Apolipoprotein A-I Conformation in Reconstituted Discoidal Lipoproteins Varying in Phospholipid and Cholesterol Content*

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The effects of the size and cholesterol content on the conformation of apolipoprotein A-I (apoA-I) have been studied in reconstituted discoidal lipoproteins containing two apoA-I per particle (Lp2A-I). The immunoreactivity of a series of 13 epitopes distributed along the apoA-I sequence has been evaluated in Lp2A-I with a phospholipid/apoA-I molar ratio ranging from 31 to 156 and in Lp2A-I with constant phospholipids but varying in cholesterol content from 0 to 22 molecules. The results are compatible with a three domain structure in apoA-I in which the central domain is located between residues 99 and 143 and postulated to be a hinged domain that responds differentially to changes in phospholipid and cholesterol contents. Increasing the phospholipid content results in significant changes of epitope immunoreactivity throughout the N-terminal and central domains of apoA-I with fewer modifications in the C-terminal domain. In contrast, increasing Lp2A-I cholesterol content modifies only the immunoreactivity of two central epitopes, A11 (residues 99–132) and 5F6 (residues 118–148), and an extreme N-terminal epitope, 4H1 (residues 2–8). Interestingly, the effects of increasing cholesterol or phospholipids on these epitopes are opposite. This suggests a specific effect of cholesterol on the central domain tertiary structure between residues 99 and 143. Competition binding assays among pairs of antibodies binding to apoA-I on Lp2A-I are best explained by invoking inter- as well as intramolecular competitions. The specificity of the intermolecular competitions suggests an N to C termini arrangement of the two apoA-I molecules around the disc. Increasing the phospholipid content of Lp2A-I mainly increases the competitions between 3G10 and antibodies binding to most adjacent epitopes. Simultaneously as Lp2A-I enlarges, several of these antibodies also enhance the binding of 3G10. This has been interpreted as evidence of a structural rearrangement of apoA-I as a result of the size increase where the α-helix (residues 99–121) that contains the 3G10 epitope is increasingly interacting with lipids resulting in the enhanced expression of this epitope. The increasing interactions of apoA-I helices with lipids in the enlarging discs are compatible with previous reports of a greater apoA-I stability in the large discs. By contrast, cholesterol has limited but specific effects on antibody competitions and decreases the interaction of the N-terminal domain with the domain containing 3G10, either by direct cholesterol protein interaction or by modification of the lipid phase packing.

Apolipoprotein A-I (apoA-I)1 is the primary protein component of plasma high density lipoproteins (HDL) (1, 2). Like several other related exchangeable apolipoproteins, apoA-I contains multiple repeats of 22 amino acids, constituting amphipathic α-helices that are the major lipid-binding domains of the protein (3). These repeats are interrupted by proline or glycine residues that may act as helix-breakers by creating β-turns, providing more flexibility to the amphipathic helices, and allowing apoA-I to conform to the surface of HDL as reviewed by Segrest et al. (4). Differential interaction between monoclonal antibodies and HDL support the hypothesis that the conformation of apoA-I is not the same in all HDL subspecies, depending on size or lipid composition (5, 6). This is in agreement with the demonstration that changes in reconstituted LpA-I composition modulate the net charge and the α-helicity of apoA-I and the stability of its helical segments (7). More recently, it has been demonstrated that a particular apoA-I conformation present in pre-β₂-HDL could have a major role in promoting the efflux of cellular cholesterol (8). In general, data from the literature suggest that apoA-I conformation is both variable and critical for HDL metabolism, but study of its precise organization on the surface of native HDL remains limited by the great heterogeneity of this class of lipoproteins (9, 10). Reconstituted complexes of isolated apoA-I with defined molar ratios of phospholipids and cholesterol have been used to prepare discoidal or spherical HDL in vitro (11–13). These complexes, similar to some HDL observed in vivo, exist as particles of very reproducible sizes and compositions and, of particular interest, as discretely sized particles containing a constant number of apoA-I/particle (reviewed in Refs. 14 and 15). As apoA-I conformation changes between particles, mobile regions (termed hinged domains) have been postulated to explain and regulate the variable size, apolipoprotein composition, and shape of HDL (10, 16). This hypothesis is supported by physical and chemical modification and immunochemical studies of apoA-I within reconstituted complexes of different

1 The abbreviations used are: apoA-I, apolipoprotein A-I; HDL, high density lipoprotein; LpA-I, apoA-I containing reconstituted lipoprotein; mAb, monoclonal antibody; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; RIA, radioimmunoassay; Lp2A-I, reconstituted discoidal lipoproteins containing two apoA-I per particle; PBS, phosphate-buffered saline.

*This work was supported by a group grant from the Medical Research Council of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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lipid composition (reviewed in Ref. 17) and more recently, by analogy to other apolipoproteins whose crystal structure has been solved (18, 19). Nevertheless, little is known about the specific position of the domains and residues involved in the binding of phospholipids or cholesterol in these lipoproteins, and the existence of a hinge domain area is still subject of speculation.

Epitope expression studies using reconstituted LpA-I have shown that binding to lipids produces drastic changes in apoA-I conformation and that particle size can also modify specific domains, such as a central hinged domain constituted by two adjacent antiparallel $\alpha$-helices (16, 20, 21). The inhibitory or enhancing effects of anti-apoA-I mAbs on lectin:cholesterol acyltransferase reaction with LpA-I also demonstrate the importance of the central domain of apoA-I (22). Although characterized by well defined molar ratios, the particles used in previous studies often vary in more than one component at a time, making the interpretation difficult. Recently, a new approach to prepare reconstituted HDL has been published (23).

Simpler than previous methods, this approach is particularly accommodating for compositional manipulations of the particles and gives a larger scale for varying some components than that previously obtained (23). Using this method, we have undertaken a study of the immunoreactivity of apoA-I epitopes in order to define the changes of apoA-I conformation specifically associated with variations in either phospholipid or cholesterol contents of the particles. This is the first study to relate the contribution of individual lipids in reconstituted HDL to apoA-I epitope expression. The results confirm the mobility of the central area of apoA-I, which responds differentially and specifically to changes in phospholipid or cholesterol concentration. An unexpected implication of the N terminus region of the protein during size changes has also been observed, a region previously described by a complex tertiary structure (20).

**EXPERIMENTAL PROCEDURES**

Materials—Cholesterol and sodium cholate were purchased from Sigma, 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) and guanidine HCl (GdnHCl) were obtained from Avanti Polar Lipids (Birmingham, AL) and from Life Technologies, Inc., respectively. All other reagents were analytical grade.

Purification of Apolipoprotein A-I—Human HDL was isolated from pooled plasma from normal volunteers by sequential ultracentrifugation as described previously (24). HDL was delipidated, and the apoA-I was prepared according to the method of Brewer et al. (25). The purity of the apoA-I preparations was confirmed by SDS-polyacrylamide gel electrophoresis on 12% acrylamide gels (Pharmacia Biotech Inc.) and reference globular protein standards 2G11 and 4A12, first characterized by Petit et al. (30), were purchased from SANOFI Inc (Paris, France), whereas antibodies A05, A51, A16, A11, A17, A03, A07, and A44, also previously reported (20), were provided by the Institut Pasteur, Lille. The control mAb 2H2 is an antibody against rat synthetic atrial natriuretic factor (31). All mAbs were murine IgG, purified on protein G-Sepharose or protein A-Sepharose (Pharmacia) and proven free of murine apoA-I (not shown). The location of epitopes recognized by all these mAbs is summarized in Fig. 1.

Solid Phase Radioimmunoassay of apoA-I—Solid phase radioimmunoassays were done as described previously (21). Briefly, Immulon II Removawells were coated with 0.2 $\mu$g of apoA-I in N, N', N'-trimethyl-1-propanol (125 mM NaHCO$_3$, 0.1% Tween and 0.02% NaN$_3$) containing 0.02% NaN$_3$ diluted, and then saturated with 250 $\mu$l of gelatin (0.5% in PBS, pH 7.2, and 0.02% Na$_3$PO$_4$). Anti-apoA-I mAb (predetermined dilution) was mixed with serial dilutions of the competitive antigen (LpA-I) in reaction buffer (0.1% gelatin/0.02% Na$_3$PO$_4$ in PBS, pH 7.2) and incubated in the coated and saturated wells for 1 h at room temperature. After three washes with 0.05% Tween and 0.02% Na$_3$PO$_4$ in PBS, pH 7.2, 100 $\mu$l of 125$I$-labeled rabbit anti-mouse IgG in reaction buffer was added, and the wells were incubated for another 1 h at room temperature. Finally, the wells were washed (3 times) and the cpm counted. Results were expressed as B/B$_0$, where B and B$_0$ represent the cpm in the presence and absence of competing antibody, respectively, at various molar ratios between competitor and capturing mAbs.

**RESULTS**

Characterization of Discoidal Lp2A-I Varying in Lipid Composition—Five discoidal Lp2A-I particles were prepared using different starting POPC/apoA-I ratios and isolated as described by others (23). After isolation, they were analyzed in terms of homogeneity, size, and lipid composition (Table I). Chemical cross-linking of apoA-I shows that all particles contain two molecules of apoA-I (data not shown); all particles also exhibit only one band on nondenaturing gel electrophoresis, which corresponds to homogeneous LpA-I preparations (not illustrated). As expected, the increase of POPC content from 31 to 156 molecules/apoA-I is positively correlated with the particle size, ranging from 8.8 to 10.2 nm (Table I). The sizes obtained are in general agreement with those reported for particles prepared with the same method (23).

Five other particles, varying in cholesterol content, have also been prepared (Table I). They show homogeneity on gradient gel electrophoresis (not illustrated). As shown in Table I, only...
the cholesterol composition is significantly different, ranging from 0 to 11 molecules/apoA-I; their sizes are not statistically different from what is obtained with similar POPC content alone.

Immunoreactivity of apoA-I in Discoidal Lp2A-I Varying in Size—The immunoreactivities of 13 antibodies, directed against epitopes spanning most of the apoA-I sequence with isolated Lp2A-I varying in size are shown in Fig. 2A. The ED_{50} calculated from displacement curves obtained with each Lp2A-I demonstrate significant difference in all domains of apoA-I.

In the N-terminal domain, the epitope for mAb 4H1 (residues 2–8) is progressively less reactive as the particle size of Lp2A-I increases. In contrast, four antibodies, A16, A51, A05, and 2G11, which are specific for epitopes further in the N-terminal region and which overlap differently between residues 8 and 100 (Fig. 1), are more immunoreactive with large than small Lp2A-I.

In the middle of apoA-I, out of four epitopes tested (3G10, A11, 5F6, and A03), only the two central ones are modified when the particle size increases. These are the epitopes for mAbs A11 and 5F6, which overlap between residues 118 and 132 and show a decreased immunoreactivity (Fig. 2A). Interestingly and logically, the two other epitopes (3G10 and A03) surrounding that region are unchanged. This is in agreement with what we observed previously for the same epitopes of apoA-I with another panel of reconstituted discoidal Lp2A-I, which, however, differed both in size and composition (21).

In the C-terminal half of apoA-I, we have tested three antibodies, two (A07 and A44) that react with overlapping epitopes situated between residues 149–186 and 4A12, which recognizes the sequence 173–205. Despite the significant overlap of the two first epitopes, A07 is significantly less immunoreactive with the larger LpA-I as compared with A44, which is more reactive, a phenomenon previously reported (21). The epitope for mAb 4A12 is clearly unaffected by the size. As a consequence of this observation, we can now attribute the changes in reactivity of 4A12 with Lp2A-I particles observed in previous experiments (6, 21) to changes in lipid composition rather than to changes in particle size.

The results obtained with the competitive RIA demonstrate that increasing the size of the discoidal particles induces a major change in the conformation of the entire apoA-I molecule as specific domains distributed along most of the sequence show modifications of their immunoreactivity. However, each domain appears differently affected at different stages of particle enlargement (Fig. 2A). Starting with changes occurring between the small discs (ratios 31/1 to 94/1), the central part of apoA-I represented by the epitopes 5F6 and A11 seems modified with the initial size increments; with the larger discs (122/1 and 156/1), the changes of immunoreactivity appear predominantly in the N-terminal domain (A16, A51, A05, and 2G11), where we observe high increases in immunoreactivities, but excluding the extreme N-terminal epitope, 4H1, which exhibits a very significant decrease in immunoreactivity. The epitopes (A44 and A07) toward the C terminus show less drastic and more progressive modification of their immunoreactivity than epitopes in other domains. Furthermore, related to those changes of immunoreactivity, few RIAs have displayed significant differences in the calculated slopes, reflecting little differences in binding affinities of most mAbs for their specific epitopes with the various particles (Table II). Only in the largest particles (ratio 156/1) do we note significant changes in antibody affinities (mAbs 4H1, 2F1, and A16). A comparison of the Immunoreactivity of apoA-I in Lp2A-I Varying in Cholesterol Content—Analysis of the immunoreactivities of this Lp2A-I panel shows very different results compared with what are obtained with varying POPC content. In the N-terminal region, the immunoreactivity of the epitope for 4H1 increases significantly with the addition of 2 mol or more of cholesterol, and in the central domain, we observed highly significant increases in immunoreactivity for only two overlapping epitopes, 5F6 and A11 (Fig. 2B). All of these changes of immunoreactivity are opposite to those that were obtained for

**Table I**

| Size (nm) | POPC/Chol/apoA-I a,b |
|----------|----------------------|
| Lp2A-I with varying POPC |
| 8.8 (0.1) | 31.1 (1.0):0:1 |
| 9.4 (0.2) | 69.6 (4.7):0:1 |
| 9.8 (0.3) | 93.8 (5.1):0:1 |
| 9.9 (0.3) | 122.1 (7.5):0:1 |
| 10.2 (0.2) | 156.1 (10.6):0:1 |
| 9.7 (0.2) | 85.9 (3.7):0:1 |
| 9.9 (0.1) | 86.3 (10.7):0:1 |
| 9.8 (0.2) | 88.8 (4.9):3.1 (0.3):1 |
| 9.7 (0.2) | 87.4 (9.4):7.2 (0.4):1 |
| 10.0 (0.2) | 84.4 (7.2):11.1 (1.3):1 |

**a** Hydrodynamic diameters from nondenaturing gradient gel electrophoresis.

**b** Discoidal complexes prepared by cholate dispersion/Bio-Beads removal method; composition after purification by Superose 6 chromatography.

The number of apoA-I molecules/particle obtained by crosslinking with dimethyl suberimidate was two for each preparation.

**Fig. 1. Epitope map of apolipoprotein A-I.** The positions of epitopes recognized by these mAbs have been previously defined (20). The names of mAbs used in this study are placed above the solid bars, which represent the sequences recognized by these mAbs. The dashed lines on either side of the bars indicate that the antigenic recognition at this site may extend further.
the same epitopes when Lp2A-I phospholipid content increases (Fig. 2A). The immunoreactivities reached by 5F6, 4H1, and A11 with the discs containing the highest amount of cholesterol are of the same magnitude as those obtained with the small (4H1 and 5F6) and medium (A11) size discs, respectively, without any cholesterol. Except for 4A12, which does not show any change in immunoreactivity, the effect of cholesterol on those epitopes is quite similar to data that we obtained with native spherical HDL (6). Finally, none of the RIAs carried out with epitopes is quite similar to data that we obtained with native spherical HDL (6). The immunoreactivities reached by 5F6, 4H1, and A11 epitopes when Lp2A-I phospholipid content increases are of the same magnitude as those obtained with the small (4H1 and 5F6) and medium (A11) sizediscs, respectively, with few and rather limited structural modifications within the N-terminal domain. There is an increase in the competition of A07 binding to the C-terminal domain. It is also the case for the competitions between mAbs binding to the central domain (3G10, 5F6, and A03) and mAbs binding to epitopes of the adjacent C-terminal region (A07, A44, and 4A12). However to logically interpret the competitions between A05 and A16 binding to the N-terminal domain and A07 and 4A12 binding to the C-terminal domain, we need to take into account not only intramolecular competitions but also the presence of two apoA-I in Lp2A-I and the possibility of intermolecular competitions (see "Discussion").

Effect of Increasing the Lp2A-I Phospholipid/ApoA-I Ratio on the Competition between Antibodies—The competitions among pairs of mAbs for binding to Lp2A-I have been studied in particles where the POPC/apoA-I ratio increases progressively from 31/1 to 156/1, and the results are summarized in Fig. 3 (A–E). As expected the changes in the phospholipid to apoA-I ratio cause specific modifications in the competitions, reflecting the structural modifications induced on apoA-I bound to a progressively enlarging lipoprotein. The increasing phospholipid content causes either decreases or increases in the competition between specific mAbs, and in the absence of any major change in the affinity of these mAbs (Table II), these changes are largely related to lipid-induced modifications of apoA-I tertiary structure that change the relative position of epitopes. However, in some cases, as we will discuss below, the binding of mAbs to Lp2A-I also appears to contribute to the conformational modification of apoA-I. There are few and rather limited structural modifications within the N-terminal domain. There is an increase in the competition of 2F1 with A05 (Fig. 3B) Further downstream, the central domain represented by the epitope for 3G10 appears to move closer to the N terminus as competitions increase between 3G10 and A05 or 2F1 (Fig. 3A and B) and between 4H1 or A16 and 5F6 (Fig. 3D). At the same time there are increasing

![Fig. 2. Immunoreactivity of apoA-I epitopes in Lp2A-I as a function of increasing POPC content (A) or increasing cholesterol content (B). The composition of each Lp2A-I preparation is given in the insets, and the epitopes are positioned on the x axis as they appear in apoA-I sequence. They can be grouped as representative of the N-terminal (4H1 to 2G11), the central (3G10 to A03), and the C-terminal regions (A07 to 4A12). Each bar represents the ED50 (versely proportional to immunoreactivity) with its standard deviation (a, b, c, and d indicate the decreasing degree of significance relative to ED50 for the first particle. a, p < 0.05; b, p < 0.01; c, p < 0.001; d, p < 0.001).](http://www.jbc.org/)

### Table II

Antibodies affinity for apoA-I in Lp2A-I with varying POPC content

| mAbs (epitopes)* | 31/1 | 70/1 | 94/1 | 122/1 | 156/1 |
|-----------------|------|------|------|-------|-------|
| 4H1             | -2.0 ± 0.1 | -1.5 ± 0.3 | -1.4 ± 0.5 | -1.4 ± 0.6 | -1.3 ± 0.2** |
| 2F1             | nd | -5.0 ± 1.3 | -5.9 ± 1.2 | -4.2 ± 0.7 | -2.7 ± 0.4* |
| A16             | -0.8 ± 0.1 | -0.8 ± 0.1 | -0.9 ± 0.1 | -0.9 ± 0.1 | -1.0 ± 0.1 |
| A51             | -0.4 ± ND | -0.5 ± ND | -0.5 ± ND | -0.9 ± ND | -0.6 ± ND |
| A05             | -0.9 ± 0.3 | -1.2 ± 0.3 | -1.2 ± 0.5 | -1.2 ± 0.4 | -1.4 ± 0.5 |
| 2G11            | -1.0 ± 0.1 | -0.8 ± 0.2 | -1.0 ± 0.3 | -1.1 ± 0.2 | -1.5 ± 0.7 |
| 3G10            | -2.3 ± 0.5 | -3.0 ± 1.1 | -2.4 ± 1.1 | -2.1 ± 0.7 | -1.3 ± 0.2** |
| A11             | -1.2 ± 0.1 | -1.2 ± 0.1 | -1.2 ± 0.2 | -1.3 ± 0.1 | -1.0 ± 0.1 |
| 5F6             | -2.2 ± 1.0 | -1.7 ± 0.9 | -1.4 ± 0.5 | -1.4 ± 0.7 | -1.4 ± 0.7 |
| A03             | -1.7 ± 0.5 | -2.1 ± 0.4 | -2.0 ± 0.3 | -1.8 ± 0.4 | -1.6 ± 0.3 |
| A07             | -2.1 ± 1.1 | -1.4 ± 0.2 | -1.3 ± 0.4 | -1.1 ± 0.3 | -1.6 ± 0.6 |
| A44             | -1.1 ± 0.1 | -1.5 ± 0.4 | -1.5 ± 0.3 | -1.7 ± 0.3* | -1.6 ± 0.3 |
| 4A12            | -1.9 ± 0.4 | -2.5 ± 0.6 | -2.2 ± 0.8 | -2.7 ± 0.5 | -2.3 ± 0.5 |

* mAbs are separated in three groups representing the N-terminal region (4H1 to 2G11), the central region (3G10 to A03), and the C-terminal region (A07 to 4A12).

** p < 0.01, comparison versus 31/1 Lp2A-I.

* p < 0.05, comparison versus 31/1 Lp2A-I.
competitions between the mAbs reacting to the central epitope 3G10 and the C-terminal epitopes A07 (not illustrated) and 4A12 (Fig. 3E), suggesting an increasing proximity for these domains as well.

The above evidence, which indicates that Lp2A-I size increase appears to be accompanied by a conformational change of the molecule where the central domain appears to come closer to both the N-terminal and the C-terminal domains, is somewhat unexpected. However, as we will analyze these reactivities of the antibodies against the central epitope 3G10 to 11 mol/apoA-I while maintaining POPC content and size of Lp2A-I constant modifies only the immunoreactivity of 4H1, A11, and 5F6 (Fig. 2B). When we compared the competition between mAbs for binding to Lp2A-I with or without cholesterol, very unique changes were observed (Tables IV and V). Most remarkably, the presence of cholesterol causes absolutely no change in the competition between most pairs of mAbs but provokes a significant decrease in the enhancement normally exerted by four mAbs reacting to overlapping N-terminal epitopes on 3G10 binding (Table V). This appears to reflect a specific change in the relative position or interaction of the N-terminal domain in relation to the central domain where 3G10 is located, maybe an as a result of a direct interaction of cholesterol with these domains or modified packing of the lipid phase.

**DISCUSSION**

Previous immunochemical studies from this laboratory on the effect of lipid composition on apoA-I structure in native HDL (6) and in discoidal reconstituted LpA-I (21) have demonstrated the significant effects of phospholipids and cholesterol in distinct domains. In discoidal LpA-I, the variation in phospholipid/apoA-I ratio causes an increase in epitope expression in two large domains of the N-terminal and C-terminal regions (spanning residues 14–90 and 148–209, respectively). Between these domains, the epitopes of a central region (residues 99–143) decreased in immunoreactivity as the particle size increased (21). These observations first suggested to us (20) that these regions might constitute the two lipid-binding domains separating a hinge domain postulated by others to explain the existence of discrete size populations in HDL particles (16).

Here the progressive changes in the concentrations of either phospholipids or cholesterol in defined Lp2A-I provide a precise analysis of their respective effect on apoA-I conformation and confirm the notion of its three domain structure.

Effect of Phospholipids on the Immunoreactivity and Conformation of ApoA-I—As the POPC content and size of Lp2A-I increase, significant changes in immunoreactivity are observed for almost all epitopes. These results demonstrate that multiple regions are implicated in the structural adaptability of apoA-I in particles of increasing size. A summary of the immunoreactivity of the various epitopes as a function of the particle composition is presented in Fig. 4. Numerous epitopes increase in immunoreactivity with increasing POPC content; they are mostly located in the N-terminal end (A16, A51, A05, and 2G11), with another one in the C-terminal domain (A44). Two epitopes located in the middle area of apoA-I decrease in immunoreactivity (A11 and 5F6). Interestingly, the boundaries of this area are represented by two epitopes (3G10 and A03) that show no change in their immunoreactivity. Previous results (20, 21) and the confirmation obtained here both support the

**TABLE III**

| mAbs (epitopes) | POPC/A-I ratio |
|----------------|----------------|
|               | 80/1 | 80/4 | 80/8 | 80/12 |
| 4H1           | −1.9 ± 0.2 | −1.7 ± 0.2 | −2.1 ± 0.9 | −2.2 ± 0.8 |
| 2F1           | −4.0 ± 0.6 | −3.6 ± 0.7 | −4.0 ± 0.3 | −4.4 ± 0.8 |
| A16           | −1.0 ± 0.2 | −0.7 ± 0.2 | −0.9 ± 0.3 | −0.8 ± 0.1 |
| A51           | −0.8 ± 0.3 | −0.9 ± 0.4 | −1.2 ± 0.4 | −1.3 ± 0.7 |
| A05           | −1.3 ± 0.3 | −1.5 ± 0.5 | −1.4 ± 0.4 | −1.1 ± 0.1 |
| 2G11          | −1.1 ± 0.6 | −0.9 ± 0.2 | −1.0 ± 0.2 | −1.3 ± 0.4 |
| 3G10          | −3.4 ± 1.0 | −3.4 ± 1.0 | −3.1 ± 0.9 | −3.1 ± 0.9 |
| A11           | −1.4 ± 0.2 | −1.5 ± 0.4 | −1.3 ± 0.2 | −1.3 ± 0.2 |
| 5F6           | −1.7 ± 0.3 | −1.5 ± 0.2 | −1.4 ± 0.3 | −1.7 ± 0.4 |
| A03           | −2.8 ± 0.7 | −2.4 ± 1.0 | −2.5 ± 0.7 | −2.3 ± 0.5 |
| A07           | −1.2 ± 0.7 | ND    | −1.1 ± 0.7 | −1.2 ± 0.1 |
| A44           | −1.8 ± 0.6 | −1.9 ± 0.8 | −1.7 ± 0.5 | −1.8 ± 0.6 |
| 4A12          | −2.1 ± 0.3 | −2.5 ± 0.7 | −2.2 ± 0.6 | −2.3 ± 0.4 |

a mAbs are separated in three groups representing the N-terminal region (4H1 to 2G11), the central region (3G10 to A03), and the C-terminal region (A07 to 4A12).

**TABLE IV**

| Summary of the competitions among pairs of antibody binding to Lp2A-I without cholesterol: Lp2A-I (POPC/A-I, 80/1) |

| Competing mAb | Capture and competing mAb |
|---------------|---------------------------|
| 4H1           | 2F1 | A05 | 3G10 | 5F6 | A07 | 4A12 |
| 11 (3)        | 26 (7) | −30 (13) | 35 (5) | 29 (10) | 16 (4) |
| 99 (1)        | 97 (1) | −24 (9) | 4 (5) | −29 (15) | 14 (7) |
| 27 (5)        | 33 (5) | −41 (28) | 27 (4) | 1 (5) | 74 (5) |
| 83 (1)        | 98 (1) | −63 (8) | 16 (5) | 41 (6) | 32 (11) |
| 48 (3)        | 79 (2) | −62 (19) | 5 (3) | 14 (3) | 9 (10) |
| 49 (3)        | 37 (5) | −80 (16) | 22 (17) | −6 (16) | 80 (8) |
| 7 (4)         | 10 (8) | −4 (15) | −3 (5) | 6 (4) | 1 (3) |
| 5F6           | −1 (4) | 5 (3) | 8 (15) | 99 (1) | 16 (4) | 5 (5) |
| A03           | 14 (4) | 14 (4) | −9 (18) | 67 (3) | 34 (5) | 19 (2) |
| A07           | 16 (3) | 16 (3) | −9 (24) | 20 (9) | 95 (2) | 62 (3) |
| A44           | 24 (3) | 24 (3) | 72 (12) | 43 (5) | 101 (4) | 90 (6) |
| 4A12          | 10 (7) | 7 (3) | 52 (3) | 4 (6) | 46 (7) | 99 (1) |
| 2H2           | 5 (1) | −1 (8) | 2 (7) | 1 (6) | 6 (4) | 0 (4) |

a The results are expressed as the percentages of competition (+) or enhancement of the binding (−) and are presented as the means ± S.D.
concept that the two central epitopes A11 and 5F6 probably identify a central mobile domain, constituted by a hinged pair of helices and located between the epitopes 3G10 and A03 (adjacent to β-turns), which seems involved in the process of disc expansion. For reasons still not understood, the expression of the extreme N-terminal epitope 4H1 parallels the variation in immunoreactivity of the epitopes of the central domain (A11 and 5F6), suggesting, as we discuss below, that the two share some structural and functional characteristics.

In the N-terminal third of apoA-I, for which we earlier proposed a complex and condensed structure based on its high content of multiple overlapping discontinuous epitopes (20), all epitopes display very significant increases in immunoreactivity as the size of Lp2A-I increases. This effect could be mediated by the formation of short helices to accommodate the increasing disc size. Several studies have documented the increase in α-helix content with the size increase (33), which can be related to increase in the number of helical repeats (34) or to the formation of new short segments (23). The changes in lysine pKₐ reported by Sparks et al. (23) also implicated major changes in the N-terminal lysines that reflect the structural rearrangements of this domain with the increase in particle size.

**Fig. 3.** Variation in competitions among pairs of mAbs binding to Lp2A-I as a function of the increase in POPC content. Each panel represents the results of the competitions with a given capture antibody: A, 2F1; B, A05; C, 3G10; D, 5F6; E, 4A12. The competing mAbs are presented on the x axis in the order in which they appear in apoA-I sequence, and for each mAb the competitions are given for each Lp2A-I preparation (see the insets). The competitions are expressed as the percentage of the maximum Lp2A-I bound in the absence of competitor; error bars represent S.D. of three separate experiments. b, c, e, f, and p < 0.01; d, p < 0.05 (comparison with POPC/A-I, 31/1).
by increasing the phospholipid content, the changes in epitope immunoreactivity observed with increasing cholesterol content are very limited to a short area of the central domain, identified by epitopes A11 and 5F6, and to the extreme N-terminal epitope, 4H1 (Fig. 4). All increases in immunoreactivity of these epitopes are proportional to the cholesterol content. It must be noted that the change of immunoreactivity in response to cholesterol is opposite to that observed with POPC, demonstrating that the two lipid have distinct interactions with or effects on the protein resulting in different conformations (Fig. 4). Cholesterol can act directly by interaction with apoA-I and/or indirectly by modification of the physical state of the phospholipids. Our observations could be compatible with the suggestion of others (35, 36) that cholesterol exerts its effect directly by binding to apoA-I, and this should help us to localize the site of this interaction. Sparks and colleagues (37) have observed that although the increase of the phospholipid/apoA-I ratio in LpA-I increases the $\alpha$-helicity of apoA-I, the addition of a small amount of cholesterol decreases $\alpha$-helicity but increases the stability of the remaining helices. Also the addition of 1 mol of cholesterol/apoA-I is sufficient to induce a reduction in the surface potential of the LpA-I (37). This evidence of conformation changes exerted by cholesterol have been interpreted as the result of specific interaction of cholesterol with apoA-I polypeptide chain as first suggested by the energy transfer experiments of Massey et al. (35). Furthermore, titration of lysine pK$_a$ values using NMR spectroscopy is also in accord with our present results and demonstrates the differential effect of phospholipid and cholesterol (23, 37). Indeed, increase in phospholipid content correlates with widespread lysine modifications, whereas the addition of cholesterol changes the pK$_a$ of only one lysine group, tentatively identified as Lys$_{106}$, Lys$_{107}$, Lys$_{238}$, and Lys$_{239}$; in particles containing 2 mol of cholesterol/apoA-I, only some of the four lysines listed above are affected, whereas in particles with 10 mol of cholesterol/apoA-I, the titrations of all four lysines are modified. Taken together with these results, the effect of cholesterol on the immunoreactivity of mAbs A11 and 5F6 with LpA-I suggests that their epitopes, which overlap between residues 99 and 121, have responded to the same modifications that involved Lys$_{106}$, Lys$_{107}$, Lys$_{238}$, and Lys$_{239}$; in particles containing 2 mol of cholesterol/apoA-I, only some of the four lysines listed above are affected, whereas in particles with 10 mol of cholesterol/apoA-I, the titrations of all four lysines are modified. Taken together with these results, the effect of cholesterol on the immunoreactivity of mAbs A11 and 5F6 with LpA-I suggests that their epitopes, which overlap between residues 99 and 141, have responded to the same modifications that involved Lys$_{106}$ and Lys$_{107}$. In the absence of reporter epitopes for the C terminus, we cannot confirm the putative involvement of lysines 238 and 239. Two naturally occurring mutants involving apoA-I Lys$_{107}$ have been described, Lys$_{107}$$\rightarrow$Met, which has a normal ability to interact with lipids, forms discoidal LpA-I and reacts with lecithin:cholesterol acyltransferase (38), and Lys$_{107}$$\rightarrow$0, which forms abnormal LpA-I particles with decreased lecithin:cholesterol acyltransferase substrate activity (39) and decreased binding affinity to adipocytes (40). Therefore, the loss of the charge associated with Lys$_{107}$ is not essential to lipid binding properties of apoA-I, but deletion of Lys$_{107}$ changes the orientation of side chains in the $\alpha$-helix and disrupts the lipid binding properties. The informative value of the observations cited above has been hindered by the susceptibility of apoA-I to oxidation and denaturation during purification (29, 39), especially in the region of residues 99–121 where multiple epitopes generated by oxidative processes have been demonstrated (29). Others have also shown the importance of apoA-I central domain in cellular cholesterol efflux (8) and in cholesterol esterification (41). The linkage of the epitope at the N terminus, 4H1, with those in the central domain, A11 and 5F6, based on the similarity of their response to cholesterol is now well documented as a result of this and previous studies (21, 42). The specificity of the

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**Table V**

Summary of the competitions among pairs of antibody binding to Lp2A-I with cholesterol: Lp2A-I (POPC/Chol/A-I, 80/8/1)

| Competing mAb | Capture and competing mAb |
|---------------|----------------------------|
| 4H1 | 11 (8)$^a$ | 26 (8) | 16 (1) | 36 (8) | 23 (14) | 11 (5) |
| 2F1 | 98 (1) | 95 (2) | 4 (13) | 5 (8) | 29 (6) | 17 (3) |
| A16 | 27 (5) | 31 (7) | 14 (4) | 26 (8) | 13 (9) | 66 (7) |
| A05 | 82 (3) | 98 (1) | 26 (16)$^b$ | 16 (4) | 34 (2) | 34 (7) |
| 2G11 | 46 (4) | 76 (3) | 1 (9) | 11 (10) | 14 (4) | 26 (4) |
| 3G10 | 49 (4) | 38 (7) | 82 (6) | 23 (13) | 3 (22) | 83 (10) |
| A11 | 6 (7) | 2 (5) | 1 (5) | 2 (6) | 8 (2) | 7 (5) |
| SF6 | 13 (3) | 15 (5) | 1 (5) | 19 (15) | 44 (4) | 26 (4) |
| A07 | 17 (5) | 19 (6) | 1 (8) | 20 (7) | 98 (2) | 65 (2) |
| A44 | 26 (4) | 38 (8) | 64 (11) | 48 (3) | 98 (9) | 90 (4) |
| 4A12 | 10 (9) | 1 (11) | 51 (1) | 1 (4) | 49 (7) | 99 (0) |
| 2H2 | 2 (5) | 5 (11) | 4 (7) | 8 (5) | 1 (5) | 4 (7) |

$^a$The results are expressed as the percentages of competition (+) or enhancement of the binding (−) and are presented as the means ± S.D.

$^b$Indicates a significant difference (p < 0.01) compared with Lp2A-I without cholesterol in Table IV.
The competition assay has been modified to include only two competing mAbs without the complication of the possible competitions between the capture mAb and the other two antibodies.

For the interpretation of these competitions, we can consider that the observed competitions are either exclusively intramolecular or both intramolecular and intermolecular depending on the position of the epitopes and the relative position of the two apoA-I at the surface of the particle (Fig. 5). Considering first the competitions observed in a constant composition mode with the median size Lp2A-I, the results are consistent with the known position of epitopes in the primary sequence and suggest a rather compact structure with competitions within the N-terminal region, competitions between epitopes in the central domain and epitopes in the N- or C-terminal regions, and competitions within the C-terminal region. Most of these competitions can be logically interpreted as intramolecular competitions as presented in the model of Lp2A-I (Fig. 5). However, the competitions between mAbs A16 and A05, which react in the N-terminal domain, and mAb 4A12, which reacts with the farthest epitope in the C-terminal domain, are not compatible with an intramolecular competition in the apoA-I model of Fig. 5. However, in this Lp2A-I model where we only assume that the apoA-I follow one another in an N- to C-terminal arrangement around the discoidal particle, we can easily propose that the competitions between these mAbs are intermolecular. This model does not require a particular orien-
ApoA-I Conformation in Discoidal LpA-I

The amount of POPC increases, the interaction of that pair of helices with lipids is enhanced and a larger disc is formed also causing a reorganization of the N-terminal region. In contrast with increasing cholesterol, the α-helix content decreases (37) and the central domain is probably unstable, giving a higher expression of the central epitopes (more evident with 5F6 than A11, Fig. 2B).

Other evidence for the conformational sensitivity of the α-helix 99–121 containing the epitope for 3G10 is the effect of increasing cholesterol content in Lp2A-I, which decreases the normal enhancement of 3G10 binding by mAbs binding to the N-terminal domain. Indeed, cholesterol would be expected to increase the acyl chain order and thus decrease the mobility of the lipid-associated protein domains (11, 44).

In conclusion, this study confirms the notion that we previously introduced of a three domain structure for apoA-I, an N-terminal domain extending up to residue 99, a central domain centered between residues 99 and 121 but possibly extending to residue 143, and a C-terminal domain. We have demonstrated that variations in the lipid components of discoidal reconstituted Lp2A-I can have significant effects on the conformation of the entire apoA-I, particularly in its central domain. Cholesterol and phospholipid contents have specific and opposite effects on the conformation of this region of apoA-I, which has been suggested to be important in lecithin:cholesterol acyltransferase activation (22, 41) and may also be implicated in apoA-I-mediated efflux of cellular cholesterol (8). Thus the lipid composition of LpA-I can modulate its metabolism through changes of apoA-I conformation. This appears to be mediated not only by the amount of cholesterol but also by the size of the disc. We also confirm here the existence of a link (structural, functional, or both) between the extreme N terminus, represented by the epitope for 4H1, and the central domain. Both appear significantly involved or affected by cholesterol. Finally, the domain for which we observed the least modification of conformation in this study is located in the C-terminal half of the protein, a region involved in lipid binding and possibly also in interactions with cellular binding sites. Thus, apoA-I conformation appears to be crucial in HDL metabolism, with different domains having specific functions.

Acknowledgments—We thank Drs. Ross Milne and Daniel Sparks for friendly advice and criticism in the course of this work, Vivian Franklin for technical assistance, and Anne Buie for secretarial assistance.

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