The role of apoproteins AI and AII in binding of high-density lipoprotein₃ to membranes derived from bovine aortic endothelial cells

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Although binding of high-density lipoproteins (HDL) to a variety of cells in culture has been widely reported, the mechanism of this binding has yet to be fully elucidated. The aim of the current studies was to explore the roles of apoproteins (apo) AI and AII in HDL₃ binding to membranes derived from bovine aortic endothelial cells. Binding studies showed that HDL₃ (which contains both apo AI and apo AII) and AII-HDL₃ (which contain only apo AII) bound to membranes with similar affinity (44 ± 6 and 41 ± 9 $\mu\text{g}/\text{ml}$ respectively) and capacity (673 ± 97 and 969 ± 101 ng bound/mg of membrane protein respectively). In contrast with these results, HDL₃ [AI w/o AII] (which contain apo AI, but not apo AII) bound to the membranes with a significantly higher capacity (2228 ± 206 ng bound/mg of membrane protein) and lower affinity (65 ± 3 $\mu\text{g}/\text{ml}$) as compared with HDL₃ or AII-HDL₃. Therefore, although both apo AI and apo AII appear capable of facilitating HDL₃ binding, the mechanisms involved probably differ. A model which fits the data postulates that a common receptor exists which binds both apo AI and apo AII, and that a particle containing AII can occupy up to four receptors (partly owing to each AII molecule containing two binding domains), whereas an HDL₃ [AI w/o AII] particle can occupy only one.

INTRODUCTION

High-density lipoprotein (HDL) plays an important role in the transport of lipid throughout the body, and has achieved recent prominence owing to its reputed protective nature against the development of atherosclerosis [for reviews see Hopkins & Williams (1981) and Gordon (1989)]. Consequently, the principal apoprotein moieties of HDL, apo AI and apo AII, have been the subject of intense investigation in attempts to gain further knowledge about their part in determining the metabolic fate of this important plasma lipoprotein.

HDL has been observed to bind to a variety of cells in culture (Koschinsky *et al.*, 1977; Fidge *et al.*, 1983; Biesbroeck *et al.*, 1983; Schmitz *et al.*, 1985). Although there is a general consensus that apo AI is involved (Rifici & Eder, 1984; Fong *et al.*, 1985, 1987; Hwang & Menon, 1985; Fidge & Nestel, 1985; Schmitz *et al.*, 1985), there are conflicting opinions about the ability of apo AII to mediate the interaction, with some studies reporting that apo AII is able to facilitate binding (Fong *et al.* 1985; Hwang & Menon, 1985; Fidge & Nestel, 1985; Vadiveloo & Fidge, 1990), and others concluding to the contrary (Schmitz *et al.*, 1985; Fong *et al.*, 1987; Jurgens *et al.*, 1989). Furthermore, some studies which showed that both apo AI and AII contributed to HDL binding reported that apo AI particles bound with a higher affinity and lower capacity than apo AII particles (Hwang & Menon, 1985), whereas in others these observations were reversed (Barbaras *et al.*, 1986; Steinmetz *et al.*, 1990). Such discrepancies in the parameters describing binding of apo AI and apo AII raise additional uncertainty about the roles of these apoproteins in the interaction of HDL₃ with cells.

Recently our laboratory reported the results of a study which examined the role of apo AII in HDL₃ binding (Vadiveloo & Fidge, 1990). In that study HDL₃ particles enriched in apo AII and depleted of apo AI (i.e. AII-HDL₃) were used, thereby avoiding the use of artificial apo-lipid complexes prepared by

using phospholipids such as dimyristoyl phosphatidylcholine (DMPC). In contrast with such apo-lipid complexes, AII-HDL₃ particles resembled the native HDL₃ particle with respect to size, shape and lipid content. It was found that these AII-HDL₃ particles bound with the same capacity and affinity as native HDL₃, suggesting that apo AII alone was capable of mediating HDL₃ binding to bovine aortic endothelial (BAE) cells.

In the present work, examination of the roles of apo AI and AII in HDL₃ binding was extended by the use of HDL₃ particles which contained AI but not AII (HDL₃ [AI w/o AII]). The use of such particles, isolated from native HDL₃ by immunochemical means, was consistent with the approach described above, since they resembled native HDL₃ in all respects except apoprotein content. Binding of these particles to membranes derived from BAE cells was compared with the binding of AII-HDL₃ and native HDL₃. Any differences between the binding of HDL₃ [AI w/o AII], AII-HDL₃ and native HDL₃ should only reflect the difference in apoprotein content, since all particles resemble each other in size, shape and lipid composition.

METHODS

Preparation of HDL₃ [AI w/o AII]

Purification of apo AII. Apo AII was purified by ion-exchange chromatography on DEAE-Sephacel, under conditions previously described by Jackson & Holdsworth (1986). Briefly, about 200 mg of apo HDL prepared as described previously (Vadiveloo & Fidge, 1990) was dissolved in 15 ml of column running buffer (0.02 M-Tris/6 M-urea, pH 8) and applied to a DEAE-Sephacel column (15 cm $\times$ 1.5 cm), which was pre-equilibrated in the running buffer. Apoproteins were eluted with a 0–0.125 M-NaCl gradient (25 ml/h), and eluted fractions (8 ml) were analysed by SDS/PAGE. Fractions containing only apo AII were pooled, dialysed against 0.05 M-NH₄HCO₃ (pH 8.0),

Abbreviations used: HDL, high-density lipoprotein; LDL, low-density lipoprotein; BAE, bovine aortic endothelial; apo, apolipoprotein; DMPC, dimyristoyl phosphatidylcholine; HDL₃ [AI w/o AII], HDL₃ containing apo AI but not apo AII.

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freeze-dried and resuspended in Buffer A (10 mM-Tris/150 mM-NaCl/1 mM-EDTA), pH 7.4.

Preparation of apo AII-Sepharose gel. Pure apo AII (80 mg) was covalently coupled to CNBr-activated Sepharose 4B (10 ml) according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). The gel was stored at 4 °C in Buffer A containing 0.02 % merthiolate.

Preparation of anti-apo AII IgG. Rabbits were primarily immunized with 200 µg of apo AII (1 ml) mixed with Freund's complete adjuvant at a 1:1 (v/v) ratio, followed by monthly immunizations with 200 µg of apo AII in Freund's incomplete adjuvant, again at a 1:1 ratio. Then 3–4 weeks after immunization, rabbits were bled and the sera (25 ml) collected. Anti-apo AII antibodies were isolated by affinity chromatography by incubating the sera with apo AII-Sepharose for 2 h at 25 °C. The gel was packed into a column and washed extensively with Buffer A to remove non-bound material. Anti-apo AII antibodies were eluted with 0.1 M-acetic acid, pH 3.0, immediately adjusted to pH 7.0 with 2 M-Trizma base (Sigma Chemical Co., St. Louis, MO, U.S.A.) and dialysed against Buffer A.

The IgG fraction of the anti-apo AII antibody preparation was isolated by affinity chromatography using Protein A-Sepharose (Pharmacia). The antibody solution was adjusted to 1.5 M-glycine/3 M-NaCl, pH 8, and incubated with Protein A-Sepharose for 1 h at 25 °C. These salt concentrations were optimal for binding of IgG to Protein A (Pharmacia, 1986). The gel was packed into a column and washed in pH 8.0 buffer to remove non-bound material. The IgG fraction was eluted with 0.1 M-sodium citrate, pH 3.0, and immediately adjusted to pH 7.0 with 2 M-Trizma base, and then dialysed against Buffer A.

The antigen-binding activity of the anti-apo AII IgG was assessed by e.l.i.s.a. on 96-well plates. Plates were coated with 100 µl of apo AII (0.2 µg/ml) in 0.05 M-Na₂CO₃ buffer, pH 9.6. After overnight incubation at 25 °C, plates were washed and blocked with 0.05 % Tween 20/1 % BSA in phosphate-buffered saline (PBS; 7.5 mM-Na₂HPO₄, 2.5 mM-NaH₂PO₄, 150 mM-NaCl), pH 7.5, for 30 min at 37 °C, and then further washed with 0.05 % Tween 20 in PBS, pH 7.5. Fractions containing anti-apo AII IgG were serially diluted across the plate (final volume 100 µl/well) and incubated for 1 h at 37 °C. After washing (Tween 20 in PBS), 100 µl of a horseradish-peroxidase-conjugated second antibody (goat anti-rabbit) was added (1:2000 dilution) and incubated for 30 min at 37 °C. Plates were washed three times (Tween 20 in PBS), after which 100 µl of the colour reagent ABTS [0.1 % 2,2'-azinodi-(3-ethylbenzthiazoline sulphonic acid) in 0.02 % H₂O₂/0.1 M-citrate, pH 4.0] was added. Colour development proceeded for about 15 min and was stopped by addition of 2.5 % (w/v) NaF (dissolved in water). The colour formation was quantified with a Titertek Multiscan MC instrument (Flow Laboratories), with a filter setting of 414 nm. Typical e.l.i.s.a. results are given in Fig. 1, showing that the anti-AII activity in the original serum reached zero when diluted to a protein concentration of about 0.1 µg/ml, whereas the Protein-A-eluted fraction (anti-apo AII IgG) did not lose activity until it was diluted to about 5 ng/ml, indicating a 20-fold purification of activity. In this preparation there was a 24-fold protein purification (50 mg of IgG was isolated from 1200 mg of antiserum), indicating no major loss of activity during isolation of the IgG.

A flow chart outlining the procedure of HDL₃ [AI w/o AII] preparation is shown in Fig. 2. Ultracentrifugally isolated HDL₃ (*d* 1.125–1.21), prepared as described previously (Vadiveloo & Fidge, 1990), was incubated with anti-apo AII IgG at a ratio of 1 mg of HDL₃ to 8 mg of IgG, for 2 h at 25 °C. The mixture was centrifuged at 5000 *g* for 10 min to pellet the immunoprecipitate (HDL₃ [AI w AII] and IgG), and the supernatant was collected

Fig. 1. Determination of anti-apo AII activity by e.l.i.s.a.

Rabbits were injected with apo AII, and the sera were collected and incubated with apo AII-Sepharose. After removal of non-bound material, bound antibodies were eluted at pH 3. The eluted antibodies were incubated with Protein A-Sepharose to isolate the IgG. After washing to remove non-IgG antibodies, bound IgG was eluted at pH 3. Original sera (□), non-bound (■, apo AII-Sepharose; ▲, Protein A-Sepharose) and eluted (●, apo AII-Sepharose; ○, Protein A-Sepharose) fractions were assayed directly for anti-apo AII activity on 96-well plates coated with pure apo AII (see the Methods section for details).

and adjusted to 1.5 M-glycine/3 M-NaCl, pH 8.0, and incubated with Protein A-Sepharose (1 h, 25 °C) to remove excess IgG. This mixture was packed into a column and the non-bound material (HDL₃ [AI w/o AII]) was collected and concentrated under pressurised N₂ in an Amicon stirred-cell ultrafiltration apparatus (model 12) with a YM 5 membrane installed. The concentrate was adjusted to *d* = 1.21 and centrifuged in a Beckman TL 100.2 rotor at 80000 rev./min for 22 h at 4 °C. The infranatant (HDL₃ [AI w/o AII]) was collected, dialysed in Buffer A and analysed as described below.

Analysis of HDL₃ [AI w/o AII]. Protein composition was determined by PAGE in either 8 % or 15 % acrylamide gels in the presence of 0.1 % SDS (Laemmli, 1970). After electrophoresis, the gels were fixed and stained with Coomassie Blue R-250. The amount of apo AI and apo AII present on the particles was quantified by using specific antisera directed against each of the apoproteins in an immunoturbidimetric assay described previously (Vadiveloo & Fidge, 1990).

Lipoprotein particle size was determined by PAGE in 4–30 % native polyacrylamide gels (Pharmacia-LKB, Uppsala, Sweden) as described previously by Hopkins & Barter (1984). Briefly, samples (25 µg) were applied to the gels and separated at 250 V for 20 h, after which they were fixed (10 % sulphosalicylic acid), stained (0.04 % Coomassie Blue G-250 in 3.5 % HClO₄) and then destained (5 % acetic acid).

Preparation of AII-HDL₃

AII-HDL₃ was prepared and characterized as described previously (Vadiveloo & Fidge, 1990). Briefly, HDL₃ was incubated with apo-HDL at a ratio of 1 mg of HDL₃ to 10 mg of apo-HDL, for 2 h at 25 °C. The mixture was then adjusted to *d* = 1.21 with KBr and centrifuged at 60000 rev./min for 20 h in a Beckman 70.1 Ti rotor, and the top fraction containing AII-HDL₃ was dialysed against Buffer A. Immunoturbidimetric analysis indicated that the apo AII/AI + AII mass ratio was greater than 98 %, and SDS/PAGE analysis showed that apo AII was the only protein detectable on AII-HDL₃ (results not shown).

Lipoproteins were radioiodinated with ¹²⁵I as previously described (Fidge *et al.*, 1984) and showed specific radioactivities in the range 300–500 c.p.m./ng.

Preparation of BAE cell membranes

Cultured BAE cells were prepared and maintained as described previously (Vadiveloo & Fidge, 1990). Membranes

Fig. 2. Isolation of HDL₃ [AI w/o AII] from HDL₃.

HDL₃ was incubated with anti-apo AII IgG, and the immunoprecipitate (containing IgG and HDL₃ [AI w AII]) was pelleted by low-speed centrifugation and discarded. The supernatant was incubated with Protein A-Sepharose to remove excess IgG. The non-bound solution containing HDL₃ [AI w/o AII] was then concentrated, ultracentrifuged and dialysed in Buffer A. For full details see the Methods section.

were prepared essentially as described by Basu *et al.* (1978), from cells which had undergone between 5 and 25 passages. Briefly, confluent cells grown on 90 mm-diam. dishes were scraped with a rubber policeman and stored in Buffer B (Buffer A plus 1 mM-CaCl₂ and 1 mM-phenylmethanesulphonyl fluoride) at -80 °C until required. Upon thawing, cells were disrupted on ice in Buffer B with a Polytron homogenizer for 20 s on setting 4. The homogenate was centrifuged at 1000 *g* for 5 min at 4 °C. The supernatant was collected and examined by light microscopy, which showed the presence of membrane structures, but not whole cells. The pellet was homogenized and centrifuged again, and the supernatants of the 1000 *g* centrifugations were pooled. These supernatants were further centrifuged for 60 min at 100000 *g* at 4 °C to pellet the membranes, which were then resuspended in Buffer A by passage through 18-, 20- and 25-gauge needles consecutively. Portions of the membranes were stored at -20 °C.

Binding studies

Procedure. Both time-course and concentration-dependent saturation binding studies were performed. In each case the incubation mixture comprised Buffer A (pH 7.4) containing 0.1% casein, 0.5 mg of membrane protein/ml and the concentrations of ligand indicated in the Figures (final incubation volumes are given in the Figure legends). At the times indicated in the text, mixtures were applied to Whatman GF/C glass microfibre filters (pre-soaked in 0.1% casein) positioned on a vacuum manifold. Suction was immediately applied to separate unbound ligand from the membranes, which remained trapped on the filters. Filters were then washed with 5 × 1 ml of 0.1% casein in Buffer A.

In each experiment, 'no membrane' control incubations were performed simultaneously to measure the amount of radioactivity bound non-specifically to the filters in the absence of membranes. The amount of radioactivity bound on these filters was subtracted from that bound to 'test' filters.

For time-course studies, non-specific binding of ligands to BAE cell membranes was determined by measuring binding in the presence of excess unlabelled ligand, whereas for saturation binding studies non-specific binding was calculated from total binding data (see below).

Determination of binding parameters (K_d and B_{max}) from concentration-dependent binding data. The binding parameters and Scatchard plots were derived by analysis of concentration-dependent binding data by using the LIGAND computer program (Munson & Rodbard, 1980; McPherson, 1985), LIGAND deduced the 'non-specific' (NS) binding component from the 'total' binding curve. Subtracting NS from total binding provided 'specific' binding, from which binding parameters (K_d and B_{max}) were derived. Binding parameters obtained by this method were compared with those obtained by direct measurement of NS binding in the presence of excess unlabelled ligand. Both methods gave similar parameters ($n = 3$). Control HDL₃ preparations were routinely subjected to similar conditions as those used to prepare HDL₃ [AI w/o AII] (e.g. room-temperature incubations, additional ultracentrifugation and dialysis). The binding parameters of such 'control' HDL₃ were identical with those of untreated HDL₃.

Other methods. To test for possible significant differences between binding parameters, the data were analysed by Student's *t* test. The level of significance was set at $P < 0.05$. Protein concentrations were determined by the method of Lowry *et al.* (1951).

RESULTS

Preparation of HDL₃ [AI w/o AII]

A flow chart detailing the isolation of HDL₃ [AI w/o AII] from HDL₃ is shown in Fig. 2. The first step was incubation of HDL₃ with anti-apo AII IgG at a mass ratio of 1:8 for 2 h at 25 °C in order to immunoprecipitate HDL₃ [AI w AII] particles. The precipitate was pelleted by centrifugation, and the supernatant containing HDL₃ [AI w/o AII] was incubated with Protein A-Sepharose to remove any excess IgG. The data presented in Fig. 3(a) show that, before incubation with Protein A-Sepharose, the major proteins present in the supernatant were apo AI and IgG (lane 2), and after exposure to Protein A-Sepharose the contaminating IgG was removed (lane 3).

Analysis of HDL₃ [AI w/o AII] by SDS/PAGE (15% gel), which retains apo AII, showed that the only protein detectable was apo AI, providing evidence for removal of apo-AII-containing particles (HDL₃ [AI w AII]) (Fig. 3b). These findings were independently confirmed by an immunoturbidometric apo-protein assay, which showed that HDL₃ [AI w/o AII] had an apo AI/AI + AII mass ratio of > 99%, compared with HDL₃, which had a ratio of $73 \pm 1.5\%$ (mean $\pm$ S.D., $n = 3$; results not shown).

It was important to ensure that aggregation of HDL₃ [AI w/o AII] particles had not occurred during preparation, since their existence would considerably alter the interpretation of binding data obtained from the use of these particles. Analysis of the particles by PAGE in 4–30% native gels showed that the major HDL₃ [AI w/o AII] population size was the same as that of the HDL₃ from which they were isolated (Fig. 3c). A minor HDL₃ [AI w/o AII] population of larger size was also detected, again within the size range for native HDL₃. These data indicated that no particle aggregation had occurred during preparation of HDL₃ [AI w/o AII].

Fig. 3. Electrophoretic analysis of HDL₃ [AI w/o AII] particles

(a) Apoprotein composition of HDL₃ [AI w/o AII]; detection of anti-apo AII IgG. HDL₃ was incubated with anti-apo AII IgG to immunoprecipitate HDL₃ [AI w/o AII]. The supernatant containing HDL₃ [AI w/o AII] and excess IgG was then incubated with Protein A-Sepharose to remove IgG. Samples were analysed by SDS/PAGE (8% gel) before and after incubation with Protein A-Sepharose. Lanes: 1, Pharmacia low-molecular-mass markers (kDa); 2, HDL₃ [AI w/o AII] before incubation with Protein A-Sepharose; 3, HDL₃ [AI w/o AII] after incubation with Protein A-Sepharose. The gel was stained with Coomassie Blue. (b) Apoprotein composition of HDL₃ [AI w/o AII]; detection of apo AII. Samples were analysed by SDS/PAGE (15% gels) to detect the presence of apo AII. Lanes: 1, Pharmacia low-molecular-mass markers (kDa); 2, HDL₃ [AI w/o AII]; 3, HDL₃. The gel was stained with Coomassie Blue. (c) HDL₃ [AI w/o AII] particle size; detection of aggregates. Samples were analysed by PAGE in native 4–30% gels. Lanes: 1, HDL₃; 2, Pharmacia high-molecular-mass standards (radius in nm shown to left of panel); 3, HDL₃ [AI w/o AII]. Samples were electrophoresed and stained as in the Methods section.

Fig. 4. Time-course study illustrating the specific binding of ¹²⁵I-HDL₃ to BAEC membranes

Membranes were incubated with 2.5 µg/ml of ¹²⁵I-HDL₃/ml at 25 °C. The incubation volume was 2 ml, and at the times indicated duplicate 50 µl samples were removed and processed as described in the Methods section. Non-specific binding was measured as binding which occurred in the presence of 500 µg of unlabelled HDL₃/ml, and ranged between 6 and 20% of total binding. The experiment is representative of three such studies performed with ¹²⁵I-HDL₃ concentrations of 2.5–10 µg/ml.

Binding studies

To ensure accurate determination of binding parameters, it was essential to measure binding at equilibrium (Rodbard *et al.*, 1971; Bennett 1978; Mendel *et al.*, 1986). Fig. 4 shows specific binding data obtained from studies performed to determine the time required for HDL₃ binding to BAE cell membranes to reach equilibrium at 25 °C. Equilibrium was reached after approx. 15 h, and the bound ¹²⁵I-HDL₃ was displaceable by addition of excess unlabelled HDL₃ (results not shown). On the basis of these time-course data, all saturation studies were performed for 15–18 h at 25 °C.

Fig. 5. Concentration-dependent binding of HDL₃, AII-HDL₃ and HDL₃ [AI w/o AII] to BAE cell membranes

Radiolabelled ligands were incubated with membranes for 15–18 h at 25 °C, and incubation mixtures (100 µl) were then processed as described in the Methods section. (a) Total (■) and specific (○) binding of ¹²⁵I-HDL₃; (b) Scatchard transformation of the specific binding component of graph (a). (c) Total (■) and specific (○) binding of ¹²⁵I-HDL₃ [AI w/o AII]; (d) Scatchard transformation of the specific binding component of graph (c). (e) Total (■) and specific (○) binding of ¹²⁵I-AII-HDL₃; (f) Scatchard transformation of the specific binding component of graph (e). Total-binding data points in graphs (a), (c) and (e) represent means and ranges of duplicate determinations. Data points in Scatchard plots (b) (d) and (f) represent specific binding as determined by LIGAND (see the Methods section). Abbreviations and units: B = Bound = ng of ligand protein bound/mg of membrane protein; F = concn. of non-bound ligand (µg/ml).

Concentration-dependent binding studies were performed to compare binding affinities and capacities of three types of HDL₃: native HDL₃, HDL₃ which contained apo AI only (i.e. HDL₃ [AI w/o AII]), and HDL₃ which contained apo AII only (i.e. AII-HDL₃) to BAE cell membranes. After incubation with increasing concentrations of ¹²⁵I-labelled ligand, membranes were processed as described in the Methods section. Representative experiments illustrating total and specific binding of the three different ligands are shown in Figs. 5(a), 5(c) and 5(e). The specific binding component of those curves (determined by using LIGAND) are expressed as Scatchard plots shown in Figs. 5(b), 5(d) and 5(f) (note different scales on the axes). A number of such studies were performed, using different preparations of the lipoproteins on each occasion, and the binding parameters obtained from those studies are given in Table 1. Analysis of these data showed that HDL₃ and AII-HDL₃ displayed similar binding affinities and capacities ($P > 0.10$ for both parameter comparisons), whereas HDL₃ [AI w/o AII] bound with a markedly higher capacity and a lower affinity than either HDL₃ or AII-HDL₃ ($P < 0.04$). The

Table 1. Parameters describing specific binding of HDL₃, AII-HDL₃ and HDL₃ [AI w/o AII] to BAE cell membranes

Membranes were incubated with increasing concentrations of radiolabelled ligands for 15–18 h at 25 °C, and then processed as described in the Methods section. K_d and B_{max} are expressed as $\mu\text{g/ml}$ and ng of ligand protein bound/mg of membrane protein respectively. Data represent means $\pm$ S.E.M.

	HDL ₃	AII-HDL ₃	HDL ₃ [AI w/o AII]
K_d	44 $\pm$ 6	41 $\pm$ 9	65 $\pm$ 3
B_{max}	673 $\pm$ 97	969 $\pm$ 101	2228 $\pm$ 206
n	6	3	4

Fig. 6. Specific binding of ^{125}I -HDL₃ [AI w/o AII] ($\square$), ^{125}I -AII-HDL₃ ($\bullet$) and ^{125}I -HDL₃ ($\blacksquare$)

The curves illustrate the binding parameters shown in Table 1 after their insertion into the formula $B = B_{max} \cdot F/(F + K_d)$.

binding data for each ligand best fitted a model describing binding to one class of non-interacting sites.

To illustrate the different binding characteristics of the three ligands, binding curves were generated from the parameters shown in Table 1, according to the formula:

$$B = B_{max} \cdot F/(F + K_d)$$

where B = amount of ligand bound; B_{max} = capacity of binding, F = concentration of non-bound ligand, and K_d = equilibrium dissociation constant. This formula is a derivation of the classic Law of Mass Action that governs receptor–ligand interactions (Bennett, 1978). These data, shown in Fig. 6, emphasize that, although HDL₃ and AII-HDL₃ bound with a similar capacity, HDL₃ [AI w/o AII] bound to membranes with a significantly greater capacity.

DISCUSSION

The aim of these studies was to determine more precisely the role of apo AI and AII in binding of HDL₃ to endothelial cells by comparing binding of HDL₃ which contained either apo AI or apo AII. It was considered important to use particles which resembled the native lipoprotein, rather than apo-lipid complexes (e.g. AI- or AII-DMPC) which markedly differ from HDL₃ in lipid composition, particle size and shape (Blanche *et al.*, 1988; Nichols *et al.*, 1983). Such differences have been shown to affect apoprotein conformation (Brouillette *et al.*, 1984; Kunitake *et al.*, 1985; Silberman *et al.*, 1987), which in turn apparently affected their ability to activate lecithin–cholesterol acyltransferase (Jonas *et al.*, 1987, 1989). It is therefore reasonable to conclude that differences in conformation may also affect the ability of the apoprotein to recognize binding sites or receptors, and would thus affect measurement of binding parameters such as affinity (K_d) and capacity (B_{max}). Furthermore, studies by Sviridov *et al.* (1988) indicated that the lipid composition of the particle may

influence apoprotein conformation and hence binding, since the addition of cholesterol to apo AI-DMPC particles resulted in an 8-fold decrease in binding. Further evidence that apo-lipid complexes may not accurately reflect the metabolism of the native particle was provided by studies by Barbaras *et al.* (1987a), who found that artificial complexes containing both apo AI and apo AII were able to promote cholesterol efflux from cells, whereas native HDL particles which also contained both apo AI and apo AII were unable to perform such a function.

HDL₃ binding to BAE cell membranes took about 15 h to reach equilibrium at 25 °C. Most studies have reported equilibrium times of between 1 and 2 h (Chen *et al.*, 1980; Kagami *et al.*, 1984; Fong *et al.*, 1985; Barbaras *et al.*, 1985, 1986; Hwang & Menon, 1985; Hoeg *et al.*, 1985), although Mendel *et al.* (1986) reported times of 18–24 h. These variations may reflect different affinities of the binding sites present in the different tissue preparations used in each of the studies. Supporting this suggestion is the observation that the time required to reach equilibrium binding to BAE cell membranes was about 7 times longer than that required for binding to BAE cells (Vadiveloo & Fidge, 1990). These differences could reflect alterations to the binding sites on the membranes during preparation and storage, which may have resulted in a slower rate of association. Alternatively, different binding sites may have been exposed on membranes from those displayed on the surface of whole cells.

Analysis of the concentration-dependent binding data showed specific and saturable binding of all three lipoproteins to BAE cell membranes, suggesting that both apo AI and apo AII were determinants of HDL₃ binding. Both HDL₃ and AII-HDL₃ bound to membranes with a similar affinity and capacity, confirming the results reported for binding to whole BAE cells (Vadiveloo & Fidge, 1990). In addition, the binding affinities of both HDL₃ and AII-HDL₃ to membranes (Table 1) were similar to those observed for these same ligands binding to whole BAE cells (Vadiveloo & Fidge, 1990). Such a result could be expected, given that most receptors are integral membrane proteins and thus would not have been separated from their native lipid bilayer environment as a result of homogenization, since in these studies no detergents were used. Therefore it is likely that the conformation of HDL-binding proteins in isolated membranes would be the same as in the intact cell, which would explain the finding that the affinity of the ligand for the binding protein in either isolated membranes or whole cells was similar.

A striking distinction between the binding parameters of the three ligands was that the B_{max} of HDL₃ [AI w/o AII] was 3 times the B_{max} of HDL₃, and more than twice that of AII-HDL₃ (see Table 1). This indicated that BAE cell membranes were able to bind about 3 times as many apo AI-only particles (i.e. HDL₃ [AI w/o AII]) as compared with apo AII-containing particles (i.e. HDL₃ and AII-HDL₃). The other major difference in binding was that the affinity of HDL₃ [AI w/o AII] for the membrane binding sites was significantly less than that of the apo AII-containing populations.

The following section describes a model proposed to explain the binding data. Let it be assumed that the HDL₃-binding sites are cell receptors. The model suggests that both apo AI and apo AII bind to the same class of receptors, as indicated by the results of competition studies using whole BAE cells (Vadiveloo & Fidge, 1990). The model further postulates that apo-AII-containing particles (i.e. HDL₃ [AI w AII] and AII-HDL₃) contain more binding domains per particle compared with apo AI-containing particles, and therefore occupy more receptors per particle. A possible mechanism for this model is shown in Fig. 7. Consider that the area of the HDL particle exposed to the membrane surface was approximately the size of one apo AI molecule, and that each apo AI molecule had only one binding

Fig. 7. Schematic representation of HDL₃ [AI w/o AII] particles and apo AII-containing particles binding to a single class of putative HDL receptors

The model proposes that each apo AI molecule contains only one binding domain which binds only one receptor, whereas each apo AII molecule can bind two receptors as a result of each monomer containing a binding domain (see the text for details). Such a mechanism would explain how BAE cells membranes bind about 3 times as many HDL₃ [AI w/o AII] particles compared with apo AII-containing particles (HDL₃ and AII-HDL₃).

domain which interacted with only one receptor. As shown previously, two apo AII molecules can occupy approximately the same area on the HDL particle as one apo AI molecule (Lagocki & Scanu, 1980; Van Tornout *et al.*, 1981; Vadiveloo & Fidge, 1990). It therefore follows that, within a corresponding area, approximately two apo AII molecules may have been available to interact with membrane receptors. Furthermore, since each apo AII molecule is composed of two identical peptide chains linked by a disulphide bridge at residue 6 (Brewer *et al.*, 1972; Scanu *et al.*, 1972), each monomer of apo AII may contain a binding domain capable of interacting with a receptor. In this manner, particles containing apo AII may have occupied up to 4 times as many receptors as did particles containing apo AI only. This model could thus account for the B_{max} for HDL₃ [AI w/o AII] binding being about 3 times that of apo AII-containing particles (i.e. over twice that of AII-HDL₃ and over 3 times that of HDL₃).

This model is analogous to that proposed to explain the binding of low-density lipoprotein (LDL) and HDLc to the LDL receptor (Pitas *et al.*, 1979, 1980), where four apo E molecules present on HDLc bind to one receptor each, resulting in the occupancy of four receptors by one HDLc particle. However, each LDL particle contains one apo B molecule, which occupies only one receptor; therefore 4 times the number of LDL particles are required to saturate the same number of receptors.

A method of testing whether such a mechanism applied to HDL₃ binding would involve competition studies, in which bound HDL₃ [AI w/o AII] would be incubated with increasing concentrations of either HDL₃ [AI w/o AII] or AII-HDL₃. If the above model accurately depicted HDL₃ binding, one would expect AII-HDL₃ to be a far more effective competitor than HDL₃ [AI w/o AII], since it binds with a higher affinity and would saturate the receptors at a lower concentration than HDL₃ [AI w/o AII]. Unfortunately these experiments could not be performed, since preliminary studies showed that, during the

Fig. 8. Model predicting a method by which HDL₃ [AI w AII] could inhibit HDL₃ [AI w/o AII] binding

With respect to binding of native HDL₃, HDL₃ [AI w AII] particles occupy the membrane binding sites, thus preventing the access of HDL₃ [AI w/o AII] particles. Such a situation would explain the lower binding capacity of HDL₃ compared with isolated HDL₃ [AI w/o AII] (see the text for details).

time taken to reach equilibrium binding, exchange of apoproteins between particles appeared to occur (results not shown). Such transfer resulted in particles of mixed apoprotein composition, which would have made it difficult to interpret subsequent binding data. This exchange process has also been reported by others (Havekes *et al.*, 1984), and probably explains why data from 'direct' binding experiments can conflict with data obtained by 'competition' studies (Fidge & Nestel, 1985).

A conclusion that could be drawn from the binding data is that HDL₃ [AI w/o AII] present in HDL₃ was not contributing to HDL₃ binding. As shown in Fig. 6, at an HDL₃ concentration of 500 µg/ml, which contains about 125 µg of HDL₃ [AI w/o AII]/ml (James *et al.*, 1989; Atmeh *et al.*, 1983), the amount bound was 618 ng/mg of membrane protein. However, 125 µg of isolated HDL₃ [AI w/o AII]/ml bound approx. 2.5-fold higher, suggesting HDL₃ binding would have been greater had HDL₃ [AI w/o AII] been contributing to the interaction. The data therefore suggest that when it was a component of HDL₃, HDL₃ [AI w/o AII] bound with a lower capacity. As illustrated in Fig. 8, this situation may have resulted had HDL₃ [AI w AII] (also a component of HDL₃) been inhibiting access of HDL₃ [AI w/o AII] particles to the receptors, resulting in an overall lower capacity of whole HDL₃ binding compared with HDL₃ [AI w/o AII]. The possibility of such inhibition occurring is strengthened by the observation that apo AII appeared to bind with a higher affinity than apo AI (compare the affinities of HDL₃ [AI w/o AII] and AII-HDL₃, shown in Table 1).

Previous studies have shown that HDL [AI w/o AII], but not HDL [AI w AII], was capable of mediating cholesterol efflux from cells (Barbaras *et al.*, 1987a). Since cholesterol efflux from cells to HDL may be dependent on HDL binding (Oppenheimer *et al.*, 1987; Barbaras *et al.*, 1987b; Slotte *et al.*, 1987; Bierman *et al.*, 1989), the proposition that HDL₃ [AI w AII] present in HDL₃ may have inhibited binding of HDL₃ [AI w/o AII] could have important physiological consequences. Inhibition of HDL₃ [AI w/o AII] binding by HDL₃ [AI w AII] may result in a decrease in sterol efflux from cells to HDL. As a result, there would be a decrease in the amount of cholesterol entering the 'reverse cholesterol transport' pathway. This may explain why

elevated levels of plasma HDL₃ [AI w/o AII] apparently protect against the development of heart disease (Puchois *et al.*, 1987), since high concentrations of HDL₃ [AI w/o AII] would counteract the postulated inhibitory effect of HDL₃ [AI w/o AII] on HDL₃ [AI w/o AII] binding, thus allowing reverse cholesterol transport to function effectively.

During the preparation of this manuscript, Kilsdonk *et al.* (1990) reported on the binding of lipoproteins AI and AI/AII to Hep G2 cells. These authors concluded that specific binding of both ligands was similar, whereas according to the above model one would have expected lipoprotein AI/AII to bind with a lower capacity. However, since they were unable to report $B_{\max}$ and K_d values, owing to the limited number of measurements performed, it is difficult to compare their data adequately with those reported here.

In summary, the results of the present study suggested both apo AI and apo AII were capable of mediating binding of HDL₃, confirming previous studies from this laboratory (Fidge & Nestel, 1985; Vadiveloo & Fidge, 1990). However, the mode of the interaction appeared to differ for each of the apoproteins, since particles which contained apo AI only bound with a higher capacity and lower affinity as compared with HDL₃, whereas particles which contained apo AII only showed similar binding characteristics to HDL₃. The data fit a model which proposes that apo AII can occupy more binding sites than apo AI, possibly owing to each apo AII monomer being able to bind to one receptor.

We are grateful to the Red Cross Blood Bank of Victoria for supplying plasma, and to the National Heart Foundation of Australia for funds to support this research.

REFERENCES

- Atmeh, R. F., Shepherd, J. & Packard, C. J. (1983) *Biochim Biophys. Acta* **751**, 175–188
- Barbaras, R., Grimaldi, P., Negrel, R. & Ailhaud, G. (1985) *Biochim. Biophys. Acta* **845**, 492–501
- Barbaras, R., Grimaldi, P., Negrel, R. & Ailhaud, G. (1986) *Biochim. Biophys. Acta* **888**, 143–156
- Barbaras, R., Puchois, P., Fruchart, J. C. & Ailhaud, G. (1978a) *Biochem. Biophys. Res. Commun.* **142**, 63–69
- Barbaras, R., Puchois, P., Grimaldi, P., Barkia, A., Fruchart, J. C. & Ailhaud, G. (1978b) *Biochem. Biophys. Res. Commun.* **149**, 545–554
- Basu, S. K., Goldstein, J. L. & Brown, M. S. (1978) *J. Biol. Chem.* **253**, 3852–3856
- Bennett, J. P., Jr. (1978) in *Neurotransmitter Receptor Binding* (Yamamura, H. I., Enna, S. J. & Kuhar, M. J., eds.), pp. 57–90, Raven Press, New York
- Bierman, E. L., Oppenheimer, M. & Oram, J. F. (1989) in *Atherosclerosis VIII* (Crepaldi, G., Gotto, A. M., Manzato, E. & Baggio, G., eds.) pp. 297–300, Elsevier, Amsterdam
- Biesbroeck, R., Oram, J. F., Albers, J. J. & Bierman, E. L. (1983) *J. Clin. Invest.* **71**, 525–539
- Blanche, P. J., Nichols, A. V., Forte, T. M. & Gong, E. L. (1988) *Biochim. Biophys. Acta* **958**, 143–152
- Brewer, H. B., Jr., Lux, S. E., Ronan, R. & John, K. M. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1304–1308
- Brouillette, C. G., Jones, J. L., Ng, T. C., Kercret, H., Chung, B. H. & Segrest, J. P. (1984) *Biochemistry* **23**, 359–367
- Chen, Y.-D. I., Kraemer, F. B. & Reaven, G. M. (1980) *J. Biol. Chem.* **255**, 9162–9167
- Fidge, N. H. & Nestel, P. J. (1985) *J. Biol. Chem.* **260**, 3570–3575
- Fidge, N. H., Nestel, P. J. & Suzuki, N. (1983) *Biochim. Biophys. Acta* **753**, 14–21
- Fidge, N. H., Leonard-Kanevsky, M. & Nestel, P. (1984) *Biochim. Biophys. Acta* **793**, 180–186
- Fong, B. S., Rodrigues, P. O., Salter, A. M., Yip, B. P., Depres, J. P. & Angel, A. (1985) *J. Clin. Invest.* **75**, 1804–1812
- Fong, B. S., Salter, A. M., Jimenez, J. & Angel, A. (1987) *Biochim. Biophys. Acta* **920**, 105–113
- Gordon, D. J., (1989) in *High Density Lipoproteins and Atherosclerosis II* (Miller N. E., ed.), pp. 3–10, Elsevier, Amsterdam
- Havekes, L., Schouten, D., van Hinsbergh, V. & de Wit, E. (1984) *Biochem. Biophys. Res. Commun.* **122**, 785–790
- Hoeg, J. M., Demosky, S. J., Jr., Edge, S. B., Gregg, R. E., Osborne J. C., Jr., & Brewer, H. B., Jr. (1985) *Arteriosclerosis* **5**, 228–237
- Hopkins, G. J. & Barter, P. J. (1984) *Biochim. Biophys. Acta* **794**, 31–40
- Hopkins, P. N. & Williams, R. R. (1981) *Atherosclerosis* **40**, 1–52
- Hwang, J. & Menon, K. M. J. (1985) *J. Biol. Chem.* **260**, 5660–5668
- Jackson, R. L. & Holdsworth, G. (1986) *Methods Enzymol.* **128**, 288–297
- James, R. W., Proudfoot, A. & Pometta, D. (1989) *Biochim. Biophys. Acta* **1002**, 292–301
- Jonas, A., Zorich, N. L., Kezdy, K. E. & Trick, W. E. (1987) *J. Biol. Chem.* **262**, 3969–3974
- Jonas, A., Kezdy, K. E. & Wald, J. H. (1989) *J. Biol. Chem.* **264**, 4818–4824
- Jurgens, G., Xu, Q., Huber, L. A., Bock, G., Howanietz, H., Wick, G. & Traill, K. N. (1989) *J. Biol. Chem.* **264**, 8549–8556
- Kagami, A., Fidge, N., Suzuki, N. & Nestel, P. (1984) *Biochim. Biophys. Acta* **795**, 179–190
- Kilsdonk, E. P. C., Van Gent, T. & Van Tol, A. (1990) *Biochim. Biophys. Acta* **1045**, 205–212
- Koschinsky, T., Carew, T. E. & Steinberg, D. (1977) *J. Lipid Res.* **18**, 451–458
- Kunitake, S. T., La Sala, K. J. & Kane, J. P. (1985) *J. Lipid Res.* **26**, 549–555
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lagocki, P. A. & Scanu, A. M. (1980) *J. Biol. Chem.* **255**, 3701–3706
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. Biol. Chem.* **193**, 265–275
- McPherson, G. A. (1985) *J. Pharmacol. Methods* **14**, 213–228
- Mendel, C. M., Kunitake, S. T. & Kane, J. P. (1986) *Biochim. Biophys. Acta* **875**, 59–68
- Munson, P. J. & Rodbard, D. (1980) *Anal. Biochem.* **107**, 220–239
- Nichols, A. V., Gong, E. L., Blanche, P. J. & Forte, T. M. (1983) *Biochim. Biophys. Acta* **750**, 353–364
- Oppenheimer, M. J., Oram, J. F. & Bierman, E. L. (1987) *Arteriosclerosis* **7**, 325–332
- Pharmacia (1986) *Separation News* **13/5**
- Pitas, R. E., Innerarity, T. L., Arnold, K. S. & Mahley, R. W. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2311–2315
- Pitas, R. E., Innerarity, T. L. & Mahley, R. W. (1980) *J. Biol. Chem.* **255**, 5454–5460
- Puchois, P., Kandoussi, A., Fievet, P., Fourrier, J. L., Bertrand, M., Koren, E. & Fruchart, J. C. (1987) *Atherosclerosis* **68**, 35–40
- Rifici, V. A. & Eder, H. A. (1984) *J. Biol. Chem.* **259**, 13814–13818
- Rodbard, D., Ruder, H. J., Vaitukaitis, J. & Jacobs, H. S. (1971) *J. Clin. Endocrinol. Metab.* **33**, 343–355
- Scanu, A. M., Lim, C. T. & Edelstein, C. (1972) *J. Biol. Chem.* **247**, 5850–5855
- Schmitz, G., Niemann, R., Brennhäusen, B., Krause, R. & Assmann, G. (1985) *EMBO J.* **4**, 2773–2779
- Silberman, S. R., Bernini, F., Sparrow, J. T., Gotto, A. M. & Smith, L. C. (1987) *Biochemistry* **26**, 5833–5843
- Slotte, J. P., Oram, J. F. & Bierman, E. L. (1987) *J. Biol. Chem.* **262**, 12904–12907
- Steinmetz, A., Barbaras, R., Ghalim, N., Clavely, V., Fruschart, J.-C. & Ailhaud, G. (1990) *J. Biol. Chem.* **265**, 7859–7863
- Sviridov, D. D., Misharin, A. Y., Safonova, I. G., Bushmakina, N. G., Repin, V. S. & Smirnov, V. N. (1988) *Biochim. Biophys. Acta* **963**, 119–125
- Vadiveloo, P. K. & Fidge, N. H. (1990) *Biochim. Biophys. Acta* **1045**, 135–141
- Van Tornhout, P., Caster, H., Lievens, M., Rosseneu, M. & Assmann, G. (1981) *Biochim. Biophys. Acta* **663**, 630–636