Modulation of Apolipoprotein B Antigenic Determinants in Human Low Density Lipoprotein Subclasses*

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To investigate the effect of low density lipoprotein (LDL) heterogeneity on the conformation of LDL apolipoprotein B (apo-B), the immunoreactivities of 6 monoclonal antibodies against LDL apo-B were measured in 3 LDL subfractions isolated by equilibrium density gradient ultracentrifugation. To ensure a broad range of LDL particles, the LDL subfractions were prepared from normal subjects and patients with hyperapobetalipoproteinemia. With 3 of the antibodies, 1D 5E11, and 3A10, LDL fractions (the most buoyant), 2 (the intermediate), and 3 (the densest) were equally immunoreactive and competed similarly with reference whole LDL. In contrast, with 3 other antibodies, 2D8, 3F5, and 4G3, fraction 1 was significantly more reactive than fraction 3; that is for each in turn, 290, 250, and 150% more of the densest LDL protein was required to achieve the same displacement as with fraction 1. Further, the immunoreactivities of the 3 LDL fractions with antibodies 2D8, 3F5, and 4G3 were negatively correlated with their LDL cholesterol to LDL protein ratio with r values of 0.727, 0.898, and 0.870, respectively, suggesting that as LDL particle size decreases, the conformation of the LDL apo-B changes progressively. It is of interest that the antigenic determinants recognized by 3F5 and 4G3 are close to the LDL receptor recognition site on LDL apo-B. Therefore, it is possible that the reduced immunoreactivity of these determinants in dense LDL may be the in vitro correlate of the reduced fractional catabolic rate of dense LDL compared to buoyant LDL previously observed in vivo.

Most cholesterol in plasma is contained in the LDL.1 However, these cholesterol-rich particles are not made up uniformly but differ in size, hydrated density, and chemical composition. Thus, equilibrium density gradient ultracentrifugation studies in normal subjects (1, 2) have shown that as LDL particle density increases a series of related changes occur: peak flotation rate decreases, mean particle diameter decreases, and protein to phospholipid ratio increases while, at the same time, core lipidd to protein ratio decreases. These studies in normal subjects have been extended by the demonstration that disease may alter the composition of LDL subfractions; for example, in familial hypercholesterolemia (3), LDL mass is increased and a cholesterol ester-enriched fraction is present whereas in hyperapobetalipoproteinemia (4) and familial combined hyperlipoproteinemia (5), LDL mass is also increased but an LDL subfraction is present that is smaller, denser, depleted in cholesterol ester, and relatively enriched in protein (3, 5).

All these observations are consistent with a spherical model of LDL (6) in which cholesterol ester and triglyceride are confined principally to the core with phospholipid, free cholesterol, and apo-B forming a surface coat of constant thickness. We speculated that this plus the decreasing diameter of the particle and possibly changes in core lipid as well might alter the surface configuration of the apo-B. Accordingly the present study examines whether the immunoreactivity of a series of apo-B antigenic determinants is altered predictably in LDL particles of different composition and density.

EXPERIMENTAL PROCEDURES

Subject Studies—LDL was isolated from 14 adults (13 males, 1 female, average age 57 ± 7 years). Nine had suffered a documented myocardial infarction at least 3 months previously while five had no history of coronary artery disease. Plasma lipid, lipoprotein lipid, plasma LDL apo-B levels and apo-E phenotype are given in Table I. On the basis of laboratory and clinical evaluations, none of the subjects presented with secondary causes of dyslipoproteinemia, such as hypothyroidism or nephrosis, or with the E2/E2 phenotype.

Discontinuous density gradient ultracentrifugation was then performed as follows. To a 2-inch (5-cm) cellulose nitrate tube, 1 ml of the following solutions was added in succession: 1.1300 g/ml, 1.0645 g/ml, and 1.050 g/ml. 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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The abbreviations used are: LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein; DL, intermediate density lipoproteins; SBS, sodium dodecyl sulfate; BSA, bovine serum albumin; PBS, phosphate-buffered saline.
the measurement of LDL apo-B was performed in Renovacells, Alexandria, VA) as follows. The wells were coated by an overnight incubation with 200 μl of reference LDL (30 μg/ml in 5 mM glycine, pH 9.2) and subsequently saturated by incubation for 1 h with 250 μl of 1% BSA in PBS, pH 7.4. Each antibody appropriately diluted in BSA-PBS solution was incubated overnight with dilutions of either the standard reference LDL or the various LDL subfractions at final concentrations ranging from 0.1 to 10 μg of LDL protein/ml in BSA-PBS in disposable culture tubes. 200-μl aliquots of these mixtures were then added to the wells which had been washed with 0.15 M NaCl containing 0.025% Tween 20. The wells were incubated overnight and again washed with the Tween-saline solution as above. Rabbit anti-mouse IgG was labeled, purified as described earlier (7), and diluted in BSA-PBS before use. 200 μl of this solution representing about 60,000 cpm was added to each well and incubated overnight. The wells were washed with the Tween-saline solution as above and counted for radioactivity. The intra-assay coefficient of variation for the measurement of LDL apo-B was 10% or less regardless of the antibody used.

Analyses—Protein concentration of the LDL subfractions was measured by the method of Lowry et al. (9) using bovine serum albumin as a standard. Triglyceride and cholesterol concentrations were measured by enzymatic methods (4). LDL cholesterol (10), HDL cholesterol (11), and plasma LDL apo-B (12) were determined as described earlier. SDS-polyacrylamide gel electrophoresis was carried out according to Kane et al. (13).

RESULTS

In each subject, LDL was recovered in only the last three fractions separated after equilibrium density gradient ultra-centrifugation. These were labeled as fraction 1 (the least dense and located just at the meniscus), fraction 2 (intermediate density), and fraction 3 (the most dense). The cholesterol to apo-B ratio decreased. The average density of the LDL subfraction increased within a subject, and in this subject, there is considerable difference in the displacement obtained with the LDL subfractions whereas with other antibodies, for example 5E11 and 3A10, there is little difference.

From these displacement curves, the immunoreactivity of

### TABLE I

| Patient no. | Age | Sex | Clinical status | Apo-E phenotype | Total cholesterol | Triglycerides | Plasma | LDL apo-B | HDL cholesterol | LDL cholesterol |
|-------------|-----|-----|-----------------|-----------------|-------------------|---------------|--------|-----------|----------------|-----------------|
| 1           | 53  | M   | A               | E4/3            | 197               | 201           | 142    | 35        | 122            |                 |
| 2           | 49  | M   | N               | E3/2            | 208               | 135           | 110    | 49        | 132            |                 |
| 3           | 64  | F   | A               | E3/3            | 171               | 108           | 108    | 55        | 94             |                 |
| 4           | 63  | M   | A               | E3/2            | 153               | 109           | 89     | 46        | 85             |                 |
| 5           | 59  | M   | A               | E3/3            | 218               | 326           | 163    | 18        | 135            |                 |
| 6           | 59  | M   | A               | E3/3            | 190               | 110           | 114    | 38        | 130            |                 |
| 7           | 59  | M   | A               | E3/3            | 290               | 334           | 146    | 20        | 203            |                 |
| 8           | 60  | M   | A               | E3/3            | 291               | 150           | 142    | 70        | 195            |                 |
| 9           | 62  | M   | A               | E3/3            | 192               | 146           | 104    | 48        | 135            |                 |
| 10          | 61  | M   | A               | E3/2            | 242               | 245           | 135    | 35        | 158            |                 |
| 11          | 65  | M   | A               | E3/3            | 212               | 93            | 101    | 45        | 148            |                 |
| 12          | 47  | M   | N               | E4/3            | 167               | 101           | 85     | 44        | 103            |                 |
| 13          | 63  | M   | N               | E3/2            | 157               | 123           | 77     | 69        | 63             |                 |
| 14          | 42  | M   | N               | E3/3            | 204               | 113           | 86     | 41        | 140            |                 |

* HDL, high density lipoprotein.

* A, clinical coronary artery disease; N, no clinical coronary artery disease.

The LDL cholesterol values are calculated from the plasma cholesterol, minus HDL cholesterol, and minus triglyceride level divided by 5 according to Ref. 10.

### TABLE II

| Patient no. | LDL subfractions |
|-------------|------------------|
|             | 1                | 2                | 3                |
| 1           | 1.51             | 1.27             | 0.88             |
| 2           | 1.66             | 1.37             | 1.00             |
| 3           | 1.06             | 0.89             | 0.78             |
| 4           | 1.68             | 1.33             | 0.80             |
| 5           | 1.52             | 0.95             | 0.75             |
| 6           | 1.63             | 1.28             | 1.00             |
| 7           | 1.60             | 1.25             | 0.74             |
| 8           | 1.56             | 1.41             | 1.08             |
| 9           | 1.65             | 1.28             | 0.78             |
| 10          | 1.53             | 1.14             | 0.71             |
| 11          | 1.73             | 1.41             | 1.04             |
| 12          | 1.57             | 1.32             | 1.05             |
| 13          | 1.66             | 1.43             | 0.89             |
| 14          | 1.64             | 1.32             | 1.24             |

Cholesterol to apo-B ratio in LDL subfractions

Cholesterol to apo-B ratio is calculated from the cholesterol (10) and protein (8) values which are measured directly on the LDL subfractions.
than fraction plotted against the amount of LDL protein necessary for 66% displacement of maximal binding of the monoclonal antibody to the immobilized control LDL.

There are strong significant differences in immunoreactivity among individuals as well. Indeed, about 290, 250, and 150% more LDL protein was required to obtain the same displacement with fraction 3 as fraction 1 using 2D8, 3F5, and 4G3, respectively. Fraction 2 tended to be less immunoreactive than fraction 1 using 2D8, 3F5, and 4G3, respectively.

This type of analysis presumes each of the 3 subfractions is the same in each of the 14 individuals, but as the data in Table II indicate this is clearly not the case. That is, while LDL particles differ within an individual, there are differences among individuals as well.

Therefore, in Fig. 2, the LDL cholesterol to apo-B ratio is plotted against the amount of LDL protein necessary for 66% displacement. For 1D1, 5E11, and 3A10, no strong correlation is apparent and the p values are >0.05. But for 2D8, and particularly for 3F5 and 4G3, there are strong significant (p < 0.05) inverse correlations between LDL immunoreactivity and LDL cholesterol to apo-B ratio indicating that as the LDL particles become denser, these specific determinants become proportionately less immunoreactive. These differences in immunoreactivity could not be ascribed to any apparent differences among the LDL fractions of a given subject in their respective apoprotein compositions as judged by SDS-polyacrylamide gel electrophoresis and densitometric scanning of the stained gels. When in some subjects, apo-B74 and apo-B26 (13) were noted in addition to B100, these same fragments of apo-B were present in all of the 3 LDL subfractions (Fig. 3). Also, the presence of apo-B fragments was not associated with any lipoprotein phenotype of the patients, that is normal type IV and/or hyperapoB27apoproteinemia, and, therefore, was not more prevalent in subjects with the densest LDL. In addition, these electrophoreses demonstrated that none of the LDL fractions were contaminated with lower molecular weight proteins.

### DISCUSSION

LDL particles are heterogeneous, differing in lipid content and, therefore, differing in size and density. The present study indicates that the immunoreactivity of certain specific apo-B antigenic determinants varies as a function of LDL composition and, therefore, suggests a possible linkage between altered LDL composition and LDL metabolism.

We chose LDL cholesterol to apo-B ratio as our index of LDL heterogeneity since there is general agreement that this ratio varies inversely with LDL size and density (1-3). If apo-B content/LDL particle is constant, as most evidence indicates (14), then as LDL size decreases, the configuration of the protein would be expected to change. In addition, because surface lipid to protein ratios decrease (1-3), the spatial relationship of the phospholipids and free cholesterol to apo-B antigenic determinants must also change.

Among the 6 antigenic determinants studied, the strength of the relationship between immunoreactivity and LDL composition varied, indicating that specific conformational
 changes occurred which differentially affected certain determinants as particle size diminished. Two of the determinants showing the strongest relationship (3F5 and 4G3) have been shown to be adjacent to one another in cotitration experiments (7) and on tryptic LDL fragments (15), while 2D8 and 4G3 have been found together on small fragments of soluble apo-B obtained by CNBr cleavage, thus demonstrating the proximity of these 3 determinants on LDL apo-B. Furthermore, these 3 determinants in general have exhibited the same requirements for lipids when solubilized apo-B was incorporated into recombinant lipid vesicles (16). The antibodies 2D8, 3F5, and 4G3 were all antigenically active upon incorporation of apo-B into lecithin-cholesterol olate microemulsions (16), which are characterized by a hydrophobic core and so mimic LDL structure (17, 18). Thus it appears that it is the conformation of a specific region of apo-B that changes as the LDL particles become smaller and denser.

It is possible that changes in lipoprotein or apolipoprotein structure associated with preparation of the lipoprotein fractions could have contributed to the differences in immunoreactivity shown here. However, with SDS electrophoresis we have not noted differences in the major apolipoprotein B components among the fractions and have found no peptide fragments indicative of possible proteolysis. While the denser LDL fraction may have been contaminated with lipoprotein Lp(a), previous studies have shown that is unlikely to be a significant component in fractions of d < 1.050 g/ml (2), and, therefore, such contamination could not account for the differences in immunoreactivity between fractions 1 and 2 or the linear relationship of immunoreactivity with cholesterol to apo-B ratio across the particle spectrum.

Because the antibodies directed against 3F5 and 4G3 are also capable of interfering with the binding of LDL to the apo-B/E receptor of fibroblasts (7), this region may also be important in regulating LDL catabolism. Turnover studies in human using 125I-VLDL and 125I-LDL have shown a precursor-product relationship between VLDL and IDL, between IDL and buoyant LDL, and between buoyant LDL and dense LDL (19). This is the case both in normal subjects and in patients with hyperapobetalipoproteinemia. In both situations as well, the fractional catabolic rate of dense LDL is significantly less than that of buoyant LDL while the absolute

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**Fig. 2.** Correlations between the LDL cholesterol to LDL apo-B ratio of each LDL subfraction of each subject and their respective immunoreactivity with the different antibodies expressed as the amount of protein of each LDL subfraction necessary for 66% displacement of a given antibody to the immobilized reference LDL.

**Fig. 3.** SDS-gel electrophoresis of apo-B present in LDL subfractions from 3 representative patients. Buoyant, intermediate, and dense LDL subfractions from, respectively, a normolipemic and two hyperapobetalipoproteinemic with or without hypertriglyceridemia patients are applied in lanes 1 to 9 (50 μg of protein each); lane 10 contains a series of molecular weight standards.
The synthetic rate of buoyant LDL exceeds that of dense LDL, suggesting preferential clearance of buoyant LDL (19). It would, therefore, appear that as LDL particles become lipid depleted and denser, there are conformational modifications of certain determinants of apo-B, especially 2D8, 3F5, and 4G3, which consequently may decrease the fractional catabolic rate of the dense LDL.

The antigenic determinants recognized by antibodies 5E11 and 3A10 are also located close to the receptor recognition site on the LDL apo-B (7); however, the reactivities of these determinants show no correlation with the cholesterol to apo-B ratio of LDL. In previous studies designed to characterize apo-B antigenic determinants, these 2 determinants were found to be poorly immunoreactive upon delipidation of apo-B (8). Upon equilibration of apo-B with lecithin-cholesteryl oleate microemulsions, 5E11 regained only partial activity while 3A10 remained inactive (16). It appears, therefore, that these determinants are highly susceptible to modifications of the LDL apo-B conformation, and it may be that the labile nature of their immunoreactivity does not allow observation of correlation with LDL size and density.

This modulation of apo-B antigenic determinants in LDL subclasses may be a consequence of either conformational modification of apo-B by lipoprotein structure and composition or due to genetic polymorphism of apo-B. Several reports link apo-B immunoreactivity to the composition of apo-B-containing lipoproteins. Polyclonal antisera directed against apo-B were first reported (20) able to differentiate VLDL of various sizes, and certain monoclonal antibodies were also identified which distinguish among VLDL, IDL, and LDL (21-23). Most of the determinants studied by Tikkanen et al. (21) exhibited increased immunoreactivities with decreasing VLDL flotation rates and were also more reactive with LDL. Tsao et al. (22) using a different battery of monoclonal antibodies defined distinct patterns of antigenic determinant expression in VLDL, IDL, and LDL. These observations suggest that apo-B conformation in VLDL, IDL, and LDL is similar but not identical. We ourselves observed earlier (23) that the determinants recognized by antibodies 1D1, 3F5, 4G3, 5E11, and 3A10 reacted less well with VLDL than with LDL and that their immunoreactivity could be increased by partial delipidation. Therefore, it appears that a number of apo-B antigenic determinants are either masked or structurally modified by the configuration taken by apo-B in VLDL and that their immunoreactivity increases as VLDL particles are transformed into LDL through the normal metabolic sequence. In keeping with the data presented here, the immunoreactivity of determinants such as 3F5 and 4G3 reaches a maximum with LDL particles of a defined hydrated density and thereafter decreases as these particles become denser, possibly as a result of the configuration constraints exerted on apo-B. This interpretation is supported by the results of Mao et al. (24) who found LDL immunoreactivity to vary as a function of temperature, an observation compatible with modulation of LDL immunoreactivity by lipid composition and content, since temperature changes should result in modification of lipoprotein conformation and fluidity. Finally it is unlikely that partial proteolysis may be the cause of the decreased immunoreactivity of 2D8, 3F5, and 4G3 with increasing LDL density since apo-B74 and apo-B26, when present in a given subject LDL, were equally distributed in the 3 LDL subclasses. In addition, the determinants recognized by polyclonal antibodies were not found to be susceptible to proteolysis (15).

Alternatively, or simultaneously, we must also consider that this modulation of apo-B antigenic determinants could be related to genetic polymorphism of apo-B. Immunochemical polymorphism of human apo-B was demonstrated by Blumberg et al. (25) and by Butler and Brunner (26) while Fisher et al. (27) reported that LDL sizes are determined genetically. More recently (28), immunochemical polymorphism of LDL apo-B was demonstrated by the reduced binding of 3 monoclonal antibodies which allowed LDL donors from different families to be separated. Therefore, genetic polymorphism must also be included as a possible interpretation of the reduced immunoreactivity of antibodies 2D8, 3F5, and 4G3 with dense LDL, especially in the densest LDL found in hyperapobetalipoproteinemia (4).

In conclusion, the present study has shown that the immunoreactivity of certain specific antigenic determinants varies directly with LDL composition and more specifically with a parameter proportional to LDL size and density. Of special interest is the fact that two of these determinants which are predictably affected by LDL apo-B configuration are located near the receptor recognition site on LDL apo-B (7). This observation together with the differential fractional clearance rates from plasma which have been demonstrated for buoyant versus dense LDL (19) leads to the hypothesis that LDL affinity for the apo-B/E receptor may be affected by the density and size of LDL particles. Future experiments must be designed to test this hypothesis and to verify whether a correlation may be found between the immunoreactivity of the determinants 3F5 and 4G3 and LDL affinity for its cellular receptors.

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