Enzyme-Linked Immunoabsorbant Assay of Apolipoprotein AII in Plasma, with Use of a Monclonal Antibody

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We produced a monoclonal antibody (C2-22) to human apolipoprotein (Apo) AII and describe its use in an enzyme-linked immunoabsorbant assay (ELISA) for Apo AII in human plasma and lipoprotein subfractions. No cross reactivity of the antibody with Apo CI, CII, CIII, E, or albumin was detected. Apo AI and low- and very low-density lipoprotein cross reacted by 0.25%, <0.2%, and <0.3%, respectively. Whole plasma high-density lipoprotein (HDL) and HDL subfractions (HDL2 and HDL3) produced parallel displacement curves. This quantitative ELISA is based on competition between solid-phase-bound Apo AII and free Apo AII. Bound C2-22 is detected by alkaline-phosphatase-labeled second antibody. The standard curve for the assay is linear for plasma diluted 500-fold originally containing 140 to 1140 mg of Apo AII per liter. Delipidation of plasma samples exposed no additional antigenic sites. Within- and between-run CVs were respectively 8.4% and 8.7% at 327 mg/L of Apo AII, and 6.8% and 7.4% at 587 mg/L. Results correlated well with those by a polyclonal-antiserum-based RIA procedure: \( r = 0.916, \ p < 0.01, \ RIA = 0.896 \) ELISA – 19.1 mg/L.

Additional Keyphrases: RIA compared · heart disease · lipoproteins · vascular disease · sex-related differences

Numerous studies demonstrate an inverse relationship between concentration of apolipoprotein (Apo) AII in plasma and both coronary artery disease (I–3) and peripheral vascular disease (4). According to Heiss and Tyroler (5), measurement of plasma Apo AII demonstrates several associations with ischaemic heart disease, including strength, independence, biological consistency, observational consistency, and measurement validity, making this analyte worthy of continued and additional investigation. Two additional criteria necessary for proving a causal relationship between Apo AII and atherosclerosis—temporal relationship and the effect of intervention—remain to be assessed, the latter not yet having been established for high-density lipoprotein cholesterol (HDLc).

Apo AII is found in both HDL2 (d 1.063 to 1.125) and HDL3 (d 1.125 to 1.21) and is the major apolipoprotein in the latter. Longstanding epidemiological studies suggest that HDL2C is the "protective" subfraction of the HDL species (6, 7). Isolation and measurement of HDL2 and HDL3 are either tedious and expensive (7) or are still undergoing evaluation for simpler procedures (8). Consequently they are not yet available for routine clinical or clinical-research purposes. However, because the ratio of Apo AII to Apo AII is higher in HDL2 than in HDL3, increased ratios of plasma Apo AII/AII usually reflect higher concentrations of HDL2 than of HDL3 (9). In addition to its use in epidemiological and diagnostic situations, the Apo AII/AII ratio may be useful in assessing therapy. Studying patients treated with bile-acid-binding resins, Witzum et al. (10) found no changes in the HDL cholesterol, but noted an increase in the Apo AII/AII ratio, suggestive of a redistribution of cholesterol from HDL3 to HDL2.

Among the immunoassay techniques used for measuring Apo AII are radioimmunoassay (11–13), electromunnoassay (14), radial immunodiffusion (15), and, more recently, enzyme-linked immunoabsorbent assays (ELISA) (16–18). The differences in concentrations in serum reported for Apo AII probably reflect differences in techniques, standardization, antibody specificity, and patient population studied. In a recently reported competitive ELISA system for Apo AII, Dufaux et al. (18) used an enzyme-labeled purified Apo AII tracer and solid-phase-bound anti-Apo AII polyclonal rabbit antiserum.

We describe here an improved competitive inhibition ELISA system for quantifying Apo AII in human plasma. We use a monoclonal antibody to Apo AII, and purified Apo AII is not required for labeling as "tracer" or for standards.

Materials and Methods

Preparatory Procedures

Isolation and purification of Apo AII: HDL was isolated from the plasma of normal volunteers by ultracentrifugal flotation between densities of 1.090 and 1.21 kg/L in a Model L5-50 ultracentrifuge with a Model 50.3ti rotor (both from Beckman Instruments, Fullerton, CA). The HDL was dialyzed against NaHCO3 buffer (0.1 mol/l, pH 8.2), lyophilized, and delipidated with an equimolar mixture of ethanol and acetone. Pure apoprotein AII was isolated by flat-bed isoelectric focusing (10) and its purity confirmed by electrophoresis on polyacrylamide gel and by analysis for amino acids (10).

Preparation of rabbit antiserum to Apo AII: Female New Zealand White rabbits (Dutchland Laboratories, Denver, PA) were immunized, according to a standard protocol, with purified Apo AII mixed with complete Freund's adjuvant (Calbiochem–Behring Corp., La Jolla, CA). The resulting antiserum was collected and stored at −20 °C. We checked the specificity of the antiserum by reacting it with known purified apolipoproteins, selected lipoprotein fractions, and albumin.

Radioimmunoassay procedure for Apo AII measurement: We iodinated purified Apo AII by the Chloramine T method
Purified Apo AII standards or plasma samples, diluted rabbit or mouse antiserum to Apo AII (or monoclonal antibody to AII), and 32P-labeled Apo AII were combined and incubated overnight. The next day, we added antiserum to rabbit or mouse IgG (Miles Laboratory, Elkhart, IN) to all samples, to separate bound and free tracer. We counted the radioactivity of the bound fraction in a gamma counter (Packard "Multi-Prias 1"; United Technologies, Downers Grove, IL).

Development of the monoclonal antibody to Apo AII: The procedures used were adapted from those of Kohler and Milstein (21). Spleen cells from C57/Balb-C F1 mice that had been immunized with purified Apo AII were fused with SP/2 myeloma cells, and the resulting hybridomas were screened for antibody to Apo AII. This procedure was similar to the RIA procedure described above except that we used Protein A (Sigma Chemical Co., St. Louis, MO) to precipitate the immunoreactants. Hybridoma clones that were positive for anti-Apo AII antibody activity were subcultured and injected into the peritoneal cavity of "pristine primed" Balb-C mice. The ascitic fluid produced contained approximately 20 g of the monoclonal antibody per liter. The anti-Apo AII-producing cell line (clone 2-22) that we used in subsequent studies produced IgG monoclonal antibodies with kappa light chains.

Testing the specificity of monoclonal antibody C2-22: We used a competitive binding ELISA to assess the specificity of the monoclonal antibody. We coated 96-well flexible polyvinyl chloride microtiter plates (M.A. Bioproducts, Walkersville, MD) with a 600 mg/L Apo AII solution, dispensing 100 μL into each well and incubating overnight at room temperature (23°C). The wells were washed three times with a phosphate-buffered isotonic saline (PBS) containing 5 g of gelatin per liter, shaken dry, and stored at 4°C. The PBS used in these experiments contained, per liter, 8.10 mmol of Na₂HPO₄, 1.47 mmol of KH₂PO₄, 137 mmol of NaCl, 2.68 mmol of KCl, 0.90 mmol of CaCl₂, 0.49 mmol of MgCl₂·6H₂O, 3.85 mmol of Na₃SO₄, 0.5 mmol of Tween-20 detergent, and 5 g of gelatin. Into each well we placed 50 μL of PBS and 50 μL of 10,000-fold diluted ascitic fluid (containing the monoclonal antibody) and incubated for 2 h at 23°C. After washing the wells three times with PBS and resuspending them dry, we added 100 μL of rabbit anti-mouse IgG conjugated with alkaline phosphatase (EC 3.1.3.1) (Sigma Chemical Co., St. Louis, MO) to each well and incubated the plate for 1 h at 23°C. The wells were again washed three times with PBS, suctioned dry, and 100 μL of a 1 g/L p-nitrophenyl phosphate solution was added to each well. After a 30-min incubation at 23°C, color development was stopped by adding 50 μL of a 0.2 mol/L NaOH solution to each well. The most intense color (greatest absorbance) was measured in a microtiter plate reader (Flow Laboratories, McLean, VA), and stored at 4°C. We used a spectrophotometer (model 110, Bio-Rad Laboratories) to determine the absorbance at 450 nm. A standard curve was constructed by plotting absorbance (y-axis) vs log Apo AII (x-axis).

Collection and storage of plasma samples: Venous blood from fasting (12–14 h) subjects was drawn in Vacutainer Tubes (Becton, Dickinson and Co., Rutherford, NJ) containing 10 mg of disodium EDTA per liter. Until analysis, separated plasma was stored at 4°C with 500 mg of added sodium azide per liter.

Apo AII was measured in 38 healthy laboratory workers, 18 subjects with type II phenotype, and 176 subjects who were undergoing lipid and lipoprotein evaluation and (or) treatment for various lipid/lipoprotein disorders.

Obelipidation of plasma samples: To determine if delipidation exposed additional Apo AII binding sites, samples were delipidated by three washes with ethanol:acetone (1:1) and two washes with diethyl ether. Apo AII, by both the ELISA and RIA methods, and total protein were measured in...
treated and untreated samples (Table 1).

**Precision studies:** We assessed within- and between-run assay precision, using pooled plasma stored at −70°C, selected to test analytical performance at different concentrations.

**Lipid and Apo AI and B measurements:** Total HDL cholesterol was measured by a microenzymatic assay (22), standardized to the Lipid Research Clinics procedure by using a serum calibrator supplied by the Centers for Disease Control (23). HDL was isolated by use of the modified LRCLRC menaquinone procedure (24), adapted to use with 200 μL of plasma (22). Triglycerides were measured by a microenzymatic procedure as previously described (22). Apo AI and B were quantified by electroimmunoassay (19).

**Results**

**Specificity of monoclonal antibody C2-22 to Apo AI:** Displacement of the monoclonal antibody was assessed by use of increasing concentrations of Apo AI. Displacement was minimal (10%) with less than 0.10 mg of pure Apo AI per liter (5 mg per well) and maximal (100%) with 1.5 mg/L (75 mg per well). There was no detectable displacement (0% cross reactivity) with Apo CI, CII, CIII, E, or albumin. Apo AI produced 50% displacement at a concentration approximately 400 times that of Apo AI (0.25% cross reactivity). LDL and VLDL produced displacement of Apo AI only at very high concentrations (5% cross reactivities of <0.2% and <0.3%, respectively). Whole plasma and HDL and its subfractions HDL 

**Assessment of the ELISA procedure:** Figure 1 illustrates a typical standard curve obtained with the plasma calibrator (ApoCal All). We determined the Apo II content of ApoCal All by repeated measurements in the ELISA system, using a primary standard of purified Apo II isolated by isoelectric focussing for which the mass was quantified by amino acid analysis. Although a seven-point curve was run, shown in Figure 1, we only used values falling within the rectilinear center five points for the “standard” curve. We re-analyzed samples with values falling outside of this range, using different dilutions. The effective working range for plasma samples for the assay was therefore 140 to 1140 mg/L (equal to 0.3 to 2.3 mg/L after patients’ samples are diluted 500-fold).

For all samples, delipidation resulted in a decrease of 32–70% in Apo AI concentration measurable by either the ELISA or the polyvalent RIA procedure (Table 1). There was also a consistent loss of protein (as measured by the Lowry method) in the delipidation procedure, although this loss was proportionately much less (6–10%).

The within-run CVs at 327 and 587 mg of Apo AI per liter were respectively 8.4 and 6.8%. Between-run precision showed only slightly higher CVs for the two pools (Table 2).

**Correlation of monoclonal ELISA with RIA:** In Figure 1 we compare the displacement (standard) curves for the ELISA procedure with those obtained by two RIA procedures. The polyclonal RIA is more sensitive and the curve has a different slope, indicating either recognition of different or multiple antigenic sites or a different affinity for Apo AI. RIA with use of the same monoclonal antibody as in the ELISA procedure gave a parallel curve with ELISA (Figure 1).

We also compared the monoclonal ELISA and polyvalent RIA, using 41 patients’ samples. The correlation coefficient (r) was 0.916 (p < 0.01), the regression equation RIA = 0.896 ELISA − 19.1 mg/L. The mean by ELISA was 329.9 (SD 79.1) mg/L; by RIA it was 315 (SD 77.4) mg/L.

**Plasma Apo AI concentrations in patients’ samples:** Plasma Apo AI values in 38 laboratory staff, ages 22–49 years, and 18 subjects with type II phenotype are shown in Table 3, together with their lipid and Apo AI and B concentrations. For Apo AI, no statistically significant sex-related differences were seen; HDL cholesterol and Apo AI tended to be higher and LDL cholesterol and Apo B slightly lower in women. The type II subjects had markedly increased LDL cholesterol and Apo B, and decreased values for HDL cholesterol, Apo AI, and Apo AI.

Table 4 shows results for the 176 fasting subjects who were undergoing routine plasma lipid/lipoprotein evaluations, several of whom were known to have lipoprotein abnormalities.

**Discussion**

This ELISA technique has several advantages over previously described procedures (II–15). The amount of antisera used in radial immunodiffusion and electroimmunoassay is

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**Table 1. Effects of Delipidation of Apo AI in Seven Plasma Samples on Results by ELISA and RIA (Polyvalent Antibody)**

<table>
<thead>
<tr>
<th>Total protein, mg/L</th>
<th>Apo AI, mg/L</th>
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<tr>
<td>71</td>
<td>348</td>
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<td>64</td>
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<td>68</td>
<td>436</td>
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<td>64</td>
<td>159</td>
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*By procedure of Lowry et al. (25). A whole plasma; B, delipidated plasma.*
much greater than that necessary for RIA, ELISA, or nephelometry. No detectable precipitate need be formed with the antigen. The rate of passive diffusion in radial immunodiffusion varies with different lipoprotein species, and extensive sample treatment may be necessary if one is to quantify all the apolipoprotein present. A comparatively short assay time is required for the present assay, and, unlike radial immunodiffusion and electrophoresis, no difficult to detect and imprecise endpoint is involved. Although nephelometry requires less analytical time than the present procedure, and also is potentially automatable, it is subject to significant inherent light-scattering interference, especially with lipemic samples (26). RIA is extremely sensitive, subject to minimal interference, and allows simultaneous analysis of many samples, but it requires substantial time and effort to maintain, owing to the short-lived labeled antigen tracer. In addition, the low-level radiation is potentially hazardous and logistically awkward to handle.

Our ELISA system differs from that described by Dufaux et al. (18): it requires no enzyme labeling of purified Apo AII antigen, which is time consuming and may result in aggregation or alteration of the immunogenic properties of the apolipoprotein; and our use of a high-affinity monoclonal antibody ensures long-term uniformity for the procedure. Apparently, the monoclonal antibody (C2-22) recognizes a "surface" antigenic site (pan-antibody) and no additional epitopes are exposed by delipidation. This ability to quantify total plasma Apo AII without sample treatment is shared by assays in which polyvalent antisera are used (11–15, 18).

Disadvantages of our assay are the additional washing steps and the second-antibody addition. The reported intra-assay precision is similar for both procedures, but our between-assay precision represents an almost 50% improvement over that reported by Dufaux et al. (18). This may in part be related to the inherent "stability" of our system (no enzyme-labeled antigen, monoclonal antibody) and the fact that our assays are run in triplicate, vs duplicate for the procedure of Dufaux et al. Small variations in antibody coating appear to affect the procedure of Dufaux et al. notably, but have no such effect on our assay.

Our studies on whole and delipidated sera confirm those of others (11, 18, 27) in that no increase in Apo AII was measurable after delipidation. The decreased values for Apo AII as measured by both ELISA and RIA probably are a result of apoprotein loss during the delipidation procedure, because total protein also was decreased—and immunochromatographic alteration resulting from delipidation may also partly explain these results. Possibly, alternative delipidation procedures such as those involving ethanol-ether (26) or trimethylurea—urea (29) would have been preferable, because their use is not yet associated with loss of Apo AII.

Apo AII in whole serum, HDL, HDL2, and HDL3 react similarly in our system. These findings differ from those by the RIA procedure of Goldberg et al. (12)—who reported a lack of parallelism in their dilution curves for Apo AII and HDL—but are consistent with the report of Dufaux et al. (18). This parallelism lends validity to the use of a serum calibrator or secondary standard in ELISA systems.

The values for Apo AII in plasma of patients with lipid abnormalities (Table 4) and of normal laboratory volunteers (Table 3) are somewhat higher than those previously reported in the literature (11–15). However, the Apo AII and HDL cholesterol values for the volunteers are also well above those usually reported, while their Apo AII/HDL ratio of 2.6 (for men) and 2.92 (for women) is about average. These higher values may be biased by the small size of the group of subjects or, alternatively, by the assay calibration procedure as a consequence of the assigned value of the serum calibrator, ApoCAl AII. The specific (type II) and general patient population had lower values for Apo AII and HDL cholesterol.
ol, as would be expected. The lack of sex-related difference is consistent with the lack of a sex-related difference in HDL cholesterol. The general group of patients did, however, include subjects with known lipid abnormalities, some of whom were undergoing therapy with diet or drugs. Thus, their lack of conformity to previously published lipid and apolipoprotein population distributions might be expected.

There was, as expected, a strong statistical correlation between HDL cholesterol and Apo AII (r = 0.501; p < 0.001) and no relationship between Apo AII and either LDL cholesterol (r = −0.098; p = 0.223) or triglycerides (r = −0.118; p = 0.118).

The within- and between-run precision studies demonstrated CVs of <10% for both the lower concentration of Apo AII (327 mg/L) and the higher (587 mg/L). There are numerous manual steps involved in this procedure, and automation would most likely increase the precision.

The present monoclonal ELISA is not a sensitive as our own polyclonal RIA or the RIAs reported by others (11, 12, 30), but more than sufficient for quantification of Apo AII in plasma or lipoprotein subfractions (e.g., HDL2 or HDL3).

In conclusion: the system described here is sensitive, specific, and precise. It requires no pretreatment of plasma, a serum-based calibrator can be used, and no labeling of Apo AII or anti-AII is necessary. Maintenance is minimal, and the assay is stable for many months, and it is rapid and fairly simple to perform. Finally, because it is potentially automatable, large numbers of samples potentially may be quickly and inexpensively assayed.

References

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