

Measurement of apolipoprotein A-I concentration in nonhuman primate serum by enzyme-linked immunosorbent assay (ELISA)

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Abstract A sensitive and specific enzyme-linked immunosorbent assay (ELISA) for nonhuman primate serum apolipoprotein A-I (apoA-I) is described. The assay is a noncompetitive, sandwich ELISA in which polystyrene microtiter plates were used with purified, monospecific goat anti-monkey apoA-I antibodies adsorbed on the wells. The serum samples were added to the coated wells, incubated, and after washing, antibodies conjugated to horseradish peroxidase were added. After further washing, the bound label was assayed. A heat treatment step, 52°C for 3 hr, was used to maximize the apoA-I immunoreactive sites in diluted serum. Serum samples extracted with chloroform-methanol, delipidated with tetramethylurea, or denatured by heating gave essentially equivalent results. The working range of the apoA-I standards was 0.5 to 5 ng and parallel responses were observed for apoA-I in serum, in isolated HDL, and in buffer as a purified apoprotein. Recovery of apoA-I added to serum was quantitative (106 ± 3%). The intra- and interassay coefficients of variation were 6.2 and 6.9%, respectively. The enzyme immunoassay yielded values that compared favorably with those obtained by radial immunodiffusion ($r = 0.84$). ApoA-I concentration in African green monkey serum was highly correlated with the HDL cholesterol concentration ($r = 0.86$). It is concluded that this ELISA is an accurate and precise method for determination of apoA-I concentrations in primate serum.—**Koritnik, D. L., and L. L. Rudel.** Measurement of apolipoprotein A-I concentration in nonhuman primate serum by enzyme-linked immunosorbent assay (ELISA). *J. Lipid Res.* 1983. **24**: 1639–1645.

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Since the advent of the quantitative enzyme-linked immunosorbent assay (ELISA) described by Engvall and

Perlmann (1) and van Weeman and Schuurs (2), various modifications of the assay have been in widespread use for measurement of specific antibodies, haptens, and antigens in body fluids and tissue extracts. The technology involved in ELISA is based on the same principles that are used in radioimmunoassay (RIA). The precision and sensitivity of many enzyme immunoassays are also similar to those of RIA (3). However, in addition to maintaining the advantages of RIA, enzyme immunoassay is free from many of the limitations set by radioisotope use. With the enzyme label there are no expensive radioisotope purchase and disposal costs, no license and bookkeeping requirements, and no special handling techniques. The shelf-life of the enzyme-reagent is long, capital investments for equipment to measure the enzyme are considerably less than for radioisotope counting procedures, and the actual colorimetric determination requires less time than radioactivity counting. Another advantage is that the ELISA can also be used for in vivo turnover studies without concern that the radiolabel in the sample will cause interference in the enzyme immunoassay. It therefore seemed appropriate to try this technique for the determination of the apolipoprotein A-I concentration in serum. ApoA-I is the major protein of the high density

Abbreviations: HRPO, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; RIA, radioimmunoassay; HDL, high density lipoprotein; LDL, low density lipoprotein; TMU, tetramethylurea; PAGE, polyacrylamide gel electrophoresis; RID, radial immunodiffusion.

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lipoproteins, the cholesterol level of which has been found to have a strong negative correlation to increased risk of coronary heart disease (4). This important relationship suggests that direct assays of serum apoA-I levels can provide important diagnostic information.

This study describes a sensitive and specific noncompetitive 'sandwich' ELISA for measuring apolipoprotein A-I in African green monkey serum. With the sandwich type assay, the molecule to be measured does not have to be altered by labeling nor does the assay depend on equivalent adsorption to polystyrene for apoA-I-containing lipoprotein particles of variable composition. The procedure incorporates a heat treatment step for the direct estimation of apoA-I in unextracted, diluted serum (5). To the best of our knowledge, this is the first enzyme immunoassay procedure reported in the literature for the determination of serum apoA-I in any species.

MATERIALS AND METHODS

Isolation of apoA-I and other apoproteins

The apoA-I used for assay standards was prepared from pooled fresh plasma obtained from African green monkeys (*Cercopithecus aethiops*) or cynomolgus monkeys (*Macaca fascicularis*) that had been fasted overnight. The lipoproteins were first isolated from plasma by ultracentrifugation at d 1.225 g/ml and then separated by size into major lipoprotein classes using agarose column chromatography on Bio-Gel A 15-m (Bio-Rad, Inc., Richmond, CA) (6). The material from the HDL region was delipidated with ethanol-ether (7) before being fractionated into its major components by column chromatography on Ultrogel ACA34 (LKB Instruments, Bromma, Sweden) in 6 M urea-0.1 M Tris buffer, pH 8.6 (8). The peak II material containing apoA-I was rechromatographed, and small aliquots were then lyophilized and stored frozen until use. The purified apoA-I did not cross-react in Ouchterlony double immunodiffusion (9) with antisera prepared by our published method (10) to apoA-II, apoB, apoE, or albumin. The purified apoA-I migrated as a single band during polyacrylamide gel electrophoresis (PAGE) in SDS (8). ApoE was isolated from $d < 1.03$ g/ml lipoproteins of cholesterol-fed cynomolgus monkeys using the heparin-Sepharose affinity column method of Shelburne and Quarfordt (11). ApoA-II was purified from peak III of the ACA34 column eluate of apoHDL as described previously (8). The ACA34 column peak III region (8) from a preparation of lymph chylomicron apoproteins, containing mostly apoC and apoA-II as seen on SDS PAGE, was also studied. ApoB was purified from LDL using the sodium deoxycholate procedure of Helenius and Simons (12). LDL was purified using ultra-

centrifugation and agarose column chromatography (6). ApoA-I-free human serum albumin from Cutter Laboratories, Inc. (Berkeley, CA) was also used as an antigen. Protein concentrations were measured by the method of Lowry et al. (13) using bovine serum albumin (BSA), Cohn fraction V (Sigma Chemical Co., St. Louis, MO) as the standard.

Purification of antibodies to apoA-I

Antiserum to apoA-I from African green or cynomolgus monkeys was prepared as previously described (10). IgG was separated from whole antiserum by precipitation with ammonium sulfate (14). Purified goat antibodies to monkey apoA-I were then isolated from the IgG fraction on an immunosorbent column of glutaraldehyde-fixed normal African green monkey serum (15). The isolated apoA-I antibody fractions were pooled, neutralized, and concentrated by dialysis against Dextran T-500 (Pharmacia Fine Chemical Co., Piscataway, NJ) and stored at 4°C in 0.01 M phosphate-buffered saline (PBS), pH 7.0, containing 0.1% azide.

Conjugation of purified antibody to horseradish peroxidase

Purified antibody was coupled to horseradish peroxidase (HRPO, Sigma, Type VI) using a procedure described by Nakane and Kawaoi (16). The immunoreactive conjugate was isolated on Sepharose CL-4B (1 × 120 cm) in 0.01 M PBS, pH 7.0. It was diluted with an equal volume of glycerol, filtered through a 0.22- μ m Millipore membrane, stored at 4°C for up to 4 months.

Serum samples

Sera were collected from adult male African green monkeys of two diet groups, A-2 and B-2. Diet A-2 contained saturated fat, P/S ratio = 0.34, and diet B-2 contained polyunsaturated fat, P/S ratio = 2.2. Each diet contained 0.75 mg of cholesterol/Kcal and 40% of the total calories was from fat. The animals were fed the prepared diets for a period of over 2 years. Samples were collected monthly and were stored frozen until analysis.

Serum concentrations of HDL cholesterol (17) and total cholesterol (18) were determined on fresh serum by the Lipid Analytical Laboratory of our center. This laboratory is in the surveillance phase of the standardization program of the Center for Disease Control, Atlanta, Georgia. Measurement by radial immunodiffusion (RID) of apoA-I concentration in serum was done by the method of Albers et al. (19). The antibodies and apoA-I standards used in the RID were the same as those used in the ELISA. The range of the standards in the RID was from 375 to 1200 ng/5 μ l per well. A reference whole serum pool whose apoA-I concentration had been reasonably approximated by quantitative isolation of HDL

and measurement of HDL apoA-I by RID was used as the reference standard for calculation of serum sample apoA-I concentrations.

Treatment of serum samples for analysis

Three separate methods were compared to expose all of the immunoreactive sites on apoA-I in serum. Phosphate-buffered saline, 0.01 M, pH 7.0, containing 0.1% BSA and 0.1% Tween-20 (Sample Buffer) was used to dilute standards and samples. Serum samples were diluted a least 100,000-fold with Sample Buffer and then heated at 52°C for 3 hr in a water bath (5). Dilutions of apoA-I within the concentration range of the assay were also treated in the same manner. Sera were delipidated with chloroform-methanol 2:1, (one part serum to 20 parts solvent), after which the dry protein residues were resolubilized in 8 M urea-0.01 M Tris buffer, pH 8.6, before dilution with the Sample Buffer. Delipidation with freshly redistilled 1,1,4,4-tetramethylurea (TMU) was also tested (20). One part of serum was mixed with one part of TMU and this solution was held at room temperature for 1 hr. Two parts of 8 M urea-0.01 M Tris, pH 8.6, were then added after which subsequent dilution with the Sample Buffer was carried out.

The ELISA procedure

Antibodies to apoA-I of cynomolgus monkeys were adsorbed to the wells of rigid polystyrene microtiter plates (Corning #25855) by pipetting 200 μ l of a 0.1 M sodium carbonate buffer, pH 9.6, containing 500 ng of antibodies into each well and placing the microtiter plate in a humid chamber at 4°C for 18-24 hr. Then the antibody solution was aspirated from the wells and the plates were washed three times with 0.01 M PBS-0.1% Tween buffer, pH 7.0. After the third wash, the plates were either used immediately or frozen and stored at -20°C for up to 1 month before use. Standards or samples (dilutions determined by previous titration studies) were diluted with the Sample Buffer, heated at 52°C for 3 hr, and 200- μ l volumes were added to the microtiter plate wells. The antigens were incubated in a humid chamber for 24 hr at 4°C. The wells were then aspirated and washed as before. The peroxidase-antibody conjugate was diluted with Sample Buffer (dilutions determined by previous titration studies) and 200- μ l aliquots were added to each well. The plates were again incubated at 4°C for 24 hr and then aspirated and washed as before. The enzymatic color reaction was carried out next.

Color reaction

The chromogen, o-dianisidine \cdot 2HCl (Sigma), 1% in methanol-water 1:1 was freshly prepared before each use. Hydrogen peroxide (30%, Fisher Scientific Co., Pittsburgh, PA) was the substrate. To 60 ml of 0.1 M

sodium phosphate-sodium citrate buffer, pH 5.0, were added 12 μ l of 30% H₂O₂ and 500 μ l of the 1% o-dianisidine (21). Then 200 μ l of the chromogen-substrate solution was pipetted into each microtiter plate well. The color was developed in the dark for 30 min and the enzyme reaction was then stopped by addition of 25 μ l of 2 N HCl per well. The reaction medium was thoroughly mixed, and the O.D. was read at 405 nm on a Dynatech MR580 Microtiter Plate Reader. The color was stable for several hours.

RESULTS

Specificity of the antiserum

The immunosorbent column-isolated antibodies of goat antiserum to apoA-I of cynomolgus monkeys were characterized by Ouchterlony double immunodiffusion (Fig. 1). Single precipitin bands giving reactions of identity were seen when African green monkey purified apoA-I, isolated HDL, and serum were compared. No reaction was seen with purified apoA-II, apoC's, apoE, or isolated LDL. Immunoelectrophoresis of the whole antiserum defined a single precipitin arc with African green monkey serum. Further documentation of the specificity of the apoA-I antibodies was done using the ELISA itself. Fig. 2 shows the comparison of reactivity of these antibodies between apoA-I and LDL, apoE, apoA-II, serum albumin, and partially purified chylomicron apoC's. A curve parallel to apoA-I was obtained with isolated LDL. In the LDL, apoA-I accounted for less than 1% of the protein and probably represents a small amount of contamination with HDL and/or apoA-I. The curve obtained using the preparation of chylomicron apoC's was not parallel to the apoA-I curve; it appeared that the apoA-I may have been present as approximately 1% of the total protein. The presence of a small amount of apoA-I in this ACA34 column preparation was confirmed by SDS-PAGE. The purified apoE and apoA-II did not exhibit a response comparable with the ED₅₀ [estimated dose at 50% O.D. response range] response of apoA-I even at 10,000-fold excess protein concentrations. No reactivity of the apoA-I antibodies with human serum albumin was detectable.

Immunoassay validation

Fig. 3 shows the titration curve for the isolated antibodies adsorbed to the microtiter plate. To optimize our assay conditions we chose to use 500 ng of antibody protein per well. A standard curve with purified apoA-I from African green monkeys is shown in Fig. 4. The working range of the assay was between 0.5 and 5 ng of apoA-I. Parallel curves to the apoA-I standard were demonstrated with isolated HDL fractions and serum, both of which

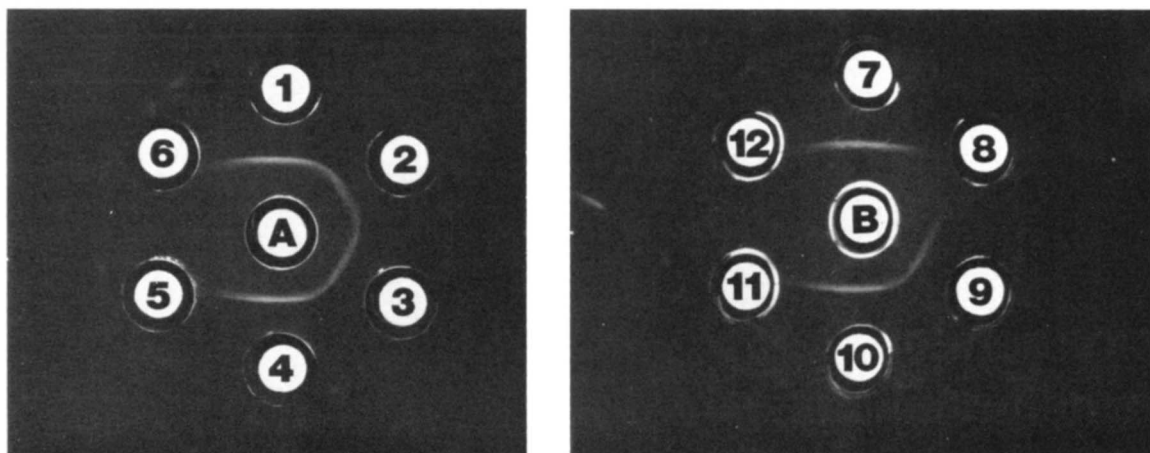


Fig. 1. Ouchterlony double immunodiffusion of isolated antibodies to *M. fascicularis* apoA-I against several apolipoproteins: (A) 100 μ g isolated antiA-I antibodies (1) African green serum, (2) HDL, (3) apoA-I, (4) African green serum, (5) chylomicron apoA-II + apoC's, (6) apoA-II; (B) 100 μ g isolated antiA-I, (7) African green serum, (8) apoE, (9) apoA-I, (10) African green serum, (11) LDL, (12) apoA-II.

had been heat-treated (Fig. 4). The apoA-I content of the HDL fraction of Fig. 4 was found to be 64% of the total protein. The solvent and TMU-delipidated sera gave curves parallel to the heated serum. The ED₅₀ values for heated, TMU- and solvent-delipidated sera were 1.65, 1.73, and 1.70 ng, respectively. Unheated control sera

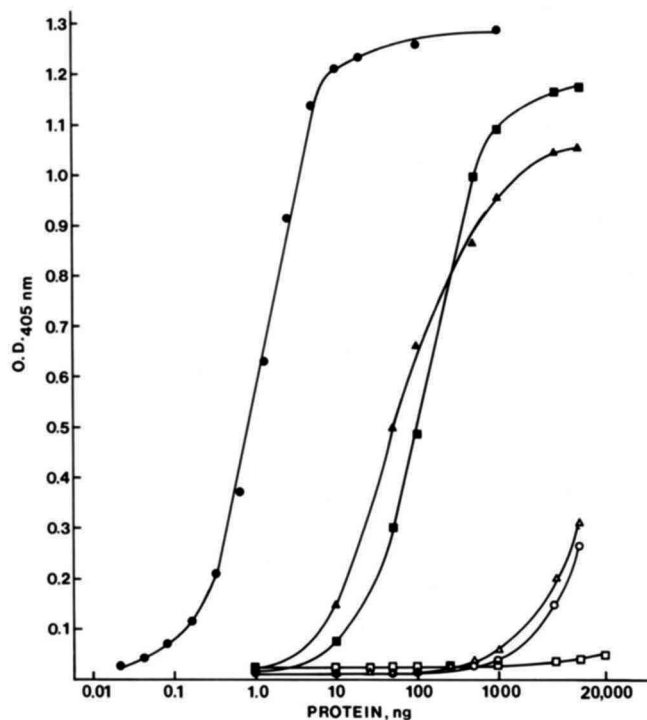


Fig. 2. Comparison of the immunoreactivity of the isolated anti-apoA-I antibodies with apolipoproteins apoA-I (●), LDL (■), chylomicron apoA-II + apoC's (▲), apoE (Δ), and apoA-II (○) and delipidated human serum albumin (□). Protein concentrations of all preparations were determined by the method of Lowry et al. (13).

were studied with their heated counterparts for immunoreactivity comparisons. The unheated sera gave values $82.3 \pm 3.5\%$ ($\bar{x} \pm S.D.$, $n = 8$) lower than the heated samples. This was somewhat greater than that reported by Karlin et al. (5); however, our Sample Buffer contains 0.1% Tween-20 which probably exposes some of the apoA-I immunoreactive sites. Purified apoA-I was added to whole serum after which the assay was performed. Recovery of apoA-I was $106 \pm 3\%$, $n = 6$, a value similar to that reported by Karlin et al. (5) for human apoA-I determined by RIA.

The intrassay coefficient of variation for our standard serum pool was 6.2%, $n = 35$, and over a period of 3 months the interassay coefficient of variation was 6.9%, $n = 10$.

Quantitation of apoA-I concentration in serum samples as measured by the ELISA method was compared to an independent estimation of the concentration of this apoprotein in the same 78 serum samples by radialimmunodiffusion (Fig. 5). The Pearson's correlation coefficient, r , equalled 0.84. In order to determine if either of the two methods consistently estimated the apoA-I concentrations to be higher, a paired t -test was used to evaluate the data. No statistically significant difference was found ($P > 0.1$).

ApoA-I in African green monkey sera

Fig. 6 depicts the mean values of apoA-I and HDL cholesterol for each animal. ApoA-I was highly correlated with HDL cholesterol ($r = 0.86$), and the relationship was the same for both diet groups. On the average, polyunsaturated fat-fed animals had lower HDL cholesterol and apoA-I levels than did the saturated fat-fed animals. For the polyunsaturated vs. saturated fat groups, the mean HDL cholesterol levels were 98 mg/dl vs. 112 mg/dl

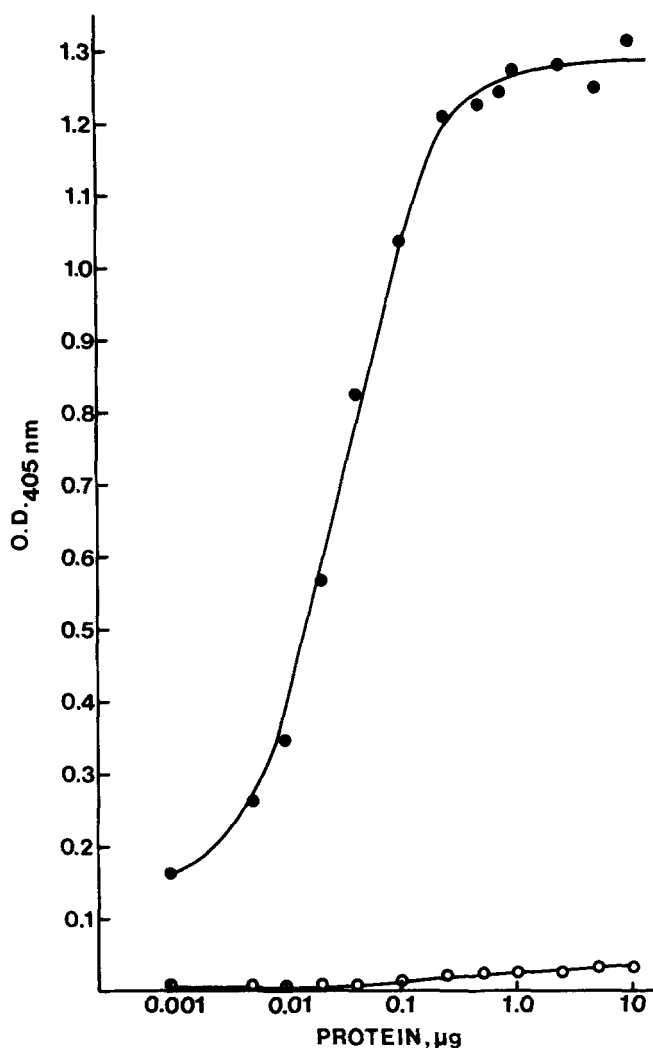


Fig. 3. Titration of the amount of binding of antibodies to apoA-I isolated from HDL of *M. fascicularis*. The various dilutions of purified antibodies were coated on the wells of the microtiter plates. An excess of apoA-I (1 $\mu\text{g}/200\ \mu\text{l}$) was added to each well and then HRPO-conjugated antibodies were added to measure the specific binding (●). Nonspecific binding of the conjugated antibody preparation (○) was also determined at each antibody concentration by adding Sample Buffer free of apoA-I to the respective wells. Each point represents the mean of three measurements.

and the apoA-I concentrations were 247 mg/dl vs. 299 mg/dl, respectively. The ratio of apoA-I to HDL cholesterol was the same for both groups.

DISCUSSION

Since apoA-I is known to be a polyvalent antigen (13, 22), the 'sandwich' technique was chosen for use in the ELISA. With the possibility of binding several enzyme-labeled antibody molecules to a single apoA-I molecule, this provided an element of amplification and, thus, an

increase in assay sensitivity (21). In preparing an enzyme-antibody conjugate, horseradish peroxidase (HRPO) was the enzyme of choice for several reasons. It has a high turnover number and is thus detectable at low levels; it is very stable and is easily obtainable in a relatively pure form at a low cost. Since, HRPO contained a carbohydrate moiety, it was possible to use the periodate procedure for selectively coupling enzymes to proteins. This procedure was chosen as it incorporated a greater amount of enzyme label into the conjugate than did most other coupling procedures while retaining the enzyme activity and immunoreactivity in the preparation (16). As a result, the ELISA presented here is among the most sensitive assays that have been described for apoA-I with a working range of 0.5 to 5 ng of apoA-I. This compares favorably with the range found for most radioimmunoassays, of 1 to 20 ng of apoA-I (5, 22).

The method has been validated for use with serum samples in several ways. The parallel responses seen across

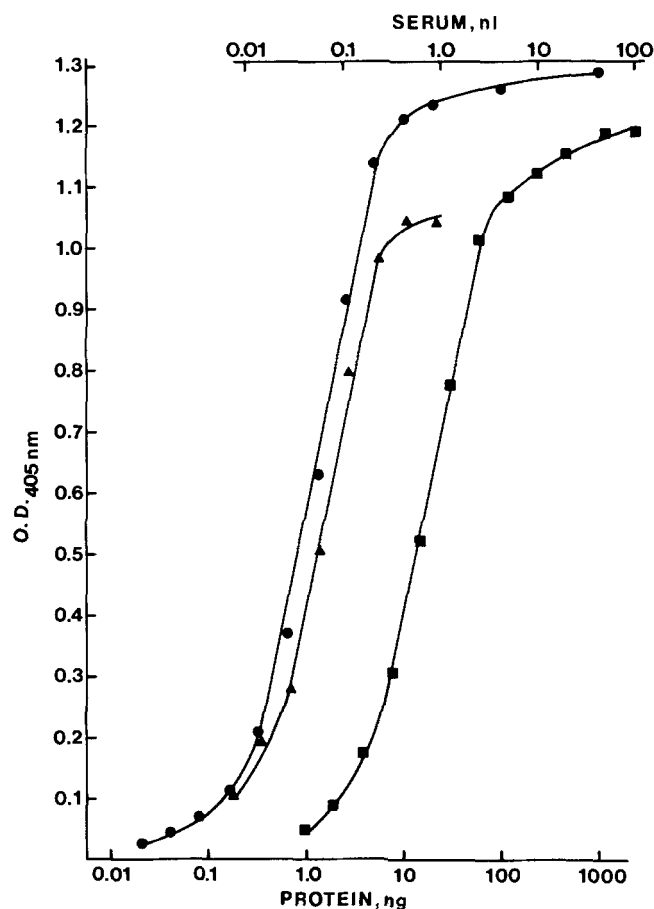


Fig. 4. Dose response curves for the two-site sandwich ELISA for nonhuman primate apolipoprotein A-I, showing a comparison of purified apoA-I (●), isolated HDL (▲), and dilutions of serum (■). The colorimetric response was directly proportional to the amount of antigen in the sample that bound to the immobilized antibody.

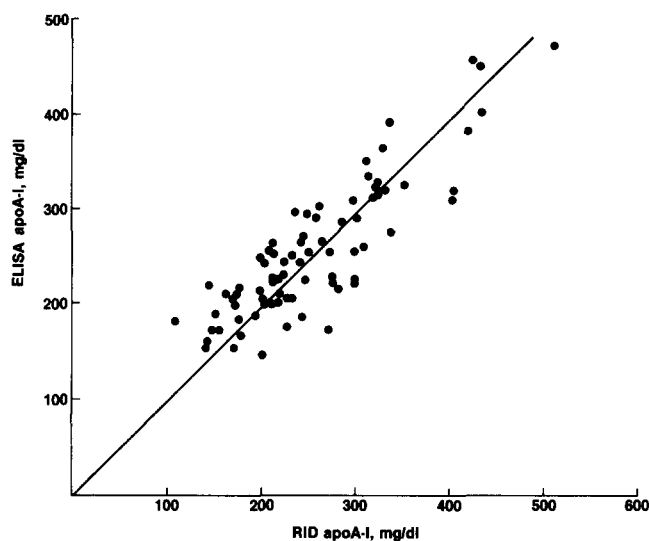


Fig. 5. Comparison of apoA-I measurements in sera by two independent methods, radialimmunodiffusion (RID) vs. the two-site sandwich ELISA. The comparison was made for 78 serum samples from 16 adult male African green monkeys; each point represents the values for duplicate determinations with each assay. The Pearson's correlation coefficient was $r = 0.84$.

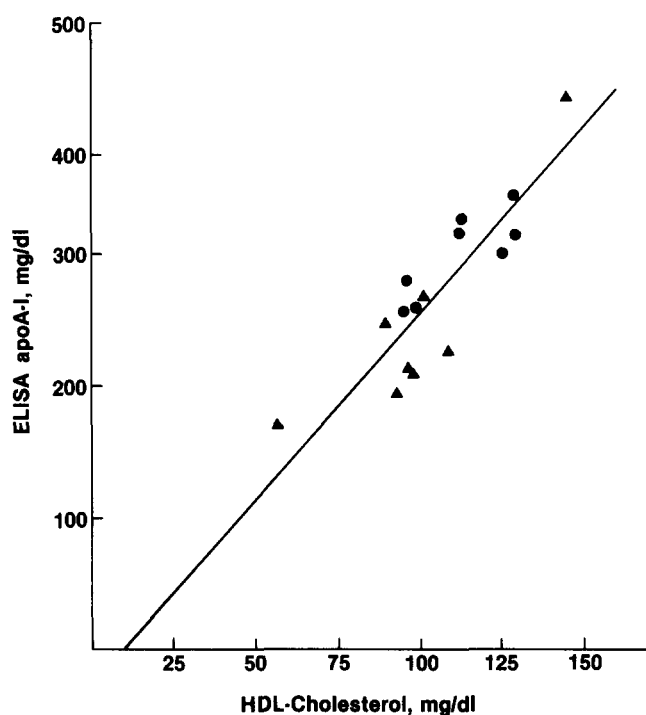


Fig. 6. The relationship between HDL cholesterol and apoA-I concentrations in sera from adult male African green monkeys fed diets containing saturated fat (●) or unsaturated fat (▲). Each point represents the value for a single animal and is the mean of the determination on six serum samples collected over a 20-month period. Sixteen animals were studied. The Pearson's correlation coefficient was $r = 0.86$. The average value for the saturated fat-fed animals was higher than that for the polyunsaturated fat-fed animals ($P < 0.01$) but the relationship between apoA-I and HDL cholesterol concentrations was the same for both diet groups.

a series of dilutions for purified apoA-I, HDL, and serum (Fig. 4) are the best indicators of accuracy and are typical of behavior also seen in the radioimmunoassay of apoA-I (5). It was difficult to demonstrate these parallel responses between a wide range of dilutions of the purified standard and samples using the RID and rocket electroimmunoassay techniques that have been used in this laboratory in the past for apoA-I determination. With the ELISA technique it was possible to add purified apoA-I to plasma and to quantitatively determine the amount added. Serum apoA-I concentrations determined by ELISA were compared to apoA-I concentrations measured by RID and excellent agreement was found (Fig. 5). This agreement with an independent method confirms that the ELISA determination of apoA-I is accurate. The precision of the ELISA was also excellent as seen by the intra- and interassay coefficients of variation. In our experience the precision of the ELISA is better than that of the RID. Interassay coefficients of variation were 7% vs. 12% for ELISA vs. RID, respectively. In summary, the results show that the ELISA procedure described herein gives an accurate and precise measurement of serum apoA-I concentration.

The comparison of the three methods of serum delipidation produced lower apoA-I values for the solvent and TMU procedures than for the heat procedure. This was not entirely unexpected as protein can be lost in the solvent delipidation methods and previously reported recoveries of apoA-I added to serum and analyzed by RIA after solvent delipidation were approximately 95% (5). It is also possible that TMU may not delipidate apoA-I of serum as effectively as does the heat treatment, but recovery of apoA-I from TMU-treated serum was not measured to evaluate this. However, comparison of the ED_{50} values of the dilution curves produced by these three delipidation techniques gave similar results, indicating that the heat treatment is a comparable method for exposing the apoA-I immunoreactive sites.

The procedure was developed using nonhuman primate samples. This provided the opportunity to examine multiple samples from the same individuals for both apoA-I and HDL cholesterol and to compare samples from animals in separate diet groups. The variations across time for apoA-I and for HDL cholesterol concentrations among samples from a single animal were similar and there was a high correlation between apoA-I concentration measured by the ELISA procedure and HDL cholesterol concentration (Fig. 6). The antibodies prepared to apoA-I from cynomolgus monkeys were used to assay apoA-I levels in serum from African green monkeys as well as that from cynomolgus monkeys. The results were equivalent as long as the serum and the apoA-I standard used in the assay were both from the same species. The diet group comparison was between saturated and poly-

unsaturated fat-fed African green monkeys, a situation for which a polyunsaturated fat-induced lowering of apoA-I and HDL cholesterol concentrations has been previously found (23, 24); the diet effect determined here by ELISA was the same. All of this information serves as a 'biological check' on the assay and helps to establish the validity of the method. ■■

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