Lipid Peroxidation Changes the Expression of Specific Epitopes of Apolipoprotein A-I*

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Incubation of human serum or high density lipoprotein (HDL) at 37 °C in the presence of Fe²⁺, Fe³⁺/Fe⁴⁺, or Mn²⁺ results in the increased immunoreactivity (up to 12-, 40-, and 80-fold, respectively) of specific apoA-I epitopes identified as 3D4 and 6B8, while Mg²⁺, Ca²⁺, or Cu²⁺ have minimal or nonsignificant effects. The effect of Mn²⁺ on the 3D4 epitope requires a specific association with lipids since it can be observed with HDL but not with apoHDL, even in the presence of other lipoproteins. The increase in immunoreactivity noted with Fe²⁺/Fe³⁺ or Mn²⁺ can be blocked with either EDTA or antioxidants (GSH and ascorbic acid), suggesting that it takes place during a peroxidative reaction of the lipids. The peroxidation of lipids which accompanies the increase in immunoreactivity does cross-link apoA-I both with itself and with apoA-II but does not cleave the molecule. The apoA-I-containing lipoproteins which float between 1.18 and 1.22 g/ml and have a pre B-electrophoretic migration are characterized by a very low immunoreactivity with monoclonal antibody 3D4 but are 10-fold or more responsive to Mn²⁺ treatment than other lipoprotein subfractions, thus demonstrating heterogeneity under oxidative conditions. Proteoliposomes containing apoA-I, cholesterol, and dilinoleyl-lecithin are sensitive to Mn²⁺ treatment, but not those made with dioleyl- or dimyristoyl-lecithins. However, the increase in 3D4 immunoreactivity is weak and transient and is followed by the disappearance of the epitope caused by cross-linking. We conclude that lipid peroxidation can specifically cross-link apoA-I and change its conformation and antigenicity.

Apolipoprotein A-I (apoA-I) is the major protein of high density lipoprotein (HDL) and our experience has shown that apoA-I elicits different immune responses when the antigen is either purified apoA-I (1, 2) or fresh HDL (3). This is a general phenomenon which has also been observed by other authors (4, 5). Antibodies generated by immunization with purified apoA-I react with epitopes which become more immunoreactive when either serum or HDL are kept in vitro (2, 5). However, these epitopes are not created by an in vitro reaction but exist in freshly isolated serum or HDL (2, 5), and we have shown that the proportion of HDL particles expressing certain of these epitopes can increase from 30% in the fresh samples to 70% upon storage at 4 °C (6). The main type of in vitro modification reported for apoA-I has been deamidation (7–9), although the acidic conditions which normally favor deamidation did not generate both the apoA-I isoforms and the increase in immunoreactivity expected (2). We have presented preliminary evidence that the process of increase in immunoreactivity at the sites recognized by mAbs 3D4, 6B8, and 5G6 upon storage of serum can be partially inhibited by EDTA and antioxidants (6). In the present report, we demonstrate that this process takes place under the conditions where the peroxidation of polyunsaturated lipids occurs and that it requires a specific association between apoA-I and the lipids. We have also observed that a specific subfraction of VHDL is most sensitive to the change in immunoreactivity.

These studies are conducted with 3D4, a mAb which reacts with such a labile epitope located between residues 86 and 112 (1) and which is representative of two other mAbs, 5G6 and 6B8, which are similarly affected by oxidative conditions and recognize different epitopes localized on the CNBr fragments 1–2 and 3, respectively (1). The control experiments are conducted with 4H1, a control mAb reacting between residues 1 and 86 at an epitope unchanged by the treatments.

The oxidation of specific lipoproteins, reviewed recently (10), was first observed by Schuh et al. (11) who reported the cleavage of LDL apoB under conditions of lipid oxidation. These modifications of LDL can be produced by endothelial cells and smooth muscle cells in culture (12, 13) and promoted by micromolar concentrations of Cu²⁺ or Fe³⁺ (13). Early work also demonstrated that such a process could be inhibited by HDL (14). However, no direct evidence has been presented to date for the occurrence of peroxidative modifications of HDL, apolipoproteins, and the present report demonstrates for the first time that apoA-I immunoreactivity is altered specifically under conditions of lipid peroxidation, possibly as a result of chemical cross-linking. There are now multiple reports on the importance of modified and especially of oxidized LDL in atherosclerosis (10), but it remains to be seen if some of the protective effects of HDL could be related to their interference in LDL oxidation.

EXPERIMENTAL PROCEDURES

Materials
Phenylmethylsulfonyl fluoride, cholesteryl, sodium cholate, dimyristoylphosphatidylcholine, dioleoylphosphatidylcholine, dilinoleyl-

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The abbreviations used are: apoA-I, apolipoprotein A-I; HDL, high density lipoproteins; VHDL, very high density lipoproteins; LDL, low density lipoproteins; mAb, monoclonal antibody; RIA, radioimmunoassay; TBAR, thiobarbituric acid-reactive substances; MDA, malondialdehyde; SDS, sodium dodecyl sulfate; DOPC, dioleoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DLPC, dilinoleylphosphatidylcholine; PBS, phosphate-buffered saline.

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phosphatidylcholine, 1,1,3,3-tetramethoxypropane, and gelatin were obtained from Sigma. Egg phosphatidylcholine was purchased from Serdary Research Inc. (London, Canada). Reagents for SDS and Tris-urea polyacrylamide gel electrophoresis, polyvinylpyrolidone of molecular weight 44,000, disodium EDTA, and NaN₃ were purchased from BDH Canada Inc. (Montreal, Canada). Tris, urea-urate grade, was purchased from Aldrich. All other chemicals were purchased from Anachemia Chemicals (Montreal, Canada).

Preparation of Serum and Plasma

Normolipidemic healthy subjects who had expressed informed consent gave blood which was collected by venupuncture into Vacutainer tubes. Serum was prepared by drawing blood into tubes without anticoagulant. The blood was allowed to clot for 1-2 h at 20 °C. Plasma was prepared by drawing blood into tubes containing EDTA (1 mg/ml). The blood was clarified by centrifugation at 1000 x g for 15 min at 5 °C. Sodium azide and phenylmethylsulfonyl fluoride were added to final concentrations of 0.02% and 1 mM, respectively.

To study the effect of dialysis of serum and plasma on the immunoreactivity of anti-apoA-I monoclonal antibody 3D4, aliquots of serum and plasma were dialyzed against 10 mM NH₄HCO₃, 0.02% NaN₃, pH 7, at 4 °C overnight.

Preparation of Lipoprotein Fractions and Deipilized HDL

The technique of Terpstra et al. (15) was used for density gradient ultracentrifugation as described previously (16). Samples were centrifuged in an SW 41 rotor at 200,000 rpm for 20 h at 4 °C. A blank gradient was included where a KBr solution of density 1.006 g/ml containing 1 mM EDTA and 0.02% NaN₃ replaced the plasma.

For the experiments in Table III, the HDL fraction collected contained the HDL-colored band and extended midway between this band and the bottom fraction. From the blank gradient, 1-ml fractions were removed and the density and position of each determined to give the cholate dialysis technique as described by Chen and Albers (20). The concentration of each pool was determined by RIA as described above. The starting antibody dilution was 1/500. A further 100 pl of reaction mixture was added to an aliquot of HDL to give final concentrations of 100 pg/ml each. The aliquots were then placed at the appropriate temperature for 72 h.

Modification of HDL with MDA and Determination of TBAR

MDA was prepared from 1,1,3,3-tetramethoxypropane by acid hydrolysis according to the method of Kwon and Watts (20). To 7.5 x 10⁻³ mol of 1,1,3,3-tetramethoxypropane in 200 ml of distilled water, 7.4 ml of 1 M HCl was added. The flask was stoppered and placed in a water bath at 50 °C for 60 min. Stopcock grease was applied to the stopper to prevent loss of MDA. The flask was cooled and the volume adjusted to 250 ml to give a 30 mM MDA solution.

HDL was modified by the addition of 10 amol of MDA/mg of total HDL protein and incubation at 37 °C for 3 h, an adequate time to modify 92% of the lysine residues in HDL (21). To a 100-μl aliquot of dialyzed water filtered HDL samples were then dialyzed against PBS, pH 7.2 (0.9% NaCl, 0.1 M Na₂HPO₄, 7H₂O; 0.05 M NaH₂PO₄·H₂O) containing 1 mM EDTA and 0.02% NaN₃ at 4 °C overnight to remove any unreacted MDA. The effect of this modification on the immunoreactivity of apoA-I with mAbs was then studied by solid phase radioimmunoassay.

In order to assess the occurrence of lipid peroxidation during incubation of plasma and HDL fractions at +4 °C and 37 °C in the presence or absence of 1 mM MnCl₂ for 48 h, the thiobarbituric acid assay was performed as described by Buege and Aust (22). The TBAR products were measured in 200-μl aliquots of plasma and 1800-μl aliquots of HDL fractions. These samples were made up to 2.0 ml and contained 0.01 M sodium phosphate, pH 7, and the following antioxidants: 0.5 mg/ml glutathione, 1 mM ascorbic acid, 1 μM EDTA, and 0.1 mg/ml butylated hydroxytoluene. A standard curve using 1,1,3,3-tetramethoxypropane was prepared under the same conditions to cover the range of 0-4.5 nmol. In this range, there is a linear relationship between absorbance and concentration. To this, 1.0 ml of 30% trichloracetic acid, 0.75% 2-thiobarbituric acid, 0.5 N HCl was added. The mixture was heated in a boiling water bath for 15 min. The tubes were left to cool and then centrifuged at 1000 x g for 10 min. The absorbance of the clear pink supernatant was read at 555 nm.

Preparation of Proteoliposomes

HDL from several gradient centrifugations were combined, dialyzed, and deipilated as described above for the preparation of apoHDL proteoliposomes. ApoHDL proteoliposomes were prepared by the fractionation technique as described by Chen and Albers (19) where apoHDL-lecithin:cholesterol are associated in the molar ratio of 0.8:250:12.5. The proteoliposomes were prepared by pipetting 3.85 mg of phosphatidylcholine and 0.155 mg of cholesterol (1 mg/ml in ethanol) into a glass tube. The lipid mixture was evaporated to dryness under a gentle stream of nitrogen at room temperature. To the lipids, 0.44 mg of apoHDL, 0.15 ml of sodium cholate (725 mM in 10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4), ascorbic acid (final concentration of 1 mM), and glutathione (final concentration of 0.5 mg/ml) were added. Tris/NaCl/EDTA, pH 7.4, was added to complete the volume to 2 ml. The solution was mixed on a vortex for 1 min and then incubated at room temperature for 30 min on an orbital shaker. The proteoliposomes were then dialyzed at 4 °C overnight against several changes of 10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4, and 10 mM NaCl, pH 7.4, to remove the EDTA. The final protein concentration of these preparations was found to be approximately 200 μg/ml.

Solid Phase Radioimmunoassay of ApoA-I

The solid phase radioimmunoassay of apoA-I used was that previously described (6). Immunol II removing wells (Dynatek Laboratories Inc., Alexandria, VA) were coated with 100 μl of 2 μg/ml apoHDL in 15 mM Na₂CO₃, 35 mM NaH₂CO₃, 0.02%NaN₃, pH 9.6. The wells were placed in a moist chamber for 2 h and all incubations were carried out at room temperature. The coating solution was discarded and the wells washed once with PBS, pH 7.2, 0.02% NaN₃ (wash I). The wells were then saturated with 250 μl of 0.5% gelatin in wash I for 1 h, the solution was discarded, and the wells washed once with wash I.

Titration of Monoclonal Antibodies—Serial doubling dilutions of monoclonal antibodies 3D4 and 4H1 were prepared in 0.5% gelatin, 0.05% Tween, 20, 0.02% NaN₃ in PBS, pH 7.2 (reaction buffer) in a volume of 100 μl in microtiter plates using a multichannel pipette. The coating antibody dilutions and the immunoreactive monoclonal antibodies were added to all wells, the solution mixed, and 100 μl transferred immediately to the coated wells. The wells were then incubated for 1 h in a moist chamber. The antibody solution was discarded and the wells washed three times with 0.05% Tween 20, 0.02% NaN₃ in PBS, pH 7.2 (wash II). Labeled rabbit anti-mouse IgG₁ was added as described below.

Incubation Conditions

The effect of incubation of serum and plasma, lipoprotein fractions, and proteoliposomes at 4 °C and at 37 °C in the absence and presence of divalent cations on the immunoreactivity of monoclonal antibodies was studied by solid phase radioimmunoassay. Three aliquots of each sample were prepared. One aliquot was placed at 4 °C (control) and one at 37 °C for the time specified (48 h in most experiments). To the third aliquot, 0.01 volume of 100 mM MnCl₂ was added to give a final concentration of 1 mM and the aliquot placed at 37 °C for the time specified (48 h in most experiments). In initial experiments, controls were incubated at +4 °C and -80 °C. No difference was observed between them, so subsequent control aliquots were stored at 4 °C.

To study the effect of Fe²⁺ and Fe³⁺, 10 mM solutions were prepared fresh, and 0.01 volume of each was added to an aliquot of HDL to give final concentrations of 100 μM each. The aliquots were then placed at the appropriate temperature for 72 h.
Competitive Assays—The antibody dilution chosen for competitive assays was that which gave 60% maximum binding in the above titration. Serial doubling dilutions of competing antigen were prepared in reaction buffer in a volume of 100 µl as above. To the antigen dilutions, 100 µl of the appropriate antibody dilution was added; the solution mixed, and 100 µl immediately transferred to the coated wells. The wells were incubated for 1 h at room temperature as above. The solution was discarded and the wells washed three times with wash II.

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

Polyacrylamide Gel Electrophoresis and Immunobots

HDL and apoHDL proteoliposomes were analyzed by SDS (15%) polyacrylamide gels as described by Neville (23) and Tris-urea (10%) polyacrylamide gels as described by Kane (24). After electrophoresis, the separated proteins were either stained with silver reagent or transferred to nitrocellulose overnight at 100 mA in 30% methanol in 42.5 mM sodium borate, pH 8. The nitrocellulose was saturated with 5% polyvinylpyrrolidone (44,000) in TBS, pH 7.4 (150 mM NaCl, 10 mM Tris base, 0.02% NaN3), for 60 min at 37 °C. To detect the 3D4 immunoreactive bands, the nitrocellulose was incubated with 3D4 diluted to 1/200,000 and 10 × 10^6 cpm of 125I-labeled goat-anti-mouse IgG-Fc in 100 ml of 1% bovine serum albumin (fraction V) in TBS, pH 7.4, for 3.5 h at 37 °C. The nitrocellulose was then washed six times with TBS, pH 7.4, 5 min wash. The nitrocellulose was then air-dried and exposed to XAR-5 Kodak film.

RESULTS

Effect of Metal Ions on ApoA-I Immunoreactivity in HDL, Serum, and Plasma—We first tested the effect of the optimum ratio of Fe2+/Fe3+ for lipid peroxidation as defined by Braughler et al. (25) on HDL apoA-I immunoreactivity (Table I). While incubation of HDL alone did not modify the immunoreactivity of any of the mAbs tested here, incubation in the presence of Fe2+ and Fe3+ caused more than a 10-fold increase in HDL immunoreactivity with 3D4 and 6B8 but no change with 4H1. Similar results were obtained with a combination of Fe2+ and H2O2 (not illustrated). Because lipid peroxidation does generate MDA, which is known to react with free amino groups of proteins causing both intra- and intermolecular cross-linking, we also sought to test the effect of that chemical on apoA-I. For this purpose, it is evident that exogenously added MDA had no significant effect on the immunoreactivity of HDL apoA-I with either 3D4 or 6B8, whereas it caused an unexpected increase with 4H1 (Table I). The latter effect was verified with a number of other cross-linkers and represents a different phenomenon of epitope modification.

In other experiments, we tested the effect of various divalent ions on the immunoreactivity of HDL apoA-I with mAb 3D4. When the incubation was carried out at 37 °C in PBS, pH 7.2, Cu2+, Mg2+, and Ca2+ (1 mM final concentration) had minimal effects (1-, 2-, and 4-fold increases, respectively, over the control kept at 4 °C), while Fe2+ (1 mM), Fe3+/Fe2+ (100 µM/100 µM), Fe3+/H2O2 (200 µM/100 µM), and Mn2+ (1 mM) had significantly higher effects (12-, 46-, 54-, and 78-fold increases, respectively).

Given this unexpected potency of Mn2+, its effect on the immunoreactivity of 3D4 with normal sera and plasma before and after dialysis in the presence of 10 mM NH4CO3, 0.02% NaN3, was evaluated (Table II). After incubation at 37 °C in the absence of Mn2+, serum and plasma were equally immunoreactive. However, the corresponding dialyzed serum and plasma were less immunoreactive with 3D4 as indicated by

### TABLE I

| Treatment* of HDL | 3D4 | 6B8 | 4H1 |
|------------------|-----|-----|-----|
| 37 °C, 72 h, Fe2+/Fe3+ | 0.6 (15.7) | 2.4 (10.9) | 2.8 (1.3) |
| 37 °C, 72 h | 5.6 (1.6) | 17.4 (1.5) | 4.2 (0.9) |
| 37 °C, 5 h, MDA | 6.5 (1.4) | 14.8 (1.8) | 0.7 (4.5) |
| 37 °C, 5 h | 8.5 (1.1) | 25.0 (1.1) | 3.8 (0.9) |

* Concentration of protein (µg/ml) necessary for B/Bo = 0.5 (ED20).

The relative immunoreactivity is expressed as the ratio of ED60 for HDL at 4 °C over the ED60 of treated HDL.

Effect of Chemical Modifications on HDL apoA-I Immunoreactivity with different mAbs

The results with Mn2+ are significantly greater (13-fold greater increases, respectively). It must be noted that the large standard deviations of the ED50 measured indicate important variations between donors. While some of the increases in immunoreactivity observed upon incubation at 37 °C may be a result of the reactions of lecithin:cholesterol acyltransferase and lipid transfer proteins, there is no evidence in the literature that these reactions could be affected by dialysis. After incubation at 37 °C in the presence of Mn2+, the immunoreactivity of serum, dialyzed serum, and dialyzed plasma with 3D4 increased significantly compared to that observed without Mn2+ (13-, 35-, and 8.6-fold, respectively); but as expected, the effect of Mn2+ was abolished by EDTA in native plasma (Table II). However, it must also be noted that extensive dialysis of plasma was not sufficient to completely restore the full effect of Mn2+ treatment as compared to dialyzed serum, suggesting incomplete removal of EDTA and/or heterogeneous reactions.

Role of Lipids in the Immunoreactivity of Serum Subfractions Treated with Mn2+—When lipoprotein fractions isolated from serum by sequential ultracentrifugation are treated with Mn2+ as described above, the observed change in immunoreactivity with 3D4 of the apo A-I containing lipoproteins varied with the density of the fraction. Since these results indicated that the increase in 3D4 immunoreactivity might depend upon the proportion of lipids associated with apoA-I, we proceeded to compare the effect of Mn2+ on HDL and apoHDL (Table III); the result were unambiguous: whereas 3D4 immunoreactivity did not change upon treatment of apoHDL with Mn2+, it increased on the average 13-fold upon treatment of HDL. Since there was no change in the immunoreactivity of these fractions with the control mAb 4H1, it is clear that the effect of Mn2+ on apoA-I requires the presence of lipids. However, when apoHDL is incubated with various other lipoproteins in the presence or absence of Mn2+, there is no change in the immunoreactivity of apoA-I with either 3D4 or 4H1 (not illustrated). When HDL is incubated without Mn2+ there is a small basal effect due to the reactions of endogenous lecithin:cholesterol acyltransferase and lipid transfer proteins with HDL (1.7-fold increase); in contrast, the results with Mn2+ are significantly greater (13-fold greater than without Mn2+).

Identification of a Dense ApoA-I Containing Fraction Highly Sensitive to Mn2+ Treatment—Since the change in 3D4 immunoreactivity depends upon the presence of lipids, we have evaluated whether apoA-I containing lipoproteins of different densities, determined by different ratios of apoA-I and lipids, have different sensitivities to Mn2+ treatment. For this pur-

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2. E. Raffai and Y. L. Marcel, unpublished results.
Lipid Peroxidation and ApoA-I Epitopes

TABLE II
Effect of dialysis with or without Mn2+ addition on the immunoreactivity of serum and plasma with 3D4

| Fraction* | Relative change in 3D4 immunoreactivity upon incubation at 37 and 4 °C | Ratio of relative immunoreactivity with/without Mn2+ |
|-----------|-------------------------------------------------|--------------------------------------------------|
| Serum     | Without Mn2+ 10.8 ± 6.7$^a$                      | With Mn2+ 139 ± 86$^a$                           | 13 |
| Serum dialyzed | 2.2 ± 1.4                          | 77 ± 51                                       | 35 |
| Plasma    | 11.6 ± 8.0                                      | 16 ± 13                                       | 1.4 |
| Plasma dialyzed | 3.5 ± 1.7                          | 30 ± 19                                       | 8.6 |

* Each fraction, either native or dialyzed against 10 mM NH4HC03, 0.02% NaN3 using a membrane with a 3500-Da cutoff, was incubated at 37 °C for 48 h in the absence or presence of 1 mM Mn2+. The relative immunoreactivity is given as the ratio of ED50 measured in competitive RIA of serum or plasma incubated at 37 °C compared to that kept at 4 °C. Each ED50 is expressed in dilution (x-fold) of the tested fraction compared to the dilution (x-fold) of the control at 4 °C. The results are the mean (± S.D.) obtained with six normolipemic subjects (three males, three females).

TABLE III
Role of lipids and lipoproteins in Mn2+-mediated changes in immunoreactivity of apoA-I

Comparison of the immunoreactivity of 3D4 and 4H1 with lipoprotein fractions incubated at 37 and 4 °C for 48 h. The lipoproteins isolated by gradient ultracentrifugation of EDTA-containing serum, dialyzed against 10 mM NH4HC03, have been incubated in the presence or absence of 1 mM Mn2+ as described under "Experimental Procedures."

| Incubated fraction* | Change in 3D4 immunoreactivity | Ratio of relative immunoreactivity with/without Mn2+ | Change in 4H1 immunoreactivity | Ratio of relative immunoreactivity with/without Mn2+ |
|---------------------|-------------------------------|------------------------------------------|--------------------------------|--------------------------------------------------|
| HDL                 | Without Mn2+ 1.7 ± 0.8$^a$    | With Mn2+ 22 ± 4.6                      | 13                            | Without Mn2+ 1.0 ± 0.0                           | 1.0 |
| apoHDL              | 1.4 ± 0.3                     | 0.9 ± 0.2                               | 0.6                            | 0.9 ± 0.2                                       | 1.1 ± 0.2 |

* The same amount of HDL and apoHDL protein were used in these experiments and recombinations were made in proportion to the original serum concentrations.

The relative change in immunoreactivity is the ratio of the ED50 measured with the fraction incubated for 48 h at 37 °C to that observed with the same fraction kept at 4 °C. Each ED50 is the result of quadruplicate determinations, means and S.D. are presented for three or more experiments, and means only are the average of two experiments.

pose, we submitted a serum sample to density gradient centrifugation and collected 0.5-ml fractions which were dialyzed individually and then incubated in the presence or absence of Mn2+. While there was no change in the immunoreactivity of each fraction with control mAb 4H1 upon Mn2+ treatment, the immunoreactivity of the fractions floating between 1.18 and 1.22 g/ml with mAb 3D4 increased significantly in the presence of Mn2+ (Fig. 1). These fractions constituted a distinct peak within the centrifugation gradient and corresponded to apoA-I containing lipoproteins which are a subset of dense HDL2 and VHDL.

These fractions were pooled (pool III, 1.189–1.224 g/ml) and compared to others (pool I, 1.059–1.121 g/ml; pool II, 1.121–1.185 g/ml; pool IV, d > 1.224 g/ml). Each pool was equally immunoreactive with mAb 3D4 after incubation in the presence of Mn2+, but it is clear that initially pool III was much less immunoreactive than any of the other fractions kept at 4 °C (Table IV). Therefore, the expression of the 3D4 epitope is optimized in all fractions after exposure to Mn2+ but differs initially as a function of density and especially in the range of 1.189–1.224 g/ml. This characteristic was constantly observed in all plasma tested. We cannot exclude the possibility that the existence of the fraction in pool III is in part related to an effect of ultracentrifugation.

Fig. 1. Density gradient ultracentrifugation of plasma. Individual fractions of 0.5 ml were collected and apoA-I concentration measured using mAb 4H1 (●). Each fraction was dialyzed and then incubated at 37 °C in the presence of Mn2+ and the resulting change in 3D4 immunoreactivity measured (+—+). The density of fractions 19–21 ranged on average from 1.185 to 1.232 g/ml.
TABLE IV

| Fractions* assayed | EDsD | Change in 3D4 immunoreactivity before and after Mn2+ treatment |
|-------------------|------|-------------------------------------------------------------|
|                   | μg apoA-I/ml | -fold |
| Pool I             | 5.9  | 0.5  | 12 |
| Pool II            | 12.7 | 0.5  | 26 |
| Pool III           | 164  | 0.4  | 427 |
| Pool IV            | 6.5  | 0.9  | 7  |

* Pools I-IV represent the fractions pooled from the gradient ultracentrifugation of plasma after determination of immunoreactivity with mAb 4H1 and 3D4 as illustrated in Fig. 1: pool I, 1.069-1.121 g/ml; pool II, 1.121-1.185 g/ml; pool III, 1.185-1.224 g/ml; pool IV, d > 1.224 g/ml.

In order to define the type of apoA-I containing lipoproteins present in pool III, these subfractions were analyzed by gradient gel electrophoresis and agarose gel electrophoresis both followed by immunoblotting with mAbs against apoA-I. The lipoproteins present in pool III were found to be heterogeneous in size but were almost exclusively of pre B- and/or pre B-like electrophoretic mobility (not illustrated). In an attempt to verify whether an enzyme or protein factor was responsible for the major change in 3D4 immunoreactivity of pool III, we also evaluated its heat stability as a function of time at 80 °C. However, not only was the effect observed at that temperature, but pretreatment of the sample by heating at 80 °C for 10 min in the absence of Mn2+ produced the same increase in 3D4 immunoreactivity as incubation at 37 °C with Mn2+ for 24 h and further incubation of the sample at 37 °C with or without Mn2+ did not bring any additional change. Therefore, these results suggest that the factor (if any) which modulates the change in the 3D4 epitope is thermostable and that the reaction which takes place is most likely chemical in nature rather than enzymatic.

Finally, to demonstrate that the effects of Mn2+ are accompanied by an oxidative reaction, probably involving the lipids, we evaluated the production of TBAR substances during treatment of pools II and III; in the presence of Mn2+, the levels of TBAR substances expressed relatively to the ED50 with 3D4 increased significantly in comparison with the corresponding fractions kept at 4 °C (21- and 96-fold increases for pools II and III, respectively) while the increases were lower in the control experiment without Mn2+ (12- and 10-fold for pools II and III, respectively). It is therefore clear that incubation at 37 °C with Mn2+ causes the peroxidation of lipids which results in the generation of TBAR substances and in the concomitant modifications of apoA-I immunoreactivity.

Immunoreactivity of ApoA-I Containing Lipoproteins before and after Mn2+ Treatment—Since the modification of apoA-I immunoreactivity upon treatment with Mn2+ was only observed with HDL but not with apoHDL, we undertook to evaluate this effect of Mn2+ on apoHDL incorporated into proteoliposomes containing cholesterol and lecithins of different levels of unsaturation which were prepared by the cholate dialysis method (19) as described under “Experimental Procedures.”

In competitive RIA, the different proteoliposomes were equally reactive with mAb 4H1, independent of the degree of unsaturation of their lecithins, and all were slightly less immunoreactive than apoHDL alone (Fig. 2). In contrast, each proteoliposome exhibited a different immunoreactivity with mAb 3D4 after incubation at 37 °C in the presence of antioxidants (Fig. 2): ApoHDL-DOPC liposomes were as immunoreactive as apoHDL alone, while apoHDL-DMPC liposomes competed with the solid phase antigen in a biphasic manner, being less immunoreactive than DOPC-proteoliposomes at protein concentrations above 20 μg/ml but equally immunoreactive at lower concentrations (Fig. 2). These similarities and differences in immunoreactivity of 3D4 with apoHDL and apoHDL-DMPC or -DOPC were observed consistently in several experiments and were statistically significant. The biphasic displacement curve observed with apoHDL-DMPC is most likely due to a self-aggregation of the proteoliposome particles at high concentrations. In all experiments, the immunoreactivity of 3D4 with apoHDL-DLPC was at least 10-fold greater than that observed with other proteoliposomes. This property reflects the importance of lipid fluidity and/or liposome size on apoA-I conformation.

When the proteoliposomes were incubated in the presence of Mn2+, there was no difference in immunoreactivity with mAb 4H1 before and after treatment. Likewise, there was no effect of Mn2+ on the immunoreactivity of mAb 3D4 with either apoHDL-DMPC or apoHDL-DOPC, but the Mn2+ treatment caused a greater than 100-fold decrease in 3D4 reactivity with apoHDL-DLPC (Fig. 2). The same effect (100-fold or more) on 3D4 immunoreactivity was noted in several experiments with different apoHDL-DLPC liposome preparations; however, when proteoliposomes were made with batches of oxidized DLPC (as judged by the yellow color of the sample) the loss of immunoreactivity with 3D4 became minimal and statistically nonsignificant (not illustrated). These experiments demonstrated that incubation under oxidative conditions of apoHDL incorporated into liposomes containing lecithin with polyunsaturated acyl groups but not monounsaturated or saturated acyl groups, would rapidly result in both peroxidation of the lipids and the specific destruction of the 3D4 epitope. Since this result was the opposite of the large increase in 3D4 immunoreactivity caused by Mn2+ treatment of either serum or HDL, we proceeded to evaluate the effect of shorter times of incubation with or without Mn2+ (Fig. 3). In the absence of Mn2+, incubation of apoHDL-DLPC caused a gradual increase in 3D4 immunoreactivity which leveled out at 24 h (4.0-fold increase compared to 0 h). When apoHDL-DLPC was incubated with Mn2+, there was a 1.4-fold increase at 3 h, a value analogous to that obtained without Mn2+, but thereafter immunoreactivity with 3D4 started to decrease (2.9- and >50-fold decrease, respectively, at 24 and 48 h).

It appears, therefore, that the model system of proteoliposomes, which we used, generated upon Mn2+ treatment a modification of apoA-I immunoreactivity which is different from that observed with serum or HDL. Incubation of apoHDL-DLPC liposomes at 37 °C caused a small and limited increase in 3D4 immunoreactivity, a process which competed with the stronger decrease in 3D4 immunoreactivity related to Mn2+ treatment. The effect of Mn2+ is exerted via the oxidation of the linoleyl groups since it does not occur with myristoyl or oleyl groups and can be prevented by addition of antioxidants together with Mn2+ (Table V). When antioxidants were included during the preparation of the proteoliposomes and dialyzed out immediately before incubation, the immunoreactivity of the resulting proteoliposomes with 3D4 was significantly lower than that of the proteoliposomes prepared without antioxidants (not illustrated). This demonstrated that oxidation also takes place, albeit more slowly, at 4 °C and contributes to an increase in 3D4 immunoreactivity.

Electrophoretic Evidence of ApoA-I Modification upon Treatment with Mn2+—When HDL were treated with Mn2+ for different periods of time, SDS-polyacrylamide gel electro-
FIG. 2. Competitive RIA with mAbs 3D4 and 4H1 of proteoliposomes containing apoHDL, cholesterol, and either dimyristoyl-lecithin (●-●), dioleyl-lecithin (□-□), or dilinoleyl-lecithin (■-■) after a 48-h incubation with antioxidants or in the presence of Mn²⁺ as indicated. The competitive displacement curve obtained with apoHDL alone is indicated by a dashed line.

FIG. 3. Effect of time of incubation at 37 °C in the presence or absence of Mn²⁺ on the immunoreactivity of proteoliposomes containing apoHDL, cholesterol, and dilinoleyl-lecithin with mAb 3D4.

phoresis and immunoblots of their apoHDL with 3D4 (Fig. 4A) showed a slight increase of the apparent molecular mass of the apoA-I band at 24 h followed by its appearance at about 38 kDa at 48 h. There was also evidence of an increase with time of the proportion of immunoreactive bands of higher M, which probably corresponds to the multiple apoA-I copies normally present in HDL particles which are cross-linked together or with other apolipoproteins. Under these conditions, there was no evidence of apoA-I cleavage either by silver stain (Fig. 4B) or by immunoblots with 3D4 or 4H1. Immunoblots of the same fractions with antibodies against apoA-II showed that the band immunoreactive with 3D4 at 38 kDa was also immunoreactive with anti-apoA-II and apoA-II. When the same apoHDL samples were analyzed by Tris-urea polyacrylamide gel electrophoresis and immunoblotted with 3D4, the RF of the band which normally corresponds to apoA-I was seen to change progressively with time as the immunoreactive protein and the silver-stained protein (not illustrated) became less positively charged. These bands are diffuse and probably represent a heterogeneous population of chemically modified apoA-I molecules.

In the case of apoHDL-DLPC liposomes treated with Mn²⁺
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Fig. 4. HDL incubated in the presence or absence of Mn$^{2+}$ for the time indicated were analyzed by SDS-polyacrylamide gel electrophoresis and either immunoblotted with mAb 3D4 (A) or stained with silver (B).

as a function of time, the results of SDS-polyacrylamide gel electrophoresis and immunoblots with 3D4 were significantly different from those seen with HDL. The slight increase in the apparent $M_r$ of the immunoreactive apoA-I band was minor but significant after 24 h of treatment as for HDL, but all signal for 3D4 disappeared at 48 h (Fig. 5A). By silver staining of the SDS-polyacrylamide gel electrophoresis, this coincided with the disappearance of the 28-kDa protein and the appearance of a broad band at 30-50 kDa (Fig. 5B). However, this fraction still reacted with other mAbs such as 4H1 (not illustrated) and reacted in RIA with 4H1 (Fig. 2). Again there was no evidence of proteolytic cleavage of apoA-I. When the same apoHDL-DLPC fractions treated with Mn$^{2+}$ were electrophoresed in Tris-urea buffers and immunoblotted, only the 24-h sample was characterized by the appearance of a diffuse 3D4-immunoreactive band, while again all signal disappeared at 48 h (not illustrated). We suggest that the slight increase in molecular weight of apoA-I which occurs up to 24 h of treatment is the result of intramolecular cross-linking, while both intramolecular and intermolecular cross-linkings occur with prolonged treatment, causing the disappearance of the 3D4 signal and the appearance of heterogeneous bands between 30 and 50 kDa (Fig. 5, A and B).

Fig. 5. Proteoliposomes containing apoHDL, cholesterol, and dilauroyl-lecithin and incubated in the presence or absence of Mn$^{2+}$ were analyzed by SDS-polyacrylamide gel electrophoresis and either immunoblotted with mAb 3D4 (A) or stained with silver (B).

DISCUSSION

We initially observed that one series of mAbs obtained by immunization with purified apoA-I (1) did react with epitopes which increased in immunoreactivity with storage of serum at 4 °C (2). Treatment of serum with NaOH did stimulate the effect of storage but only partially and it partially degraded apoA-I. The same observations have been made by Curtiss and Smith (5) with a different series of mAbs also generated by immunization with apoA-I (4). Ghiselli et al. (9) have shown that deamidation occurred in vivo at a slow rate,
making it unlikely that deamidation would be responsible for the formation of the 30% of HDL particles which were shown to express the 3D4 epitope in freshly prepared HDL (6). Furthermore, deamidation causes the formation of the acidic isoforms of apoA-I but these do not express any increased immunoreactivity with mAbs 3D4 or 6B8 compared to A-I (2). Therefore, deamidation can affect any of the glutamine and asparagine residues, but only a few of these could be implicated in the modification of the apoA-I epitopes making it at best an epiphenomenon of deamidation.

The results of the experiments described here are consistent with a lipid peroxidation pathway that would account for the formation of labile epitopes in apoA-I. The role of iron in initiating lipid peroxidation reactions within biological membranes is well established and it has been shown that the ratio of Fe$^{3+}$ to Fe$^{2+}$ is the primary determining factor for the initiation of lipid peroxidation reactions (25). Fe$^{3+}$ ions may initiate lipid peroxidation provided an oxidant is present to oxidize Fe$^{3+}$ to Fe$^{2+}$ or alternatively peroxidation can be initiated by Fe$^{2+}$ provided a reductant is present to form Fe$^{3+}$ (25, 26). We propose that the Mn$^{2+}$-mediated peroxidation which we noted here depends on the presence of a small amount of endogenous Mn$^{3+}$ or on its formation by an oxidant.

Such a mechanism would explain the high levels of Mn$^{3+}$ required: in typical experiments, incubation of HDL at 37°C for 48 h with 0.01, 0.1, and 1 mM Mn$^{2+}$ can increase 3D4 immunoreactivity 2-, 4-, and 10-fold, respectively. It would also be consistent with the inhibitory effect of ascorbate which at a high level (1 mM) will prevent the reduction of Mn$^{3+}$ or Fe$^{3+}$ (26). Such a mechanism is clearly different from the Cu$^{2+}$-mediated peroxidation which was found to operate optimally at 5 μM for the oxidation of LDL apoB (27) and which, as we observed here, is without effect on HDL apoA-I even at 1 mM. However, one cannot rule out other mechanisms for the effect of Mn$^{2+}$ such as production of superoxide or hydrogen peroxide.

We have demonstrated that the Mn$^{2+}$-mediated peroxidation requires the presence of lipids and further that the lipids must be in specific association with apoA-I as is the case in HDL (Table III). One of the main products of lipid peroxidation is MDA which reacts with free amino groups and causes cross-linking and derivatization of peptides; however, exogenously generated MDA failed to produce any modification of 3D4 immunoreactivity despite the evidence that it reacted elsewhere in the molecule and caused an increase in 4H1 immunoreactivity (Table I). Likewise, the specific increase in 3D4 immunoreactivity observed with HDL cannot be duplicated by treatment of apoHDL in the presence of VLDL and LDL (results not illustrated). We can therefore conclude that for lipid peroxidation to result in specific modification of apoA-I, such as a change in 3D4 epitope, lipids, and apoA-I must be in close contact as in HDL, and under these conditions the production of MDA can cause a progressive cross-linking of apoA-I, resulting in a subtle and progressive increase in apparent Mr (Fig. 4A). We have hypothesized that such a slight increase in Mr is the result of intramolecular cross-linking.

The attempt to produce apoA-I proteoliposomes that could be used as a reconstituant system to study in vitro the details of apoA-I modification has been only partially successful (Figs. 2 and 3). While we demonstrated that, as expected, dimyristoyl- or dioleoyl-lecithin could not sustain a lipid peroxidative pathway, dilinoleyl-lecithin was too sensitive to oxidation, and the reaction resulted in a weak and transient increase in 3D4 immunoreactivity before leading to the destruction of the epitope (Fig. 3). We hypothesize that the difference in response of the HDL and dilinoleyl-lecithin to Mn$^{2+}$ treatment is related to the greater dilution of polyunsaturated acyl groups among the mixed and varied acyl groups of the phospholipid species of HDL and to the presence of endogenous lipid soluble antioxidants (a-tocopherol and retinol). This prevents the chain reaction of peroxidation which is likely to occur in dilinoleyl-lecithin. While such a hypothesis could be tested with natural mixed lecithins such as egg lecithins, we were unable to obtain results similar to those with HDL, owing we believe to the evidence of auto-oxidation in all the preparations (not illustrated).

We have demonstrated that apoA-I-containing lipoproteins express differently the epitope for 3D4. This is especially striking for those floating in the density range of 1.18-1.22 g/ml. We have shown that this VHDL fraction is characterized by pre B-electrophoretic migration and heterogeneous Mr. Such pre B-migrating lipoproteins containing apoA-I have been described by others (28, 29). These lipoproteins contain free cholesterol and phospholipids and may be important in the egress of cellular cholesterol (30). It may be of importance in relation to the susceptibility of these fractions to the peroxidative conditions, that they were also found enriched in relation to plasma in arterial intima (31, 32). Modified and oxidized LDL are thought to be generated by vascular endothelial cells, and this process may also affect the specific subclass of apoA-I lipoproteins that are considered here. It is possible that a certain ratio of lipids to apoA-I and probably a specific composition of the lipids, both characteristics of the pre B-migrating VHDL fraction, are predisposing factors for lipid peroxidation leading to the modification of the 3D4 epitope. Other interpretations are also possible including the absence of antioxidants in that fraction or finally the existence of a heat-stable factor mediating the peroxidation. Further experiments are required to sort out these possibilities.

In conclusion, although the extent of its biological occurrence is difficult to assess, lipid peroxidation is a general phenomenon (33) which has general implications in cellular damage, in tumorigenesis and in atherosclerosis. The antiatherogenic effect of Probucol, an antioxidant hypocholesterolemic drug, has received much attention as it appears to act by preventing oxidation of LDL apoB (34, 35). Since it works as a fat-soluble antioxidant, it is certainly an antioxidant for HDL as well and may prevent HDL apoA-I modification. Such considerations are interesting as the cholesterol lowering effect of Probucol has been shown to be greater on HDL than on LDL cholesterol levels (36). There is no evident interpretation for the mechanisms responsible for these effects of Probucol which in addition to oxidative reactions, may include altered lipoprotein catabolism and increased lipid transfer. This important difference in the response of LDL and HDL levels to Probucol therapy may also be related to the very different susceptibility of LDL apoB and HDL apoA-I to oxidative degradation, a subject requiring additional investigations.

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