

An *Apa*LI restriction site polymorphism is associated with the MB19 polymorphism in apolipoprotein B

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Abstract An apolipoprotein (apo) B-specific monoclonal antibody, MB19, detects a commonly occurring two-allele genetic polymorphism in human apoB (Young, S. G., S. J. Bertics, L. K. Curtiss, D. C. Casal, and J. L. Witztum. 1986. *Proc. Natl. Acad. Sci. USA.* **83**: 1101-1105). Antibody MB19 binds to two different allotypes of apoB, MB19₁ and MB19₂, with high and low affinity, respectively. The epitope for antibody MB19 is located within apoB-100 thrombolytic fragment T4 (apoB-100 amino acid residues 1-1297). In this study, we examined the relationship of the MB19 polymorphism to a C→T nucleotide substitution at apoB cDNA nucleotide 421. This nucleotide substitution results in a Thr→Ile substitution at apoB-100 amino acid 71, and it changes an *Apa*LI restriction endonuclease site in the apoB gene. The nucleotide substitution was easily detectable by *Apa*LI digestion of a 141-base pair fragment of the apoB gene obtained by enzymatic amplification of genomic DNA. In 62 subjects, the MB19 phenotype, as determined by radioimmunoassays, correlated perfectly with the *Apa*LI restriction site polymorphism in the amplified DNA. The apoB allotype MB19₁ is associated with an Ile at residue 71 and the absence of the *Apa*LI site, whereas the apoB allotype MB19₂ is associated with a Thr at residue 71 and the presence of the *Apa*LI site. We conclude that the amino acid substitution at residue 71 probably accounts for the MB19 polymorphism in apoB.—Young, S. G., and S. T. Hubl. An *Apa*LI restriction site polymorphism is associated with the MB19 polymorphism in apolipoprotein B. *J. Lipid Res.* 1989. **30**: 443-449.

Supplementary key words apolipoprotein B • low density lipoproteins • radioimmunoassay • restriction fragment length polymorphism

In 1986, our laboratory reported that an apoB-specific monoclonal antibody, MB19, detects a commonly occurring two-allele genetic polymorphism in apolipoprotein (apo) B (1). Monoclonal antibody MB19 was originally developed and characterized by Curtiss and Edgington (2). We demonstrated that antibody MB19 binds to low density lipoprotein (LDL) samples from different individuals with one of three distinct patterns of immunoreactivity: strong, intermediate, or weak (1). We showed that these three binding patterns result from the inheritance of two co-dominant apoB alleles that code for apoB allotypes MB19₁ and MB19₂, which have high and low affinity, respectively, for antibody MB19. Thus, in-

dividuals whose LDL have strong or weak MB19 binding patterns are homozygous for the alleles coding for apoB allotypes MB19₁ and MB19₂, respectively, whereas the intermediate binding pattern is due to heterozygosity for the two alleles. Recently, Tikkanen et al. (3, 4) have demonstrated that the MB19₁ and MB19₂ alleles are related to the Ag(c) and Ag(g) alleles, respectively. The latter pair of alleles was originally detected by human antisera from multiply transfused thalassemic patients (5). Similar data have been reported by Schumaker and co-workers (6) and Robinson et al. (7).

The MB19 polymorphism is very common. In several different human populations, the frequency of the MB19₁ and MB19₂ alleles has been shown to be approximately 0.36 and 0.64, respectively (1, 5, 8). Because the MB19 polymorphism is easily detected and because it is so common in the general population, it has proven to be useful in genetic studies of apoB mutations (9).

Several groups have reported finding positive associations between plasma cholesterol and triglyceride levels and several commonly occurring restriction fragment length polymorphisms in the apoB gene (10-12). The effect of genetic variations in apoB (and other apolipoproteins) on plasma lipoprotein levels has recently been reviewed by Lusis (13). Our laboratory recently determined the MB19 phenotype of over 330 human subjects, but we were unable to detect any relationship between plasma lipoprotein levels and MB19 phenotype (8). However, Tikkanen and co-workers (14) found that Finnish school children homozygous for the MB19₂ allele had slightly lower LDL cholesterol and apoB levels in plasma than did children who were MB19₁/MB19₁ homozygotes or MB19₁/MB19₂ heterozygotes.

Abbreviations: apo, apolipoprotein; bp, base pair; LDL, low density lipoproteins; RIA, radioimmunoassay.

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The epitope for antibody MB19 is located in apoB-100 thrombolytic fragment T4 (15), which contains apoB-100 amino acids 1–1297 (16). We have previously reported that the MB19 epitope is sensitive to disulfide reducing agents (17); this suggested to us the possibility that the epitope might be located in the amino-terminal 500 amino acids of apoB-100, a region enriched in cysteine residues. The first 500 amino acids contain 12 of its 25 cysteines (16). Two groups of investigators have reported only an incomplete, or imperfect, association between the MB19 polymorphism (or the Ag(c)/Ag(g) polymorphism) and restriction fragment length polymorphisms in the carboxy-terminal portion of the apoB gene (18, 19).

The complete or partial sequence of the apoB cDNA or gene has been determined by six independent groups (16, 20–25). Ludwig and co-workers (25) have recently compiled a list of nucleotide and amino acid sequence differences between the reported apoB sequences. Within the first 500 amino acids of apoB, only one amino acid substitution was identified by more than one group of investigators. Four groups reported Thr at amino acid 71, whereas two others reported Ile at amino acid 71 (25). The substitution of Thr by Ile is caused by a C→T nucleotide substitution at apoB cDNA nucleotide 421; this single base substitution changes an *Apa*LI restriction site in the apoB gene. In this study, enzymatic amplification of the apoB gene was used to study this single nucleotide substitution. In 62 individuals, we found a perfect association between this single nucleotide substitution and the MB19 immunochemical polymorphism in apoB.

METHODS

Human subjects

Blood samples were obtained from 62 human subjects, including 59 samples from subjects in lipid clinics and 3 samples from normal laboratory personnel, under Human Use Protocols approved by the University of California, San Diego, and the University of California, San Francisco. Thirty six of the 59 lipid clinic blood samples were obtained from members of a single large kindred. The MB19 binding pattern of each individual in this kindred has been previously reported (9).

MB19 phenotyping of plasma samples

The MB19 binding pattern, or phenotype, was determined by solid-phase radioimmunoassay (RIA) using monoclonal antibodies MB19, MB3, and MB47 exactly as previously described (1, 9).² This assay system identified each individual as being an MB19₁/MB19₂ heterozygote, an MB19₁/MB19₁ homozygote, or an MB19₂/MB19₂ homozygote.

Enzymatic amplification of DNA

A Bluescribe™ subclone of λ5C (26) containing the amino-terminal portion of the apoB gene was given to us by Drs. Blackhart, McCarthy, and Levy-Wilson of the Gladstone Foundation Laboratories, and plasmid DNA was obtained by alkaline lysis of an overnight bacterial culture (27). The genomic DNA of human subjects was isolated from peripheral blood leukocytes (28). Enzymatic amplification of DNA was performed using *Thermus aquaticus* DNA polymerase, according to the instructions of the Geneamp kit (Perkin Elmer Cetus, Part No. N801-0043). Oligonucleotide primers for the amplification reactions were as follows: MB19-1 (5'-TTGATCTaGAGGTTCCCCAGCTC-3', apoB cDNA nucleotides 367–389) and MB19-2 (5'-ACATGGaTcCAGCAAACCTCTCA-3', complementary to apoB cDNA nucleotides 507–485). Two base substitutions (lower case letters) were introduced into the 5' ends of the oligonucleotides MB19-1 and MB19-2 to create *Xba*I and *Bam*HI restriction endonuclease sites, respectively. For each amplification reaction, 500 ng of genomic DNA (or 10 ng of plasmid DNA) and 50 pmol of each oligonucleotide were used. Amplification reactions were performed for 35 cycles, with annealing, extension, and denaturation temperatures of 62°, 72°, and 94°C, respectively. Following the amplification reaction, one-tenth of the amplified DNA was examined on an ethidium bromide-stained 8% polyacrylamide gel (27). A single band of the expected size (141 base pairs [bp]) was observed.

Subcloning amplified DNA into M13

The 141-bp band amplified from the genomic DNA of several subjects was removed from the polyacrylamide gel, and the DNA in the band was eluted into a buffer containing 0.01 M Tris-HCl (pH 8.0), 2.0 mM EDTA, and 3.25 M ammonium acetate for 16 hr at 37°C. The DNA was then precipitated with 70% ethanol, and a portion of it was cut with *Xba*I and *Bam*HI for 16 hr at 37°C. After restriction endonuclease digestion, the DNA was extracted with phenol-chloroform and then precipitated with ethanol. The *Xba*I- and *Bam*HI-cut amplified DNA was then "force cloned" into *Xba*I- and *Bam*HI-cut M13 (27, 28). DNA sequencing of single-stranded M13 templates was performed according to the dideoxynucleotide chain termination technique of Sanger et al. (29).

²These monoclonal antibodies may be obtained by writing to Linda K. Curtiss, Ph.D., Department of Immunology, Research Institute of Scripps Clinic, La Jolla, CA 92037, or to Joseph L. Witztum, M.D., Division of Endocrinology/Metabolism, University of California, San Diego, La Jolla, CA 92093, or to the authors of this paper.

Examining amplified DNA by allele-specific oligonucleotide probes

To detect the nucleotide substitution at apoB cDNA nucleotide 421, we used slot blots and allele-specific oligonucleotide probes. One-tenth of the amplification reactions were loaded onto nitrocellulose membranes, using a slot blot apparatus. After the membranes were baked for 2 hr at 80°C, they were incubated in a prehybridization buffer (5 × SSPE, 5 × Denhardt's solution, 0.1% SDS, and 200 μg/ml herring sperm DNA) for 16 hr at 37°C. The slot blots were then probed with either ³²P-labeled oligonucleotide MB19-Thr₇₁ (5'-CAGTGCACCCTGAAA-3') (apoB cDNA nucleotides 414–428, and coding for Thr at amino acid 71) or ³²P-labeled oligonucleotide MB19-Ile₇₁ (5'-CAGTGCATCCTGAAA-3') (apoB cDNA nucleotides 414–428 and coding for Ile at amino acid 71). Oligonucleotides were end-labeled with ³²P as described (27). The slot blots were incubated with the labeled oligonucleotide probes in hybridization buffer (identical to prehybridization buffer) for 16 hr at 37°C, then washed at 40°C for 1 hr in 2 × SSC, containing 0.1% SDS, and then exposed on X-ray film for 20 min.

ApaLI restriction endonuclease digestion of amplified DNA

One-tenth of each amplification reaction was digested with 10 units of *Apa*LI (New England Biolabs) for 16 hr at 37°C in the low-salt buffer recommended by the supplier. The *Apa*LI-digested amplified DNA was subjected to electrophoresis on an 8% polyacrylamide gel, and the gel was stained with ethidium bromide. The 141-bp band amplified from the genomic DNA of some individuals was cut completely into 91- and 50-bp fragments (*Apa*LI +/+); in other individuals, the 141-bp band was not cut at all (*Apa*LI -/-); in other individuals, only one-half of the 141-bp fragment was cut into 91- and 50-bp fragments (*Apa*LI +/-).

RESULTS

MB19 binding phenotype

The MB19 binding pattern of each of the 62 individuals was determined by RIA (1, 8). Thirty two were MB19₁/MB19₂ heterozygotes, 9 were MB19₁/MB19₁ homozygotes, and 21 were MB19₂/MB19₂ homozygotes.

Enzymatic amplification of the apoB gene

The polymerase chain reaction technique has proven to be very useful for genetic analysis of mutations in single copy genes (30–32). In this study, we used the polymerase chain reaction technique to amplify almost the entire fourth exon of the apoB gene, which codes for apoB-100

amino acid residues 53–101. Our attention was directed to this region of the apoB gene because four research groups had reported a Thr residue at amino acid 71, whereas two others had reported an Ile residue at amino acid 71 (25). Enzymatic amplification of this region of the gene was successful; without exception, a single band of the expected size (141 bp) was observed on ethidium bromide-stained polyacrylamide gels (see Fig. 3 below).

Our initial approach was to subclone the amplified DNA into M13 for DNA sequence analysis (27). Subcloning the amplified DNA was greatly facilitated by inclusion of restriction endonuclease sites in the oligonucleotide primers, as suggested by Scharf, Horn, and Erlich (33). Two M13 subclones derived from the DNA amplified from λ5C DNA were sequenced; each yielded Thr at amino acid 71, as previously reported (25). Three M13 subclones from an MB19₂/MB19₂ homozygote were sequenced; each yielded Thr at amino acid 71 (Fig. 1B). Two subclones of an MB19₁/MB19₁ homozygote were sequenced, and each yielded Ile at amino acid 71 (Fig. 1A). We identified and sequenced both “Thr” and “Ile” subclones from the amplified DNA of an MB19₁/MB19₂ heterozygote.

We next tested the ability of allele-specific oligonucleotide probes to bind to the enzymatically amplified DNA. Oligonucleotide MB19-Thr₇₁ bound to the DNA amplified from λ5C DNA, and the DNA amplified from the genomic DNA of MB19₂/MB19₂ homozygotes and MB19₁/MB19₂ heterozygotes. It failed to bind to the DNA amplified from MB19₁/MB19₁ genomic DNA (Fig. 2A). In contrast, oligonucleotide MB19-Ile₇₁ bound to the DNA amplified from MB19₁/MB19₂ heterozygotes and MB19₁/MB19₁ homozygotes, but not to the DNA amplified from λ5C or MB19₂/MB19₂ homozygotes (Fig. 2B).

Although probing blots with allele-specific oligonucleotides enabled us to document the existence of the nucleotide substitution at apoB cDNA nucleotide 421, *Apa*LI digestion of amplified DNA proved to be a simpler means of screening samples. The “Thr₇₁ allele” has an *Apa*LI restriction site; the C → T substitution at nucleotide 421 in the “Ile₇₁ allele” eliminates this *Apa*LI restriction site. As shown in Fig. 3, the 141-bp band amplified from λ5C or from genomic DNA of MB19₂/MB19₂ homozygotes was cut completely by *Apa*LI into 91- and 50-bp fragments (*Apa*LI +/+). In contrast, amplified DNA from MB19₁/MB19₁ homozygotes was not cut (*Apa*LI -/-). Only one-half of the 141-bp band amplified from MB19₁/MB19₂ heterozygotes was cut (*Apa*LI +/-).

Of the 62 subjects, 32 had an *Apa*LI +/- pattern. Each of these subjects was an MB19₁/MB19₂ heterozygote by plasma RIA. Twenty one had an *Apa*LI +/+ pattern, and each of these individuals was an MB19₂/MB19₂ homozygote by plasma RIA. Nine had an *Apa*LI -/- pattern, and each of these subjects was an MB19₁/MB19₁ homozygote by plasma RIA.

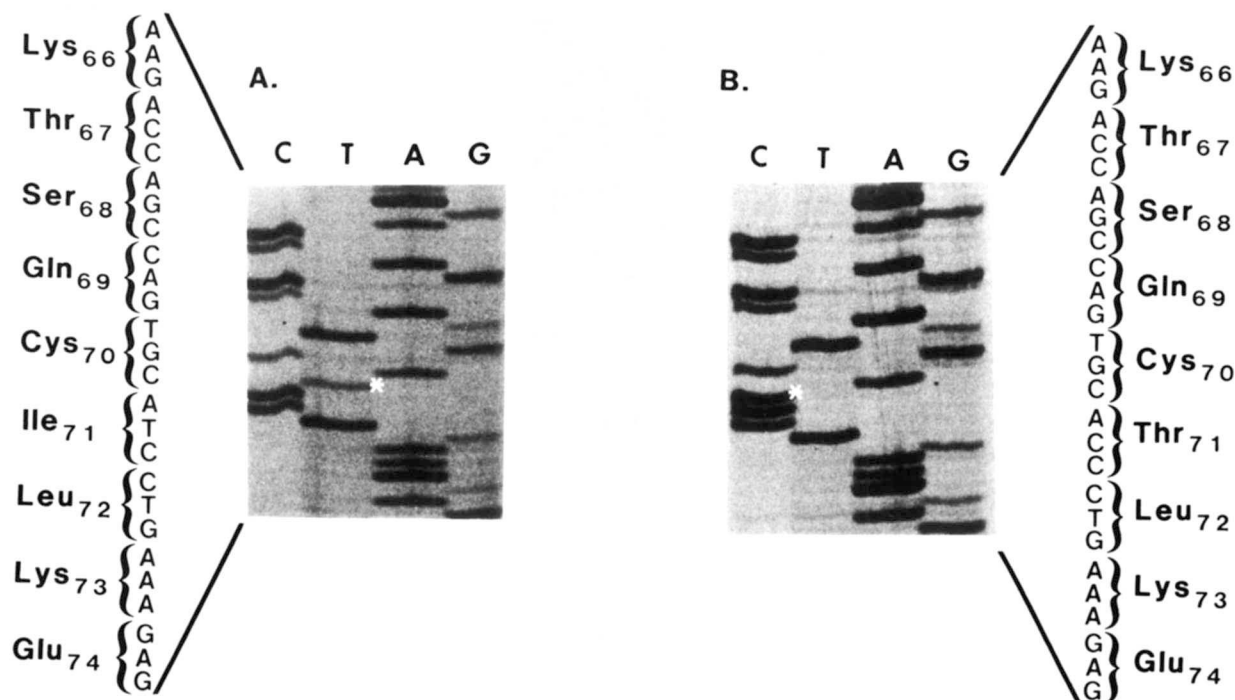


Fig. 1. Autoradiograms of sequencing gels of apoB clones derived from the amplified genomic DNA of an MB19₁/MB19₁ homozygote (panel A) and an MB19₂/MB19₂ homozygote (panel B). The sequence of the clone in panel A predicts an Ile at residue 71, whereas the sequence of the clone in panel B predicts a Thr at residue 71. The asterisk in both autoradiograms designates apoB cDNA nucleotide 421.

DISCUSSION

In this report, we show that a previously described nucleotide substitution in the apoB gene is associated with the MB19 immunochemical polymorphism in apoB. In 62 individuals, there was a perfect association between the MB19 polymorphism and the C→T nucleotide substitution at apoB cDNA nucleotide 421. The MB19₁ allele is associated with a T at nucleotide 421 and an isoleucine residue at amino acid residue 71. The MB19₂ allele is associated with a C at nucleotide 421 and a threonine residue at amino acid 71. Thus far, four groups of investigators studying the apoB sequence have found Thr at residue 71, whereas two have reported Ile (25). Because the MB19 polymorphism is very common, it would not be surprising if the apoB gene polymorphism responsible for the MB19 polymorphism had already been documented by DNA sequence analysis.

The current data are consistent with the hypothesis that the amino acid substitution at residue 71 causes the MB19 polymorphism. First, there was a perfect association between the amino acid substitution at residue 71 and the MB19 immunochemical polymorphism. It is certainly conceivable that the substitution of a polar amino acid residue, threonine, for a nonpolar residue, isoleucine, could lead to a significant structural alteration in the epi-

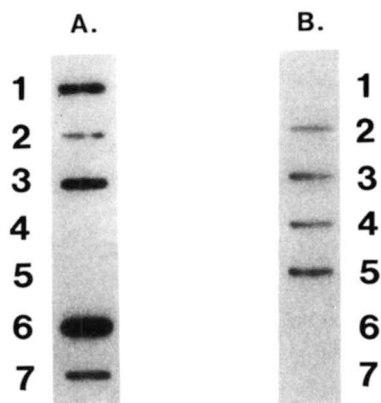


Fig. 2. Detection of the Thr₇₁→Ile substitution in enzymatically amplified DNA samples using allele-specific oligonucleotide probes. The region of the apoB gene flanking amino acid 71 was enzymatically amplified, as described under Methods. One-tenth of each of the amplification reactions was loaded onto nitrocellulose membranes using a slot blot apparatus. The nitrocellulose membranes were then probed with ³²P-labeled oligonucleotide MB19-Thr₇₁ or ³²P-labeled oligonucleotide MB19-Ile₇₁, as described under Methods. Panel A shows the autoradiogram of the slot blot probed with [³²P]MB19-Thr₇₁; panel B, the autoradiogram of the slot blot probed with [³²P]MB19-Ile₇₁. Slot 1 shows the DNA amplified from λ5C DNA, previously reported to have a Thr residue at amino acid 71 (25). Slots 2 and 3 show the amplified DNA of two subjects determined to be MB19₁/MB19₂ heterozygotes by plasma RIA; slots 4 and 5, the amplified DNA of two subjects shown to be MB19₁/MB19₁ homozygotes by plasma RIA; lanes 6 and 7, the amplified DNA of two subjects shown to be MB19₂/MB19₂ homozygotes by plasma RIA.

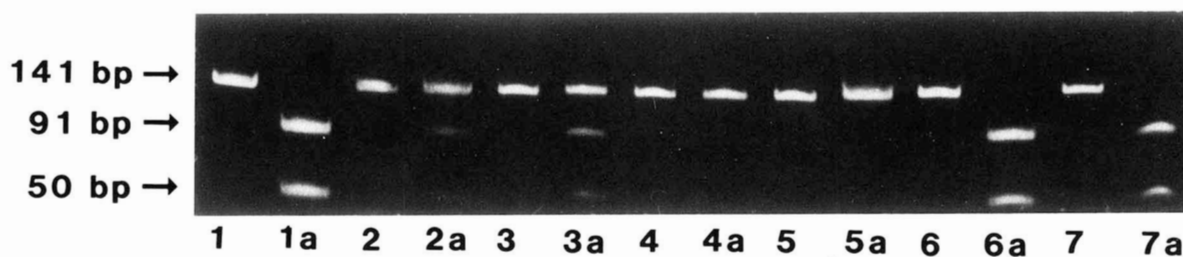


Fig. 3. Digestion of enzymatically amplified DNA with *Apa*LI. Amplification of genomic DNA was performed as outlined under Methods. One-tenth of the amplification reaction was loaded directly (no restriction endonuclease digestion) onto an 8% polyacrylamide gel (lanes 1–7); one-tenth of each of the seven amplification reactions was subjected to an overnight digestion with *Apa*LI and then loaded onto the gel (lanes 1a–7a). The gel was stained with ethidium bromide. Lane 1 shows the DNA amplified from λ 5C DNA; lanes 2 and 3; and DNA amplified from the genomic DNA of two MB19₁/MB19₂ heterozygotes; lanes 4 and 5, the DNA amplified from the genomic DNA of two MB19₁/MB19₁ homozygotes; lanes 6 and 7, the DNA amplified from the genomic DNA of two MB19₂/MB19₂ homozygotes.

tope for a monoclonal antibody. Furthermore, we know that antibody MB19 binds within residues 1–1297, and the observed amino acid substitution is at residue 71. Also, we know that the epitope for antibody MB19 is changed by disulfide reducing agents (15), and amino acid 71 is in a cysteine-enriched portion of apoB. Indeed, there is a pair of cysteine residues at apoB amino acid residues 61 and 70 (16). Furthermore, by using enzymatic amplification and restriction endonuclease digestion, we have recently analyzed the relationship between the MB19 polymorphism and a nucleotide substitution at apoB-100 cDNA nucleotide 1981. This nucleotide substitution results in an Ala→Val amino acid substitution at amino acid residue 591. We found that the substitution at residue 591 is definitely not related to the MB19 polymorphism (unpublished observations, S. G. Young and S. T. Hubl). Recently, Wang and co-workers (34) have demonstrated a perfect relationship between the amino acid substitution at residue 591 and the Ag(a₁)/Ag(d) polymorphism, which is an apoB genetic polymorphism unrelated to the Ag(c)/Ag(g) (or MB19) polymorphism.

All of these data suggest strongly that the amino acid polymorphism at residue 71 is synonymous with the MB19 polymorphism. However, we recognize that the data do not definitively exclude an alternative hypothesis: that the MB19 polymorphism is actually caused by another, as-yet-undiscovered amino acid sequence polymorphism that is invariably linked to the amino acid substitution at residue 71. We believe that the perfect association between the substitution at residue 71 and the MB19 polymorphism in 62 subjects makes the latter hypothesis unlikely. Furthermore, Schumaker and co-workers have independently found a perfect association between the Ag(g)/Ag(c) (or MB19) polymorphism and the amino acid substitution at residue 71 in 17 individuals. They also found that this immunochemical polymorphism was not related to amino acid substitutions at apoB-100 residues 302, 591, and 766 (personal communication, V. Schumaker).

Demonstrating that apoB-100 amino acid residue 71 is contained within the epitope for antibody MB19 would strongly support the hypothesis that this amino acid substitution causes the MB19 polymorphism. One means of localizing the MB19 epitope on apoB-100 would be to isolate and purify an apoB-100 proteolytic peptide containing the MB19 epitope from an MB19₁/MB19₁ homozygote and from an MB19₂/MB19₂ homozygote. Once the two peptides were purified, it would be necessary to sequence them completely. If the substitution at residue 71 was the only sequence difference identified, one could logically infer that it was the biochemical basis of the MB19 polymorphism. However, we have found that the epitope for antibody MB19 is destroyed by subjecting apoB to denaturing conditions and then cleaving it into small fragments. For example, on Western blots, antibody MB19 does not bind to any peptide in a cyanogen bromide digest of apoB-100. Nor does antibody MB19 bind to any of the small peptides formed when LDL particles are incubated with sodium deoxycholate and then extensively digested with trypsin (unpublished observations, S. G. Young). Thus, identifying and purifying a small peptide containing the MB19 epitope would be quite difficult.

With the data presented in this paper and the data contained in personal communication by Schumaker, it is our hypothesis that the substitution at amino acid 71 causes the MB19 polymorphism. It is our hope that the association between the MB19 polymorphism and this amino acid substitution will be examined in other laboratories. If a linkage between the two polymorphisms is invariably observed in large numbers of subjects in different human populations, it would strongly support a causal link between them. Fortunately, the procedure for MB19 phenotyping by RIA and the method for assessing the amino acid substitution at residue 71 (enzymatic amplification and *Apa*LI digestion) can be performed simply and rapidly.

Other laboratories have independently developed apoB-specific monoclonal antibodies that detect the same epitope as antibody MB19 (35, 36). Duriez and co-workers

(35) reported that an apoB-specific antibody, BIP 45, detects the MB19 polymorphism, and they are using antibody BIP 45 to investigate the possible role of apoB allotypes as genetic risk factors in atherosclerosis. Schlapfer and his co-workers (36) have reported that 20 of 128 hybridomas producing antibodies against LDL appeared to detect genetic variations in apoB. One of these antibodies, D2E1, detects the Ag(c)-Ag(g) polymorphism, or MB19 polymorphism, in apoB. Because of the apparent ease of developing murine monoclonal antibodies capable of detecting the MB19 polymorphism, it seems likely that the MB19 epitope on human apoB is very immunogenic in mice. ■

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