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GENERATION OF ANTI-HUMAN APOLIPOPROTEIN E ISOFORM-SPECIFIC MONOCLONAL ANTIBODIES

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Abstract:

Human apolipoprotein (apo)E is a 299 amino acid protein which is polymorphic in terms of its primary structure. As a ligand for the low density lipoprotein (LDL) receptor, it plays an important role in lipoprotein metabolism. The most common apoE isoforms, apoE2, apoE3 and apoE4 are encoded by 3 co-dominantly expressed alleles and differ by single amino acid substitutions. ApoE2 is defective in its ability to bind to the LDL receptor and homozygous inheritance of the allele encoding apoE2 is associated with hyperlipidemia. ApoE genetic polymorphism also influences plasma lipid levels, postprandial lipemia, apoE distribution in plasma lipoprotein subclasses and susceptibility to atherosclerosis and Alzheimer's disease.

The objectives of the present study were: i) to establish a novel immunization protocol for the preparation of monoclonal antibodies (mAbs) specific for epitopes that are polymorphic in humans, ii) to use this protocol to prepare anti-apoE mAbs that are either apoE isoform-specific or are antibody mimetics of the LDL receptor, i.e. that recognize the same conformational epitope of apoE as is recognized by the LDL receptor. To obtain mAbs of the desired specificities, we have immunized mice that carry a transgene encoding apoE of one isoform with a second isoform of human apoE. As transgenic mice normally treat the product of the transgene as self, the immune response should be restricted to epitopes that are present on the apoE used for immunization and absent from the apoE encoded by the transgene. Variations of two basic immunization protocols were used. In protocol 1, mice that carried an apoE3 (Cys^{112},Arg^{142},Arg^{158}) transgene were immunized with either apoE4(Arg^{112},Arg^{142},Arg^{158}), a natural apoE variant

apoE(Arg^{112},Cys^{142},Arg^{158}) that is defective in binding to the LDL receptor, or both. Using protocol 1 we hoped to obtain anti-apoE4-specific mAbs. In protocol 2, apoE (Arg^{112},Cys^{142},Arg^{158}) transgenic mice were immunized with apoE3 and apoE4. We anticipated that we would generate mAbs that recognize apoE3 but not apoE4 and, as apoE (Arg^{112},Cys^{142},Arg^{158}) does not bind to the LDL receptor, whereas both apoE3 and apoE4 bind well, we hoped to obtain mAbs that recognize the same conformational apoE epitope that is recognized by the LDL receptor.

In general, mice showed a weak anti-apoE immune response that had the predicted isoform specificity. Only one of the nine fusions that were performed generated stable, apoE-specific hybridomas (protocol 1). Of the 7 anti-apoE mAbs that were obtained, none possessed the isoform-specificity that would have been predicted from immunization protocol 1. The specificity of two of the mAbs, 9F3 and 7D4 were, however, analyzed in some detail. Both 9F3 and 7D4 reacted well with both apoE3 and apoE4 but failed to react with apoE2. The epitopes for both mAbs were mapped to a region that coincides with the apoE LDL receptor-binding site (residues 136-158). The reactivities of 7D4 and 9F3 with a series of apoE variants indicate that the fine specificities of the mAbs are identical to that of the LDL receptor. These two mAbs can, therefore, be considered as candidates for mAb mimetics of the LDL receptor. As would be expected, other anti-apoE mAbs, whose epitopes had been previously mapped to apoE residues 140-150, could effectively compete with 7D4 and 9F3 for binding to immobilized apoE. Surprisingly, however, a mAb specific for an epitope in the amino terminal 15 amino acids

was also an effective competitor. I propose a model of apoE tertiary structure that is consistent with these observations.

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I would like to thank the staff and students in the Lipoprotein Group in the Heart Institute of Civic Hospital and especially to Xingyu Wang.

I would like to dedicate my thesis to my father for providing an enriched and lovely environment, to my mother who taught me patience and faith, to my fantastic children, and especially to my husband for his encouragement and support.

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AD	Alzheimer's disease
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
Arg	Arginine
ARP	Arginine rich lipoproteins
CE	Cholesterol esters
CH	Cholesterol
cDNA	Complementary DNA
CMR	Chylomicron remnants
Cys	Cystein
Glu	Glutamine
HDL	High density lipoproteins
HTG	Hypertriglyceridemia
IDL	Intermediate density lipoproteins
kDa	killo Dalton
LDL	Low density lipoproteins
LDLr	Low density lipoprotein receptors
LP	Lipoproteins
Lys	Lysine
PL	Phospholipids
TG	Triglycerides

VLDL Very low density lipoproteins

CHAPTER I. INTRODUCTION

Water insoluble lipids in human plasma are cholesterol (CH), triglycerides (TG), phospholipids (PL) and cholesterol esters (CE) which are transported in blood as macromolecular complexes of lipids and proteins known as plasma lipoproteins (LP). Plasma LP are classified according to their hydrated densities into subclasses that include the high density lipoproteins (HDL), low density lipoproteins (LDL), intermediate density lipoproteins (IDL), and very low density lipoproteins (VLDL) and chylomicrons (CM). Plasma apolipoproteins are responsible for transport and metabolism of LP among different tissues and cells. Apolipoproteins play three important roles. First, apolipoproteins stabilize the pseudomicellar structure of LP particles by binding to lipids. Second, apolipoproteins accelerate lipid metabolism by activating different enzymes or lipid transport proteins involved in remodeling of LP. A third important role for apolipoproteins is the ability to function as ligands that mediate LP binding to cell surface LP receptors. The best characterized apolipoproteins ligands are apoE and apoB. After binding of apoE and apoB to the receptors, the LP are targeted to intracellular lysosomes where they are enzymatically degraded. Therefore, apoE and apoB have a key role in LP metabolism and in the control of the CH homeostasis (Brown and Goldstein, 1986).

The importance of apoE in LP metabolism is very apparent in mice who have been made deficient in apoE by gene targeting. These mice are characterized by both hyperlipoproteinemia and premature atherosclerosis (Breslow et al., 1993). ApoE also appears to have functions that are unrelated to the transport of plasma LP, most notably, in the peripheral and central nervous systems. In subsequent sections, I will discuss the

structure, metabolism and genetics of apoE and its role in physiological and pathophysiological situations.

ApoE Structure and the Genetic Basis of ApoE Polymorphism:

In 1973, Shore and Shore identified apoE as a component of triglyceride-rich VLDL. They named this protein the "arginine-rich" protein (ARP) because of its high content of arginine compared to other apolipoproteins that were known at that time. The molecular weight of apoE was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as a broad band between 33,000 and 39,000 (Shelburne and Quarfordt, 1974). The observation that apoE eluted in multiple peaks following anion-exchange chromatography was the first indication of apoE heterogeneity (Shore and Shore, 1973). Isoelectric focusing of apoE on polyacrylamide yields a number of distinct bands with pI values ranging from 5 to 6 (Utermann et al., 1975), again suggestive of charge heterogeneity. ApoE polymorphism was shown to be an inherited trait and a two allele model was initially presented to explain the genetic basis for the inheritance (Utermann et al., 1977). Based on two-dimensional polyacrylamide gel electrophoretic (PAGE) analysis of apoE heterogeneity, Zannis and Breslow (1981) subsequently proposed a three-allele model of apoE inheritance which has proved to be essentially correct. In the model of Zannis and Breslow, the apoE polymorphism was explained by the presence of three co-dominantly expressed alleles at a single gene locus. Additional heterogeneity in terms of both size and charge is introduced by the addition of one or more sialic acid residues to the product of each allele. The three alleles, designated as ε2, ε3, and ε4, reflect the relative pI of the respective gene products, apoE2, apoE3 and apoE4, on isoelectric focusing gels, with apoE2 being the most acidic isoform (PI~5.7)

and apoE4 the most basic (PI~6.1); the apoE1 species was considered to result from sialylated isoforms (Zannis et al., 1982). The three homozygous phenotypes were designated E2/2, E3/3, and E4/4; the heterozygous phenotypes were designated E4/3, E4/2, and E3/2. The allelic frequencies are similar in all populations studied, with apoE3 being the most common form (Hallman et al., 1991).

In 1982, Rall et al. determined the amino acid sequence of apoE by direct sequencing of the 299 amino acid protein. Cysteine-arginine interchanges at two positions in apoE structure were shown to be the molecular basis of apoE polymorphism (Weisgraber et al., 1981). ApoE3 which is the most common apoE isoform has a single cysteine residue located at position 112 and an arginine at position 158. Residues 112 and 158 are both cysteines in apoE2 whereas apoE4 has arginines at both positions. The primary structures of apoE from 10 species have been determined. It has been shown that residues 29-61 compose the most conserved region in these species (Chan and Li, 1991), suggesting that this region is important in structure and function of apoE. The region of apoE that is responsible for binding to the LDLr is also highly conserved amongst species. The sequencing of the complete apoE gene which, in humans, is located on chromosome 19 (Olaisen et al., 1982; Das et al., 1985), revealed that the gene was composed of 3597 nucleotides and contained four exons and three introns (Paik et al., 1985). Based on amino acid and nucleotide homologies and on similarities in the exon / intron organization of the genes between apoE and other apolipoproteins, it is thought that apoE is a member of an apolipoprotein multigene family (Luo, Li, Moore, and Chan, 1986).

Structural Domains and Physical Properties of ApoE:

Soluble apolipoproteins tend to be monomers at low concentration but, at higher concentration, they self-associate and form high molecular weight oligomers in a concentration-dependent manner. Similar to other LP, apoE was demonstrated to be self-associated in the absence of lipid (Yokoyama et al., 1985; Aggerbecket al., 1988). However, unlike other apolipoproteins in which oligomerization was concentration-dependent, apoE formed tetramers over a wide range of concentrations (50 μg / ml to 15 mg / ml) (Yokoyama et al., 1985; Aggerbeck et al., 1988).

When apoE was subjected to increasing concentrations of guanidine and protein unfolding of apoE was monitored by circular dichroism (Wetterau et al., 1988), apo E displayed two transitions with the first transition midpoint occuring at the 0.7 M guanidine, a point at which all other apolipoproteins are completely unfolded. The second midpoint which produces a free energy of stabilization of ~12 Kcal / mol occurs at a high concentration of guanidine (2.4M). These results suggest that apoE contains two independently folded structural domains with very different stabilities. The second transition is characteristic of stable, soluble, globular proteins. Having two structural domains, especially one with such a high stability, is unique among plasma Lps. The two domain stucture of apoE was confirmed when apoE was subjected to limited proteolysis by using five proteases which had widely varying specificities (Wetterau et al., 1988). The results demonstrated that, in the amino-terminal (residues 20-165) and carboxyl-terminal (residues 225-299) portion of apoE, there are two protease-resistant regions, suggesting that these regions compose the structural domains. The random structure of apoE

(residues 165-210) in the central portion of protein is the portion which is highly susceptible to proteolysis.

Thrombin digestion of apoE produces a 22-kDa N-terminal fragment and a 10-kDa C-terminal fragment by cleavage at residues 191 and 215 (Bradley et al., 1982; Innerarity et al., 1983). HPLC analysis revealed that the 22-KDa fragment was monomeric and the 10-KDa fragment was tetrameric (Aggerbeck et al., 1988). These results suggest that the carboxyl-terminal domain mediates the tetramerization of the intact protein and also that the domains did not interact with each other when the protein is free in solution. Another hydrodynamic property of apoE is that the 22-kDa fragment is compact and globular and that the 10-kDa fragment is elongated in shape (Aggerbeck et al., 1988). Thus, based on these physical chemical measurements, apoE appears to be composed of two domains, a stable and globular N-terminal domain which is connected to an elongated C-terminal domain by a "hinge region" which is protease-susceptible. Circular dichroism demonstrated the helical structure of the intact protein (Shore and Shore, 1974; Roth et al., 1977; Mims et al., 1990) as well as the individual domains (Aggerbeck et al., 1988). Considering the α-helical nature of the C-terminal domain and the fact that these helices are predicted to be amphipatic (Rall et al., 1982), it was proposed that the C-terminal domain forms a tetrameric α-helical bundle by self-association (Aggerbeck et al., 1988). In an aqueous environment, the hydrophobic surfaces of the amphipatic helices are predicted to be sequestered within the interior of the bundle and hydrophilic helices are toward the aqueous solution.

The 3-dimensional crystal structures of the lipid-free N-terminal 22-KDa fragments of apoE2 (Wilson,et al., 1994), apoE3 (Wilson,et al., 1991), apoE4 (Dong, et al., 1994)

have been solved. The ribbon representation of the 22-KDa fragment shown in Figure 1 demonstrates the helical structure of the domain. Four of the helices are arranged in an antiparallel bundle with the receptor-binding region located in the helix fourth (Wilson et al., 1991). The helices in the apoE3 22-KDa fragments are amphipatic in nature but their properties and charge distributions are different from other typical apolipoprotein helices (Segrest et al., 1992). The hydrophobic residues are clustered in the interior of the helix bundle and the hydrophilic residues are exposed to the solvent which probably contributes to the stability of the structure. There are 24 acidic and 24 basic residues in the 22-KDa structure which most of them are involved in intra- and interhelical salt brides that probably causes the unusual stability of this fragment compared to other apolipoproteins. The basic residues (except Arg-147 and Arg-150) within the receptor-binding region (residues 136-150) of apoE3 are not involved in salt bridges and are exposed to the solvent and should, therefore, be free to interact with the LDLr (Wilson et al, 1991). All three isoforms of apoE share the four helix bundle motif. The amino acid substitutions that characterize the isoforms do, however, impart subtle changes in apoE tertiary structure which have important manifestations in terms of function. These will be discussed below.

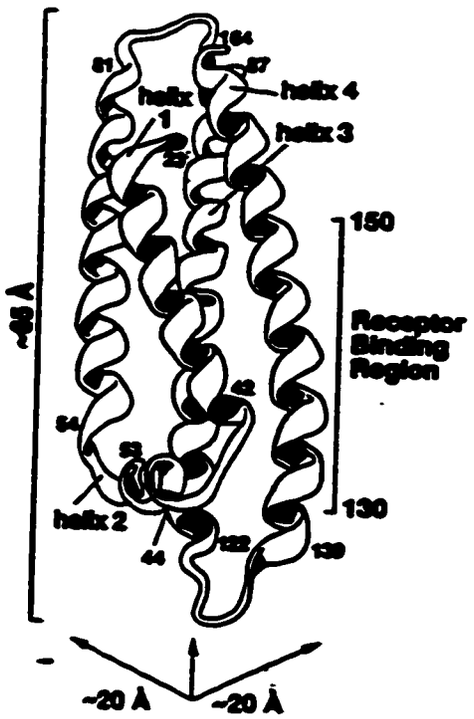


Figure 1- The structure of the 22-kDa domain of apoE. Taken from Wilson et al., 1991. For details, see text.

The Structural Basis for Binding of LDL to the LDL receptor:

By selective chemical modification, lysyl and arginyl residues of apoE were shown to be important in the binding of apoE to the LDLr (Mahley et al. 1977; Weisgraber et al., 1978). Since the cysteine-rich repeats that constitute the ligand-binding domain of the LDLr are enriched in acidic residues (Yamamoto et al., 1984), it was suggested that the basic residues in the receptor-binding domain of apoE bind to the acidic residues of the LDLr via ionic interactions (Mahley et al., 1986). When isolated thrombolytic apoE fragments were recombined with PL and examined for their ability to bind to the LDLr on the surface of cultured human fibroblasts, the amino-terminal 22-KDa fragment (residues 1-191) retained binding activity whereas the 10-kDa carboxy-terminal fragment was

completely inactive (Innerarity et al., 1983). Of the 4 fragments that are generated by cyanogen bromide cleavage of apoE, only that composed of residues 126-218 could mediate binding to the LDLr (Innerarity et al., 1983). Taken together, these results showed that the receptor-binding site of apo E is located in the middle of the primary structure of the protein. This is consistent with the finding that monoclonal antibodies (mAbs) specific for epitopes located between apoE residues 140-150 could inhibit apoE-mediated binding to the LDLr (Weisgraber et al., 1983; Raffaï et al., 1995). Finally, natural apoE variants and variants generated by *in vitro* mutagenesis that are defective with respect to receptor-binding are characterized by amino acid substitutions that replace basic amino acids by neutral amino acids or disrupt secondary structure in the region of residues 136-158 (Lalazar et al., 1989).

It is now believed that only the basic residues between residues 136-150 directly interact with the LDLr (Mahley et al., 1990). In the crystal structure of the lipid-free N-terminal domain of apoE these residues are clustered on the forth helix and are exposed to the solvent (Wilson et al., 1991). The apoE2 isoform in which arginine 158 is replaced by cysteine has very low affinity for the LDLr. Nevertheless, Arg^{158} is not thought to interact directly with the LDLr, but rather to help to maintain the receptor-binding region in a proper conformation for binding (Innerarity et al., 1984). Removal of the C-terminus (residues 192-299) of apoE2 by thrombin digestion increased its binding activity approximately 10-fold (Innerarity et al., 1984). Introduction of a positive charge onto Cys^{158} of apoE2 by cysteamine modification also resulted in an increase in its affinity for the LDLr and, when the positive charge on the modified cysteine was subsequently removed, the loss of binding activity (slow) and the loss of the positive charge on residue

158 (rapid) could be temporally dissociated. Although circumstantial, both of these observations would be consistent with residue 158 having an indirect effect in the interaction of apoE and the LDLr. A very recent study has demonstrated the structural basis for the defective-LDLr binding property of apoE2. Replacement of Arg^{158} with Cys, disrupts a naturally occurring salt bridge between Asp^{154} and Arg^{158} and a new salt bridge forms between Asp^{154} and Arg^{150} which causes the shifting of Arg^{150} out of the receptor binding region (Wilson et al., 1994; Dong et al., 1996a). Disruption of the Asp^{154}-Arg^{150} salt bridge in apoE2 by site directed mutagenesis of Asp^{154} to Ala increased the binding activity to near normal levels. The X-ray crystal structure of apoE2 Ala^{154} showed that Arg^{150} was relocated into the receptor-binding domain, suggesting that the replacement of one salt bridge with another is a novel mechanism for the defective receptor binding of apoE2.

While the residues of apoE that are directly implicated in the interaction with the LDLr are situated between positions 136-150, other regions of apoE also appear to contribute indirectly to the binding. To determine the minimal residues necessary for LDL receptor-binding activity of apoE, a series of C-terminal truncations was produced by site-directed mutagenesis and expressed in *E.coli* (Lalazar et al., 1989). The 1-171 and the 1-183 variants possessed 19% and 85% normal binding activity, respectively, and no other shorter truncated proteins were active, suggesting that residues 171-183 possess sequences critical to receptor binding, probably through modulation of the conformation of the receptor binding region (residues 136-150). Other studies indicated that residues 1-126 also are required for full activity of apoE (Dyer et al., 1991), suggesting that a critical length of sequence (1-135) is also required to maintain receptor-binding epitope (136-150)

in a proper conformation for high affinity interaction with the LDLr. Finally, a $Glu^3 \rightarrow Lys$ apoE variant showed a significantly increased affinity for the LDLr suggesting that the extreme amino terminus could influence binding of apoE to the LDLr (Dong et al., 1990; Wardell et al., 1991).

ApoE must be associated with lipid to mediate high affinity binding to the LDLr (Innerarity et al, 1979). The hydrophobic residues of apoE 22-KDa are thought to interact with the hydrophobic acyl chains of phospholipids. It has been postulated that when apoE associates with lipid, the amino terminal four-helix bundle undergoes a structural reorganization at the interface surface. The bundle opens without a major disruption of the α-helical structure (Weisgraber et al., 1992) (Figure 2). In this reorganization, the hydrophobic faces of helices, would be directed toward lipid, maintaining a hydrophobic environment.

ApoE-mediated binding of a lipoprotein to the LDLr appears to involve multiple interactions between apoE molecules and receptors. HDLc, obtained from CH/fat-fed dogs, that only contain apoE and no other apolipoproteins, bind to the LDLr with a higher affinity than does LDL (Pitas et al., 1979). Furthermore, four times as many LDL particles compared to HDLc particles are required for saturation of the available receptor sites which implies that HDLc bind to multiple (up to four) sites on LDLr, whereas one LDL particle binds to one site (Pitas et al., 1979). When discoidal apoE-dimyristoylphosphatidylcholine (DMPC) complexes (4 molecules of apoE / complex) containing various ratios of receptor-active and receptor-inactive (chemically modified) apoE were prepared and tested for their ability to bind to the LDLr on cultured human fibroblasts, it was shown that a single apoE bound to the LDLr with an affinity similar to

that of LDL (Pitas et al., 1980). These results suggested that the higher affinity of apoE-containing LP, compared to that of LDL, resulted from the interaction of four molecules of apoE with four LDLr. Addition of more than four molecules of apoE to the particles does not further increase binding activity (Funahashi et al., 1989).

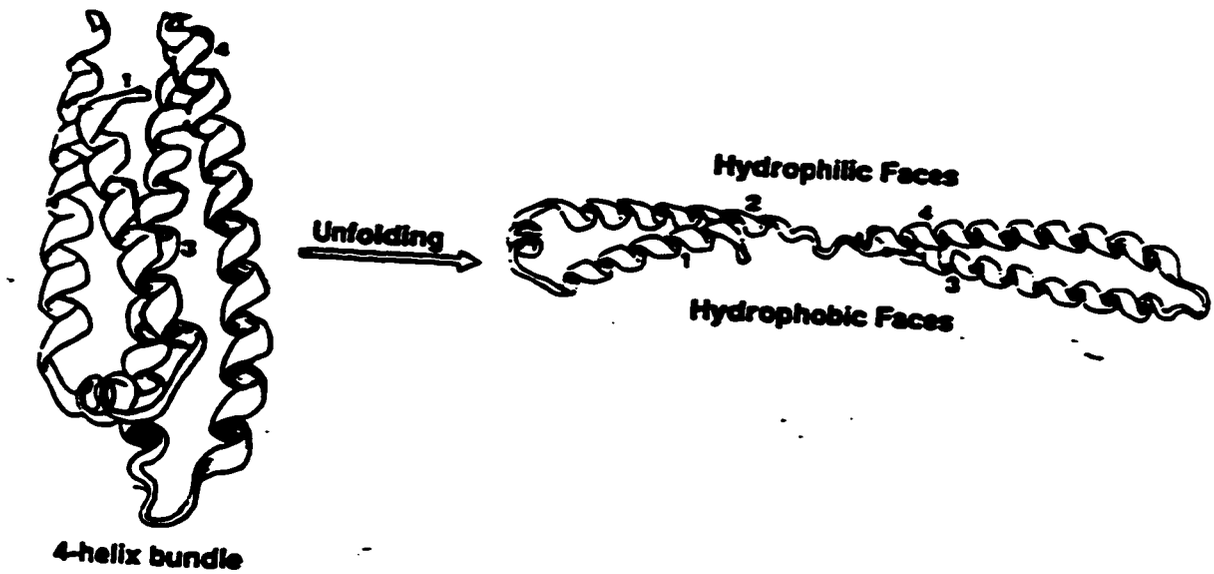


Figure 2- The interaction of the four-helix bundle structure of the apoE3 22-kDa fragment with lipid is illustrated in this model. On the left, the four-helix boundle structure of apoE in solution is shown. Interaction of apoE with lipid is illustrated on the right, the boundle "opens" without disrupting α helices, exposing the hydrophobic core of the boundle and making it available to interact with lipid (Weisgraber et al., 1992).

Interaction of ApoE with Heparin:

One of the important properties of apoE in LP metabolism is its ability to bind to various glycosaminoglycans, including heparin (Mahley et al., 1979; Weisgraber et al., 1986). It has been proposed that the proteoglycans of the arterial wall interact with LP and, through this interaction, CH is deposited and may promote atherosclerosis (Srinivasan et al., 1972). Furthermore, lipoprotein lipase (LPL) which is responsible for the intravascular hydrolysis of triglyceride -rich LP (TGRLP) is present on the endothelial cell, associated with heparin-like proteoglycans. ApoE-mediated binding of TGRLP to the proteoglycans may facilitate access of LPL to the TG core (Landis et al., 1987). Addition of apoE to TG emulsion particles have been shown to cause an increased hydrolysis of these emulsions by LPL that were bound to a heparin-sepharose matrix core (Landis et al., 1987). The heparin sulfate proteoglycans in the liver are also postulated to be involved in the initial binding of chylomicron remnants in the space of disse (Mahley and Hussain, 1991). ApoE was found to have two heparin-binding sites (Cardin et al., 1986 ;Weisgraber et al., 1986). The primary heparin-binding site is composed of residues 142-147 and thus coincides with the epitope recognized by the human apoE monoclonal antibody (mAb), 1D7, which is specific for an epitope situated between residues 140 and 150 (Weisgraber et al., 1986; Raffaï et al., 1995). The second heparin-binding site, which is not well defined, is only exposed on lipid-free apoE and is partially blocked with anti-apoE mAb 3H1, whose epitope is between residues 243-272 (Weisgraber et al., 1986). This second heparin-binding site may be responsible for the increased affinity for cell surface proteoglycans that was observed with a natural apoE variant, in which Glu^{212} is replaced by Lys (Feussner et al., 1996).

Association of ApoE with Lipoproteins:

Although the N-terminal and C-terminal domains of apoE seem to be independently folded in a lipid-free state, there are clearly interdomain interactions in the intact molecule. Carboxy-terminal truncation studies of apoE have shown that the important residues for LP association are located at the extreme C-terminus of apoE (residues 225-266) (Westerlund and Wiesgraber, 1993). Nevertheless, amino acid substitutions in the amino-terminal domain can modulate the relative affinities of apoE for LP subclasses. Whereas apoE4 associates preferentially with VLDL, apoE2 and apoE3 are primarily associated with HDL (Gregg et al., 1986; Weisgraber, 1990). When the 22-kDa fragments of apoE3 and apoE4 were mixed with LP and then subjected to gel filtration chromatography, both species eluted in the lipid-poor region of the column and did not distribute with any of the major LP classes (Weisgraber, 1990). The distribution of the 10-kDa carboxy-terminal fragment, which appears to constitute the major lipid-binding region of apoE, did not resemble that of either apoE3 or apoE4 and displayed a unique LP association of its own, associated primarily with LDL and small HDL (Weisgraber, 1990). These results demonstrate that residue 112 in the N-terminal domain, influences the lipid-binding properties of the C-terminal domain, suggesting the interaction between two domains (Weisgraber, 1990).

While these results would suggest that the preferential association of apoE4 with VLDL and the inter-domain interactions may be the result of a positively charged arginine residue at position 112 , recent results show clearly that this charged residue does not interact directly with the C-terminal domain but, on the contrary, causes a change in the position of the Arg^{61} side chain compared to its position on apoE3 (Agard et al., 1994).

Replacement of the Arg^{61} with threonine changes the lipoprotein preference of apoE4 from VLDL to HDL (Agard et al., 1994). These results suggest two possibilities: 1) the change in the conformation of the Arg^{61} results in VLDL preference in apoE4 or 2) Arg^{61} is a key mediator of inter-molecular domain interaction in apoE4, and interacts with one or more acidic residues in the C-terminal domain, probably by salt bridge formation. Replacement of Arg^{61} with Lys did not alter the preference of apoE4 for VLDL, demonstrating that a positive charge is critical for domain interaction rather than the requirement of Arg at position 61 (Dong and Weisgraber, 1996). To identify the acidic residues in C-terminal domain with which Arg^{61} interacts, the six acidic residues known to be important for both LP association and isoform-specific preferences were substituted individually with Ala in apoE4. Only substitution of Glu^{255} altered the preference of apoE4 from VLDL to HDL, demonstrating the importance of this residue in interaction with Arg^{61}(Dong and Weisgraber, 1996). Deletion of the hinge region had no effect on the apoE4 preference for VLDL, suggesting that, the hinge region may act simply as a spacer that connects the two domain (Dong and Weisgaber, 1996).

Conformational Heterogeneity of ApoE in Lipoproteins:

The conformation of apoE on the surface of a LP particle can be modulated by different factors including lipid composition. ApoE-associated with large VLDL isolated from hypertriglyceridemic (HTG) patients binds with a higher affinity to LDLr than to smaller HTG VLDL (Gianturco et al., 1983). Moreover, the large VLDLs were more thrombin-sensitive than smaller particles (Gianturco et al., 1983). Binding of VLDL to the LDLr was shown to be mediated by a subpopulation of apoE molecules that were thrombin- accessible (Gianturco et al., 1983; Bradley et al., 1984). The conformation of

apoE in the vicinity of the receptor-binding region was studied by a panel of apoE mAbs on different LP classes, and their interaction with LDLr was examined (Krul et al., 1988). Heterogeneity in the expression of these apoE epitopes in different LP classes correlated with differences that were observed in the receptor-binding activities. ApoE binding to the LDLr is also affected by lipolysis of VLDL. There is 2-20-fold increase in the ability of the lipolysed particles to interact with receptors and deliver CH to cells after lipolysis (Sehayek et al., 1991), suggesting that the receptor inactive form of apoE is changing to a receptor-active form during hydrolysis of core TG. Other factors, such as protein-protein interactions or the loss of apoC during the lipolysis, also could contribute to these conformational changes. Circular dichroism of apoE on PL discs, on HDLc and on spherical artificial microemulsion particles illustrated the effect of CH on the conformation of apoE (Mims et al., 1990). When apoE was bound to spherical particles, the α-helical content was lower compared to discoidal particles (Mims et al., 1990). It has, therefore, been proposed that apoE-mediated uptake of LP depends on the exposure of apoE at the surface of the particle, its conformation, its interaction with other apolipoproteins and its extent of self association (Dergunov and Rosseneu, 1994). In addition, manipulation of the lipid composition of VLDL by lipolysis and lipid transfer proteins has demonstrated that the lipid composition of VLDL is also an important factor in the association of apoE with the particles themselves (Ishikawa et al., 1988).

Mediator of Lipoprotein Metabolism:

ApoE plays a key role in the plasma LP metabolism and contributes to the interaction of LP with at least five different cellular receptors, the LDLr, the LDLr-related protein (LRP), the VLDL receptor (VLDLr), the macrophage receptor for

hypertriglyceridemic VLDL, and the scavenger receptor (Dergunov and Rosseneu, 1994). Apart from the LDLr, the best characterized of these are the LRP and VLDLr, both of which are related to the LDLr in terms of structure and evolution. The LRP, which can bind a number of structurally unrelated ligands, is thought to be at least partially responsible for the hepatic clearance of chylomicron remenants (CMR) from plasma through its ability to bind apoE and LPL (Hussain et al., 1989 and 1990). In apoE-deficient mice, the removal of plasma remnants was totally impeded, while in LDLr-deficient mice, the remnant clearance was somewhat slower than in control mice (Mortimer et al., 1995). This suggests that CMR are rapidly internalized by the LDLr and catabolized in hepatocytes and that apoE is required for these activities. In LDLr-deficiency, remnants are taken up by a second apoE-dependent pathway, presumably mediated by the LRP. The binding of apoE with LRP is inhibited by a mixture of the apoC proteins and it was shown that apoC-I was the most effective inhibitor (Kowal et al., 1990). It is thought that the extent of apoE self-association, which is dependent upon the degree of hydrolysis of the TG-rich particles, may control their interaction with the LRP.

ApoE is also a ligand for the VLDLr. This receptor is expressed in heart, muscle and adipose tissue and in a human monocyte-macrophage cell line. The VLDLr binds and internalizes VLDL and IDL. Binding of TGRLP to the VLDLr on Chinese hamster ovary cells transfected with the rabbit VLDLr cDNA, is enhanced by apoE and LPL (Takahashi et al ., 1995). It has been proposed that the VLDLr may function as a receptor for remnants of TGRLP in extrahepatic tissues.

Chylomicrons (CM) are synthesized in the intestine after exogenous fat absorption and transport dietary TG and cholesterol. Initially, CM do not contain apoE ,but as they

undergo lipolysis and lipid transfer in the circulation, they become relatively depleted in TG and enriched cholesteryl esters and also acquire apoE from other LP classes (Blum et al., 1982). CMR are cleared by hepatocytes, probably through the interaction of apoE with the LDLr and the LRP (Beisiegel et al., 1989). ApoE is also implicated in the metabolism of hepatically-derived VLDL. ApoE is essential for the efficient LPL-catalyzed conversion of VLDL to LDL (Ehnholm et al., 1984) and for the hepatic capture of VLDL remnants. The role of apoE in major metabolic pathways and the central importance of the liver in LP metabolism is shown in a simple scheme (figure 3).

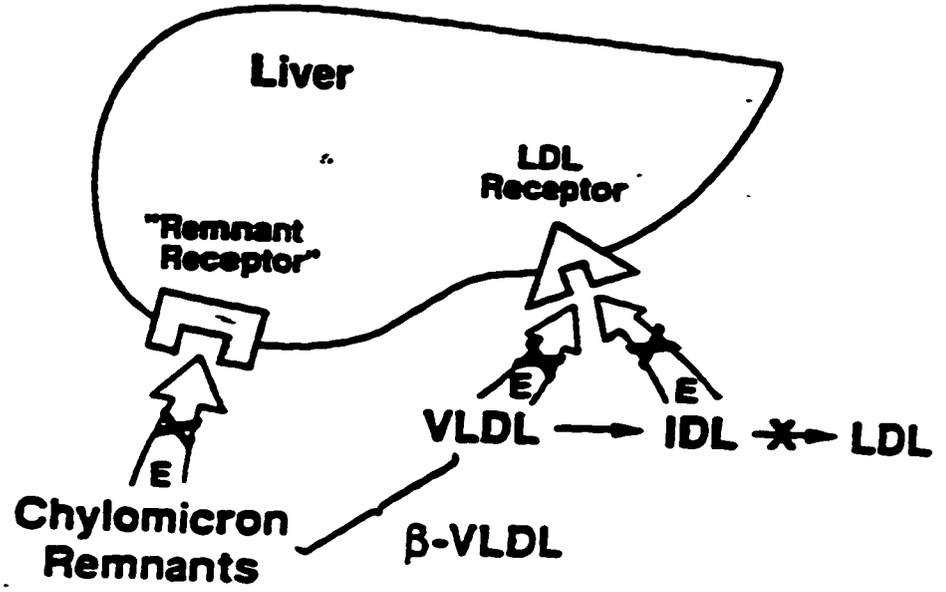


Figure 3- The importance of apoE in major metabolic processes and the central role of the liver in lipoprotein metabolism (Weisgraber, 1993).

Infusion of apoE into CH/fat-fed rabbits which highly elevates circulating apoE levels, results in the lowering of plasma CH concentrations, suggesting that plasma apoE

concentration may be rate limiting for LP clearance (Mahley et al., 1989). These results were confirmed by overexpression of apoE in transgenic mice which resulted in decreased plasma CH levels in chow-fed animals (Shimnano et al., 1991) and a resistance to hypercholesterolemia when overexpressors were fed a high-CH/fat diet (de Silva et al., 1992; Shimnano et al., 1992). The importance of apoE in LP metabolism is underscored in human apoE deficiency (Schaefer et al., 1986) and in mice whose apoE gene had been inactivated by gene targeting (Zhang et al., 1992; Plump et al., 1992). Both exhibit an accumulation of remnant Lps in their plasma. Nevertheless, if apoE is, in fact, limiting for LP clearance, it is surprising that plasma CH levels were normalized in lethally-irradiated, apoE-deficient mice which had been injected with apoE-expressing bone marrow cells from normal mice (Linton et al., 1995). The bone marrow-reconstituted mice were also protected from diet-induced atherosclerosis in spite of the fact that circulating apoE levels were only 12% of those of normal mice.

The role of apoE in hepatic uptake and metabolism of CMR was studied in transgenic mice overexpressing apoE in the liver (Shimano et al., 1994). Five-fold faster plasma clearance of human CM was detected in transgenic mice than in controls. Immunohistochemistry determined that apoE was specifically localized at the basolateral surface of hepatocytes and vesicular staining was detected in the cytoplasm after injection of a bolus of CM, suggesting that the cell surface apoE was used for hepatic endocytosis of CM and CMR. The data provide evidence for the secretion-recapture process of apoE (Ji, Fazio, Lee, and Mahley, 1994a) in which CMR enter the sinusoidal space, acquire apoE molecules, and eventually are endocytosed. The efficiency of normal marrow cells to normalize plasma lipid levels and CM clearance in apoE-deficient mice would,

however, seem to be at odds with the secretion-capture model for apoE-mediated hepatic uptake of remnants as few of the donor cells were detected in the livers of the hosts (Linton et al., 1995).

In a number of CH/fat-fed animal models there is accumulation of HDL that is greatly enriched in apoE. This apoE-containing HDL, referred to as HDL_1 or HDLc, is a prominent LP class in several species, including dogs (Mahley and Holcombe, 1977), pigs (Mahley et al., 1975), and mice (de Silva et al., 1992) and represents an excellent ligand for the LDLr. In humans, apoE-enriched HDL is less prominent but may transport as much as 20% of plasma CH (Weisgraber and Mahley, 1980). Studies of the hepatic uptake of the CH moiety of rabbit apoE-rich and apoE-poor HDL indicate that apoE is not directly involved in the selective uptake of HDL cholesterol by the liver (Fragoso and Skinner, 1995). It is suggested that an alternative mechanism might exist for the clearance of HDL cholesterol from plasma.

Effect of Apo E Heterogeneity on Plasma Lipid Concentrations:

In certain studies the ε4 allele has been associated with high plasma CH levels , while the ε2 and ε3 alleles are associated with low and intermediate levels of plasma CH, respectively (Davignon et al., 1988). These associations between plasma lipid levels and apoE genetic polymorphism do, however, appear to be dependent upon ethnic origin, gender and lifestyle (de Knijff, and Havekes, 1996). In individuals who are homozygous for the ε2 allele, remnant LP are cleared at a slower rate than normal and the conversion of VLDL to LDL is also retarded which leads to the up-regulation of hepatic LDLr, which causes a further lowering of plasma LDL concentrations (Davignon et al., 1988). The accumulation of β-VLDL-like particles and high concentrations of apoE in plasma are

characteristic apoE2/E2 subjects (Utermann et al., 1985). Remnants are cleared more efficiently in subjects who are apoE4/E4 than those with the apoE3 phenotype, although both of the isoforms have identical affinities for binding to the LDLr (Wientraub et al., 1987; Weisgraber et al., 1982). This effect is probably due to the preferential association of apoE4 with TGRLP in plasma (Gregg et al., 1986; Weisgraber, 1990). It has been suggested that the effective clearance of remnant particles causes the down regulation of hepatic LDLr which is responsible for elevation of plasma LDL concentrations in individuals who have inherited an ε4 allele (Gregg et al., 1986; Weintraub et al., 1987; Davignon et al., 1988).

The importance of apoE isoforms on the response curve of different TGRLP and the in 37 normolipemics was studied after a fatty meal (Orth et al., 1996). A delay in CMR clearance was detected only in apoE2 homozygotes which was neither related to plasma lipids nor to peak LP concentrations. In contrast, in other non-apoE2/2 individuals, the CMR response was clearly related to the magnitude of the CM and VLDL responses. This suggests that, the CMR response curve is an indicator of the extent of postprandial lipemia in non-apoE2/2 individuals only.

Reverse CH transport is thought to be initiated by the uptake of the CH into HDL which contain either apoA-I or apoE. Minor HDL subclasses, pre β1-lipoprotein A-1 and γ-lipoprotein E (γ–LpE), are initial acceptors of cell-derived CH. The effect of apoE polymorphism on the formation of γ-LpE, uptake and transfer of cell-derived CH to LDL was studied (Assman et al., 1995). Results demonstrated that, 30% less CH was released into plasma of apoE2/2 and apoE4/4-individuals as compared with plasma of apoE3/3 subjects. Furthermore, plasma of individuals with apoE3/3 accumulated 50% and 65%

more cell-derived CH in α-LPA-I than plasmas of apoE4/4 and apoE2/2 subjects, respectively. These results suggest that apoE polymorphism is an important determinant of efflux and transfer of cell-derived CH in plasma.

ApoE Polymorphism and Disease:

Type III hyperlipoproteinemia is a disease characterized by delayed CM and VLDL remnant catabolism and is usually associated with homozygosity for the ε2 allele (Utermann et al., 1977). Although apoE2 has been shown to be functionally defective both, *in vivo,* in mediating clearance of LP in plasma (Gregg et al., 1981), and, *in vitro,* in binding to the LDLr (Weisgraber et al., 1982), a second genetic or environmental factor, in addition to homozygocity for the ε2 allele, is also required for full expression of the type III phenotype (Davignon et al., 1988). A similar phenotype is seen in subjects who have inherited certain rare apoE alleles and, in some cases, unlike that of the ε2 allele, there appears to be a dominant form of inheritance (Fazio et al., 1993). While most of these rare apoE variants are defective with respect to binding to the LDLr, the severity of the associated type III hyperlipidemia and the dominant pattern of inheritance appears to correlate more closely with the decreased affinity for proteoglycans (Ji et al., 1994b; Mann et al., 1995). As would be expected, subjects with a complete apoE deficiency show a severe type III phenotype (Schaeffer et al., 1986).

As discussed in the preceding section, inheritance of an ε4 allele can be associated with high serum CH levels. As was observed for the association between apoE genetic polymorphism and plasma lipid levels, the association between the inheritance of the ε4 allele and an increased risk for atherosclerosis appears to be dependent upon the population studied (de Knijff and Havekes, 1996).

Inheritance of the ε4 allele appears to be a risk factor for Alzheimer's disease (AD) (Strittmatter et al., 1993a; Roses et al., 1993), an irreversible neurodegenerative disorder that results in progressive dementia (Mckhann et al., 1984). There is presently a major effort in many laboratories to determine the mechanism responsible for the association between apoE genetic polymorphism and susceptibility to AD. Brains of AD patients have massive extracellular deposits of β amyloid, known as senile plaques, and intracellular neurofibrillary tangles. Immunoreactive apoE can be observed within both the neurofibrillary tangles and the β-amyloid senile plaques (Beyreuther et al., 1991). While apoE has been shown to bind to β-amyloid peptide *in vitro*, the isoform specificity of the association is controversial (Stritmatter et al., 1993b; LaDu et al., 1994; Golabek et al., 1996). Binding of apoE to the tau protein, the principal component of the neurofibrillary tangles, has been reported to be isoform-specific (Strimatter et al., 1994) with apoE3 having higher affinity than apoE4. It was proposed that apoE binding may prevent polymerization of the tau protein. Lipid-associated apoE stimulates neurite outgrowth from cultured neurons derived from the peripheral (Nathan et al., 1994; Nathan et al., 1995) and central (Holtzman et al., 1995; Fagan et al., 1996) nervous system in an isoform-specific manner, with apoE3 being more effective than apoE4. The effects of the apoE appear to be mediated by the LRP (Holtzman et al., 1995; Fagan et al., 1996).

Amyloid β peptides are toxic to neuronal cells and this cytotoxicity is mediated by hydrogen peroxide and can be inhibited by certain anti-oxidants (Behl et al., 1992 and 1994). Recently, apoE has been shown to have an isoform-specific antioxidant activity that decreases the cytotoxicity caused by oxidative insults and β-amyloid peptides with the following order of efficacy; E2>E3>E4 (Miyata and Smith, 1996). The apoE had a

biphasic dose response, however, providing protection at low concentrations but causing increasing cytotoxicity at high concentrations (Miyata and Smith, 1996). It was proposed that the decreased antioxidant activity of apoE4 may have a role in its association with AD, cardiovascular disease and decreased longevity.

Other Biological Functions of ApoE:

ApoE has been proposed to be a modulator of immune function. It is a powerful suppresser of interleukin-2-dependent T lymphocyte proliferation. The region of apoE that is responsible for immuno-regulataion has been mapped to residues 141-149 (Clay et al., 1995). The cytostatic and cytotoxic activity of this immunoregulatory sequence, depend on nonpolar face hydrophobicity and polar face positive charge density, respectively.

ApoE can inhibit the proliferation of endothelial cells and tumor cells in a dose- and time-dependent manner (Vogel et al., 1994). Maximal inhibition of cell growth by apoE was observed under conditions where proliferation is dependent on heparin-binding growth factors. The inhibition of cell proliferation by apoE was reversible. It has been proposed that apoE may be effective in modulating angiogenesis, tumor cell growth, and metastasis.

The LDL Receptor:

The LDLr is a cell-surface glycoprotein that recognizes clusters of positively-charged amino acids of apoB100 and apoE and serves both to deliver lipids to cells and to remove LDL particles from the extracellular environment. In extrahepatic tissues, the LDLr functions to deliver CH into cells to be utilized as structural components of membranes. In the liver, however, LDLr also serves to clear LDL particles from the

plasma, targeting the CH either for resecretion back into the plasma or excretion from the body as bile acids. As a consequence, the hepatic LDLr pathway is largely responsible for maintenance of the steady-state plasma concentration of LDL cholesterol (Brown and Goldstein, 1986).

The ligand-binding domain of the LDLr is composed of 7 imperfect repeats of a 40 amino acid motif that comprises the amino terminus of the protein (Goldstein et al., 1985). Ionic interactions between basic residues of the ligands and acidic residues of the receptor are probably responsible for the binding of apoE and apoB with the LDLr (Brown et al., 1978; Basu et al., 1977). Natural LDLr mutants are responsible for familial hypercholesterolemia (Hobbs et al., 1990). Analysis of the binding properties of natural LDLr variants as well as variants produced by *in vitro* mutagenesis, have given some insight into the structural aspects of the receptor-ligand interaction (Esser et al., 1988; Russell et al., 1989). Results demonstrated that, the deletion of the first repeat has no effect on the binding activity of LDLr with βVLDL (apoE-mediated binding) or LDL (apoB-mediated binding). Binding of LDL is reduced by elimination of, or substitution of conserved residues within, any of the other repeats, whereas binding of βVLDL is reduced only by the deletion of, or replacement of conserved residues within, repeat 5. Recent modeling studies suggest that each of the seven repeats is composed of two loops which are separated by a β–turn in such a way that acidic amino acids are accessible on the outer face of the repeat and thus available for interaction with the basic residues of apoB or apoE (Rosseneu et al., 1995).

The Statement of Objectives:

While cells use cholesterol acquired from plasma lipoproteins for essential cell functions such as synthesis of membranes and steroid hormones, excessive concentrations of cholesterol in the circulation can lead to its deposition in the arterial wall and, eventually, to arteriosclerosis. Normally, the human body tightly controls plasma cholesterol levels through cell surface receptors that specifically bind cholesterol-carrying proteins of the blood. The best characterized of these cell surface receptors is the LDLr. Individuals who lack functional LDLr have high plasma cholesterol levels, and typically suffer severe premature coronary artery disease.

As one of the ligands for the LDLr, apoE is an important modulator of plasma lipoprotein metabolism. Its significance is manifested in the dramatic hyperlipidemia and atherosclerosis that is seen in mice whose apoE gene has been inactivated by gene targeting. Human apoE is a 299 amino acid protein which is polymorphic in terms of its primary structure. The most common apoE isoforms, apoE2, apoE3 and apoE4 are encoded by 3 co-dominantly expressed alleles and differ by single amino acid substitutions. ApoE2 is defective in its ability to bind to the LDLr and homozygous inheritance of the allele encoding apoE2 is associated with hyperlipidemia. ApoE genetic polymorphism also influences plasma lipid levels, postprandial lipemia, apoE distribution in plasma lipoprotein subclasses and susceptibility to atherosclerosis and Alzheimer's disease. In addition to polymorphism in terms of primary structure, apoE also shows conformational heterogeneity on the surface of lipoproteins which affects its ability to mediate lipoprotein binding to the LDLr.

The primary goal of this study was to develop new anti-apoE monoclonal antibodies (mAbs) that could be used as probes to study apoE structure and function, as reagents for an antibody-based method for rapid determination of apoE genotype and as reagents for immunhistology to help define the role of individual apoE isoforms in disease etiology. Specifically, we proposed, on the one hand, to generate anti-apoE mAbs that could discriminate between apoE isoforms and, on the other hand, anti-apoE mAbs that were specific for the same conformational epitope on apoE as is recognized by the LDLr. To this end, we have tried to establish a novel immunization protocol that takes advantage of the ability of the immune system to discriminate between self and non-self in order to generate mAbs that are specific for predefined epitopes within apoE. Mice that carry a human transgene that encodes one apoE isoform are immunized with apoE of a second isoform. The immune response should, therefore, be restricted to epitopes that differ between the two isoforms. If the immunization strategy were effective, it could have applications for other proteins beyond the scope of the present project.

During the course of my project, we were obliged to euthanize all of our transgenic animals due to an infection with murine hepatitis virus. It took almost a year to acquire new, non-infected, founder animals and to establish new colonies. During this time, I began a second project in which I attempted to separate VLDL lipoproteins by anti-apoE immunoaffinity chromatography into a subfraction that contained apoE in a conformation that is capable of mediating binding to the LDLr and a subfraction in which the apoE is poorly reactive with the LDLr. While these experiments proved to be very interesting and should, when completed, serve as the basis of a manuscript, I have not included the results from the second project in the thesis.

CHAPTER II. METHODS

GENERATION OF ANTI-HUMAN APOE HYBRIDOMAS

Transgenic Mice:

A breeding pair of ICR mice, heterozygous for a transgene encoding human apoE3 were obtained from Dr. John Taylor (Gladstone Institute for Cardiovascular Research, San Francisco CA) (Simonet et al., 1993). A breeding pair of C57BL6 X SLJ mice that were heterozygous for a transgene encoding the human apoE Arg^{112}, Cys^{142} variant were obtained from Drs. Stanley Rall and Sergio Fazio (Gladstone Institute for Cardiovascular Research, San Francisco CA) (Fasio et al., 1993). Offspring were screened for inheritance of the transgenes by testing plasma for the presence of human apoE using a solid phase sandwich radioimmunometric assay with 6C5 as the capture antibody and ^{125}I-3H1 as the detection antibody (See below).

Preparation of Antigen:

The total lipoprotein fraction of transgenic mice expressing different apoE isoforms were isolated from plasma by ultracentrifigation at a density of 1.21 g / ml. The d < 1.21 g / ml fraction was dialyzed against PBS and its protein concentration was measured (Lowry et al. 1951). In some experiments, the purified amino terminal 22 kDa fragment (Wardell et al., 1991) that was generated by thrombin digestion of human plasma apoE4 (apoE4-22kDa) was also used as an immunogen and for detection of antibody. This was a gift from Dr. Karl Weisgraber of the Gladstone Institute of Cardiovascular Research, San Francisco CA.

Immunization:

BALB/C female mice were immunized intraperitonealy with 50-100 μg of antigen in 100 μl of PBS emulsified in 100 μl of complete Freund's adjuvant. At intervals of at least 3 weeks, two to five intraperitoneal boosts were given with antigen emulsified in incomplete Freund's adjuvant. The mice were injected intravenously with 10 μg of antigen in 100 μl of PBS 3-4 days before fusion (Milne et al., 1992).

Screening of Immunized Mice Plasma for Anti-apoE Antibodies:

Prior to undertaking a cell fusion, blood samples were taken from anesthetized mice by puncture of the retro-orbital sinus and sera were prepared for the measurement of anti-apoE antibodies. The fusion would be performed only when the serum had significant levels of anti-apoE antibodies. A solid RIA was used for this purpose (Milne et al. 1981). Immulon II Removawells (Dynatech) were coated by an overnight incubation at room temperature with 50 μl of antigen that had been diluted in 5mM glycine pH 9.2 to 2 μg/ml in the case of the apoE4-22kDa and to 10 μg/ml in the case of transgenic mouse lipoproteins. The wells were washed 3 times with phosphate buffered saline (PBS) containing 0.025% Tween20 (v/v) (PBS-Tween) and unoccupied binding sites on the plastic were then blocked by a 30 minute incubation with 250 μl of PBS containing 1% BSA (PBS-BSA).

Serial dilutions (50 μl) of sera from immunized or control mice were prepared in PBS-BSA, added to the antigen-coated wells and incubated for 3 h at room temperature. After washing with PBS-Tween, bound mouse antibody was detected by a 3 hour incubation with ^{125}I labeled-affinity-purified rabbit anti-mouse IgG (BioCan) that had been

appropriately diluted in PBS-BSA. Plates were washed in PBS-Tween and bound radioactivity was determined in a gamma spectrophotometer.

Radioiodination of Purified Immunoglobulins:

Affinity-purified rabbit anti-mouse immunoglobulin or mouse monoclonal antibodies were iodinated using a modification of the Chloramine T technique (Mellman and Unkless). Immunoglobulin (50 μg) in 0.3 M phosphate buffer pH 7.0 were mixed with 10 μl ^{125}I (1 mCi / μl, Amersham) 10 μl of the stock solution of ^{125}I added to the diluted protein followed by 20 μl of a 9 mg/ml Chloramine T solution. After a 30 minute incubation on ice, the reaction was stopped by the sequential addition of 20 μl of a 1M KI solution and 100 μl of a PBS/BSA. Free and protein-bound ^{125}I were immediately separated by passage on a Dowex AG1X8 column (Biorad). Specific activities normally range from 1-2 x 10^4 cpm / ng.

Plasmacytoma Fusion Cell Partner :

The mouse myeloma cell line, SP2-O was used for all cell fusions (Harlow et al., 1988). This cell line was negative for the expression of the enzyme hypoxanthineguanine phosphoribosyltransferase and for endogenous immunoglobulin synthesis. As the cell line lacks hypoxanthineguanine phosphoribosyltransferase activity, SP2-O cells, are killed in a medium supplemented with hypoxanthine, aminopterine, and thymidine (HAT) (Littlefield, J.W, 1964). SP2-O cells were maintained in Dulbecco's Modified Eagles Medium containing 4.5 g/l glucose and supplemented with non-essential amino acids (Gibco), 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 40 μM 2-β mercaptoethanol (DMEM), that was further supplemented with 5% heat inactivated Fetal Bovine Serum (FBS). Sp2-O cells were split 24 hours prior to cell fusion so that, on the

day of the fusion, the cells would be rapidly growing at a density of about 4 x 10^5 cells / ml with >95% viability as monitored by trypan blue exclusion.

Cell Fusion:

On the day of the fusion the immunized mice were sacrificed and the spleens were removed aseptically and placed into DMEM. Spleen cells were dissociated by gentle teasing with sterile forceps and aggregates were allowed to settle by gravity. The cells that remained in suspension were washed twice in DMEM and viable cells were counted in a haemocytometer. SP2-O cells were also washed twice in DMEM and viable cells counted. Splenocytes and SP2-O cells were mixed at a ratio of 4:1, centrifuged and the supernatant removed. Sterile polyethylene glycol (1300-1600 Da) (American Type Culture Collection (ATCC) was used to promote cell fusion. Just before the fusion, the polyethylene glycol was melted and diluted to 47% (v/v) with prewarmed (37°C) DMEM. The polyethylene glycol solution was slowly added to the cell pellet and then sequentially diluted with DMEM and DMEM containing 30% fetal calf serum and HAT (DMEM/30% FBS/HAT) to a final volume of 60 ml as has been described in detail (Milne et al., 1992). DMEM/30% FBS/HAT was prepared on the day of the fusion using premade 100x HAT (ATCC) that had been aliquoted and stored frozen. The following day, a further 100 µl DMEM/30%FBS/HAT were added to each of the wells which would later be screened.

Screening of Hybridoma Supernatants:

Seven to ten days after fusion, when hybridoma clones occupied about 10% of the surface of the well, 50 µl aliquots of supernatants were tested for the presence of specific anti-human apoE antibodies using a solid phase antibody-capture assay essentially as described above. Wells that gave bound counts at least 3 times over background or in the

range of the positive control (an anti-human apoE monoclonal antibody) were considered to be indicative of the presence of a hybridoma that secretes an anti-human apoE monoclonal antibody.

Subcloning of Hybridomas:

As the microculture wells that contain specific hybridomas could also contain different irrelevant clones or cells that had lost the ability to secrete antibody and might quickly overgrow the clones of interest, it was necessary to rapidly reclone cells from positive wells and identify those subclones that produce large amounts of specific antibody. The clones were screened by the limiting dilution method (Milne et al., 1992). The cells which were positive in several screenings were expanded, viable cells were counted in a haemocytometer, diluted to give 10 cells / ml in FBS containing 30% FBS and 100 µl aliquots were distributed in wells of a 96-well culture plate. In wells in which growth had occurred, the culture supernatants were tested for the presence of specific antibody by solid phase RIA as described above. Several subclones which showed the highest secretion of specific antibody were expanded, retested and the highest antibody-secreting subclone was recloned, as above, at a concentration of 5 cells per ml. Subcloning was repeated using increasing dilutions of cells until growth occurred in less than 10% of wells and all subclones were positive for antibody secretion. By this criteria, hybridomas were considered to be monoclonal.

Freezing and Thawing of Hybridomas:

Hybridomas that were deemed to be monoclonal were frozen in DMEM supplemented with 10% FBS and 10% dimethylsulphoxide at a density of 2 X 10^6 cells / ml as has been described (Milne et al., 1992). Frozen cells were maintained in liquid

nitrogen. As a precaution against contamination during the subcloning etc., hybridomas determined to be positive for antibody secretion were also frozen before recloning or after successive subclonings. When necessary frozen cells were rapidly thawed in a 37^{0}C water bath (Milne et al., 1992).

Purification of mAbs:

The IgG subclass containing the mAbs were purified by affinity chromatography on Staphylococcal protein A sepharose (Pharmacia) using a step-wise pH elution protocol as was described by Ey et al., 1982.

CHARACTERIZATION OF ANTI-APOE MABS

Previously Characterized mAbs:

Preparation and characterization of anti-human apoE mAbs 1D7, 2E8, 6C5, and 3H1 have been previously described (Milne et al., 1981; Weisgraber et al., 1983; Weisgraber et al., 1986; Raffaï et al., 1995). The mAbs 1D7 and 2E8 are able to block apoE-mediated binding to the LDLr and have been shown to be specific for epitopes between apoE residues 140 and 150 (Weisgraber et al., 1983; Raffaï et al., 1995). The mAb 6C5 has been shown to be specific for an epitope within the first 13 residues of mature apoE (Weisgraber et al., 1983) and 3H1 for an epitope between apoE residues 242-272 in the C-terminal domain (Weisgraber et al., 1986). The apoE epitope map is shown schematically in figure 4.

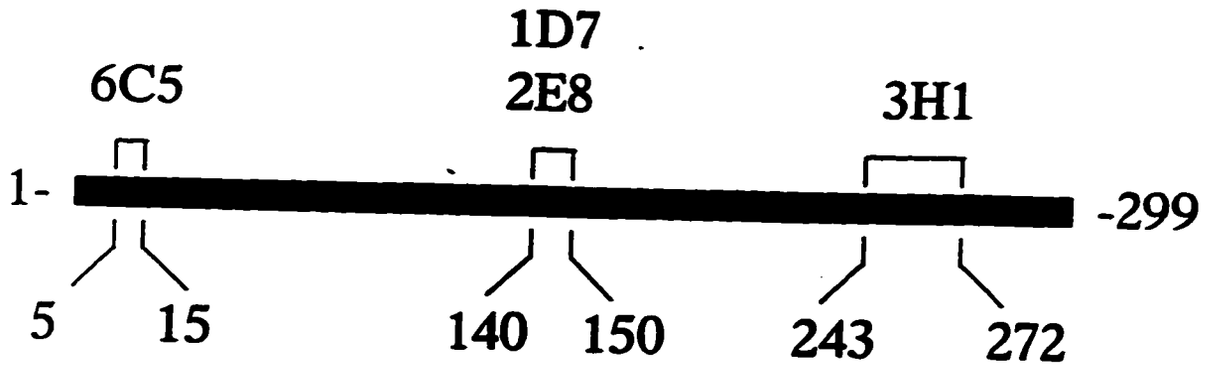


Figure 4- Epitope map of apoE monoclonal antibodies: 6C5 (residues 5-15), 1D7 (140-150), and 3H1 (243-272).

Antibody Competition assay:

To determine if anti-apoE mAbs react with the same or different apoE epitopes, individual antibodies were tested in pairs for their abilities to mutually compete for binding to immobilized apoE. Serial dilutions (100 µl) of individual anti-apoE or control mAbs (competitor) in PBS/BSA were prepared in a 96 well microtitre plate. One hundred µl of an ^{125}I-labeled anti-apoE mAb that had been diluted in PBS/BSA to contain about 40 ng of IgG protein was added to the wells containing the diluted competitors. A 50 µl aliquot of the mixture were then transferred to Removawells that had been coated with 50 µl of antigen (apoE4-22kDa or human VLDL) or BSA and saturated with BSA as described above. Results are presented as specifically bound radioactivity in the presence of competitor (B) divided by specifically bound radioactivity in the absence of competitor

(Bo). Non-specifically bound radioactivity was estimated as bound radioactivity on BSA-coated plates. New anti-apoE mAbs were tested for their ability to mutually compete amongst themselves, with previously characterized anti-apoE mAbs and with an irrelevant mAb (anti human cholesterol ester transfer protein (CETP).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting:

SDS-PAGE of apolipoproteins or lipoproteins was carried out according to Kane et al., 1980) on 12% polyacrylamide gels and the electrophoretically separated proteins were transferred to nitrocellulose membranes according to Towbin et al., 1979. Conditions for the immunodetection of the transferred proteins on the nitrocellulose membranes has been described (Théolis et al., 1984).

Sandwich Radioimmunometric Assay (SRIA):

In the SRIA, capture antibody was adsorbed to Immulon II Removawells by an overnight incubation with 50 µl of purified IgG at a concentration of 10 µg / ml in 5 mM glycine, pH 9.2 and, after washing in PBS-Tween, the wells were saturated with PBS-BSA as described above. Fifty µl of antigen (apoE4-22kDa, transgenic mouse plasma, human plasma, human VLDL, human apoE variants) diluted in PBS/BSA were added to the wells and incubated for 3h at room temperature. The wells were washed with PBS-Tween, as above, and bound antigen was detected by a 3 hour incubation at room temperature with a second, radiolabeled, anti-human apoE mAb. Wells were washed, as above, and bound radioactivity was determined.

For certain experiments a variation of this sandwich assay was used in order to avoid purification and labeling of the detection mAb. In this modification, Fab fragments,

instead of complete IgG, were used as the immobilized anti-apoE capture antibody. The anti-apoE detection antibody (complete IgG) was not radiolabeled but was detected by an additional incubation with an affinity purified, radiolabeled, rabbit anti-mouse Fc antibody (Jackson Research Laboratories). The ^{125}I-anti-Fc would recognize an epitope present on the detection antibody (IgG) but would not react with the capture antibody (Fab fragment).

Prepration of Fab Fragment:

IgG (all subtypes) that had been purified by affinity chromatography on Protein A was dialyzed against 0.075 M phosphate, 0.075M NaCl, and 0.002M EDTA PH 7.0 and protein concentration was estimated by absorption at 280 nm (EC_{280} 13.8). The digestion was started by addition of cysteine (final concentration 0.01M) and papain (Sigma) at a protein:papain ration of 100/1 (W/W). Digestion was allowed to proceed for 5 h at 37 °C. The reaction was stopped by the addition of iodoacetamide at a final concentration of 0.01 M and the sample was dialysed against PBS. Undigested IgG and Fc fragments were removed from the preparation by Protein A-Sepharose (Pharmacia) and Fab fragments in the non-retained fractions were then further purified by ion exchange chromatography on DEAE Sephacel (Pharmacia). Bound antibodies were eluted by a 16 hour linear gradient (0-100%) of Tris-HCl 50 mM, PH 8.5 containing NaCl 0.2 M at a flow rate of 12 ml/h. Purity of the Fab was verified by SDS PAGE (12%) and by reactivity of the Fab fragments with ^{125}I-anti-mouse IgG but not with ^{125}I-anti-mouse Fc.

CHAPTER III. Results

Immunization of Transgenic Mice:

To produce apoE isoform-specific mAbs we have attempted to develop a novel immunization strategy that favours the generation of an immune response that is limited to epitopes of the immunogen that are polymorphic in the species. The protocol is based on the premise that, when one immunizes a mouse that carries a transgene which encodes human apoE of one isoform, with a different human apoE isoform, only B lymphocytes that are specific for epitopes that differ between the apoE encoded by the transgene and the apoE used for immunization will be stimulated to proliferate and differentiate into plasmacytes.

From Dr. John Taylor at the Gladstone Institute for Cardiovascular Research, of the University of California, San Francisco, we obtained a breeding pair of mice that were heterozygous for a transgene that encoded human apoE3 and, from Drs. Stanley Rall and Sergio Fazio, at the same institution, we received a breeding pair of mice that were heterozygous for a transgene that encodes a natural, human apoE variant, $apoE^{Arg112,Cys142}$. The two lines were expanded by breeding. Offspring were screened for the presence of human apoE in plasma by a solid phase sandwich radioimmunometric assay. Purified IgG of the anti-human apoE mAb, 6C5, specific for an epitope mapped to the amino terminus of apoE, was used as the solid phase capture antibody. Dilutions of plasma from control mice or from the offspring of matings of transgenic mice were added to the 6C5-coated wells and bound human apoE was subsequently detected with ^{125}I-3H1, an anti-human apoE mAb that is specific for an epitope in the apoE carboxy-terminus. Offspring that

carry the human apoE transgene could be easily differentiated from those that did not inherit a human allele by this assay (Figure 5). Figure 5 shows the human apoE levels in plasma from 4 pups from a litter that resulted from breeding of the original human apoE3 mice that were received from Dr. Taylor. In this case, mouse 1 and mouse 3 had high levels of apoE in the plasma whereas mouse 2 and mouse 4 were negative. Both transgenes were expressed at equivalent levels. and human apoE concentrations in the plasma of positive offspring were estimated to be about 100 times higher than those found in the human plasma that was used as a positive control (note the difference in dilutions used for human and mouse plasma in figure 5). The concentration of apoE in normal human plasma varies between 40 and 80 μg / ml as measured in our laboratory (Milne et al., 1981). Human apoE concentrations were always higher in the plasma of the pups than in the plasma of the parents which may indicate that there is decrease in the expression of the transgene, or a change in the metabolism of mouse lipoproteins containing human apoE, with age. Two-fold and greater differences in the human apoE titre were commonly seen between positive offspring of the same litter. Subsequent breeding of the pups showed, however, that the relative human apoE concentration did not necessarily reflect the homozygous / heterozygous inheritance of the transgene.

Mice were immunized according to the protocols shown in figure 6. In immunization protocol 1, human apoE3 mice were immunized with the lipoprotein fraction of human apoE^{Arg112,Cys142} transgenic mice, with purified apoE4-22kDa, or with both antigens. In immunization protocol 2, apoE^{Arg112,Cys142} transgenic mice were

Figure 5- **Screening the offspring for the inheritance of the human apoE3 transgene. A solid phase sandwich RIA was performed to test the plasma for the presence of human** apoE. **In this assay serial dilutions of plasma from apoE (Arg ^{112}, Cys ^{142}) were incubated with previously coated 6C5 as an capture antibody and detected with ^{125} I-3H1 mAbs.** Similar results were obtained for screening of pups of the apoE$^{Arg112, Cys142}$ transgenic line. NSH; normal human serum.

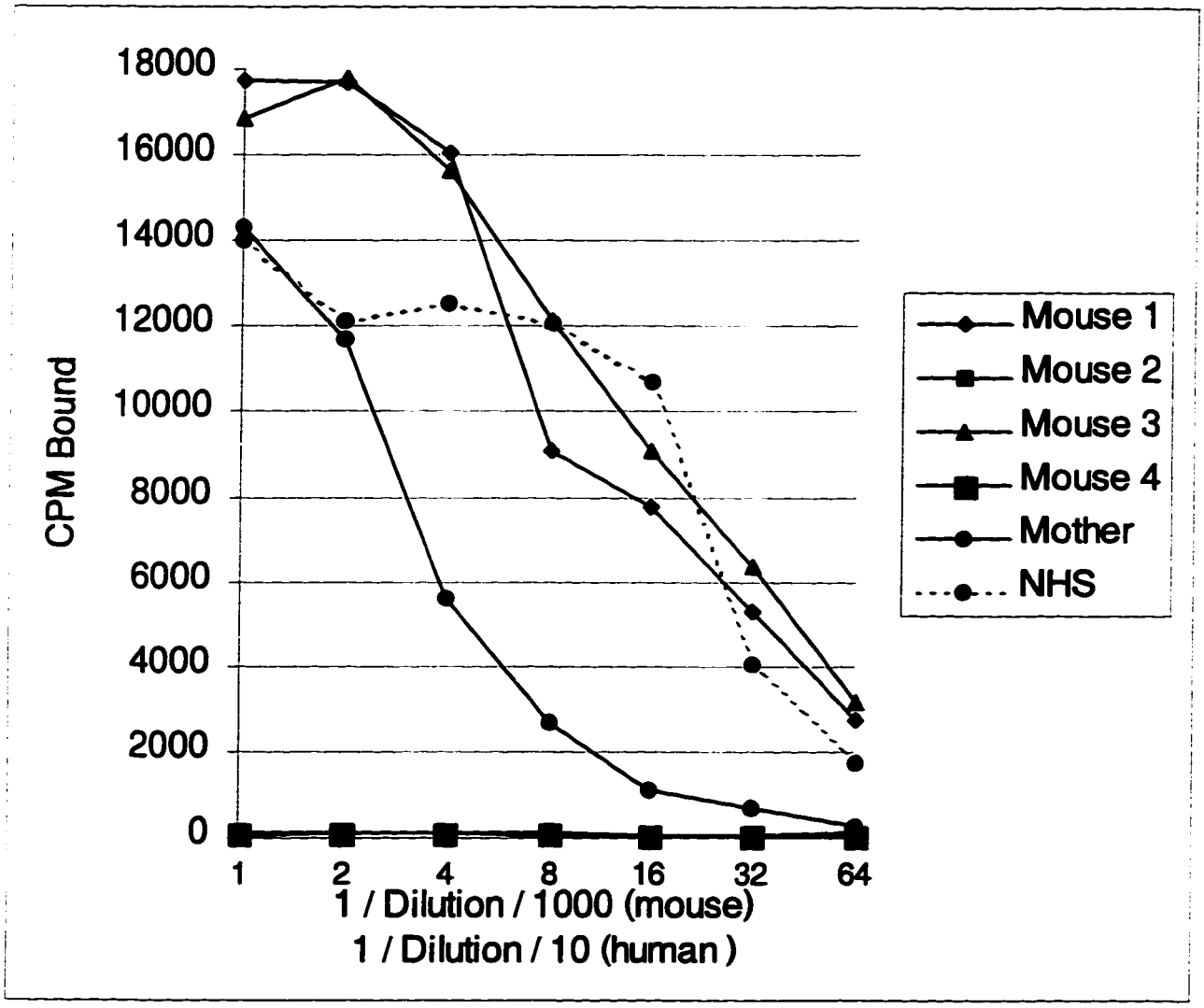
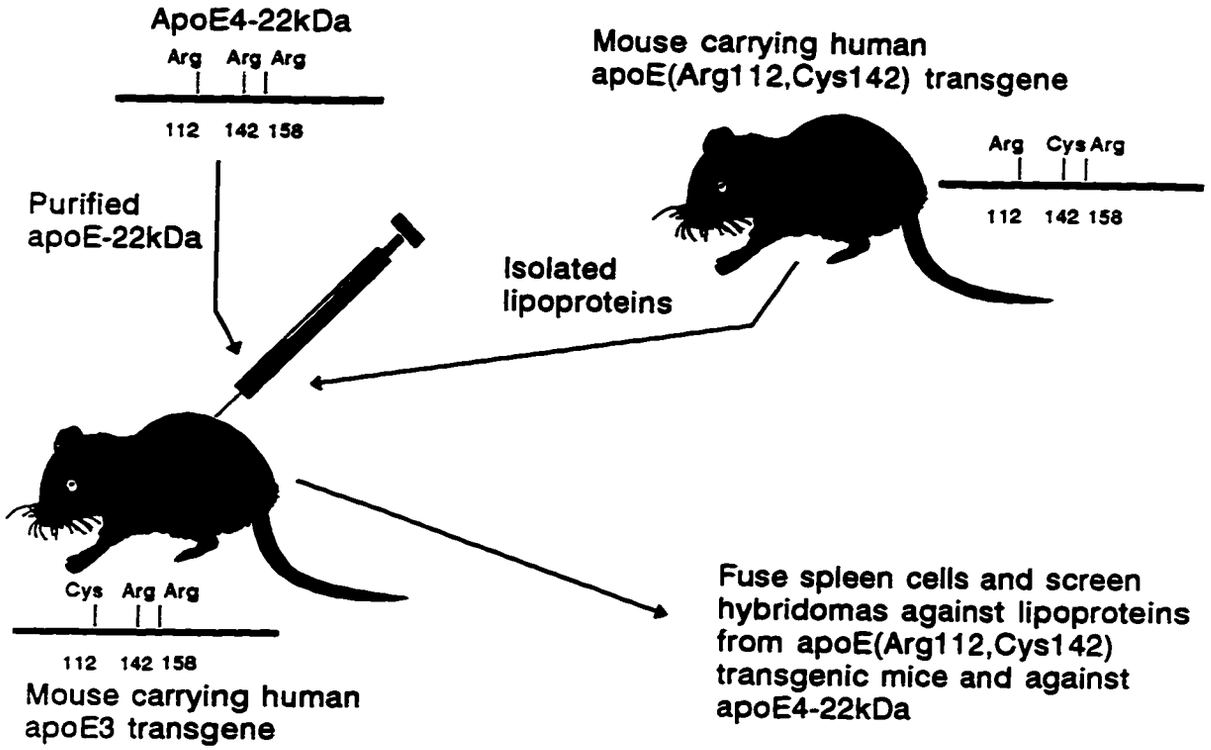
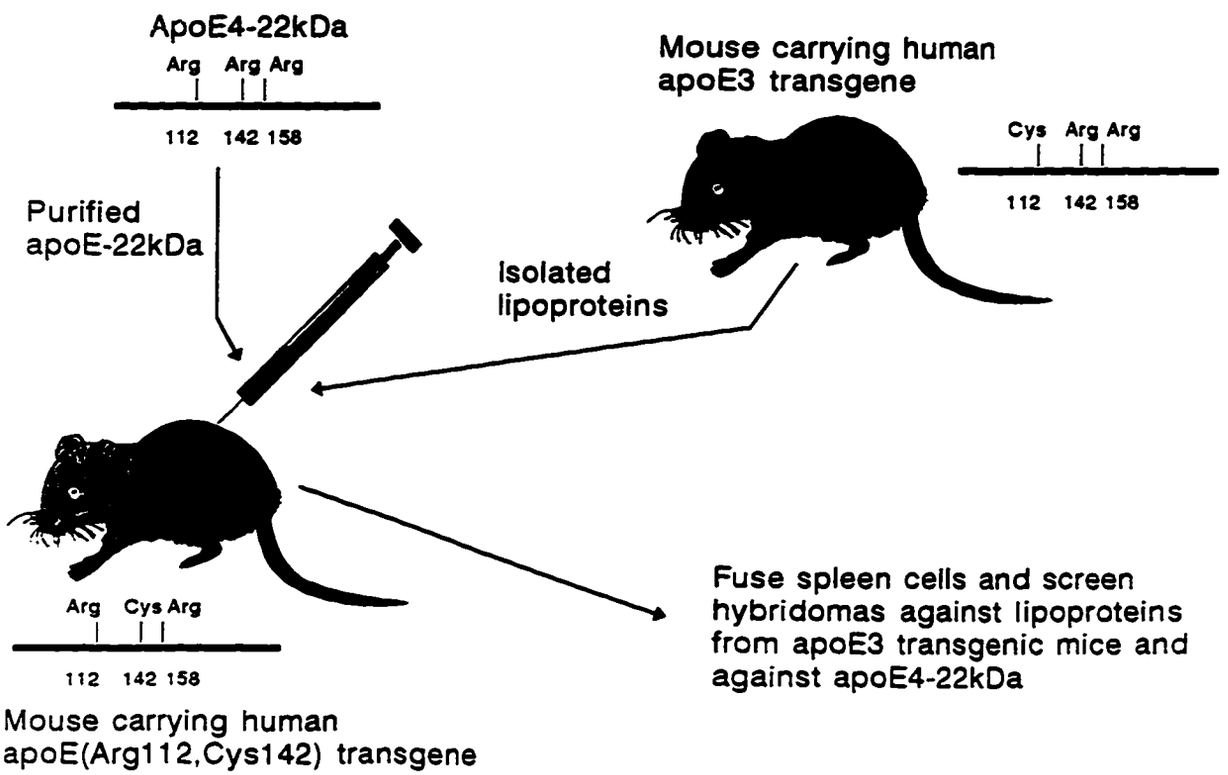

18000
16000
14000
12000
10000
8000
6000
4000
2000
0
CPM Bound
1
2
4
8
16
32
64
1 / Dilution / 1000 (mouse)
1 / Dilution / 10 (human)
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mother
NHS

Figure 6- Schematic representation of immunization protocols used to raise apoE isoform-specific immune responses. For details, see text.

IMMUNIZATION PROTOCOL 1



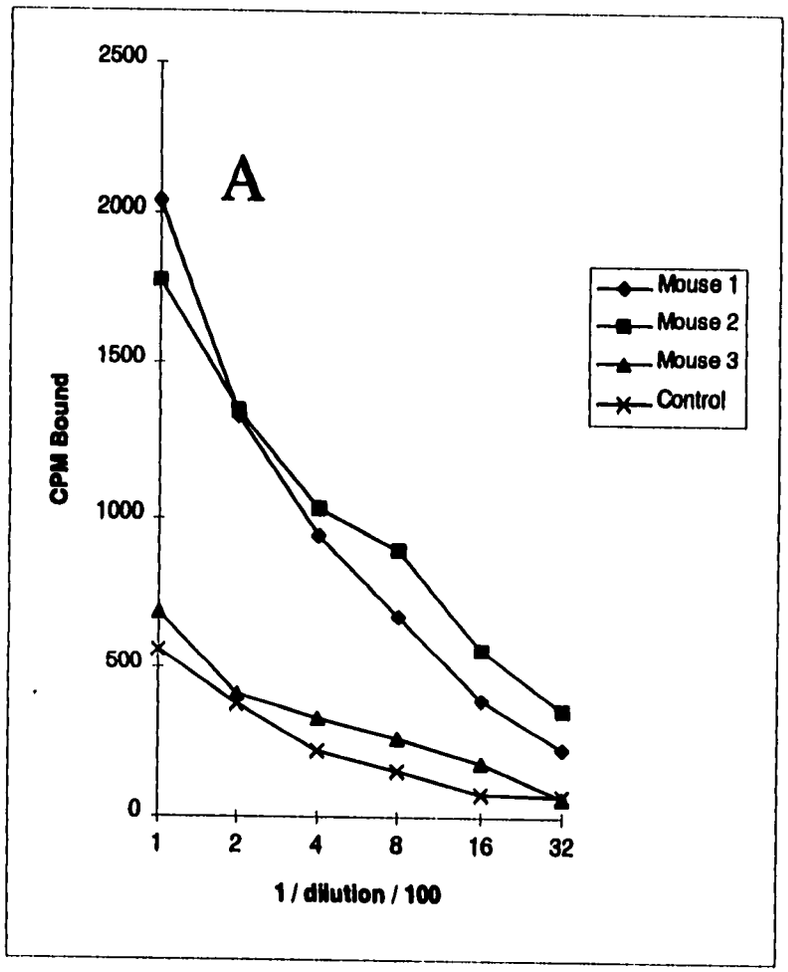
IMMUNIZATION PROTOCOL 2

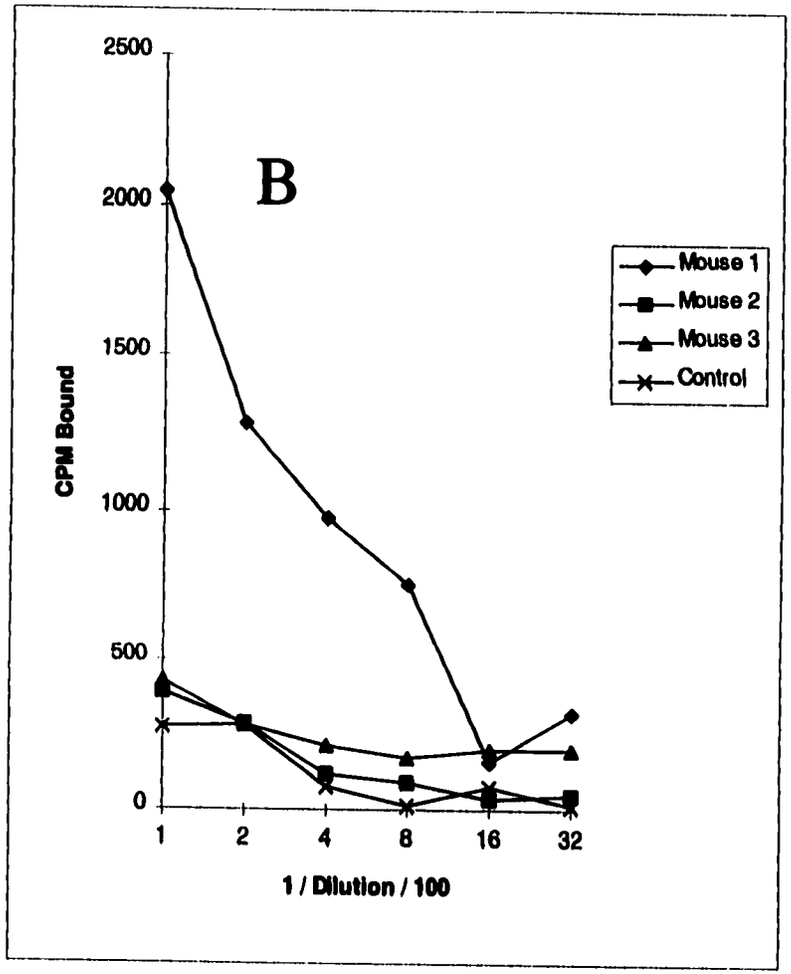


immunized with lipoproteins from apoE3 transgenic mice, with purified apoE4-22kDa or both.

Sera from immunized mice were screened for the presence of antibody by a solid phase, antibody-capture assay. The anti-apoE titres of 3, apoE3 transgenic mice that had been given a primary immunization and 3 subsequent boosts with lipoproteins isolated from apoE^{Arg112,Cys142} transgenic mice are shown in figures 7A, and 7B to illustrate the variety of responses that were elicited by the immunization protocol. Mouse 1 had a relatively high titre of anti-human apoE antibodies in the serum but there was no apparent isoform specificity in the response. In contrast, the antibodies in the serum of mouse 2 reacted well with immobilized lipoproteins isolated from apoE^{Arg112,Cys142} mice but did not react with lipoproteins from apoE3 transgenics. While the immunization protocol would appear to have elicited the anticipated immune response in the case of mouse 2, apoE isoform specificity is difficult to assess in the serum of the transgenic mice due to the high concentration of circulating human apoE, in this case apoE3; i.e. the circulating apoE3 could potentially compete with the immobilized apoE for binding to the antibodies. In a minority of mice (e.g. mouse 3 in figure 7A, and 7B) no anti-human apoE antibodies could be detected in the serum. Again, however, the immune response may have been masked by circulating human apoE In general, low titres of anti-apoE antibody were detected in the sera of immunized mice and there appeared to be partial isoform specificity. Mice with the highest titres of antibodies to human apoE or mice which appeared to have mounted an apoE-isoform-specific immune response were chosen for subsequent cell fusions.

Figure 7A and 7B- Screening of immunized mice plasma for circulating anti-apoE antibodies. 7A. A solid RIA was performed in which a serial dilution of sera from immunized or control mice were incubated with the coated apoE ^{Arg112,Cys142}. The bound mouse antibody was detected by addition of ^{125} I-anti mouse IgG. 7B. A solid RIA in which a serial dilution of sera from immunized or control mice were incubated with the coated apoE3. The bound mouse antibody was detected by addition of ^{125} I-anti mouse IgG.


A
2500
2000
1500
1000
500
0
CPM Bound
1
2
4
8
16
32
1 / dilution / 100
Mouse 1
Mouse 2
Mouse 3
Control


B
2500
2000
1500
1000
500
0
CPM Bound
1
2
4
8
16
32
1 / Dilution / 100
Mouse 1
Mouse 2
Mouse 3
Control

Generation of Anti-human ApoE Hybridomas:

A total of 9 fusions were performed and the results are summarized in table 1. Anti-apoE antibodies in hybridoma supernatants were detected by either a solid phase antibody-capture assay or a solid phase sandwich assay. I developed the latter assay to avoid any denaturation of the antigen that might occur upon its direct adsorption to the solid phase. In this assay, Fab fragments of the capture antibody (6C5) are adsorbed to polystyrene wells. The immobilized Fab fragments are sequentially exposed to antigen, hybridoma supernatant and ^{125}I-anti-mouse Fc. The solid phase antibody capture assay was used to screen hybridoma supernatants in fusions 1-4, the sandwich assay for fusions 7 and 8, and both assays were used for fusions 5 and 6. In general, in fusions 5 and 6, there was a good correlation between the results obtained with the two assay formats.

In primary screening of most fusions, potentially positive hybridomas were identifed according to the criteria described in the methods section. However, in most cases, when these clones were subsequently expanded, specific antibody reactivity was lost, before the cells could be subjected to recloning. There is a number of possibilities for the apparent loss of reactive cells. The initial positive reactions could be due to the presence of specific anti-apoE antibody-producing plasma cells that would not be killed in the selective HAT medium and that can survive up to 14 days in culture. A second possibility is that the hybridomas are intrinsically unstable. As hybridomas initially contain a double complement of chromosomes, from the splenocyte and the myeloma, respectively, they must rapidly lose chromosomes if they are to continue to survive and

proliferate. This segregation of chromosomes can lead to loss of function including antibody secretion. It is known that inter-species cell hybrids are often much less stable than intra-species hybrids and the chromosomes from one species can be selectively lost. While the cells partners used in the present study were both of murine origin, the SP2-0 myeloma was of BALB/c origin whereas the transgenic mice used as a source of splenocytes were either outbred mice (apoE3 transgenics) or C57BL6 X SLJ hybrids ($apoE^{Arg112,Cys142}$ transgenics). It is possible that the inter-strain fusions may have contributed to instability of the hybridomas. A third possibility is that cells growing in individual wells were not monoclonal and that irrelevant hybrid cells overgrew the specific hybridomas before the hybridomas could be recloned. This may have accounted for the loss of certain hybridomas. However, in several experiments, the fusion efficiency was poor and growth occurred in a low percentage of the wells that were seeded after the fusion (e.g. fusions 1 and 8). In these experiments, it is probable that the HAT-resistant cells in any individual well were monoclonal.

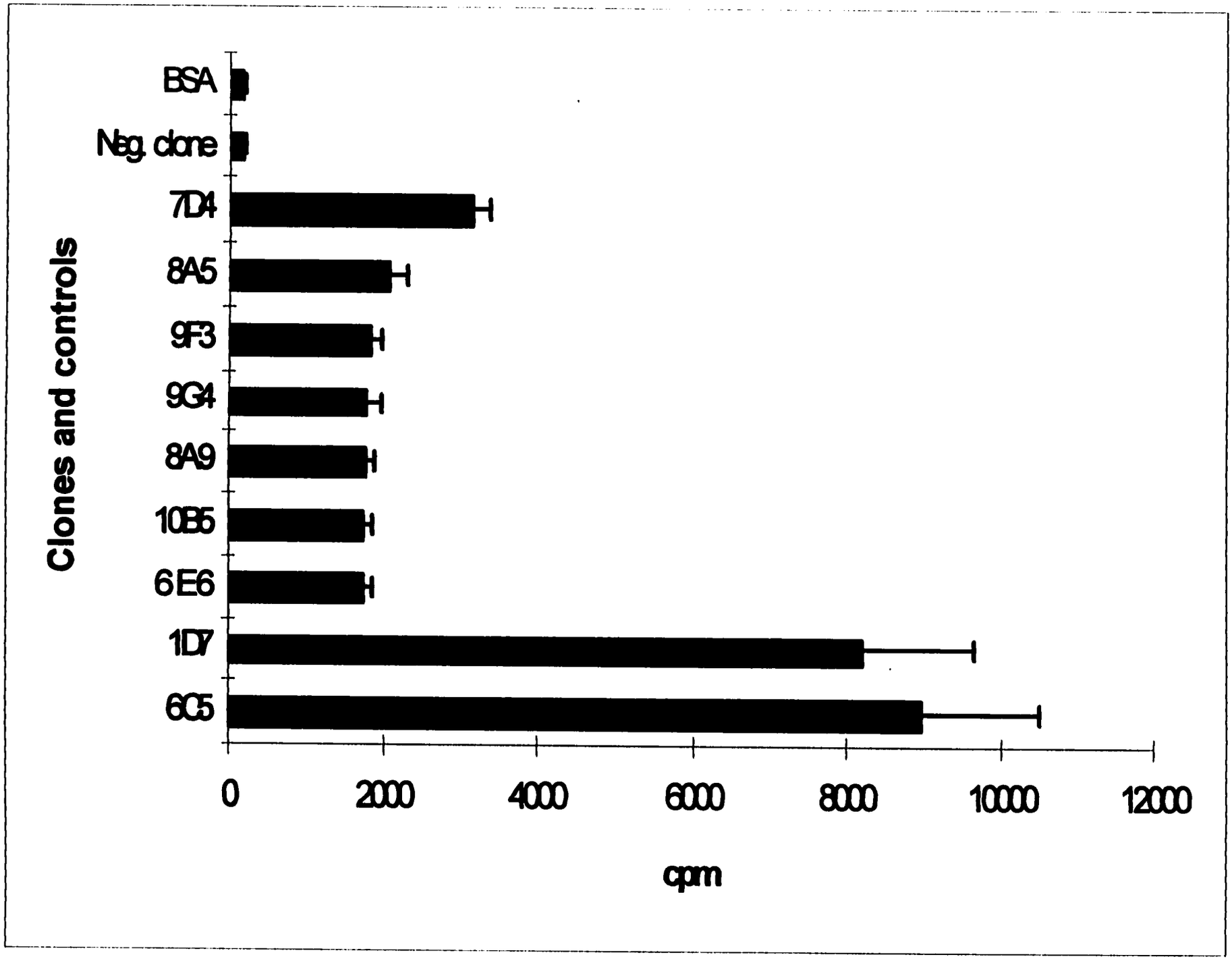
Seven stable hybridomas that secrete antibodies which are reactive with human apoE were obtained from fusion 9. After detection in the initial screening, the positive hybridomas were expanded, recloned 3 times by limiting dilution and were deemed to be monoclonal according to the criteria described in the preceding chapter. In the solid phase antibody capture assay culture supernatants from the hybridomas, before recloning, showed binding that was clearly higher than background although lower than that with purified 6C5 and 1D7 IgG (figure 8).

Table 1: Summary of the results of fusions to produce anti-apoE isoform-specific mAbs.

Fusion	Immunized mouse	Immunogen	Screening antigen	Wells with growth	Positive wells	Stable hybridomas
1	apoE^{Arg112Cys142} Tg	apoE3 Lp	apoE3 Lp	84	8	0
2	apoE3 Tg	apoE^{Arg112,Cys142} Lp	apoE^{Arg112,Cys142} Lp	420	22	0
3	apoE^{Arg112,Cys142} Tg	apoE3 Lp	apoE3 Lp	422	0	0
4	apoE^{Arg112,Cys142} Tg	apoE3 Lp	apoE3 Lp	140	0	0
5	apoE3 Tg	apoE^{Arg112,Cys142} Lp	apoE^{Arg112,Cys142} Lp	179	40	0
6	apoE3 Tg	apoE^{Arg112,Cys142} Lp	apoE^{Arg112,Cys142} Lp	182	5	0
7	apoE^{Arg112,Cys142} Tg	apoE3 Lp / apoE4-22kDa	apoE4-22kDa	160	8	0
8	apoE3 Tg	apoE4-22kDa	apoE4-22kDa	80	2	0
9	apoE3 Tg	apoE^{Arg112,Cys142} Lp / apoE4-22kDa	apoE4-22kDa	433	35	7

Tg: transgenic mice, Lp: lipoproteins

Figure 8- Summary of the screening of hybridoma supernatants for the presence of anti-apoE mAbs. Immunoreactivity of hybridoma supernatants was determined by a solid RIA. Supernatants were added to plates coated with apoE4 fragment and antigen-antibody complexes were detected by addition of ^{125}I-anti-mouse IgG. 6C5 and 1D7 are previously characterized anti-apoE mAbs and were used as positive controls. Clones 6E6, 10B5, 8A9, 9G4, 9F3, 8A5, and 7D4 were positive clones in these assays. BSA and Neg. clones (negative clones) are also shown.


BSA
Neg. clone
7D4
8A5
9F3
9G4
8A9
10B5
6E6
1D7
6C5
Clones and controls
0
2000
4000
6000
8000
10000
12000
cpm

Characterization of the Anti-human ApoE mAbs:

To confirm their human apoE specificity, culture supernatants of the from 7 recloned hybridomas were tested for reactivity with purified apoE4-22kDa that had been subjected to SDS-PAGE and transferred to nitrocellulose membranes. Two hybridomas, 7D4 and 9F3, secreted antibodies that reacted with the apoE-22kDa (figure 9). While the other mAbs may react with native apoE-22kDa but not with apoE-22kDa that been subjected to SDS-PAGE, only 7D4 and 9F3 have been characterized further, to date.

As the 7D4 and 9F3 hybridomas were generated from a fusion using spleen cells from an apoE3 transgenic mouse that had immunized with lipoproteins from an $apoE^{Arg112,Cys142}$ transgenic mouse and boosted with apoE4-22kDa, we anticipated that the antibodies may be specific for an epitope centered on Arg^{112} and thus be specific for apoE4. To test the isoform specificity of the two mAbs, they were tested in a sandwich radioimmunometric assay for their reactivity with apoE in the plasma of human subjects whose apoE phenotype had been determined. Purified 3H1 IgG was used to capture apoE containing lipoproteins in the human plasma and bound apoE was then detected with ^{125}I-7D4, ^{125}I-9F3 or ^{125}I-6C5. As can be seen in figure , both 7D4 and 9F3 reacted well with apoE in the plasma of apoE3/E3 and apoE3/E4 subjects but showed little reactivity with the apoE of an apoE2/E2 subject (figures 10A, and 10B). As expected mAb 6C5 showed no isoform specificity and reacted well with the apoE of all 3 subjects (figure 10C). Thus, 7D4 and 9F3 appeared to be specific for an epitope that required the presence of an arginine at position 158 and their reactivity was independent of an arginine / cysteine

Figure 9- A densitometric scan of a western blot that demonstrates the specific interaction of mAbs 7D4 and 9F3 with apoE4-22kDa fragment. The apoE4 fragment was subjected to a 12% SDS-PAGE and the migrated protein was electrophoretically transferred to a nitrocellulose membrane. Incubations with mAbs were followed by addition of ^{125}I-anti-mouse IgG. The interactions were then revealed by exposure of the membrane to a film that was subsequently analyzed by densitometric scanning.

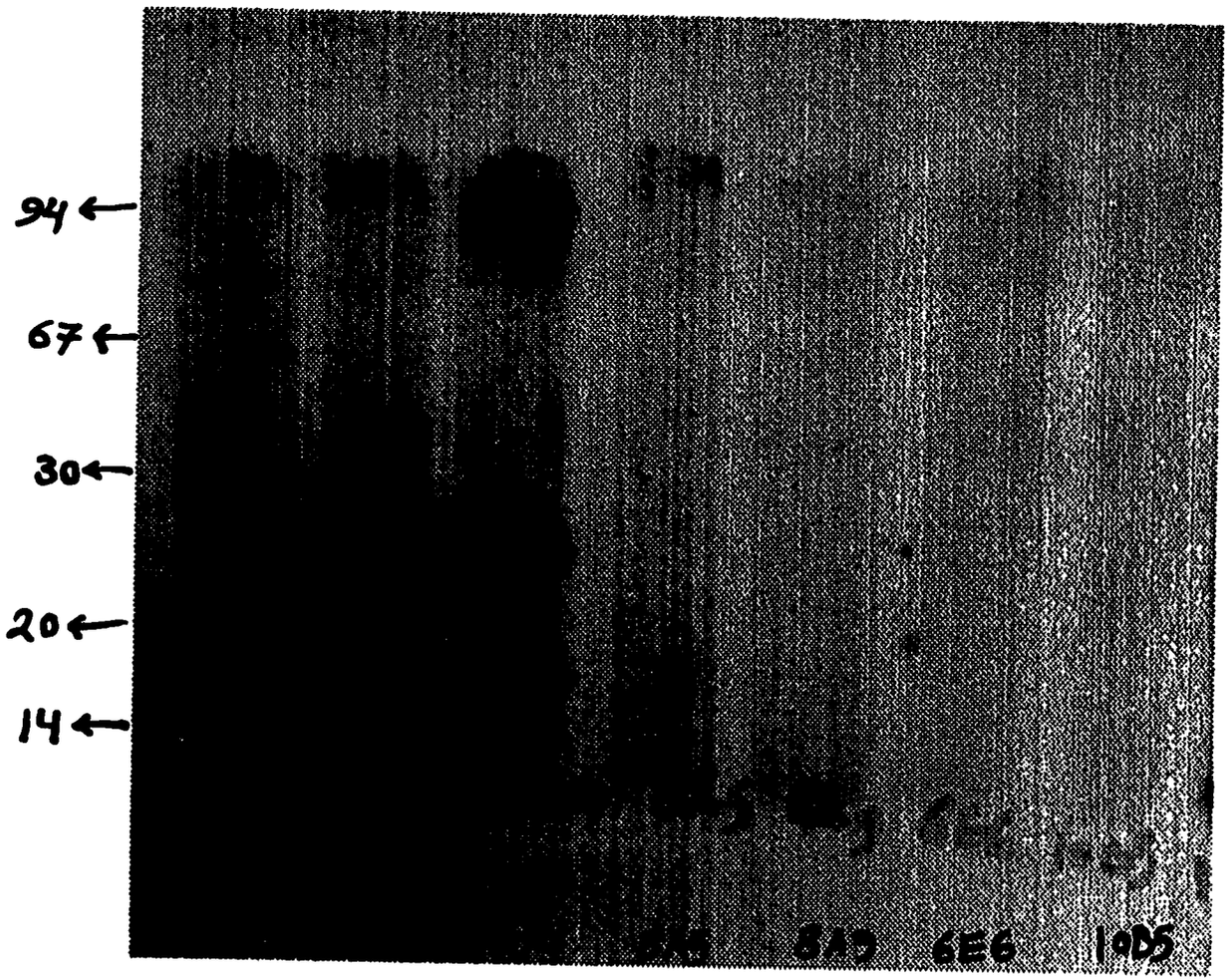

94
67
30
20
14
6AD
6E6
19D5

Figure 10A- Isoform specificity of mAb 7D4. Polystyrene wells coated with the anti-apoE capture mAB, 3H1. Serial dilution of plasma from individuals of known apoE phenotype (E2/2, E3/3, E3/4) were added to the 3H1-coated wells. Bound apoE was detected by the subsequent addition of ^{125}I-7D4.

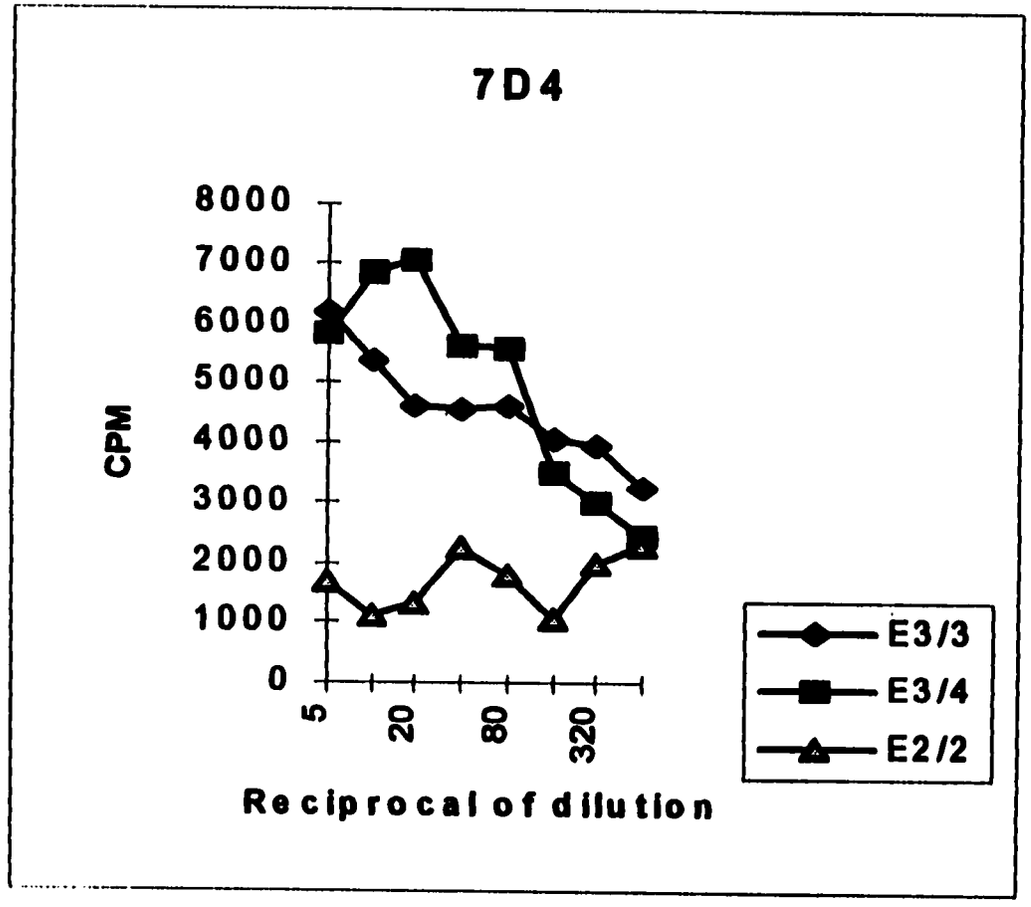

7D4
8000
7000
6000
5000
4000
3000
2000
1000
0
CPM
5
20
80
320
Reciprocal of dilution
E3/3
E3/4
E2/2

Figure 10B- Isoform specificity of mAb 9F3. Polystyrene wells coated with the anti-apoE capture mAB, 3H1. Serial dilution of plasma from individuals of known apoE phenotype (E2/2, E3/3, E3/4) were added to the 3H1-coated wells. Bound apoE was detected by the subsequent addition of ^{125}I-9F3.

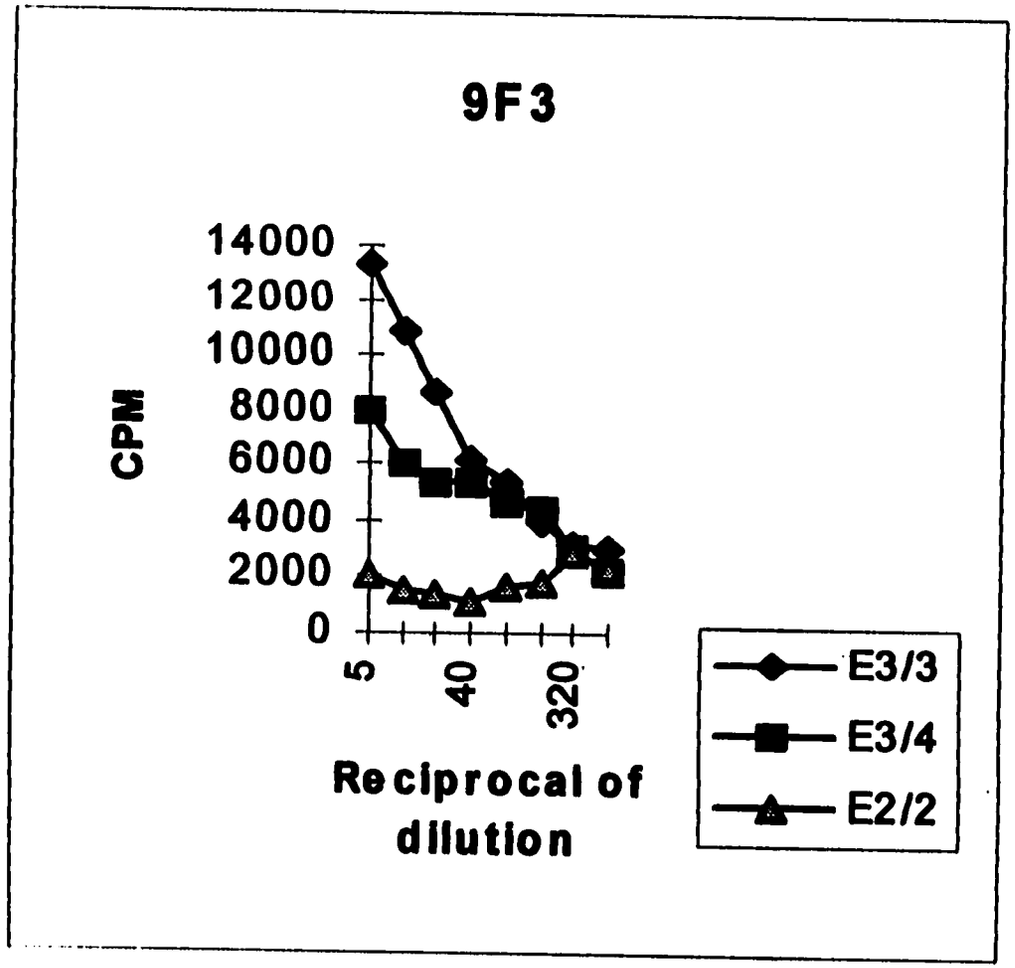
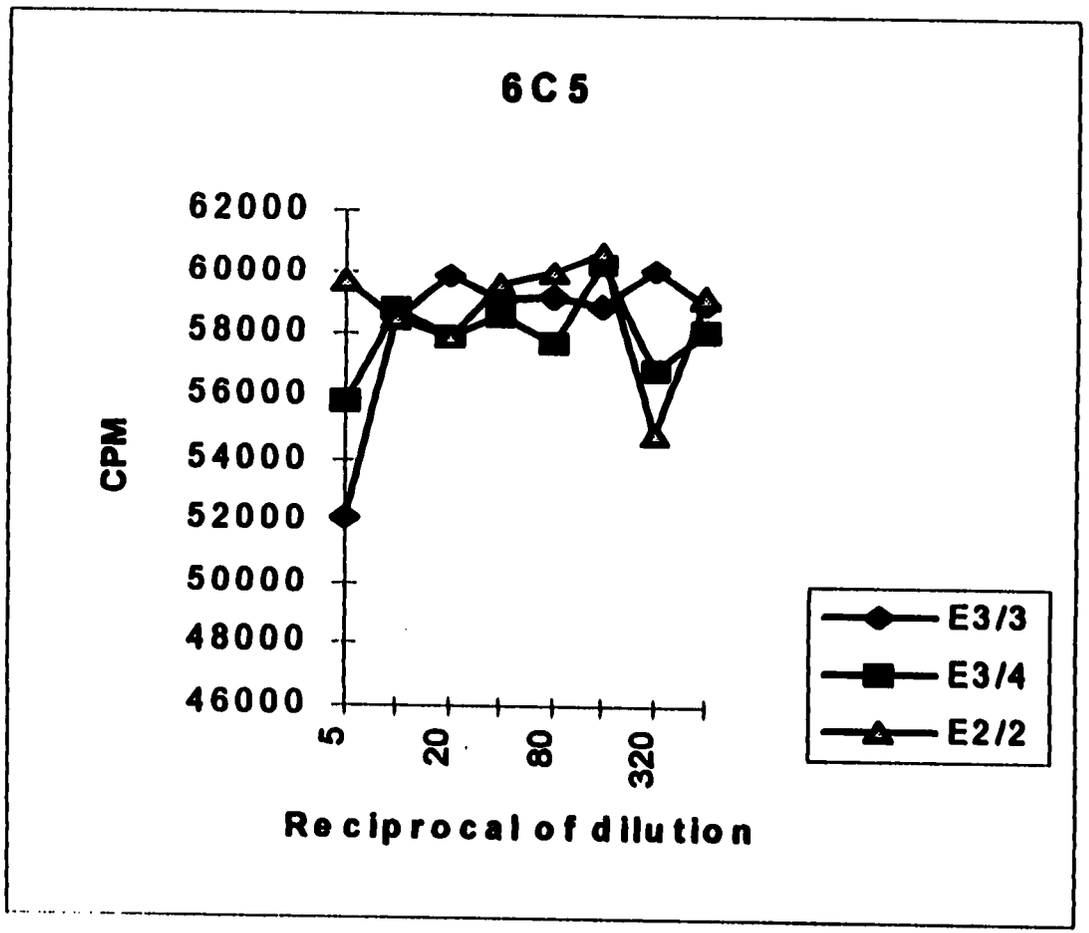

9F3
14000
12000
10000
8000
6000
4000
2000
0
CPM
5
40
320
Reciprocal of dilution
E3/3
E3/4
E2/2

Figure 10C- Interaction of 6C5 with different apoE isoforms. Polystyrene wells coated with the anti-apoE capture mAB, 3H1. Serial dilution of plasma from individuals of known apoE phenotype (E2/2, E3/3, E3/4) were added to the 3H1-coated wells. Bound apoE was detected by the subsequent addition of ^{125}I-6C5.


6C5
62000
60000
58000
56000
54000
52000
50000
48000
46000
CPM
5
20
80
320
Reciprocal of dilution
E3/3
E3/4
E2/2

interchange at residue 112. The isoform specificity was confirmed with plasma of other subjects of known apoE phenotype (not shown).

To establish the fine specificity of the mAbs, 7D4, 9F3 and the previously characterized anti-human apoE mAb, 3H1, were tested in a solid phase antibody-capture radioimmunometric assay for their reactivity with a series of apoE variants that are characterized by amino acid substitutions in the region of residues 136-158. These apoE proteins had been expressed in *E.coli* and represent natural apoE variants that are associated with type III hyperlipidemia and that show reduced affinity for the LDLr. In a previous study from the laboratory, it has been shown that mAb 3H1, specific for an epitope between apoE residues 243-272, reacts equally with all of the variants (Raffaï et al., 1995). This was confirmed in the present study and, as a consequence, all results are presented in figures 11A, and 11B relative to the 3H1 reactivity in order to compensate for any minor errors in the protein determinations etc. The strong reactivity of 7D4 and 9F3 with apoE3 and the low reactivity with apoE2 confirmed the results presented in figures 10A, and 10B. Both mAbs reacted poorly with apoE variants having substitutions of neutral amino acids for basic residues at positions 136, 140, 143, and 150. The 7D4 and 9F3 epitopes appear to coincide with the LDLr-binding site of apoE and overlap the epitopes of the previously described anti-apoE mAbs, 1D7 and 2E8 (Weisgraber et al., 1983; Raffaï et al., 1995).

The specificity of 7D4 and 9F3 for the common apoE isoforms and for the rare apoE variants suggest that they react with the same or overlapping epitopes. In an attempt to confirm the common specificity of the two mAbs or to discriminate between their respective specificities, we have tested the ability of the two mAbs to compete for

binding to immobilized apoE4-22kDa. To further define the 7D4 and 9F3 epitopes, we have also tested the ability of mAbs 1D7, 2E8 and 6C5 to compete with 7D4 and 9F3 for binding to apoE4-22kDa. For these experiments, dilutions of the unlabeled competing mAb was mixed with about 10 ng of ^{125}I-7D4 or ^{125}I-9F3 before addition to apoE4-22kDa coated wells. Results are presented in figures 12A, and 12B. Mutual competition was observed between 7D4 and 9F3 which would be consistent with the two mAbs having identical or overlapping epitopes. As expected from experiments described above, both 1D7 and 2E8 (not shown) competed with both ^{125}I-7D4 and ^{125}I-9F3. Totally unexpected, however, was the observation that 6C5, specific for an epitope close to the amino terminus of apoE, also competed effectively with both mAbs for binding to apoE4-22kDa. An irrelevant mAb (anti-human cholesteryl ester transfer protein) showed no ability to compete, indicating the specificity of the competition.

Figure 11A- Immunoreactivity of 7D4 with different apoE mutants. Interactions of different apoE mutants (see materials and methods) with 7D4 were evaluated in a solid RIA. Plates coated with apoE mutants were incubated with ^{125}I-7D4, ^{125}I-1D7, and ^{125}I-3H1 and the immunoreactivity was then measured by a gamma counter. Relative immunoreactivity was calculated by dividing 7D4 cpm bound by 3H1 cpm bound.

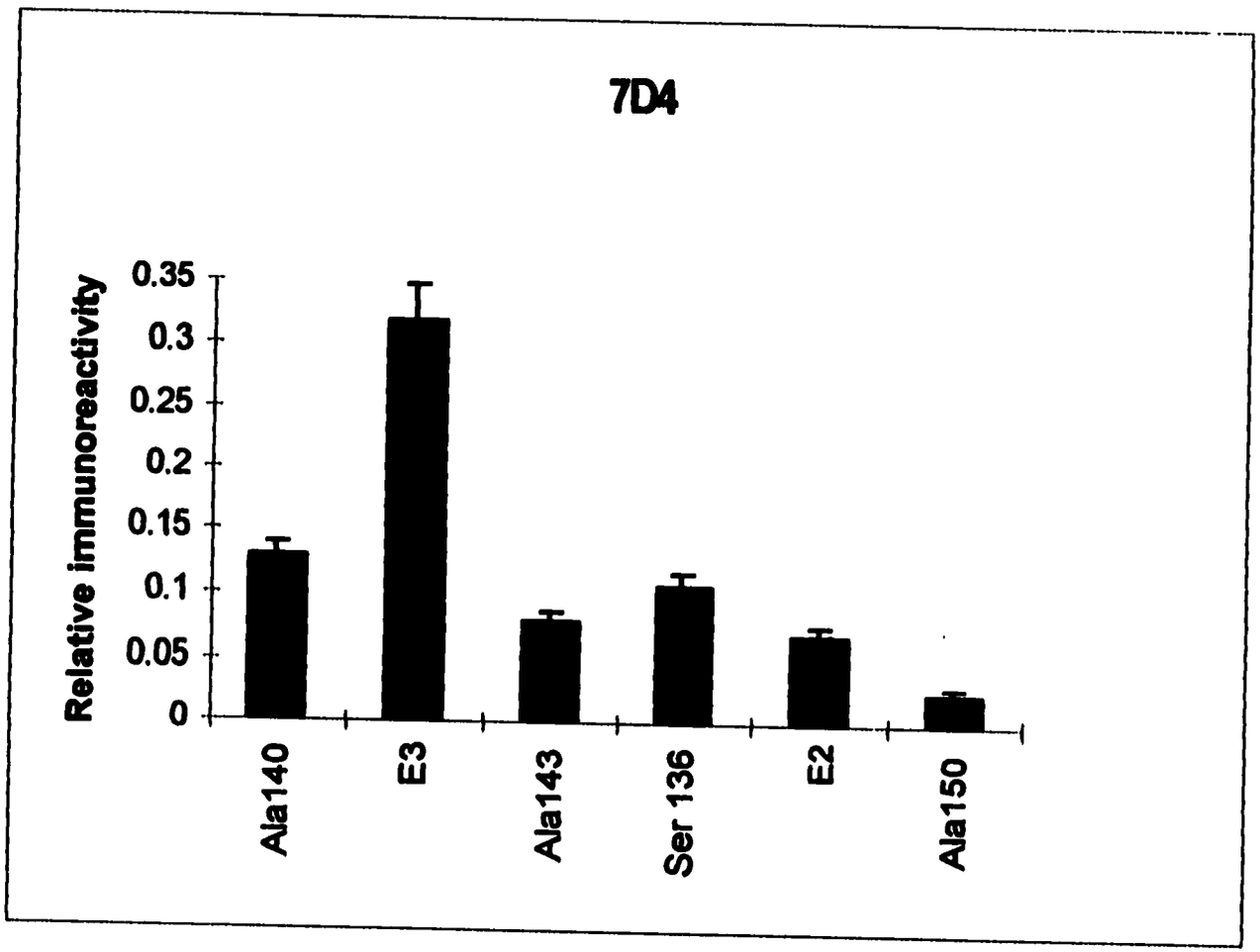

7D4
Relative immunoreactivity
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Ala140
E3
Ala143
Ser 136
E2
Ala150

Figure 11B- Immunoreactivity of 9F3 with different apoE mutants. Interactions of different apoE mutants (see materials and methods) with 9F3 were evaluated in a solid RIA. Plates coated with apoE mutants were incubated with ^{125}I-9F3, ^{125}I-1D7, and ^{125}I-3H1 and the immunoreactivity was then measured by a gamma counter. Relative immunoreactivity was calculated by dividing 9F3 cpm bound by 3H1 cpm bound.

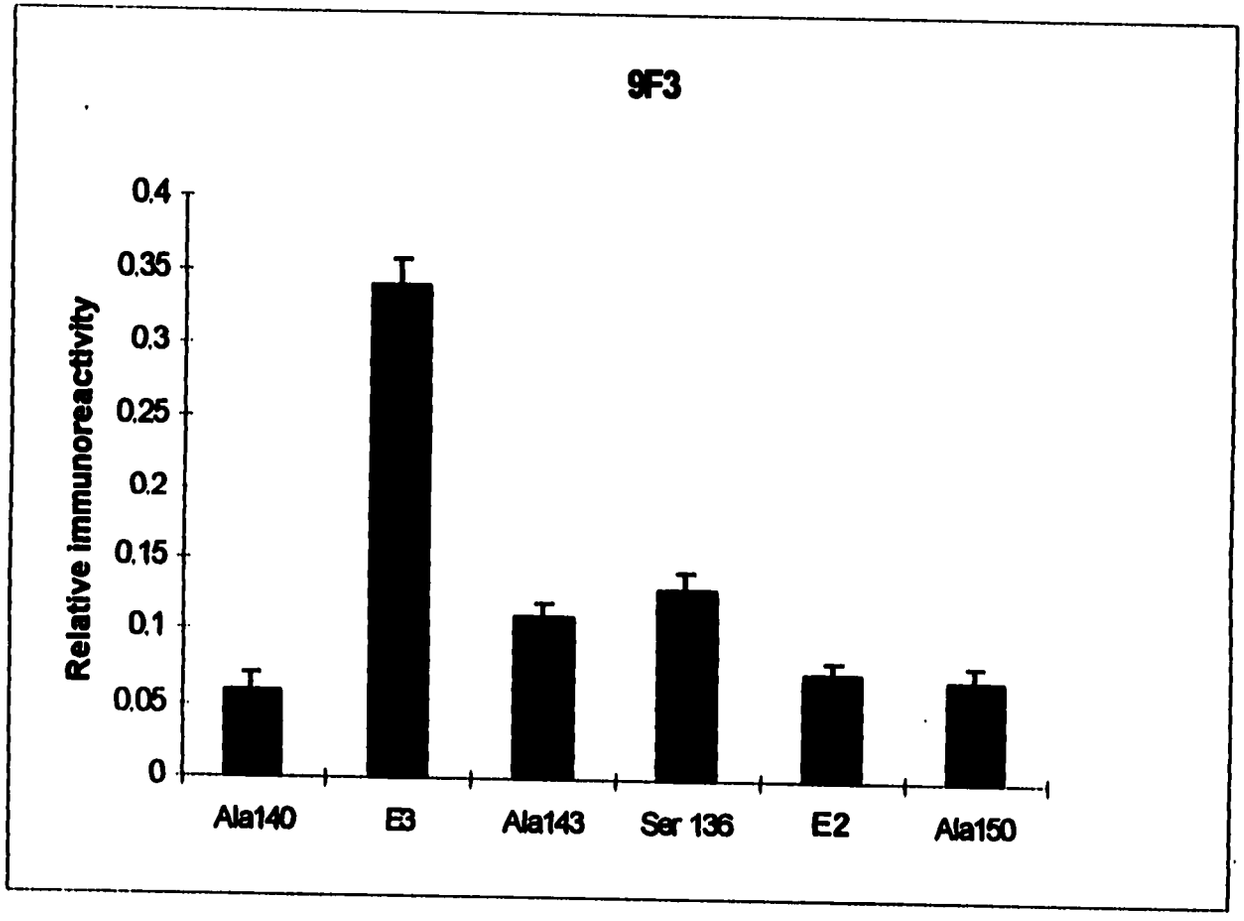

9F3
Relative immunoreactivity
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Ala140
E3
Ala143
Ser 136
E2
Ala150

Figure 12A- **Competition of 9F3 with different mAbs. Equal amounts of ^{125}I-9F3 were incubated with different concentration of 6C5, 1D7, 7D4, 9F3, and anti-CETP. A mixture of ^{125}I-9F3 and each of these mAbs was then added to plates coated with apoE4 fragment. Bound radioactivity was determined in a gamma counter. Results are presented as counts bound in the presence of competitor (B) divided by counts bound in the absence of competitor (Bo).**

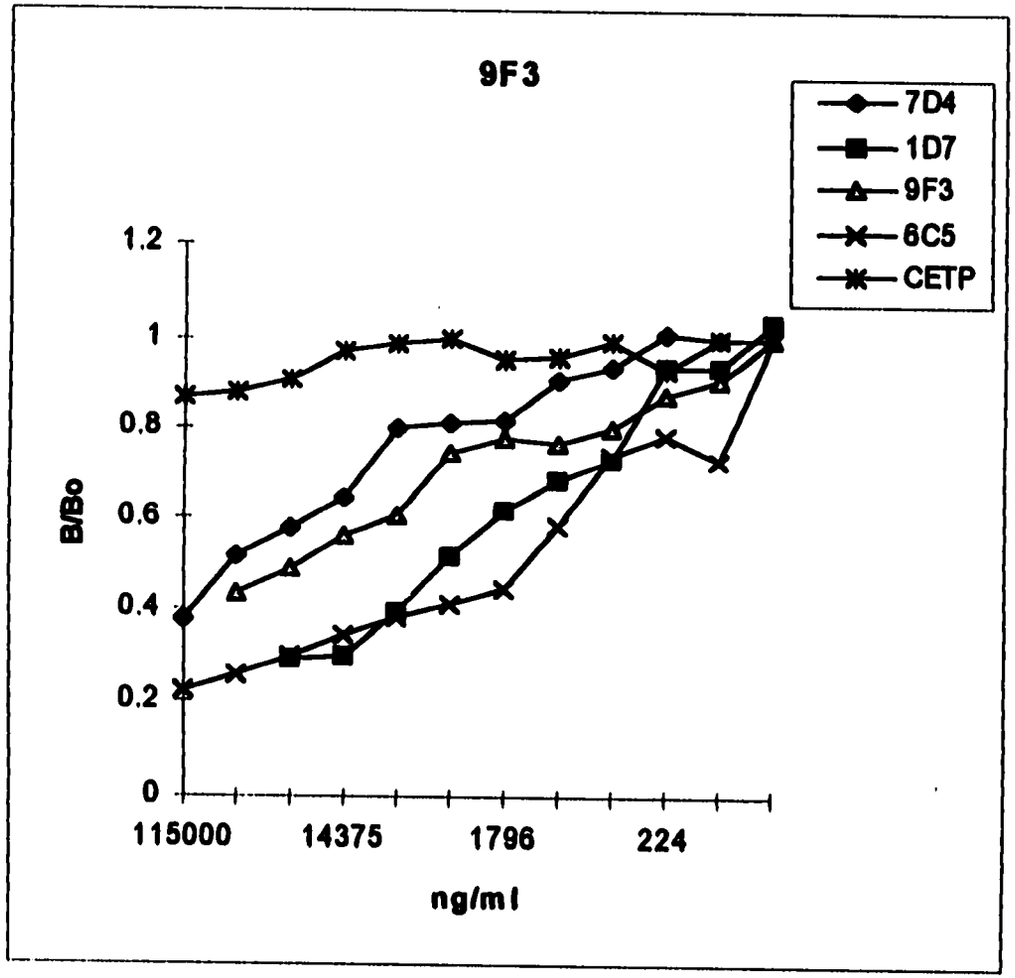
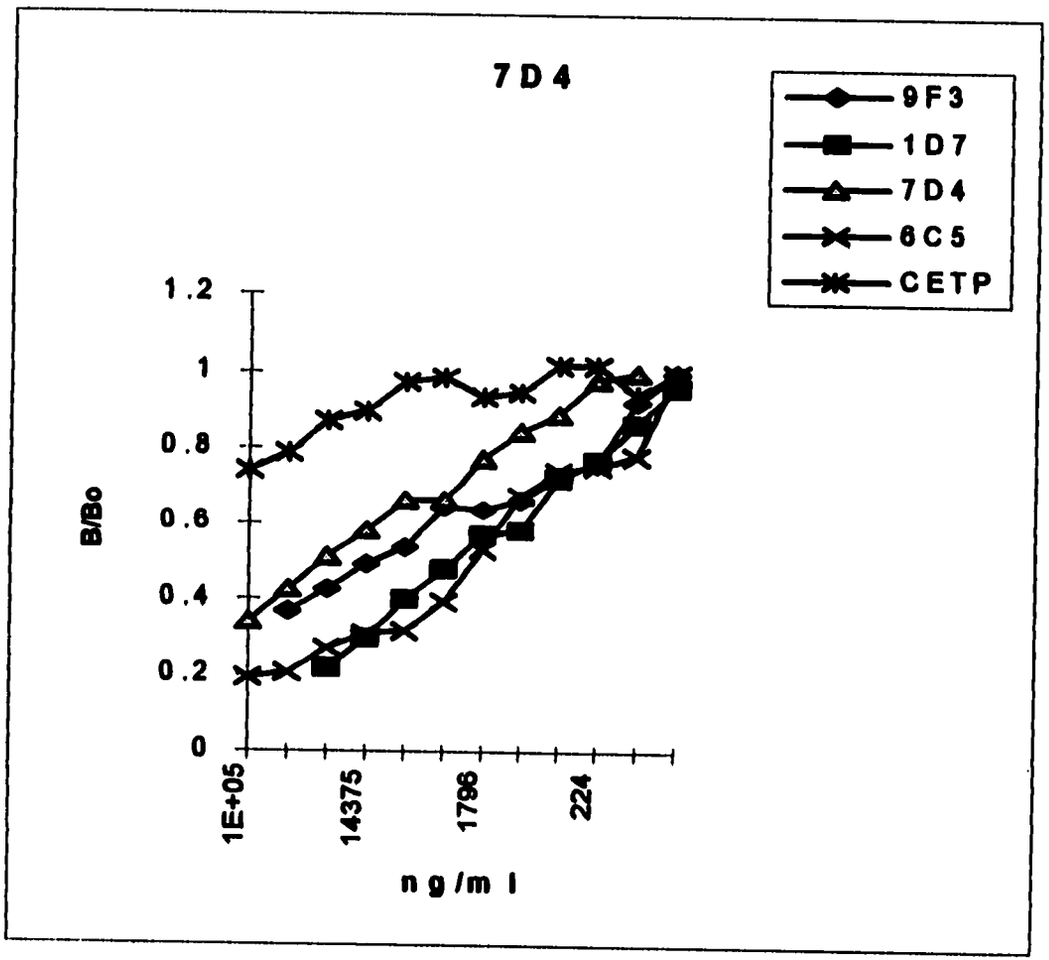

9F3
7D4
1D7
9F3
6C5
CETP
1.2
1
0.8
0.6
0.4
0.2
0
B/Bo
115000
14375
1796
224
ng/ml

Figure 12B- **Competition of 7D4 with different mAbs. Equal amounts of ^{125}I-7D4 were incubated with different concentration of 6C5, 1D7, 7D4, 9F3, and anti-CETP. A mixture of ^{125}I-9F3 and each of these mAbs was then added to plates coated with apoE4 fragment. Bound radioactivity was determined in a gamma counter. Results are presented as counts bound in the presence of competitor (B) divided by counts bound in the absence of competitor (Bo).**


7D4
9F3
1D7
7D4
6C5
CETP
B/Bo
1.2
1
0.8
0.6
0.4
0.2
0
1E+05
14375
1796
224
ng/ml

The abilities of mAbs 6C5, 2E8 and 1D7 to compete with ^{125}I-7D4 and ^{125}I-9F3 for binding to apoE could be due to steric hindrance between antibodies specific for epitopes that are spatially close. These results help to explain one of our early observations (not shown). We found that neither ^{125}I-7D4 nor ^{125}I-9F3 could detect apoE that had been captured in a sandwich radioimmunometic assay with either 6C5 or 2E8 (1D7 was not tested). We had originally attributed this inability to bind to 6C5- and 2E8-captured apoE to a lack of reactivity of the two mAbs with native epitopes of apoE.

It is proposed that the amino terminal domain of apoE undergoes a major conformational change when it binds to lipids. It is thought that the four-helix bundle opens up on the lipid surface. As such a conformational change could alter the relative positions of the epitopes on apoE, we have repeated the antibody competition experiments using VLDL from an apoE3/E3 donor as the solid phase antigen rather than apoE4-22kDa. As can be seen in figures 13A and 13B, 6C5 and 1D7 could compete with ^{125}I-7D4 and ^{125}I-9F3 for binding to lipoprotein bound apoE.

Figure 13A- **Competition of 7D4 with different mAbs. Equal amounts of ^{125}I-9F3 were incubated with different concentration of 6C5, 1D7, and anti-CETP. A mixture of ^{125}I-9F3 and each of these mAbs was then added to plates coated with human VLDL. Bound radioactivity was determined in a gamma counter. Results are presented as counts bound in the presence of competitor (B) divided by counts bound in the absence of competitor (Bo).**

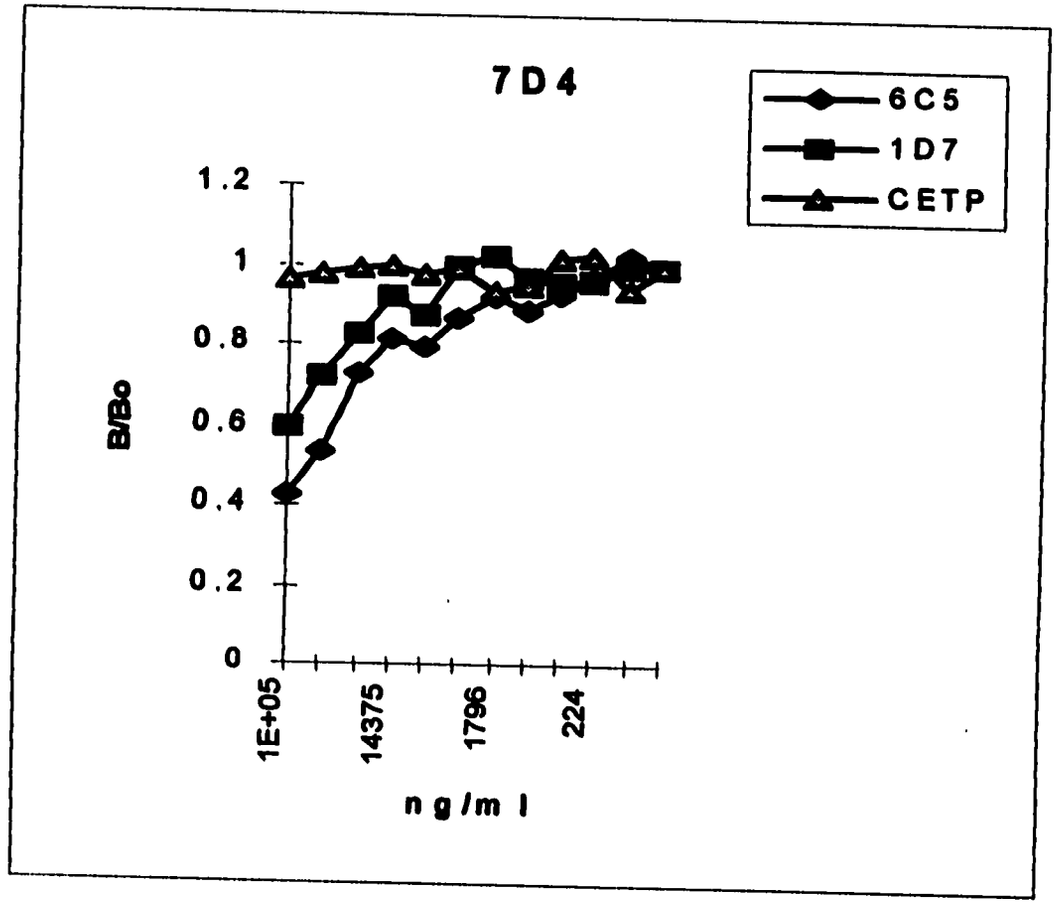
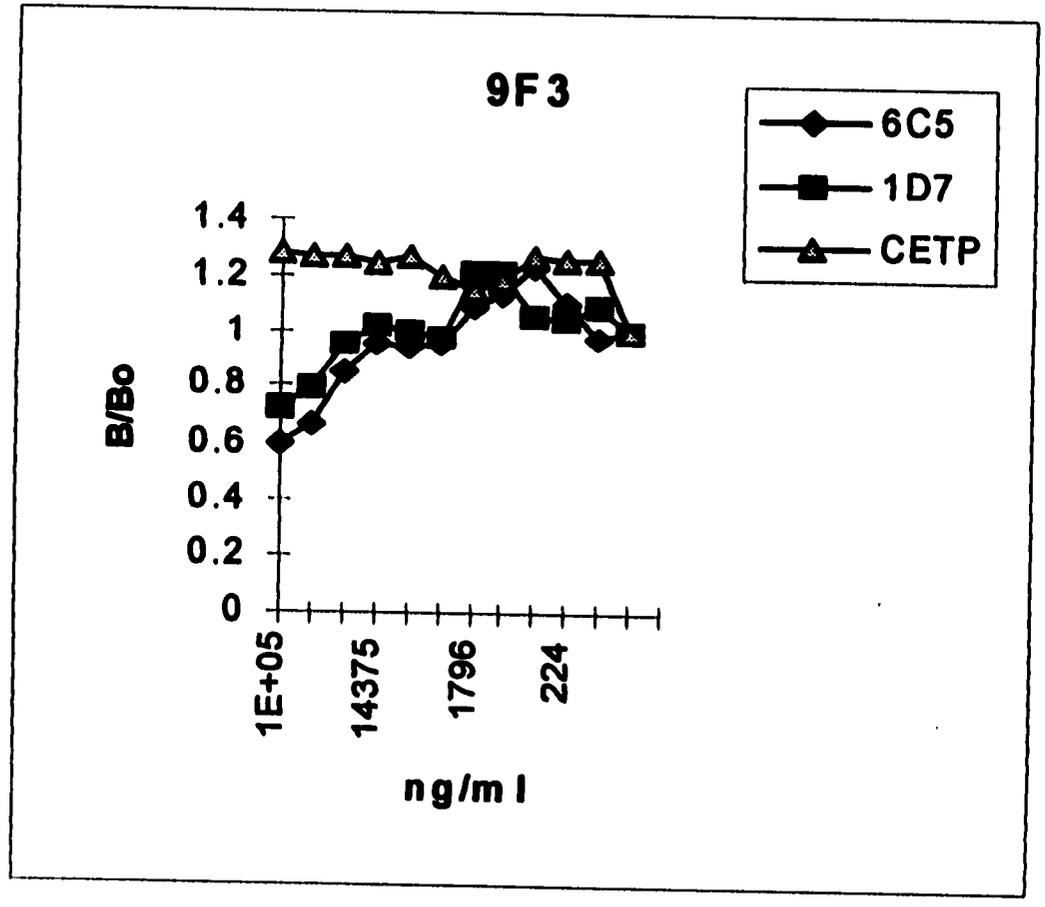

7D4
6C5
1D7
CETP
1.2
1
0.8
0.6
0.4
0.2
0
B/Bo
1E+05
14375
1796
224
ng/ml

Figure 13b- Competition of 9F3 with different mAbs. Equal amounts of ^{125}I-9F3 were incubated with different concentration of 6C5, 1D7, and anti-CETP. A mixture of ^{125}I-9F3 and each of these mAbs was then added to plates coated with human VLDL. Bound radioactivity was determined in a gamma counter. Results are presented as counts bound in the presence of competitor (B) divided by counts bound in the absence of competitor (Bo).


9F3
6C5
1D7
CETP
B/Bo
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1E+05
14375
1796
224
ng/ml

CHAPTER IV. DISCUSSION

Through its ability to mediate binding of lipoproteins to cell surface receptors and to heparin sulfate proteoglycans, apoE plays a key role in plasma lipoprotein metabolism. The importance of this function is clearly evident in natural and experimental apoE deficiency. ApoE-deficient humans show severe type III dyslipoproteinemia (Schaeffer et al., 1986) and mice that lack apoE, as a result of gene targeting, are hyperlipidemic and suffer from premature atherosclerosis (Breslow et al., 1993). ApoE function is also modulated by changes in its primary structure that result from genetic polymorphism at the apoE locus. ApoE polymorphism has been shown to influence plasma lipid levels (Davignon et al., 1988), interactions of lipoproteins with cell surface receptors (Demant et al., 1991), and proteoglycans (Ji et al., 1994a), postprandial lipemia (Brenninkmeijer, et al., 1987), distribution of apoE between lipoprotein subclasses (Gregg et al., 1986), anti-oxidant properties of apoE (Miyata and Smith, 1996) and susceptibility to atherosclerosis (De Knijff and Havekes, 1996) and AD (Beyreuther et al., 1991).

One goal of the present project was to develop mAbs that would be capable of discriminating between apoE isoforms. There are presently two approaches that are commonly used to determine the apoE phenotype / genotype of individuals. Isoelectric focusing, usually on isolated VLDL, has been the traditional method for apoE isotyping of individuals (Bouthillier et al., 1983). Recently, PCR-based apoE genotyping has gained increased acceptance (Hixson and Vernier, 1990). Although both of these methods have proved reliable, an antibody based method for the determination of apoE phenotype could offer advantages in terms of both speed and cost. Moreover, in addition to their use as reagents for the determination of apoE phenotypes, isoform-specific anti-apoE mAbs could also be useful reagents to define the distribution of apoE isoforms

in tissues and lesions of subjects with AD and to elucidate the mechanisms responsible for the observed association between inheritance of the ε4 allele and susceptibility to AD.

In addition to apoE heterogeneity due to genetic polymorphism, apoE on the lipoprotein surface is also heterogeneous in terms of conformation. This conformational heterogeneity of apoE is, in turn, manifested in heterogeneity in the expression of epitopes that are recognized by anti-apoE mAbs (Krul et al., 1988), in the susceptibility of apoE to cleavage by proteases and, most importantly, in the ability of apoE to mediate binding of lipoproteins to the LDLr (Gianturco et al., 1983; Bradley et al., 1984). It is unclear which physical and / or compositional properties of lipoproteins are responsible for the conformational and functional heterogeneity of apoE. The second goal of the project was to prepare anti-apoE mAbs that could be used as conformational probes of lipoprotein-bound apoE. Specifically, we hoped to generate a mAb that was an antibody mimetic of the LDLr, i.e. a mAb that recognizes the same conformational epitope on apoE as is recognized by the LDLr. The primary criteria that would be used to identify an anti-apoE mAb with the same specificity as the LDLr would be its ability to specifically bind apoE isoforms that are recognized by the LDLr and its inability to bind receptor-defective apoE isoforms. Such an antibody would be used as a probe to identify the specific physical and chemical properties (particle diameter, surface charge, lipid and apolipoprotein composition etc.) of triglyceride-rich lipoproteins that modulate the conformation of the apoE-LDLr-binding epitope. An anti-apoE mAb mimetic of the LDLr would also be used as an immunogen to produce antibodies specific for the apoE-binding site of the LDLr, the LRP and the VLDLr. These anti-idiotypic antibodies would, in turn, be used to study the structure-function relationships of the members of the LDLr family of cell surface proteins.

Since apoE isoforms vary from one another by single amino acid substitutions, generation of isoform-specific anti-apoE mAbs could prove to be very difficult. Immunization with a foreign protein generally elicits the production of high affinity antibodies to epitopes that are unique to the immunogen but not to epitopes that are shared between the immunogen and the host. As a consequence, inter-species immunization generally results in the production of antibodies against species-specific epitopes that rarely distinguish between polymorphic forms of the antigen whereas, inter-strain or intra-species immunization favors production of antibodies specific for allogeneic or polymorphic epitopes, respectively. An example of the latter is the antigen polymorphisms of human apoB that were originally detected and defined by antibodies present in the sera of multi-transfused individuals (Butler et al., 1974). Because the immune system does not normally produce antibodies that are reactive with self proteins, the LDL present in the transfused blood presumably elicited an immune response in the patients which was limited to apoB epitopes that differed from those present on the patient's own LDL. While it was not tested, it is possible that the serum of these multi-transfused individuals also contained isoform-specific anti-apoE antibodies.

While intra-species immunization does favor the production of isoform-specific antibodies, it also presents ethical and technical problems when applied to humans. Compared to animals, the immunization protocols that one can use with humans are much more restrictive. One is also usually limited to peripheral blood as a source of lymphocytes for the fusion where the frequency of antigen-specific B cells is much lower than in the spleen. In the protocol that we have chosen for production of isoform-specific anti-apoE mAbs, we have tried to incorporate the conditions of intra-species immunization that favor the generation of an isoform-specific immune response, all

the while, maintaining the advantages of using experimental animals for immunization. Under most circumstances, transgenic mice recognize the product of the transgene as self and are non-responsive to immunization with this antigen (Goodnow et al., 1992). Therefore, a mouse carrying a human apoE transgene should produce an isoform-specific immune response when immunized with a human apoE isoform which is different from that encoded by the apoE transgene. As this strategy could be used for production of isoform-specific antibodies against any antigen for which the appropriate transgenic mouse lines exist, if it were effective, it could have potential applications beyond the scope of the present project (e.g. adhesion molecules and enzymes).

When I began this project, we possessed two lines of transgenic mice, one that carried a transgene which encoded human apoE3 and one that carried a transgene which encoded the human apoE$^{Arg112, Cys142}$ variant that is defective in mediating binding of lipoproteins to the LDLr. The immunization protocols that we used are shown in figure 6. In initial experiments (fusions 2, 5, 6, 8, and 9), using protocol 1, human apoE3 mice were immunized with the lipoprotein fraction of human apoE^{Arg112,Cys142} transgenic mice. The apoE used for immunization differed from the human apoE3 expressed by the immunized transgenic mice at two positions, residues 112 and 142. In principle, the immune response should have been specific for epitopes centered on Arg^{112} and on Cys^{142}, or for conformational changes in apoE that result from one or both of these substitutions. In certain experiments, using protocol 1, the apoE3 transgenic mice were also immunized with the apoE4-22 kDa fragment. In this case the immune response should be centered on Arg^{112}. Therefore, from fusions using protocol 1, we hoped to obtain mAbs specific for apoE4 and for variants with an Arg→Cys substitution at residue 142. Using a similar

rationale, the immune response elicited in apoE^{Arg112,Cys142} transgenic mice using immunization protocol 2 should be limited to epitopes directly or indirectly resulting from the presence of Cys^{112} and Arg^{142} in apoE3 or Arg^{142} in apoE4-22kDa. We anticipated that, with protocol 2, we would generate mAbs that would react with apoE2 and apoE3 but not with apoE4 and mAbs that would be reactive with the common isoforms of apoE but would be unreactive with the apoE^{Arg112,Cys142} variant. Furthermore, as the apoE^{Arg112,Cys142} variant is defective in mediating lipoprotein binding to the LDLr, the immune response elicited by protocol 2 could include antibodies that recognize the same conformational epitope on apoE as is recognized by the LDLr.

Most human apoE transgenic mice that were immunized did show a weak isoform-specific immune response when their plasma was analyzed for antibodies using a solid phase antibody-capture radioimmunometric assay. Nevertheless, when we used these mice as a source of spleen cells for cell fusions, we were unable to subsequently isolate hybridomas that secrete mAbs having the anticipated anti-apoE isoform specificities. In most fusions we failed to detect any hybridomas that secreted antibodies reactive with the antigen used for immunization. For several of the fusions, we screened the culture supernatants using two different assay formats, a solid phase antibody capture assay and a solid phase sandwich format. Therefore, the inability to identify positive hybridomas does not likely reflect deficiencies in the format of the assay used for screening. One possibility is that we failed to detect anti-human apoE specific antibodies in the hybridoma supernatants because they cross-reacted with bovine apoE present in the FBS that was used as a supplement in the culture medium. In the solid phase antibody capture assay, the bovine apoE could potentially compete with the immobilized human apoE for binding the anti-apoE antibody. Again this is unlikely, as cross-reactivity between human and bovine apoE would not

present a problem in the sandwich assay as it has been shown that the capture antibody used in the sandwich assay, 6C5, does not bind to bovine apoE (Maurice, Collet, and Milne, manuscript in preparation). Given the low anti-apoE titers in the plasmas of the immunized mice, the most probable explanation for our failure to generate specific hybridomas is that the frequency of antigen-specific B cells was extremely low in the spleens of the immunized mice and our fusion efficiency was not sufficiently high to allow the generation of specific hybridomas.

From one fusion, in which apoE3 transgenic mice were immunized initially with lipoproteins from an apoE^{Arg112,Cys142} transgenic mice and subsequently with purified apoE4-22kDa (protocol 1), a series of anti-apoE specific hybridomas were identified and cloned. By such an immunization schedule, we hoped to generate an anti-apoE4 immune response. While the mAbs secreted by these hybridomas bound to apoE, they did not show the expected isoform specificity. None of the mAbs discriminated between apoE3 and apoE4. Most of the mAbs appeared to recognize epitopes that were exposed on apoE that was adsorbed to plastic but not on native apoE in solution or on lipoprotein-bound apoE. Specificity for non-native epitopes is commonly seen with mAbs (e.g. Roy et al., 1996) and selection for B-lymphocytes with such a specificity may have been favored by an immunization protocol in which the diversity of the immune response was limited by the self-tolerance imposed by the presence of the human apoE transgene. Two of the mAbs (7D4 and 9F3) did, however, react with native apoE3 in solution and on the surface of lipoproteins. As the hybridomas were generated in mice carrying the human apoE3 transgene, these hybridomas may be considered to have arisen from autoreactive B cell clones. Introduction of a foreign transgene has been shown to produce different outcomes with respect to the immune status of the host. Goodnow et al.,1988 have described transgenic mice in which

antigen-specific B cells are present but which are refractory to activation by both specific antigen and non-specific mitogens (*clonal anergy*) whereas, there are several reports that the presence of the transgene can result in the complete elimination of all antigen-specific B cells (*clonal deletion)* (Nemazee and Burki, 1989). In some cases, B cells specific for the product of the transgene are present and can be stimulated to differentiate into antibody-secreting plasma cells (Goodnow et al., 1989). The animal does not, therefore, appear to recognize the product of the transgene as self. This has been called *clonal ignorance* and has been noted in situations in which the transgene is expressed at low levels. The fact we obtained hybridomas that secreted antibodies that react with the product of the transgene may suggest that the transgenic animals were in a state of clonal ignorance. However, as can be seen in figure 5, this is not the result of poor expression of the transgene in the mice. Moreover, it should be noted that antibodies with self-reactivity can be elicited in experimental animals and, in at least some cases, with no apparent associated pathology (e.g. Milne et al., 1987).

While the mAbs 7D4 and 9F3 did not possess the apoE isoform specificity that we would have anticipated from apoE3 transgenic mice that had been immunized according to protocol 1, both mAbs did show apoE isoform specificity in that neither recognized apoE2 (Figures 10A, and 10B). ApoE2 differs from apoE3 by the substitution of a cysteine for an arginine at residue 158 and differs from apoE4 by substitutions of cysteine for arginine at residues 112 and 158. Therefore, in addition to its effects on the ability to mediate binding of lipoproteins to the LDLr, replacement of Arg^{158} by Cys in apoE2 eliminates the expression of the 7D4 and 9F3 epitopes. The low affinity of apoE2 for the LDLr is thought to reflect changes in the intra-molecular salt bridges that result from the absence of a positive charge at residue 158. In the crystal structure of

the amino terminal domain of apoE3, Arg^{158} is located at the C-terminal end of the fourth helix, and forms salt bridges with Glu^{96} and Asp^{154} that may help to stabilize the pairing of helices 3 and 4 (Wilson, et al., 1994). In apoE2, Arg^{150} forms a salt bridge with Asp^{154} (Wilson et al., 1994) which shifts Arg^{150} out of the receptor binding site. Disruption of the Arg^{150}-Asp^{154} salt bridge in apoE2 by replacing Asp^{154} by Ala restores receptor-binding activity (Dong et al., 1996). The low immunoreactivity of 7D4 and 9F3 with apoE2 could reflect this altered conformation of apoE2 relative to apoE3 or, alternatively, Arg^{158} could directly participate in the epitope(s) recognized by the two mAbs. Determination of the respective reactivities of 7D4 and 9F3 with apoE2 ^{Ala154} could potentially differentiate between the two possibilities.

In addition to showing reduced reactivity with apoE2, mAbs 7D4 and 9F3 also bind a number of other apoE variants poorly (Figures 11 A, and 11B). These apoE variants $apoE^{Ser136}$, $apoE^{Ala140}$,$apoE^{Ala143}$,$apoE^{Ala150}$ are characterized by substitutions of basic amino acids by neutral resdues within the apoE LDLr-binding site and all have reduced affinity for the LDLr. It would appear, therefore, that the 7D4 and 9F3 epitopes coincide with the apoE LDLr-binding site. Moreover, the 7D4 and 9F3 epitope(s) appear to overlap epitopes that are recognized by two anti-human apoE mAbs, 1D7 and 2E8, that have been previously characterized in this laboratory (Milne et al., 1981; Weisgraber et al., 1983; Raffaï et al., 1995). As would be expected, both 1D7 and 2E8 effectively compete with 7D4 and 9F3 for binding to immobilized apoE (Figures 12A, and 12B). The fine specificities of 7D4 and 9F3 show some similarity to these two previously described anti-apoE mAbs. Anti-apoE mAb 1D7 blocks apoE-mediated lipoprotein binding to the LDLr, reacts with a synthetic peptide composed of apoE residues 139-169 (Weisgraber et al., 1983) and has low affinity with the apoE variants $apoE^{Arg112,Cys142}$, $apoE^{Ala143}$, $apoE^{Pro144}$,

apoE^{Cys145}, apoE^{Gln146}, apoEAla^{150}, and apoE^{Pro152}. Unlike 7D4 and 9F3, 1D7 shows normal binding activity with apoE2, apoE^{Ser136}, and apoE^{Ala140} (Weisgraber et al., 1983; Horie et al., 1992; Raffaï et al., 1995). Antibody 2E8, which also neutralizes apoE-mediated binding to the LDLr, is similar to mAbs 7D4 and 9F3 in binding poorly to apoE2, apoE^{Ala143}, and apoE^{Ala150} but differs in its normal binding to apoE^{Ser136} and apoE^{ala140}. Therefore, in terms of the fine specificity of binding of apoE variants, mAbs 7D4 and 9F3 resemble, more closely, the LDLr than do either 2E8 or 1D7.

The specificity of 7D4 and 9F3 for apoE3 and apoE4, the LDLr-active apoE isoforms, and the lack of reactivity with apoE2 and the other apoE variants that are defective in binding to the LDLr suggest that these two mAbs may be candidates for antibody mimetics of the LDLr. What other criteria must these mAbs fulfill to be considered as LDLr mimetics? Lipid-free apoE does not bind to the LDLr and it has been proposed that apoE undergoes a conformational change upon binding lipid which renders it receptor-active (Innerarity et al., 1979). If 7D4 and 9F3 are specific for the same conformational epitope as recognized by the LDLr, one would predict that the mAbs would react with lipid-bound apoE but not with lipid-free apoE. This is not the case as both mAbs bind well to apoE3 and apoE4-22kDa fragment that are not bound to lipid (figures11A, 11B,12A, and 12B). The human LDLr shows little species specificity with respect to apoE, and the apoE of certain species e.g. rat and mouse, bind with higher affinity to the human LDLr than does human apoE. If 7D4 and 9F3 are LDLr mimetics, they should bind to heterologous apoEs that have high affinity for the human LDLr. This should be tested. It is known that lipid-bound apoE and LDL can mutually compete for binding to the LDLr which would be consistent with the existance of a common ligand-binding site on the LDLr for apoE and

apoB. On the other hand, analysis of apoE- and apoB-mediated binding to LDLr variants having deletions or point mutations in the ligand-binding domain indicates that separate binding sites for apoE and apoB could potentially exist. Thus, it remains unclear whether cross-reactivity of 7D4 and 9F3 with apoB should be considered a necessary criteria to demonstrate identity to the LDLr in terms of specificity. Finally, based on other experimental systems (Williams et al., 1988), one might expect that the anti-idiotypic immune response following immunization with 7D4 and 9F3 would include antibodies that cross-react with the LDLr. Generation of such antibodies remains one of the long term goals of the project.

The ability of anti-apoE mAbs 1D7 and 2E8 to compete with 7D4 and 9F3 for binding to immobilized apoE (Figures 12A, and 12bB) and the fine specificity of 7D4 and 9F3, as defined by their respective reactivities with apoE variants (Figures 11A and 11B), strongly suggest that the 7D4 and 9F3 epitopes are situated on the fourth helix of the amino terminal domain of apoE. It was unexpected, therefore, that anti-human apoE mAb, 6C5, which is specific for an epitope at the extreme amino terminus of apoE, competed effectively with 7D4 and 9F3 for binding to immobilized apoE (Figures 12A, and 12B). It has been demonstrated that removal of the amino terminal 13 residues of apoE eliminates its immunoreactivity with 6C5 (Weisgraber et al., 1983) and, more recently, it was shown that apoE residues 10-14 compose the core of the 6C5 epitope (Maurice, Collet, and Milne, manuscript in preparation). In the crystal structure of the 22kDa fragment of apoE, the amino terminal 22 amino acids could not be resolved and so the position of this region relative to the putative 7D4 and 9F3 epitope(s) within apoE tertiary structure is unknown. One explanation for the results in Figures 12A, and 12B is that the 6C5 epitope and that of 7D4 and 9F3 are spatially close in native apoE. This is illustrated in the form of a model

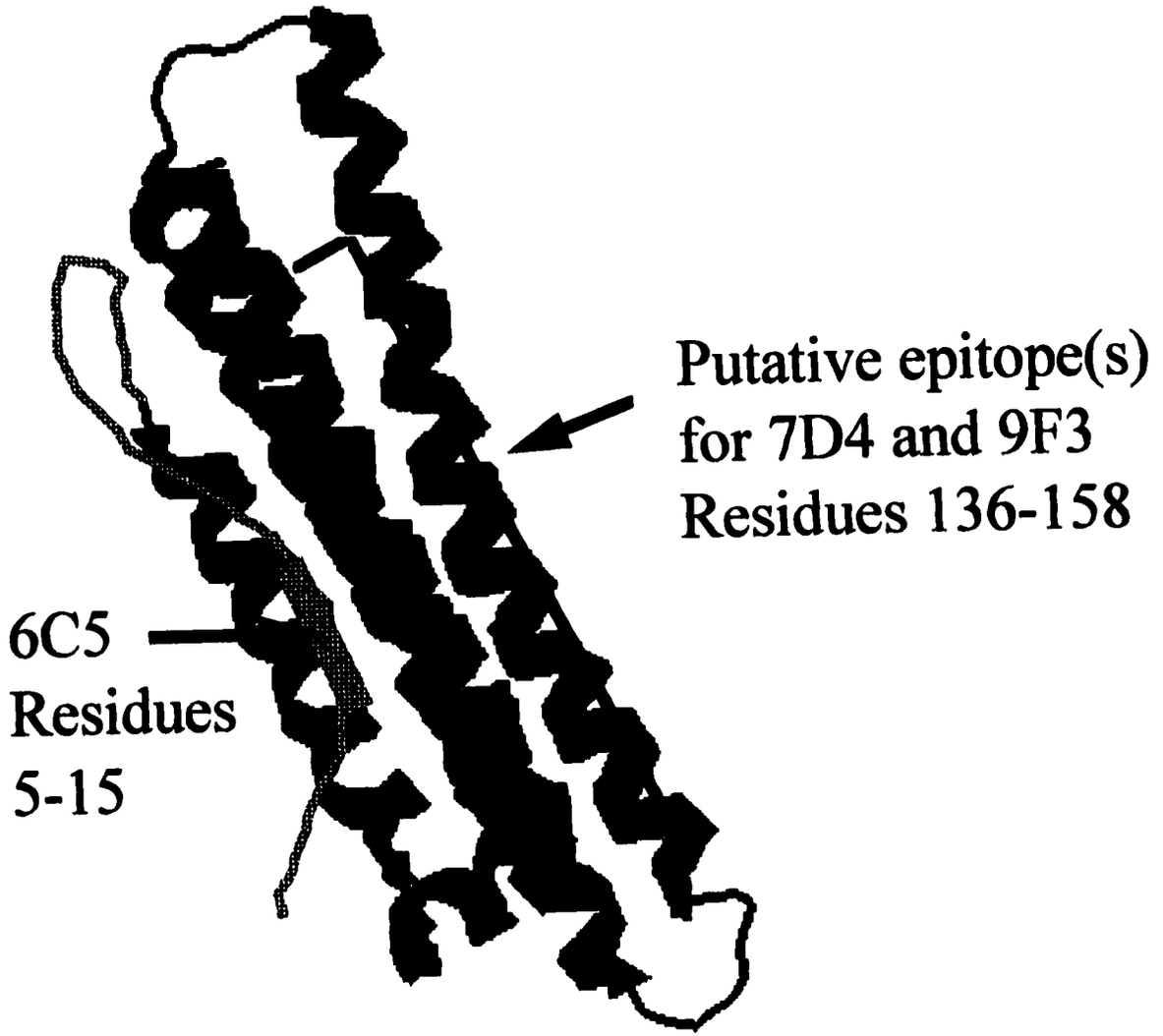
presented in Figure 14. In this model binding of 6C5 could sterically impair the binding of 7D4 and 9F3 because of the proximity of the respective epitopes. We have observed that neither ^{125}I-7D4 nor ^{125}I-9F3 could detect apoE that had been captured by immobilized 6C5 Fab fragments (This format was used to screen hybridomas in fusion 9). This observation would also argue against a mechanism by which binding of 6C5 to apoE induces a conformational change in the 7D4 / 9F3 epitope.

Analysis of the structure of a number of antigen-antibody complexes indicate that many epitopes are discontinuous, composed of several short segments that are separated in the primary structure of the protein but brought together in the tertiary structure. In an immune complex, as many as 17 residues of the antigen be in contact with residues of the antibody, and the surface of antigen that is inaccessible in the complex is usually about 700 A^{o2}. One can not, therefore, exclude the possibility that the 7D4 / 9F3 epitope(s) is not only close to, but actually includes, residues from the amino terminus of apoE.

Structural studies of apoE have shown that there are intra-domain and intra-helical interactions that have important functional consequences that include LDLr-binding activity (Wilson et al., 1994; Dong et al., 1996a) and the affinity of apoE for the specific lipoprotein subclasses (Dong et al., 1994; Dong et al., 1996b). One observation suggests that there may be interactions between the amino terminus of apoE and the apoE LDLr-binding site that may have relevance to the present study. It has been demonstrated that (Dong et al., 1990; Wardell et al., 1991) a natural apoE variant which is characterized by a $Glu^3 \rightarrow Lys$ substitution shows increased LDLr-binding activity compared to wild type apoE3. The Lys^3 residue of the variant could potentially directly participate in binding to the LDLr by forming an ionic interaction with an

Figure 14- A rebon model to illustrate the proximity of the putative epitopes for 7D4, 9F3, and 6C5 on apoE. Putative epitopes for 7D4 and 9F3 are shown by red and that of 6C5 is shown by yellow. (see text for details)

MODEL OF APOE IN WHICH THE AMINO TERMINUS IS SPATIALLY CLOSE TO THE LDLr-BINDNG SITE



acidic residue of the LDLr as has been proposed for other basic residues within the apoE LDLr-binding site. This would imply that residue 3, at least in the variant, is spatially close to the basic residues of helix 4 that have been implicated in binding to the LDLr. Alternatively, Glu^3 in wild type apoE3 may form a salt bridge with one or more basic residues in the LDLr binding site, and when this interaction is disrupted by a $Glu^3 \rightarrow$Lys substitution, there is a direct (e.g. an increase in the local positive surface potential in the apoE LDL-receptor binding site) or indirect (e.g. a local rearrangement of salt bridges) that results in an increased affinity for the LDLr. Again this would imply a spatial proximity of the amino terminus and the apoE LDLr-binding site. On the other hand, the effect of the $Glu^3 \rightarrow$Lys substitution on the affinity for the LDLr may be much more indirect (e.g. inducing a cascade-like rearrangement in salt bridges) and would not require propinquity of the amino terminus and the LDLr-binding site.

It has been proposed that, upon binding to the surface of a lipoprotein, the four-helix bundle that composes the apoE amino terminal domain opens up as is shown in figure 2. While the helices are predicted to remain intact, the model would imply a major alteration in the spatial relationships of individual structural elements of apoE. It has been reported (Weisgraber et al. 1986) that, in lipid free apoE, the 6C5 epitope may be close to the 3H1 epitope (243-272) but when apoE is bound to a lipid surface the two epitopes are separated. The results shown in Figures (12A, and 12B), and figure (13A, and 13B) indicate that 6C5 effectively competes with 7D4 and 9F3 for binding to immobilized apoE whether it be free or bound to the surface of a VLDL molecule. If the competition does, in fact, reflect proximity of the 6C5 and 7D4 / 9F3 epitope(s), this spatial relationship does not appear to be changed when apoE binds to a lipid surface.

Conclusions and perspectives:

While the mAbs 7D4 and 9F3 did not possess the apoE isoform specificity that we would have predicted from immunization strategy that was used, both mAbs did show apoE isoform-specificity in that neither recognized apoE2. Both mAbs could, therefore, potentially be useful reagents for inclusion in an antibody-based test to determine apoE phenotype. The 7D4 and 9F3 epitopes do appear to coincide with the apoE LDLr-binding site. Moreover, the striking similarity in the fine specificities between the two mAbs and that of the LDLr in terms of binding apoE variants suggests that 7D4 and 9F3 may be mAb mimetics of the LDLr. It will, of course, be important to demonstrate that both mAbs can, in fact, block apoE-mediated binding to the LDLr on cultured human fibroblasts. The ability of the mAbs to also meet the other criteria for mAb LDLr mimetics as described above should also be verified.

Recently, another anti-human apoE mAb, 2E8, that has been characterized in our laboratory, was shown to have binding specificity similar to the LDLr (Raffaï et al., 1995). When the cDNAs encoding the heavy and light chains of 2E8 were cloned and sequenced, partial homology was seen between the second hypervariable region of the 2E8 heavy chain and the ligand binding domain of the LDLr. It was proposed that the 2E8 and the LDLr may use a similar mechanism to recognize apoE. It will be interesting to determine the primary structure of the 7D4 and 9F3 mAbs to see if the similarity in specificity between the mAbs and the LDLr is also reflected in homology of primary structure. Whether or not homology between the mAbs and the LDLr is found, 7D4 and 9F3 should be used as antigens to elicit an anti-idiotypic immune response that may include internal image antibodies that are cross-reactive with the apoE ligand-binding site of the LDLr. The anti-idiotypic/ LDLr mAbs will, in turn, be used to probe the

binding domain of the LDLr and to examine structural relationships between the ligand-binding domains of the other receptors related to the LDLr, e.g. the LRP and the VLDLr.

Of particular interest was the unexpected observation that mAb 6C5, which is specific for an epitope close to the amino terminus of apoE, competed effectively with 7D4 and 9F3 for binding to immobilized apoE. This suggests that the amino terminus may be spatially close to the LDLr-binding site in native apoE, although other mechanisms may be envisaged. In collaboration with Dr. Karl Weisgraber, at the Gladstone Institute for Cardiovascular Research, the structure of the 2E8 Fab fragment has recently been solved (Weisgraber et al., manuscript in preparation) and we are continuing this collaboration to determine the crystal structure of the 2E8-apoE4-22kDa immune complex. Determination of the crystal structure of the 7D4-apoE4-22kDa or 9F3-apoE4-22kDa immune complexes could unambiguously define residues of apoE that are in contact with the antibody. Moreover, the antibody may help to stabilize the conformation of the amino terminus of apoE and an analysis of antibody-apoE-22kDa co-crystals may permit resolution of this region which could not be resolved from the diffraction data obtained from crystals of the apoE22-kDa fragment, itself. In the mean time, we will compare the affinities of 7D4 and 9F3 with an apoE variant that lacks the first 15 amino acids (obtained from Dr. Weisgraber) to see if amino terminal residues could participate in the respective epitopes. A method is currently being developed in the laboratory to specifically biotinylate residues that are inaccessible in immune complexes. Immune complexes are subjected to chemical modification (e.g. reductive methylation of lysine residues). The immune complex is then dissociated and lysine residues of the antigen that were protected from reductive methylation by the antibody are then biotinylated. The antigen is subjected to proteolysis and biotinylated peptides are isolated on

streptavidin-sepharose, further purified by high pressure liquid chromatography and sequenced. This approach could potentially be adapted for 7D4-apoE or 9F3-apoE immune complexes to identify residues of apoE (perhaps in the amino terminus) that are inaccessible to the chemical modifying reagents.

A principal goal of my project was to develop a novel immunization strategy using transgenic mice that favors the generation of mAbs that can discriminate between epitopes that are polymorphic in a species. While we did obtain anti-apoE mAbs that show isoform specificity, it is difficult to attribute this success to the immunization strategy that was used. We are, therefore, left with the question of whether or not the immunization strategy has merit and should be further studied. The immunization schedule may not have been optimal. In some cases, no immune response was observed and, in other cases, the immune tolerance to the product of the transgene appeared to have been broken. Different immunization schedules and different adjuvants should be tested. Based on the antibodies present in the sera of the immunized mice, an isoform-specific response appeared to have been elicited in certain cases. Isoform-specific B lymphocytes may have been present at low frequencies but our fusion efficiency may have been too low to obtain specific hybridomas. Other fusion protocols that are more efficient (e.g. antigen-mediated electrofusion) could be tried. Finally, the human apoE immune response of the transgenic mice is limited, not only by the self-tolerance that is imposed by the human transgene, but also by tolerance due to the endogenous mouse apoE genes. As an example, the mouse is an "apoE4" animal, as is it has an arginine at a position that corresponds to human residue 112. The presence of the endogenous gene may have restricted the immune response of human apoE3 transgenic mice to immunization with human apoE4. To see if the endogenous mouse apoE

genes do limit the immune response to immunization with human apoE. We have begun to backcross human apoE3 transgenic mice with apoE mice whose endogenous apoE genes have been inactivated by gene targeting. The human apoE3 transgenic mice that lack endogenous apoE genes will be immunized with different human apoE isoforms.

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