

Monoclonal antibodies specific for different regions of human apolipoprotein A-I. Characterization of an antibody that does not bind to a genetic variant of apoA-I (Glu₁₃₆ → Lys)

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Abstract Three monoclonal mouse hybridoma antibodies, designated 2AI, 4AI, and 5AI, specific for human plasma apolipoprotein A-I (apoA-I) were characterized. In an enzyme-linked immunosorbent assay (ELISA) each of the antibodies reacted with purified apoA-I and with A-I in normal human serum. Immunoblotting of apoA-I subjected to isoelectric focusing revealed that the three antibodies reacted with all the charge isomorphs of apoA-I and with proapoA-I. Using a solid phase competitive displacement assay, the antigenic determinant for antibody 5AI could be localized to cyanogen bromide fragment 3 of apoA-I (residues 113–148), while the epitope for antibody 4AI resided in cyanogen bromide fragment 4. Dot blot experiments and data obtained by the competitive displacement assay revealed that antibody 2AI reacts with high affinity with CNBr fragment 2 but that it also reacts with lower affinity with fragments 1 and 4. The antibody 5AI did not bind to a genetic variant of apoA-I (Glu₁₃₆ → Lys), demonstrating that the substitution of a single amino acid in human apoA-I can cause the loss of an antigenic determinant.—Ehnholm, C., M. Lukka, I. Rostedt, and K. Harper. Monoclonal antibodies specific for different regions of human apolipoprotein A-I. Characterization of an antibody that does not bind to a genetic variant of apoA-I (Glu₁₃₆ → Lys). *J. Lipid Res.* 1986. 27: 1259–1264.

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Epidemiological studies have implicated high density lipoproteins (HDL) in the pathogenesis of atherosclerosis (1, 2). However, the mechanism underlying the inverse relationship between their concentration in plasma and the incidence of coronary heart disease is unknown. The major apolipoproteins of HDL, A-I and A-II, are well characterized (3). Apolipoprotein A-I (apoA-I) is a polypeptide of 243 amino acids with a known sequence (4). It is an activator of lecithin:cholesterol acyltransferase (5) and in vitro experiments have suggested that it may be involved in the delivery of cholesterol from peripheral tissues to the liver (6). Thus apoA-I plays a central role in lipoprotein metabolism.

In order to study the structure–function relationship of apoA-I, we prepared monoclonal antibodies using the mouse hybridoma technique. In this report we describe the specificities of three monoclonal apoA-I antibodies.¹

MATERIAL AND METHODS

Chemicals for polyacrylamide gel isoelectric focusing and 0.45-μm nitrocellulose sheets were from Bio-Rad (Richmond, CA). Ampholytes and equipment (LKB 2001) for isoelectric focusing were from LKB (Bromma, Sweden). Rabbit anti-mouse, peroxidase-conjugated second antibody was from Dakopatts (Denmark). Rabbit anti-mouse, alkaline phosphatase-conjugated second antibody was from Orion Diagnostica (Espoo, Finland). The microtitration plates (Immunoplate I) were from Nunc (Denmark). Titertek Multiskan apparatus (EFLAB Oy, Helsinki, Finland) was used for reading of microtitration plates. Protein A-Sepharose CL-4B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden).

Blood was collected from the antecubital vein into tubes containing EDTA (1 mg/ml). The plasma was separated after centrifugation at 2500 rpm for 15 min.

Isolation of lipoproteins, apolipoprotein A-I, and cyanogen bromide fragments

HDL (density 1.12–1.21 g/ml) was isolated by sequential ultracentrifugation as described by Havel, Eder, and Bragdon (7). ApoA-I was purified as described (8). The cyanogen bromide fragments were prepared, purified,

Abbreviations: HDL, high density lipoproteins; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

¹ Limited amounts of the monoclonal antibodies can be obtained upon request from Dr. C. Ehnholm, National Public Health Institute, Helsinki, Finland.

and characterized as described by Rall et al. (9, 10). Cyanogen bromide fragments characterized by high performance liquid chromatography and sequencing were a kind gift from Dr. Stanley Rall and Dr. Karl Weisgraber, Gladstone Foundation Laboratories, San Francisco, CA.

Isoelectric focusing

Isoelectric focusing of purified apoA-I and serum (5 μ l) was done as described earlier using pH 4–6 ampholyte (11). After electrophoresis, the separated proteins were transferred to nitrocellulose sheets as described (11), and visualized using the monoclonal antibodies in a dilution of 1:1000 and peroxidase-conjugated rabbit anti-mouse (Dakopatts RAM P260) as second antibody.

Production of monoclonal antibodies

Twelve-week-old female BALB/c mice were injected intraperitoneally with human apoA-I in Freund's complete adjuvant. The protein content and volume per mouse was 80 μ g and 0.5 ml, respectively. Four weeks later a second intraperitoneal injection, 80 μ g of apoA-I in saline per mouse, was given. After 1 week, a booster of 25 μ g apoA-I in saline was given intraperitoneally and cell fusion was performed 3 days later (10^8 spleen cells + 10^7 SP-2 myeloma cells in 0.5 ml of 50% polyethylene glycol).

After 10 days in culture, 62 wells containing hybrid cells were found and 12 wells showed anti-A-I activity in ELISA. Five antibody-producing cell lines were established of which lines 2AI, 4AI, and 5AI were studied further.

As culture medium, Earle's minimum essential medium (MEM) with added glucose, glutamine, antibiotics, and sodium bicarbonate was used throughout. The content of fetal calf serum was 5–10%.

Enzyme-linked immunosorbent assay (ELISA)

Coating. The purified apoA-I was diluted in coating buffer (0.05 M carbonate buffer, pH 9.6) to a final concentration of 1 μ g/ml. Of this dilution, 150- μ l aliquots were pipetted into the wells of microtitration plates. For the binding of apoA-I to well walls, the plates were incubated for 4 hr at room temperature. The wells were thereafter emptied and the remaining binding sites were saturated with 3% bovine serum albumin in coating buffer for 3 hr at room temperature. The wells were emptied, covered with sealing tape, and stored at -20°C .

Screening for anti-A-I-producing clones

The apoA-I-coated wells were washed with phosphate-buffered saline, pH 7.4 (PBS), containing 0.05% Tween (washing buffer). To the wells 100 μ l of growth medium from the hybridoma cultures was added. After incubation for 3 hr at room temperature, the wells were emptied

and washed with washing buffer. They were then incubated for 1 hr with 100 μ l of a 1:100 dilution of alkaline phosphatase-conjugated anti-mouse IgG. After this incubation the plates were rinsed three times with washing buffer, whereafter 100 μ l of 0.1% disodium *p*-nitrophenyl phosphate in 0.05 M carbonate buffer, pH 9.8, containing 1 mM MgCl_2 was added to each well. After 30 min the reaction was stopped by adding 100 μ l of 1 N NaOH to the wells. The plates were then read at 405 nm.

ELISA displacement assay

The apoA-I-coated wells were washed with washing buffer. To each well a constant amount of monoclonal apoA-I antibody in 50 μ l of PBS containing 1% bovine serum albumin and 4% polyethylene glycol 6000 (dilution buffer) was added. Thereafter, 50 μ l of dilution buffer containing increasing amounts of purified apoA-I, serum, or cyanogen bromide fragments of apoA-I were pipetted into the wells. After a 3-hr incubation at room temperature, the wells were washed, treated with second antibody, and read as described above. The results were expressed as B/B_0 (where B = optical density in the presence of competing apoA-I, cyanogen bromide fragment of apoA-I or serum and B_0 = optical density in the absence of competitor).

DOT-immunobinding assay

This was done essentially as described (12). Approximately 150 pmol of antigen (1–3 μ l) was placed on nitrocellulose paper. After drying, the paper was handled as described for immunoblotting after isoelectric focusing.

Purification of antibodies

The monoclonal antibodies were purified from the cell culture medium using affinity chromatography on protein A-Sepharose. To identify the immunoglobulin class and subclass, the antibodies were tested by double diffusion against antisera specific for mouse immunoglobulin classes. (Miles Laboratories, USA).

RESULTS

Three different mouse hybridomas that secrete antibodies binding to human apolipoprotein A-I were the products of cell fusion between the cell line SP-2 and spleen cells of BALB/c mice immunized with human apoA-I. They were selected by enzyme-linked immunosorbent assay (ELISA) and designated 2AI, 4AI, and 5AI. The antibodies were purified from the culture medium using protein A affinity chromatography. All three antibodies were of the IgG-1 subclass.

In order to study the binding of these antibodies to the different isomorphs of apoA-I, isoelectric focusing of

normal human serum (NHS) and serum from persons having a genetic variant form of apoA-I (Glu₁₃₆ → Lys) was performed. The reaction patterns after immunoblotting using the different monoclonal antibodies are shown in Fig. 1. Each of the three antibodies reacted with all isomeric forms of apoA-I and with proapoA-I. Two of the antibodies, 2AI and 4AI, also detected the isoforms of the A-I genetic variant. However, one of the antibodies, 5AI, did not bind to the isoforms of the variant apoA-I.

Immunoblotting of normal and mutant apoA-I after separation by SDS-gel electrophoresis revealed that antibody 5AI bound to normal apoA-I but not to the mutant form.

To study whether this difference in the reactivity of antibody 5AI with the mutant apoA-I (Glu₁₃₆ → Lys) also was evident in plasma, inhibition experiments using the ELISA method were performed. Plasma from subjects with normal apoA-I inhibited the binding of all three antibodies to immobilized apoA-I in a dose-dependent manner as shown for 4AI and 5AI in Fig. 2. When, however, plasma from a subject with the mutant apoA-I was used as competitor, the binding of 2AI and 4AI was inhibited in a normal fashion while that of 5AI was not (Fig. 2). To demonstrate that the lack of immunoreactivity of the mutant apoA-I with 5AI was not related to a change in apoA-I conformation, the inhibition studies were repeated with plasma partially delipidated using tetramethylurea and with heat-denatured (+52°C, 30 min) plasma. The results were almost identical to those obtained with nonheated plasma (data not shown). As the molecular difference in the variant A-I, as compared to normal A-I, is a substitution of one amino acid (Glu₁₃₆ → Lys) (10), these data

indicated that the antigenic determinant for 5AI might include this residue.

In an attempt to localize the epitopes for the three monoclonal antibodies, the four cyanogen bromide fragments of apoA-I (CNBr 1 residues 1–86, CNBr 2 residues 87–112, CNBr 3 113–148, CNBr 4 149–243) were prepared and separated. Dot-immunobinding assays employing purified CNBr-fragments immobilized on nitrocellulose indicated that the antibody 2AI reacted strongly with apoA-I and CNBr 2, but faint staining was also observed with CNBr fragments 1 and 4. Antibody 5AI recognized apoA-I and CNBr 3, while antibody 4AI recognized apoA-I and CNBr 4. Using the ELISA displacement assay, it could be demonstrated that CNBr 3 prepared from normal apoA-I specifically inhibited the binding of antibody 5AI to immobilized apoA-I, while CNBr 3 from mutant apoA-I did not (Fig. 3). The binding of antibody 4AI was specifically inhibited by CNBr 4, Fig. 4A, indicating that the epitope for this antibody resides in the C-terminal CNBr fragment of apoA-I. As is evident from Fig. 4A, CNBr 4 is not as good a competitor as apoA-I; thus, at a concentration of 1 nmol of CNBr 4/ml, the inhibition is about 40% while apoA-I at 1 nmol/ml gives about 90% inhibition. The inhibition can be further increased from 40 to 60% by increasing the concentration of CNBr 4 from 1 to 2 nmol/ml. The difference between CNBr 4 and apoA-I may result from the fact that it is not known how much of the purified CNBr 4 fragment is in the proper configuration to be immunoreactive. It is also technically difficult to reach high enough concentrations to obtain complete inhibition. The results obtained using antibody 2AI were not as clear. Although CNBr 2 showed

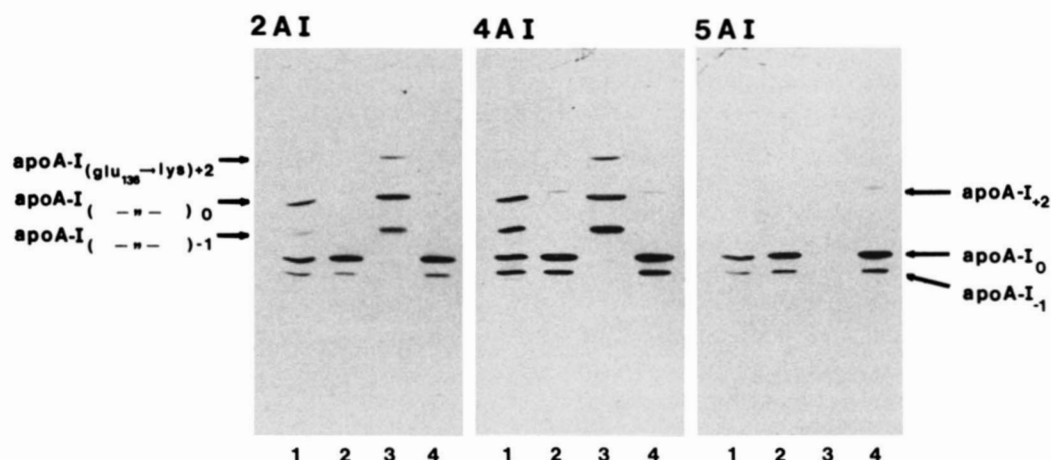


Fig. 1. Identification of apolipoprotein A-I from normal human serum and from sera of subjects with a variant apoA-I by immunoblotting with monoclonal antibodies after isoelectric focusing. Isoelectric focusing and immunoblotting were performed as described in Materials and Methods. The monoclonal antisera used are as indicated in the figure. The samples were: serum from a subject heterozygous for the apolipoprotein variant A-I (Glu₁₃₆ → Lys), lane 1; normal human serum, lanes 2 and 4; serum from a subject homozygous for the genetic apoprotein variant A-I (Glu₁₃₆ → Lys), lane 3. Five μ l of serum was applied to each lane. The isoforms of the variant A-I are indicated on the left and those of normal A-I on the right side.

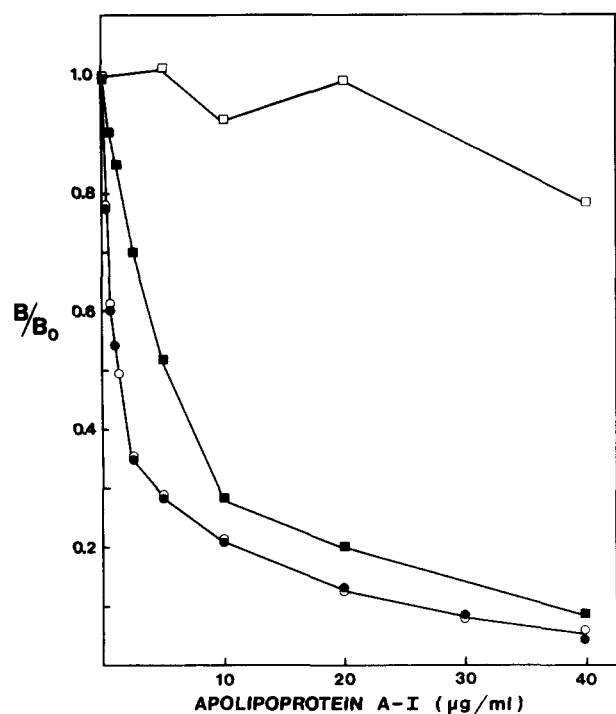


Fig. 2. Ability of plasma from a subject homozygous for a genetic variant of apolipoprotein A-I, apoA-I (Glu \rightarrow Lys), to compete with the monoclonal antibodies 5AI and 4AI for binding to normal apoA-I. Dilutions of each plasma, expressed as the amount of apolipoprotein A-I added (measured using polyclonal antibodies), were added as competitors to the solid phase ELISA assay. Using the monoclonal antibody 4AI, each serum, (●, normal human serum; ○, serum from a homozygous apoA-I (Glu \rightarrow Lys)) had identical competition curves. With the antibody 5AI, normal human serum (■) showed a similar inhibition pattern, while the addition of serum containing the variant A-I (□) gave rise to very little inhibition of antibody binding.

the best inhibitory capacity, inhibition of binding was also observed using CNBr fragments 1 and 4, Fig. 4B. Thus antibody 2AI demonstrates strong immunoreactivity with CNBr 2 and weak immunoreactivity with CNBr 1 and 4.

DISCUSSION

Previous studies (13, 14) have indicated that monoclonal antibodies recognizing different epitopes of apoprotein A-I, the main apoprotein of HDL, can be prepared by immunizing mice with HDL or purified apoA-I. We have generated three mouse hybridomas that produce antibodies recognizing different epitopes on apoA-I. Our strategy for localizing the antibody binding region on the apoprotein was based on digestion of apoA-I with cyanogen bromide and purifying the four cyanogen bromide fragments of apoA-I. The fragments were then immobilized on nitrocellulose sheets and visualized by incubation

with monoclonal antibody followed by enzyme-linked second antibody. This method is convenient and should be useful in mapping epitopes in other proteins also. The results were verified using an ELISA inhibition assay. The epitope for the antibody 5AI could be demonstrated to reside on CNBr fragment 3. Our finding that a point mutation in the mid-portion of CNBr 3 (Glu \rightarrow Lys) dramatically alters the epitope for 5AI localizes this antigenic determinant to the center of CNBr 3. Point mutations that effect the immunological behavior of proteins have been reported previously for haptoglobins (15) and hemoglobins (16). Whether the single amino acid mutation as such can effect the binding of an antibody or whether it leads to changes in secondary structure which then abolish binding is not known. However, our observation that the isolated cyanogen bromide fragment derived from mutant apoA-I does not compete for antibody binding to A-I also suggests that Glu is important for binding. Previous studies have indicated that the variant A-I may be correlated to a defect in the cholesteryl ester transfer process (10). Therefore, more subjects with this apoA-I

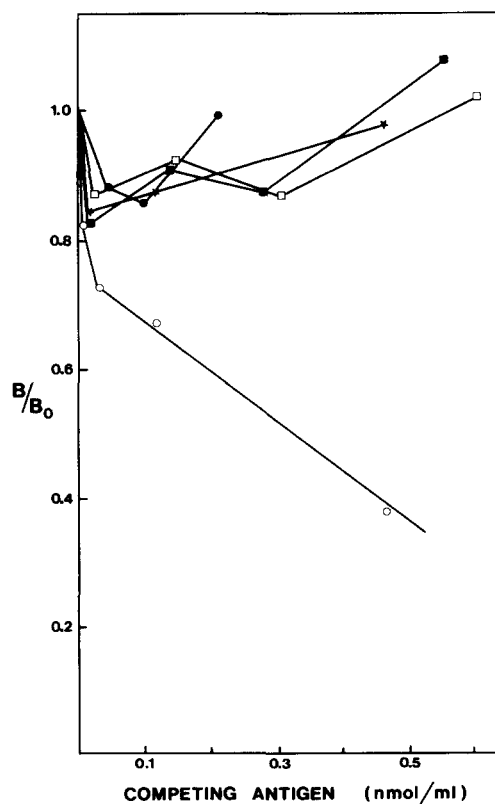


Fig. 3. Ability of cyanogen bromide fragments of apoA-I to compete with normal apoA-I for monoclonal antibody (5AI) binding. Microtiter wells were coated with apoA-I, as described in Material and Methods. Increasing amounts of purified cyanogen bromide fragments (CNBr) were used as competitors. CNBr 1, □; CNBr 2, ●; CNBr 3, ○, fragment prepared from normal A-I; CNBr 3, *, fragment prepared from apoA-I (Glu \rightarrow Lys); CNBr 4, ■.

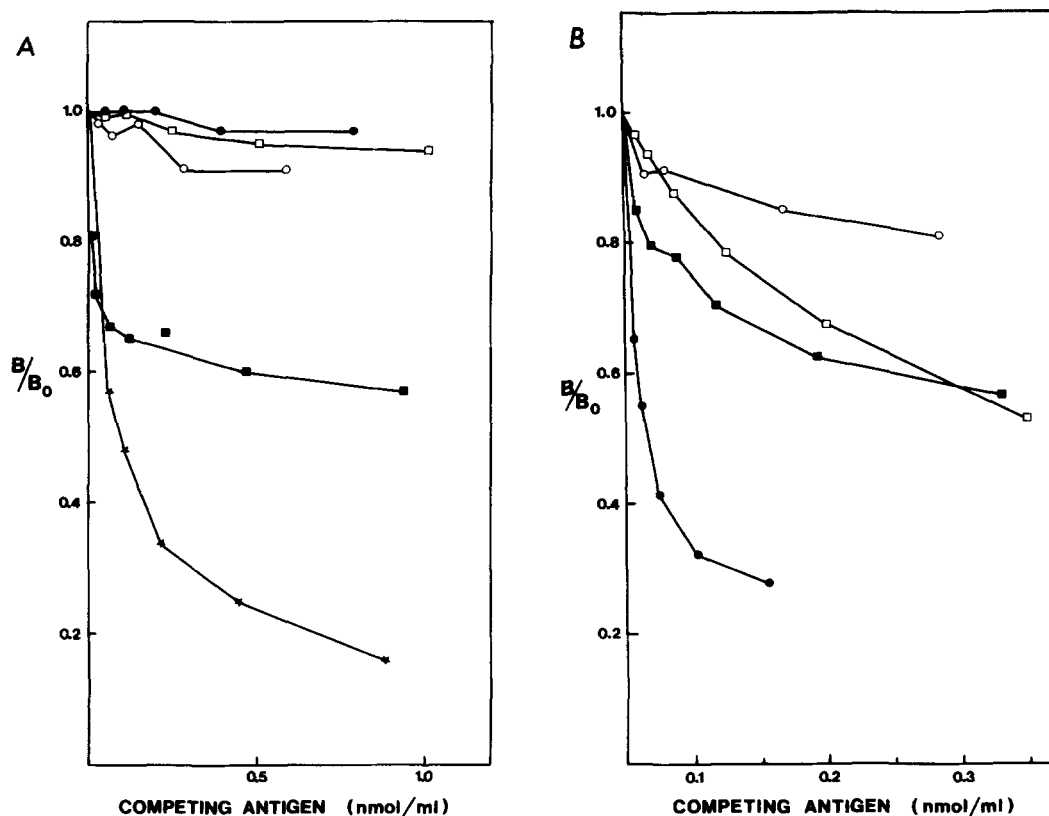


Fig. 4. Ability of cyanogen bromide fragments of apoA-I to compete with normal apoA-I for binding of monoclonal antibody 4AI (panel A) and 2AI (panel B). The conditions for the assay are as stated in the legend of Fig. 3. CNBr 1, □; CNBr 2, ●; CNBr 3, ○; CNBr 4, ■; apoA-I, *.

variant should be studied in order to clarify whether this correlation is valid. The antibody 5AI should be useful in screening for this apoA-I mutant.

The antibody binding site for 4AI could be localized to the carboxy terminal cyanogen bromide fragment of apoA-I. To our knowledge, no monoclonal antibody specific for this region has been reported so far. The unpublished observations by Weech et al. (14) that monoclonal antibodies directed against CNBr fragments 1, 2, and 3 do not inhibit apoA-I-mediated activation of lecithin:cholesterol acyltransferase (LCAT) may mean that this antibody could be helpful in elucidating the mechanism of LCAT activation. The localization of the antigenic site for the antibody 2AI using CNBr fragments of apoA-I was not equally clear. Although the binding of 2AI was preferentially inhibited by CNBr 2, the fragments 1 and 4 also caused inhibition. The epitope for 2AI, which should be common for these three fragments, is at present not known.

The apoA-I monoclonals reported in this study reacted with all of the charge isomorphs of apoA-I and with proapoA-I, as was also observed by Curtiss and Edgington (13) and Weech et al. (14) using their monoclonal antibodies. Thus all of the isoforms, as studied by isoelectric

focusing, seem to carry all three epitopes. The isoelectric focusing pattern of serum from a subject heterozygous for the A-I variant allele revealed that normal and variant A-I are present in equal amounts and that similar charge isomorphs occur for both A-I populations, the only difference being that all the variant isomorphs have a more cathodal isoelectric point relative to normal A-I.

For better standardization of the immunochemical quantitation of apolipoproteins, the use of monoclonal antibodies has been suggested. However, our results clearly demonstrate that this approach has its limitations. For example, if the antibody 5AI were to be used to quantitate the apoA-I in plasma of a subject homozygous for A-I (Glu₁₃₆ → Lys), a totally wrong result would be obtained. Therefore, if monoclonal antibodies are to be used for quantitative analysis, they should be well characterized and mixtures of several antibodies should be used.

In conclusion, our study demonstrates that mouse hybridoma antibodies specific for different regions of apoA-I can be produced and that these should be powerful tools for elucidating the structure-function relationships of apoA-I. It also illustrates the exquisite specificity of the immunological recognition, as the substitution of a single amino acid in the amino acid sequence of human apoli-

poprotein A-I containing 243 amino acids can cause the loss of an antigenic determinant. *IB*

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