibroblasts (24). DIDS treatment almost completely inhibited the release of radio-labeled cholesterol to the culture medium containing either HDL or AP-HDL (Fig. 4A). However, this treatment did not alter the effects of AP-HDL on both ACAT and CEH activities (Fig. 1A and 3A, respectively). Liposomes, protein-free or containing either 2 μmol SAA1.1, SAA2.1, or apoA-I also promoted cholesterol efflux in cholesterol-loaded J774 cells (Fig. 4B). Protein-free liposomes and those containing SAA1.1 withdrew 30.0 ± 2.0% of the cellular cholesterol when compared with DMEM/BSA alone. Cells cultured for 24 h in the presence of apoA-I or SAA2.1 liposomes exported 43.0 ± 2.6% and 68.1 ± 3.4% of total cholesterol counts, respectively (Fig. 4B). On a molar basis, apoA-I liposomes were 1.5-fold more efficient than those containing SAA1.1. However, liposomes containing SAA2.1 were 1.7-fold more effective than apoA-I liposomes.

Cholesterol efflux from cholesterol-loaded J774 cells that had been pre-incubated with AP-HDL

We have demonstrated that AP-HDL is more effective than native HDL in mobilizing of free cholesterol from cholesterol-loaded macrophages (Fig. 4A). There exist at least three possibilities for this result. First, AP-HDL treatment causes both an inhibition of ACAT and a stimulation of CEH activity in J774 cells, thus providing more free cholesterol available for efflux. Second, AP-HDL might be a more effective acceptor of cholesterol. Third, the result may be a combination of both effects. To distinguish among these possibilities, we carried out the following experiments. Cholesterol-loaded macrophages labeled with [^{3}H]cholesterol were pre-incubated with either 50 μg/ml HDL or AP-HDL for 4 h. Following incubation, the cells were washed extensively with DMEM/BSA to remove all radioactivity in the pre-incubation medium. The chase efflux media consisted of DMEM/BSA alone or medium containing the equivalent amount of HDL or AP-HDL. At various time points, the efflux media were collected and analyzed for [^{3}H]cholesterol radioactivity and free cholesterol mass. The results shown in **Fig. 5** indicate that pretreatment with HDL did not cause any significant changes in the rate of [^{3}H]cholesterol efflux into the medium containing equivalent amount of HDL or AP-HDL when compared with the results obtained from the cells without preincubation with HDL, respectively (Fig. 4A). However, when cholesterol-laden J774 cells labeled with [^{3}H]cholesterol were pre-incubated with AP-HDL, it was observed that similar amounts of [^{3}H]cholesterol were released into the medium when the cells were subsequently cultured in the presence of either HDL or AP-HDL (Fig. 5). This suggests that pretreatment with AP-HDL, but not HDL, resulted in readily available free cholesterol for efflux independent of which acceptor (HDL or AP-HDL) was present.

Cholesterol export studies in vivo

To examine cholesterol export in vivo, five million [^{3}H]cholesterol-loaded J774 cells were injected intravenously into noninflamed and inflamed mice. Cholesterol

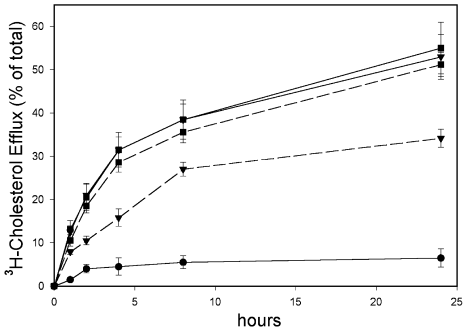

Fig. 5. Cholesterol export from cholesterol-loaded macrophages that had been pre-incubated with AP-HDL. J774 cells were cholesterol loaded and [^{3}H]cholesterol labeled as described for Figure 4. The cells were then pre-incubated with either 50 μg/ml HDL (dashed line) or AP-HDL (solid line) for 4 h. After this incubation period, the cells were washed extensively with DMEM/BSA to remove all radioactivity in the incubation medium. The chase efflux medium consisted of DMEM/BSA alone (closed circles) or the same medium plus 50 μg/ml HDL (closed down triangles) or AP-HDL (closed boxes). At the indicated time points, the efflux media were removed and analyzed for [^{3}H]cholesterol radioactivity and free cholesterol mass as described in Materials and Methods. Total [^{3}H]cholesterol mass was (1.0–1.3) × 10^6 dpm/mg cell protein. Results are the mean ± SEM of four determinations.

export was determined over a 96 h period by measuring the appearance of [^{3}H]cholesterol in plasma. The amount of radio-labeled cholesterol released to plasma was 3- to 3.5-fold greater in mice with an acute-phase response than in control animals (**Fig. 6**). The [^{3}H]cholesterol peaked between 24–48 h after injection of cholesterol-laden cells in inflamed mice. To determine whether export of cholesterol from J774 cells to plasma is mediated by the ABCA1 transport pathway or endogenous destruction of the injected cells, we repeated the above procedure, but prior to injection into mice, we incubated the cells overnight with DIDS. This treatment almost completely blocked the release of radio-labeled cholesterol to plasma in both uninflamed and inflamed mice (Fig. 6).

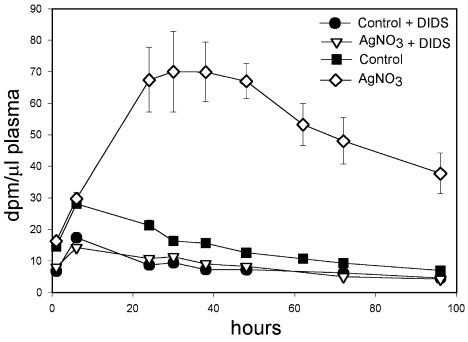

Fig. 6. Cholesterol export studies in vivo. J774 cells were cholesterol loaded and labeled with [^{3}H]cholesterol as described in Figure 4. Cells were then washed extensively and detached from the culture dishes. Five million cells in 200 μl DMEM were injected into control and inflamed mice through the tail vein. At the indicated time points, approximately 25 μl blood was collected from the tail vein of each animal. The blood samples were centrifuged to separate the RBC from plasma and the [^{3}H]cholesterol in plasma of control (closed box) and inflamed animals (open diamonds) determined by scintillation counting. To examine whether efflux of cholesterol from J774 cells to plasma is mediated by the ABCA1 transporter pathway, the radio-labeled cholesterol-laden cells were incubated overnight with 400 μM DIDS, an inhibitor of the anion transport activities of ABC1, prior to injecting into uninflamed (closed circles) and inflamed (open down triangles) animals. Results are the mean ± SEM of five animals and are representative of two independent experiments. Error bars not shown are within the symbol dimensions.

The following experiments were preformed to determine whether the increase in cholesterol export in vivo in inflamed mice is due to a general nonspecific aspect of the inflammatory response. Cholesterol-laden macrophages, labeled with [^{3}H]cholesterol, were pre-incubated with medium alone, HDL, or AP-HDL for 4 h, followed by extensive washing and detachment from the culture dishes. Five million cells from each treatment were then injected into the tail vein of uninflamed animals. As shown in **Fig. 7**, only the cells that have been pretreated with AP-HDL release approximately 3-fold more [^{3}H]cholesterol to plasma in control mice, a result not seen with HDL pretreatment. The radioactivity peaks at 24 h as observed in Fig. 6 where non-pre-treated cells were injected into animals with an acute inflammatory process. The foregoing experiment was repeated with protein-free liposomes or with liposomes containing 2 μmol of apoA-I, SAA1.1, or SAA2.1 to determine which apolipoprotein was responsible for the increase in cholesterol export from cholesterol-loaded macrophage to plasma of uninflamed mice. Among these various liposomes, only pretreatment of cells with liposomes containing SAA2.1 resulted in a 3-fold increase in cholesterol efflux to plasma of uninflamed animals (**Fig. 8**).

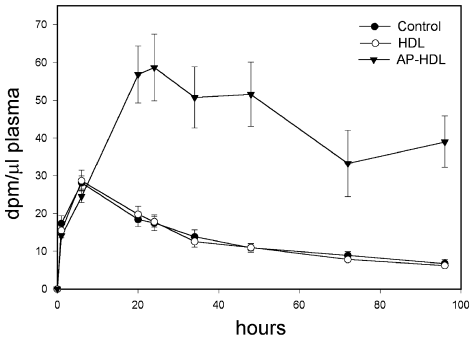

Fig. 7. Effects of pre-incubating cholesterol-laden J774 cells with either HDL or AP-HDL on cholesterol efflux in uninflamed mice. J774 cells were cholesterol loaded and labeled with [^{3}H]cholesterol as described in Figure 4. The cells were then incubated further with medium alone (control) or medium containing either HDL (50 μg/ml) or AP-HDL for 4 h. Cells were then washed extensively and detached from the culture dishes. Five million cells in 200 μl DMEM from each preparation were injected into uninflamed mice through the tail vein. At the indicated time intervals, export of labeled cholesterol from control J774 cells (closed circles), cells pre-incubated with either HDL (open circles) or AP-HDL (closed down triangles) to plasma were determined as described in Materials and Methods. Results are the mean ± SEM of five animals and are representative of two independent experiments. Error bars not shown are within the symbol dimensions.

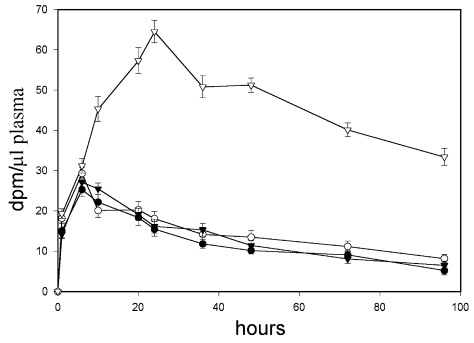

Fig. 8. Effects of pre-incubating cholesterol-laden J774 cells with protein-free liposomes or with liposomes containing either apoA-I, SAA1.1, or SAA2.1 on cholesterol efflux in uninflamed mice. J774 cells were cholesterol loaded and labeled with [^{3}H]cholesterol as described in Figure 3. The cells were then incubated further with medium supplemented with protein-free liposomes or liposomes containing either 2 μmol of apoA-I, SAA1.1, or SAA2.1 for 4 h. Cells were then washed extensively and detached from the culture dishes. Five million cells in 200 μl DMEM from each preparation were injected into uninflamed mice through the tail vein. At the indicated time intervals, export of labeled cholesterol from J774 cells pre-incubated with either protein-free liposomes (PC, closed circles), or liposomes containing apoA-I (open circles), SAA1.1 (closed down triangles), or SAA2.1 (open down triangles) to plasma were determined as described in Materials and Methods. Results are the mean ± SEM of five animals and are representative of two independent experiments. Error bars not shown are within the symbol dimensions.

DISCUSSION

SAA was first identified as a circulating plasma protein using antibodies directed against the inflammation-associated amyloid peptide (AA amyloid) (26). It was quickly demonstrated that SAA had the characteristics of an apolipoprotein (7), was associated primarily with HDL and its plasma concentration increased dramatically (500- to 1,000-fold) within 24 h of an inflammatory stimulus. Furthermore, the evolutionary conservation of its amino acid sequence over a period of 600×10^6 years suggested that it had some unidentified important function related to events that follow tissue injury. A prominent feature of the acute inflammatory reaction, a process that follows virtually all forms of acute tissue injury, is the infiltration of the affected tissue site by macrophages that ingest the tissue debris (2). Macrophages thereby acquire a significant cholesterol load from the cell membranes so ingested, becoming foam cells (2), and therefore require a mechanism to export this cholesterol load. Furthermore, HDL/SAA is known to be internalized by macrophages where it enters the endosomal/lysosomal compartment, as shown both by tissue culture and in vivo data (27–30). Based on the foregoing findings, and HDL's role as a "reverse cholesterol transporter" (31, 32), we postulated that SAA played a major role in modulating HDL's function and in mobilizing macrophage cholesterol during acute inflammation. Previous tissue culture studies exploring the effect of AP-HDL on macrophage cholesterol export observed either no effect or an inhibition of macrophage cholesterol efflux (33–36) and concluded that AP-HDL is impaired in its ability to promote cholesterol efflux and may actually cause cholesterol loading of macrophages. All these past studies put trace quantities of radio-labeled cholesterol into macrophages, and, unfortunately, failed to use macrophages frankly laden with cholesterol, the physiologic setting in which AP-HDL and SAA are found and in which their effects should have been assessed. A difference in physiological behavior of cells laden with cholesterol (present experiments) and those relatively empty of cholesterol (published experiments) vis a vis their response to HDL and AP-HDL would not be surprising. A more recent report using cholesterol-laden macrophages demonstrated an AP-HDL enhancement of cholesterol export (37), but SAA's role was not considered.

Using J774 mouse macrophages laden with RBC membrane fragments as a source of radio-labeled cholesterol, we have demonstrated that in mice with an acute inflammatory process such cells release substantially more radio-labeled cholesterol to plasma than in noninflamed animals. This effect can be abrogated by the ABCA1 transport pathway inhibitor, DIDS, which indicates that this enhanced release during inflammation, *i*) is not a function of macrophage cell destruction that may occur during inflammation, *ii*) is an aspect of the inflammatory process that primes these macrophages to release cholesterol, and *iii*) is in some manner coupled to the ABCA1 cholesterol transport process. The reasons for reaching these conclusions are as follows. If immune or inflammatory destruction of J774 cells were responsible for the release of the radio-labeled cholesterol then this should also have taken place with J774 cells treated with HDL and the non-SAA2.1-prepared liposomes. This was not seen. Therefore this possibility would have to be postulated as occurring only with cells treated with SAA2.1-containing particles (i.e., AP-HDL and SAA2.1 liposomes). If this were the case, then this should also have occurred with the cells that were treated with AP-HDL and followed by DIDS. This was precisely the reason we used DIDS in the in vivo experiments (i.e., an agent that blocks the ATP'ase action of the ABCA1 transporter should not block immune/inflammation destruction of macrophages but should block release of cholesterol from viable cells). DIDS inhibited the AP-HDL promotion of cholesterol release consistent with its role as an inhibitor of the ABCA1 transporter, and SAA2.1's action as described in this manuscript. If one were still willing to entertain the possibility of immune or inflammatory destruction of J774 cells, one would have to postulate that DIDS, an ATPase inhibitor, is now also an inhibitor of the immune/inflammation destruction specifically of SAA2.1-treated cells. We feel such varied and diverse postulations, i.e., that SAA2.1 promotes destruction of macrophage and DIDS specifically blocks such destruction of macrophages, is much less consistent with our data than the established role of DIDS vis a vis the ABCA1 transporter and SAA2.1's direct action on ACAT and nCEH demonstrated previously (13). Furthermore, such cells treated with AP-HDL and then injected into nonin-

flamed mice also release substantially more radio-labeled cholesterol to plasma in vivo, an effect not observed with HDL (Fig. 7). Such findings indicate that an AP-HDL component (e.g., SAA) is responsible for the enhanced cholesterol export, one not found on native HDL. This hypothesis is confirmed by pre-incubating cholesterol-laden macrophages, labeled with [^{3}H]cholesterol, with protein-free liposomes or liposomes containing apoA-I, SAA1.1, or SAA2.1 prior to injection to noninflamed mice. Only pretreatment with liposomes containing SAA2.1 resulted in a 3-fold increase in cholesterol export to plasma (Fig. 8). Moreover, as shown previously (38) and in the present studies, the peak of cholesterol release from a site of inflammation and the injected J774 cells occurs within 24–48 h. This correlates with the time course of, and peak of, plasma SAA concentration during acute inflammation. Such cholesterol (as free cholesterol) is found predominantly in the HDL fraction (38).

In tissue culture using the J774 mouse macrophage cell line and mouse peritoneal macrophages (the latter only for the initial ACAT experiments), we have demonstrated that such cells ingesting plasma membrane fragments experience a major increase in ACAT activity and a resultant 12-fold increase in cellular cholesterol esters (Table 1). This enhancement of ACAT activity (to be expected with the cell's acquisition of a large cholesterol load) can be significantly inhibited by the subsequent exposure of the cells to AP-HDL or liposomes containing the 2.1 isoform of SAA, but not HDL, nor liposomes carrying apoA-I or SAA1.1. Although we have not examined the uptake of [^{14}C]oleate under the various whole-cell experimental conditions, it is not likely that the differences seen with AP-HDL and SAA2.1 liposomes (see below) are due to alterations in [^{14}C]oleate transport. The identical results with AP-HDL and SAA2.1 liposomes are seen when using postnuclear homogenates, a situation in which transport of [^{14}C]oleate does not play a role (13). Why apoA-I, or native HDL, has previously been shown to affect cholesterol esterification, but is not seen in the present studies, is not fully understood. One reason may be that the results from these previous studies were performed mainly with cholesterol-loaded fibroblasts (17, 39) rather than macrophage. Mahlberg and coworkers (40) have used J774 and mouse peritoneal macrophages loaded with cholesteryl ester through the phagocytosis of sonicated lipid droplets. Such cells when exposed to liposomes containing apoA-I, apoA-II, or apoC experience little hydrolysis of cholesteryl ester or esterification of membrane or lysosomal cholesterol. Our results are in good agreement with this finding. Here we also show that mild trypsin treatment of AP-HDL almost completely abolishes its ability to inhibit ACAT activity in cholesterol-loaded macrophages, indicating that intact AP-HDL apoliproteins and not lipids are responsible for this process. It has been reported previously by Mendez and Oram (18) that this mild treatment digested less than 20% of HDL apolipoproteins and did not alter the lipid composition, size distrubution, or electrophoretic mobility of the particles. Furthermore, the effect of AP-HDL on cholesterol-laden macrophage ACAT and CEH activity is not influenced by DIDS, indicating that such AP-HDLs effects are not mediated by cholesterol efflux.

The ACAT inhibitory effect of SAA2.1 (particularly its N-terminal 16 residues) can also be demonstrated with macrophage postnuclear cell homogenates (13), suggesting that this isoform does not operate through a cell signaling pathway, but has a direct effect on ACAT. Of further interest is a comparison of the 16 residue N-terminal sequence of isoforms 1.1 and 2.1, the inactive and active isoforms, respectively. They differ only at positions 6 and 7 where there is an IG substitution for VH, respectively (5). The lack of ACAT inhibitory properties of isoform 1.1 presumably resides in these amino acid substitutions.

The first 16 residues of murine SAA2.1 and SAA1.1 are as follows:

SAA2.1$_{(1–16)}$ GFFSFVHEAFQGAGDM
SAA1.1$_{(1–16)}$ GFFSF**IG**EAFQGAGDM

The differences in sequences relative to SAA2.1 are bold. Macrophage CEH activity is not significantly affected by the ingestion of RBC membrane fragments (data not shown). However, CEH activity in such membrane-laden cells is significantly stimulated by AP-HDL and liposomes containing SAA2.1, but not HDL, T-AP-HDL, protein-free liposomes, nor those containing SAA1.1 or apoA-I. Analogous CEH data have been obtained with macrophage post-uclear cell homogenates (13). Furthermore, the active domain vis a vis the enhancement of CEH activity resides in the carboxy-terminal 80 residues of SAA2.1 (13). The precise mechanism by which SAA2.1 enhances CEH activity is not completely clear but may depend on product removal of cholesterol ester cleavage (probably cholesterol) by SAA2.1. The complementary but opposite effects of AP-HDL on macrophage ACAT and CEH activity appears to reside in SAA2.1 domains found at opposite ends of this protein. Whether it is the intact protein or proteolytically processed frag-

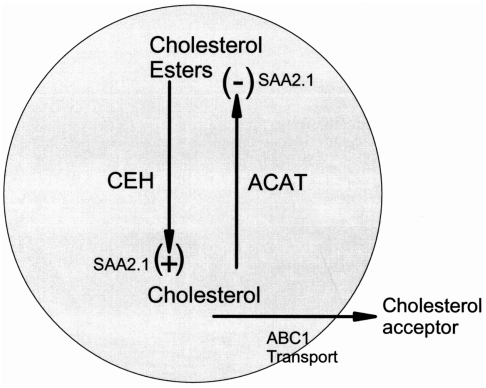

Fig. 9. Schematic summary of the role of SAA2.1 in mobilizing macrophage cholesterol for export. SAA2.1 inhibits (−) ACAT activity and enhances (+) cholesterol esterase activity, promoting a shift in the cellular cholesterol balance to unesterified (transportable) cholesterol. The latter may then be exported by the ABCA1 transport pathway to an extracellular cholesterol acceptor.

ments of SAA2.1 that are the active intracellular components remains to be determined. The net effect of SAA2.1's action on cholesterol-loaded macrophages is to drive cholesterol into its transportable form, and in this manner prime the cell for cholesterol export in the presence of a functional cholesterol transporter and an extracellular cholesterol acceptor (**Fig. 9**).

SAA2.1 may also provide interesting therapeutic possibilities. To the best of our knowledge, the ACAT inhibitory properties of SAA2.1 is the first example of a naturally occurring ACAT inhibitor. The ACAT inhibitory domain at the amino terminus (approximately 16 residues) may serve as a model for the design and synthesis of novel ACAT inhibitors, a focus already receiving significant attention by pharmaceutical firms. In addition, to the best of our knowledge, the CEH-enhancing properties of SAA2.1 are the first example of a natural or synthetic CEH stimulator. The CEH-enhancing properties of SAA2.1 reside in an 80 residue carboxy-terminal peptide. More precise definition of this domain is in progress, and in conjunction with the ACAT inhibitory properties, may culminate in the development of an agent that is a potent promoter of cellular cholesterol export, a product that may have great utility as an anti-atherogenic compound.

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