Apolipoprotein E phenotyping with a single gel method: application to the study of informative matings[1]

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Abstract Two-dimensional gel electrophoresis or isoelectric focusing before and after treatment with cysteamine are currently used to determine the six apolipoprotein E isomorphic phenotypes from isolated very low density lipoproteins. A technique is described that makes this possible by performing isoelectric focusing on a single polyacrylamide cylindrical gel under standardized conditions. The technique is simple and accurate enough to obtain 99.5% concordance when the gels are interpreted independently by four different skilled and unskilled observers in the absence of any knowledge of the origin of the samples. There was complete agreement between our technique and the bidimensional method carried out independently in another laboratory on 74 aliquots of plasma very low density lipoproteins. Its application to 16 informative matings involving 101 subjects confirmed the recent demonstration that the apolipoprotein E phenotype inheritance is autosomal and compatible with three common alleles acting at a single genetic locus. Analyses of the contribution of apoE polymorphism to lipid and lipoprotein variability demonstrated a recessive allelic effect of $\epsilon 2$ on plasma very low density lipoprotein cholesterol and a dominant $\epsilon 4$ effect on low density lipoprotein cholesterol. As much as 30% of the variability in low density lipoprotein cholesterol was attributable to this polymorphic gene locus. A simplified scheme is proposed for the symbolic representation of the six phenotypes for clinical and genetic applications.—**Bouthillier, D., C. F. Sing, and J. Davignon.** Apolipoprotein E phenotyping with a single gel method: application to the study of informative matings. *J. Lipid Res.* 1983. **24:** 1060–1069.

Supplementary key words lipoproteins • hyperlipidemia • isoelectric focusing

Apolipoprotein E (apoE) is a normal constituent of human plasma very low density lipoproteins (VLDL) and is also present in small amounts in a subfraction of the high density lipoproteins (HDL) (1, 2). Its plasma concentration is elevated in familial dysbetalipoproteinemia (type III) (3) where it is associated largely with catabolic remnants of triglyceride-rich lipoproteins (4). The metabolic importance of this molecule stems from its role in cholesterol transport (5), its ability to interact with the apoB,E receptor of fibroblasts (6) as well as with the apoE receptor of liver cells where it mediates recognition and uptake of lipoproteins (7, 8). Isoelectric focusing separates human apoE into several immunochemically related bands (9, 10). This heterogeneity has been ascribed recently by Zannis and Breslow (11–13) both to glycosylation and to heritable alterations in the protein determined by three independent alleles acting at a single gene locus. On the other hand, Weisgraber, Rall, and Mahley (14) and Rall et al. (15), were able to demonstrate that the three major apoE isoforms differed from one another by a single substitution at either of two sites on the 299 amino acids polypeptide chain. E4, the most basic of the isoforms, has the strongest positive charge because of the presence of arginine at residues 112 and 158. The most commonly found isoform, E3, with an intermediate charge, has cysteine at the 112th residue and arginine at the 158th. Finally E2, which is the major circulating apoE isoform in type III, has a cysteine residue at both sites. The minor bands were found to be glycosylated forms of the major isoproteins in the three different homozygous subjects who served as donors for the sequencing studies of apoE. Zannis and Breslow (12) used a two-dimensional electrophoretic technique on polyacrylamide gel to establish their genetic model which predicted three homozygous and three heterozygous conditions that have now been identified. Weisgraber et al. (14) used either the same technique or carried out isoelectrofocusing in one dimension before and after treatment with cysteamine or glutathione to determine the phenotype. We wish to report here that the method of Warnick et al. (16) may

Abbreviations: VLDL, very low density lipoproteins: LDL, low density lipoproteins; HDL, high density lipoproteins; C, cholesterol; TG, triglyceride.

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be readily adapted to identify the six apolipoprotein E phenotypes with a single isoelectric focusing run on a straight polyacrylamide gel. Application of this simple method to the study of the inheritance of apoE isoforms in 16 different family units confirmed the genetic model proposed by Zannis and Breslow (12). We also describe a simplified scheme for the symbolic representation of the six phenotypes for clinical and genetic applications.

MATERIALS AND METHODS

Laboratory procedures

Plasma samples were obtained from patients followed at our lipid clinic and members of their family. Blood was collected in the morning after a 12- to 13-hr fast from an antecubital vein into Vacutainer Tubes (Becton-Dickinson) containing dry disodium ethylenediamine tetraacetate for a final concentration of 1 mg/ml. After thorough mixing it was cooled to 4°C and within 12 hr the plasma was separated in a refrigerated centrifuge (2500 rpm, 15 min) and kept at 4°C until processed.

Cholesterol (17) and triglycerides (18) were measured enzymatically with an autoanalyzer (ABA-100 bichromatic analyzer, Abbott Laboratories, Pasadena, CA). The plasma lipoprotein profile was examined by paper electrophoresis according to the method of Lees and Hatch (19). Plasma lipoprotein cholesterol content was measured after separation of the lipoproteins according to the Lipid Research Clinic protocol (20). The very low density lipoprotein (VLDL) fraction thus obtained after ultracentrifugation at 10°C for 20 hr at 104,000 *g* (40.3 Rotor and Model L5-65 ultracentrifuge, Beckman Instruments, Palo Alto, CA) was semi-quantitatively recovered from the top of the tube, transferred to a 6.5 ml cellulose nitrate tube, and the volume was adjusted to 6 ml with 0.15 mol/l NaCl for a second ultracentrifugation under the same conditions. For normolipidemic subjects, three tubes of plasma (5 ml overlayered with 1.5 ml of saline) were spun in the first run and the VLDL fractions were pooled in a single tube for the wash run. For hypertriglyceridemic patients, only one tube was used in the first run. The washed VLDL fraction recovered from the top in a volume less than 2 ml was used for isoelectric focusing; it could be kept at 4°C for up to 3 weeks before processing without any major alteration in the isoform profile. We preferred to aspirate the VLDL fraction with a pipette without cutting the tube; we found that the cutting knife and gaskets may be a source of contamination if not perfectly clean.

An aliquot of the VLDL fraction containing 0.9 mg of triglycerides (equivalent to about 150 μg of protein) was delipidized by its dropwise transfer into 1 to 5 ml of a freshly prepared mixture of acetone–ethanol 1:1 (v/v) at −15°C in a 12 ml conical centrifuge tube. The contents of the tube was thoroughly mixed with a vortex-type mixer and allowed to stand at −15°C for at least 4 hr before the protein was sedimented by centrifugation (15 min, 2500 rpm) and the solvent was aspirated. The protein was re-suspended in the same volume of solvent mixture used for delipidation and stored at −15°C for 2 hr, after which the protein was again sedimented and the solvent was aspirated. Finally the protein was re-suspended in 1 ml of diethyl ether at −15°C for 1 hr, after which it was centrifuged, the solvent was aspirated, and the protein was dried under a weak stream of nitrogen. The protein was immediately dissolved into 200 μl of 8 M urea containing Tris (hydroxymethyl) aminomethane-HCl (10 mmol/l, pH 8.6, reagent grade, Sigma Chemical, St. Louis, MO) and dithiothreitol (10 mmol/l, electrophoresis purity reagent, Bio-Rad).

The gel isoelectric focusing method was essentially carried out according to Warnick et al. (16). With this method the whole gel is prepared in one step, 3.0 g of acrylamide (the method stipulates 30.0 g but this is a misprint) is added to 80 mg of bisacrylamide, 19.2 g of urea, and 2.0 ml of carrier ampholyte. We favor the use of a stock solution made of 50 g of acrylamide ("Purity Reagent", Bio-Rad Laboratories, Richmond, CA) and 1.3 g of N,N′-methylenebisacrylamide ("Purity Reagent", Bio-Rad Laboratories) dissolved in bidistilled deionized (bd) water to a final volume of 100 ml. Six ml of this solution are added to 19.2 g of urea ("Purity Reagent", Bio-Rad Laboratories) and dissolved in 15 ml of bd water with a magnetic stirrer. To this solution 2.0 ml of carrier ampholyte, 400 g/l solution, pH 4–6 (BioLyte; Bio-Rad Laboratories) was added and the volume was adjusted to 40 ml with bd water. Without filtering, 40 μl of N,N-tetramethylene diamine (TEMED "Purity Reagent" Bio-Rad Laboratories) and 160 μl of fresh 100 g/l ammonium persulfate solution (ACS grade, Fisher Scientific, Montreal, Quebec) were added to this solution with mixing. Thereafter the procedure of Warnick et al. (16) is followed exactly. The focusing is carried out in a Bio-Rad Model 155 gel electrophoresis cell (Bio-Rad Laboratories) in a cold room at 4°C. The Coomassie blue G 250 was obtained from Miles Laboratory (Elkhart, IN) and the perchloric acid from Fisher Scientific.

Interpretation of the isoform profile

Seventy four aliquots of VLDL dialyzed overnight against 4 l of water at 4°C were lyophilized and sent to Drs. Zannis and Breslow in Boston, MA, for two-dimensional gel electrophoretic analysis (11). The gels

were interpreted independently in each of the two laboratories. At first VLDL samples of unknown phenotype were sent to Boston; later on, in order to get independent interpretation of all six phenotypes, VLDL from selected cases already phenotyped in our laboratory were sent. These 74 samples were derived from a pool of 650 subjects followed at our lipid clinic and whose phenotype was determined in our laboratory. In no instance was the interpretation made in one laboratory with the knowledge of the phenotype obtained in the other laboratory. Ten aliquots of plasma were also sent to Dr. Robert Mahley in San Francisco for determination of the phenotype with the cysteamine technique (14).

The determination of an individual's apoE phenotype was made as follows. In each run of 18 tubes, delipidized VLDL obtained from either a normal subject with the E3/3 phenotype or a case of typical familial dysbetalipoproteinemia (type III) with the E2/2 phenotype were included for reference. The gels were aligned using the pattern of the four apoC bands at the bottom of the tube and photographed in groups of six to eight tubes to obtain 5″ × 7″ prints in scale. The number of major bands was determined visually and the E2/E3 ratio was measured from the densitometric scan (Vitatron TLD-100, Vitatron Scientific BV, Dieren, Holland) after the relative position of each major band had been assessed by comparison with the reference samples. A sample of each of the six phenotypes is illustrated in **Fig. 1.** The minor bands seen on these gels represent glycosylated products of the major isoproteins (12), so that when an "E2/E3" ratio is calculated in phenotypes other than E3/2, what is measured in fact is a ratio of glycosylated and/or major isoforms migrating in positions corresponding to the E2 and E3 areas on the gel. The E4 isoprotein moves closest to the cathode just below the C-I band, the slightly more acidic E3 band is next below, followed by the E2 band. The homozygous phenotypes have only one major band; the first three tubes of Fig. 1 represent successively the E4/4, the E3/3, and the E2/2 phenotypes where the major band is respectively E4, E3, and E2. The heterozygotes have two major bands; in Fig. 1, the E4/3, E3/2, and E4/2 phenotypes are shown in succession in the last three tubes where the two major bands are readily seen in each case.

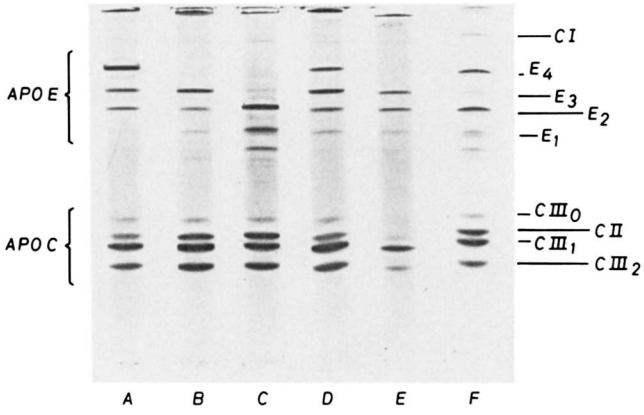

Fig. 1. The six apolipoprotein E phenotypes determined by unidimensional isoelectric focusing on polyacrylamide gel. The homozygous phenotypes are depicted in tubes A to C; they are sequentially E4/4, E3/3, and E2/2. Tubes D to F show the heterozygous phenotypes, respectively E4/3, E3/2, and E4/2. Tubes A to E were run in the same cell.

In order to test the ability of an observer to determine the phenotype, we asked two skilled (a physician and a research associate Ph.D. familiar with the technique but not performing it in the laboratory) and two unskilled invididuals (a nurse and a technician unfamiliar with the technique) to interpret the photographs of 100 gels. They were provided with the value of the E2/E3 ratio for each gel numbered 1 to 100 and given the following instructions. "Each of the photographs includes a gel where the major band is E3 (E3/3 phenotype) or one where the major band is E2 (E2/2 phenotype). The purpose is to determine whether there are one or two major bands and their relative position from one gel to the other or within the same gel. First, identify the E2 or the E3 position and then determine the six phenotypes. The six phenotypes are illustrated on the attached photograph. Below the thin line at the top which represents C-I, the closest band would be E4, then E3 below, then E2; the bands below E2 (E1 or E1′) do not serve for phenotyping. The distances between the E4 and E3 positions and between the E2 and E1 positions are usually wider than the distance between the E3 and E2 positions. The phenotypes with only one major band are E4/4 where E4 is strongest and the other two bands below are weaker and of relatively equal intensity; E3/3 where E3 dominates frankly over the nearby band at the E2 position below, and E2/2 where the lowest of the three major bands dominates with very little staining above, the more distant weaker band below representing the E1 position. Phenotypes with two major bands are of three kinds depending on which two bands are dominant E4/3, E3/2, and E4/2. Note that the E4/2 pattern is the easiest to identify and provides useful information on the relative position of the major bands when present. The figure should help you identify the phenotypes. You may also be guided by the E2/E3 ratio which is also provided for each gel. This ratio is close to 1.0 for E3/2 but also for E4/3 or E4/4. An infinite ratio confirms the E2/2 phenotype but it may in some instances be between 3.0 and infinity. When in doubt, the visual assessment of the major bands should guide your decision, not the E2/E3 ratio."

Graphic representation of the six phenotypes and genetic studies

To simplify the recording of the various phenotypes in a kindred, we used the following convention. A square (for males) or a circle (for females) is separated in four equal parts by two diagonal lines (**Fig. 2**). Each major isoform is assigned one of the upper three triangles thus formed: E4 in the left, E3 in the upper middle and E2 in the right triangle. The lower triangle is left empty when the subject is normolipidemic or is occupied by a black dot when the subject is hyperlipidemic. For each major isoprotein band on the gel the corresponding triangle is blackened, thus the six phenotypes may be clearly and simply visualized.

In the genetic model proposed by Zannis and Breslow (12), there are 21 different matings possible. Starting from hyperlipidemic probands followed at our lipid clinic, whose apoE phenotype had been determined, and phenotyping their spouse and first degree relatives, we were able to select 15 families where 16 different matings had occurred. The purpose was to verify the three allele-one gene locus model using as many different matings as possible. The following matings were examined; E3/3 by E4/4, E3/3, E2/2, E4/3, E3/2, and E4/2; E2/2 by E4/4, E4/3, and E3/2; E4/2 by E3/2 and E4/2; E3/2 by E3/2, E4/4, and E4/3; and finally E4/3 by E4/3 and E4/2.

In **Fig. 3,** each family is represented by a Roman numeral. The place in the kindred is given by the Arabic numeral at the lower right end of each symbol representing an individual. The age of each subject is given at the upper right end of the symbol. The graphic representation described above is used for the apoE phenotype. The presence of hyperlipidemia is represented by a dot in the lower triangle. The nature of the hyperlipidemia in the probands was established during a previous visit by an extensive clinical, dietary, familial, and laboratory evaluation in the light of a complete lipid and lipoprotein profile obtained on a normal diet during a control period, care being taken to exclude secondary causes (21). All family members sampled were examined for clinical manifestations of hyperlipidemia and their lipid and lipoprotein profiles were determined. Fig. 3 was constructed from the data obtained at the time of sampling for determination of the apoE phenotype. The following cut-off points were used to define hyperlipidemia in this figure: a cholesterol of 240 mg/dl or more and/or triglycerides of 150 mg/dl or more, with corresponding upper limits of 35 mg/dl for VLDL-cholesterol and 190 mg/dl for LDL-cholesterol. Low cut-off points were selected in order to identify those normolipidemic offspring that would be the most appropriate subsample for estimating the independent influence of the apoE genotypes on plasma lipid levels. The lower cut-off points exclude most, if not all, of those individuals that share the genetically determined etiology represented by the proband that identified the family. The proband is indicated by an arrow, he (she) happened to be the father (or mother) of the first degree relatives tested in 13 instances. In one of these, the proband was also the offspring of another informative mating (family XV). In one case the proband was the daughter resulting from such a mating (family V) and in another he was the brother of one of the mates (family VIII). All probands had hyperlipidemia with the following distribution of the lipoprotein electrophoretic types: six type IV (subjects IV-1, VI-2, VIII-6, XI-1, XIII-1, XIV-4)—no attempt was made here to distinguish between familial hypertriglyceridemia or familial combined hyperlipidemia (FCH); two type IIb, one with FCH (I-1) and one with xanthomatous familial hypercholesterolemia (FH) (II-1); one type IIa with homozygous receptor-negative FH (V-7); and six type III (on the basis of the presence of a broad beta and/or βVLDL), three with severe xanthomatosis typical of familial dysbetalipoproteinemia (III-1, IX-1, XV-1) and three without (VII-1, X-1, XII-1).

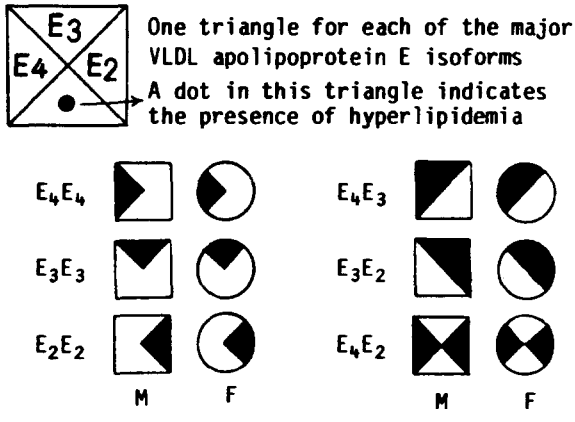

Fig. 2. Graphic representation of the six apolipoprotein E isomorphic phenotypes for genetic studies (M: males, F: females).

RESULTS

For the 74 samples examined by both the single gel method in our laboratory and the two-dimensional gel electrophoresis technique by Zannis and Breslow (11–13), there was 100% concordance in the identification of the phenotypes. They included 9 E4/4, 14 E3/3, 12 E2/2, 14 E4/3, 14 E3/2 and 11 E4/2. This relatively equal distribution of subjects among the various phenotypes resulted from the mode of selection of the al-

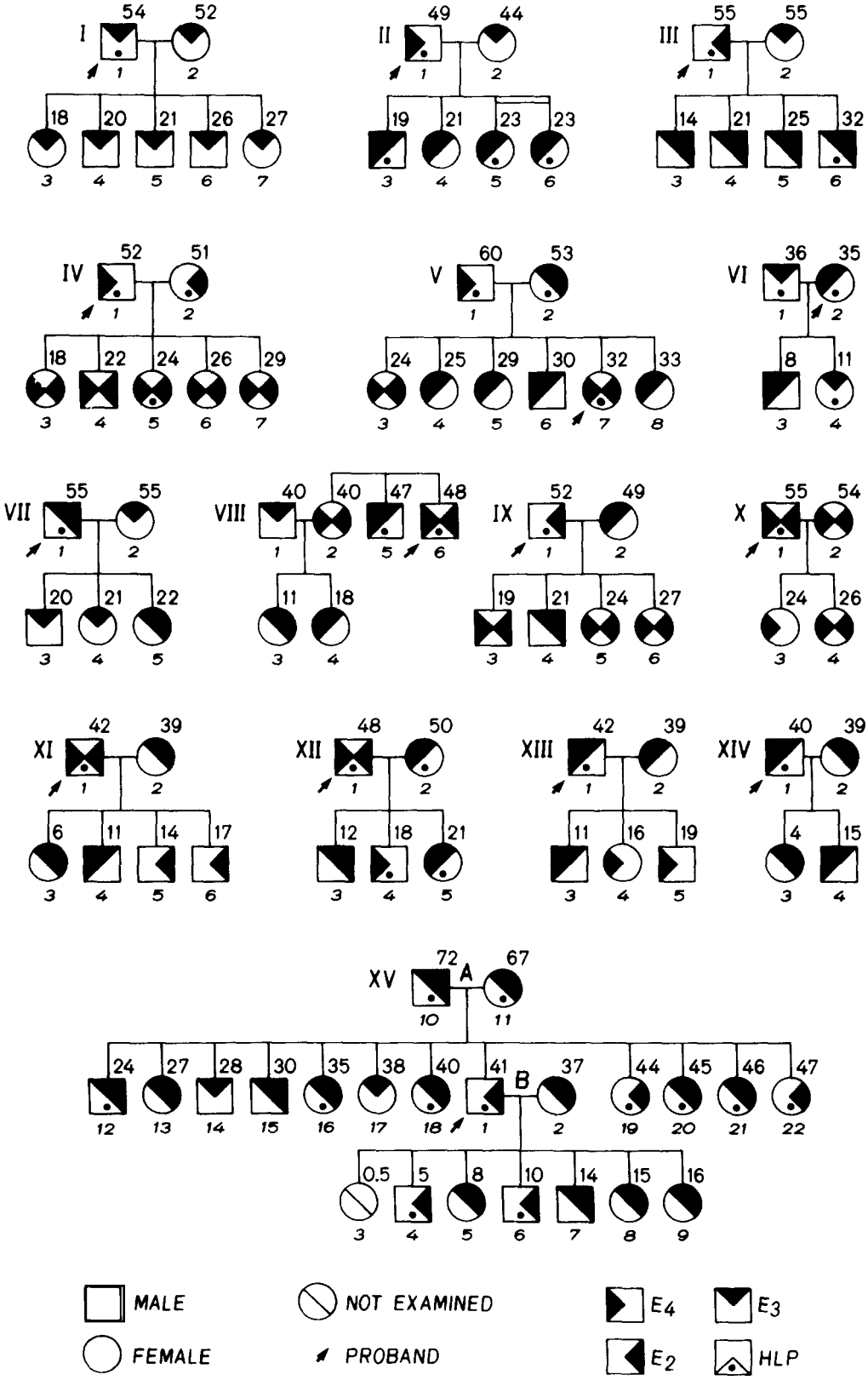

Fig. 3. ApoE phenotypes in 16 different matings that occurred in 15 families. For each individual, the upper figure gives the age and the lower figure gives the place in the kindred. Each family is identified by a Roman numeral.

TABLE 1. Interpretation of the gels for the determination of the six apolipoprotein E phenotypes by two skilled and two unskilled observers.

Phenotype	N	E2/3 Mean ± SD	E2/3 (Range)	Concordance Ratio	%
E4/4	3	0.82 ± 0.32	(0.53–1.16)	12/12	100
E3/3	41	0.49 ± 0.13	(0.16–0.71)	162/164	98.7
E2/2	16	4.00[a]	(3.00–∞)	64/64	100
E4/3	14	0.52 ± 0.17	(0.22–0.77)	56/56	100
E3/2	22	1.19 ± 0.32	(0.78–1.68)	88/88	100
E4/2	4	3.61 ± 1.40	(2.6–5.68)	16/16	100
Total	100			398/400	99.5

[a] Only two subjects did not have an infinite ratio.

iquots to be tested in both laboratories and is not a representative sample of the general population (12). There was also complete agreement between our technique and the cysteamine method of Weisgraber et al. (14) for the ten samples sent to Dr. Mahley's laboratory as part of another study. These included eight E2/2, one E3/3, and one E3/2.

The result of the study aimed at determining the ability of "skilled" and "unskilled" observers to interpret the gels correctly is given in **Table 1.** In this test, the interpretation was made slightly more difficult than in the standard situation described in the technique; although the observers knew that there was either an E3/3 or an E2/2 gel on each photograph, they were not told which of the two was present and the known gel was not pointed out to them. In spite of this added difficulty we obtained a 99.5% concordance between the four observers for the phenotyping of the gels. This sample of 100 gels was enriched in the E2/2 phenotype because of an ongoing review of all familial dysbetalipoproteinemia (type III) patients seen at our lipid clinic over the past 13 years.[3] Only one gel was misinterpreted. The two unskilled observers mistook an E3/3 pattern for an E3/2 phenotype; the E2/E3 ratio was 0.71 and this particular gel was slightly overloaded. In Table 1, the E2/E3 ratio that was formerly used by Utermann, Hess, and Steinmetz (22) to separate three apoE phenotypes (apoE-N, apoE-ND, and apoE-D) is averaged for all six phenotypes. It is interesting that a ratio close to 1.0, characteristic of apoE-ND subjects, is seen not only for E3/2 (1.19) but also for E4/4 (0.82). Similarly, an E2/E3 ratio in the range of the apoE-D phenotype is not only seen with E2/2 but also with the E4/2 profile (3.61). Finally this ratio is close to 0.5 in both the homozygous E3/3 (0.49) and the heterozygous E4/3 (0.52).

[3] Luc, G., D. Bouthillier, and J. Davignon. Unpublished data.

We gathered family data on 16 of the 21 possible different mating types for a total of 101 subjects. In every case the phenotypes of the offspring were consistent with the three allele-one gene model proposed by Zannis and Breslow (11). Marriages of homozygous individuals (families I (E3/3 × E3/3), II (E4/4 × E3/3), III (E2/2 × E3/3), and IV (E4/4 × E2/2) produced the expected single phenotype. The matings between homozygous and heterozygous individuals in families V (E4/4 × E3/2), VI (E3/3 × E4/3), VII (E3/2 × E3/3), VIII (E3/3 × E4/2), IX (E2/2 × E4/3), and XVB (E2/2 × E3/2) each gave the expected segregation of two different phenotypes. Finally, the three possible phenotypes expected from matings of heterozygotes were observed in families X through XIV and XVA.

An analysis of the effects of allelic variation at the apoE gene locus on lipid levels is presented in **Table 2.** Only the offspring of the 16 matings excluding all probands and spouses are considered. Individual XV-9 was also excluded because plasma lipoprotein concentration was not available. The means and standard deviations for age, total plasma cholesterol, triglycerides, VLDL-cholesterol, LDL-cholesterol, HDL-cholesterol, and the ratios: VLDL-C to triglycerides and LDL-C to VLDL-C, are given in Table 2 for each of the six phenotypes. Also presented are *1*) the results of the one-way analysis of variance (23) that tests the null hypothesis that the mean of a variable does not vary significantly among apoE phenotypes, and *2*) the estimate of the fraction of variance attributable to apoE variability. Mean age did not vary significantly among phenotypes, hence an age correction of these data was not considered necessary. Statistically significant differences among phenotype means were observed for total plasma cholesterol, VLDL-C, LDL-C, and HDL-C. The one-way analysis of variance was used to estimate the component of the sample variance of each trait attributable to differences among apoE phenotype class means. The contribution

TABLE 2. Statistical summary of apolipoprotein E phenotypes of offspring

Phenotype	N	(δ)	Age	Variable (mean ± SD) Cholesterol	Triglycerides	VLDL-C	LDL-C	HDL-C	VLDL-C / TG	LDL-C / VLDL-C
			yr			*mg/dl*				
All offspring										
E4/4	4	(2)	19 ± 3.4	194 ± 20	138 ± 81	38 ± 25	115 ± 24	42 ± 8	0.28 ± 0.10	4.5 ± 3.7
E3/3	10	(5)	23 ± 7.7	155 ± 21	96 ± 45	22 ± 10	89 ± 21	43 ± 12	0.25 ± 0.15	6.2 ± 6.2
E2/2	6	(4)	23 ± 18.0	151 ± 58	246 ± 207	69 ± 43	48 ± 23	34 ± 8	0.30 ± 0.11	0.8 ± 0.22
E4/3	14	(6)	20 ± 7.5	219 ± 95	112 ± 45	32 ± 16	146 ± 81	45 ± 10	0.30 ± 0.10	4.9 ± 1.8
E3/2	20	(9)	22 ± 12.1	150 ± 33	150 ± 145	34 ± 29	76 ± 17	40 ± 10	0.27 ± 0.22	3.7 ± 2.5
E4/2	10	(2)	24 ± 3.4	186 ± 58	107 ± 53	26 ± 17	104 ± 33	56 ± 20	0.24 ± 0.07	4.8 ± 2.0
All	64	(28)	22 ± 9.8	174 ± 62	135 ± 113	34 ± 26	98 ± 52	44 ± 13	0.27 ± 0.15	4.3 ± 3.4
F test[a]			N.S.	**	N.S.	**	**	**	N.S.	*
% Variance attributable to apoE				17.0	7.6	18.2	34.4	19.2	0.0	12.5
Excluding hyperlipidemics										
All	46		20 ± 7.4	153 ± 33	87 ± 29	21 ± 9	89 ± 32	45 ± 10	0.27 ± 0.17	5.12 ± 3.5
F test[a]			N.S.	**	N.S.	N.S.	**	N.S.	N.S.	N.S.
% Variance attributable to apoE				42.8	0.0	0.0	32.7	0.0	0.0	2.5

[a] *, Significant at the 0.05 level of probability; **, significant at the 0.01 level of probability.

of discrete electrophoretic differences in the apoE molecule to the total variance in this sample ranges from 7.6% for triglycerides to 34% for LDL-C. ApoE variability explains approximately 18% of the quantitative variation in cholesterol, VLDL-C, and HDL-C each.

The simultaneous pairwise comparisons among phenotype means for each of the lipid variables summarized in Table 2 were computed. Two effects of the allelic differences at the apoE gene locus on levels were revealed. The E2/2 phenotype accounts for the major effect on the levels of VLDL-C and TG, all other pairs of phenotypes are not significantly different for these traits at the 0.05 level of probability. The effect of the ε2 allele to elevate VLDL-C and TG is expressed virtually only in homozygotes, suggesting that a single dose of either the ε3 or the ε4 allele is necessary for normal processing of triglycerides. The significant effects of the apoE allelic variability on LDL-C and total serum cholesterol presented in Table 2 involve primarily those phenotypes with the ε4 allele. Phenotypes with the ε4 allele have mean values of 204 and 127 mg/dl for total cholesterol and LDL-C, respectively, compared to values of 151 and 75 mg/dl for the remaining classes. These differences are significant at the 0.001 level of probability. As expected, the ranking of the means of all phenotypes is similar for LDL-C and total cholesterol. (The correlation between LDL-C and total cholesterol in the sample, excluding those over 240 mg/dl cholesterol and over 150 mg/dl triglycerides, is 0.896).

The observation that higher LDL-C and total cholesterol concentrations are found in those who are heterozygous E4/3 and E4/2 suggests that the effect of ε4 is dominant to the effect of ε3 and ε2 on cholesterol metabolism. Segregation analysis to determine whether this apoE effect is homogeneous among pedigrees ascertained by different types of probands using a strategy developed by Moll et al.[4] is in progress.

Consideration of the ratio of LDL-C to VLDL-C (Table 2) reveals that the E2/2 phenotype stands out from all the others by its very low value. VLDL-C contributes most to total cholesterol in this phenotype. This is to be expected because of the cholesterol-enriched apoB- and apoE-containing remnants present in the VLDL fraction in the type III subjects included in this group. However, exclusion of the hyperlipidemics does not alter this pattern; the ratio is 1.0 ± 0.1 for E2/2 and varies between 4.5 ± 2.3 (E3/2) and 7.0 ± 6.7 (E3/3) for the other phenotypes. This implies that intermediate density lipoproteins (IDL) or cholesterol-enriched VLDL are present in circulation in the normolipidemic E2/2 subject. In contrast, the effect of the ε4 allele does not involve a redistribution of cholesterol

[4] Moll, P. P., et al. Unpublished data (personal communication).

among particle sizes, as the ratios of LDL-C to VLDL-C (with and without the hyperlipidemics considered) are very similar in all phenotypes bearing the $\epsilon 4$ allele and not significantly different from ratios of E3/3 and E2/2 subjects (Table 2). The VLDL-C to TG ratio, which has been used as a criterion in the past for the diagnosis of type III ($r \geq 0.30$) (24), did not differ significantly among the various phenotypes. It was, however, unexpectedly high in the E4/3 subjects who had a mean ratio equal to that found in the E2/2 phenotype.

The concordance of the apoE allele effects on lipid levels and the determination of the clinical condition of hyperlipidemia defined by exceeding 240 mg/dl cholesterol and/or 150 mg/dl triglycerides is illustrated by excluding the hyperlipidemic individuals from consideration. Hypertriglyceridemia occurs in every phenotypic class. Exclusion of the hyperlipidemic individuals lowers the mean VLDL-C plus TG of the E2/2 and E3/2 phenotypes the most. There are no significant differences among apoE phenotypes for HDL-C, VLDL-C, and TG after removing the hyperlipidemic individuals. These data are consistent with the suggestion that hyperlipidemia is more severe in the E2/2 and E3/2 phenotypes. In contrast, the effects of the apoE phenotypes on LDL-C and cholesterol in determining hyperlipidemia are distributed nearly uniformly among apoE phenotypes. That is, the rankings of the means remain essentially unchanged after removing the hyperlipidemic subjects. This comparison further supports the argument that the apoE variability studied here is associated with both the production of cholesterol and its distribution between LDL and VLDL.

DISCUSSION

The results obtained indicate that the method of Warnick et al. (16) may be readily adapted to permit an assessment of the apoE phenotypes from a single gel reading. The amount of material loaded on the gel is a critical aspect of the modified method since overloaded gels may give rise to erroneous readings. It is important to start from a larger volume of plasma as described for normolipidemic individuals and to keep the volume of delipidation low at all times to obtain the desirable staining characteristics of the bands. Too large a delipidation volume will result in loss of material. We found that it was unnecessary to filter the acrylamide in the cold which often introduces unwanted bubbles. On the other hand, the washing step in the ultracentrifuge, although not absolutely necessary, will insure cleaner bands in the apoE area and avoid contamination with albumin as reported by Warnick et al. (16). The visual assessment of the gels is highly reliable when it is done from a photograph that includes a known phenotype and where the apoC bands of each gel are properly aligned. The most readily recognizable pattern is that of the E4/2 phenotype, the presence of which greatly facilitates the interpretation of the other gels. Because of minor variations from batch to batch, only gels coming from a single electrophoresis cell should be compared and interpreted at a time, and in no instance should two different preparations of acrylamide be used in the same cell.

The apoE2/E3 ratio formerly used to separate three distinct phenotypes (apoE-N, apoE-ND, and apoE-D) (22, 25) may be misleading. First, this ratio measures major isomorphs in only two phenotypes, E3/2 and E2/2, when there is no band moving cathodic to E2. In all other instances it is a ratio of bands representing glycosylated products of a major isomorph alone (E4/4) or in combination with a major isomorph (E3/3, E4/3, and E4/2). Second, it may be seen from the data presented in Table 1 that densitometric measurements of the bands migrating in the E3 and E2 regions without taking into account the presence of E4 would have led to an erroneous phenotyping when the former criteria were used, i.e., an E4/4 would be taken in some instances for an apoE-ND (E3/2) phenotype since the ratio may be near one in some cases (the A gel in Fig. 1 is a good example), and an E4/2 phenotype would have been interpreted as an apoE-D (E2/2) phenotype since in some instances this ratio may be greater than 3.0. Finally an E4/3 would have been interpreted as an apoE-N phenotype almost systematically. Third, we found that overloading the gels could markedly affect the value of this ratio. This indicates that previous studies using the E2/E3 ratio as a major criterion for phenotyping could have introduced mistakes in the allocation of some subjects into a given phenotype category. With practice we found that the E2/E3 ratio added little for the interpretation of the gels with our own method.

Our study confirms the work of Zannis and Breslow (12) demonstrating that the major isoforms of apoE are inherited according to a model whereby three alleles ($\epsilon 4$, $\epsilon 3$, $\epsilon 2$) are acting at a single autosomal genetic locus. Recently, using a different method involving neuraminidase treatment of the gels, Utermann, Steinmetz, and Weber (26) also confirmed this model. After screening a large population of hyperlipidemic subjects, we assembled 16 of the 21 possible matings of the various phenotypes that could provide the most information about genetic segregation. Because in all instances only the phenotypes predicted by the model were observed among the offsprings, it is highly unlikely that an alternative genetic model is appropriate. All probands in these nuclear families were hyperlipidemic and our data provided the opportunity to examine the distribution

of the hyperlipidemia among offspring of various mating types (Fig. 3). One of the striking findings was the variable degree of expressivity of the hyperlipidemia. For instance, in family XI, the 14- and 17-year-old E2/2 children of an E4/2 × E3/2 mating were normolipidemic. In contrast, hypertriglyceridemia was expressed at the ages of 5 and 10 years in the E2/2 children of the proband in family XV. Furthermore, when several phenotypes were present among the children of hyperlipidemic parents, some phenotypes appeared to be spared and others were systematically affected. This is the case, for instance, in family XV-A where both parents of the proband had the E3/2 phenotype. As expected, this mating resulted in three different phenotypes among the offspring whose ages ranged between 24 and 47 years. None of the E3/3 children had hyperlipidemia but all E2/2 offspring had hyperlipidemia including the proband who was the only one to exhibit severe xanthomatosis, whereas five out of seven subjects with the E3/2 phenotype (including the youngest) were hyperlipidemic. In another situation where all offspring had the same phenotype, the hyperlipidemia was expressed in the eldest only in one family (III) and in none of five siblings in another (family I). These wide variations in expressivity illustrate the complexity of the genetic basis for the development of hyperlipidemia and ultimately of its atherosclerotic complications. Because of the obvious heterogeneity among the probands that identify these pedigrees, it is possible that variable expressivity in their first degree relatives is a consequence of the interaction of primary genetic effects with the segregation of inherited modifying factors. Studies in progress by our group will establish whether the apoE polymorphism may be one such factor. Hence it became apparent to us from these preliminary observations that a first approach to the problem should be to establish the contribution of the apoE locus to the variability in plasma lipid and lipoprotein levels in this sample of available pedigrees. Estimation of the mean lipid and lipoprotein levels for each of the apoE phenotypes (Table 2) clearly establishes the significant contribution of allelic polymorphism at the apoE locus. This locus acts in the pleotropic manner on LDL-C and VLDL-C levels. The $\epsilon 2$ allele acts as a recessive gene in determining the elevation of VLDL-C (and triglycerides). The $\epsilon 4$ allele acts as a dominant gene in determining elevated plasma LDL-C concentration. This is the first demonstration that the $\epsilon 4$ allele correlates with higher plasma cholesterol and LDL-cholesterol. The fact that the $\epsilon 2$ effect is primarily associated with hypertriglyceridemia while the magnitude of the $\epsilon 4$ effect is consistent in both normals and hyperlipidemics suggests that the influence of this apoE gene locus on LDL-C is a more direct general effect, whereas the VLDL-C response in only a fraction of the E2/2 individuals argues for the role of other genetic and environmental factors that promote hypertriglyceridemia as proposed earlier by Utermann, Pruin, and Steinmetz (27). It is surmised that by looking at the normolipidemic offspring using low cut-off points for the definition of the hyperlipidemia (and of course excluding probands from the calculation), we are excluding most if not all of the monogenetically determined hyperlipidemia from this group and hence reducing any bias that might be introduced by an interaction of such genetic loci with the contribution that the apoE gene locus makes to determining lipid levels. Although, the possibility that the apoE differences are influenced by an aggregation of polygenic effects in these families remains, experience suggests that interactions among genetic loci with small effects are expected to be far less of a factor than those known to occur among the monogenetically determined hyperlipidemias (27).

It is not possible to infer from our study the reasons for the existence of a genetic polymorphism at a locus that apparently has such a large impact on lipid metabolism. It is reasonable to suggest that the relatively high frequency of alleles ($\epsilon 2$, $\epsilon 4$) that promote hyperlipidemia may be attributable to the relatively greater advantage the heterozygous individuals have in coping with environmental challenges.

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