Identification of a Sequence of Apolipoprotein A-I Associated with the Efflux of Intracellular Cholesterol to Human Serum and Apolipoprotein A-I Containing Particles†

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ABSTRACT: The effect of monoclonal antibodies against apolipoprotein A-I (apoA-I) on the efflux of intracellular and plasma membrane cholesterol from HepG2 cells to human serum, high-density lipoprotein (HDL), apoA-I, and apoA-I/phosphatidylcholine complex (apoA-I/PC) was studied. Fab fragments of two monoclonal antibodies, AI-3 (residues 140−147) and Al-4.2 (residues 149−150), inhibited the efflux of intracellular cholesterol to serum in a dose-dependent manner. In combination, these antibodies were twice as effective than when used alone. None of the antibodies tested inhibited efflux of the plasma membrane cholesterol. When different types of acceptors were compared for their ability to promote intracellular cholesterol efflux, they were effective in the following order: serum > HDL > apoA-I/PC > pure apoA-I. Antibody AI-3 inhibited efflux of intracellular cholesterol to serum, HDL, and pure apoA-I, but not to apoA-I/PC. Antibody Al-4.2 inhibited efflux to serum, apoA-I/PC, and pure apoA-I, but not to HDL. An explanation for this is that antibody AI-4.2 reacts poorly with isolated α-HDL in an immunoprecipitation assay and has higher affinity for preβ_2-HDL and preβ_3-HDL particles than antibody AI-3 in nondenaturing two-dimensional electrophoresis. In conclusion, we have demonstrated that a region of apoA-I within or adjacent to residues 140−150 determines the ability of apoA-I to promote intracellular cholesterol efflux.

High-density lipoprotein (HDL)[1] is the primary acceptor of cellular cholesterol during cholesterol efflux, the first step of the reverse cholesterol transport pathway (Barter, 1993; Forte & McCall, 1994). While HDL lipid composition is important, the protein moiety of HDL plays the crucial role in promoting cholesterol efflux (Forte & McCall, 1994). The principal apolipoproteins of HDL are apolipoprotein A-I (apoA-I) and apolipoprotein A-II (apoA-II). Numerous epidemiological studies (Genest et al., 1993; Sharrett et al., 1994), as well as more recent studies using transgenic animal models (Breslow, 1993; Paszty et al., 1994; Warden et al., 1993), have demonstrated that a high concentration of apoA-I, but not apoA-II, is associated with the antiatherogenic role of HDL. It has also been suggested that HDL particles containing apoA-I without apoA-II could be equally effective (Johnson et al., 1991a; Oikawa et al., 1993) or better (Barkia et al., 1991) acceptors of cholesterol than HDL particles containing both apoA-I and apoA-II. These data point to the conclusion that apoA-I is the major determinant of the capacity of HDL particles to promote cholesterol efflux.

Two hypotheses have been proposed to explain the mechanism of apoA-I action. Oram and Bierman (Oram et al., 1991; Slotte et al., 1987) suggested that apoA-I specifically interacts with a putative HDL receptor and promotes cholesterol translocation from intracellular compartments to the plasma membrane where efflux occurs. Rothblat and Johnson (Johnson et al., 1991b; Rothblat et al., 1992) proposed a less specific process whereby the α-helical regions of apoA-I, especially the "hinge domain", are anchored in the plasma membrane, where they modify cholesterol packing and thereby promote the transfer of cholesterol from the plasma membrane to HDL. To find clues to mechanisms involved in HDL-mediated removal of cellular cholesterol, investigators have focused attention on the structural features of apoA-I that may influence cholesterol efflux. Several recent studies have exploited specific monoclonal antibodies to identify candidate regions of apoA-I involved in cholesterol efflux (Banka et al., 1994; Fielding et al., 1994; Luchoomun et al., 1994). These studies, however, have not attempted to distinguish the changes in cholesterol removal from intracellular versus plasma membrane pools and have employed different types of acceptor particles containing conformationally variable apoA-I. In the present work, we have studied the effect of monoclonal antibodies (MABs) against different epitopes of apoA-I, including a previously unstudied C-terminal epitope, on cholesterol efflux. We have compared the effect of these antibodies on the efflux of intracellular versus plasma membrane cholesterol in the presence of four different acceptors: human serum, isolated HDL, apoA-I/phosphatidylcholine complex (apoA-I/PC), and pure apoA-I. We used HepG2 cells since the pathway of cholesterol efflux from these cells has been characterized in detail in our laboratory

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[1] Abbreviations: apo, apolipoprotein; apoA-I/PC, apolipoprotein A-I/phosphatidylcholine complex; ER, endoplasmic reticulum; FC/PC, free (unesterified) cholesterol/phosphatidylcholine liposomes; FCS, fetal calf serum; HBSS, Hanks balanced salt solution; HDL, high-density lipoprotein; LPDS, lipoprotein-deficient serum; MAB, monoclonal antibody; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PM, plasma membrane; TLC, thin-layer chromatography.

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(Sviridov & Fidge, 1995b), and the procedures for selective labeling of intracellular or plasma membrane cholesterol have been established (Sviridov & Fidge, 1995a).

MATERIALS AMD METHODS

Cells. Human hepatoma cells HepG2 were grown in a CO_2 incubator (5% CO_2/95% air) in 75 cm^2 flasks or six-well cell culture clusters (Costar, Cambridge, MA) coated with collagen (Dixon et al., 1991). Cultures were maintained in Dulbecco's modified minimum essential medium containing 10% fetal calf serum (FCS), 20 mM HEPES, 1% nonessential amino acids, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 3.7 mg/mL sodium bicarbonate.

Human Serum, Lipoproteins, ApoA-I, and Liposomes. Human blood was collected from healthy volunteers, immediately cooled on ice, and allowed to stand for 2−3 h on ice. After clot retraction, the blood was centrifuged for 30 min at 2000*g* to remove blood cells and the fibrin clot. The serum was removed and stored frozen at −80 °C.

HDL (d = 1.063−1.21 g/cm^3) was isolated by sequential preparative ultracentrifugation (Havel et al., 1955) and purified by additional ultracentrifugation at the corresponding density. The protein composition of HDL was checked by electrophoresis on 12% SDS−polyacrylamide gels.

Recombinant human apoA-I was expressed as a fusion protein in an *Escherichia coli*/pGEX vector expression system (Hakes & Dixon, 1992) with modifications described elsewhere (N. Fidge et al., in preparation). The apoA-I fusion protein was purified by glutathione−agarose affinity chromatography, cleaved with thrombin, and repurified to remove the glutathione *S*-transferase carrier. The final product was checked by electrophoresis on 12% SDS−polyacrylamide gels and was confirmed to be highly purified pro-apoA-I (apoA-I_{-6-243}). To characterize the monoclonal antibodies, the following truncated forms of apoA-I were also generated: apoA-I_{-6-222}, apoA-I_{-6-210}, apoA-I_{-6-150}, apoA-I_{-6-135}, and apoA-$I_{219-243}$.

Phosphatidylcholine (PC) liposomes were prepared according to Arbogast et al. (1976) with modifications. In brief, egg phosphatidylcholine (Sigma, St. Louis, MO) was dried under N_2, reconstituted to a final concentration of 1 mg/mL in PBS, sonicated, and then centrifuged for 1 h at 436000*g* to remove multilamellar vesicles. To prepare [^{14}C]-cholesterol/phosphatidylcholine liposomes ([^{14}C]FC/PC), [4-^{14}C]cholesterol (DuPont NEN, North Ryde, NSW, Australia, specific activity = 2.0 GBq/mmol) was dried under N_2, PC liposomes were added to a final ratio of 1:1 (mol/mol), and the mixture was sonicated in 1 mL of PBS.

ApoA-I/phosphatidylcholine complex (1:2.5, w/w) was prepared according to Davidson et al. (1994).

Antibody. The preparation and characterization of the monoclonal antibodies have been described previously (Allan et al., 1991, 1993). Anti-apoA-I antibodies selected against the C-terminal cyanogen bromide fragment of apoA-I (AI-4.1 and AI-4.2) were further characterized for their ability to react with variably truncated forms of recombinant apoA-I (see above) by Western blotting (Towbin et al., 1979). For the production of Fab fragments, affinity-purified antibody was subjected to papain digestion (Harlow, 1988). Fab fragments were removed from undigested IgG and Fc fragments by passage through protein A−Sepharose (Pharmacia, North Ryde, NSW, Australia), and their purity was assessed by 12% SDS−polyacrylamide gel electrophoresis.

Cholesterol Efflux. To study the effect of various antibodies on cholesterol efflux, medium without FCS and with either human serum, HDL, apoA-I/PC, or apoA-I (final apoA-I concentration = 30 μg/mL) was incubated with varying concentrations of Fab fragments of monoclonal anti-human apoA-I antibodies for 4 h at 37 °C. The maximum concentration of Fab fragments corresponded to a 12-fold (w/w) excess over apoA-I. After incubation with antibodies, the medium was used in the efflux experiments.

For labeling with [^{14}C]cholesterol, cells were washed twice with Hanks balanced salt solution (HBSS) and incubated for 3 h in serum-free medium containing 2% (v/v) Ultraser SF (Serva, Heidelberg, Germany), [^{14}C]FC/PC (final activity = 32 KBq/mL), or [1-^{14}C]acetate (Amersham, North Ryde, NSW, Australia, specific activity = 2.15 GBq/mmol, final activity = 0.23 MBq/mL). In previous experiments (Sviridov & Fidge, 1995a), we established that this method predominantly labels either the PM or ER, enabling a comparison of cholesterol efflux from either pool. In brief, cells were then fractionated in a sucrose density gradient, and PM, ER, and lysosomes were identified by their marker enzymes: 5′-nucleotidase for PM, NADPH-cytochrome C reductase for ER, and acid phosphatase for lysosomes. When the amount of [^{14}C]cholesterol comigrating with the markers was quantitated, it was found that, after labeling of HepG2 cells for 3 h with [^{14}C]FC/PC, 90% of [^{14}C]cholesterol is located on the plasma membrane and 10% in lysosomes, while incubation for 3 h with [^{14}C]acetate resulted in 70% of cholesterol being associated with the endoplasmic reticulum without any other major peaks (Sviridov & Fidge, 1995a). The average rate of [^{14}C]acetate incorporation into cholesterol was 10^5 dpm/mg of cell protein/3 h.

After labeling, cells were washed six times with HBSS and incubated further for 3 h with the medium containing various acceptors pretreated with Fab fragments of antibodies, as described earlier. The medium was then collected and centrifuged for 15 min at 4 °C at 30000*g*, and the supernatant was used for further analysis. Cells were harvested using a cell scraper, dispensed in 0.5 mL of distilled water, and dissolved by overnight incubation in 0.5 M NaOH. No changes in cell total cholesterol content were detected as a result of labeling and efflux incubations.

Aliquots of medium were saponified, lipids were extracted, and cholesterol was isolated by TLC as described previously (Sviridov & Fidge, 1995a). In some experiments, cholesterol from the band was extracted into chloroform, dried, redissolved in isopropyl alcohol, and analyzed further by reverse phase HPLC on a C-18 column using the solvent system acetonitrile/isopropyl alcohol/water (44:54:2, v/v/v) at 1 mL/min and room temperature; radioactivity was measured using an on-line radiochromatographic detector. Seventy percent of newly synthesized sterols released into medium were represented by cholesterol compared to 87% in the commercial sample of [^{14}C]cholesterol; no other sterols were detected as a peak, and the remaining radioactivity was represented by background "noise".

Immunoprecipitation. To analyze the relative reactivity of MABs toward different apoA-I containing particles, a liquid immunoassay was employed using conditions identical to those used in the efflux experiments. ApoA-I, HDL, and total serum protein were labeled with ^{125}I using IodoGene (Pierce, Rockford, IL) according to the manufacturer's

instructions. Specific radioactivity was 500−700 dpm/ng of protein for [^{125}I]apoA-I and [^{125}I]HDL. The specific radioactivity of [^{125}I]apoA-I in the serum was determined following isolation by SDS−polyacrylamide gel electrophoresis and was 0.5 dpm/ng of protein. When calculating the amount of [^{125}I]apoA-I precipitated from whole serum, the contribution of other labeled proteins, coprecipitated together with [^{125}I]apoA-I in the HDL particles, was subtracted from the labeled protein content of immunoprecipitated HDL. An [^{125}I]apoA-I/PC complex was prepared from [^{125}I]apoA-I and PC as described earlier. Human serum was treated with protein G−Sepharose 4 Fast Flow (Pharmacia, 1 mL of drained gel per 1 mL of serum) prior to experiments to remove serum [^{125}I]IgG. The incubation mixture consisted of ^{125}I-labeled human serum, [^{125}I]HDL, [^{125}I]apoA-I/PC, or [^{125}I]apoA-I at a final apoA-I concentration of 30 μg/mL plus indicated concentrations of various MABs and PBS to a final volume of 100 μL. It was established in the preliminary experiments that the binding of MABs to antigen saturates within the first 2 h incubation at 37 °C. After incubation for 4 h at 37 °C, protein G−Sepharose was added at an amount equivalent to 30 μL of drained gel, and samples were incubated for an additional 18 h at 4 °C with rotation. Further preliminary experiments established that the amount of protein G−Sepharose and the incubation time were sufficient for maximum binding of MABs. After incubation, protein G−Sepharose was pelleted by centrifugation, and the pellet was washed twice with PBS and counted in a γ-counter. A nonimmune mouse IgG was used as a control for experiments with serum, and monoclonal anti-apoB antibody was the control for experiments with the other particles.

Nondenaturing Two-Dimensional Electrophoresis. To analyze the relative reactivity of MABs toward different HDL subfractions, human serum was separated by nondenaturing two-dimensional electrophoresis according to Castro and Fielding (1988), with modifications described previously (Sviridov & Fidge, 1995b). ApoA-I containing subfractions of lipoproteins were identified by Western blotting (Towbin et al., 1979) using different anti-apoA-I MABs as the first antibody and ^{125}I-labeled anti-mouse IgG (Amersham, specific activity = 500 kBq/μg of protein) as the second antibody. It was demonstrated previously that the transfer of apoA-I containing lipoproteins to nitrocellulose is quantitative (Sviridov & Fidge, 1995b). The distribution of label among lipoprotein subfractions was quantitated by using a BAS-1000 bioimager (Fuji, Japan). The experiment was reproduced twice with similar results.

Other Methods. The protein content of cells, serum, apoA-I, IgG, and Fab fragments was determined according to Bradford (1976). The apoA-I content of serum and HDL was determined by using a Cobas-Bio analyzer. Cell cholesterol content was determined by enzymatic fluorometric assay (Gamble et al., 1978).

Statistics. All experiments were performed in triplicate or quadruplicate (i.e., determinations from three or four dishes) and reproduced 2−3 times. The specific radioactivity of cellular cholesterol was determined on washed cells following labeling, and this value was used to calculate the amount of [^{14}C]cholesterol released into the medium. Background values, i.e., the amount of [^{14}C]cholesterol released from HepG2 cells into the medium in the absence of an acceptor, accounted for 22% of intracellular and 12% of PM [^{14}C]cholesterol released in the presence of 3% serum, and these values were subtracted. Mean ± standard error of mean is presented. The statistical significance of differences was determined by a Student's two-tailed *t*-test.

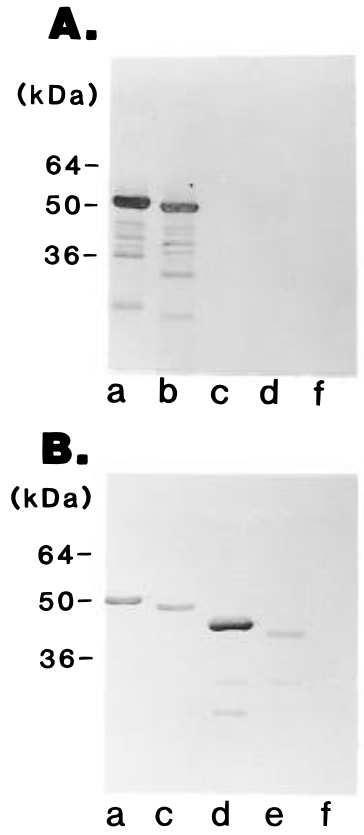

FIGURE 1: Western blot of different truncated versions of apoA-I detected with antibodies AI-4.1 (A) and AI-4.2 (B). Differently truncated forms of apoA-I fusion products were run on 0.1% SDS/12% polyacrylamide gels, transferred to nitrocellulose membranes, and incubated with monoclonal antibody AI-4.1 (A) or AI-4.2 (B) (first antibody) and then with rabbit anti-mouse IgG conjugated with horseradish peroxidase (second antibody). Lanes: a, apoA-I$_{-6-243}$; b, apo A-I$_{-6-222}$; c, apoA-I$_{-6-210}$; d, apoA-I$_{-6-150}$; e, apoA-I$_{-6-135}$; f, apoA-I$_{219-243}$.

RESULTS

Characterization of Monoclonal Antibodies. Antibodies AI-1 and AI-3 were characterized previously and were shown to interact with epitopes within residues 28−47 and 140−147, respectively (Allan et al., 1991). To characterize monoclonal antibodies AI−4.1 and AI−4.2, directed against the C-terminal end of apoA-I, differently truncated forms of apoA-I were expressed in an *E. coli*/pGEX vector expression system, and fusion protein was detected with MABs on a Western blot. Antibody AI-4.1 failed to react with apoA-I when truncation was extended from residue 222 to 210 (Figure 1A, lanes b and c). The reaction of antibody AI-4.2 decreased dramatically when apoA-I truncation was extended from residue 150 to 135 (Figure 1B, lanes d and e); neither antibody reacted with fusion peptide apoA-I$_{219-243}$ (Figure 1A,B, lane f). However, as was shown previously (Allan et al., 1993), the antibody Al-4.2, which was produced against the C-terminal CNBr fragment of apoA-I, CF4 (residues 149−243), did not react with CNBr fragment CF3, which ends with the residue 148. It was concluded, therefore, that MAB Al-4.2 interacts with the epitope apoA-I$_{149-150}$ (Arg-Asp) and Al-4.1 interacts with the epitope apoA-I$_{211-222}$. It should be noted, however, that there was a weak reactivity of MAB AI-4.2 with the N-terminal portion of

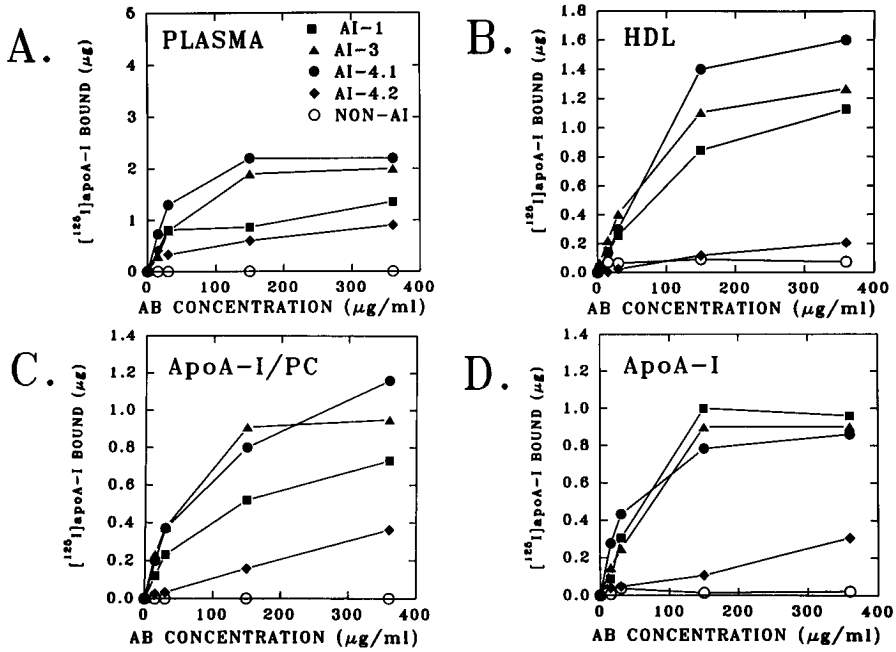

FIGURE 2: Immunoprecipitation of apoA-I containing particles from serum (A), HDL (B), apoA-I/PC complex (C), or apoA-I (D) with monoclonal antibodies. The incubation mixture consisted of ^{125}I-labeled human serum, [^{125}I]HDL, [^{125}I]apoA-I/PC, or [^{125}I]apoA-I at the final apoA-I concentration of 30 μg/mL, indicated concentrations of various MABs, and PBS to the final volume of 100 μL. After incubation for 4 h at 37 °C, protein G−Sepharose was added and samples were incubated for an additional 18 h at 4 °C with rotation. Protein G−Sepharose was then pelleted by centrifugation, washed twice with 0.4 mL of PBS, and counted. Each point represents the mean of duplicate determinations.

apoA-I (Figure 1, lane e), apparently toward another Arg-Asp sequence in apoA-I$_{27-28}$.

To investigate the ability of different MABs to bind apoA-I contained within different particles, antibodies were incubated with ^{125}I-labeled serum, HDL, apoA-I/PC, or apoA-I and then precipitated with protein G−Sepharose. Antibody AI-4.1 had the strongest reactivity with apoA-I in plasma, HDL, and apoA-I/PC, followed by AI-3 and AI-1 (Figure 2A−C). The binding of all three antibodies to free apoA-I was very similar (Figure 2D). Antibody AI-4.2 showed a weaker reaction with all apoA-I containing particles, especially with HDL where binding was only marginally different from the binding of the control, unrelated antibody (Figure 2A−D). The amount of apoA-I bound by the antibodies varied from 70% in the serum to 30% in apoA-I and apoA-I/PC (Figure 2A−D).

To study the ability of different MABs to interact with different HDL subfractions, human serum was separated by nondenaturing two-dimensional electrophoresis. After transfer to nitrocellulose, filters were incubated with different MABs, and the HDL subfractions were visualized and quantitated by incubation with ^{125}I-labeled anti-mouse IgG antibody. As shown in Figure 3, all MABs showed some reactivity with the α-HDL subfraction, as well as with all preβ-HDL subfractions. However, the avidity of the reaction of MABs with different subfractions varied as shown in Table 1. The reactivity of MABs AI-4.1 and AI-4.2 toward preβ_2-HDL and preβ_3-HDL relative to their reactivity toward α-HDL was 2−3 times higher than for both MABs AI-l and AI-3. No difference was found between antibodies in their relative reactivity toward preβ_1-HDL. The lower avidity of MAB AI-4.2 to α-HDL is consistent with its poor binding to isolated HDL, which consists of only α-HDL (Sviridov & Fidge, 1995b), in immunoprecipitation assay (Figure 2B).

Effect of MABs on Cholesterol Efflux Using Human Serum as Acceptor. To study the effect of different MABs on

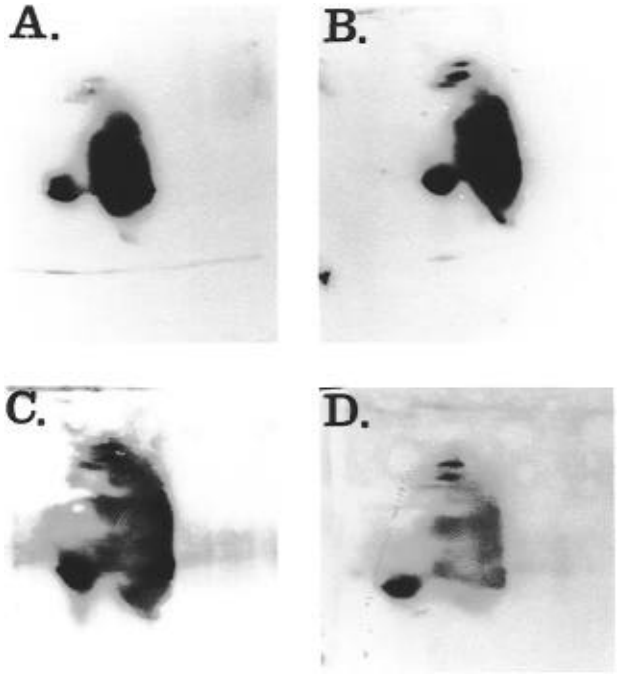

FIGURE 3: Wester blot of human serum separated by nondenaturing two-dimensional electrophoresis and detected with monoclonal antibodies AI-1 (A), AI-3 (B), AI-4.1 (C), and AI-4.2 (D).

cholesterol efflux in the presence of human serum, serum was treated with Fab fragments of MABs before addition to HepG2 cells. When plasma membrane cholesterol of HepG2 cells was labeled with [^{14}C]FC/PC, none of the MABs affected the ability of serum to promote cholesterol efflux (Figure 4A). However, when intracellular cholesterol of HepG2 cells was labeled with [^{14}C]acetate, treatment of serum with MABs AI-3 and AI-4.2 resulted in a dose-dependent and statistically significant ($p < 0.001$) inhibition of efflux by up to 40−45% (Figure 4B). MABs AI-1 and AI-4.1 had no effect on intracellular cholesterol efflux. In

Table 1: Relative Binding of MABs to HDL Subfractions[a]

	binding to HDL subfractions (PSL[b]/mm^2)			
MAB	αHDL	preβ_1-HDL	preβ_2-HDL	preβ_3-HDL
Al-1	66.3 (1)	79.2 (1.2)	17.0 (0.26)	9.9 (0.15)
Al-3	83.3 (1)	99.4 (1.2)	19.5 (0.23)	8.1 (0.10)
Al-4.1	45.4 (1)	52.8 (1.2)	20.2 (0.44)	8.7 (0.20)
Al-4.2	21.9 (1)	25.9 (1.2)	16.1 (0.74)	7.9 (0.36)

[a] Human serum was separated by two-dimensional nondenaturing electrophoresis as described in Materials and Methods. After transblotting, filters were incubated with different MABs and then with ^{125}I-labeled anti-mouse IgG. The amount of radioactivity in each subfraction was quantitated with the bioimager. The nomenclature of the preβ-HDL species is taken from Miida et al. (1990). The values presented are "specific binding", i.e., radioactivity of a region divided by the area of the region; values in parentheses are relatve to the "specific binding" to the α-HDL subfraction. [b] Photostimulated luminescence units per 2 h.

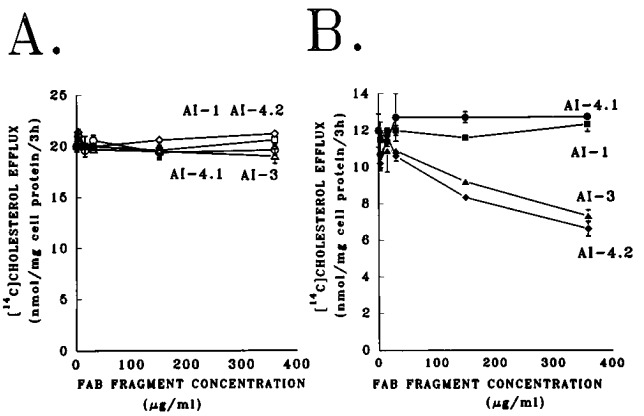

FIGURE 4: Effect of monoclonal antibodies on the efflux of either plasma membrane (A) or intracellular (B) cholesterol to human serum. Efflux medium consisting of serum-free medium containing 3% human serum (final apoA-I concentration = 30 μg/mL) was incubated for 4 h at 37 °C with the indicated concentrations of Fab fragments of various monoclonal antibodies. HepG2 cells were incubated for 3 h with [^{14}C]FC/PC (final activity = 32 kBq/mL) to label PM (A) or with [^{14}C]acetate (final activity = 0.23 MBq/mL) to label intracellular cholesterol (B) pools. Cells were then washed with HBSS and incubated for an additional 3 h with the efflux medium. After a second incubation medium was collected and centrifuged for 15 min at 30000*g*, cholesterol was isolated by TLC as described in Materials and Methods and counted. Values of mean ± standard error of the mean of triplicate determinations are presented.

comparison, polyclonal rabbit anti-human apoA-I antibody at the same concentration inhibited the efflux of intracellular cholesterol by 70% without affecting the efflux of plasma membrane cholesterol (Sviridov & Fidge, 1995a). To determine whether the effect of MABs AI-3 and AI-4.2 was additive, serum was pretreated with a mixture (1:1, w/w) of these antibodies and efflux was studied. The inhibition of intracellular cholesterol efflux by the mixture was twice that of each antibody separately (Figure 5).

Effect of MABs on Cholesterol Efflux to HDL, ApoA-I/PC Complex, and ApoA-I. The capacity of serum to promote intracellular cholesterol efflux was compared with that of HDL, apoA-I/PC complex, and apoA-I. Cholesterol in HepG2 cells was labeled with [^{14}C]acetate, and different cholesterol acceptors were added at the same final concentration of apoA-I. By setting efflux to serum at 100%, HDL and apoA-I/PC were, respectively, 82% and 71% as effective in promoting the efflux of intracellular cholesterol (Table 2). However, free apoA-I was less than 40% as effective as serum. Lipoprotein-deficient serum (LPDS) and PC liposomes accepted, respectively, 23% and 30% of the [^{14}C]-cholesterol released to the serum under the same conditions. It should be noted that when cells were incubated for the same period of time in medium free of added cholesterol acceptor, the amount of [^{14}C]cholesterol released to the medium was 21.6 ± 0.7% (n = 4) of the amount released in the presence of the serum (not shown). Most of this [^{14}C]-cholesterol was associated with newly synthesized lipoproteins, and their detailed analysis is reported elsewhere (Sviridov & Fidge, 1995b). The addition of Fab fragments of MABs to cells in the absence of cholesterol acceptors did not affect the rate of [^{14}C]acetate incorporation into cholesterol nor the amount of [^{14}C]cholesterol released with secreted lipoproteins (not shown).

To study the effect of MABs on HDL-mediated cholesterol efflux, intracellular cholesterol in HepG2 cells was labeled with [^{14}C]acetate, and cells were then incubated with isolated

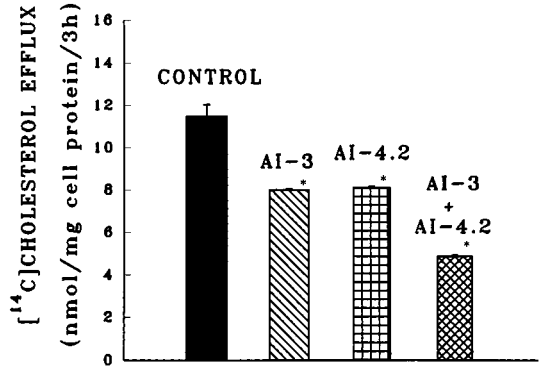

FIGURE 5: Effect of MABs AI-3 and AI-4.2 either individually or in combination on the efflux of intracellular cholesterol to human serum. Efflux medium consisting of serum-free medium containing 3% human serum (final apoA-I concentration = 30 μg/mL) was incubated for 4 h at 37 °C with the indicated concentrations of Fab fragments of antibodies AI-3 or AI-4.2 (final concentration = 360 μg/mL) or in a mixture (1:1, w/w). The intracellular cholesterol pool of HepG2 cells was labeled by incubation for 3 h with [^{14}C]-acetate (final activity = 0.23 MBq/mL), washed with HBSS, and incubated for an additional 3 h with the efflux medium. After a second incubation medium was collected and centrifuged for 15 min at 30000*g*, cholesterol was isolated by TLC as described in Materials and Methods and counted. Values of mean ± standard error of the mean of quadruplicate determinations are presented. * indicates $p < 0.01$.

Table 2: Efflux of Intracellular Cholesterol to Serum, HDL, ApoA-I/PC, ApoA-I, LPDS, and PC[a]

	[^{14}C] cholesterol efflux	
acceptor	nmol/mg of cell protein/3h	%
serum	12.5 ± 0.15	100
HDL	10.3 ± 0.26[b]	82.4
apoA-I/PC	8.9 ± 0.19[b,c]	71.2
apoA-I	4.7 ± 0.11[b,c,d]	37.6
LPDS	2.9 ± 0.26[b]	23.2
PC	3.7 ± 0.19[b,d]	29.6

[a] HepG2 cells were labeled by incubation for 3 h with [^{14}C]acetate (final activity = 0.23 MBq/mL). Cells were then washed with HBSS and incubated for an additional 3 h with the efflux medium consisting of serum-free medium containing 3% human serum, 45 μg/mL HDL, apoA-I/PC, or apoA-I (final apoA-I concentration in all four = 30 μg/mL), and 3% LPDS or PC liposomes at a final PC concentration similar to that for apoA-I/PC. After the second incubation, medium was collected and centrifuged for 15 min at 30000*g*, and cholesterol was isolated by TLC as described in Materials and Methods and counted Values of mean ± standard error of mean of quadruplicate determinations are presented. [b] $p < 0.001$ versus serum. [c] $p < 0.01$ versus HDL. [d] $p < 0.01$ versus apoA-I/PC.

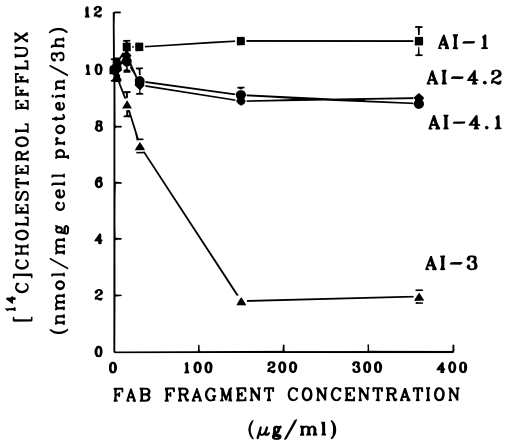

FIGURE 6: Effect of monoclonal antibodies on the efflux of intracellular cholesterol to human HDL. Efflux medium consisting of serum-free medium containing 45 μg/mL human HDL (final apoA-I concentration = 30 μg/mL) was incubated for 4 h at 37 °C with the indicated concentrations of Fab fragments of various monoclonal antibodies. The intracellular cholesterol pool of HepG2 cells was labeled as described for Figure 5. Cells were washed with HBSS and incubated for an additional 3 h with the efflux medium. After a second incubation medium was collected and centrifuged for 15 min at 30000*g*, cholesterol was isolated by TLC as described in Materials and Methods and counted. Values of mean ± standard error of the mean of triplicate determinations are presented.

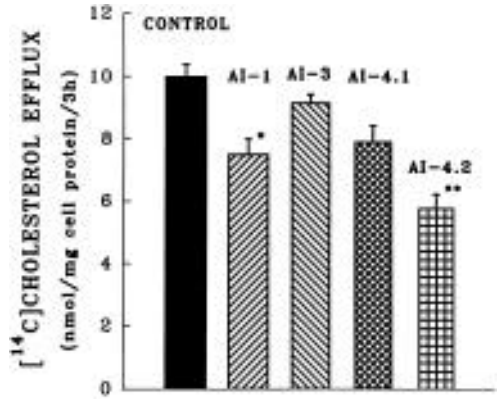

FIGURE 7: Effect of monoclonal antibodies on the efflux of intracellular cholesterol to apoA-I/PC complex. Efflux medium consisting of serum-free medium containing 30 μg/mL apoA-I/PC complex was incubated for 4 h at 37 °C with Fab fragments of the various monoclonal antibodies (final concentration = 360 μg/mL). The intracellular cholesterol pool of HepG2 cells was labeled as described for Figure 5. Cells were washed with HBSS and incubated for an additional 3 h with the efflux medium. After a second incubation medium was collected and centrifuged for 15 min at 30000*g*, cholesterol was isolated by TLC as described in Materials and Methods and counted. Values of mean ± standard error of the mean of quadruplicate determinations are presented. * indicates $p = 0.05$; ** indicates $p < 0.005$.

human HDL pretreated with various Fab fragments. Treatment of HDL with MAB AI-3 reduced its ability to promote the efflux of intracellular cholesterol in a dose-dependent manner up to a maximum 80% inhibition ($p < 0.001$) (Figure 6). Treatment with other MABs had no statistically significant effect on the efflux. However, when apoA-I/PC complex replaced HDL as the acceptor, the Fab fragments of MABs AI-3 and AI-4.1 had no inhibitory effect on cholesterol efflux even at a high concentration (360 μg/mL) ($p > 0.05$) (Figure 7). Antibody AI-1 caused a moderate inhibition [27%, with marginal statistical significance ($p = 0.05$)], while antibody AI-4.2 produced a pronounced (45%,

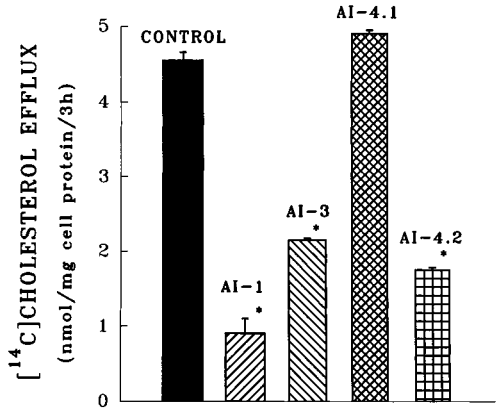

FIGURE 8: Effect of monoclonal antibodies on the efflux of intracellular cholesterol to apoA-I. Efflux medium consisting of serum-free medium containing 30 μg/mL apoA-I was incubated for 4 h at 37 °C with Fab fragments of the various monoclonal antibodies (final concentration = 360 μg/mL). The intracellular cholesterol pool of HepG2 cells was labeled as described for Figure 5. Cells were washed with HBSS and incubated for an additional 3 h with the efflux medium. After a second incubation medium was collected and centrifuged for 15 min at 30000*g*, cholesterol was isolated by TLC as described in Materials and Methods and counted. Values of mean ± standard error of the mean of quadruplicate determinations are presented. * indicates $p < 0.001$.

$p < 0.005$) inhibition of efflux of intracellular cholesterol (Figure 7).

Finally, we tested the effect of MABs on the ability of free apoA-I to promote the efflux of intracellular cholesterol. Treatment of apoA-I with Fab fragments of MABs AI-3 and AI-4.2 (360 μg/mL) caused an inhibition of efflux by 52% and 61%, respectively ($p < 0.001$), while AI-4.1 had no effect (Figure 8). Unexpectedly, incubation of antibody AI-1 with apoA-I inhibited cholesterol efflux by 80% ($p < 0.001$) (Figure 8).

DISCUSSION

It is now generally accepted that apoA-I plays a key role in cholesterol efflux [for a review, see Forte and McCall (1994)]. Although the presence of apoA-I increases cholesterol efflux compared to lipid vesicles without apolipoprotein (Davidson et al., 1994), the contributory mechanism of apoA-I action still has not been established. Two suggestions have been offered to explain the role of apoA-I in cholesterol efflux. First, it was suggested that apoA-I anchors HDL particles to the plasma membrane and then disrupts the plasma membrane lipid layer (Johnson et al., 1991b; Mahlberg & Rothblat, 1992; Rothblat et al., 1992). This action promotes the desorption of cholesterol from the plasma membrane, facilitating its passage through an aqueous layer to HDL. This mechanism does not require specific interaction between apoA-I and cellular receptors nor the existence of any specific sequences of apoA-I responsible for its function. The secondary, amphipathic structure of apoA-I was suggested to be adequate for this role. Indeed, peptides with secondary structure that mimics that of parts of apoA-I, but with no homology to the apoA-I primary structure, promote cholesterol efflux from the plasma membrane almost as effectively as apoA-I (Davidson et al., 1994; Mendez et al., 1994).

The second proposition is that apoA-I facilitates the transfer of cholesterol from the intracellular compartments to the plasma membrane for subsequent efflux (Hokland et al., 1993; Mendez et al., 1991; Slotte et al., 1987). Central

to this hypothesis is the recognition of HDL (apoA-I) by a cellular receptor, which requires the existence of high-affinity HDL binding sites and specific sequences of apoA-I that interact with the putative receptor. Some evidence exists that consistent with the second proposal. High-affinity HDL binding sites with properties similar to classical cellular receptors have been described (Barbaras et al., 1994; Morrison et al., 1992), and specific HDL binding proteins have been isolated from liver and extrahepatic cells (Barbaras et al., 1990; Hidaka & Fidge, 1992; McKnight et al., 1992; Tozuka & Fidge, 1992). It was also found that HDL activates second-messenger systems in cells (Mendez et al., 1991; Voyno-Yasenetskaya et al., 1993), which in turn promote the translocation of intracellular cholesterol to the plasma membrane (Hokland et al., 1993).

Recently, amphipathic helical peptides have been reported to promote intracellular cholesterol efflux from cholesterol-loaded cells (Mendez et al., 1994), but in this report intracellular cholesterol efflux was not monitored directly. While the two hypotheses are not mutually exclusive, one conflicting point concerns the existence of an apoA-I specific sequence(s), which regulates the process of cellular cholesterol efflux. In the present work, we have used, as have others (Banka et al., 1994; Fielding et al., 1994; Luchoomun et al., 1994), monoclonal antibodies to block specific epitopes of apoA-I to determine whether any of these regions are involved in efflux of either plasma membrane cholesterol and/or intracellular cholesterol to the provisional acceptors. The results showed that two antibodies reacting with the adjacent epitopes apoA-$I_{140-147}$ and apoA-$I_{149-150}$ inhibited the efflux of intracellular cholesterol, indicating that this region is important for the process. This effect was specific, since other anti-apoA-I antibodies, while showing even stronger binding to apoA-I in the liquid immunoprecipitation assay, had no effect on the efflux. The inhibition by these antibodies in combination was twice as effective as when each was used alone and as effective as an anti-apoA-I polyclonal antibody (Sviridov & Fidge, 1995a). None of the antibodies had an inhibitory effect on the efflux of cholesterol from the plasma membrane. This is consistent with our finding (Sviridov & Fidge, 1995a) that polyclonal anti-apoA-I antibody inhibited intracellular but not plasma membrane cholesterol efflux and also consistent with the recent conclusion that the efflux of cholesterol from the plasma membrane occurs via a nonspecific aqueous diffusion mechanism (Rothblat et al., 1992). The cellular cholesterol content in our experiments remained unchanged during both labeling and efflux incubations, and, as others have done (Banka et al., 1994; Fielding et al., 1994; Luchoomun et al., 1994), we assessed cholesterol efflux by measuring the transfer of [^{14}C]cholesterol from the cells to an acceptor. The amount of [^{14}C]cholesterol released during our relatively short experiments was about 5−10% of cellular [^{14}C]-cholesterol, i.e., the specific activity of [^{14}C]cholesterol was almost unchanged. This means that the system was in equilibrium and our data reflect the true ability of the particles to promote cholesterol efflux/exchange.

Most of the plasma apoA-I is present in the different subfractions of HDL (Forte & McCall, 1994), with minor amounts existing in the free form (Liang et al., 1994). Because the conformation of apoA-I varies among different HDL particles (Calabresi et al., 1993; Jonas et al., 1989), we compared cholesterol efflux with different types of apoA-I containing particles, including that in whole serum, which contains all types of particles, with isolated HDL, composed exclusively of spherical α-HDL, with apoA-I/PC complex, existing as discoidal particles with preβ mobility (Rye & Barter, 1994), and with free apoA-I. Antibody AI-3 effectively inhibited cholesterol efflux to serum, HDL, and free apoA-I, but not to apoA-I/PC. Antibody AI-4.2 inhibited efflux to serum, apoA-I/PC, and free apoA-I, but not to HDL. The explanation for the latter is that interaction between antibody Al-4.2 and isolated HDL in the immunoprecipitation assay was very weak, and even this residual binding could possibly be attributed to the weak reaction with the epitope apoA-I_{27-28}. Instead, following Western blotting, antibody AI-4.2 displayed a greater avidity for preβ_2-HDL and preβ_3-HDL, particles that are present in serum and similar to the apoA-I/PC particles, but are absent from isolated HDL (Sviridov & Fidge, 1995b). It is not clear, however, whether the effect of antibody AI-4.2 can be entirely explained by the interaction of this antibody with preβ_2-HDL, as was found for the antibody described by Fielding et al. (1994), which reacted with an adjacent epitope. The effect of antibody AI-3 on the efflux to HDL was much stronger than that of the other particles. If antibody AI-3 does not inhibit the efflux of cholesterol to preβ-HDL particles, an assumption that is indirectly supported by the inability of AI-3 to inhibit efflux to apoA-I/PC and its weaker reactivity with preβ_2-HDL and preβ_3-HDL particles, it follows that it would not inhibit the preβ-HDL-dependent portion of the efflux to the serum (Kawano et al., 1993), but would be more effective in inhibiting efflux to isolated HDL, which lacks preβ-HDL particles (Sviridov & Fidge, 1995b).

Unexpectedly, antibody AI-1 inhibited cholesterol efflux to free apoA-I and to some extent to apoA-I/PC (probably due to the presence of free apoA-I in the apoA-I/PC preparation). A possible explanation is that the conformation of free apoA-I in solution, which is different from that in the lipid-bound form (Calabresi et al., 1993), is such that antibody bound to the N-terminal end also blocks the region 140−150 in the center of the molecule, an interaction that is unavailable for the lipid-bound form. Alternatively, when complexed with antibody, apoA-I may assume a conformation that does not favor receptor recognition. An effect of MAB AI-1 on the binding of free apoA-I to phospholipid, a preferential state for promoting efflux (Forte et al., 1993), cannot be excluded.

The region responsible for the efflux of intracellular cholesterol found in this work overlaps regions identified by other laboratories, namely, apoA-$I_{137-144}$ and apoA-$I_{141-148}$ (Fielding et al., 1994) and apoA-$I_{135-148}$ (Luchoomun et al., 1994), and is close to another site, apoA-I_{96-111}, described by Banka et al. (1994). However, our protocol differs from that of others in distinguishing between plasma membrane and intracellular cholesterol mobilization to allow the assessment of inhibition of specific versus nonspecific processes by MABs. Our data strongly favor the involvement of a specific apoA-I site in the regulation of biochemical processes, presumably following interaction with high-affinity binding sites on the cell membrane. In this context, it is interesting that antibody AI-4.2 (apoA-$I_{149-150}$), and to a lesser extent AI-3 (apoA-$I_{140-147}$), also inhibited binding of [^{125}I]HDL_3 to liver plasma membranes (Allan et al., 1993), suggesting that apoA-I binding and cholesterol efflux are mediated by the same region of apoA-I. Residues 140−150 are characterized by a relatively low interspecies variability while lying within a highly variable domain

between residues 120−200 (Weinberg, 1994), consistent with the biological conservation of an important biological motif.

An alternative argument could be made that membrane-reactive amphipathic sites of apoA-I are disrupted following the formation of an antibody−apolipoprotein complex. This situation could apply to MABs AI-3 and AI-4.2, which recognize epitopes within one amphipathic α-helical region of apoA-I (apoA-$I_{143-164}$) (Segrest et al., 1992). This explanation is unlikely, however, since a single (Davidson et al., 1994), or at most two adjacent (Mendez et al., 1994), α-helical region is sufficient to promote cholesterol efflux. Thus, the disruption by antibody of one of six similar amphipathic helices in apoA-I (Segrest et al., 1992) is unlikely to inhibit cholesterol efflux by this nonspecific process, and blocking of a specific, biologically active site is a more likely explanation. Furthermore, epitopes of the antibodies AI-1 and AI-4.1 also overlap with the amphipathic helical regions apoA-I_{44-65} (class A_1 similar to apoA-$I_{143-164}$) and apoA-$I_{210-220}$ (class Y) (Segrest et al., 1992), yet MABs AI-1 and AI-4.1 have no effect on cholesterol efflux.

We believe that one conclusion that can be drawn from our data is that a specific sequence of ApoA-I, between or adjacent to residues 140−150, may lead to the mobilization of intracellular cholesterol, possibly through specific membrane interaction and biochemical signaling. This specific sequence apparently is not involved in any "acceptor" capacity, since the efflux of cholesterol from the plasma membrane is not affected when the region forms a complex with antibody.

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