Nephelometry of Apolipoprotein B in Human Serum

Claus C. Heuck and Günther Schlierf

We studied the development of light scattering in the reaction between anti-apolipoprotein B and apolipoprotein B in intact very-low-density lipoproteins (I) and low-density lipoproteins (II) as well as in lipoproteins treated with lipases, and found considerable differences in the kinetics of the immunoreaction for the two lipoprotein classes. Pre-incubation with triglyceride lipase and cholesterol esterase caused a decrease of final light scattering in I but only minimal changes in the reaction with II. Non-ionic detergent not only decreased the original light scattering in hyperlipemic serum samples, but also accelerated the immunoreaction. Under standardized conditions, results of quantitative nephelometry correlated highly significantly with quantitative determination of apolipoprotein B by radial immunodiffusion, both for normolipemic and hyperlipoproteinemic serum samples. The nonspecific light scattering caused by neutral lipids in intact lipoproteins could be minimized when samples were pre-incubated with lipolytic enzymes.

Additional Keyphrases: atherosclerosis · lipoproteins · lipases · hyperlipoproteinemia · heart disease · radial immunodiffusion compared

Previous investigations have confirmed that apolipoproteins, particularly apolipoprotein B, can be measured by immunological procedures such as radial immunodiffusion (1) immunoelectrophoresis (2), enzyme immunoassay (3), and radioimmunoassay (4, 5). In addition, determination of apolipoprotein B by automated light-scattering measurement has been described (6), but this method proved to be useful only for investigating normolipemic serum samples because hyperlipoproteinemic serum samples give falsely high values.

The purpose of this study was to evaluate factors influencing light scattering in the immunoreaction between apolipoprotein B antibody and apolipoprotein B in very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and serum. We examined nephelometry of apolipoprotein B and compared the results with those obtained by radial immunodiffusion.

Materials and Methods

General Procedures for Lipoprotein Isolation and Lipid Determination

Serum specimens from apparently healthy normolipemic subjects, from persons with hyperlipoproteinemia, and from hypolipidemic patients (in each case after an overnight fast) were analyzed within 24 h of receipt.

Triglyceride (triacylglycerol) and cholesterol concentrations in serum and isolated lipoprotein fractions were quantitated according to the procedure recommended by the Lipid Research Clinics program (7). VLDL and LDL were isolated by ultracentrifugation in a 40.3 rotor in a Beckmann centrifuge (d 1.006, 20 h, 105,000 X g; d 1.063, 24 h, 105,000 X g) (8).

Apolipoprotein B Determination by Radial Immunodiffusion and Electroimmunodiffusion

Apolipoprotein B concentrations were quantitated by radial immunodiffusion on commercially available agarose plates (Partigen, Behringwerke, Marburg, G.F.R.). One volume of serum or standard solution was diluted with three volumes of isotonic saline, and 5 µL of this mixture was used for each determination. The plates were kept at 23 °C for 72 h before reading (9).

For quantitation by electroimmunodiffusion we used agarose plates (16 g/L, in barbital buffer, pH 8.6) (4). Five microliters of standard or diluted serum was applied for each determination. The samples were diluted 10-fold with isotonic saline. Each run (6 to 8 V/cm) lasted 4 h, with cooling.

Quantitation of Apolipoprotein B by Light Scattering

Five microliters of serum sample was diluted with 500 µL of iso-tridecyl-polyoxyethyleneoxide-ether (BASF, Ludwigshafen, G.F.R.) 0.15 mL/L in isotonic saline, containing one IUB unit (U) of pancreatic triglyceride lipase (EC 3.1.1.3; Worthington Biochemicals, Freehold, NJ) or 67 µU of cholesterol esterase (EC 3.1.1.13; Boehringer, Mannheim, G.F.R.), or a mixture of the two lipases. Alternatively, 20 µL of serum was diluted with 2000 µL of the detergent solution. The lipases were added separately in a volume of 10 µL containing the corresponding activities. The mixture was kept at 23 °C for 30 min. After this pre-incubation, 100 µL of the solution was placed in a laser-nephelometer cuvette (Behringwerke, Marburg, G.F.R.) to measure background light scattering. With detergent solution present, the background was generally so low (<3%) that it could be neglected. Thereafter, 200 µL of a solution of an antisem towards apolipoprotein B, which had been filtered through a 0.45-µm Millex filter (Millipore, Neusenburg, G.F.R.) in a sixfold dilution with isotonic saline, was added with gentle shaking. The mixture was kept for 2 h at 23 °C, then gently re-mixed before the light scattering resulting from immunocomplex formation was measured with a laser-nephelometer (prototype; Behringwerke, Marburg). The background light scattering for the empty cuvette, which amounted to 5 to 10% of the total light scattering, was subtracted for quantitative estimations. With the commercially available Behring laser-nephelometer the light scattering for empty cuvettes is 0.14–0.22 V, which increases to 0.19–0.25 V after the cuvette is filled with serum detergent solution.

Klinisches Institut für Herzinfarktforschung, Bergheimer Str. 58, D 6900 Heidelberg, G.F.R.
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Standardization of the Procedures

A commercially available beta-lipoprotein control serum (no. 2202; Behringwerke) was used as standard for all three procedures. It was diluted two- to fourfold for radial immunodiffusion, four- to 16-fold for electroimmunodiffusion, and 11- to 161-fold for nephelometry. This control standard serum was calibrated by use of an isolated LDL fraction.

The concentration of apolipoprotein B in LDL was evaluated by estimating the water-insoluble protein fraction (10) in the presence of sodium lauryl sulfate. The reason for using a serum for calibration instead of an isolated LDL fraction (or even apolipoprotein B fraction) was that we found it impossible to obtain reproducible results by the radial immunodiffusion technique with the last two, whereas reproducibility of measurements was satisfactory with the standard serum.

Analytical Variables

Reproducibility. Reproducibility was measured in 18 to 20 aliquots of one serum sample during a day. Reproducibility from day to day was estimated during two weeks (14 assays) for the nephelometric procedure with use of native serum, which was stored at 4 °C. The reproducibility of the radial immunodiffusion technique was estimated from results for 24 assays done during 24 consecutive days.

Specificity. The monospecificity of the applied antibody was studied by the double immunodiffusion technique. Precipitates were only observed with serum, LDL, VLDL, and isolated apolipoprotein B. The antiserum did not react with HDL or apolipoproteins A₁, A₂, or C₁₋₃ (10).

Kinetic Measurements

We investigated light scattering during immunoreaction of anti-apolipoprotein B with apolipoprotein B in intact LDL, intact VLDL, and serum standard under various conditions, as follows.

(a) Effect of various antibody concentrations at constant VLDL concentrations. One volume of VLDL solution was diluted with 100 volumes of saline or a detergent solution. The antibody solution was diluted with three, five, seven, or nine volumes of saline. One hundred microliters of the standard serum solution were used with 200 μL of each antibody dilution.

(b) Effect of various VLDL concentrations at constant antibody concentrations. One volume of antibody solution was diluted with five volumes of saline. One volume of VLDL was diluted with 25, 50, 100, and 200 volumes of saline or a 0.1 mL/L detergent solution. The portions of the reactant volumes were essentially the same as in a.

(c) Effect of various detergent concentrations at constant concentrations of VLDL and LDL. LDL or VLDL were diluted with saline solutions containing 10, 1, or 0.1 mL of the detergent per liter or with saline only. To 100 μL of this dilution was added 200 μL of antibody solution as in b.

(d) Effect of preincubation of VLDL and LDL with lipases. LDL or VLDL were diluted with saline, one part of lipoprotein solution plus 100 parts of 9 g/L NaCl. To 300 μL of this dilution we added 20 μL of cholesterol esterase (200 mU) or pancreatic triglyceride lipase (3 U). After 30 min at 23 °C, antibody solution was added. The volume of reactant mixture was essentially as in b.

(e) Light scattering at various standard serum concentrations and constant antibody concentrations in the presence of detergent and pretreatment with lipases. One volume of serum standard solution was diluted with 10, 20, 40, 80, and 160 volumes of a 0.1 mL/L detergent solution. To 300 μL of the dilution we added 20 μL of a solution containing the two lipases cholesterol esterase (200 mU) and pancreatic triglyceride lipase (3 U). One hundred microliters of this solution was mixed with 200 μL of antibody serum as described under a.

Results

The kinetic study of the development of light scattering during the reaction between anti-apolipoprotein B and apolipoprotein B in LDL and VLDL shows considerable differences. The final concentration of apolipoprotein B in LDL used for this experiment was 5 mg/L; in VLDL it was 4.5 mg/L. In the reaction with LDL the maximum was reached after about 30 min (Figure 1). Thereafter it remained constant for at least 3 h. In contrast, the reaction with VLDL did not reach a maximum before 3 h. Although apolipoprotein B concentration in VLDL is lower than that in LDL, the light scattering surpassed that of LDL.
Fig. 3. Effect of detergent on light scattering of the immunoreaction between anti-apo-apolipoprotein B and native VLDL or LDL, as a function of time.
Isolated VLDL (bottom) or LDL (top) were diluted with isotonic saline or a solution of detergent at various concentrations in isotonic saline. To 100 μL of the dilutions was added 200 μL of the antibody solution diluted as described. Light scattering was measured during 3 h. (The percentages refer to the final volume concentrations of the detergent)

Lipolysis of the neutral portion of lipids with pancreatic triglyceride lipase decreased light scattering of the apo-B anti-apo-B reaction in VLDL. This was even more pronounced after a preceding treatment with cholesterol esterase (Figure 2). In corresponding experiments with LDL, only minimal changes developed (11). Pre-incubation of VLDL with pancreatic triglyceride lipase or with cholesterol esterase not only decreased final light scattering, but also reached maximum after a shorter interval as compared to the reaction with the untreated lipoprotein, though still longer than in the corresponding reaction with LDL.

The presence of various concentrations of detergent caused different effects. In those concentrations of detergent commonly used for quantitative determination of metabolites such as cholesterol or glucose (12, 13) or even for enzymatic activities (14) (0.3 mL/L final concentration) the immuno-reaction was inhibited, both in VLDL and in LDL. At final concentrations lower than 0.05 mL/L, the immunocomplex formation in LDL as estimated by light scattering was practically unchanged as compared to the reaction in the absence of the detergent. In contrast, immunocomplex formation with VLDL was still highly decreased. In addition, maximum light scattering developed earlier than in the reaction with the untreated VLDL (Figure 3).

Comparative analysis of the immunoreaction with VLDL at constant concentrations of the antibody and various concentrations of the antigen reveals considerable differences in the absence and presence of the detergent. With VLDL that has only been diluted with saline, light-scattering development was not linearly related to dilution of the antigen. At a high concentration of VLDL it was less than would be calculated from low concentrations of the antigen. At high antigen concentrations the detergent did not affect light scattering, but at low antigen concentrations it not only caused a reduction but also yielded data close to the expected values for the dilution series at various time intervals.

Fig. 4. Double-logarithmic plot of light scattering from standard serum dilutions
Light scattering was measured after 2 h for standard serum samples treated as described. The dilution is indicated on the right side of the diagram

At various concentrations of antibody and constant concentrations of antigen, light scattering increased with increasing antibody concentration. Similarly, the presence of the detergent retarded the development of light scattering. In addition, a considerably shorter interval was needed to reach the maximum than was the case for samples diluted with saline.

To standardize the nephelometric procedure and radial immunodiffusion, we used a normotriglyceridemic standard serum. The final concentration of the detergent was 50 μL/L. At this concentration, the values for the final light scattering complexes were of the same magnitude as in the absence of the detergent. However, the added detergent stabilized the solution.

At various dilutions of the standard serum with an apolipoprotein B concentration of 804 mg/L, light scattering development was very similar to that of an immunoreaction with isolated LDL. Although normolipemic serum samples reached the final value of light scattering after an hour, we used the reaction time of 2 h for routine analysis, because with serum samples rich in triglycerides the maximum was not reached sooner. Figure 4 shows a double-logarithmic presentation of light scattering after 2 h vs. apolipoprotein B concentration. This standard curve is linear within the range of 40 to 600 mg/L.

With the use of a type IIb serum sample for quality control, precision in one series was similar (Table 1). The coefficient of variation was of the same magnitude as that reported for determination by radioimmunoassay (5). The coefficient of variation for assays done over a period of 14 days was similar to that for determinations of enzymic activities in human serum.

Table 2 lists data comparing quantitation of apolipoprotein B by radial immunodiffusion with various modifications of nephelometry. Pre-incubation with triglyceride lipase or with cholesterol esterase alone did not improve the correlation as compared to estimations from serum samples diluted only with saline. The combination of both enzymes not only results

<table>
<thead>
<tr>
<th>Procedure</th>
<th>CV (within-day),%</th>
<th>n (aliquots)</th>
<th>CV (day-to-day),%</th>
<th>n (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nephelometry</td>
<td>6.7</td>
<td>20</td>
<td>7.1</td>
<td>14</td>
</tr>
<tr>
<td>Radial immunodiffusion</td>
<td>8.4</td>
<td>18</td>
<td>9.4</td>
<td>24</td>
</tr>
<tr>
<td>Electroimmuno-diffusion</td>
<td>5.8</td>
<td>18</td>
<td>7.9</td>
<td>14</td>
</tr>
</tbody>
</table>

Quality-control serum: triglyceride, 2.42 g/L; cholesterol, 3.08 g/L.

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Table 2. Comparison of Apolipoprotein B Determination by Laser Nephelometry and Radial Immunodiffusion

<table>
<thead>
<tr>
<th>Procedure (nephelometry)</th>
<th>n</th>
<th>Triglyceride min – max.</th>
<th>Cholesterol min – max.</th>
<th>a</th>
<th>b</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>176</td>
<td>470 – 43 000</td>
<td>470 – 9300</td>
<td>0.515</td>
<td>+516</td>
<td>0.727</td>
</tr>
<tr>
<td>0.005 vol % detergent (final concn)</td>
<td>176</td>
<td>470 – 43 000</td>
<td>470 – 9300</td>
<td>0.577</td>
<td>+497</td>
<td>0.758</td>
</tr>
<tr>
<td>Saline/pancr. triglyceride lipase</td>
<td>54</td>
<td>520 – 15 350</td>
<td>1330 – 7250</td>
<td>0.250</td>
<td>+634</td>
<td>0.559</td>
</tr>
<tr>
<td>Saline/cholesterol esterase</td>
<td>54</td>
<td>520 – 15 350</td>
<td>1330 – 7250</td>
<td>0.491</td>
<td>+638</td>
<td>0.613</td>
</tr>
<tr>
<td>Saline/tg-lipase + cholesterol esterase</td>
<td>72</td>
<td>490 – 1 200</td>
<td>960 – 8000</td>
<td>0.804</td>
<td>+266</td>
<td>0.820</td>
</tr>
<tr>
<td>Detergent/tg-lipase + cholesterol esterase</td>
<td>198</td>
<td>47 – 27 000</td>
<td>420 – 14 000</td>
<td>0.846</td>
<td>+0.237</td>
<td>0.926</td>
</tr>
</tbody>
</table>

n: no. serum samples; r: correlation coefficient; a: slope; b: intercept

Triglyceride lipase: 1 U/100 µL of diluted serum sample. Cholesterol esterase: 67 mlU/100 µL of diluted serum sample.

in a significantly better correlation, but also gives a regression line closer to the 45° slope. Data from normotriglyceridemic serum samples (triglyceride concentration: <1.5 g/L) obtained by the latter procedure correlate highly (r = 0.965) within a range of 0.15 and 3.5 g/L apolipoprotein B (cholesterol concn: 1.5 to 7.5 g/L, Figure 5). The correlation is not as good (r = 0.856) for serum samples rich in triglyceride (triglyceride concn: 1.5 to 27 g/L, Figure 6). This series included samples with rather extreme lipid concentrations (cholesterol concn: 0.42 to 14 g/L). The slope of the regression lines for the two series differ only slightly. The nephelometric procedure yields higher values at higher apo-B concentrations than does radial immunodiffusion, which confirms a study comparing the Mancini technique and radioimmunoassay with electroimmunodiffusion (4).

A few data on serum samples with triglyceride concentrations >6 g/L (type V, Table 3) show satisfactory results for the nephelometric procedure as compared to electroimmunodiffusion, although all of the untreated samples were highly turbid.

There are few data available on apolipoprotein B concentrations in hyperlipoproteinemic serum samples (4, 15). In our study, a correlation coefficient of 0.727 between total serum cholesterol and serum apolipoprotein B has been calculated in normolipemia, which is in good agreement with previous statements (1). It is of similar magnitude for type IV and type IIb, but slightly less for type IIa (Table 4). The portion of serum cholesterol vs. serum apolipoprotein B decreases in hyperlipoproteinemia as compared to normolipemia. Although the relation is higher than observed by electroimmunodiffusion (4), the changes are essentially the same.

Discussion

Lipoproteins are the largest non-cellular particles in serum, and may cause light scattering even without complex formation with antibody. This renders difficult the nephelometric quantitation of serum proteins, particularly individual proteins in serum lipoproteins in hyperlipemic serum samples. Immunochemical quantitation of apolipoprotein antigens by light scattering yields results that are far from estimates by conventional procedures (16). It is not even satisfactorily improved by taking the basel light dispersion of the serum sample into account, because light scattering not only depends on antigen concentration but also on lipid concentrations in lipoproteins containing the antigen. Therefore, even at constant concentrations of apolipoprotein, light-scattering development during immunoreaction differs obviously from one lipoprotein class to another.

Various methods have been published for the determination of serum proteins, such as immunoglobulins by laser nephelometry (17, 18). Polyethylene glycol has been found to induce an enhancement (19). Characteristically an increase of light scattering occurs in the presence of this polymer (20). In our experience, use of polyethylene glycol is impractical for determining apolipoprotein B in intact lipoproteins, causing non-specific light scattering in hyperlipemic serum samples. This does not allow quantitative estimation.

There are two possible ways to reduce light dispersion caused by serum lipoproteins: by adding detergents and by hydrolysis of lipids in intact lipoproteins.

Table 3. Apolipoprotein B Determination in Serum Samples of Subjects with Type V Hyperlipoproteinemia

<table>
<thead>
<tr>
<th>Triglyceride</th>
<th>Cholesterol</th>
<th>Apolipoprotein B</th>
</tr>
</thead>
<tbody>
<tr>
<td>35 900</td>
<td>14 300</td>
<td>420</td>
</tr>
<tr>
<td>17 900</td>
<td>4500</td>
<td>890</td>
</tr>
<tr>
<td>17 900</td>
<td>1800</td>
<td>940</td>
</tr>
<tr>
<td>14 750</td>
<td>3650</td>
<td>940</td>
</tr>
<tr>
<td>10 000</td>
<td>4030</td>
<td>1720</td>
</tr>
<tr>
<td>8000</td>
<td>2790</td>
<td>1180</td>
</tr>
<tr>
<td>6100</td>
<td>2910</td>
<td>1460</td>
</tr>
</tbody>
</table>

Apolipoprotein B measured by nephelometry and radial immunodiffusion.

Fig. 5. Correlation between determination of apolipoprotein B by nephelometry and by radial immunodiffusion in normotriglyceridemic serum samples (triglyceride concn, <1.5 g/L) 

\[ y_{\text{vid}} = 0.875 x_{\text{neph}} + 0.225 \]

The triangles mark the data obtained from serum samples from type Ila subjects (n = 22). The dots correspond to data obtained from normolipemic subjects.
Table 4. Correlation of Serum Cholesterol/Serum Apolipoprotein B in Normolipemia and Hyperlipidemias

<table>
<thead>
<tr>
<th>Category</th>
<th>n</th>
<th>Cholesterol</th>
<th>Apo-B</th>
<th>r</th>
<th>Chol/apo-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normolipemia</td>
<td>68</td>
<td>1970 ± 410</td>
<td>820 ± 230</td>
<td>0.727</td>
<td>2.40 ± 0.39</td>
</tr>
<tr>
<td>Type lla</td>
<td>14</td>
<td>3730 ± 620</td>
<td>1900 ± 610</td>
<td>0.644</td>
<td>2.06 ± 0.37</td>
</tr>
<tr>
<td>Type llb</td>
<td>15</td>
<td>3420 ± 410</td>
<td>1790 ± 280</td>
<td>0.803</td>
<td>1.94 ± 0.20</td>
</tr>
<tr>
<td>Type IV</td>
<td>22</td>
<td>2460 ± 520</td>
<td>1160 ± 320</td>
<td>0.728</td>
<td>2.17 ± 0.40</td>
</tr>
</tbody>
</table>

Clearing of lipemic serum samples by adding nonionic detergents has already been described (12). Commonly used concentrations >1 g/L do not impair quantitative measurements of enzymic activities or metabolites. However, according to our results the immunological reaction towards apolipoprotein B is inhibited. If the concentration of detergent is decreased, it does not impair immunocomplex formation but still has a clearing effect on light scattering. Although we do not have physical data, we believe that this effect is caused by alterations in the hydration at the lipoprotein surface, mainly of VLDL.

Additionally, the detergent accelerates immunocomplex formation with apolipoprotein B in VLDL, obviously by altering the lipoprotein surface. However, this effect does not suffice for quantitative measurements. This can be achieved by additional enzymatic lipolysis. Pre-incubation of VLDL with pancreatic triglyceride lipase or cholesterol esterase decreases light scattering in the immunoreaction. In contrast, enzymic treatment of LDL causes only minimal changes. These observations point to structural differences at the surface of these lipoprotein classes, although additional information is needed for unequivocal interpretation.

The combined action of detergent and lipases overcomes the handicaps of nephelometric quantitation of apolipoprotein B. On one hand, the enzymic hydrolysis decreases the particle size, as has already been shown by ultrastructural analysis (21). On the other hand, the detergent not only decreases basal light scattering of a lipemic serum sample but also enhances the immunoreaction and stabilizes the immunocomplexes.

Apolipoprotein B concentrations increase with increasing cholesterol concentrations, both in normolipemic individuals and in those with hyperlipoproteinemia. The ratio of serum cholesterol to serum apo-B is highest in normolipemia and lowest in hypercholesterolemia. Although the differences are statistically insignificat, this observation may indicate gradual differences of cholesterol and of apolipoprotein B metabolism in hyperlipidemia (4).

Nephelometry offers advantages as compared to other methods. Measurements do not last longer than 2.5 h, a considerably less time than for any other immunological procedure. The methodology is simpler than that for either the Laurell technique or for radioimmunoassay. The setup enables an easy quantitation within routine laboratory analysis in clinical chemistry.

This study would have been impossible without the excellent technical assistance of Mrs. J. Erbe and Mr. H. Mayer. We are indebted to Dr. Schäfer (Marburg) for a generous supply of the antibody.

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