Am. J. Hum. Genet. 45:147–154, 1989

Genetic Studies of Human Apolipoproteins. IX. Apolipoprotein D Polymorphism and Its Relation to Serum Lipoprotein Lipid Levels

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Summary

Apolipoprotein D (APO D) is a constituent of plasma high-density lipoproteins. Its precise role in lipid metabolism is not well established, though it may be involved in cholesterol esterification and cholester ester transport to the liver for catabolism. No genetic polymorphism has been reported in the APO D gene product. To investigate the extent of genetic variation at the APO D structural locus, we have developed an isoelectric focusing–immunoblotting technique and have screened a large number of plasma samples from U.S. whites, U.S. blacks, Nigerian blacks, the Aleuts of the Pribilof Islands, Eskimo groups from Kodiak Island and St. Lawrence Island, and Amerindian populations from Mexico and Canada. Except for the U.S. blacks and Nigerian blacks, the APO D locus is monomorphic in all other population groups tested. In populations with black ancestry, the products of two alleles, *APO D*1* and *APO D*2,* have been observed at respective allele frequencies .987 and .013 in U.S. blacks and .978 and .022 in Nigerian blacks. The detection of a unique protein polymorphism in blacks makes APO D a useful black marker of significance in anthropogenetics and racial admixture studies. In addition to the interindividual variation observed, APO D reveals extensive intraindividual molecular variation with a multiple banding pattern. The basis of this molecular variation is explained, in part, by variation in the number of terminal sialic acid residues. We have investigated the effect of the APO D polymorphism on triglycerides, total cholesterol, LDL-, VLDL-, HDL-, and HDL3 cholesterol in 352 Nigerian blacks (190 males and 162 females). Although no significant effect of APO D polymorphism was seen on lipoprotein lipid levels, there was indication that the *APO D*2* allele may be associated with lower triglyceride levels.

Introduction

Human apolipoprotein D (APO D) is a protein component of plasma lipoprotein particles, with its maximum concentration being localized in high-density lipoproteins (HDL) and with trace amounts in very-low-density lipoproteins, low-density lipoproteins (LDL), and very-high-density lipoproteins (Curry et al. 1977; Fielding and Fielding 1980; Albers et al. 1981). The precise role of APO D in lipid metabolism is not known. It has been suggested, however, that APO D has a stabilizing effect on lecithin-cholesterol acyltransferase (LCAT) enzyme (Steyrer and Kostner 1987) and may thereby be involved in cholesterol esterification and transport to the liver for catabolism.

APO D was first isolated and partially characterized by McConathy and Alaupovic (1973, 1976), and subsequently these findings were confirmed by Albers et al. (1981). APO D is a glycoprotein containing approximately 18% carbohydrate by weight, with an apparent molecular weight of 32,000 daltons by SDS electrophoresis, and it exhibits multiple isoforms by isoelectric focusing (IEF) (Albers et al. 1981). The plasma concentration of APO D in normolipidemic individuals is about 6 mg/dl and is significantly reduced in individuals with familial LCAT deficiency (Albers et al. 1981).

Received January 23, 1989; revision received March 8, 1989.

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0002-9297/89/4501-0018$02.00

Relatively little is known about the hormonal or environmental factors that regulate APO D levels, but smoking and the anabolic steroid stanozolol have been shown to reduce APO D levels (Albers et al. 1984; Haffner et al. 1985). Recently the complete human APO D cDNA has been cloned and sequenced, and the gene has been mapped to chromosome 3 (Drayna et al. 1986, 1987*a*). The cDNA sequence predicts a mature protein of 19,303 daltons, containing 169 amino acids. The predicted protein sequence of APO D shows no homology with other apolipoproteins, but it shows extensive homology with members of the alpha 2–microglobulin superfamily. APO D is synthesized in a variety of human tissues including liver, intestine, pancreas, kidney, placenta, adrenals, spleen, and fetal brain (Drayna et al. 1986).

In an effort to understand the role of genetic variation of apolipoprotein loci in determining lipid and lipoprotein levels in the general population, we have characterized several apolipoproteins at their gene product levels (Kamboh and Ferrell 1987: Kamboh et al. 1987, 1988*a*, 1988*b*, 1989; Sepehrnia et al. 1988*a*, 1988*b*, 1989, and in press). So far no genetic polymorphism has been reported in the APO D protein molecule. We have developed an IEF and immunoblotting method to investigate the extent of genetic polymorphism in the products of the APO D structural locus and have investigated the impact of APO D polymorphism on lipid and lipoprotein levels.

Subjects and Methods

Samples

Serum or plasma samples from a large number of ethnic groups including U.S. whites ($N = 250$), U.S. blacks ($N = 263$), Nigerian blacks ($N = 364$), the Aleuts of the Pribilof Islands ($N = 78$), Eskimo groups from Kodiak Island ($N = 96$) and St. Lawrence Island ($N = 68$), and Amerindian populations from Mexico ($N = 68$) and Canada ($N = 88$) were available in the Human Genetics Laboratory. The sources of these samples have been described elsewhere (Kamboh and Ferrell 1986*a*; Sepehrnia et al. 1988*a*). In a few selected cases, samples were dialyzed and desialylated simultaneously according to a method described by Kamboh et al. (1988*a*). Because of the availability of lipid and lipoprotein profiles (Sepehrnia et al., submitted) the Nigerian sample was used to investigate the effect of APO D polymorphism on lipoprotein concentrations. The mean ± SD height and weight of 190 Nigerian males are 168.0 ± 7.5 cm and 62.9 ± 9.44 kg, respectively. The mean ± SD height and weight of 162 Nigerian females are 159.5 ± 6.10 cm and 58.11 ± 9.74 kg, respectively. They range in age from 17 to 54 years, with a mean ± SD age of 30.2 ± 9.5 years. The concentration of triglycerides, total serum cholesterol (TC) total high-density lipoprotein cholesterol (THDL-C), HDL3 cholesterol (HDL3-C) and low-density lipoprotein cholesterol (LDL-C) were measured on fasting blood samples as described elsewhere (Sepehrnia et al., submitted).

APO D Antiserum

For preparation of the goat anti–human APO D, APO D was prepared according to a method described elsewhere (Albers et al. 1981), and 300 μg APO D in 0.5 ml was mixed with an equal volume of complete Freund's adjuvant and was injected intramuscularly in the thigh. A booster dose of about 300 μg APO D with incomplete Freund's adjuvant was injected 14 and 28 d after the initial injection. The antiserum, obtained 7 d after the last booster injection, did not react with any of the known apolipoproteins, except for APO D, in gel diffusion studies.

IEF and Immunoblotting

The T5%-C3% polyacrylamide gels (4.85% monomer, 0.15% bis), containing 6 M urea, were prepared by mixing 3.33 ml stock polyacrylamide solution (29.1% monomer, 0.9% bis), 7.2 g urea, 0.6 ml ampholine (any one of the single-pH-range ampholines covering the gradient of pH 4.2–4.9, pH 3.5–5.0, pH 4–6, or pH 4–6.5), and 20 μl riboflavin (0.1% w/v) and then adjusting it to a final volume of 20 ml with deionized water. Photopolymerization was achieved by overnight exposure to fluorescent light, and the gel was used the following morning. Electrode solutions for the cathode and anode were 1 M NaOH and 1 M H_3PO_4, respectively. Samples absorbed on 5 × 7-mm Whatman 3 MM filter-paper wicks were applied close to the cathode on pH 4.2–4.9 and pH 3.5–5 gels and 2 cm from the cathode on pH 4–6 and pH 4–6.5 gels. IEF was carried out on an LKB 2217 ultrophor or LKB 2117 multiphor II electrofocusing unit connected to a cooling unit operating at 10°C and to an LKB power supply. All pH-range gels were focused at 30 W, 2,000 V, 250 mA for 3 h, except that pH-range 4.2–4.9 gels were prefocused for 30 min at these running conditions. Following IEF, the gel was rinsed briefly in TBS buffer (0.15 M NaCl, 0.03 M Tris-HCl, pH 8.0), and the proteins were transferred to a 0.20-μm nitrocellulose filter

(Schleicher and Schuell) by simple diffusion for 30–40 min (Kamboh and Ferrell 1986*b*). The filter was then carefully removed from the gel and was washed briefly in TBS buffer, followed by a 30-min incubation with 5% (w/v) nonfat dry milk dissolved in deionized water, to saturate the remaining protein-binding sites on the filter. The filter was probed with goat anti–human APO D antiserum at 1 μl/1 ml dilution in TBS for 40 min, followed by three 10-min washes in TBS. The filter was then incubated with rabbit anti–goat IgG conjugated with alkaline phosphatase at 1 μl/5 ml dilution in TBS for 40 min, followed by three 10-min washes in TBS. Finally, the filter was stained histochemically using 25 mg β-naphthyl phosphate, 25 mg Fast Blue BB salt, and 60 mg magnesium sulphate in 50 ml stock buffer (1.8 g NaOH, 3.7 g boric acid/liter).

Statistical Methods

Allele frequencies were estimated by gene counting. For each lipid variable, to test the null hypothesis of equality of mean values between the two APO D phenotypic classes (1–1 and 2–1), we performed an analysis of covariance. Adjustment for covariates was necessitated by the fact that the individuals in the two phenotypic classes differed with respect to sex, age, height, weight, and body-mass index (BMI)—these five variables were used as covariates in the analyses of covariance. Statistical tests were also performed to check whether the slopes of the dependent variables on the covariates were equal in the two phenotypic classes; none of these tests was significant at the 5% level.

Results

Native and Asialo IEF Patterns

As mentioned in the IEF and Immunoblotting subsection, several single ampholines covering different pH ranges were tested in order to achieve maximum resolution between APO D isoforms. Of the four ampholines used (pH 4.2–4.9, pH 3.5–5, pH 4–6, and pH 4–6.5), the pH range 4.2–4.9 gave the best resolution of the APO D pattern in its native form. Figure 1 illustrates the IEF-immunoblot pattern of APO D in the pH range 4.2–4.9. The most frequently observed pattern in this pH-range gel consists of at least three or four major and several minor bands, focusing both anodal and cathodal to the major isoproteins. In addition to the common pattern, a less frequent but more complex, multibanded pattern was observed in plasma samples from individuals of African ancestry. This rarer pattern consists of at least eight major and several minor components. Four of the major bands of this phenotype correspond to the common pattern, and the other four major bands (indicated by arrows in fig. 1) are shifted toward the cathode as compared with the common pattern. The three shifted major bands fall between the common pattern, while the fourth shifted band is clearly cathodic. A repeat run on the same samples gave the exact complicated multibanded pattern in this unusual phenotype. The distribution of APO D isoforms, as based on their focusing positions and intensity differences in both the frequent and less frequent phenotypes, suggests that these two IEF-immunoblot patterns are the gene products controlled by two alleles at a single locus. We propose to refer to this structural locus as APO D, with two alleles designated as *APO D*1* and *APO D*2*. The isoprotein pattern of the most frequent phenotypes is controlled by the *APO D*1*, allele, while the cathodally shifted pattern is controlled by the *APO D*2* allele. The expression of each allele in homozygous individuals results in two phenotypes, APO D 1–1 and APO D 2–2; and the combination of these two phenotypes results in the formation of a heterozygous phenotype, APO D 2–1. These phenotypes are illustrated in figure 1. Owing to the low frequency of the *APO D*2* allele, we did not observe the APO D 2–2 phenotype in our survey.

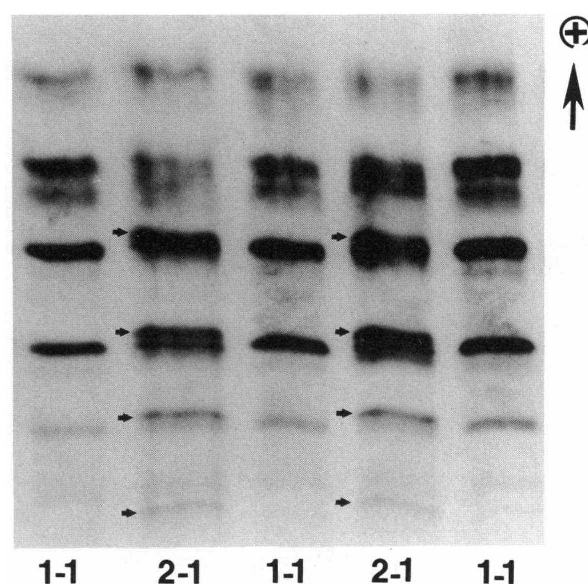

Figure 1 Native APO D IEF-immunoblot patterns in 5% polyacrylamide IEF gels containing 6 M urea, pH range 4.2–4.9. The APO D phenotypes are given beneath each sample track. The major bands of the variant type 2 are indicated by arrows.

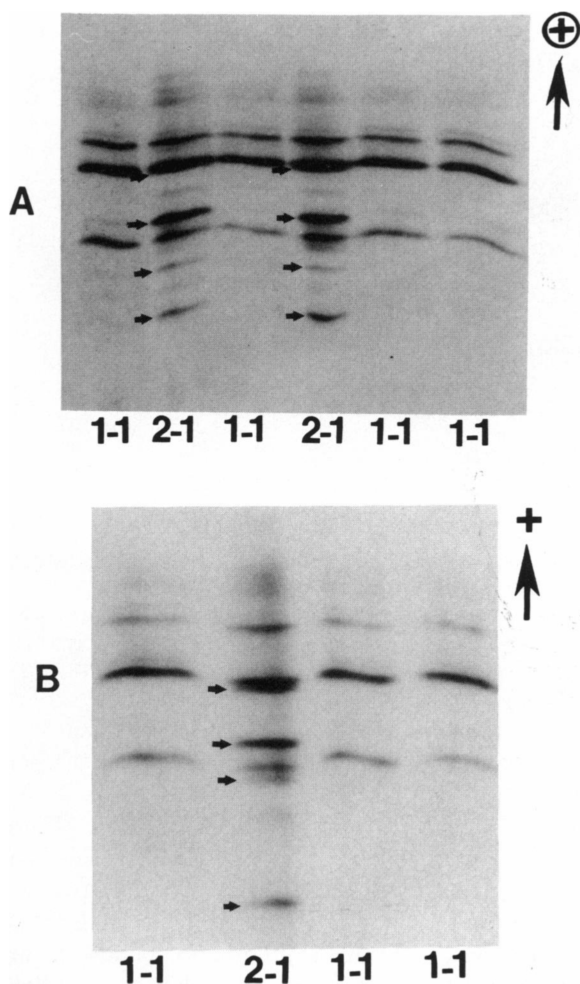

Figure 2 Asialo APO D IEF-immunoblot patterns after treatment of plasma samples with neuraminidase, in 5% IEF polyacrylamide gels containing 6 M urea, pH range 4–6 (*A*) and pH range 4–6.5 (*B*). The APO D phenotypes are given beneath each sample track. The variant banding patterns of type 2 are indicated by arrows.

The basis of the intra- and interindividual variation seen in the APO D gene products was tested further by incubating the plasma samples of proposed homozygous and heterozygous phenotypes with neuraminidase. fig. 2 shows the asialo patterns of APO D homozygous (1–1) and heterozygous (2–1) phenotypes obtained for pH range 4–6 (fig. 2*A*) and pH range 4–6.5 (fig. 2*B*) gels. In its asialo form the APO D 1–1 phenotype is represented by a single major band flanked by two minor bands. On the other hand, the APO D 2–1 phenotype consists of several bands. The asialo patterns in their homozygous forms, as compared with their corresponding complicated native forms, indicate that the basis of the intraindividual variation can be partly explained by variation in the number of terminal sialic acid residues on the APO D molecule. On the other hand, the persistent interindividual differences in isoelectric points in the presumed homo- and heterozygous phenotypes after the removal of sialic acid residues are under genetic control. It is noteworthy that, under our experimental conditions, the asialo major bands in each phenotype are associated with several minor components. This suggests either that the desialylation is incomplete or that these minor components are due to the other glycosylated products which are resistant to neuraminidase treatment. The resolution between the asialo pattern on pH 4–6 (fig. 2*A*) and pH 4–6.5 (fig. 2*B*) gels is slightly different, with optimum resolution being achieved on the latter gel system.

Table 1

Inheritance of APO D Phenotypes

Parental Matings (no. of families)	Offspring 1–1	Offspring 2–1
1–1 × 1–1 (10)	44	. . .
1–1 × 2–1 (3)	7	7

Family Study

The two-allele hypothesis for explaining the two phenotypes was tested further in family material. A total of 13 families with 58 children were screened to test the mode of inheritance of APO D phenotypes (table 1), and the genetic basis of the observed variation was confirmed in the three informative families (fig. 3). The

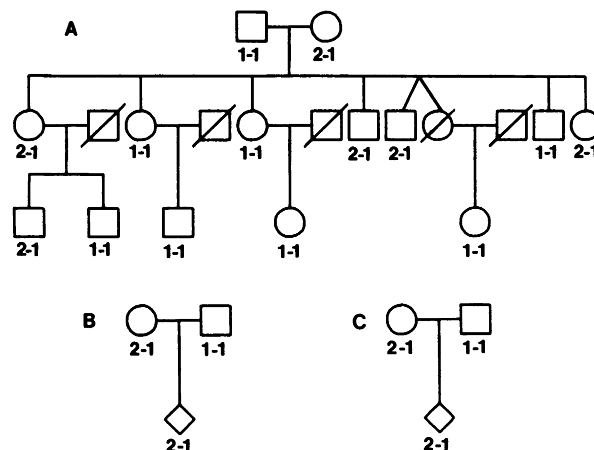

Figure 3 Inheritance of APO D phenotypes in a large pedigree (*A*) and in two trios (*B* and *C*).

Table 2

Distribution of APO D Phenotypes and Allele Frequencies in Different Population Groups

Population (N)		Phenotype 1–1	Phenotype 2–1	Allele Frequency APO D*1	Allele Frequency APO D*2
U.S. blacks:					
Pittsburgh	80	78	2	.987	.013
Houston	81	79	2	.988	.012
New York	102	99	3	.985	.015
Combined	263	256	7	.987	.013
Nigerian blacks	364	348	16	.978	.022
U.S. whites	250	250	...	1.000	.000
Dogrib Indians	88	88	...	1.000	.000
Mayan Indians	68	68	...	1.000	.000
Aleuts	78	78	...	1.000	.000
Kodiak Island Eskimos	96	96	...	1.000	.000
St. Lawrence Island Eskimos	68	68	...	1.000	.000

segregation pattern of the two APO D alleles was compatible with autosomal codominant inheritance, and no departure from this expected segregation pattern was seen.

Population Study

To estimate the extent of genetic variation at the APO D structural locus, we screened 1,275 plasma or serum samples from a large number of ethnically diverse populations (table 2). With the exception of U.S. blacks and Nigerian blacks, an invariant banding pattern corresponding to the APO D 1–1 phenotype was seen in all other population groups. A total of 23 unrelated black samples revealed the presence of the variant pattern APO D 2–1. When Hardy-Weinberg equilibrium was assumed, an excellent agreement was seen between the observed and expected values in these two phenotypic classes in black populations. The less frequent *APO D*2* allele is present at polymorphic frequency in both the U.S. blacks (1.3%) and the Nigerian blacks (2.2%).

APO D Polymorphism and Lipoprotein Levels

For the 364 Nigerian samples tested, lipid and lipoprotein levels were available on 352 subjects. These quantitative lipid data were used to determine the impact of APO D polymorphism on its variation. Analyses of covariance were performed to test equality of adjusted mean values of lipid variables among the two

Table 3

Results of Analysis of Covariance for Testing Equality of Mean Values of Lipid Variables, between APO D Phenotypes, in 352 Nigerian Blacks

Lipid Variable	Significant Covariates[a]	Adjusted Mean Lipid Level for APO D Phenotypes: 1–1[b] (n = 336)	2–1[b] (n = 16)	F-Value (1 df)	P
Total cholesterol (mg/dl)	Sex and age	163.25 ± 1.70	153.35 ± 7.81	1.53	.22
HDL cholesterol (mg/dl)	None	47.03 ± .62	45.27 ± 2.87	.36	.55
HDL3 cholesterol (mg/dl)	Height, weight, and BMI	30.23 ± .39	29.21 ± 1.78	.31	.57
LDL cholesterol (mg/dl)	Sex and age	102.33 ± 1.58	96.60 ± 7.26	.59	.44
Triglycerides (mg/dl)	Sex and age	69.41 ± 1.52	57.44 ± 6.97	2.81	.09

[a] Covariates included in analysis are sex, age, height, weight, and BMI.
[b] Values are mean ± SD.

APO D phenotype classes (1–1 and 2–1), and the results are given in table 3. No significant effect of APO D phenotypes on the quantitative levels of lipoprotein lipids was detected, at the 5% level, in the Nigerian blacks. However, the difference in the adjusted levels of triglycerides between the APO D 1–1 and APO D 2–1 phenotypic classes was significant at the 9% level. Because of the limited sample size of the APO D 2–1 phenotypic class, this result needs to be confirmed in a separate study.

Discussion

Although the APO D was identified and isolated several years ago as a minor component of the human plasma lipid transport system (McConathy and Alaupovic 1973; 1976; Curry et al. 1977, Fielding and Fielding 1980; Albers et al. 1981), its functional importance in lipid metabolism is a matter of speculation and dispute. In recent years it has been established that genetic variation at a large number of apolipoprotein loci is a major determinant of interindividual variation in plasma cholesterol levels in the general population (Breslow 1988; Davignon et al. 1988; Humphries 1988). To investigate whether APO D is polymorphic at the protein level, and whether this protein polymorphisim has any impact on quantitative lipoprotein lipid levels, we have developed a simple IEF-immunoblotting technique which enables the immunolocalization of APO D isoproteins from microliter quantities of whole plasma. The specific localization of APO D isoforms on IEF gels was confirmed by direct comparison with immunolocalized IEF patterns of all other apolipoproteins for which antisera were available [e.g., APOS A-I, A-II, A-IV, B, C-I, C-II, C-III, E, H, and Lp(a)]. No immunological cross-reaction was observed between the APO D antiserum and the other well-known apolipoprotein gene products in our IEF system. Recently Weech et al. (1986) have identified cross-reacting human plasma apolipoproteins by using several APO D monoclonal antibodies. The cross-reactive apolipoprotein species identified by Weech et al. (1986) migrated more cathodally, as compared with the APO D isoform. However, our polyclonal monospecific antiserum did not yield any major cross-reactivity, because all the immunolocalized APO D bands clustered together on acidic pH gels and no additional bands were seen on basic pH or broad pH range gels.

Our IEF-immunoblotting technique has shown APO D to be heterogeneous at both the molecular and genetic levels. At the molecular level the APO D reveals extensive microheterogeneity in its native form by having multiple major and minor isoforms, an observation that is consistent with previous reports about its molecular heterogeneity (Albers et al. 1981; Weech et al. 1986). The chemical basis of this heterogeneity can be explained, in part, by the presence of negatively charged sialic acid residues because, after treatment with neuraminidase, the APO D pattern was simplified and focused at a more basic pH region in the IEF gels. It is important to note here that, despite the treatment with neuraminidase, the asialo pattern still contained several minor components, indicating additional postsynthetic processing of the APO D molecule. The second type of heterogeneity seen in both the native and the asialo forms in different individuals' plasma samples is under genetic control, and the genetic hypothesis is supported further by family and population data which demonstrate the presence of two codominantly expressed alleles at a single APO D structural locus.

Population genetic studies of APO D indicate that this structural locus is monomorphic at the protein level in most of the human populations, the notable exception being populations of black ancestry, among whom the APO D locus is polymorphic, with the variant allele frequency ranging from 1.3% in U.S. blacks to 2.2% in Nigerian blacks. The widespread distribution of the *APO D*2* allele in native African blacks and U.S. blacks indicates that this is a unique allele of the black gene pool, one that may be of importance in admixture and evolutionary genetic studies.

In order to evaluate whether the APO D polymorphism has any impact on lipoprotein lipid levels in the general black population, we have compared the adjusted mean values of lipid variables between the observed APO D 1–1 and 2–1 phenotypes. With the possible exception of triglycerides, all lipid variables showed comparable values in these two classes. In comparison with the 1–1 phenotype, the triglyceride levels were found to be lower in the 2–1 phenotype. Although this difference was not significant at the 5% level, it was significant at the 9% level. This suggests that the *APO D*2* allele may have a small reducing effect on triglyceride levels. However, this finding requires confirmation, especially in view of the limited sample size of the 2–1 phenotype class, and the marginal effect detected in the present study may vanish on enlargement of sample size. Since APO D is mainly confined to the HDL fraction and since its levels are highly correlated with HDL-cholesterol (Albers et al. 1981), one would have expected the APO D polymorphic effect, if any, to be on HDL-cholesterol levels. However, our failure to see any effect

on HDL-cholesterol levels could also be due to the fact that the observed number in the 2–1 phenotype class is very small and to the fact that no individual with the 2–2 phenotype was observed. It may be relevant that our test sample consists of an African population whose mean cholesterol values are significantly lower than those of Western populations. Data on the effects of APO E polymorphism on lipoprotein lipid levels in different population groups have shown that the magnitude of the genetic effects can be modulated by different ethnic and dietary backgrounds (Utermann 1987; Davignon et al. 1988; Sepehrnia et al., submitted). To determine the exact role of APO D genetic variation on lipoprotein levels will require extensive black population data. Furthermore, a test of the effect of APO D polymorphism on lipoprotein lipid levels in U.S. blacks may provide a useful comparison of the possible modulating effect of dietary factors on genetic determinants of lipid metabolism. Despite their African ancestry, U.S. blacks have cholesterol values quite similar to those in U.S. whites, owing perhaps in part to dietary influences. APO D has been found to be associated with lipoprotein particles containing APO A-I and LCAT (Fielding and Fielding 1980; Albers et al. 1981) and appears to have some functional role in their metabolism. Unfortunately, we do not have the quantitative data on APO A-I and LCAT to examine the APO D phenotypic effects on the levels of APO A-I and LCAT. Unrelated to the observed protein polymorphism, recently two common DNA polymorphisms have been reported at the APO D locus (Drayna et al. 1987*b*). In future studies both the protein and DNA polymorphisms will help to identify the genetic role of APO D in lipid metabolism.

Acknowledgments

This work was supported in part by NIH grants HL 39107, HL 24489, and HL 30086. We would like to thank Lori Kelly for her excellent technical assistance and Jeanette Norbut for typing the manuscript.

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