Apolipoprotein E Phenotype Determined by Agarose Gel Electrofocusing and Immunoblotting

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Polymorphism at the apolipoprotein E (ApoE) locus is an important factor in the development of remnant (Type III) hyperlipidemia and also influences the distribution of cholesterol concentrations in the population. The new method for ApoE phenotyping described here gives good results with simple apparatus. Serum (10 μL) is digested with sialidase (EC 3.2.1.18), delipidated, and redissolved in 6 mol/L urea. Electrofocusing is carried out in agarose, followed by immunoblotting with a monoclonal antibody to ApoE and an anti-immunoglobulin–peroxidase conjugate. Sialidase-catalyzed digestion effectively removes siallated forms of ApoE, which eases interpretation. This method can be used in nonspecialist laboratories and is particularly suited for assay of large numbers of samples.

Additional Keyphrases: hyperlipidemia · sialidase

Apolipoprotein E (ApoE) is a component of the triglyceride-rich lipoproteins, very-low-density lipoprotein (VLDL) and chylomicrons, and also certain high-density lipoprotein subfractions. Chylomicron remnants are cleared from the circulation by hepatic ApoE receptors, whereas low-density lipoproteins (LDL) and VLDL remnants are cleared by the LDL receptor, which recognizes ApoE in combination with apolipoprotein B (1). There are three common allelic variants at the ApoE locus, ε2, ε3, and ε4, giving six phenotypes E2E2, E2E3, E2E4, E3E3, E3E4, E4E4. There is some variation in the frequency of the different alleles between different populations. The range of frequencies, reported from nine different population studies, is as follows: ε2 0.04–0.12, ε3 0.73–0.85, ε4 0.07–0.23 (2).

ApoE3 has a lesser affinity for the receptors than does ε2 or E4 (3). Consequently, subjects who are homozygous for the E2 allele (phenotype E2E2) are prone to accumulate lipoprotein remnant particles in the circulation. This accumulation is only slight for most E2 homozygotes. However, about 5% of E2 homozygotes will accumulate large quantities of remnant particles, owing to the presence of an additional lipid-increasing factor such as obesity, diabetes, or an independent genetic trait, thereby causing remnant hyperlipidemia (Fredrickson Classification Type III) (4). Therefore, in the presence of combined hypercholesterolemia and hypertriglyceridemia, the ApoE phenotype E2E2 indicates remnant hyperlipidemia and as such is a useful diagnostic test (4). Diagnosis of remnant hyperlipidemia is worthwhile, because it is associated with accelerated atherosclerosis of both the coronary and peripheral arteries, and it responds extremely well to treatment with diet and fibrate-class drugs (5).

Population studies have also shown that ApoE4 tends to be correlated with higher blood cholesterol concentrations although not with any specific lipoprotein disorder (2, 4, 6). The mechanism of this E4 effect is not clear.

ApoE is a glycoprotein containing 299 amino acid residues (7). The three common variants of ApoE differ in the amino acid sequence at positions 112 and 158 (E2: Cys112, Cys158; E3: Cys112, Arg158; E4: Arg112, Arg158). These amino acid substitutions enable separation of the isoforms by electrofocusing (EF), because arginine is positively charged. The apparent pI values are as follows: E2 5.57, E3 5.80, E4 6.03. Sialated forms of ApoE bear additional negative charge and focus at positions anodal to the parent protein (8, 9).

The first method for ApoE phenotyping required separation of VLDL by ultracentrifugation and protein staining of EF gels (10). The use of chemical precipitation of VLDL removed the need for an ultracentrifuge, but both methods are laborious and require at least 1 mL of serum (11). Furthermore, contamination of VLDL with pro-apolipoprotein A-1 (12) or serum amyloid A (13) may give extra bands in the ApoE region, which confuses the interpretation. In more recent methods polyacrylamide EF and immunoblotting have been used with either whole serum (14, 15) or an ApoE-rich serum protein fraction obtained by a preliminary electrophoretic step (16). In the method described here, EF in agarose is combined with immunoblotting. The method offers advantages in terms of simplicity, speed, and number of samples that can be processed concurrently.

Materials and Methods

Materials

Chloroform, methanol, and diethyl ether were "Analar" grade. GelBond, Pharmalyte (pH 4–6.5), and "Agarose IEF" were obtained from Pharmacia-LKB Ltd., Milton Keynes, U.K. Sialidase (EC 3.2.1.18; Grade V, from Clostridium perfringens), Tween 20 surfactant, diaminobenzidine hydrochloride, and cysteamine hydrochloride were obtained from Sigma Chemicals, Poole, U.K. Decyl sodium sulfate ("HPLC" grade) was obtained from Kodak Biochemicals, Liverpool, U.K. Urea ("Aristar" grade), sialidase (from Vibrio cholerae), and colored protein pI markers were from BDH, Poole, U.K. Polyvinylidenedifluoride (PVDF) membranes were obtained from Millipore Ltd., Harrow, U.K. Nitrocellulose was of 0.2-μm pore size (Schleicher & Schuell, Dassel, F.R.G.).

Antibodies: The sources of antibodies to ApoE were as follows: Mouse monoclonal antibody (code number E01) was donated by Professor J. C. Fruchtert, SERIJA, Lille, France (17). Goat polyclonal antibody was donated by Dr.
L. Havekes, TNO Gaubius Institute, The Netherlands. Sheep polyclonal antibody to ApoE (affinity-purified) was provided by Immuno Ltd., Vienna, Austria. The sources of horseradish peroxidase-conjugated antibodies were as follows: Rabbit anti-mouse IgG conjugate was obtained from Bio-Rad Labs., Richmond, CA 94804. Rabbit anti-goat-IgG conjugate (affinity-purified) was obtained from Nordic Laboratories, Maidenhead, U.K. Donkey anti-sheep IgG conjugate was obtained from the Scottish Antibody Production Unit, Carluke, Scotland.

Procedures

Sample preparation: Blood was collected into plain glass tubes without special precautions. The serum was separated from the cells within 8 h, kept at 4 °C for up to 24 h, and thereafter stored at −20 °C or −50 °C. We added 10 μL of serum to an equal volume of sialidase solution (5 kU/L in 0.1 mol/L acetate buffer, pH 4.5) in a 1.5-μL polypropylene microcentrifuge tube and incubated at 37 °C for 2 h. We then added 1 mL of a 4:2:1 (by vol) mixture of chloroform, methanol, and ether, and mixed by continuous gentle inversion on a shaker for 30 min at room temperature. After centrifugation (7 min at 12 000 × g) the supernatant fluid was decanted and 1 mL of ether was used for further delipidation for 30 min. After further centrifugation and decantation, we allowed the protein pellet to air-dry, then either promptly solubilized it or stored it at −20 °C.

Samples were solubilized by allowing them to stand in Tris buffer (50 mmol/L, pH 8.2) containing, per liter, 6 mol of urea, 10 g of decyl sodium sulfate, and 25 mmol of dithiothreitol for at least 1 h at room temperature. Because most ApoE is carried on the triglyceride-rich lipoproteins, the volume of buffer used to solubilize the protein pellet was varied according to the triglyceride concentration of the sample: 100 μL for each 1 mmol of triglyceride per liter in the sample; e.g., if triglyceride = 4.5 mmol/L, then buffer = 450 μL.

Cysteamine treatment: Cysteamine hydrochloride (40 μg/mL in Tris buffer, 0.1 mol/L, pH 10.3) was added to serum to give a final concentration of 20 μg/mL. Samples were left to stand for 1 h at room temperature, then delipidated and resolubilized as above, except that the dithiothreitol was omitted.

Electrofocusing: The agarose gel was prepared according to the method of Kane and Gowland (71). Agarose (0.5 g) and sorbitol (3.6 g), in 25 mL of de-ionized distilled water, were left for 20 min in a boiling water bath until dissolved, cooled to 65 °C in an oven, then mixed with 10.5 g of urea and 1.9 g of Pharmalyte (pH 4.0–6.5) and poured horizontally onto GelBond film. The 22 × 12 cm plate was kept humidified overnight at 4 °C until use.

For horizontal electrofocusing we used the "Multiphor System" (Pharmacia-LKB) at 10 °C. The anode buffer was 50 mmol/L H₂SO₄ and cathode buffer 1.0 mol/L NaOH. After prefocusing for 30 min at 3 W per gel, we applied 40 3.5-μL samples near the cathode with use of a plastic mask with 2 × 7 mm slots. Focusing took 3 h at 4 W per gel and was monitored by using colored protein PI markers and methyl red (pI 3.5), which moves across the gel to the anode.

Immunoblotting: PVDF was wetted in 5 g/L Tween 20 solution and washed in distilled water followed by 0.15 mol/L NaCl. Nitrocellulose was wetted with 0.15 mol/L NaCl alone. The agarose gel, on the cooling plate at 10 °C, was overlaid for 45 min by the blotting membrane, covered by a sheet of Whatman No. 1 filter paper soaked in 0.15 mol/L NaCl, a sheet of dry Whatman No. 1, six ab sets of medium-thick blotting paper, a glass plate, and a 1-kg weight. The blotting membrane was removed from the gel and incubated for 45 min in a 50 g/L solution of low-fat powdered milk in Tris-buffered isotonic saline (TBS; 0.1 mol/L Tris, pH 7.4, containing 0.15 mol of NaCl per liter) at room temperature, followed by a single 5-min wash in TBS. The membrane was incubated overnight with mouse antibody to ApoE (0.5 mg/L, in TBS), followed by anti-mouse immunoglobulin conjugate (Bio-Rad; diluted 3000-fold in TBS containing 0.5 mL of TWEEN 20 per liter) for 4 h. Both incubations were at room temperature, with gentle shaking. Five 50-mL washes (5 min each) in TBS containing TWEEN were performed at the end of each incubation. (The other antibodies and conjugates were used at the following dilutions: goat polyclonal anti-ApoE 1:1500, anti-goat conjugate 1:1000; sheep polyclonal anti-ApoE 1:1500, anti-sheep conjugate 1:1000.) After further washing as before, the peroxidase conjugate was made visible by using a solution of 0.8 g of diaminobenzidine and 0.4 g of NiCl₂ per liter of Tris buffer (0.1 mol/L, pH 7.5), which produces dark-blue bands. The membranes were washed in water and stored in the dark to prevent fading.

Results

Sialidase treatment: Figure 1 shows immunobLOTS of the six different phenotypes, with and without sialidase treatment. The sialated forms of ApoE focus closer to the anode than the parent protein, producing a step ladder pattern of minor bands. Sialidase digestion removes the sialic acid residues, thereby eliminating the minor bands and intensifying the parent protein band. A comparison of sialidase derived from Clostridium perfringens and Vibrio cholerae showed the former to be more effective (results not shown). Sialidase enzyme has a pH optimum of 4.5–5.0, and reconstitution of the enzyme in 0.1 mol/L acetate buffer, pH 4.5, was adequate to buffer the serum sample to below pH 5. The immunoblots obtained from sialidase-treated samples are clearcut and allow unambiguous determination of ApoE phenotype.

The three bands obtained can be assigned as E₂, E₃, or E₄ on the basis of their position relative to the PI markers and

![Image](image-url)

Fig. 1. Immunoblots of the six major ApoE phenotypes: (A) untreated; (B) with sialidase treatment

Cathode at top

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the preponderance of the E₄ allele.

Storage of serum samples: Serum samples can be stored for three months at −20 °C or at least six months at −50 °C without producing any change in the immunoblot pattern. However, storage for six months or longer at −20 °C results in the appearance of multiple minor bands and diminution of major bands, so that the assignment of ApoE phenotype becomes unreliable (Figure 2). Deterioration of the sample is exacerbated further by repeated freezing and thawing. The delipidated protein pellet can be stored for at least six weeks (−20 °C) without deterioration before solubilization for electrofocusing (not shown).

Solubilization buffer: We systematically investigated the composition of the solubilization buffer. Including the detergent decyl sodium sulfate (10 g/L) in the solubilizing buffer, as used by Menzel and Utermann (14), greatly increased the intensity of the ApoE bands. Other detergents, found to be less satisfactory, were dodecyl sodium sulfate, octyl sodium sulfate, octylglucoside, Nonidet P40, and cholic acid sulfobetaine. Mercaptoethanol and dithiothreitol were equally effective at reducing the sulfhydryl groups of cysteine, but the latter is preferred because of the stench and toxicity of mercaptoethanol. Redissolved specimens can be kept for up to three days at 4 °C without deterioration. However, storage of dissolved samples for more than three days resulted in subsidiary bands being produced from ApoE₂ and ApoE₂, probably because of inactivation of the sulfhydryl reducing agent. Other workers have reported extra bands being produced by carbamylation with urea solutions (15). This was not a significant problem with "Aristar"-grade urea.

Immunoblotting: PVDF and nitrocellulose were both satisfactory blotting membranes but PVDF was preferred because it gave lower background staining and was less fragile to handle. After blotting with either membrane, protein staining of the blotted agarose gel demonstrated almost complete transfer of protein to the blotting membrane (not shown).

The best antibody probe was the combination of mouse monoclonal antibody and Bio-Red's rabbit anti-mouse IgG-peroxidase conjugate. Other anti-ApoE antibodies assessed were goat polyclonal antibody, as used by Havekes et al. (16), and a sheep polyclonal antibody. Both gave good results, although with slightly increased background staining. The dilute solutions of both first and second antibodies can be re-used at least twice over a period of two weeks, if stored at 4 °C.

The addition of Tween 20 to the incubation buffers reduces the nonspecific protein binding but it also reduces specific antigen–antibody bonds. The best compromise was to omit Tween from the protein-blocking step and the first-antibody incubation but to include it in the washes and the second-antibody incubation.

Cysteamine treatment: The effect of cysteamine treatment on ApoE is shown in Figure 3. Cysteamine specifically reacts with cysteine residues to introduce an amino group, thereby increasing the net charge of the protein by one positive charge for each cysteine residue (19). The result is that E₂ (two cysteine residues) and E₄ (one cysteine residue) both focus in the same position as E₄ (two arginine residues). Cysteamine treatment can be applied to samples that have already been treated with sialidase. We dissolved the cysteamine hydrochloride in 0.1 mol/L Tris base (pH 10.3), which brings the pH of the mixture of serum plus acetate buffer above 8, facilitating the cysteamine reaction. The effect of cysteamine confirms that the observed charge differences are due to Arg-Cys substitutions; we do not use it routinely for determination of the ApoE phenotype.

Discussion

This new method for ApoE phenotyping has several advantages over established methods. The sample volume of serum required (10 μL) is a distinct advantage in comparison with ultracentrifugation and chemical precipitation methods, both of which require at least 1 mL. Sialidase treatment of all samples removes the sialated forms of ApoE, thereby greatly simplifying interpretation. For instance, previous workers had difficulty in distinguishing the E₂E₂ phenotype from the E₂E₄ phenotype when there was a considerable proportion of sialated E₄, which focused in the E₂ position (15). The removal of these additional bands has allowed unambiguous assignment of phenotype in all patients (over 500) so far tested.

The delipidation protocol used for this method is simple. All steps can be carried out at room temperature with a small bench-top centrifuge. The delipidating solvents are similar to those used by Havekes et al. (15), except that the modification of incorporating one part of diethyl ether into the chloroform–methanol mixture results in a more compact pellet after centrifugation. The use of peroxide-free distilled ether is not necessary.

The use of agarose gels, combined with blotting by pressure alone, is faster and simpler than polycrylamide gels, which require electroblotting. This horizontal electrofocusing system allows two gels, each with 40 samples, to be run simultaneously from one power pack. A large sample throughput would be of particular value in population studies.

Fig. 2. Immunoblots from six samples P(3/2), Q(3/3), R(3/3), S(4/4), T(4/3), U(4/4): (A) analyzed fresh, (B) after six months storage at −50 °C, (C) after six months storage at −20 °C, and (D) after six months storage at −20 °C plus six freeze–thaw cycles
The sample deterioration is most marked for samples S and T. Cathode at top

Fig. 3. Immunoblots of the six major ApoE phenotypes: (A) untreated, (B) after cysteamine treatment, (C) after sialidase plus cysteamine treatment
Cathode at top
In conclusion, this is a simplified and improved method for determination of ApoE phenotype. The only equipment required for this method is a horizontal electrofocusing system, a low-speed centrifuge, and a horizontal shaker. It can be performed in non-specialized laboratories and is particularly suited for processing large numbers of samples.

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References