Rapid apolipoprotein E phenotyping by immunofixation in agarose

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Summary Conventional determination of apolipoprotein E isomorphs comprises ultracentrifugation of 1–5 ml serum, delipidation of very low density lipoproteins (VLDL), and isoelectric focusing (IEF) in polyacrylamide gels. In order to reduce the sample volume and to avoid nonspecific protein bands, immunoblotting was proposed. Now we describe a methodological variant that uses 25 μl serum, replaces ultracentrifugation by precipitation of apoE-containing lipoproteins with polyethylene glycol, and delipidation by dissolution in detergent. IEF is carried out in agarose. This allows specific immunofixation of apoE-containing bands with 10 μl antiserum per sample. This method yields apoE patterns that are specific and well resolved. Also, it offers considerable savings of time and equipment involved.—**Luley, C., M. W. Baumstark, and H. Weiland.** Rapid apolipoprotein E phenotyping by immunofixation in agarose. *J. Lipid Res.* 1991. **32:** 880–883.

Supplementary key words very low density lipoprotein • isoelectric focusing

Hyperlipoproteinemia type III is strongly genetically determined and is associated with a high risk of premature atherosclerosis. An early diagnosis is necessary for both the patient and family members. The diagnosis, however, is difficult. Conventionally, it is based on the observation of a broad β-band in lipoprotein electrophoresis, on VLDL migrating in the β-position, and on an elevated cholesterol to triglyceride ratio in VLDL, respectively. These observations, however, lack both sensitivity and specificity. The most frequent prerequisite for the development of type III hyperlipoproteinemia is the apolipoprotein E2/2 homozygosity which is a necessary although not sufficient condition for this disease. Unfortunately, the methodology for determination of apoE isomorphs is somewhat complicated and time-consuming and is, therefore, carried out only in few research laboratories. It comprises several steps such as isolation of VLDL by preparative ultracentrifugation, delipidation of VLDL, and isoelectric focusing (IEF) of VLDL apolipoproteins in polyacrylamide gels. This procedure requires sometimes considerable amounts of serum (1–5 ml) and may still not yield an unequivocal band pattern due to other protein bands in the apoE region. In order to overcome both problems, immunoblotting has been proposed by several investigators (1–3) which, however, requires several steps such as transfer, blocking, washing, and reaction with substrate.

We have developed a methodological variant that simplifies several steps of the conventional procedure: *1*) ultracentrifugation is replaced by precipitation of apoE-containing lipoproteins, *2*) delipidation is replaced by dissolution of the precipitate by means of a detergent, *3*) IEF in polyacrylamide gels is replaced by IEF in agarose, which *4*) allows specific immunofixation with antiserum against apoE. This procedure is relatively easy, quick, very specific and requires 25 μl of serum and 10 μl of antiserum per sample.

METHODS

Agarose. Two hundred ml of 2.5% agarose containing 11% sorbitol is prepared in a wide Erlenmeyer flask by gentle boiling for 1 h. Since some electroendosmosis in the gel appeared to be favorable, three different agarose products were mixed as follows: 2 g agarose IEF, 2 g agarose Z, and 1 g agarose EF, respectively, all from Pharmacia, Freiburg, Germany). This agarose solution can be stored at 4°C for several months. If plates are to be prepared, pair-wise portions of 30 ml may be stored in 50-ml Erlenmeyer flasks containing a magnetic stirring bar.

Gel casting. Two IEF plates (124 × 260 mm) are prepared using the LKB Capillary Gel Casting Kit for analytical IEF, following directions in the LKB manual (4). The plates are preheated for 10 to 15 min at 50°C. Meanwhile, 7.25 g of ultrapure urea (Serva, Heidelberg, Germany) is placed in a 50-ml Erlenmeyer flask. The 30-ml agarose portion is boiled for a short period and cooled to 70°C. The addition of the melted agarose to the urea has to be carried out quickly in order to avoid formation of gel clots. Once the urea is dissolved the gelation of the agarose solution is considerably retarded. Subsequently, 2.8 ml of the ampholyte mixture is added, consisting of four ampholytes in equal proportions: 0.7 ml pH 3–10 Servalytes (Serva), 0.7 ml pH 3–10 Pharmalytes (Pharmacia), and 0.7 pH 5–6 of each producer. Also, 60 mg dithiothreitol (Sigma) in 1.8 ml H_2O is added to the mixture which then has a temperature of 40–50°C. Using a prewarmed 20-ml pipette, the agarose is poured between the warm glass plates which are held at an angle of about 45°. The agarose gels when room temperature is reached. In order to prevent drying of the agarose where it is exposed to air, both short sides of the plates are covered with Scotch tape. Plates can be stored in this condition at room temperature for up to 10 days. Storage at 4°C has to be avoided since urea crystals may form in the gel.

Sample preparation. To 25 μl of serum, 50 μl of solution B (Quantolip HDL, Immuno, Heidelberg, Germany) is

Abbreviations: VLDL, very low density lipoprotein; IEF, isoelectric focusing.

added and for a short time vortexed. This solution precipitates VLDL, LDL, and HDL_2. After an incubation period of 10 min, the mixture is centrifuged (3 min, 13,000 rpm) and the supernatant is discarded. In order to remove remainders of serum proteins, the precipitate is redissolved in 25 μl H_2O and the procedure is repeated. After discarding the supernatant, the precipitate is dissolved in 20 μl of the sample buffer. One hundred ml of the sample buffer contains in H_2O: 154 mg dithiothreitol (Sigma), 121 mg Tris (Merck), 5 ml Tween 20 (Sigma). Prior to use, 2.4 g of urea is added to 5 ml of buffer.

Two plastic sheets are used for the subsequent procedure: *1*) the application sheet that is used for the sample application and coverage of the gel during IEF, and *2*) the immunofixation sheet that is used for the subsequent immunofixation of apoE bands. Both consist of hydrophobic plastic sheets (Leitz Klarsichthülle, Nr. 4153, Germany) into which the appropriate slits have been cut by means of a slim surgical knife. The application sheet measures 220 × 95 mm and has along its long side 20 square application wells (4 × 4 mm) that are 6 mm apart from each other and form one long edge. The 20 holes in the immunofixation sheets measure 2 × 40 mm and are 8 mm apart from each other and 50 mm from the cathodic long side of the sheet.

IEF is carried out on a horizontal 2117 Multiphor II Electrophoresis Unit (LKB) chamber which keeps the plate cooled at 8°C. The gel is blotted for 20 sec with an adequately sized filter paper (Whatman No. 1) in order to remove excess moisture. It is then covered with the application sheet and the electrode wicks are placed after being soaked in 0.5 M NaOH (cathode) and 0.5 M H_3PO_4 (anode), respectively. Three μl of sample is applied to each well and focusing is started. The settings of the 2197 power supply (LKB) are: voltage at constant 2000 volts, and current at maximum while power is adjusted in order to yield a starting voltage of 850 volts. After 30 min the moisture in the application holes is removed by means of blotting with filter paper. After 60 min the voltage reaches about 1300 volts and is increased to 1700 volts again using the power adjustment. After another 60 min focusing is stopped, the electrodes and the application sheet are removed, and the gel is again very quickly blotted with filter paper.

The immunofixation sheet is placed onto the gel surface with care being taken to place the slots on the sample lanes. It is recommended that the gel be transferred into a humidity chamber. Thirty μl of an antiserum dilution (1:3 in 0.9% saline) is then pipetted into each slot of the immunofixation sheet. The polyclonal and monospecific antiserum was from Daiichi, Tokyo, Japan. Other commercial antisera can also be used with good results, e.g., from Immuno). The specificity of the antibody used can be tested with good sensitivity by the same method: there must not be additional bands at locations where potential contaminating proteins (apolipoproteins A-I, A-IV, or albumin) could appear. The incubation takes 30 min at room temperature after which the immunofixation sheet is removed. The gel is washed three times for 1 h in 0.9% saline. After drying at room temperature or in a dryer between 37° to 60°C, the gel is stained for 7 min in Serva Blue (*1* g Serva Blue in 500 ml ethanol, *70* ml acetic acid, and 430 ml H_2O) and destained in water–ethanol–acetic acid 7.5 : 2.5 : 1.

Since the formation of cyanate ions in both sample buffer and gel can cause artificial bands by protein carbamylation, this problem was addressed by additional experiments. The formation of cyanate was stimulated by two ways: *1*) in the urea-containing casting gel by preparation at higher temperatures (90°C instead of 70°C: 90°C gel); and *2*) by boiling the urea-containing sample buffer for 10 min (boiled buffer). An apoE3/4 sample was run under following conditions: *1*) under conditions as described above (normal); *2*) with normal sample buffer in the 90°C gel; *3*) in a normal gel with boiled buffer; and *4*) in a normal gel after incubation in boiled buffer for 60 min at 37°C. Only the latter procedure (incubation of the apoE-containing precipitate for 60 min at 37°C in buffer that had been boiled for 10 min) produced a band pattern that differed from the band pattern obtained under normal conditions. ApoE3 and apoE4 bands were weaker and additional bands appeared anodically.

RESULTS

Fig. 1 shows representative patterns of the six common apoE phenotypes. Some precipitated material appears above the application spot in the form of diffuse, smeared bands. This material contains proteins that precipitated nonspecifically during focusing and did not elute during the washing period after immunofixation. The immunofixed apoE bands appear above the smeared region. Since their width is determined by the width of the slot in the immunofixation sheet, they are not as wide as the application spot or the nonspecific proteins below them. The major apoE isomorphs appear as sharp and dominant bands that are well separated from each other. Samples of homozygote carriers of apoE2/2, E3/3, and E4/4 were run in lanes 1, 3, and 5. It can be seen that the major bands are accompanied by two bands that focus towards the anode and are designated ' and ", respectively. These secondary bands are considerably less intense than the bands of the main proteins below.

Samples of individuals being heterozygote for apoE2/3, E3/4, and E2/4 were applied in lanes 2, 4, and 6, respectively. They display major and minor bands according to

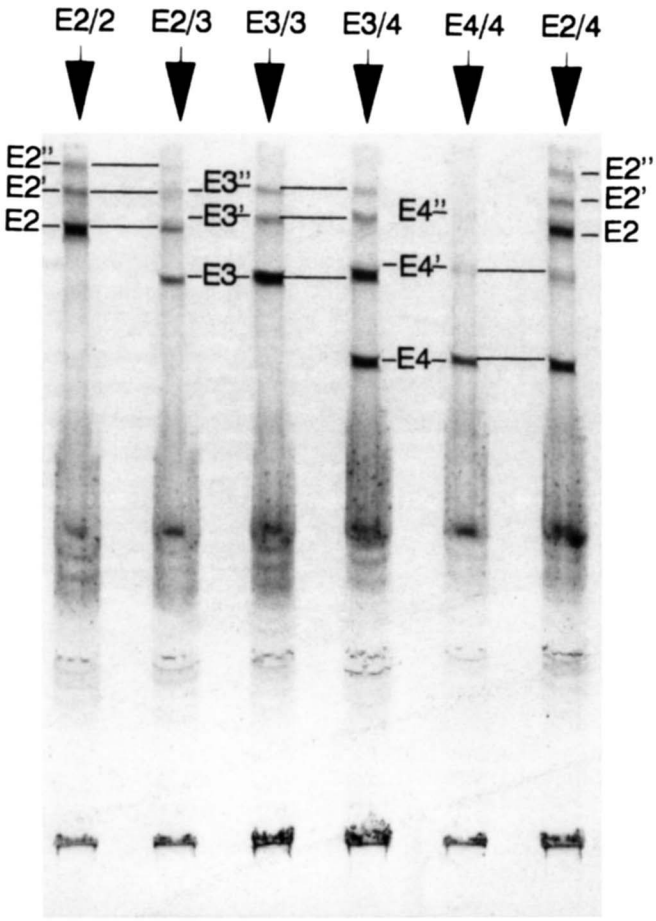

Fig. 1. Immunofixation patterns of apolipoprotein E isomorphs. Samples in lanes 1, 3, and 5 are from three homozygotes for apoE2, E3, and E4, respectively. Samples in lanes 2, 4, and 6 are from heterozygotes for apoE2/3, E3/4, and E2/4, respectively.

each of the two alleles. In phenotype E2/3 there is some overlapping of the E2 band with the E3' band and, in phenotype E3/4, of E3 with E4'. The ' bands, however, can be distinguished from major bands in their neighborhood by both band position and intensity. For example, E3' focuses more anodically than E2 and is considerably less intensive than E3 while, in phenotype E2/3, both major bands show practically equal intensity. This holds true for the differentiation between E4/4 and E3/4.

DISCUSSION

Rhigetti (5) cited several advantages of agarose over the polyacrylamide medium: *a*) agarose is nontoxic, whereas both acrylamide and N,N'-methylene bisacrylamide reagents are neurotoxic; *b*) agarose gelation does not require catalysts, whereas catalysts in the acrylamide polymerization reaction produce free radicals that can result in artefacts in the gel; *c*) it allows quick and efficient staining and destaining; and of particular importance in this application, *d*) the large pore size of agarose allows subsequent immunofixation.

The presence of urea in both agarose gel and sample buffer raises the question of cyanate formation with subsequent protein carbamylation. Generally, possible cyanate ions in the gel should not cause a problem because they will rapidly be removed as soon as focusing is started. An experiment with an agarose gel that was prepared at 90°C (in order to stimulate cyanate formation) did not show an alteration of the band patterns. Also, using urea-contaning sample buffer (8 molar) enriched with cyanate ions by boiling for 10 min did not lead to any alterations of the pattern. Only incubation of the sample for 60 min at 37°C in the boiled buffer resulted in band shifting towards the anode. We conclude that the presence of cyanate ions in agarose gel or sample buffer does not produce artefacts for this method.

The ampholyte mixture used in our procedure yields a separation of bands that allows identification of the common six apoE phenotypes. Since the apoE2 and the apoE3' bands migrate very close, problems of isotyping may occur for the differentiation of E2/3 from E3/3 phenotypes (6). As pointed out above, this differentiation can be safely done on the basis of band intensities and band position.

In our view, this methodologic variant is an alternative to other procedures for apoE phenotyping. Since ultracentrifugation and delipidation are omitted, there is a major saving of time and equipment involved. Sample preparation, focusing procedure, and immunofixation are easy and can be carried out within 3 h. If two IEF chambers are available, a technician can run 4 × 20 samples each per day. Small sample volumes are required, rendering this method appropriate for pediatric laboratories. Due to the use of a monospecific antiserum, the band identification is sensitive and specific. The required amounts of antiserum are moderate (10 μl per sample).

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