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A direct sandwich enzyme-linked immunosorbent assay for human serum apolipoprotein A-I

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Abbreviations: HDL, high-density lipoproteins; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; CV, coefficients of variation; apo A-I, apolipoprotein A-I

Abstract

Apolipoprotein (apo) A-I, an abundant protein in high-density lipoproteins (HDL), has received considerable clinical attention as a negative predictor of coronary artery disease, because the plasma concentration of apo A-I has been shown to be inversely correlated with the incidence of coronary artery disease. A direct sandwich enzyme-linked immunosorbent assay (ELISA) for determination of the level of human serum apo A-I was developed using highly purified anti-apo A-I polyclonal antibodies raised in rabbits as the capturing antibody, and as the detecting antibody as well. Microtiter plates were coated with purified anti-apo A-I polyclonal antibodies. The concentration of apo A-I in samples was determined from the peroxidase color reaction. The sensitivity of the assay was determined to be 10 ng of apo A-I per ml of serum. The working range, where the standard curve showed a linearity, was between 10 and 100 ng/ml. The mean intra-assay and inter-assay coefficients of variation (CV) were <5% and <7%, respectively. No cross reactivities with serum albumin, apo A-II, B-100, C-I, C-II and E were detected. For the evaluation of validity of the assay, 20 serum samples of domestic subjects were employed, and the serum apo A-I measurement by the sandwich ELISA of this report was compared with those of Beckman's and Kallestad's immunonephelometry. The results indicated that there is a good correlation between the data obtained by the present ELISA method and by the two commercially available immunonephelometric assay methods (r=0.95 and 0.84, respectively).

Keywords: apolipoprotein A-I, coronary artery disease, polyclonal antibodies, sandwich ELISA

Introduction

Apolipoprotein (apo) A-I is the major protein component of plasma high-density lipoproteins (HDL) which play a principal role in cholesterol transport and metabolism from peripheral tissues to the liver through so called "reverse cholesterol transport". Apo A-I represents about 70% of the total HDL proteins (Bekaert *et al.*, 1988), and appear to provide necessary structural component for HDL formation and to function as a cofactor for lecithincholesterol acyltransferase (LCAT) in the esterification of plasma free cholesterol to cholesteryl esters. Deficiencies of apo A-I are associated with alphalipoproteinemia and sometimes with premature cardiovascular disease (Naito, 1986).

Clinical interest in measuring apolipoproteins has been intensified after the recognition of their physiological importance and utility in assessing an individual's atherosclerotic risk. Results of some studies (Miller *et al.*, 1975; Heiss *et al.*, 1980; De Backer *et al.*, 1982) have demonstrated a significant negative correlation between HDL-cholesterol and coronary heart disease, and it has been suggested that apo A-I could be an even more effective predictor than HDL-cholesterol in identifying potential survivors of myocardial infarction (Avogaro *et al.*, 1980; Fager *et al.*, 1981). Apo A-I has also been suggested as a better marker for coronary heart disease than the traditional lipid marker, HDL-cholesterol (Maciejko *et al.*, 1983).

Human apo A-I has been quantified by radioimmunoassay (Schonfeld *et al.*, 1974; Fainaru *et al.*, 1975; Karlin *et al.*, 1978), immunonephelometry (Rosseneu *et al.*, 1981; Heuck *et al.*, 1983), immuno turbidimetry (Nader and King, 1986; Maciejko *et al.*, 1987) and enzyme immunoassay (Bury and Rosseneu, 1985).

Here, we developed a direct, sandwich enzyme-linked immunoassay for human apo A-I by utilizing the high titer of purified, specific polyclonal antibodies. The advantages of this technique include rapidity, ease of

the procedures, sensitivity, small antiserum requirement and absence of radioactive tracers. The validity of the presently developed ELISA was evaluated by comparison with commercially available immunonephelometric kits.

Materials and Methods

Chemicals and enzymes

Acrylamide, *N,N'*-methylene-*bis*-acrylamide, sodium dodecylsulfate, bovine serum albumin, *4*-chloro-*1*-naphtol, glycine, *o*-phenylene-diamine dihydrochloride, sodium *m*-periodate, horse radish peroxidase, Freund's adjuvant were purchased from Sigma (St. Louis, MO, U.S.A.). Protein G-Sepharose and Sephadex G-200 column chromatography media were from Pharmacia LKB Biothechnology (Uppsala, Sweden), and Matrex Cellufine Formyl, an affinity chromatography resin, was purchased from Amicon (Beverly, MA, U.S.A.). Maxisorp Nunc-Immuno Module and 96-well ELISA plates were from Nunc (Roskilde, Denmark). The reference apo A-I, A-II, B-100, C-I, C-II and E, for calibration and cross-reactivity test, were purchased from Calbiochem (La Jolla, CA, U.S.A.).

Isolation of apo A-I

Human blood samples were freshly collected from normal volunteers, allowed to clot at 4°C, and sera were recovered by low speed centrifugation. Protease inhibitors and preservatives were added to the sera, and HDL in the density range between 1.08 and 1.21 g/ml was isolated by sequential ultracentrifugation. The HDL samples were washed and dialyzed extensively against 0.9% NaCl and 0.01% EDTA (pH 7.4). Apo A-I was prepared by delipidation with ethanol/diethyl ether (3:2, v/v) (Scanu *et al.*, 1971) and, subsequently, by electroelution from gel following SDS-PAGE (Laemmli, 1970).

Preparation of anti-apo A-I antibody

Purified apo A-I (200 μg), as an antigen, was emulsified wit an equal volume of Freund's complete adjuvant and injected into rabbits subcutaneously. Four weeks later, booster injections were given intramuscularly with a freshly prepared emulsion of the antigen and the Freund's incomplete adjuvant. Ten days later, the antiserum was collected and the anti-apo A-I antibody titer in the antiserum was checked by an antigen-coated indirect ELISA. Whole blood was collected by cardiac puncture and the antiserum was separated by low speed centrifugation. Immunoglobulin G (IgG) was purified from the rabbit antiserum by protein G-Sepharose affinity chromatography. The bound IgG was eluted with 0.1 M glycine, pH 2.7. The apo A-I-specific antibodies were purified by the antigen affinity chromatography. The apo A-I antigen (10 mg/ml of resin) was coupled to preswollen Cellufine Formyl resin and the total IgG purified from protein G chromatography was loaded onto the column. After washing with the phosphate buffer, apo A-I-specific IgG, which bound to the resin, was eluted with the glycine buffer.

Western blot analysis

In order to examine antibody specificity, Western blot analysis was performed according to the method of Towbin *et al.* (1971). Briefly, proteins were separated by 10% SDS-PAGE and electroblotted onto a nitrocellulose membrane. After incubation of the proteins with anti-apo A-I antiserum, bound antibodies were detected by adding horseradish peroxidase (HRP)-goat anti-rabbit IgG conjugate, and the colors were developed by adding 4-chloro-1-naphthol and H_2O_2.

Preparation of antibody-enzyme conjugate

HRP-coupled antibodies were prepared according to the procedure described by Nakane and Kawaoi (1974). To 5 mg of HRP dissolved in 1 ml of 0.3 M sodium bicarbonate buffer (pH 8.1), 0.1 ml of 2,4-dinitrofluorobenzene (0.1 g/l) dissolved in ethanol was added and the reaction mixture was incubated for 1 h at room temperature. One ml of 80 mM sodium periodate was added and the reaction was prolonged for additional 30 min. The reaction was stopped by the addition of 1 ml of 0.16 M ethylene glycol in distilled water. After 1 h of incubation, the sample was dialyzed against 10 mM sodium carbonate (pH 9.5) and mixed with 10 mg of purified anti-apo A-I antibodies. After 3 h of incubation at room temperature, to stabilize the Schiff base formed by reaction of HRP-aldehyde with anti-apo A-I antibodies, 0.1 ml of $NaBH_4$ (5 mg/ml) was added to the HRP-antibody mixture. The reaction mixture was then dialyzed against 10 mM phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 7.8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4), and the antibody-HRP conjugate was purified in a 85 x 1.5 cm column of Sephadex G-200 equilibrated in phosphate-buffered saline (10 mM, pH 7.4).

Enzyme immunoassay

The wells of 96-well microtiter plates were coated with 100 μl of anti-apo A-I polyclonal antibodies in 0.1 M carbonate buffer (pH 9.5) by incubating at 4°C overnight. The wells were washed 4 times with PBST (137 mM NaCl, 2.7 mM KCl, 7.8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.05% Tween-20, pH 7.4), and to block non-specific binding sites, 200 μl of blocking buffer, 1% bovine serum albumin (BSA), 2% nonfat dry milk in PBS, was added. The plate was incubated at room temperature for 1 h. The wells were washed 4 times with PBST. Serially diluted reference apo A-I or human serum samples were pipetted onto the wells (100 μl per

well) and the plate was incubated at 37°C for 1 h. After washing the wells again, 100 µl of diluted antibody-HRP conjugate was added to each well, and the plate was incubated for 1 h at 37°C. After washing, 100 µl of freshly prepared substrate solution (mixture of 0.4 g/l of *o*-phenylenediamine in 0.075 M sodium phosphate-citrate buffer, pH 5.0 and 0.04 g/l of H_2O_2) was pipetted into each well, and after 30 min at room temperature, the reaction was stopped by adding 50 µl of 2.5 M H_2SO_4.The absorbances at 490 nm were measured by ELISA reader (Biomek 1000, Beckman Instrument, Columbia, MD). A calibration curve was plotted from the results of reference apo A-I and the concentration of apo A-I in human serum samples was determined accordingly.

Results and Discussion

Isolation of apo A-I

HDL of the density range of 1.08~1.21 g/ml, which was isolated from human serum by sequential preparative ultracentrifugation, contained little serum albumin. Apo A-I was purified from delipidated HDL by electroelution from SDS-PAGE. The purified apo A-I is shown in Figure 1.

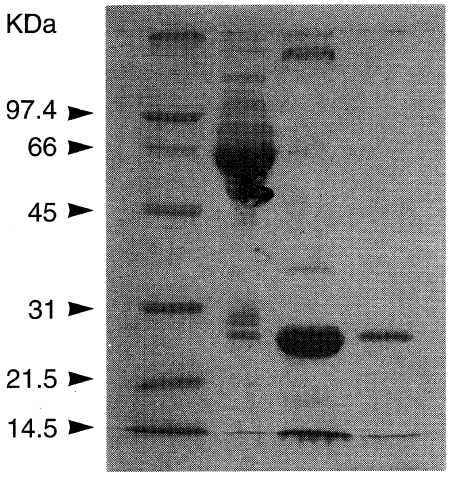

Figure 1. SDS-polyacrylamide gel electrophoretic analysis of apo A-I purified from human serum. Lanes: 1, protein molecular weight marker; 2, human serum; 3, HDL; 4, purified apo A-I. The HDL was purified by the sequencial ultracentrifugation in the density range between 1.08-1.21 g/ml and then the apo A-I was purified by the preparative SDS-polyacrylamide gel electrophoresis. The HDL and purified apo A-I were analyzed on the 10% SDS-polyacrylamide gel electrophoresis and stained with the Coomassie Blue.

Preparation of anti-apo A-I antibody

The antiserum was raised in rabbits, and anti-apo A-I polyclonal antibodies were purified from the antiserum by two successive steps of affinity chromatographies, followed by protein G and antigen chromatographies. By these separation procedures, highly purified anti-apo A-I specific antibodies were obtained. The specificity of purified antibodies was examined by Western blotting. Total human serum proteins, purified apo A-I and apo B-100 were electrophoresed on the gel and analyzed for immunoreactivity with the purified antibodies. As shown in Figure 2, the antibodies reacted with the apo A-I, however, no detectable cross-reactivities were seen with serum albumin, apo B-100 or any other proteins in whole human serum. These results essentially indicate that the polyclonal antibodies are highly specific to apo A-I and do not have cross-reactivities with any other proteins in human serum.

Development of sandwich ELISA system

Using the polyclonal antibodies as capturing antibody (Ab), and also as detecting antibody as well, a sandwich ELISA system for apo A-I was developed and evaluated as follows:

Optimization of assay conditions: Assay conditions were optimized for measuring serum apo A-I concentration by a direct sandwich ELISA. Optimal coating was obtained by applying 100 µl of 4 µg/ml solution of the purified anti-apo A-I antibodies to each well. The optimal dilution rate of the HRP-coupled antibody conjugate was obtained at the dose of 7,000-fold dilution with the high sensitivity and low background of the assay. A wide range of absorbance values (OD_{490nm}), from 0.1 to 1.7, was obtained according to apo A-I concentration in the serially diluted human sera, under the above optimal assay conditon. A standard curve was drawn from the results of the assay, where the solutions containing serially-diluted reference apo A-I (commercially obtained from Calbiochem) were employed. As a result, a wide working range of apo A-I concentrations, 10-100 ng/ml, where the calibration curve showed a linearity was obtained for the direct sandwich ELISA (Figure 3). Therefore, human serum samples were diluted to 40,000-fold and used 50, 100, 150 and 200 mg/dl to establish 4 points on calibration curve which showed good linearity (Figure 4).

Sensitivity and precision: The minimum detectable concentration (assay sensitivity) of apo A-I in the assay was 10 ng/ml, which is equivalent to 1.0 g/dl. Considering the average concentration of apo A-I in human serum as approximately 130 mg/dl, the present assay method showed a sensitivity enough for the serum apo A-I. Three serum samples with known apo A-I concentrations, between 50 and 200 mg/dl, were

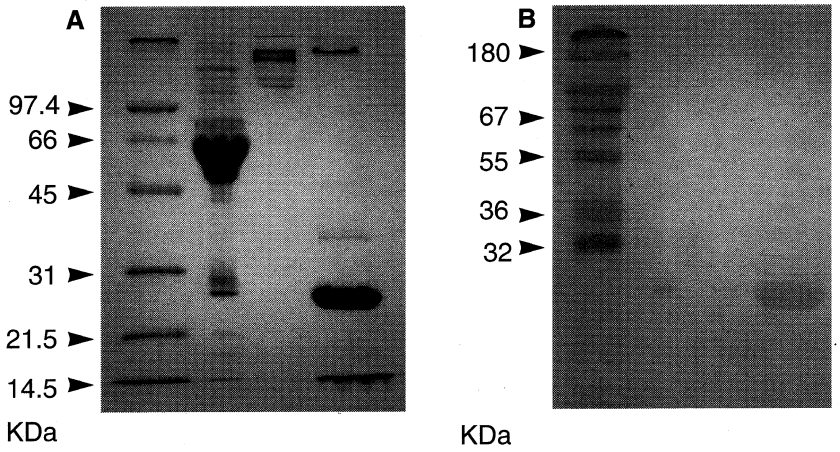

Figure 2. Analysis of the specificity of polyclonal anti-apo A-I antibodies by Western blot analysis. Various proteins were separated in a 7.5% SDS-polyacrylamide gel (**A**) for the subsequent immuno-blotting analysis employing the purified polyclonal antibodies (**B**). The blotted nitrocellulose paper was blocked with a 3% bovine serum albumin and reacted with goat anti-rabbit IgG-horseradish peroxidase conjugate. The color was developed in the 4-chloro-1-naphtol substrate solution. Lanes: 1, prestained protein molecular weight marker; 2, total human serum proteins; 3, LDL; 4, HDL. The color was deveoped only for the apo A-I band of the human serum and purified HDL.

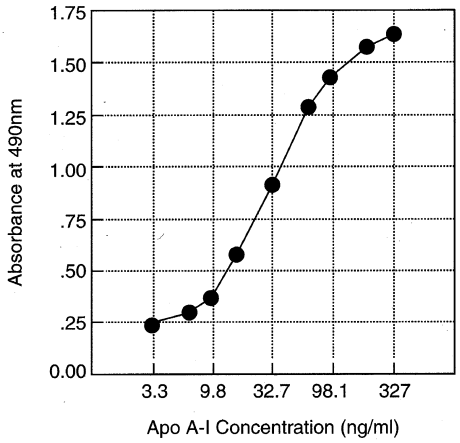

Figure 3. Determination of working range in the developed sandwich ELISA for serum apo A-I measurement. For optimal condition, the serially diluted apo A-I reference materials were used and the direct sandwich ELISA was performed. The linear range was obtained in 10-100 ng/ml of apo A-I concentration.

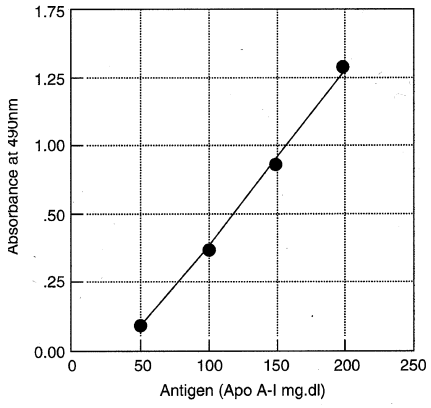

Figure 4. Standard curve of the developed sandwich ELISA for serum apo A-I measurement. The linear range of the developed sandwich ELISA was obtained in 10-100 ng/ml of apo A-I concentration. To assay apo A-I concentration of the human sera, which are normally ranged about 100-170 mg/dl, the samples were diluted to about 40,000-fold. In this condition, a good standard curve was obtained in 50, 100, 150 and 200 mg/dl calibration points.

Table 1. The precision of developed sandwich ELISA for serum apo A-I.

Serum apo A-1 (mg/dl)[a]	Intra-assay			Inter-assay		
	Mean[b]	SD	CV(%)	Mean[b]	SD	CV(%)
50	43.20	3.80	8.80	46.93	5.85	12.47
150	161.37	4.25	2.63	155.22	9.64	6.21
300	295.39	11.09	3.75	297.95	3.88	1.30
Mean CV(%)			5.06			6.66

For determination of intra-assay CV (coefficient of variation), 20 replicate assays were performed on each serum sample at the same time. For determination of inter-assay CV, 20 replicate assays for each of the serum samples were performed through 5 days of period.

[a] Measured by immunonephelometry

[b] Measured by developed sandwich ELISA

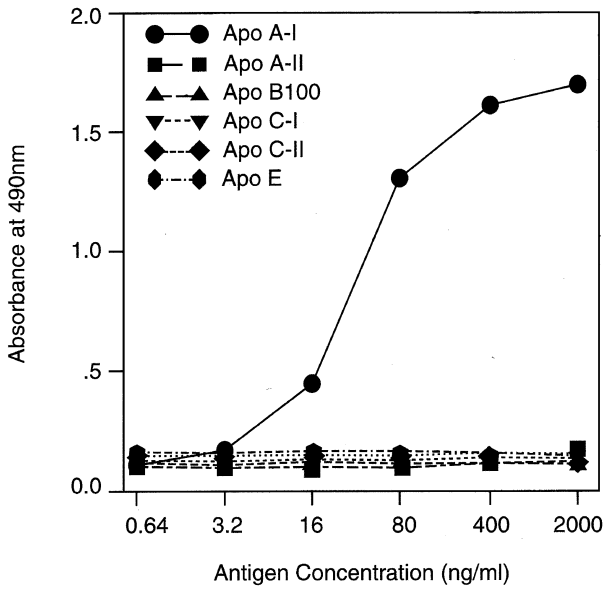

Figure 5. Analysis of the specificity for developed sandwich ELISA. Various apolipoproteins purchased from Calbiochem were used for the sandwich ELISA and the results were plotted. The assay was very specific to apo A-I and no other detectable reaction was obtained from other apolipoproteins, even at higher concentration ranges.

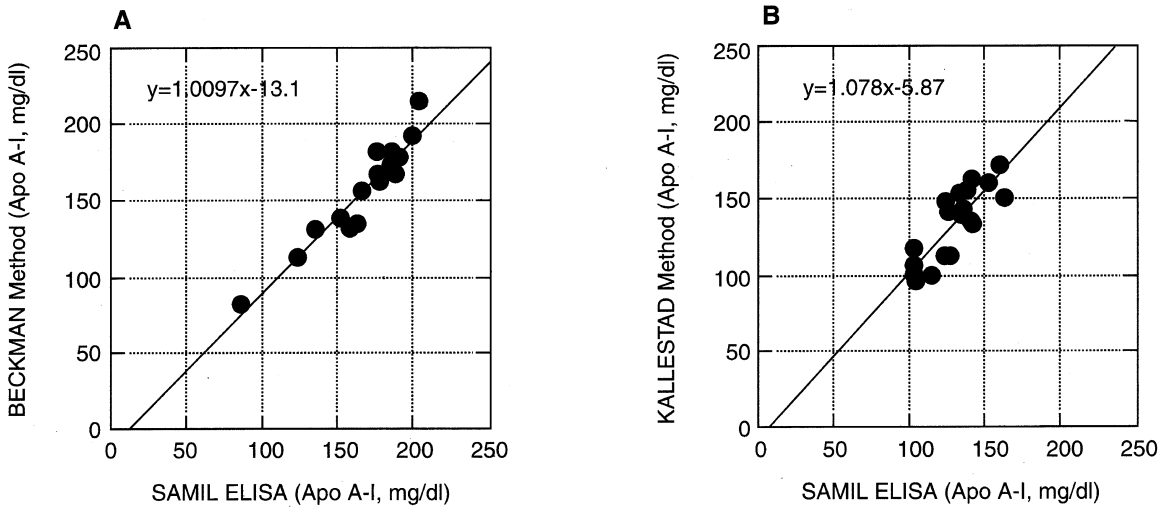

Figure 6. Correlation between Samil ELISA (this report) and Beckman's or Kallestad's immunonephelometry. Randomly selected 20 adult Koreans were employed for the Beckman's (panel A) and Kallestad's (panel B) immunonephelometry, respectively. The regression between Samil ELISA (x) and Beckman's immunonephelometry (y) was represented by an equation, y=1.0097x-13.1, n=20, r=0.95), and the regression between Samil ELISA (x) and Kallestad's immunonephelometry (y) was represented by an equation, y=1.078x-5.87 (n=20, r=0.84).

taken and the precision of assay was analyzed as the followings: For determination of intra-assay coefficient of variation (CV), 20 replicate assays were performed on each serum sample at the same time. For determination of inter-assay CV, 20 replicate assays for each of the serum samples were performed through 5 days of period. These measurements were compared. The result showed that, the mean intra-assay CV was determined to be less than 6%, and the inter-assay CV, less than 7% (Table 1).

Cross-reactivities: To test for any cross-reactivities of the presently developed assay, several apolipoproteins samples were employed for the sandwich ELISA for analyses. As shown in Figure 5, the assay

was very specific to apo A-I, and no immunoreactivity was shown with any of the other apolipoproteins, A-II, B-100, C-I, C-II and E.

Correlation with commercial immunonephelometry: Serum samples of randomly selected 20 adult Koreans were employed and apo A-I concentration was measured by the following three assay methods: Beckman's and Kallestad's immunonephelometry, and the presently developed sandwich ELISA.

These values were compared and plotted as shown in Figure 6. All values are within the range of between 50 and 250 mg/dl. Since data obtained from the two nephelometric methods were well correlated with those from our ELISA method, being the correlation coefficient (r) of 0.95 (Beckman) and 0.84 (Kallestad), respective, The presently developed sandwich ELISA confirms an accuracy which is comparable to those used commercially.

In conclusion, we have developed a direct sandwich ELISA to quantify serum apo A-I concentration, which could be utilized for domestic production of the immunodiagnostic kit. The validity of assay has been proved, by analyzing serum samples of 20 adult Koreans, and comparing with the data obtained by using commercially available immunonephelometric kits. The assay was found to be precise, specific, and is easy to use. Hence it could be well utilized for clinical purposes as well as for research applications.

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References

Avogaro, P., Bittolo Bon, G., Cazzolato, G. and Rorai, E. (1980) Relationship between apolipoproteins and chemical components of lipoproteins in survivors of myocardial infarction. *Atherosclerosis* 37: 69-76

Bekaert, E. D., Ayrault-Jarrler, M., Petit, E., Betourne, C., Robin, H. and Polonovski, J. (1988) Competitive enzyme inhibition immunoassay of apolipoprotein A-I: use of monoclonal antibodies. *Clin. Chem.* 34: 1030-1035

Bury, J. and Rosseneu, M. (1985) Quantification of human serum apolipoprotein A-I by enzyme immunoassay. *Clin. Chem.* 31: 247-251

De Backer, G., Rosseneu, M. and Deslypere, J. P. (1982) Discriminative value of lipids and apoproteins in coronary heart disease. *Atherosclerosis* 42: 197-203

Fager, G., Wiklund, O., Olofsson, S.-O., Wilhelmsen, L. and Bondjers, G. (1981) Multivariate analyses of serum apolipoproteins and risk factors in relation to acute myocardial infarction. *Arteriosclerosis* 1: 273-279

Fainaru, M., Glanegeaud, M. C. and Eisenberg, S. (1975) Radioimmunoassay of human high density lipoprotein apolipoprotein A-I. *Biochim. Biophys. Acta* 386: 432-443

Heiss, G., Johnson, N. J., Reiland, S., Davies, C. E. and Tyroler, H. A. (1980) The epidemiology of plasma high density lipoprotein cholesterol levels. The Lipid Research Clinics Prevalence Study. *Circulation* 62: 116-136

Heuck, C. C., Erbe, I. and Flint-Hansen, P. (1983) Immunonephelometric determination of apolipoprotein A-I in hyperlipoproteinemic serum. *Clin. Chem.* 29; 120-125

Karlin, J. B., Juhn, D. J., Goldberg, R. and Rubenstein, A. H. (1978) Recent progress in the development of radioimmunoassays for human serum lipoproteins. *Ann. Clin. Lab. Sci.* 8: 142-154

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 227: 680

Maciejko, J. J., Holmes, D. R., Kottke, B. A., Zinsmeister, A. B., Dinh, D. M. and Mao, S. J. T. (1983) Apolipoprotein A-I as a marker of angiographically assessed coronary artery disease. *N. Engl. J. Med.* 309: 385-389

Maciejko, J. J., Levinson, S. S., Markyvech, L., Smith, M. P. and Blevins, R. D. (1987) New assay of apolipoproteins A-I and B by rate nephelometry evaluated. *Clin. Chem.* 33: 2065-2069

Miller, G. J. and Miller, N. F. (1975) Plasma-high-density lipoprotein concentration and development of ischemic heart disease. *Lancet* 1: 16-20

Naito, H. K. (1986) The clinical significance of apolipo-protein measurements. *J. Clin. Immunoassay* 9: 11-20

Nakane, P. K. and Kawaoi, A. (1974) Peroxidase-labeled antibody. A new method of conjugation. *J. Histochem. Cytochem.* 22: 1084-1091

Rifai, N. and King, M. E. (1986) Immunoturbidimetric assays of apolipoproteins A, A-I, A-II, and B in serum. *Clin. Chem.* 32: 957-961

Rosseneu, M., Vercaemst, R., Vinaimont, N., Vantornout, P. O., Henderson, L. O. and Herbert, P. N. (1981) Quantitative determination of human plasma apolipoprotein A-I by laser immunonephelometry. *Clin. Chem.* 27: 856-859

Scanu, A. M. and Edelstein, C. (1971) Solubility in aqueous solutions of ethanol of the small molecular weight peptides of the serum very low density and high density lipoproteins: relevance to the recovery problem during delipidation of serum lipoproteins. *Anal. Biochem.* 44: 576-588

Schonfeld G. and Pfleger B. (1974) The structure of human high density lipoprotein and the levels of apolipoprotein A-I in plasma as determined by radioimmunoassay. *J. Clin. Invest.* 54: 236-246

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. *Proc. Natl. Acad. Sci. U.S.A.* 76: 4350-4354