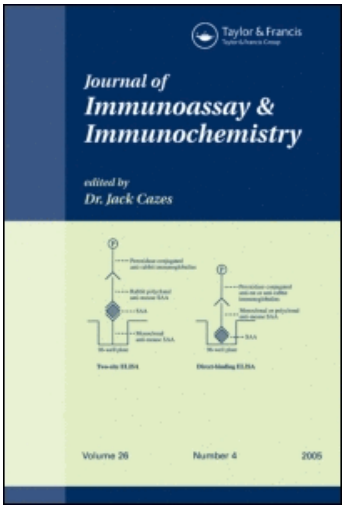

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Characterization of a Monoclonal Antibody that Binds to Apolipoprotein E and to Lipoprotein of Human Plasma Containing APO E. Applications to ELISA Quantification of Plasma APO E

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CHARACTERIZATION OF A MONOCLONAL ANTIBODY THAT BINDS TO APOLIPOPROTEIN E AND TO LIPOPROTEIN OF HUMAN PLASMA CONTAINING APO E. APPLICATIONS TO ELISA QUANTIFICATION OF PLASMA APO E

(KEYS WORDS : Non competitive enzyme-linked immunosorbent assay- Apolipoprotein E - Human plasma)

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ABSTRACT

This study describes the development of an enzyme-linked immunosorbent assay for human apolipoprotein E (apo E).

A mouse monoclonal IgG_1 antibody named E01 against apolipoprotein E was selected from five antibodies secreted by hybridomas. This antibody had a high affinity for apo E ($K = 1.2 \times 10^7$ $L.M^{-1}$ for purified apo E and $K = 1.05 \times 10^7$ $L.M^{-1}$ for native apo E in very low density lipoproteins) in liquid phase and recognized every isoform of apo E but not other proteins in VLDL. Competition experiments with ^{125}I apo E showed that its binding affinity for the E in every density class (VLDL, HDL, LDL) and in serum was the same.

This antibody was used for the quantification of human apo E in serum by enzyme linked immunoassay. E01 was coated on microtiter plates and a polyclonal peroxidase-conjugate was used as second antibody. A good correlation was observed between the values obtained for apo E using both monoclonal and polyclonal antibodies.

INTRODUCTION

Apoliprotein E (apo E), formerly known as the¨arginine rich¨apoprotein is a water soluble, glycosylated polypeptide of 299 amino acids (Mw = 34,000) originally described in 1973 by Shore and Shore (1). It represents approximately 17%,8.1%,1.0% and 0.33% of the apoprotein moiety of human VLDL, IDL, LDL and HDL, respectively (2-6). Apo E has been shown to bind with high affinity to the LDL or apo B, E receptor on cultured human skin fibroblast in vitro, and to a distinct hepatocyte apo E receptor (7). Three major isoforms of apo E are separable by isoelectric focusing, and have been designated apo E_2, apo E_3 and apo E_4 (8).

Plasma lipoproteins rich in apo E and cholesteryl esters are highly atherogenic and characteristically accumulate in the plasma of individuals with dyslipoproteinemia. Indeed such individuals usually display elevated serum apo E concentrations (9).

Various methods including radioimmunoassay (2,10,11), electroimmunoassay (9,12,13), laser nephelometry (14), competitive enzyme immunoassay (15-17) and radial immunodiffusion (18) have been employed to quantify the protein.

Several authors used monoclonal antibodies to study the immunochemical heterogenity of apoliproteins B, AI and E (19,20,21). It is possible that antigenic determinants of an apolipoprotein are expressed in different lipoprotein classes in different ways. Consequently in order to quantify

circulating apo E in the plasma with a single monoclonal antibody, it is important to establish whether this monoclonal antibody is directed against an epitope which is expressed in all isoforms of apo E (See the report of Voyta et al (22)), and reacts with all major lipoprotein classes that contain apo E.

In this paper we describe a monoclonal antibody, (E01) that binds equally to purified apo E and to lipoproteins containing apo E (i.e, VLDL, LDL and HDL). This antibody was used to quantity human apo E by enzyme linked immunoassay.

MATERIALS AND METHODS

Plasma Donors

Samples of human serum were obtained from overnight fasted normolipemic donors (cholesterol and triglyceride levels were <7.2 mM/L and <1.14mM/L, respectively).

For comparative purposes, sera from hypertriglyceridemic (>1.14 mM/L) or from hypercholesterolemic patients (>7.2mM/L) were also included in this study.

After addition of EDTA (0.27 mM/L),cis-aminocaproic acid (0.99mM/L), chloramphenicol (0.62 μM/L) and glutamine (32.54 mM/L), all serum samples were kept at 4°C or frozen at -20°C until assay.

Lipoprotein Fractionation

Lipoprotein subfractions were prepared in a L 8-70 Beckman ultracentrifuge with a 50.2.ti rotor (Beckman Instruments Palo Alto, CA).

The VLDL, LDL and HDL were isolated by sequential ultracentrifugal flotation (23) at densities of < 1.006, 1006-1.063 and 1.063 - 1.21 g/L, respectively.

Production of Monoclonal Antibodies

Male balb/c mice were immunized intraperitonally with 80 µg of apo E emulsified in 300 µL of complete Freund adjuvant. The procedure was repeated one month later using complete Freund adjuvant. Three days before the cell fusion, the animals received a 20 µg booster intravenous dose of apo E. Splenic cells were fused using Campbell's (24) modification of the procedure of Kohler and Milstein (25,26) with myeloma line SP2-O in the presence of polyethylene glycol (Merck) containing 10% dimethyl sulfoxide (Sigma) .

Hybrids were screened for the secretion of the specific antibody by ELISA using microtiter plates coated with purified apo E and horseradish peroxidase conjugated rabbit anti-mouse IgG (Nordic, Netherlands).

The monoclonal antibodies were isolated from ascitic fluid by ammonium sulphate precipitation and purified by ion exchange on DEAE-Tris acryl (IBF, France) using Tris 0.025

M/L, NaCl 0.035 M/L, buffer pH 8.8, in order to isolate the IgG fraction from the ascitic fluid.

Isotype characterization of the monoclonal antibodies was performed using the Ouchterlony technique (27) and antisera against mouse immunoglobulin subclasses (Nordic, Netherlands).

Apolipoprotein characterization

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were used for the separation of apo VLDL as described by Weber and Osborn (28). A vertical electrophoresis chamber (LKB 2100) was employed in the separation of VLDL apolipoproteins which took three hours at +15°C, a 60 mA stabilized current being applied to each gel. The electrophoresis (SDS-PAGE) was performed in discontinuous gel: 3% polyacrylamide stacking gel (pH 6.8) and 10% polyacrylamide resolving gel (pH 8.8).

The isoforms of apo E were separated by isoelectric focusing in polyacrylamide gel slabs containing 6M urea and ampholines with a pH range of 4-6 (29). One half of the slab was stained with coomassie blue R-250 while proteins on the remainder of the gel slab were transferred on nitrocellulose paper for the immunoblotting.

Immunoblotting

Western blotting was performed as described by Towbin et al(30); after electrophoretic separation, apo E isoforms were transferred to nitrocellulose films 0.2 µm (LKB Sweden). The film was washed with Tris 15mM/L, NaCl 0.15mM/L, Tween 0,05% v/v, pH = 8 (buffer A) and incubated overnight in the same buffer with the E01 antibody (5 µg/mL). After a two hours washing incubation was continued in buffer A containing 10 µl/mL of peroxidase-labeled rabbit anti-mouse IgG from Nordic, Netherlands for 90 min at room temperature. After a last washing in buffer A and reequilibration in PBS the apoE bands were then visualized in 50 mM/L tris buffer, pH 6.4, containing 90 mM/L NaCl, 0.5 µL/mL of H_2O_2, and 10 µL/mL of 1.8% 4-chloro-1-naphthol dissolved in ethanol. After washing in water, the nitrocellulose was dried.

Apo E Purification and Iodination

Apo E was purified from delipidated VLDL of hypertriglyceridemic donors by gel filtration on sephacryl S200 columns (Pharmacia, Sweden) followed by anion exchange mono Q HR 10/10 chromatography using a fast protein liquid chromatography system (Pharmacia, Sweden). Apo E obtained by this procedure displayed the typical amino acid composition of the purified protein (6).

The E protein was iodinated by a modification of the Greenwood procedure (31); 0.2 mg of purified apo E in 8M/L urea, 0.01M/L Tris (pH = 8.2) was mixed with 0.2 mCi of ^{125}I and 0.1 mg of chloramine T in 10 µL of 0.5 M/L phosphate buffer pH = 7.4. Thirty seconds later, 0.2 mg of sodium metabisulfite in 10 µL of phosphate buffer was added to stop the reaction. The specific activity of the labelled protein was 1321 DPM/ng.

Binding Studies

Microtiter plates (Falcon n°3911, Becton Dickinson and Co., USA) were coated with E01 antibody (100 µL per well at 20 µg/mL in PBS) and incubated overnight at room temperature. The plates were washed four times with PBS, 50 µL of ^{125}I apo E and 50 µL of serially diluted serum lipoproteins or delipidated VLDL were added and incubation was continued for two hours at 37°C. The plates were then washed with PBS, cut and counted in a gamma spectrometer.

The results were expressed as B/Bo, where:
B = ^{125}I apo E counts per minute bound - ^{125}I apo E nonspecifically bound, and Bo = ^{125}I apo E bound in the absence of unlabelled apo E - ^{125}I apo E nonspecifically bound. the nonspecific binding was determined by replacing E01 with normal mouse IgG fixed to the plastic plates.

The E01 affinity constant was determinated by the method of Müller (32) in a fluid phase system using 100 ng

of ^{125}I apo E per tube and different concentrations of unlabelled of apo E VLDL, LDL or HDL. The inhibition concentration was measured, and used to calculate affinity constant from the followed equation.

$$K\ (L/M) = \frac{1}{(It - Tt) \times (1 - 1.5\ b + 0.5\ b^2)}$$

Where b is the fraction of tracer bound by the antibody, Tt is the amount of tracer bound in the absence of inhibitor and It is the molar concentration of the inhibitor giving 50% inhibition of tracer antibody binding in the competitive RIA.

Enzyme Immunoassay

The immunoassay of apo E was performed using an automatic ELISA processor (Behring Institute, Marburg, West Germany) and polystyrene microtiter plates (Costar n°3590, Cambridge, USA).

In order to minimize non-specific binding to the microtiter wells, the 0.1 M/L PBS buffer (pH = 7.4) used in the assay contained 1% (w/v) of BSA (bovine serum albumin) (Sigma, USA).

The plates were washed with 0.1 M/L PBS before coating each well with 100 µL of E01 (20 µg/mL in 0.1 M/L PBS, pH = 7.4) by incubation overnight at room temperature.

After washing the coated plates four times with PBS, 100 µL of each dilution of the sample and standard were added to the wells. The plates were then covered and incubated for 2 h at 37°c.

Thereafter, washing was repeated four times with PBS and the assay developed by addition of a peroxidase-polyclonal antibody conjuguate (100 µL per well diluted 4,000 fold in PBS-BSA) followed by a further incubation of 2 h at 37°C.

The plates were washed again four times and 100µL of fresh substrate solution (see below) added to each well. The enzyme reaction was allowed to develop for 30 min at room temperature in the dark and was then terminated by addition of 100 µL of 1 N HCl per well, and the absorbance read at 492 nm. The absorbance was plotted against apo E concentration to generate the standard curve from which the apo E concentration in serum or lipoprotein subfractions could be determined.

The standard of the assay covered the range from 20 ng to 400 ng apo E per mL. Plasma samples were diluted 1,000 and 2,000 fold before assay.

Apo E Standard

Since the stability of highly purified apoliproteins might be less than that of their counterparts in plasma (33), standard curves were constructed from a secondary

plasma standard, which in this case was a plasma pool calibrated by enzyme immunoassay against purified apo E. The protein content of this primary apo E standard was determined by the method of Lowry et al, (34) using bovine serum albumin as standard (Sigma, USA).

Antiserum to Human Apo E

Antiserum to human apo E was prepared by injection of 0.3 mg of purified apo E into rabbits as previously described (35). After four additionnal injections of 0.16 mg of purified apo E, blood was collected and the specificity of the antiserum assessed by double immunodiffusion in agarose against purified lipoprotein B, apo AI, AII, C, E and whole serum and HDL subfractions.

The polyclonal antibody to apo E was purified by passage over the apo E-Sepharose 4 B column (9 x 3 mm; 1 mL/h), eluted with a 200 mM/L glycine HCL buffer at pH 2.8 and dialysed against 0.1 M/L PBS buffer.

Preparation of the Antibody-Enzyme Conjugate

Ten milligrams of purified polyclonal antibody to apo E were coupled to peroxydase (5 mg: Boehringer Mannheim, EC 1.11.1.7) as described by Nakane and Kawaai (36). The

conjugate was mixed with an equal volume of glycerol for storage in portions at -20°C.

Peroxidase Substrate Solution

O-phenylene diamine dihydrochloride (Sigma, USA) was dissolved at a concentration of 0.3% (w/v) in 0.1 M/L phosphate (3.5 mM/L) and used within 30 min of its preparation.

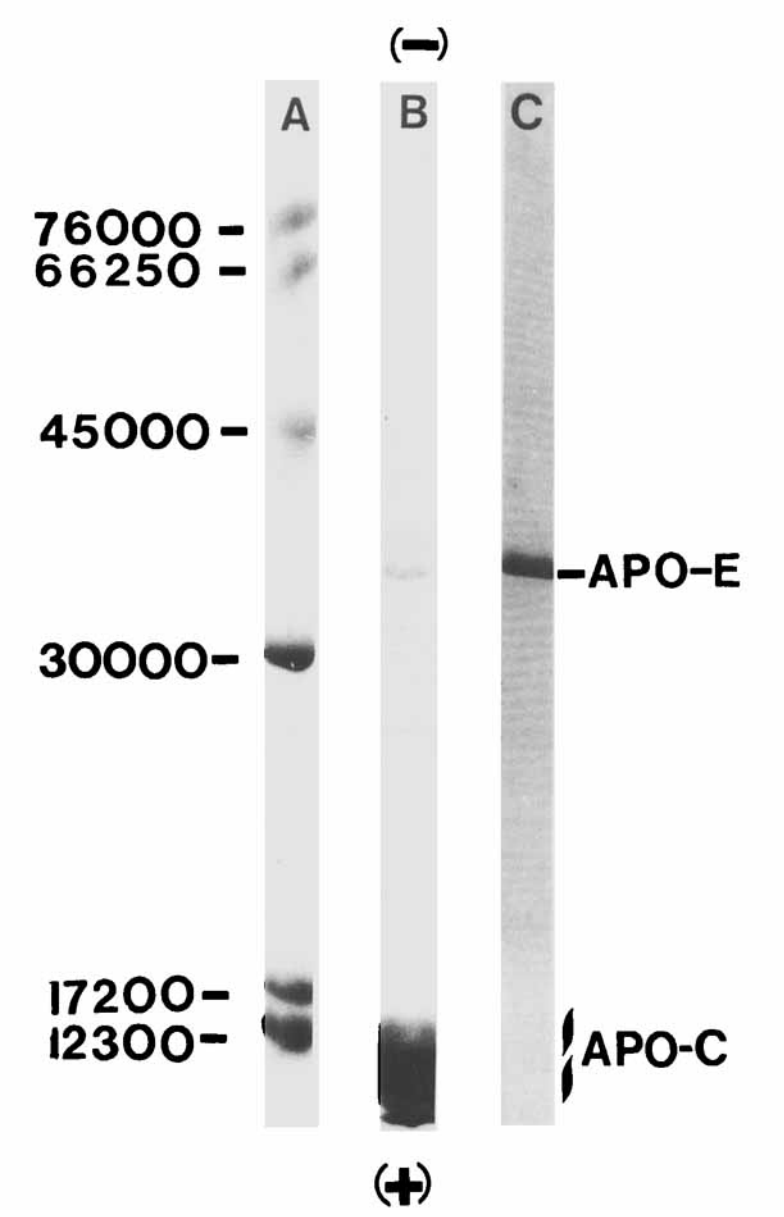
RESULTS

Five clones were obtained that secreted anti apo E antibodies but only one (the IgG1 E01) had high affinity for every class of lipoproteins and bound well to the polystyrene plates.

Specificity of E01 Antibody

Western blotting of apo VLDL clearly showed that E01 bound to a protein with the electrophoretic mobility and immunological reactivity of apo E. The antibody did not react with the other apolipoproteins in the gel (Fig.1).

The apo E isoforms were separated by isoelectric focusing from apo VLDL. E01 recognized all apo E isoforms but no other proteins from VLDL (Fig.2).



Binding Properties and Affinity Constant of E01 Antibody

The apo E concentration in lipoproteins, determined as described above showed that all the competition curves of lipoprotein classes with the E01 monoclonal antibody immmobilized on the polystyrene microtiter plates are the same as the displacement curve obtained with purified apo E or a pool of human serum (Fig 3a, 3b).

At a confidence level of 5% there is no difference between the slopes and by changing the units used (from protein concentration to apo E concentration). We obtained a single line (Fig 3b) for all lipoprotein fractions.

This was confirmed by the linear regression analysis. The equation of the regression line of Figure 3b is :

$y = -28.53\ x + 136.81;\ r = 0.990;\ n = 20$

This displacement curve is linear over the working range 500 to 10000 ng of apo E per mL.

Purified apo A-I, A-II, c-II and c-III do not compete with ^{125}I apo E.

FIGURE 1. Sodium dodecyl sulphate (SDS) polyacrylamide (10%) gel electrophoresis.

A - Molecular weight standarts

B - 34 µg of delipidated apo VLDL

C - Immunoblotting with E01 antibody of the delipidated apo VLDL.

Note : The apo B can not be seen because they stayed in the stacking gel (not showed in the Figure), the E01 did not react with the apo B.

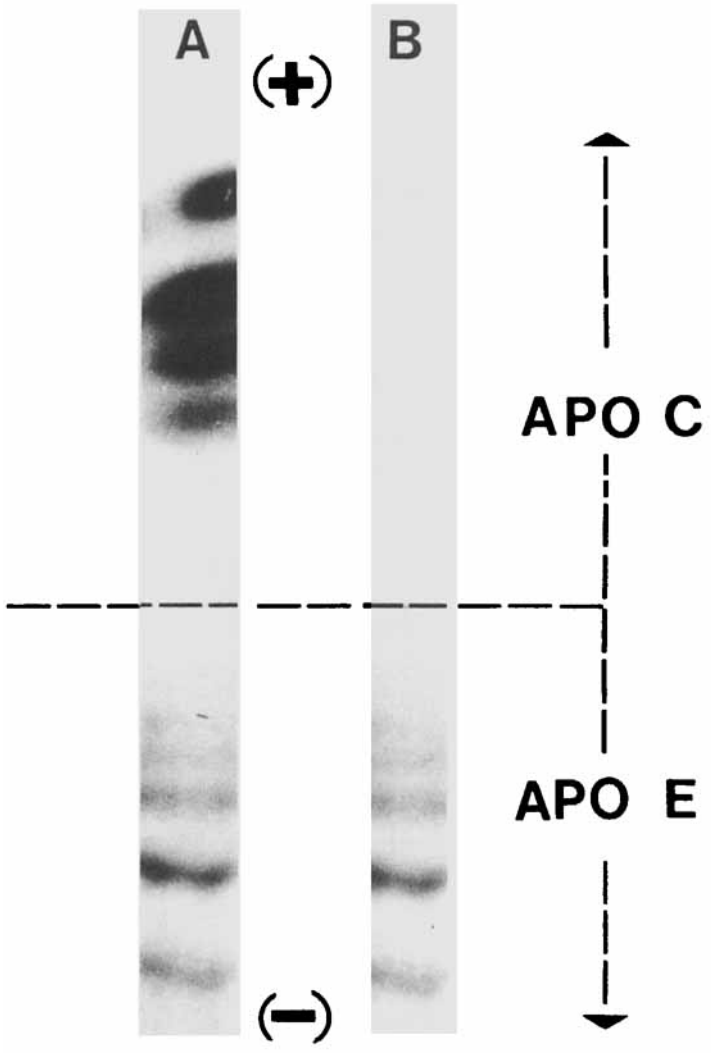


FIGURE 2. Isoelectrofusing of delipidated VLDL (15 µg well) on a pH 4-6 plate.

A - Coomassie R250 staining of apo VLDL

B - Immunoblotting with E01 antibody

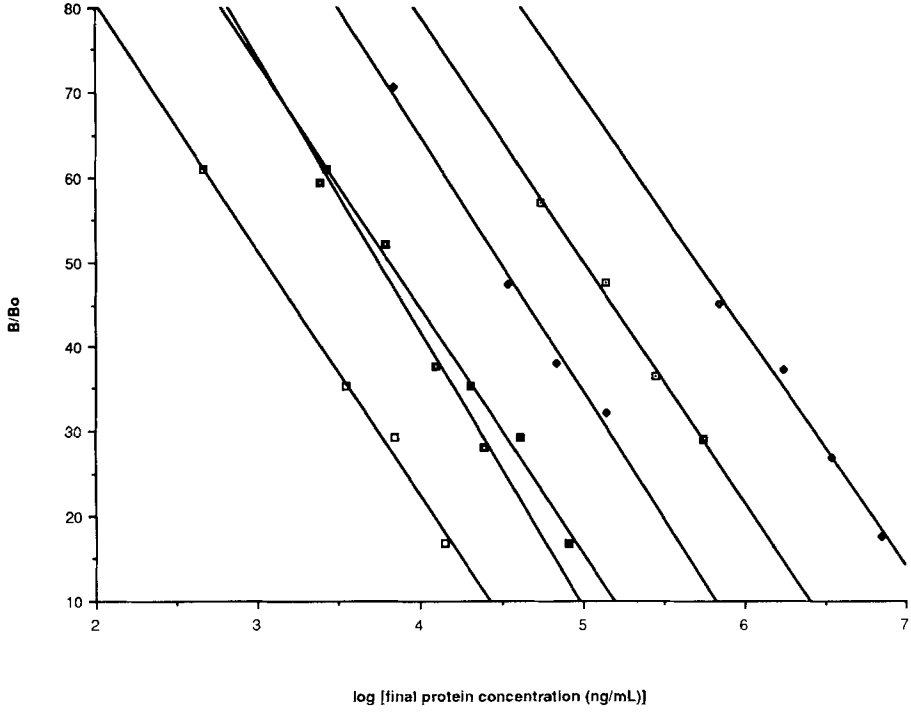


FIGURE 3a. Dilution of apo E(◘), apo VLDL(■), HDL(◘), VLDL(◘), LDL(●) and plasma(◆) expressed in protein concentration, tested for their ability to compete with ^{125}I apo E.

(Continued)

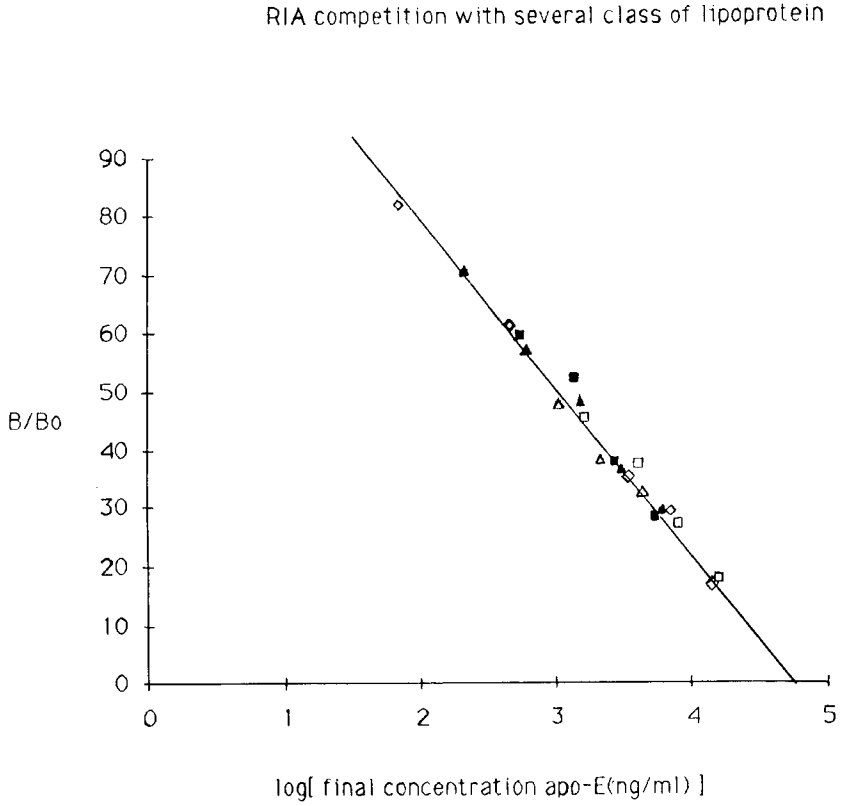


FIGURE 3b. Dilution of apo VLDL(◇), HDL(▲), VLDL(■), LDL(▲) and plasma(□) expressed in apo E concentration, tested for their ability to compete with ^{125} I apo E.

We determined the antibody affinity constant using the procedure described by Müller (32) for purified apo E and native apo E in VLDL.

The calculated antibody affinity determined with unlabelled apo E, was $K = 1.22 \times 10^7$ $L.M^{-1}$.

For apo E contained in VLDL we determined a value for K of 1.05×10^7 $L.M^{-1}$. These values are similar, indicating that delipidation of apo E does not affect antibody binding.

Enzyme Linked Immunosorbent Assay (ELISA)

When dilutions of human plasma or lipoprotein were tested in the ELISA procedure, the curves were parallel to the curve obtained with pure apo E expressed as protein concentration and superimposable when the concentration of the lipoprotein classes was expressed as apo E concentration.

Consequently linear regression analysis after linearization with log-log scale gives a single line (Fig.4). The equation of the regression line is :

$y = 0.5741 x - 1,1786$; $r = 0,990$ and $n = 26$.

These data validate the utility of the ELISA method for the estimation of apo E in lipoproteins.

The coefficient of variation within assay ($n = 60$) was 6.4% and between assay ($n = 46$), 8.2%.

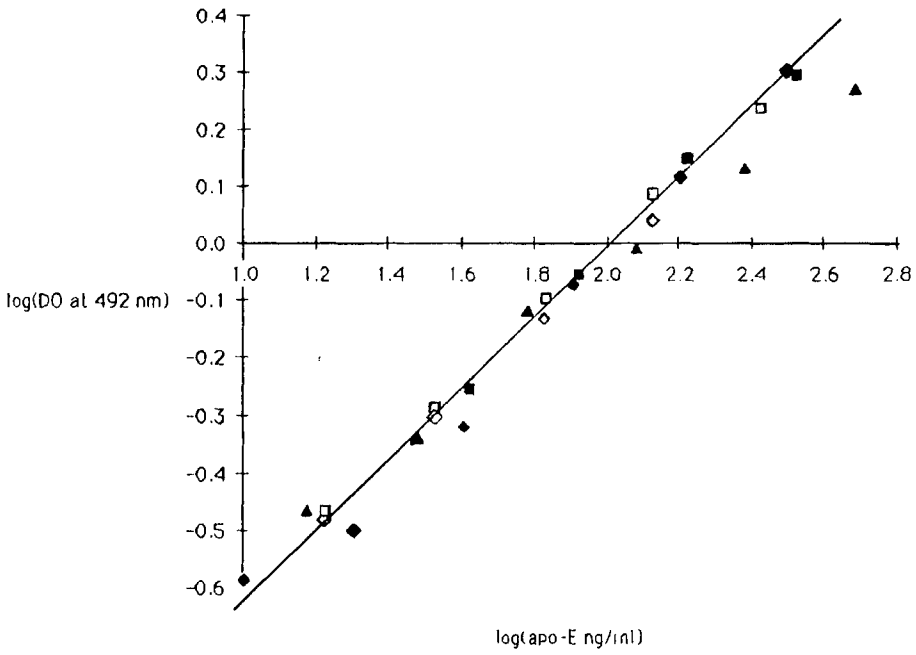


FIGURE 4. Linearization of the ELISA curve with a log-log scale for the lipoprotein subclasses VLDL(◆), LDL(◇), HDL(▲), purified apo E and serum (■ , □) expressed as apo E concentration.

$y = 0.575\ x - 1.18;\ r = 0.99;\ n = 26$

Correlation of the Monoclonal Method with a Polyclonal Method and Accuracy of the Assay

The correlation of the assay with the monoclonal and the polyclonal antibody was tested with 41 sera from normal and hypertriglyceridemic subjects.The polyclonal antibody was used

in the same conditions as the monoclonal E01 100 µL/well at 20 µg/mL in 0.1 M/L PBS for the coating; the same dilution (4000 fold in PBS-BSA) of the peroxidase-polyclonal conjuguate was used for the revelation.

The mean of apo E values obtained with the E01 monoclonal assay was similar to that found with the polyclonal antibody assay. Linear regression analysis confirmed the equivalence of both methods (Fig.5).

We also evaluated the accuracy of the assay by mixing various quantities of chylomicrons or VLDL to the infranatant at d = 1.006 g/mL of the corresponding hgypertriglyceridemic plasma ; or inversely by mixing various quantities of delipidated apo VLDL to a serum. Each fraction was quantified for apo E before and after mixing (Fig.6). The origin ordinates represent the values of residual apo E before adding. All the regression coefficients are good (r = 0.99) and the slopes are near 1.

Apo E Concentration in Normolipidemic Plasma

In our normal subjects (n = 128) the mean apo E concentration determined with our assay was 49 ± 17 mg/L.

DISCUSSION

From a single cell fusion experiment, five stable hybridomas were obtained that secrete antibodies against apo E.

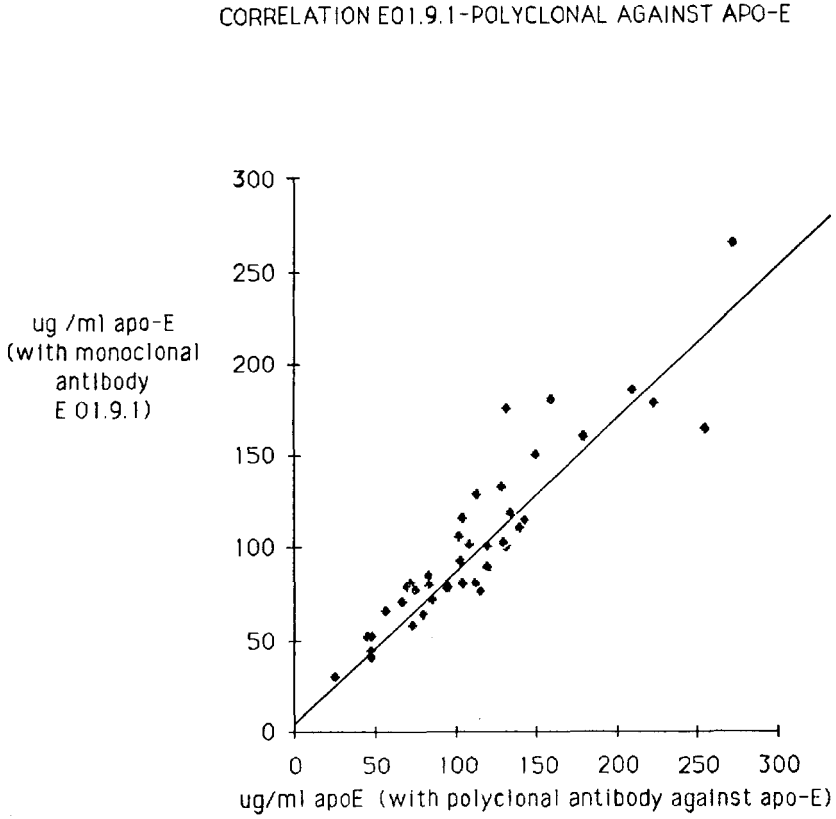


FIGURE 5. Correlation between apo E concentration determined in the polyclonal immunoassay and the monoclonal assay E01.

$y = 0.8\ x + 11.49\ \mu g/mL$; $r = 0.92$ and $n = 41$

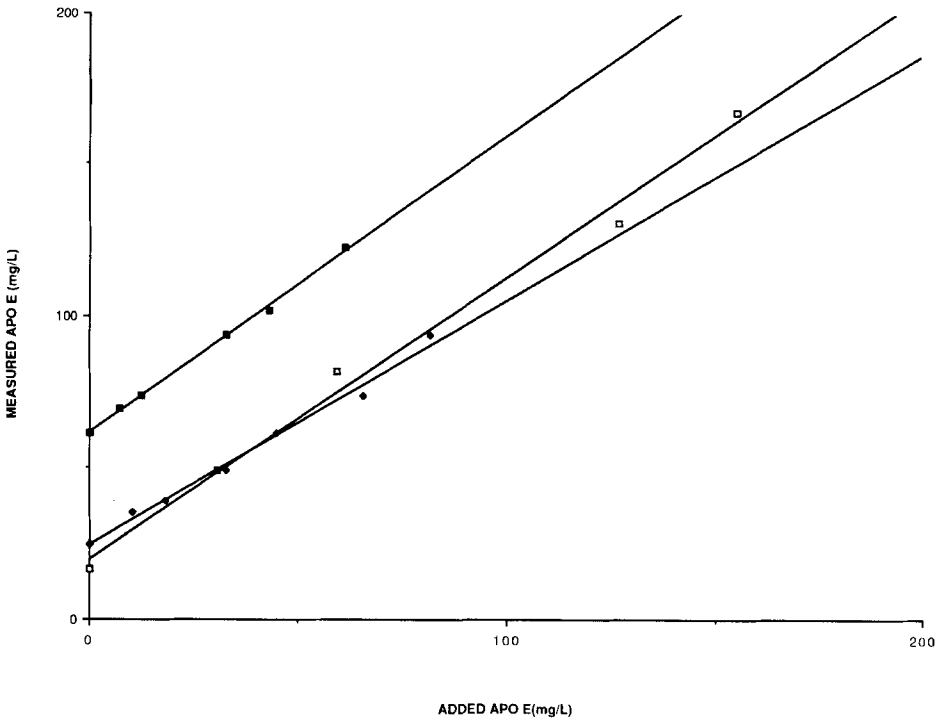


FIGURE 6. Plotting of measured apo E vs added apo E. When apo E is added as VLDL to the infranatant at d = 1.006 g/L ( ); as chylomicrons to the infranatant at d = 1.006 g/L ( ); as delipidated apo VLDL to serum ( ). The linear regressions are: y = 19.75 + 0.932 x, r = 0,99; y = 24.33 + 0.811 x, r = 0.99 and y = 61.56 + 0.981 x, r = 0.99 respectively.

In solid phase only one antibody named E01 showed a high capacity to bind lipoproteins containing apo E. This antibody recognized all isoforms of apo E and the native protein in VLDL.

It is noteworthy that delipidation did not affect the binding of the antibody confirming that the antigenic determinant recognized by E01 is clearly expressed on delipidated and native apo E and is evidently expressed on the surface of lipoproteins.

The antibody showed no cross-reactivity with other human apolipoproteins and whatever the lipoprotein class tested, E01 showed identical binding affinities towards the apo E contained in HDL, LDL, VLDL.

To date, three reports of the production of monoclonal antibodies to apo E have been published (11,22,37).

The use of these antibodies in a radioimmunoassay for plasma apo E demonstrated that they underestimated apo E levels compared with polyclonal antisera. Milne et al obtained apo E levels which were well correlated with apo E, although differences in absolute values were observed. This problem is not apparent with our assay. The use of the E01 monoclonal antibody reacting only with a surface antigenic determinant of apo E expressed on all isolated lipoproteins and on native lipoproteins in the plasma offers distinct advantages in terms of standardization.

The antibody is readily available in large quantities, measures total apo E (of whatever isoforms) in plasma, and provides in the ELISA assay a sensitive and reproducible means of apo E determination.

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