notes on methodology

Genetic studies of human apolipoproteins. V. A novel rapid procedure to screen apolipoprotein E polymorphism

M. I. Kamboh, R. E. Ferrell, and B. Kottke*

Human Genetics Division, Department of Biostatistics, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA 15261, and Division of Cardiovascular Diseases, Mayo Clinic and Mayo Foundation, Rochester, MN 55905*

Summary A simple and new method has been developed to detect apolipoprotein E polymorphism directly from plasma or serum without prior ultracentrifugation and delipidation. The method combines the use of dialyzed plasma or serum samples with or without neuraminidase treatment followed by monodimensional isoelectric focusing in simple or 3 M urea gels at a constant low power and progressively increasing voltage over a 3-hr period, and finally protein blotting to a nitrocellulose membrane. Apolipoprotein E phenotypes are identified immunologically using a double antibody reaction, the primary antibody being a monospecific, polyclonal goat anti-apolipoprotein E, and the secondary antibody being a rabbit anti-goat IgG conjugated with alkaline phosphatase. The method was employed to screen apolipoprotein E polymorphism in two white populations in the United States. The frequency values are comparable to those reported previously by other investigators using conventional detection methods. The procedure is simple, accurate, suitable for large scale epidemiologic, clinical, and genetic studies.—**Kamboh, M. I., R. E. Ferrell, and B. Kottke.** Genetic studies of human apolipoproteins. V. A novel rapid procedure to screen apolipoprotein E polymorphism. *J. Lipid Res.* 1988. **29:** 1535–1543.

Supplementary key words isoelectric focusing • immunoblotting

Apolipoprotein E (apoE) is a monomeric glycoprotein with 299 amino acid residues and a 34,145 dalton molecular mass (1). The protein is synthesized as preapoE with 317 amino acids containing an 18-residue signal peptide that is cleaved cotranslationally (2). In human plasma apoE is a normal constituent of very low density lipoproteins (VLDL), high density lipoproteins (HDL), and chylomicron remnants (3). ApoE is synthesized in hepatic and extrahepatic tissues (4). The gene for apoE has been mapped to chromosome 19 in a cluster with the apoC-I and C-II genes (5). It plays an important role in lipid metabolism and appears to be involved in the cholesterol transport between different tissues. ApoE acts as a ligand for two receptors, apoE or remnant receptor, which is expressed exclusively on hepatic cells, and the LDL (apoB/E) receptor which is found in most tissues (6). ApoE therefore mediates the uptake of apoE-containing lipoprotein particles by receptor mediated endocytosis.

ApoE exhibits genetically determined structural polymorphism using isoelectric focusing (IEF) or two-dimensional (2-D) gel electrophoresis which detect the products of three common and several rare alleles (7–9). The three common alleles designated *APOE*2, E*3,* and *E*4* control the expression of six phenotypes, E2/2, 3/3, 4/4 homozygotes, and E3/2, 4/2, and 4/3 heterozygotes. The products of these three alleles differ from each other by a single amino acid substitution (10, 11). ApoE2 differs from the common E3 isoform by the replacement of arginine by cysteine at position 158 and E4 differs from E3 by the replacement of cysteine by arginine at position 112. Several other less frequent apoE variants have been reported (8). The apoE protein polymorphism shows profound effects on lipid metabolism. ApoE2 and E4 allele products are functionally different from the most common E3 form. The apoE2 variant binds defectively to its receptors and results in reduced in vivo catabolism of triglyceride-rich remnant particles (3, 12). ApoE4 shows an increased in vivo catabolism and enhanced clearance of these particles (13). In the general population the *APOE*2* allele is found to be associated with lower levels of LDL and increased triglyceride levels whereas the *APOE*4* allele appears to be involved in a similar but opposite stepwise gene–dosage effect by elevating LDL levels (12–14). ApoE polymorphism is also associated with type III hyperlipoproteinemia. About 90% of the patients with this disorder have the homozygote phenotype E2/2 (15). However, only a fraction of the E2/2 homozygote individuals in the population develop type III hyperlipoproteinemia. This implies that, in addition to genotype at the apoE structural locus, the development of type III hyperlipoproteinemia requires the presence of another gene or genes, or a combination of apoE genotype and some specific environmental factor.

Despite the striking association of apoE polymorphism with dyslipoproteinemia and its gene dosage effect on lipid levels, the screening of apoE protein poly-

Abbreviations: IEF, isoelectric focusing; apo, apolipoprotein; TBS, Tris-buffered saline; VLDL, very low density lipoproteins; 2-D, two-dimensional; HDL, high density lipoproteins; LDL, low density lipoproteins.

morphism has been limited due to the tedious nature of the experimental procedure. Conventionally, apoE genetic heterogeneity has been determined by isolation of apoVLDL by prolonged ultracentrifugation and delipidation followed by IEF or 2-D electrophoresis and protein staining. Recently three studies (16–18) have described relatively simple immunoblotting methods for apoE phenotyping by eliminating the ultracentrifugation step. However, these methods still require serum delipidation (16) or serum treatment with guanidine–HCl (17) to dissociate apoE from lipoproteins, or charge-shift electrophoresis of hydrophobic serum proteins (18) followed by prolonged IEF. In the present study, we report the simple and rapid IEF procedure followed by immunoblotting to screen apoE polymorphism directly from plasma or serum without prior ultracentrifugation and delipidation or charge-shift electrophoresis.

MATERIALS AND METHODS

Materials

Plasma or serum samples from unrelated and healthy U.S. white subjects were available in the Human Genetics Laboratory, Graduate School of Public Health, Pittsburgh, PA. EDTA plasma was stored at −20° C or −70° C for various lengths of time ranging from 1 month to 3 years. Two sets of control plasma samples of the six common apoE phenotypes were obtained from Dr. C. F. Sing (University of Michigan, Ann Arbor) and Dr. Jean Davignon (Clinical Research Institute, Montreal). Acrylamide and N,N′-methylenebisacrylamide were purchased from Bio-Rad laboratories (Richmond, CA). Pharmalytes pH 4.5–5.4 and pH 5–8 were obtained from Pharmacia (Uppsala, Sweden) and Ampholine pH 5–7 from LKB (Bromma, Sweden). Nitrocellulose was obtained from Schleicher and Schuell (Keene, NH). Goat-anti-human apoE antiserum was purchased from Daiichi Chemicals (Tokyo, Japan) and alkaline phosphatase-conjugated rabbit-anti-goat IgG was from Pel-Freeze (Rogers, AR) or Atlantic Antibodies (Scarborough, ME). *Clostridium perfringens* type V neuraminidase and ultrapure urea were obtained from Sigma (St. Louis, MO) and Bethesda Research Laboratories (Gaithersburg, MD), respectively. All other chemicals were reagent grade.

Dialysis and neuraminidase treatment

Plasma or serum samples (20 μl) were either dialyzed directly in their native form or simultaneously dialyzed and desialylated by the addition of 10 μl of neuraminidase solution (1 U/28 μl 0.2 M disodium ethylenediaminetetraacetic acid, pH 7.2). Simple dialysis or desialylation-dialysis was carried out overnight at room temperature in a continuous flow microdialysis system (Model 1200, Bethesda Research Laboratories). The dialysis buffer was prepared by dissolving 20.9 g monobasic sodium phosphate, 16.55 g dibasic sodium phosphate, and 6.25 g ethylenediamine tetracetic acid, tetrasodium salt, in 1 liter of deionized water. The stock buffer was diluted fourfold with deionized water to give a final pH 6.8 before use.

Flat bed polyacrylamide gels and isoelectric focusing

The polyacrylamide gels of 5% by weight (monomer 4.85%; bis 0.15%) containing 3 M urea were prepared as follows: 5.4 g urea was dissolved in 5 ml of monomer solution (29.1% w/v) and 5 ml of bis acrylamide solution (0.9% w/v) and the final volume was adjusted to 30 ml with deionized water. The pH gradient was established with either 1.5 ml LKB ampholine pH 5–7 (2%) or with the combination of 0.45 ml Pharmalyte pH 4.5–5.4 (0.68%) and 0.9 ml Pharmalyte pH 5–8 (1.35%). Twenty μl of the polymerization solution, (0.1% riboflavin) was added directly to the acrylamide solution and the mixture, enough for two gels, was immediately poured between two glass plates (122 × 260 mm) separated by a 0.5-mm plastic gasket. The polymerization process was allowed to continue overnight by exposing the gel sandwich to fluorescent light. Similarly, polyacrylamide gels of 6.5% (monomer 6.305%; bis 0.195%) in 10.95% sucrose were prepared either covering the pH range 5–7 or a combination of pH 4.5–5.4 and 5–8 as described above.

Plasma samples absorbed on 5 × 7 mm Whatman 3 MM filter paper wicks were applied 0.5 cm from the catholyte end on the long side of the gel. The long catholyte and anolyte wicks were saturated with 1 M NaOH and 1 M H_3PO_4, respectively, using 6 mm × 25 cm electrode strips. The gel was placed on an LKB 2217 Ultrophor electrofocusing unit connected with a Lauda RM6 cooling unit operating at 8° C and an LKB power supply at 250 mA maximum current and 10 W constant power. At these conditions, the voltage increased progressively from initial average readings of about 600 V to the final readings of about 1900 V. After 30–60 min focusing, the sample wicks were removed and the cathode electrode wick was blotted with filter paper. The total isoelectric focusing time was 3 hr. The initial and final voltage readings varied slightly from gel to gel.

Immunoblotting

After the completion of isoelectric focusing, the gel was rinsed briefly with TBS buffer (0.25 M NaCl, 0.03

M Tris-HCl, pH 8.0) followed by protein transfer onto 0.2-μm pore size nitrocellulose membrane by simple diffusion (19); the gel–nitrocellulose contact was for 60–75 min at room temperature. After protein transfer, the filter was removed carefully from the gel and rinsed in TBS buffer, followed by 60 min incubation with 5% (w/v) nonfat dry milk dissolved in deionized water to saturate any remaining protein binding sites. The filter was then exposed for 90 min to goat-anti-human apoE polyclonal antiserum (Daiichi Chemical Co.) diluted 1:750 in TBS buffer followed by three 10-min washes in TBS. The washed filter was then incubated in a second antibody, rabbit-anti-goat IgG conjugated with the enzyme alkaline phosphatase, for 90 min in TBS at 1:5000 dilution. Subsequently the filter was washed thrice in TBS and eventually stained histochemically using 25 mg β-naphthyl phosphate, 25 mg Fast Blue BB salt and 60 mg magnesium sulfate in 50 ml stock buffer (1.8 g NaOH, 3.7 g boric acid/l).

RESULTS

Immunolocalized isoelectric focusing patterns of apoE phenotypes are shown in Figs. 1–3. The monospecificity of the polyclonal apoE antiserum was predetermined using antisera from all other apolipoproteins which showed no cross reactivity. The accuracy of the phenotype assignments was checked by running plasma samples of control apoE phenotypes, 3/3, 4/3, 4/2, 2/2, 4/4, and 3/2 that had previously been typed using mono- or two-dimensional gel electrophoresis of ultracentrifugated and delipidated plasma samples. The assignment of each apoE phenotype was, therefore, based on a direct comparison with the isoprotein bands of control phenotypes. By comparison to the reported patterns, our technique yielded superior resolution between the different apoE phenotypes which are easily recognizable based on their different banding patterns. **Fig. 1** illustrates the banding patterns of

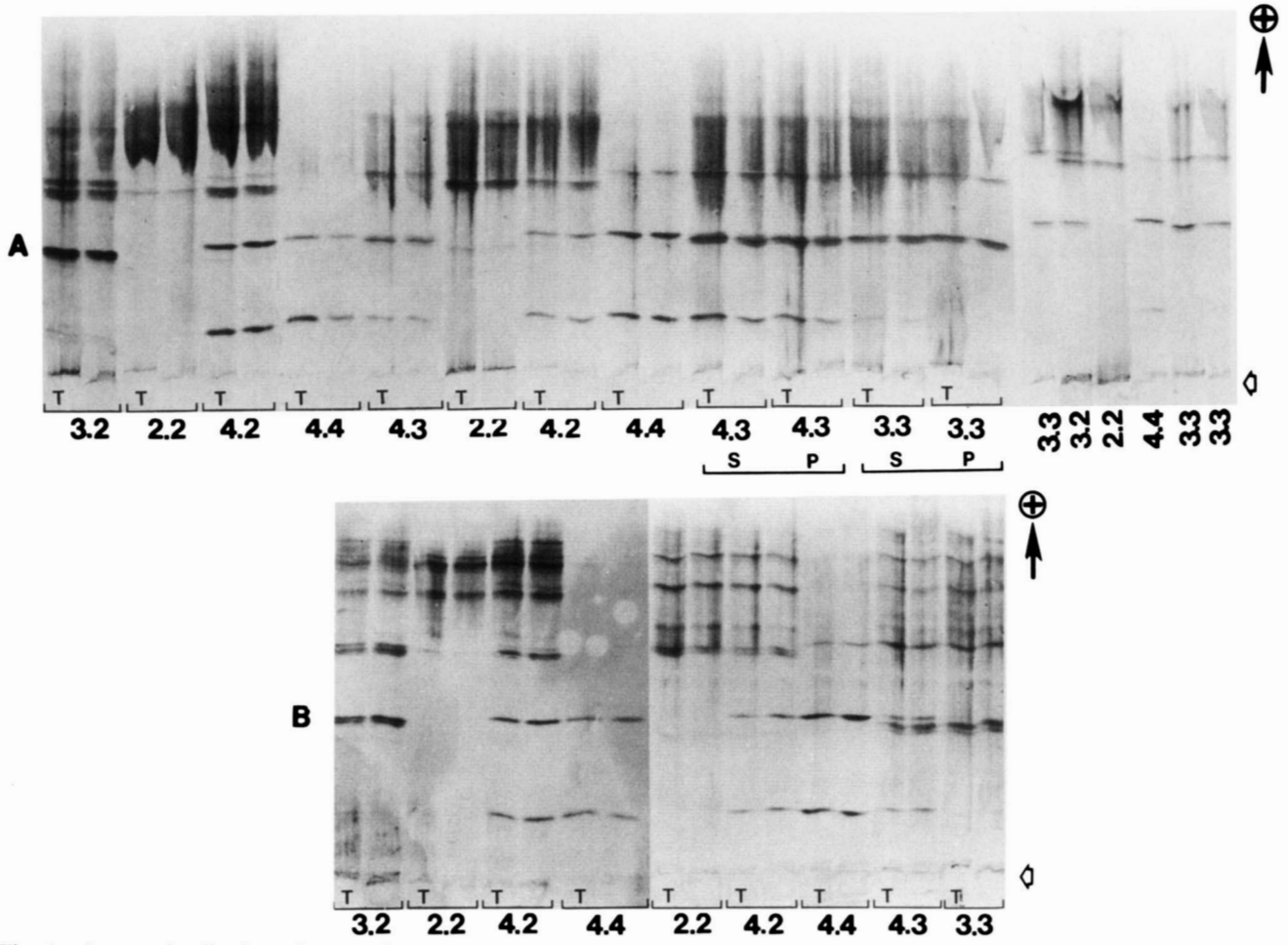

Fig. 1. Immunolocalization of apoE phenotypes in 6.5% polyacrylamide isoelectric focusing gels containing sucrose: (A) pH range combination of 4.5–5.4 and 5–8; (B) pH 5–7. Identical plasma or serum samples were dialyzed with and without neuraminidase treatment. Simple dialyzed samples are to the right of the neuraminidase-treated (T) samples. The last six samples in (A) were individually dialyzed without the neuraminidase treatment. Plasma (P) and serum (S) samples from two different individuals with E3/3 and E4/3 phenotypes were tested, and they gave identical patterns for each phenotype. ApoE phenotypes are indicated beneath the samples. The sample application area is indicated by an open arrow at the bottom of (A) and (B). Note that in these gels the banding patterns of neuraminidase-treated and untreated samples are indistinguishable.

six different apoE phenotypes obtained on two different pH range gels containing sucrose as an additive. Both the native dialyzed samples and samples treated with neuraminidase gave similar patterns in these types of IEF gels. A relatively clear and simple pattern was obtained in pH 4.5–8 gels (Fig. 1A) as compared to pH 5–7 gels which showed several additional acidic isoforms (Fig. 1B). In sucrose-containing gels, the E3/3 phenotype is characterized by two major bands. Similarly, the E4/4 phenotype is composed of two major isoproteins, but their positions are clearly cathodal and quite different to those observed in plasma from E3/3 homozygotes. A similar but more anodally focused pattern was identified as an E2/2 homozygote. The heterozygote phenotypes, where two of the three primary patterns focused together in three different combinations to give rise to E3/2, 4/2, and 4/3 phenotypes, could easily be identified. **Fig. 2** shows the comparison of apoE phenotypes using a hybrid pH range 4.5–5-4 and 5–8 (Fig. 2A) and pH 5–7 (Fig. 2B) under partial denaturing conditions in 3 M area. By comparison to Fig. 1, the anodal sialylated peptides

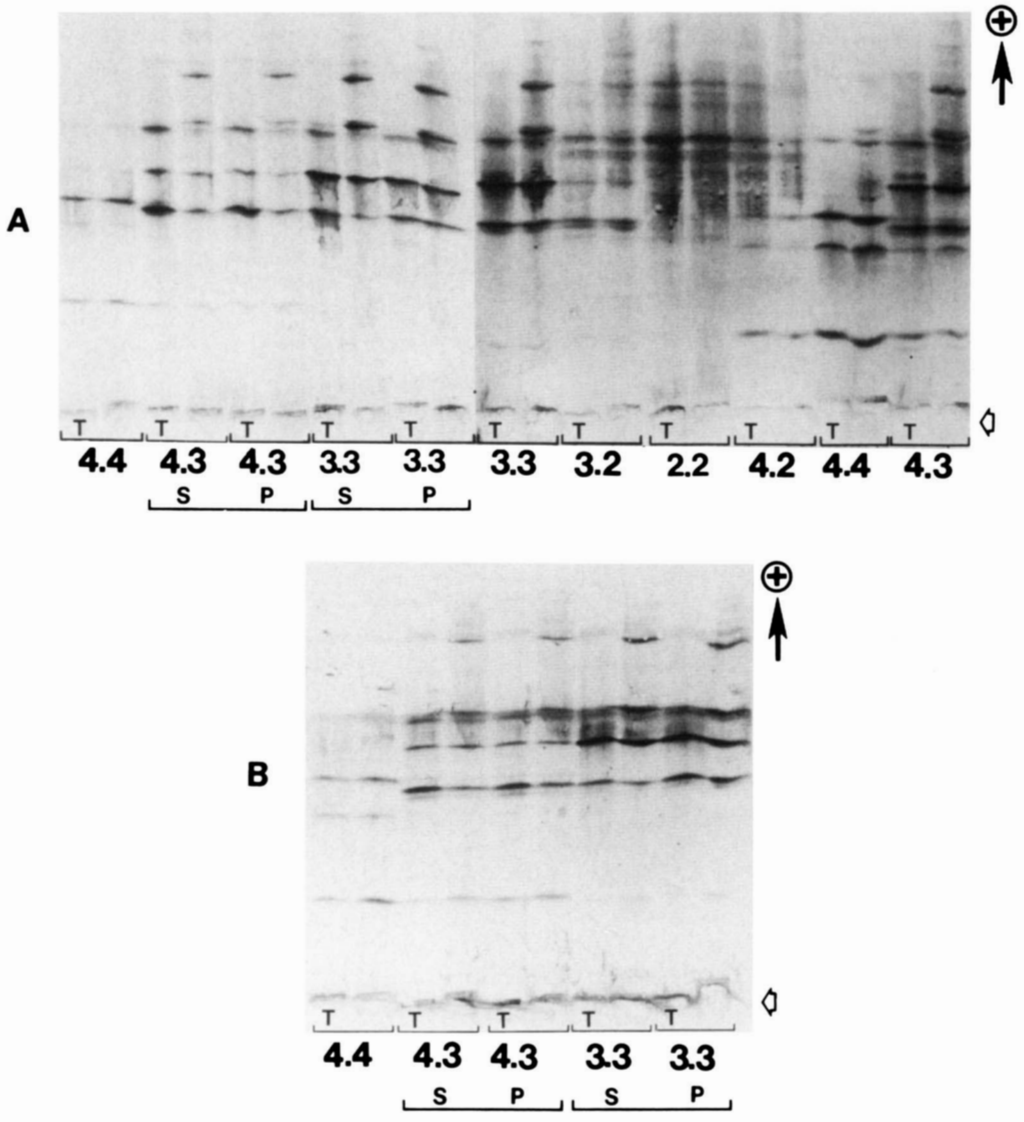

Fig. 2. Immunolocalization of apoE phenotypes in 5% polyacrylamide isoelectric focusing gels containing 3 M urea: (A) pH range combination of 4.5–5.4 and 5–8; (B) pH 5–7. Identical plasma or serum samples were dialyzed with and without neuraminidase treatment. Simple dialyzed samples are to the right of the neuraminidase-treated (T) samples. Plasma (P) and serum (S) samples from two different individuals with E3/3 and E4/3 phenotypes were tested, and gave identical patterns for each phenotype in (A) and (B) gels. ApoE phenotypes are indicated beneath the samples. The sample application area is indicated by an open arrow at the bottom of (A) and (B). Note that by comparison to Fig. 1, the sialylated isoforms are apparent in 3 M urea gels. Furthermore, these sialylated isoforms are more clearly resolved in pH range 4.5–8 (A) than in pH 5–7 gel (B).

are more clearly distinguished in 3 M urea gels which also demonstrate three major bands for the corresponding homozygous phenotypes observed in sucrose gels. The major difference between the simple and urea gels is the detection of the middle major band as well as clear anodal background in the gels containing 3 M urea. By comparison, simple IEF gels demonstrate clear background in the region consisting of major bands and therefore also enable apoE classification without any difficulty. In Fig. 2, asialo and sialo bands are more resolved in pH range 4.5–8 than in pH range 5–7. Identical patterns were observed in plasma and serum samples from the same individual as shown in Fig. 2. **Fig. 3** shows different apoE phenotypes of neuraminidase-treated samples in pH 4.5–8 and pH 5–7 gels containing 3 M urea. For better understanding, the major banding patterns of six apoE phenotypes obtained under partial denaturing conditions in 3 M urea gels of dialyzed or neuraminidase-treated samples (Figs. 2 and 3) are shown diagrammatically in **Fig. 4.** The major bands that correspond to Figs. 2 and 3 are labeled from **a** to **h** in the order of increasing isoelectric points. The E2/2 phenotype is characterized by having a, b, and c bands; the E3/3 phenotype has b, d, and f bands; and the E4/4 phenotype has e, g, and h bands. The heterozygote phenotypes consist of six major bands, except for the 3/2 phenotype which is composed of five major bands because the b band is shared by E2 and E3. It is important to note in Figs. 2 and 3 that the middle band of the apoE4 type is not present with the same intensity in all E4 carrier samples. The reason for this quantitative shift is not clear. Under our experimental conditions identical major patterns were observed in both fresh and stored samples. However, stored samples of the E3 phenotype tend to show additional minor bands. In a few such

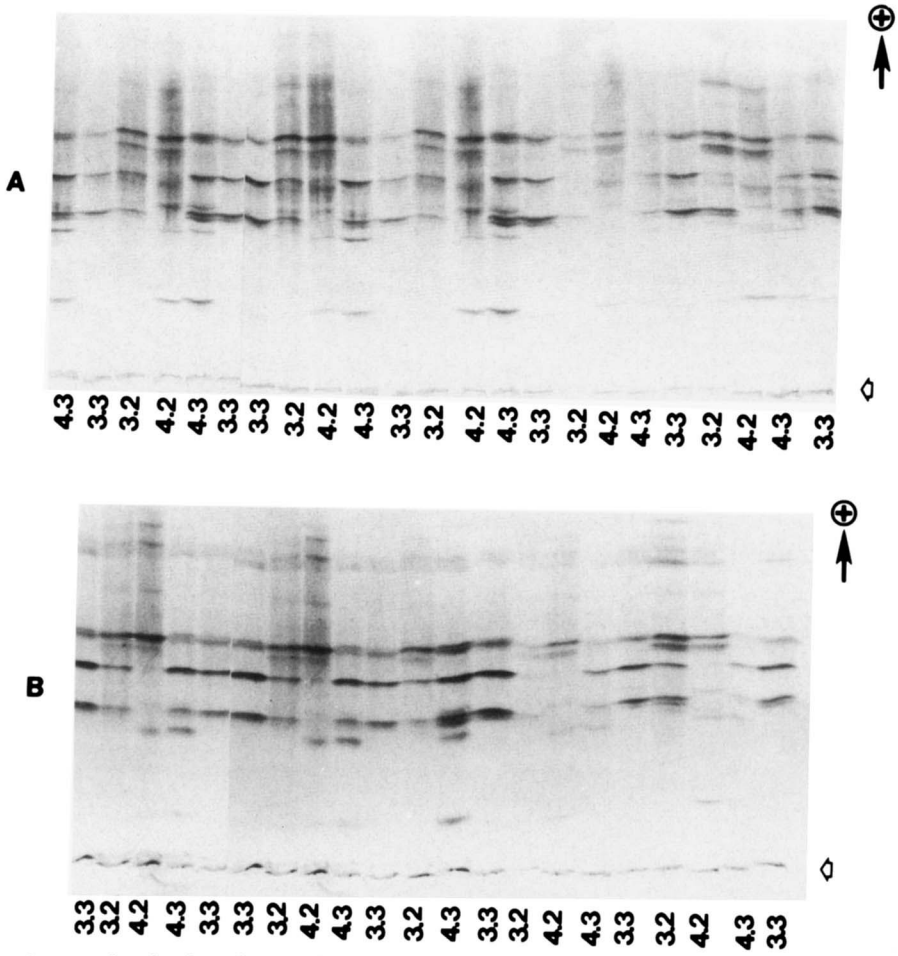

Fig. 3. Immunolocalization of apoE phenotypes in 5% polyacrylamide isoelectric focusing gels containing 3 M urea; (A) pH range combination of 4.5–5.4 and 5–8; (B) pH 5–7. All plasma or serum samples were dialyzed with the addition of neuraminidase. ApoE phenotypes are indicated beneath each sample track. The sample application area is indicated by an open arrow at the bottom of (A) and (B). Note that in (A) and (B) the phenotypic patterns in the 6th and 7th lanes from the right are light. This probably reflects the low level of apoE in these two individuals. However, on the actual immunoblot these patterns were strong enough to classify them reliably.

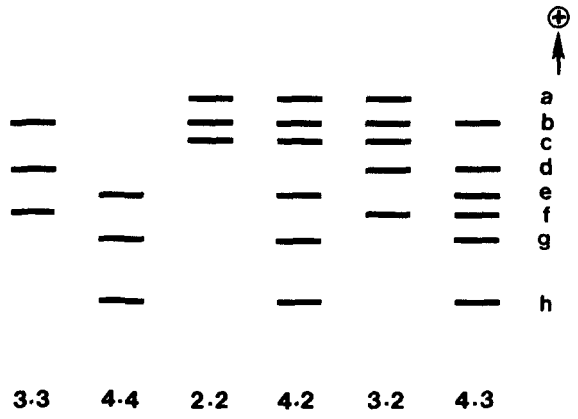

Fig. 4. Diagrammatic representation of major banding patterns of six apoE phenotypes obtained on 5% polyacrylamide isoelectric focusing gels containing 3 M urea, pH range 4.5–8.

samples, the middle band of apoE3 was comparatively weaker or missing altogether. Despite these additional minor components and quantitative shift, no major problem was encountered in scoring the exact phenotypes.

The validity of this simple procedure was tested in family material (data not shown), which agreed with previous reports of its autosomal codominant inheritance. Furthermore, a large number of unrelated individuals from two U.S. white populations were tested for apoE phenotypes. **Table 1** shows apoE phenotypes and allele frequencies in these two populations. The observed and expected values based on Hardy-Weinberg equilibrium showed an excellent agreement. The remarkable similar allele frequencies in two U.S. white populations and their similarity to other mixed Caucasian populations provide further support in favor of the specificity and accuracy of this proposed method. The data generated in Table 1 were obtained simultaneously by using all different conditions described above (i.e., IEF in sucrose and 3 M urea gels of digested and undigested plasma samples). The classification of various apoE phenotypes based on these systems was identical. However, as illustrated in Figs. 1–3 and mentioned above, variable glycosylation and major bands were observed in the sucrose and 3 M urea gels. We recommend the use of simple dialysis and 3 M urea gels as the sialo bands are more apparent in this system and this may be useful in the identification of rare APOE alleles and in characterizing posttranslational modifications in the apoE molecule.

DISCUSSION

ApoE plays a pivotal role in lipoprotein metabolism, and genetically controlled structural variation in this molecule appears to influence lipid concentrations in the general population (12, 14, 20). In order to evaluate in depth the effect of APOE genotypes on lipoprotein metabolism in ethnically and environmentally diverse populations, we need a simple, rapid, and accurate method for large-scale screening. We believe the method presented here is the simplest and most reproducible method as compared to those reported previously. It provides an opportunity to extend our observation on this already established gene locus, which alone contributes about one-sixth of the total variation in total plasma and LDL cholesterol levels (20, 21).

In order to develop a simple and rapid monodimensional isoelectric focusing method to screen apoE polymorphism directly from plasma or serum, we had to test and optimize several experimental variables. The conventional use of 6–8 M urea, which is normally incorporated in apolipoprotein gel systems, yielded monomorphic apoE patterns when whole plasma was subjected to isoelectric focusing over a single ampholine range (e.g., pH 4–6.5 or pH 5–7). Reduction of the urea concentration to 3 M, selection

TABLE 1. ApoE phenotype and allele frequencies in two white populations in the United States

	Rochester		Utah		Combined	
Phenotype	Obs.	(Exp.)	Obs.	(Exp.)	Obs.	(Exp.)
E3/3	127	(126.62)	47	(48.27)	174	(174.61)
E4/3	48	(46.19)	20	(18.51)	68	(64.92)
E3/2	21	(23.58)	11	(10.00)	32	(33.58)
E4/2	7	(4.30)	2	(1.92)	9	(6.24)
E4/4	2	(4.21)	1	(1.78)	3	(6.03)
E2/2	1	(1.10)	0	(0.52)	1	(1.62)
Total	206		81		287	
Allele frequencies						
*APOE*2*	0.073		0.080		0.075	
*APOE*3*	0.784		0.772		0.780	
*APOE*4*	0.143		0.148		0.145	

of a hybrid ampholine range, and maintaining a low voltage gradient did affect the overall pattern of apoE and made it possible to demonstrate interindividual variation in plasma samples. However, high, nonspecific background staining observed on the immunoblot made it difficult to reliably assess the resulting apoE phenotypes. This problem was overcome by simple dialysis or neuraminidase treatment of plasma samples, which significantly reduced the staining background and produced a simpler, more discrete isoprotein pattern. In early experiments neuraminidase treatment was thought to be necessary. However, in later studies this step was replaced by simple dialysis (data not shown) which is cost-effective and, like neuraminidase-treated samples, provides unequivocal classification of various apoE phenotypes. The important aspects of the present method are as follows: i) simple dialysis of plasma or serum samples in a dialysis apparatus; ii) IEF under simple or partial denaturing conditions containing 3 M urea; iii) 1:2 mixture of carrier ampholines pH 4.5–5.4 and pH 5–8; and iv) constant low power (10 W) with progressively increasing voltage over 3 hr, rather than fixing the voltage at a constant reading. For routine apoE typing, we recommend the use of either simple or 3 M urea gels. However, if some difficulties are encountered due to sample conditions, either gel system can be supplemented by the another. The only example we observed was one case of the 2/2 homozygous phenotype, whose plasma sample gave a strong background in 3 M urea gels (Fig. 2A). However, when the same sample was run on sucrose gels, the pattern was very clear (Fig. 1A). According to our experience, the best results were obtained using the mixture of pH 4.5–5.4 and pH 5–8 ampholines. Other combinations of carrier ampholines were tested but the results were not consistent, and especially when using the mixture of pH 4–6.5 and pH 5–8 ampholines, the product of E4 was hardly detectable. Using a single carrier ampholine, the best patterns were detected in pH 5–7 range gel. However, in the pH 5–7 range gel, variable banding intensity was observed in the E3/2 phenotype and this made its reliable classification difficult. We assume these phenomena occurred because of conductivity gaps and poor buffering capacity in different commercial carrier ampholines due to their different physiochemical composition. Therefore, one has to be very careful in choosing the appropriate concentration, pH range, and the commercial source. In recent years many useful oligoamines have been excluded in the preparation of carrier ampholines due to their carcinogenic properties (22). Because of this, most commercial carrier ampholines show conductivity gaps in and around the pH 6 region, which ultimately causes poor buffering capacity. This leads to the highest potential drop in this particular pH region, and underfocusing in other parts of the gradient. Under high voltage conditions, the pH 6 and neighboring areas are subject to localized heating which distorts the pH gradient and has a deleterious affect on heat-labile proteins. In this context Bier, Mosher, and Palusinski (23) have emphasized the use of extremely low voltage to obtain a useful pH gradient in simple mixtures of ampholines. A similar mechanism may occur in the separation of apoE isoforms that focus around isoelectric point (pI) 6. In its native form, at high voltage the resolution of apoE isoforms appears to be affected by the nonuniform distribution of ampholines which create conductivity gaps and prevent the passage of protein ions. In our experience, this phenomenon is not unique to apoE and has been observed while separating other proteins having pI values between 5 and 6.5 on IEF gels (24). The other important consideration of this method is the selection of the optimum concentration of urea to detect different apoE phenotypes. Previously apoE heterogeneity has been screened using high concentration of urea from delipidated apoVLDL. However, under our experimental conditions, the effect of high concentration of urea on the native apoE molecule in plasma is profound and appears to be directly related to changes in configuration that modify its net charge.

To date, our method is the simplest and most economical for detection of the apoE polymorphism directly from plasma or serum without the prior ultracentrifugation and delipidation steps. Recently two methods have been described (16–17) to determine apoE phenotypes without purification of apoE by ultracentrifugation. These methods still require several steps to dissociate apoE from lipoprotein lipids either by delipidation by organic extraction or treatment with guanidine–HCl. Another recently described method of apoE typing from whole plasma requires extensive pretreatment of plasma samples with DTT, nonionic, and cationic detergents followed by both the charge-shift electrophoresis and IEF (18). The present method eliminates the need for sample delipidation, requires only a few microliters of plasma, and up to 45 samples can easily be applied on a single gel. The significance of this method is the reliable classification of different apoE phenotypes that can easily be categorized based on their different banding positions rather than relying on intensity ratios of various bands in heterozygote phenotypes. Possibilities of misclassification have been noted previously using other methods of detection. For example, quantitative shifts in bands may cause problems of discriminating between phenotypes E4/4 and 4/3 as well as between E3/3 and 3/2. Such possibilities of false scoring of phenotypes have been circumvented by the development of this new procedure because both the homozygote and het-

erozygote phenotypes are clearly recognizable based upon their different focusing positions.

Our method gives multiple banding patterns for each homozygote state; these are comparable to some reported patterns and differ from others in which only one major band in each homozygote phenotype was found. The differences in the reported focusing bands appear to be variations in the purification of apoVLDL and delipidation steps (18, 25). Since we are not using delipidated plasma samples, it is likely that some of the intraindividual variation we observe upon IEF is due to interaction between apoE (or ampholines) and lipid components of the plasma. However, the phenotypic patterns are not influenced by the broad range of lipoprotein levels included in the random sample of 287 individuals reported here. In addition to the random sample used to validate the method, we have typed a variety of samples from patients with clinically defined hyperlipemia (data not shown), and the apoE phenotypes observed are qualitatively identical to those obtained from normolipemic samples. Another possibility for multiple patterns could be the preservation of the terminal sialic acid content of apoE in its native form in whole plasma as compared to the relative and preferential loss of carbohydrate moiety as a result of the ultracentrifugation and delipidation processes. The reliability of the present method was examined first by analyzing plasma samples of control apoE phenotypes obtained from two different laboratories and then screening apoE polymorphism in 287 plasma samples from two different populations. The combined observed frequency of the *APOE*2*, *E*3*, and *E*4* alleles are 0.075, 0.780, and 0.145, respectively. These values are comparable to those reported previously in Caucasian whites (7, 12, 20, 21). Recently, using delipidated apoVLDL followed by combination of minigel electrophoresis and laser densitometric scanning, Ordovas et al. (26) screened more than 1200 plasma samples from a U.S. white population and their frequency values for the three alleles (*E*2*, 0.075; *E*3*, 0.786; *E*4*, 0.135) are remarkably similar to our own values. The close resemblance of these results provides further support in favor of the specificity, sensitivity, and accuracy of this new method. The other advantage of this method is that fresh and stored samples give equally good results and frequent thawing and freezing did not affect the major bands. Although we have identified additional minor components in such samples, these do not lead to problems of misclassification. This procedure is also cost-effective and does not require electroblotting, as suggested in the literature.

In conclusion, this procedure should significantly expedite the population screening of the apoE genetic polymorphism and will contribute to a detailed understanding of the genetic mechanism involved in determining interindividual differences in lipid metabolism.

This research was supported in part by N.I.H. grants AM-24799 and HL-39107. We wish to thank Drs. C. F. Sing, Eric Boerwinkle, and J. Davignon for providing plasma samples of known apoE phenotype. We thank Lina D'Amico for preparing the manuscript.

Manuscript received 23 February 1988 and in revised form 10 May 1988.

REFERENCES

1. Rall, S. C., K. H. Weisgraber, and R. W. Mahley. 1982. Human apolipoprotein E. The complete amino acid sequence. *J. Biol. Chem.* **257:** 4171–4178.
2. Zannis, V. I., J. McPherson, G. Goldberger, S. K. Karathanasis, and J. L. Breslow. 1984. Synthesis, intracellular processing, and signal peptide of human apolipoprotein E. *J. Biol. Chem.* **259:** 5495–5499.
3. Mahley, R. W., and T. L. Innerarity. 1983. Lipoprotein receptors and cholesterol homeostasis. *Biochim. Biophys. Acta.* **737:** 197–222.
4. Williams, D. L., P. A. Dowson, T. C. Newman, and L. L. Rudel. 1985. Synthesis of apolipoprotein by peripheral tissues. *Ann. NY Acad. Sci.* **455:** 222–229.
5. Scott, J., T. J. Knott, D. J. Shaw, and J. D. Brook. 1985. Localization of genes encoding apolipoproteins C-I, C-II, and E in the p13 → cen region of human chromosome 19. *Hum. Genet.* **71:** 144–146.
6. Brown, M. S., and J. L. Goldstein. 1985. The receptor model for transport of cholesterol in plasma. *Ann. NY Acad. Sci.* **454:** 178–182.
7. Ehnholm, C., M. Lukka, T. Kuusi, E. Nikkilä, and G. Utermann. 1986. Apolipoprotein E polymorphism in the Finnish population: gene frequencies and relation to lipoprotein concentrations. *J. Lipid Res.* **27:** 227–235.
8. Wardell, M. R., S. O. Brennan, E. D. Janus, R. Fraser, and R. W. Carrell. 1987. Apolipoprotein E2-Christchurch (136 Arg → Ser). New variant of human apolipoprotein E in a patient with type III hyperlipoproteinemia. *J. Clin. Invest.* **80:** 483–490.
9. Zannis, V. I., P. W. Just, and J. L. Breslow. 1981. Human apolipoprotein E isoprotein subclasses are genetically determined. *Am. J. Hum. Genet.* **33:** 11–24.
10. Mahley, R. W., and B. Angelin. 1984. Type III hyperlipoproteinemia: recent insights into the genetic defects of familial dysbetalipoproteinemia. *Adv. Intern. Med.* **29:** 385–411.
11. Weisgraber, K. H., S. C. Rall, and R. W. Mahley. 1981. Human E apoliprotein heterogeneity. Cysteine–arginine interchanges in the amino acid sequence of the apoE isoform. *J. Biol. Chem.* **256:** 9077–9083.
12. Utermann, G. 1987. Apolipoprotein E polymorphism in health and disease. *Am. Heart J.* **113:** 433–440.
13. Gregg, R. E., L. A. Zech, E. J. Schaefer, D. Stark, D. Wilson, and H. B. Brewer, Jr. 1986. Abnormal in vivo metabolism of apolipoprotein E_4 in humans. *J. Clin. Invest.* **78:** 815–821.
14. Hegele, R. A., and J. L. Breslow. 1987. Apolipoprotein

genetic variation in the assessment of atherosclerosis susceptibility. *Genet. Epidemiol.* **4:** 163–184.
15. Breslow, J. D., V. I. Zannis, T. R. SanGiacomo, J. L. H. C. Third., T. Tracy, and G. J. Glueck. 1982. Studies of familial type III hyperlipoproteinemia using a genetic marker the apoE phenotype E2/2. *J. Lipid Res.* **23:** 1224–1235.
16. Menzel, H. J., and G. Utermann. 1986. Apolipoprotein E phenotyping from serum by Western blotting. *Electrophoresis.* **7:** 492–495.
17. Havekes, L. M., P. de Knijff, U. Beisiegel, J. Havinga, M. Smit, and E. Klasen. 1987. A rapid micromethod for apolipoprotein E phenotyping directly in serum. *J. Lipid Res.* **28:** 455–463.
18. Steinmetz, A. 1987. Phenotyping of human apolipoprotein E from whole blood plasma by immunoblotting. *J. Lipid Res.* **28:** 1364–1370.
19. Kamboh, M. I., and R. E. Ferrell. 1986. A sensitive immunoblotting technique to identify thyroxin-binding globulin protein heterogeneity after isoelectric focusing. *Biochem. Genet.* **24:** 273–280.
20. Davignon, J., R. E. Gregg, and C. F. Sing. 1988. Apolipoprotein E polymorphism and atherosclerosis. *Arteriosclerosis.* **8:** 1–21.
21. Sing, C. F., and J. Davignon. 1985. Role of the apolipoprotein E polymorphism in determining normal plasma lipid and lipoprotein variation. *Am. J. Hum. Genet.* **37:** 268–285.
22. Righetti, P. G. 1983. Isoelectric Focusing: Theory, Methodology and Applications. Elsevier Biomedical Press, Amsterdam. 44–83.
23. Bier, M., R. A. Mosher, and O. A. Palusinski. 1981. Computer simulation and experimental validation of isoelectric focusing in ampholine-free systems. *J. Chromatogr.* **211:** 313–335.
24. Kamboh, M. I., R. E. Ferrell, and B. Sepehrnia. 1988. Genetic studies of human apolipoproteins. IV. Structural heterogeneity of apolipoprotein H (B_2-glycoprotein I). *Am. J. Hum. Genet.* **42:** 452–457.
25. Weidman, S. W., B. Suarez, J. M. Falko, J. L. Witztum, J. Kolar, M. Raben, and G. Schonfeld. 1979. Type III hyperlipoproteinemia: development of a VLDL apoE gel isoelectric focusing technique and application in family studies. *J. Lab. Clin. Med.* **93:** 549–569.
26. Ordovas, J. M., L. Litwack-Klein, P. W. F. Wilson, M. M. Schaefer, and E. J. Schaefer. 1987. Apolipoprotein E isoform phenotyping methodology and population frequency with identification of apoE1 and apoE5 isoforms. *J. Lipid Res.* **28:** 371–380.