Characterization of Antigenic Determinants of Human Apolipoprotein B

Distribution on Tryptic Fragments of Low Density Lipoprotein

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In the hope of obtaining useful probes to study the structure of human apolipoprotein B (apo B), we characterized monoclonal antibodies against low density lipoprotein (LDL). We examined the distribution of their corresponding antigenic determinants on tryptic fragments of LDL separated by monodimensional (SDS) or two-dimensional electrophoresis. Each antibody reacted with several different fragments even when the proteolysis was apparently complete. A peptide of 125,000 daltons was the smallest fragment recognized by all the antibodies. The antibody, 2D8, which cross-reacts with apo B-48 and 3A8 which blocks the LDL pathway both reacted with the same 43,000 dalton fragment. Two other antibodies, 3F5 and 4G3, previously shown to be close together in LDL, also appeared close together in the primary structure of apo B. A determinant present on apo B-26 (1D1) was dissociated from all others examined on fragments of less than 125,000. Similarities in the patterns of reactivities with LDL-tryptic fragments between certain monoclonal antibodies and the lectins Concanavalin A and Limax flavus agglutinin indicated the proximity of the corresponding antigenic determinants to carbohydrate moieties. Competition studies suggested that the two major carbohydrate chains of LDL do not participate in the determinants themselves. (Arteriosclerosis 4:498-509, September/October 1984)
Methods

Preparation of LDL

Blood from normal, nonfasting donors was collected into EDTA. Red blood cells were immediately removed by centrifugation, and the plasma was supplemented with NaCl (final concentration 0.02%) and phenylmethyl sulfonl fluoride (150 mg in 0.5 ml dimethylsulfoxide per 100 ml plasma). Lipoprotein subfractions were prepared in a Beckman L5-65 ultracentrifuge with a 50.2 Ti rotor (Beckman Instruments, Spinco Division, Palo Alto, California). Density was adjusted with solid KBr. LDL was isolated at 4°C by sequential preparative ultracentrifugation between densities of 1.020 and 1.05013 and then dialyzed exhaustively against phosphate-buffered saline (PBS) (pH 7) containing 0.02% NaN3 and 0.9 µg of Chloramine T (Calbiochem, La Jolla, California) was performed according to Mellman and Unkeless.15 The iodination of protein in 0.3 M NaP, (pH 7), 0.5 mCi of 125I (Amerham Corporation, Arlington Heights, Illinois) and 0.9 µg of Chloramine T in a total volume of 0.1 ml. The mixture was incubated for 30 minutes at 4°C and the reaction was terminated as described in the following sections.

Tryptic Digestion of LDL

The LDL samples were adjusted to 2 mg/ml protein and centrifuged for 5 minutes at 12,000 g to remove any aggregated material. The digestion was initiated by the addition of an appropriate volume of a freshly prepared solution of bovine trypsin (8 mg/ml, specific activity 8170 SU/ml) (Calbiochem, La Jolla, California, Catalog No. 64852) in PBS (pH 7) containing 0.02% NaN3 and 0.001% EDTA, sterilized by ultrafiltration, and stored at 4°C. For all of these experiments, LDL was obtained from a single donor.

Monoclonal Antibodies against LDL

Hybridomas that secrete monoclonal antihuman LDL were the product of a cell fusion between cells of the plasmacytoma cell line SP2-014 and isolated spleen cells from BALB/c mice previously immunized with human LDL. Details of the cell fusion and characterization of the monoclonal antibodies have been described elsewhere.7 9

Iodination of Protein

Iodination of affinity-purified rabbit antianmouse immunoglobulin (Ig) (Kirkegaard and Perry Laboratories Incorporated, Gaithersburg, Maryland), low and high molecular weight standards for electrophoresis (Pharmacia, Uppsala, Sweden), concanavalin A (Con A) type V (Sigma Chemical Company, St. Louis, Missouri) and Limax Flavus agglutinin (LFA) (Calbiochem, La Jolla, California) was performed according to Mellman and Unkeless.15 The iodination mixture consisted of 25 µg of protein in 0.3 M NaP, (pH 7), 0.5 mCi of 125I (Amerham Corporation, Arlington Heights, Illinois) and 0.9 µg of Chloramine T in a total volume of 0.1 ml. The mixture was incubated for 30 minutes at 4°C and the reaction was terminated by the sequential addition of 10 µl of 1 M KI and 100 µl of 0.1% BSA in PBS. Free 125I was removed by passage of the reaction mixture on a 1.5 ml column of AG1-X8 resin (Bio-Rad Laboratories, Richmond, California). Labeled proteins were stored at 4°C in PBS containing 0.1% bovine serum albumin (BSA) and 0.02% NaN3. Immediately after labeling, more than 90% of the radioactivity was precipitable with 10% trichloroacetic acid and specific activities ranged from 4 to 8 µCi/µg. For all experiments with 125I-Con A and 125I-LFA, the BSA solution (0.1% in PBS) had been prepassed on Con A-Sepharose 4-B (Pharmacia, Uppsala, Sweden) to remove contaminating glycoproteins.

Electrophoretic Procedures

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Whole tryptic digests were analyzed on SDS-PAGE by using the discontinuous buffer system of Leammli16 on 1.5 mm thick slab gels consisting of a 3% stacking gel and a separating gel composed of a linear gradient from 5% to 15% acrylamide. For these analyses, the digestion was stopped by the addition of 1 volume of a mixture of 0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol; the sample was immediately heated at 100°C for 5 minutes. The samples could then be stored for at least 1 week at 4°C without affecting the resulting electrophoretic pattern. For the electrophoresis, the equivalent of 70 µg of whole tryptic digested protein was applied per centimeter of gel. 125I-low and high molecular weight standards treated identically to the samples were applied respectively on the right and left hand side of the gel. Gels were routinely run at 20 mA/gel for about 4 hours. The migrated proteins were either stained with Coomassie brilliant blue R-25017 or silver stain18 or were transferred electrophoretically to nitrocellulose paper (NCP) as described below.

Two-Dimensional Gel Electrophoresis

The method used for two-dimensional gel electrophoresis was essentially that described by O‘Farrell19 with a modification for the solubilization of the samples.20 For these studies, proteolytic digestion was terminated by adding to the reaction mixture a stock solution of 10% SDS to give a final ratio of SDS/protein of 2.5 (wt/wt) and of 2-mercaptoethanol to give a final concentration of 8%. The mixture was immediately heated at 100°C for 5 minutes. After the sample cooled, 900 mg of solid urea was dissolved per milliter of sample and a sample dilution buffer containing 9.5 M urea; 0.4% (vol/vol) amphotilin (pH 3.5 to 10); 1.6% (vol/vol) amphotilin (pH 5 to 7) (LKB, Bromma, Sweden); and 8% (wt/vol) Nonidet P40 (BDH Chemicals, Montreal, Canada) was added to give a ratio of Nonidet P40/SDS of 8 and a final ampholine concentration of 1.7%. Immediately after this treatment, samples were applied (170 µg of total...
proteins/tube) on cylindrical isoelectrofocusing gels (10.5 cm × 5.5 mm). The gels contained 9.5 M urea, 4% acrylamide, 2% Nonidet P-40, 1.6% (vol/vol) ampholines (pH 5 to 7), and 0.4% (vol/vol) ampholines (pH 3.5 to 10). Gels were preelectrophoresed before the samples were applied according to the method of O’Farrell,19 The proteins were allowed to migrate for 18 hours at 400 volts and then for 1 hour at 800 volts. Gels were removed from the tubes and stored frozen in 10 ml of 0.0625 M Tris HCl (pH 6.8), 10% (wt/vol) glycerol, 2% SDS, and 5% 2-mercaptoethanol. Occasionally gels were stained with Coomassie blue G-250 0.04% in 3.5% perchloric acid and destained in 5% acetic acid. Before the second-dimension gel electrophoresis, isoelectrofocusing gels were thawed in water at room temperature and allowed to equilibrate for 10 to 15 minutes before loading on the second-dimension gel.

First-dimension isoelectrofocusing gels were loaded on the top of the second-dimension SDS polyacrylamide slab gels (1.5 mm thick) as described by O’Farrell.19 A warm solution of 0.5% Agarose (Aga rose C, low electroendosmosis, Pharmacia Fine Chemicals) containing 0.0625 M Tris HCl (pH 6.8) and 0.1% SDS was used to embed isoelectrofocusing gels. A teflon strip was used to form a well in Agarose for including 125I-low molecular weight standards in the second dimension. The composition of the second-dimension gel consisted of a linear gradient from 5% to 15% acrylamide and a 3% stacking gel. Gels were run at 25 mA/gel for about 4 hours. The migrated proteins were either stained with Coomassie blue or transferred electrophoretically to NCP.

Electrophoretic Transfer to Nitrocellulose Paper and Detection of Peptides

Electrophoretic transfer from SDS-PAGE to NCP (0.45 μm pore size, Millipore) was done according to the method of Towbin et al.21 After SDS-PAGE, the slab gels were equilibrated for 15 minutes at room temperature in the electrophoretic transfer buffer (20 mM Tris, 150 mM glycine pH 8.3, 20% methanol, vol/vol) and then the gels were mounted in a Trans-Blot cell (Bio-Rad Laboratories). Blotting was performed at 350 mA for 3 hours. After the transfer, the NCP prints were stored in 10 mM Tris, 150 mM NaCl, 0.02% NaN3, pH 7.4 (Buffer A) at 4°C for more than 3 days.

For immunological detection of peptides generated by proteolytic digestion of LDL, all incubations and washes were done in Buffer A containing 3% BSA (Buffer B) in heat-sealable plastic bags and under rotary agitation at 37°C. Free protein-binding sites on NCP were blocked by incubation in Buffer B for 2 hours. After saturation, NCP were incubated with monoclonal ascitic fluid diluted 1:1000 in Buffer B for 1 hour followed by two rinses, two washes (30 minutes each), and two more rinses. The NCP was then incubated for 1 hour with 125I-rabbit antimouse Ig diluted in Buffer B to a concentration of 1.5 × 106 cpm/ml, was washed as described above, and was dried. Autoradiography was performed on KAR-5 Kodak films with an intensifier screen (Cronex, Dupont).

For detection of glycopeptides liberated by proteolysis, NCP were saturated as above in Buffer B, which had been prepurified on Con A-Sepharose 4-B. NCP were then incubated with 125I-Con A or 125I-LFA at a concentration of 2 × 106 cpm per ml in Buffer A containing 1 mM Ca2+, Mn2+, Mg2+ and 0.5% triton X-100 (Buffer C) for 1 hour at room temperature. Excess 125I-lectin was removed by washing (5 × 10 minutes) in Buffer C; the NCP was dried before autoradiography.

Solid Phase Assay to Determine Lectin-Monoclonal Antibody Competition for Apo B

LDL (200 μl) at 30 μg/ml protein/ml in 5 mM glycine was added to Removawells (Dynatech Laboratories Incorporated, Alexandria, Virginia) and left overnight at room temperature. The following day the wells were washed in 0.15 M NaCl containing 0.025% Tween 20 and then exposed for 30 minutes at room temperature to 300 μl of 1% BSA which had been prepassed on Con A-Sepharose. Aliquots of Con A (200 μl) diluted in Buffer C containing 0.1% BSA (prepassed on Con A-Sepharose) were added to the wells and were incubated overnight at room temperature. The wells were washed as above and free Con A binding sites were saturated by a 1-hour incubation with 1% ovalbumin. The wells were then exposed for 3 hours to 200 μl of monoclonal antibody diluted in 0.1% BSA. After washing in 0.025% Tween-20, 200 μl of 125I-antimouse IgG diluted in 0.1% BSA was added to the wells and incubated overnight. The wells were washed as above and the bound radioactivity was determined. The results are expressed as B/Bo where B = cpm bound in the presence of unlabelled lectin and Bo = cpm bound in the absence of unlabelled lectin.

Protein Determination

The protein was measured according to the method of Lowry et al.22 using BSA as a standard.

Results

Tryptic Digestion of LDL

Time-Course Analysis by SDS-Polyacrylamide Gel Electrophoresis

Human LDL that had been exposed to trypsin for 0.5, 3, 6, 18, or 24 hours was subjected to polyacrylamide gel electrophoresis in the presence of SDS. The migrated proteins were stained either by Coomassie Blue or silver stain or transferred electrophoretically to nitrocellulose paper and treated sequentially with a monoclonal anti-apo B antibody and 125I-rabbit antimouse immunoglobulin. A gel stained with
Coomassie Blue (Figure 1) illustrates the time course of tryptic cleavage of apo B. After 30 minutes of proteolysis, intact apo B (band 1) had virtually disappeared and approximately 40 different peptides having molecular weights from 17,000 to 190,000 had been generated. In addition, a nonresolved mass of peptides having molecular weights of less than 15,000 was detected with silver stain (not shown). In spite of the rapid disappearance of the apo B, a progressive digestion of the fragments occurred over a period of 18 hours. Little difference was seen between the patterns of the fragments in the 18-hour and 24-hour digests, which indicated that (by this criteria) at 18 hours the reaction had stopped. It was not, however, determined if the reaction was truly complete or if autoproteolysis of the trypsin had limited the digestion.

Over the period of the proteolysis, the appearance and/or cleavage of 46 different fragments was detected. A series of fragments of over 75,000 daltons were detected in the 30-minute digest which, in general, were subsequently cleaved; an exception was band 16 (78,000 daltons) which showed a progressive accumulation with time. The rates of production and destruction of fragments of lower molecular weight (<75,000 daltons) were diverse. Certain fragments (e.g., bands 18 and 27) increased as a function of the proteolysis, whereas others appeared at 30 minutes and either showed no apparent subsequent change (e.g., bands 20, 28, and 33) or underwent further cleavage (e.g., bands 21, 22, and 36). When samples of LDL prepared from four different subjects were digested individually with trypsin and analyzed by SDS gel electrophoresis, the patterns were indistinguishable (not shown).

 Autoradiographs of the NCP blot treated with the antibodies 1D1, 2D8, 3F5 and 4G3 are shown in Figure 2. An attempt was made to correlate the bands present on the autoradiographs to those of the stained gel. This was complicated by the fact that during equilibration with the transfer buffer, there was a shrinkage of the polyacrylamide gel. This seemed to be greater in the upper part of the gel which contained a lower percentage of acrylamide. Therefore, in addition to position, the shape, production rates, and destruction rates were also used as criteria for identification of the bands. The Rf values were calculated from the stained gel, and a summary of the most important antibody reactions is shown in Table 1. It is evident from Table 1 and Figure 2 that it is possible to make groupings of antibodies. The reaction patterns of 3F5 and 4G3 are very similar, as are those of 2D8 and 3A8 (the latter not shown in Figure 2), although subtle differences were apparent between members of each pair. The detection in the autoradiographs of reactions with the antibodies 3A8 and 5E11 required extended exposure times (up to 10 times that of other antibodies). We had previously noted7 that these two antibodies reacted poorly with apo B that had been subjected to SDS electrophoresis and transferred to nitrocellulose paper. In control experiments using a monoclonal antibody against an irrelevant antigen (apo A-I), no reactions were observed.

All antibodies appeared to react with a 124,000 dalton fragment (band 8). Band 8 had a characteristic doublet form on both the autoradiographs and the stained gel which facilitated identification. This appeared to be the smallest band that included all the antigenic determinants. In smaller fragments, the determinant recognized by 1D1 was clearly dissociated from all other determinants. Within the resolution of the technique, the determinants 2D8, 3A8, and 5E11 seem to be present together on a major 72,000 dalton fragment (band 18), and 2D8 and 3A8 are on a 43,000 dalton fragment (band 28a). The determinants 3F5 and 4G3 appear very rapidly on several fragments having molecular weights between 60,000 and 70,000 (bands 18, 20b, 21) and on a minor fragment with a molecular weight of 43,000 (band 28b).

**Two-Dimensional Electrophoresis**

In the hope of increasing the resolution of the separation of the tryptic fragments of apo B, we subjected the digested LDL to isoelectrofocusing in polyacrylamide followed by SDS gel electrophoresis in a second dimension. This procedure is complicated by the well-documented insolubility of delipidated apo B in aqueous buffers. Based on the results of preliminary trials, we chose a technique originally de-
Figure 2. LDL treated with trypsin and separated as in Figure 1 was transferred to nitrocellulose paper (NCP) and exposed to the indicated monoclonal antibodies followed by $^{125}$I-antimouse Ig. Lanes 2 to 8 show LDL that had been exposed to trypsin for 0, 0.5, 1, 3, 6, 18, or 24 hours, respectively. The LDL of Lane 9 had been incubated for 24 hours in the absence of trypsin. Lanes 1 and 10 show, respectively, the high (albumin 67,000, catalase subunit 60,000, and lactate dehydrogenase 36,000) and low (albumin 67,000, ovalbumin 43,000, carbonic anhydrase 30,000, and trypsin inhibitor 20,100) molecular weight standards which had been labelled with $^{125}$I. The other proteins in the mixture of molecular weight standards were not detected on NCP. The bands that are discussed in the text are identified on the figure.

scribed$^{20}$ for the two-dimensional separation of membrane proteins, in which the sample is solubilized in a buffer containing both SDS and a nonionic detergent, Nonidet P40. During the course of the isoelectrofocusing step, the SDS bound to the protein is displaced by the Nonidet P40 which permits a separation based on the isoelectric point of the individual peptides. In spite of these precautions, we observed aggregated material in the overlying buffer that had failed to penetrate the gel. The relative solubility of the tryptic fragments during the isoelectrofocusing step may, therefore, introduce a selectivity with respect to the peptides which can be subsequently detected on the NCP.

Thus, human LDL was exposed to trypsin for either 0.5 or 24 hours and was analyzed by two-dimensional electrophoresis. The gels stained by Coomassie Blue are shown in Figure 3. After 30 minutes of proteolysis (Figure 3 A) the separation of the fragments by isoelectrofocusing yielded approximately 20 bands, most of which had isoelectric points between 5.5 and 6.5 (Figure 3 A inset). In addition, we saw a major band in the alkaline end of the gel as well as several barely visible bands in the region of the gel with a pH below 5.5. Because of the presence of SDS during the solubilization step, the position of the fragment in the gel may not reflect the true isoelectric points of the fragments.$^{20}$ On the two-dimensional gel of the 30-minute digest, about 30 well resolved spots were detected in the region of the gel corresponding to peptides having apparent isoelectric points ranging from 5.5 to 6.5 and molecular
Table 1. Summary of Reactivities of Monoclonal Antibodies with Apo B Tryptic Fragments

<table>
<thead>
<tr>
<th>Band</th>
<th>RI</th>
<th>Molecular weight $\times 10^{-3}$</th>
<th>Antibodies reactive</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>0.279</td>
<td>124</td>
<td>1D1</td>
</tr>
<tr>
<td>9</td>
<td>0.299</td>
<td>115</td>
<td>2D8</td>
</tr>
<tr>
<td>13</td>
<td>0.374</td>
<td>87</td>
<td>3F5</td>
</tr>
<tr>
<td>16</td>
<td>0.405</td>
<td>78</td>
<td>4G3</td>
</tr>
<tr>
<td>17</td>
<td>0.415</td>
<td>75</td>
<td>3A8</td>
</tr>
<tr>
<td>18</td>
<td>0.425</td>
<td>72</td>
<td>5E11</td>
</tr>
<tr>
<td>19a</td>
<td>0.442</td>
<td>68</td>
<td>2D8</td>
</tr>
<tr>
<td>19a or b</td>
<td>0.442</td>
<td>68</td>
<td>3F5</td>
</tr>
<tr>
<td>20a</td>
<td>0.456</td>
<td>64</td>
<td>4G3</td>
</tr>
<tr>
<td>20b</td>
<td>0.456</td>
<td>64</td>
<td>3A8</td>
</tr>
<tr>
<td>21</td>
<td>0.469</td>
<td>61</td>
<td>5E11</td>
</tr>
<tr>
<td>25</td>
<td>0.520</td>
<td>51</td>
<td>1D1</td>
</tr>
<tr>
<td>27</td>
<td>0.537</td>
<td>48</td>
<td>1D1</td>
</tr>
<tr>
<td>28a</td>
<td>0.565</td>
<td>43</td>
<td>2D8</td>
</tr>
<tr>
<td>28b</td>
<td>0.565</td>
<td>43</td>
<td>3F5</td>
</tr>
<tr>
<td>32</td>
<td>0.605</td>
<td>37</td>
<td>4G3</td>
</tr>
<tr>
<td>34</td>
<td>0.626</td>
<td>34</td>
<td>3A8</td>
</tr>
<tr>
<td>35</td>
<td>0.653</td>
<td>30</td>
<td>5E11</td>
</tr>
</tbody>
</table>

Bands were distinguished by RI, rates of appearance and disappearance, and by their characteristic shape.

Figure 3. Human LDL was exposed to trypsin for either 30 minutes (A) or 24 hours (B). The fragments were separated in a first dimension by isoelectrofocusing and in a second dimension by SDS polyacrylamide electrophoresis and then stained with Coomassie Blue. The molecular weights standards (phosphorylase B 94,000, albumin 67,000, ovalbumin 43,000, carbonic anhydrase 30,000, trypsin inhibitor 20,100 and $\alpha$-lactalbumin 14,400) can be seen on the right-hand side of the gels. Insets. The isoelectrofocusing gel and the observed pH gradient.
weights from 45,000 to 150,000 and including major fragments between 60,000 and 70,000 daltons. Upon SDS electrophoresis, the major band seen at the alkaline end of the isoelectric focusing gel yielded a vertical streak from the top of the second-dimension gel to a position equivalent to a peptide of 50,000 daltons, as well as five major bands having molecular weights from 120,000 to 60,000 and several minor bands with lower molecular weight. The abnormal behavior of these peptides may have been the result of precipitation of certain fragments during isoelectric focusing when the peptide-bound SDS was displaced by the Nonidet. The vertical streak may reflect an incomplete resolubilization of aggregated fragments by SDS during SDS electrophoresis. A similar phenomenon was seen when the proportion of ampholytes was changed to give a pH gradient between 3.5 and 10.

The stained isoelectric focusing gel of the 24-hour digest (Figure 3 B inset) revealed approximately 12 bands. Longer exposure of LDL to trypsin yielded more fragments in the pH region below 5.5, a decrease in fragments of an apparent pH between 5.5 and 6.5, and an almost complete disappearance of the major band at the alkaline end of the gel which had been prominent in the 30-minute digest. The two-dimensional gel of the LDL digested for 24 hours (Figure 3 B) revealed no peptides of greater than 90,000 daltons. The vertical streak that had been observed after 30 minutes of digestion had almost disappeared at 24 hours and only two of the associated bands (70,000 and 80,000 daltons) could still be detected. About 20 peptides with apparent pHs of between 5.5 and 6.5 and molecular weights of between 18,000 and 70,000 were seen, including several major fragments around 40,000 daltons. Those cleavage products with apparent pIs below 5.5 were of relatively low molecular weight (less than 30,000).

Fragments from LDL that had been digested for 0.5 or 24 hours and had been separated by twodimensional electrophoresis were transferred electrophoretically to NCP and were exposed to monoclonal antibodies as in the time-course analysis. Autoradiographs of the NCP blot treated with antibodies 1D1, 2D8, 3F5, and 4G3 are shown in Figure 4. A summary of all the reactions is graphically represented in Figure 5.

At 30 minutes, a cluster of spots having molecular weights of 45,000 to 50,000 and apparent pIs around 6 reacted with 1D1 (Figure 4). These probably correspond to bands 25 and 27 in the time-course study. These reactions were no longer seen at 24 hours and instead were replaced by a large, poorly defined spot probably consisting of several nonresolved spots with molecular weights from 30,000 to 37,000 and pIs similar to those of the reactive peptides of the 30-minute digests. After the 30-minute digestion, 2D8 reacted with the vertical streak and with a series of nine adjacent fragments of molecular weights analogous to the major 2D8-reactive bands in the time-course study. These 2D8-reactive spots correspond to fragments that penetrated the IEF gel poorly. At 24 hours, 2D8 reacted with two bands (probably identical to bands 16 and 18 of Table 1) and with the residual vertical streak seen in the Coomassie-stained gel. Similarly to 2D8, the antibodies 3F5 and 4G3 reacted with material that entered the gel poorly. In the 30-minute digest, this consisted of a reaction with the vertical streak and with four associated fragments; in the 24-hour digest, reaction was with one or more fragments of 60,000 daltons that were seen as two overlapping spots on the autoradiograph. The latter were not detected with 2D8. With both antibodies, a cluster of spots corresponding to fragments having molecular weights of 65,000 to 70,000 and apparent pIs of 5.8 to 6.1 were seen at 30 minutes and were even more evident in the 24-hour digests. A peptide of 95,000 daltons was also faintly visible in the 24-hour digest. 3F5 reacted with a group of peptides of molecular weights of 35,000 to 40,000 in both the 30-minute and 24-hour digests; it reacted with a fragment of approximately 105,000 and an apparent pI of 5.9 in the 30-minute digest. Several barely visible spots were seen in the reaction of the 24-hour digest with antibody 4G3, indicating a weak reaction with several fragments of about 35,000 daltons which clearly could be dissociated from the 3F5-reactive peptides of low molecular weight. Antibody 3A8 reacted weakly as a single spot with a 70,000 dalton fragment (pI 6) in the 30-minute tryptic LDL sample (not detected by 2D8), but this antibody showed no reactivity with fragments generated after 24 hours of digestion. Antibody 5E11 showed no reaction with tryptic fragments in either the 30-minute or the 24-hour digest.

Relation between Carbohydrate and Antigenic Determinants of LDL

LDL is composed of 5% to 9% carbohydrate which is largely present in the form of two glycopeptide chains. Glycopeptide 1D1 includes a terminal mannosyl residue which is in α 1→2 linkage with the penultimate sugar which is also mannosyl. Glycopeptide 2 is a branched chain each branch of which has a terminal sialic acid residue. It has been estimated that there are approximately 20 units per mole of LDL.

To determine if any of the antibodies reacted with either of the two glycopeptides, LDL that had been exposed to trypsin for 30 minutes or 24 hours was subjected to two-dimensional electrophoresis and was electrophoretically transferred to NCP. The NCP blot was then exposed to 125I-Con A, a lectin specific for terminal glycopyranosyl, manno-pyranosyl, and certain sterically related residues or to 125I-LFA, a lectin specific for sialic acid. The two lectins should distinguish between the two chains. The resulting autoradiographs are shown in Figure 6 and a summary of the reaction is included in Figure 5. The pattern seen with 125I-Con A showed a striking resemblance to that seen with the antibody 2D8 (Figure 4). This was true with both the 30-minute and the
Figure 4. LDL treated with trypsin for 30 minutes (A) and 24 hours (B) and separated as in Figure 3 was transferred to nitrocellulose paper and exposed to the indicated monoclonal antibodies followed by $^{125}$I-anti-mouse IgG. The $^{125}$I-labeled molecular weight standards (albumin 67,000, ovalbumin 43,000, carbonic anhydrase 30,000, and trypsin inhibitor 20,100) are seen on the right side of the gel.
24-hour digest. In addition, Con A reacted in the 30-minute digest with three fragments of 120,000, 80,000, and 65,000 daltons having apparent pI's between 5.9 and 6.2 that were not detected with our monoclonal antibodies. In the 24-hour digest, Con A reacted with two 60,000 dalton peptides that poorly penetrated the isoelectrofocusing gel to give two overlapping spots on the autoradiograph which corresponded to fragments detected by the antibodies 3F5 and 4G3, as well as to fragments not detected by any of the monoclonal antibodies (Figure 5). The 125I-LFA also reacted with a series of peptides that penetrated the IEF gel poorly and appeared identical to fragments reactive with 2D8. In addition, the LFA reacted in the 30-minute digest with several fragments having molecular weights between 60,000 and 70,000 with an apparent pI of 5.5. The autoradiograph of the 24-hour LDL tryptic digest exposed to 125I-LFA revealed a number of diffuse spots corresponding to fragments of 60,000 daltons and with apparent pI of 5.8.

Because of the similarities in reactivities of the Con A and one of the antibodies (2D8), we did a second experiment to examine the relationship between the antigenic determinants and the carbohydrate moieties of LDL recognized by Con A. LDL were insolubilized on polystyrene removal wells and were exposed to dilutions of Con A. After washing and saturating the unoccupied Con A binding sites with ovalbumin, we exposed the LDL sequentially to the monoclonal antibodies and to 125I-antimouse Ig. The Con A failed to prevent the binding of the antibodies to the LDL. The results obtained with the antibody 2D8 are shown in Figure 7. Figure 7 also shows a control experiment which demonstrated that Con A could prevent the binding of 125I-Con A to plastic-bound LDL. In the latter experiment, the ovalbumin saturation step and incubation with 125I-antimouse Ig were not included. To ensure that the antibodies were not merely displacing the Con A due to their higher affinities (approximately $10^6$ for the antibodies compared to $10^4$ for Con A<sup>2</sup>), 125I-Con A was incubated with immobilized LDL and was exposed to the diluted monoclonal antibodies. None of the antibodies were capable of displacing the 125I-Con A from the LDL (results not shown).
Figure 6. LDL treated with trypsin for 30 minutes (A) or 24 hours (B) and separated as in Figure 3 was transferred to nitrocellulose paper and exposed to $^{125}$I-Con A or $^{125}$I-LFA. The $^{125}$I-labeled molecular weight standards (albumin 67,000, ovalbumin 43,000, carbonic anhydrase 30,000, and trypsin inhibitor 20,100) are seen on the right-hand side of the gel.

Figure 7. In a solid-phase radioimmunoassay, dilutions of Con A were tested for their ability to block the binding of $^{125}$I-Con A (•) or the monoclonal antibody 2D8 (x) to immobilized human LDL. The bound antibody was detected by the addition of $^{125}$I-rabbit antimouse Ig.

Discussion

In previous studies we characterized a series of monoclonal antibodies against human LDL using several criteria. In the present report we extended this characterization by identifying determinants recognized by the monoclonal antibodies on tryptic fragments of LDL and by demonstrating the relationship of these determinants to the major carbohydrate moieties of LDL.

When LDL was digested with trypsin and subjected to SDS-polyacrylamide gel electrophoresis, a progressive digestion occurred over the 24-hour period. All monoclonal antibodies showed characteristic reactivities with the tryptic apo B fragments in the time-course analysis. Several different sequences of cleavages could be proposed to explain the kinetics of appearance and disappearance during the digestion of fragments bearing the antigenic determinants. Several complicating factors must be considered in such an interpretation: 1) the possible anomalous migration of apo B fragments in SDS-polyacrylamide gel electrophoresis that has been reported for intact
apo B, and 2) differences among the fragments in the efficiency with which they transfer to the nitrocellulose paper. We have seen that the monoclonal antibodies differ in their ability to detect their corresponding antigens fixed to the NCP. In SDS-polyacrylamide gel electrophoresis, we are also limited by our ability to resolve and identify the bands.

Similarities in the distribution of the antigenic determinants recognized by the antibodies 3F5 and 4G3 were evident in both the time-course study and the experiments using two-dimensional electrophoresis. In cotitration experiments, we had previously shown that the determinants recognized by 3F5 and 4G3 were so close together in LDL that their corresponding antibodies could not bind simultaneously to the same LDL particle. Both determinants were absent from apo B-100 and apo B-26, but were present on apo B-74 as well as on the previously uncharacterized apo B species or fragment that we have called apo B-50. While Fab fragments of both 3F5 and 4G3 inhibited the LDL pathway in cultured fibroblasts, this inhibition only reached 70% in the case of 3F5. The reactivities of the two antibodies showed different susceptibilities to chemical modification of LDL. In the present study, there were subtle differences in the reactivities of the two antibodies with fragments of less than 35,000 daltons. However, because some of these reactions were very weak, the differences may only reflect differences in the sensitivity of detection. The determinants, 3F5 and 4G3, appeared together on a fragment of 43,000 daltons (Table 1, band 28b), which would represent the maximum separation of the two determinants. The cotitration experiments, however, indicate that the two determinants would be much closer, at least topographically, in LDL.

Although there were differences between 2D8 and 3A8 in reactivity with fragments in the 30-minute digest separated by two-dimensional electrophoresis, the similarity between the two antibodies in the time-course study was surprising. Previously, these antibodies were shown to differ both in their reactivities with apo B-48 and in their ability to inhibit the LDL pathway; their corresponding determinants were shown to be sufficiently far apart in LDL to allow the mutual binding of the two antibodies to the same LDL particle. The present observation that the two determinants are present as a small fragment as 43,000 daltons (Table 1, band 28a) is not necessarily inconsistent with the previous observations. This fragment apparently contains at least one domain that cross-reacts with apo B-48, possibly the domain recognized by the LDL receptor. The reactions of 3A8 (and also of 5E11) were quite weak, which may indicate that tertiary or quaternary structure plays an important role in these determinants.

The fact that 1D1 could be clearly differentiated from all the other antibodies might have been expected, because it had been previously shown that this is the only antibody that reacts with apo B-26. Moreover, 1D1 was also the only antibody capable of reacting in radioimmunoassay with delipidated apo B that had been solubilized by the method of Cardin et al. The 1D1 determinant was present together with those recognized by all the other antibodies on a fragment of 124,000 daltons (Table 1, band 8). The characteristic appearance of band 8 on both the autoradiographs and the stained gel allowed an unambiguous identification. The observation that all of the determinants were present on a single fragment of under 125,000 daltons, which may represent less than 25% of intact apo B, may indicate that the majority of the molecule is hidden in LDL and is thus nonimmunogenic.

Carbohydrates are an important component of LDL. Two different major glycopeptides having mannose and sialic acid, respectively, as their terminal sugars have been identified. The reactivity of lectins specific for the two glycopeptides with the tryptic fragments of apo B indicated similarities to the pattern seen with the antibody 2D8. Although this suggested association of certain glycopeptides with the determinant recognized by 2D8, it is unlikely that this determinant includes the glycopeptide with terminal mannose, because the lectin Con A failed to block the binding of 2D8 to LDL. Participation of the sialic acid-containing glycopeptide in the 2D8 determinant has not been rigorously excluded, although it is considered unlikely because of the reactions seen with LFA and are not with 2D8. We have also not excluded the participation of sugars, which are not part of the two major side chains and which may be as much as 50% LDL carbohydrate.

In characterizing monoclonal antibodies against LDL, we attempted to identify antigenic determinants that are close to or form part of functionally important domains of apo B. We hope to eventually isolate and characterize apo B fragments bearing these determinants. In the field of lipoproteins, this has been recently used successfully in identifying the domain of apo E responsible for binding to the LDL receptor. The present paper extends the characterization of the anti-LDL monoclonal antibodies by showing the distribution of antigenic determinants, as well as carbohydrate moieties, on apo B tryptic fragments.

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