Expression of human apolipoprotein A-I epitopes in high density lipoproteins and in serum

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Abstract The expression and immunoreactivity of apolipoprotein (apo) A-I epitopes in high density lipoproteins (HDL) and serum has been investigated using two series of monoclonal antibodies (Mabs) which have been described elsewhere. Series 1 Mabs, identified as 3D4, 6B8, and 5G6, were obtained by immunization and screening with apoA-I, and series 2 Mabs, identified as 2F1, 4H1, 3G10, 4F7, and 5F6, were obtained by immunization and screening with HDL. These Mabs were characterized with respect to their binding to HDL particles in solution. In series 2 Mabs, 2F1, 3G10, and 4F7, which react with apoA-I CNBr-fragments 1 and 2, could precipitate 100% of ^{125}I-labeled HDL, while 4H1 and 5F6, which react with CNBr fragments 1 and 3, precipitated 90 and 60% of ^{125}I-labeled HDL, respectively. Therefore, three distinct epitopes mapped to CNBr fragments 1 and 2 have been identified which are expressed on all HDL particles, indicating that several antigenic do mains exist on apoA-I which have the same conformation on all apoA-I-containing lipoproteins. The Mabs reacting at these sites have significantly higher affinity constants for ^{125}I-labeled HDL than those that failed to precipitate 100% of HDL. This suggests that the high affinity Mabs react with apoA-I epitopes that are both expressed on all lipoproteins and located in thermo-dynamically stable regions of the molecules. All Mabs from series 1 precipitated 35% or less of ^{125}I-labeled HDL prepared from freshly collected serum, but the proportion of HDL particles expressing the epitopes for these Mabs doubled or more upon serum storage at 4°C. The time course of the alteration of apoA-I antigen in vitro was measured in three normolipemic donors. Upon storage of serum at 4°C, the immunoreactivity of series 2 Mabs (4H1, 3G10) remained unchanged. However, the immunoreactivity of series 1 Mab 3D4 increased linearly at 38%/day for 4 weeks and by 12 weeks had plateaued at about 280-fold compared to day 1. The immunoreactivity of other series 1 Mabs also increased significantly with time in vitro. This process was partially inhibited in the presence of EDTA and by addition of antioxidants, however, the exact molecular nature of this in vitro alteration of apoA-I antigen was not identified.—**Marcel, Y. L., D. Jewer, C. Vézina, P. Milthorp, and P. K. Weech.** Expression of human apolipoprotein A-I epitopes in high density lipoproteins and in serum. *J. Lipid Res.* 1987. **28:** 768—777.

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Apo A-I is the major protein constituent of high density lipoproteins (HDL) and lymph chylomicrons and represents the major apolipoprotein in normal subjects. As such apoA-I has been extensively studied and well characterized both in terms of its molecular structure and metabolism and of its role in lipid transport (1–3). The immunochemical properties of apoA-I have also been the object of many investigations (4) and several panels of monoclonal antibodies (Mabs) directed against apoA-I have been described (4–8). Our first series of Mab was obtained from mice immunized with purified apoA-I and after screening with purified apoA-I. These series 1 Mabs identify three distinct epitopes that have been mapped to the CNBr fragments 1, 2, and 3 of apoA-I (6) and which are better expressed on stored than on freshly collected sera (4). Our series 2 Mabs were obtained by immunization and screening using freshly prepared HDL, and they react with another set of five distinct epitopes that are also distributed on CNBr fragments 1, 2, and 3 of apoA-I (8) and which appear unaffected by storage of the antigen (4).

Curtiss and Edgington (5) have also reported a panel of anti-apoA-I Mab that identify three distinct epitopes and were characterized by their inability to precipitate more than 60% of ^{125}I-labeled HDL when used alone or more than 80% when used in combination (5). This finding was interpreted as evidence that HDL particles are heterogeneous and that none of these three epitopes were expressed on all HDL species. The possible dominance of such antigenic determinants on apoA-I appears to be supported by the preliminary report of Krul et al. (7) who described seven different anti-apoA-I Mabs that could be divided into two groups as a function of their capacity to bind to ^{125}I-labeled HDL: those that bound 75% of the HDL and those that bound only 40%. However, in the present studies, we demonstrate that three Mabs from our

Abbreviations: apo, apolipoprotein; HDL, high density lipoproteins; Mab, monoclonal antibody; EDTA, ethylenediaminetetraacetate; BHT, butylated hydroxytoluene; PBS, phosphate-buffered saline; RIA, radioimmunoassay; PMSF, phenylmethylsulfonylfluoride.

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series 2 that identify three distinct epitopes can singly be used to immunoprecipitate the totality of ^{125}I-labeled HDL. This demonstrates, therefore, that while apoA-I conformation may be heterogeneous in different apoA-I-containing particles, domains exist within the molecule that express the same epitopes an all particles.

In contrast, none of the Mabs from our series 1 could precipitate more than about 40% of freshly prepared HDL, but the percentage of bound particles increased with storage. We also report here on the time course of the in vitro alteration of apoA-I epitopes and present evidence that this process can be minimized by the presence of EDTA and antioxidants.

MATERIALS AND METHODS

Preparation of serum and plasma

Blood was drawn from normolipemic subjects by venipuncture and collected into Vacutainer tubes. Serum was prepared by drawing blood into tubes without anticoagulant. The blood was allowed to clot at room temperature for 1 to 2 hr. Plasma was prepared by drawing blood into tubes containing EDTA (1 mg/ml). Sodium azide and phenylmethylsulfonyl fluoride were added to final concentrations of 0.02% and 1 mM, respectively.

To study the effect of storage on immunoreactivity of 4H1, 3G10, and 3D4 by radioimmunoassay, serum samples were aliquoted and stored at either 4°C, −20°C, or −80°C.

To study the effect of serum additives on the immunoreactivity of series 1 and series 2 anti-apoA-I Mabs, all samples contained 1 mM PMSF and 0.02% NaN_3. Plasma (1 mg/ml EDTA) and sera samples of three donors were aliquoted and stored at 4°C or −80°C either as such, or as sera to which were added antioxidants (0.05% α-tocopherol, 500 μg/ml glutathione, 10 ng/ml butylated hydroxytoluene), or antioxidants plus an antibacterial agent (1000 units/ml penicillin–streptomycin) and antiproteases: aprotinin (100 kallikrein-inhibitory units/ml) and soybean trypsin inhibitor (10 μg/ml).

Solid phase radioimmunoassay of apoA-I

Immulon II Removawells were coated with 100 μl apoHDL diluted to a concentration of 2μg protein/ml with 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% NaN_3, pH 9.6. The wells were placed in a moist chamber at room temperature for 2 hr. The coating solution was discarded and the wells were washed once with phosphate-buffered saline (PBS), pH 7.2 (wash I). The wells were then saturated with 300 μl of 0.5% gelatin, 0.02% NaN_3 in PBS, pH 7.2, for 30 min; the solution was discarded, and the wells were washed once with wash I.

Titration of monoclonal antibodies. Serial doubling dilutions of series 1 antibodies 5G6, 3D4, and 6B8 and series 2 antibodies 2F1, 4H1, 3G10, 4F7, and 5F6 were prepared in 0.5% gelatin, 0.05% Tween 20, 0.02% NaN_3 in PBS, pH 7.2 (reaction buffer), in a volume of 100 μl in microtiter plates (Linbro) using a multichannel pipet (Titertek). The starting antibody dilution was 1/500. A further 100 μl of reaction buffer was added to all wells and 100 μl of this mixture was transferred to the coated wells and incubated for 1 hr at room temperature in a moist chamber. The antibody solution was discarded and the wells were washed three times with 0.05% Tween 20, 0.02% NaN_3 in PBS, pH 7.2 (wash II). Labeled rabbit anti-mouse IgG was added as described below.

Competitive assays. The antibody dilution chosen for competitive assays was that which gave 60% maximum binding in the titration above. Serial doubling dilutions of competing antigen (serum) were prepared in reaction buffer in a volume of 100 μl as above. To the serum dilutions, 100 μl of the appropriate antibody dilution was added, the solution was mixed, and 100 μl was immediately transferred to the coated wells. The wells were incubated for 1 hr. The solution was discarded and the wells were washed three times with wash II.

To each of the wells, 100 μl containing 200,000 cpm of ^{125}I-labeled rabbit-anti-mouse IgG diluted in reaction buffer was added. The wells were incubated for 1 hr. The solution was discarded, the wells were washed three times with wash II, and each well was counted in a gamma counter. Results were expressed as B/Bo where B and Bo represent the cpm bound in the presence and absence of competing antigen, respectively.

Preparation of HDL and delipidated HDL

HDL were prepared by discontinuous density gradient centrifugation as described by Terpstra, Woodward, and Sanchez-Muniz (9) but without prestaining (4). HDL between densities 1.09 g/ml and 1.16 g/ml were identified by color, carefully removed from the gradient, and dialyzed overnight against 10 mM NH_4HCO_3, 0.02% NaN_3, pH 7. The protein concentration was determined by the method of Lowry et al. (10). HDL were delipidated by the chloroform–methanol 2:1 (vol/vol) procedure of Olofsson, McConathy, and Alaupovic (11).

Labeling of HDL with ^{125}I for immunoprecipitation

HDL was labeled with ^{125}I using single reaction Enzymobead radioiodination reagent purchased from Bio-Rad (Chemical Division, Richmond, CA) as previously described (12). To 25 μl of Enzymobead reagent, the following were added: 100 μg of protein in a minimum volume of PBS, pH 7.2, containing 1 mM EDTA, 25 μl of 0.2 M phosphate buffer, pH 7.2, 1.0 mCi of Na^{125}I (Amersham Corporation), and 25 μl of D-glucose. The mixture was incubated at room temperature for 15 min. The sample

was diluted with 200 μl of 1 mM EDTA, 0.02% NaN_3 in PBS, pH 7.2, and applied to a Sephadex G25 column (20 cm × 1.0 cm) equilibrated with 1 mM EDTA, 0.02% NaN_3 in PBS, pH 7.2, to remove free ^{125}I. Fractions of 1 ml were collected. The peak fractions were pooled and dialyzed overnight against 1 mM EDTA, 0.02% NaN_3 in PBS, pH 7.2. Preparations of ^{125}I-labeled HDL had specific activities from 0.78 to 1.51 μCi/μg of protein. The radioactivity precipitable by 12% trichloroacetic acid ranged from 67% to 88%.

Preparation of Pansorbin cells armed with anti-mouse IgG

Pansorbin cells (pickled *Staphylococcus aureus* cells, Calbiochem, CA) were washed three times with 1% BSA, 1mM EDTA, 0.02% NaN_3 in PBS, pH 7.2 (reaction buffer). Between washings, the cells were centrifuged at 3,000 *g* for 15 min at 5°C. The prewashed Pansorbin was armed with rabbit anti-mouse immunoglobulin (Cedarlane Laboratories, Hornsby, Ontario) at a concentration sufficient to saturate the Pansorbin cells and the mixture was left to incubate for 24 hr at 4°C (13). The armed cells were washed three times as above.

Immunoprecipitation of ^{125}I-labeled HDL with monoclonal antibodies

Various anti-apoA-I Mabs and an anti-apoA-II Mab (a generous gift from Linda K. Curtiss, La Jolla, CA) were used in these experiments. To determine nonspecific precipitation, an unrelated Mab 2H2 (anti-rat atrial natriuretic factor) was used. Antibody dilutions were prepared in reaction buffer in a volume of 450 μl, mixed with 50 μl of ^{125}I-labeled HDL (25 ng of protein), diluted in reaction buffer, and incubated for 24 hr at 4°C. To this mixture, Pansorbin cells armed with rabbit anti-mouse immunoglobulin (50 μl) were added and the mixture was further incubated for 18 to 24 hr at 4°C. The Pansorbin was spun down by centrifugation at 3000 *g* for 15 min at 5°C. The pellet was washed once with reaction buffer and then counted in a gamma counter. Several controls were included which contained 450 μl of reaction buffer and 50 μl (25 ng of protein) of ^{125}I-labeled HDL, but no Pansorbin was added. To precipitate the protein, an equal volume of 24% trichloroacetic was added, and the precipitate was spun down by centrifugation at 13000 *g* for 4 min; the supernatant was removed and the pellet was counted. Results were expressed as percentage of radioactivity bound, B/Bo × 100, where B = cpm precipitated by Pansorbin cells and Bo = cpm precipitated in controls by trichloroacetic acid.

Determinations of affinity constants

Competitions between ^{125}I-labeled HDL and HDL were used to determine the affinity constants of series 1 monoclonal antibody 3D4 and series 2 monoclonal antibodies 4H1, 3G10, and 2F1. Serial doubling dilutions of competing antigen unlabeled HDL were prepared in volumes of 100 μl to which were added 100 μl of ^{125}I-labeled HDL (20 ng of protein) and 100 μl of the Mab at the dilution required for approximately 50% maximum binding. Maximum binding in the absence of competing antigen was also determined. The tubes were incubated at 4°C for 18 hr, after which 50 μl of Pansorbin armed with rabbit anti-mouse immunoglobulin was added and the mixtures were incubated for another 4 hr at 4°C. The Pansorbin was spun down and counted as above. The affinity constant, K_a of the antibodies was calculated from the formula (14):

$$K_a = \frac{1}{(C - L)(1 - 1.5b + 0.5b^2)}$$

where C is the molar concentration of competing antigen required for 50% inhibition of ^{125}I-labeled HDL binding to the antibody, L is the molar concentration of ^{125}I-labeled HDL added, and b is the maximum binding of ^{125}I-labeled HDL in the absence of competitor calculated as the proportion of radioactivity bound, B/Bo, as above. The molar concentration of HDL was calculated from the protein mass of HDL (2.5×10^5 g/mol) (12).

RESULTS

Immunoprecipitation of ^{125}I-labeled HDL prepared from fresh and stored serum

Under conditions of antibody excess and of second antibody excess, most Mab from series 2 could precipitate the totality of ^{125}I-labeled HDL **(Fig. 1)**: 2Fl, 3G10, and 4F7 were the most effective while 4Hl could precipitate only 90% of the labeled HDL at dilutions below 1/1000. Labeled HDL prepared from either fresh or stored sera were equally and totally precipitated by each of these Mabs (Fig. 1). Therefore most of the apoA-I epitopes that are identified by Mabs from series 2 are expressed on all apoA-I-containing lipoproteins and, in agreement with the results of RIA experiments described subsequently, these epitopes are unaffected by storage of the serum at 4°C. These results were reproducible, although in some experiments **(Table 1)** the immunoprecipitation by series 2 Mabs of stored HDL was decreased as compared with the corresponding fresh HDL, possibly reflecting an undefined alteration of the stored fraction. Mab 5F6 could precipitate only 50 to 60% of ^{125}I-labeled HDL and was the only Mab from series 2 that precipitated significantly more stored than fresh HDL **(Fig. 2).**

None of the series 1 Mabs was as effective as the series 2 Mabs in precipitating ^{125}I-labeled HDL prepared from fresh serum (Fig. 1). In addition, even at higher antibody concentrations, 3D4 and 6B8 could not precipitate all fresh HDL and the plateau could only be obtained by ex-

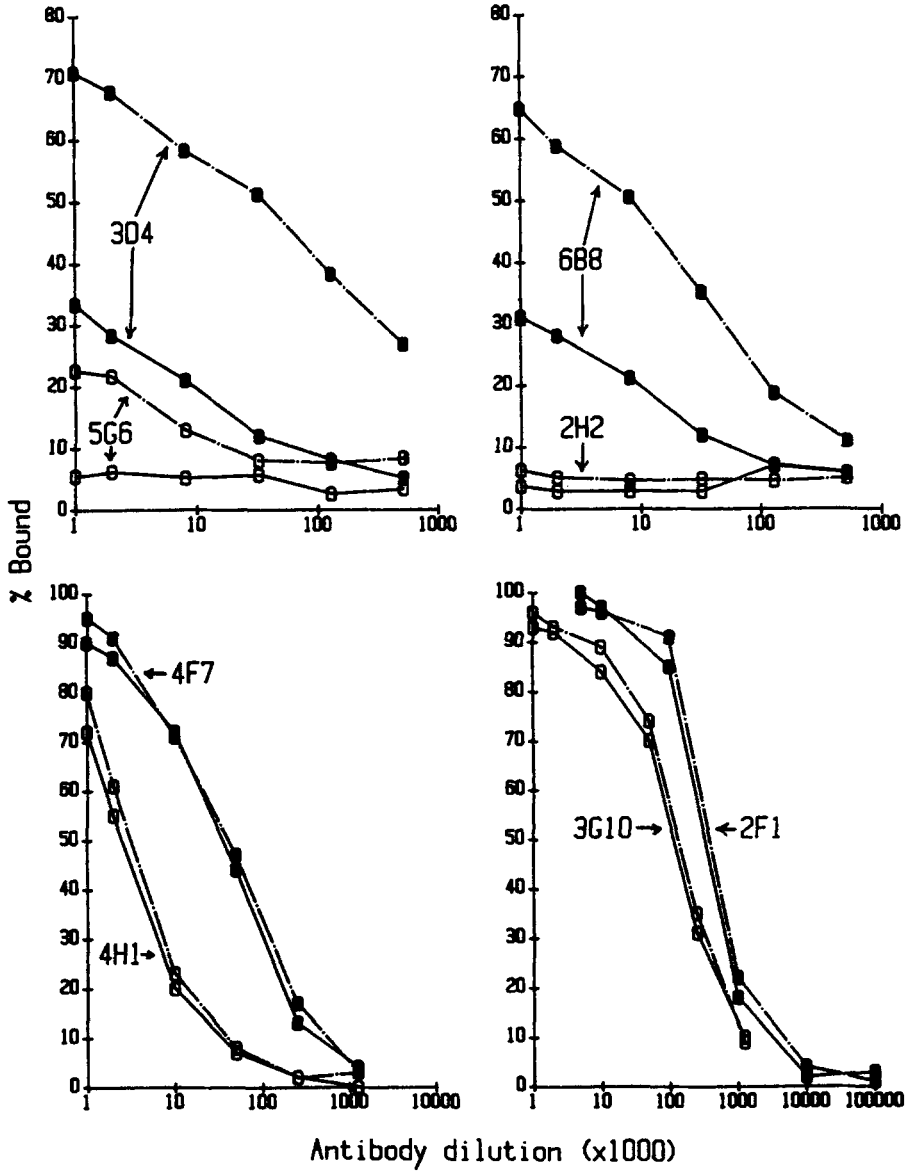

Fig. 1. Immunoprecipitation of fresh and stored ^{125}I-labeled HDL by series 1 and series 2 antibodies. The percentage of trichloroacetic acid-precipitable radioactivity that was bound by each antibody is shown as a function of antibody dilution. For each antibody the solid line shows the result obtained with HDL isolated from fresh serum; HDL from stored serum is shown by the broken line. A control experiment to measure nonspecific binding was made with an unrelated antibody, 2H2. The experiments with antibodies 3D4, 5G6, 6B8, and 2H2 used HDL from fresh serum or serum stored for 46 days at 4°C; both HDL preparations were subsequently stored for 14 days at 4°C prior to the experiment. The experiments with antibodies 4H1, 4F7, 3G10, and 2F1 used HDL from fresh or stored (27 days, 4°C) serum; both HDL were subsequently stored 11 days at 4°C before the experiment.

trapolation (Fig. 2). However, series 1 Mabs reacted significantly better with stored HDL than they did with fresh HDL (Figs. 1 and 2), highlighting the contrast between series 1 and series 2 Mabs: the latter, with the exception of 5F6, having precipitated uniformly well both fresh and stored HDL. Nevertheless, none of the series 1 Mabs could precipitate more than 75% of the labeled fresh HDL. Although the proportion of labeled HDL that expressed the epitopes of series 1 Mabs increased consistently with time in vitro, the phenomenon that produced these effects did not affect all HDL particles and was probably incomplete.

Immunoprecipitation of ^{125}I-labeled HDL by combination of antibodies

In these experiments, we wanted to determine whether different epitopes of apoA-I were heterogeneously expressed in different populations of apoA-I containing lipopro-

TABLE 1. Immunoprecipitation of ^{125}I-labeled HDL by different Mab against apoA-I

Mab	Experiment I		Experiment II	
	Fresh (20 d) HDL	Stored (66 d) HDL	Fresh (11 d) HDL	Stored (38 d) HDL
Series 2				
2F1	90 (2)[a]	71 (1)	99	97
3G10	88 (1)	70 (1)	93	96
4F7	79 (1)	57 (1)	90	95
4H1	74 (5)	74 (5)	72	80
5F6	55 (0.4)	71 (2)	56	59
Series 1				
3D4	55 (1)	75 (1)	46	59
6B8	46 (1)	68 (0.5)	44	46
5G6	5.9 (1.1)	29 (2)	3	5
Anti-apoA-II	47 (1)	40 (1)	45	55

In experiment I, the labeled HDL were prepared from fresh and stored (46 days at 4°C) sera from the same subject (n = 3); in experiment II, the labeled HDL were prepared from fresh and stored sera (27 days at 4°C) from the same subject (n = 2).

[a]Values are the percentage of radioactivity bound to Pansorbin-IgG at the plateau, with the standard deviation in parentheses.

teins. If this was the case, additive effects would be observed by immunoprecipitation with certain Mab combinations. The additivity of immunoprecipitation by pairs of Mabs from series 2 was investigated under conditions of antibody excess for each individual Mab but using a particular preparation of ^{125}I-labeled HDL that was not totally precipitated by any single Mab, **(Table 2).** Most antibody combinations gave an additive effect in immunoprecipitation and, as should be expected, the effect was most pronounced for Mabs such as 5F6 and 4H1 which were the least effective when used alone, but in this particular experiment the precipitation never exceeded

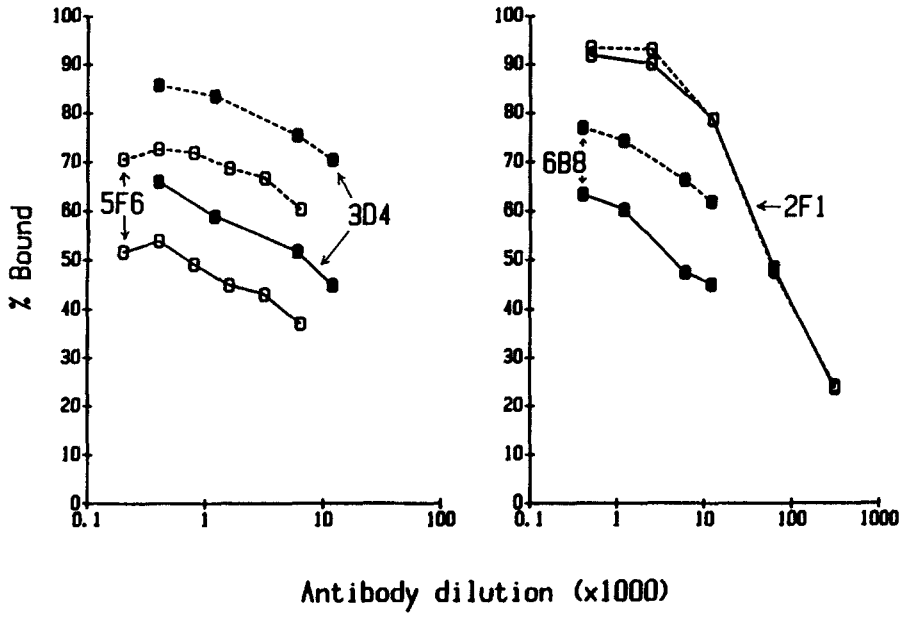

Fig. 2. Immunoprecipitation of fresh and stored ^{125}I-labeled HDL by series 1 and 2 antibodies. The legend is the same as that for Fig. 1; however in these experiments the added amount of anti-mouse IgG armed-Pansorbin was increased to 150 μl to ensure saturation at the higher concentrations of antibodies used. The experiments used HDL prepared from fresh or stored (70 days, 4°C) serum; both were kept for 10 days at 4°C (5F6) or for 17 days at 4°C (3D4, 6B8 and 2F1) before the experiment.

TABLE 2. Immunoprecipitation of ^{125}I-labeled HDL by combination of Mab against apoA-I (series 2) and apoA-II

	4H1	2F1	4F7	3G10	5F6	Anti apoA-II
4H1	65[a]	91	87	89	79	90
2F1		85	94	90	94	96
4F7			67	90	86	88
3G10				76	64	90
5F6					63	
Anti apoA-II						41

The HDL used in this experiment were prepared from fresh serum and had been stored 30 days at the time of the experiment.

[a]Percentage of radioactivity bound to Pansorbin-IgG at the plateau (SD ≤ 10%).

95%. Similar results were also observed when series 2 Mabs were combined with anti-apoA-II (Table 2). These experiments, therefore, demonstrate that apoA-I epitopes are indeed not homogeneously expressed on all lipoproteins and that they may reflect a heterogeneous conformation of apoA-I itself or steric hindrance by lipids or other apoproteins.

In contrast, no significant additivity could be observed with pairs of Mabs from series 1 such as 5G6, 3D4, and 6B8 **(Table 3),** a result that indicates that the determinants for these Mabs tend to be expressed on the same lipoproteins and from which we could conclude that the same apoA-I molecule expresses each of these three epitopes. However there were additive effects when pairs of Mabs from series 1 were combined with an anti-apoA-II, and in this case immunoprecipitation of ^{125}I-labeled HDL reached more than 90% (Table 3).

Since the heterogeneity of apoA-I-containing lipoproteins is well documented (15–17) and since the partition of apoA-I between lipoproteins with or without apoA-II has been quantified, it was of interest to determine whether the epitopes for 3D4 and 4H1 were preferentially expressed

TABLE 3. Immunoprecipitation of ^{125}I-labeled HDL by combination of Mab against apoA-I (series 1) and apoA-II

	5G6	3D4	6B8	anti-A-II	5G6 3D4	5G6 6B8	3D4 6B8	3D4 5G6 6B8
5G6	13[a]	63	59	55				
5G6	16	68	57					
3D4		70	74	85				
3D4		76	75	89				
6B8			59	84	71			
6B8			56	87	72			
Anti-A-II				50	82	82	92	89
Anti-A-II				54	83	83	93	91

Both HDL preparations used in this experiment were obtained from blood of the same donor, but drawn on different days.

[a]The results are expressed as percent of radioactivity bound to Pansorbin-IgG at the plateau (SD < 10%). The two values given for each Mab combination represent the results obtained with each ^{125}I-labeled HDL preparation.

in specific apoA-I-containing lipoproteins with or without apoA-II. To evaluate the nature of the labeled lipoproteins that were immunoprecipitated, the distribution of radioactivity in the apolipoproteins of the supernatants and starting ^{125}I-labeled HDL were analyzed and compared **(Table 4).** While 4H1 could precipitate 91% and 68% of the labeled apoA-I and apoA-II, respectively, the anti-apoA-II precipitated 42% and 79% of apoA-I and apoA-II, respectively, results that are compatible with those of Cheung and Albers (15) who reported that the percentage of plasma apoA-I not associated with apoA-II varied between 25 and 51%. Both 3D4 and 4H1 precipitated a similar ratio of apoA-I to apoA-II, and in the case of 3D4, when labeled HDL prepared from stored and fresh serum were compared, the proportion of precipitated apoA-I increased together with that of apoA-II (Table 4). Therefore both 3D4 and 4H1 epitopes are expressed on apoA-I-containing lipoproteins with or without apoA-II, but the lower proportion of apoA-II precipitated by either Mab indicates that the immunoreactivity of these epitopes is lower in lipoproteins with apoA-I and apoA-II compared to lipoproteins with apoA-I and no apoA-II.

Effect of storage time on serum apoA-I immunoreactivity

As we had shown earlier that the immunoreactivity of 3D4 but not that of 4H1 increased significantly with storage of serum at 4°C (4), we undertook here to quantify this process precisely. Aliquots of sera from three normolipemic subjects were stored at 4°, −20°, and −80°C for different periods of time up to 84 days and were assayed weekly by solid phase RIA as described under Methods using three different Mabs: 4H1, 3G10, and 3D4. There was no significant change in serum apoA-I immunoreactivity with any of the Mabs studied when the samples were stored in the frozen state at −80° or −20°C **(Table 5),** and under these conditions, the interassay coefficient of variation was calculated to be 9.1%,

TABLE 4. Immunoprecipitation of ^{125}I-labeled HDL (Percentage of precipitated apolipoproteins)

Precipitated Apoprotein	Precipitating Mab: 3D4 Fresh	3D4 Stored	4H1 Fresh	4H1 Stored	Anti-ApoA-II Fresh	Anti-ApoA-II Stored
ApoA-I	58 (11)[a]	83	91 (4)	92	42 (5)	38
ApoA-II	40 (9)	62	68 (10)	71	79 (4)	75

^{125}I-labeled HDL prepared from fresh serum (n = 4) or from stored serum (n = 2-, 27-, and 46-days-old) were precipitated by excess antibody against apoA-I or apo A-II as indicated under Methods. An aliquot of the unbound supernatant was separated by electrophoresis on 15% polyacrylamide gels in the presence of SDS. After migration, the gel was dried and the regions corresponding to apoA-I and apoA-II were cut and counted for radioactivity.

[a]The results are expressed as the percentage of apoA-I and apoA-II radioactivity that were precipitated relative to that present in the starting material, with the standard deviation in parentheses.

TABLE 5. Effect of storage time and temperature on serum apoA-I immunoreactivity

Mab	Storage temp.	Storage Time (days) 1	7	14	21	28	35	49	63	84
4H1	4°C	1.00	1.14 (0.29)[a]	1.33 (0.30)	1.35 (0.38)	1.48 (0.16)	1.39 (0.15)	1.44 (0.03)	1.78 (0.12)	1.78 (0.45)
4H1	−20°C		1.17 (0.23)	1.34 (0.34)	1.26 (0.55)	1.00 (6.03)	1.13 (0.13)	1.14 (0.18)	1.22 (0.23)	1.24 (0.21)
4H1	−80°C		1.09 (0.37)	1.17 (0.30)	1.17 (0.41)	0.94 (0.10)	1.00 (0.13)	1.17 (0.16)	1.14 (0.15)	1.09 (0.15)
3G10	4°C	1.00	1.41 (0.50)	1.33 (0.48)	1.66 (0.69)	1.64 (0.40)	1.85 (0.53)	1.81 (0.46)	2.04 (0.76)	1.97 (0.81)
3G10	−20°C		1.31 (0.44)	1.34 (0.51)	1.19 (0.51)	1.17 (0.31)	1.17 (0.41)	1.11 (0.19)	1.17 (0.29)	1.13 (0.31)
3G10	−80°C		1.29 (0.47)	1.25 (0.46)	1.27 (0.59)	1.05 (0.33)	1.17 (0.25)	1.14 (0.26)	1.16 (0.31)	1.14 (0.20)
3D4	4°C	1.00	2.84 (1.38)	6.55 (3.25)	25.4 (21.8)	44.2 (13.6)	71.0 (32.2)	112 (29)	205 (26)	271 (31)
3D4	−20°C		1.21 (0.85)	0.98 (0.43)	1.24 (0.86)	0.86 (0.40)	1.00 (0.52)	0.80 (0.46)	1.25 (0.95)	0.96 (0.23)
3D4	−80°C		1.17 (0.61)	1.36 (0.90)	1.29 (0.86)	1.07 (0.35)	0.95 (0.50)	0.95 (0.49)	0.95 (0.50)	1.07 (0.61)

[a]The results (average of three donors, with standard deviation in parentheses) are expressed as the ratio of serum dilutions required to obtain a B/Bo = 0.5 on day n relative to that on day 1. The sera contained additives as described under Methods.

8.6%, and 12.2% for Mab 4H1, 3G10, and 3D4, respectively. In contrast, there were highly significant increases in immunoreactivity of serum with 3D4 during storage at 4°C, but not with the other antibodies. The immunoreactivity with 3D4 increased linearly during the first 4 weeks at an average increment of 38%/day and reached a mean increase of 278-fold for the three donors at 12 weeks (Table 5). In contrast, the immunoreactivity of the same samples assayed on the same days with 4H1 and 3G10 changed very little, although a significant linear regression was calculated over the 12-week period using the data in Table 5 and represented average increases of 0.80 and 0.75%/week for 4H1 and 3G10, respectively.

Some of these results with series 2 Mabs contradict certain of our preliminary observations (4) which indicated that 3G10 and 4F7 reacted better with fresh sera, although we had also noted that these Mabs reacted better with NaOH-treated HDL, a model of antigen in vitro modification. In addition, 2F1 was also initially reported as reacting better with control HDL than with NaOH-treated HDL, although it appeared to react better with a stored serum (4). However, it is clear now from the results of both the competitive RIA and the immunoprecipitations that Mabs of series 2, such as 4H1, 3G10, 4F7, and 2F1, react equally with the fresh and stored antigen, whether in serum or in HDL.

The changes in apoA-I immunoreactivity with series 1 Mabs as a function of antigen storage at 4°C were not related to any evident degradation of the antigen. The serum samples stored for the longest periods were electrophoresed on SDS polyacrylamide gel and submitted to immunoblots with each of the three tested Mabs. There was no evidence of proteolysis of apoA-I, as indicated by the absence of immunoreactive fragments (not illustrated), in agreement with our previous studies (4). It is important to note that all Mabs of series 1 could detect important increases in apoA-I immunoreactivity, an observation that indicates that the phenomenon is not related to a modification of apoA-I at a single site in the molecule.

The expression of apoA-I epitopes on HDL prepared from fresh or stored serum was evaluated by equilibrium competitive inhibition analyses in which the ability of homologous HDL to compete with ^{125}I-labeled HDL for binding with Mabs 3D4, 2F1, 4H1, and 3G10 was studied. Full displacement of ^{125}I-labeled HDL (fresh or stored) by HDL (fresh or stored, respectively) was obtained with each Mab and the K_a were calculated **(Table 6).** In the cited experiments where the HDL preparations were stored at 4°C for different periods of time, K_a for 2F1 did not change, and the K_a for 3G10 tended to decrease with storage, whereas the K_a for 4H1 was unchanged or slightly increased. In contrast, the K_a for Mab 3D4 of series 1 increased with storage in both experiments and especially with the HDL stored for 76 days, indicating that the affinity of 3D4 for its epitope was significantly enhanced upon prolonged time in vitro. However, the changes in K_a for 3D4 were most pronounced only upon prolonged storage and may not be sufficient to explain all of the changes in immunoreactivity noted with this antibody.

Effect of EDTA and antioxidants on apoA-I immunoreactivity

Blood from three normolipemic donors was used to prepare serum or EDTA-plasma and the serum samples were further separated in aliquots containing mixtures of either antioxidants alone, or antioxidants, antibacterial, and antiproteolytic agents, as described under Methods. The different samples were stored for about 50 days at −80°C or 4°C and then assayed by RIA with Mabs from series 1 and 2. As noted before, the immunoreactivity of all sera stored at 4°C compared to the frozen samples increased greatly with Mabs 3D4, 6B8, and 5G6 of series 1, whereas the immunoreactivity with 4H1 and 3G10 of series 2 was not significantly changed **(Table 7).** In contrast, the increase in reactivity with Mabs 3D4, 6B8, and

TABLE 6. Effect of storage on the affinity constants (K_a) of anti-apoA-I with ^{125}I-labeled HDL

	Experiment I		Experiment II		
	HDL (30 d)	HDL (76 d)	HDL (8 d)	HDL (14 d)	HDL (41 d)
2F1			14.4	15.1	12.3
3G10	4.13[a]	1.98	10.8	6.8	5.1
4H1	0.132	0.226	0.086	0.064	0.090
3D4	0.055	0.704	0.035	0.029	0.076

[a]The affinity constants ($\times$ 10^9 M $^{-1}$) were calculated as described under Methods.

3G10 of the plasma stored at 4°C compared to the frozen plasma was also important and significant, but on the average about three times less than that of the corresponding serum samples (Table 7).

When a mixture of antioxidants was added to the serum samples, the increase in immunoreactivity with Mabs 3D4, 6B8 and 5G6 related to storage at 4°C was diminished but did not reach the levels observed with plasma (Table 7). When antioxidants, antiproteases, and antibacterial agents were added to the different sera, the relative increase in immunoreactivity with Mabs 3D4, 6B8, and 5G6 was further diminished and reached the levels observed with most of the plasma samples (Table 7). These experiments, therefore, demonstrate that a significant component of the mechanism that accounts for the increase in apoA-I immunoreactivity observed in vitro at 4°C can be inhibited by the chelation of divalent cations and may be related to oxidative processes.

DISCUSSION

The great majority of plasma apoA-I is found in HDL which comprise lipoproteins heterogeneous in their physical properties, and within each density subclass there is further polydispersity. While the lipid composition and the lipid to protein ratio of HDL subclasses vary as a function of density, an even greater heterogeneity exists which is brought about by the presence of diverse apolipoproteins that associate in various combinations to yield the complex apoA-I-containing lipoproteins. The best illustration of this heterogeneity is probably that obtained by isoelectric focusing on agarose films which demonstrates the existence of at least ten lipoprotein species (16) and which are characterized by the association of two or more apoproteins (A-I, A-II, C-I, C-II, C-III, D, and E) on the same particle. The apoA-I-containing lipoproteins can also be separated into two major subclasses, the particles with A-I and A-II and those with A-I but no A-II (15, 17).

In view of this well-documented heterogeneity of apoA-I containing lipoproteins, it is not surprising that the early immunochemical studies of apoA-I disposition on the HDL surface had demonstrated the heterogeneity of the antigen (18, 19); evidence was presented that some antigenic sites of apoA-I in HDL are more accessible to antibodies than others. This concept appears to be supported by the first reported studies with Mabs (5), where none of the three epitopes identified were expressed on more than 60% of the HDL particles. In addition, the relative expression of these three epitopes was found to vary in HDL

TABLE 7. Evolution of apoA-I immunoreactivity upon storage of plasma, sera, and sera containing either or both antioxidants, antiproteases and antibacterial agent

	Antibody														
	3D4			6B8			5G6			4H1			3G10		
Fraction Assayed	A	B	C[a]	A	B	C	A	B	C	A	B	C	A	B	C
Serum	517[b]	193	320	36	20	23	10	3.9	6.7	1.9	1.8	1.8	1.58	1.9	1.8
Serum + antioxidant	283	82	210	16	7	15	5.3	2.5	4.8	1.3	1.4	1.5	1.9	1.6	1.8
Serum + antioxidant + antibacterial + antiproteases	394	67	131	11	5	7	5.3	2.3	3.3	1.4	1.5	1.4	1.7	1.6	1.7
Plasma	157	118		11	6		2.6	2.6		1.5	1.4		1.7	2.1	
Serum − 80°C / Plasma − 80°C	0.9	1.7		1.1	0.8		0.8	1.2		1.0	1.1		1.0	1.1	

[a]Plasma and sera samples from donors A, B, and C were stored at 4° or − 80°C under the conditions described for 45, 52, and 47 days, respectively.

[b]Ratio of the immunoreactivity of the assayed fraction stored at 4°C relative to that of the corresponding control stored at − 80°C. The immunoreactivity for each fraction and its corresponding control is measured as the antigen dilution necessary for B/Bo = 0.5.

subfractions obtained by density gradient ultracentrifugation and by chromatofocusing. These different observations indicate that some domains of apoA-I are sterically cryptic in lipoproteins bearing the antigen and that the immunoreactivity of these domains may vary in individual lipoproteins. The difficulties encountered in the standardization of plasma apoA-I immunoassays have been ascribed for the most part to such immunological properties of apoA-I (20).

In this context, the present demonstration that several distinct epitopes exist on apoA-I, which are expressed on the totality of apoA-I-containing lipoproteins, is most important. First conceptually, this indicates that despite the heterogeneity of apoA-I-containing lipoproteins, defined regions of the molecule exist that have the same conformation and the same antigenicity. Second practically, the existence of such epitopes allows the development of accurate immunoassays and of immunodepletion techniques for apoA-I in serum and lipoprotein fractions. Mabs 2F1, 3G10, and 4F7, which react at three distinct sites mapped to CNBr fragments 1 and 2, respectively (8), could consistently precipitate up to 100% of ^{125}I-labeled HDL prepared from different donors (Figs. 1, 2 and Table 1). Another Mab 4H1, which reacts at a different site on CNBr fragment 1 (8), could precipitate up to 90% of ^{125}I-labeled HDL. In addition, none of the epitopes for 2F1, 3G10, 4F7, and 4H1 were found to be affected by the in vitro alteration of the apoA-I antigen. Among the Mabs of series 2, only 5F6 precipitated a lower proportion (50–60%) of ^{125}I-labeled HDL. Mabs 3D4 and 6B8 from series 1 also precipitated the same percentage of labeled HDL, but the percent bound by these Mabs increased with the storage of the HDL preparation. Finally, the most significant observation with respect to the definition of epitopes that are expressed on all HDL particles is that Mabs that react at these sites have a significantly higher K_a than Mabs reacting at sites not expressed on all apoA-I molecules (Table 6). This is clearly the case for 2F1 and 3G10 which bind 100% of the HDL compared to 4H1 and 3D4 which bind 90 and 60%, respectively. These results would imply that the epitopes for 2F1, 3G10, and 4F7 are located in thermodynamically stable regions of apoA-I which favor high affinity interactions with the respective Mabs.

The immunoprecipitation experiments have also demonstrated that the number of HDL particles that express the epitopes identified by series 1 Mabs increases with time in vitro (Figs. 1 and 2). This indicates that the increase in immunoreactivity for 3D4 and 6B8 is not due to the generation of a few highly reactive A-I molecules but to a doubling or more in the number of the apoA-I-containing lipoproteins expressing these determinants. The alteration of the serum apoA-I antigen as a function of time at 4°C was rapid and linear for the first 4 weeks reaching an average 278-fold increase for the three donors at 84 days (Table 5). Because these Mabs recognize three distinct epitopes localized close to the cleavage site for CNBr fragments 1 and 2 for 5G6, on CNBr fragment 2 for 3D4, and on CNBr fragment 3 for 6B8 (6), this alteration of the antigen clearly affects several regions of apoA-I and may result from modifications of apoA-I at multiple sites on the molecule. However, it is also important to note that these modifications, which probably occur at multiple sites, have a limited spatial effect since the epitopes for Mabs of series 2, which are interspaced with those for Mabs of series 1, are not affected by storage at 4°C. Because the process can be significantly decreased by the presence of EDTA and by the additions of antioxidants (Table 7), the modification of the apoA-I antigen may be mediated, at least in part, by oxidation reactions requiring divalent cations. Such oxidation could be effected by hydroperoxides of polyunsaturated fatty acids and be concurrent with the deamidation reaction which we proposed earlier (4) to explain this in vitro alteration.

Several types of in vitro modifications have been reported for apoA-I and apoA-I-containing lipoproteins and the best described is certainly deamidation (21–23). This reaction affecting apoA-I is accelerated at alkaline pH (4) and, with the liberation of ammonia, results in the formation of the apoA-I acidic isoforms (23). Interestingly, it has also been found that an antioxidant (BHT) could prevent this reaction and this was taken to suggest that some of the acidic isoforms could be formed by the derivatization of free amino groups with lipid peroxides (24). Although the acidic isoforms of apoA-I may be heterogeneous in nature, this polymorphism does not appear related to the in vitro alteration of apoA-I immunoreactivity in vitro since each isoform reacts with the different anti-apoA-I Mab that we have studied (4, 6). It has also been noted that upon storage of serum samples at 4°C, there was a marked decrease in the mass of HDL_3 with no change in HDL_2 and that this process could be prevented by high salt concentration (25). This may represent a rearrangement of serum lipoproteins that occurs slowly upon separation of serum from blood cells and upon drawing of blood. There is no evidence that the in vitro alteration of the apoA-I antigen, which can be observed in apoHDL as well as in HDL (7), can be related to the above-mentioned observations, but it is probably related to a combination of chemical modifications of apoA-I such as deamidation, oxidation, or reaction with lipid peroxides. Finally, it is of interest that the recently reported modification of growth hormone immunoreactivity, as a function of storage in vitro, is correlated with both deamidation and changes in circular dichroism measurements (26). Presumably deamidation can modify the antigen conformation and uncover normally buried or nonaccessible epitopes.

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