Production and characterisation of monoclonal antibodies against human plasma apolipoproteins

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by

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I hereby declare that this thesis is based upon my own work

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DEIRDRE BREEN

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SUMMARY

144 antibody-secreting hybridomas were produced over a total of 16 fusions using human apolipoprotein as immunogen The cells were screened both by an ELISA technique (fusions 1-10) and by an immunodotting technique (fusions 9-16) which utilised a small amount of supernatant (5µl) which was spotted onto nitrocellulose-immobilised antigen The ELISA also screened for antibodies against non-specific antigens and 52 of the 144 hybridomas were discovered to be of this type 7 antibodies were selected for further study 3 of them, 5B10, 6C9 and 6G10A4, were raised against total apolipoprotein A further three, 2B6A12, 3F12D12 and 4G1B4 were raised against VLDL These six antibodies were directed against apolipoprotein B The seventh antibody, 3H9, was raised against delipidated HDL and was directed against a low molecular weight protein from that fraction The immunoreactivities of the seven antibodies were examined on immunoblots of total apolipoprotein, apo VLDL, apo LDL, apo HDL_2 and apo HDL_3 after electrophoresis under both denaturing and non-denaturing conditions
The immunoreactivities of the anti-apoB antibodies were examined after chemical or enzymic modification of the apolipoprotein antigen, following immunoblotting from a non-denaturing gel These experiments yielded information on the molecular nature of the antigenic determinants on apoB Antibodies 5B10, 6C9 and 6G10A4 bind to epitopes

which are sensitive to lipid removal 5B10 also has lowered immunoreactivity after treatment of the antigen with trypsin, while 6G10A4 binds to a pepsin-sensitive epitope 5B10, 6C9 and 6G10A4 react with epitopes which are sensitive to reductive methylation treatment

5 of the antibodies, 5B10, 6C9, 4G1B4, 6G10A4 and 3H9 were purified from ascites fluid by DEAE Affi-Gel blue chromatography They were tested for their ability to prevent LDL-receptor binding in a ligand blotting experiment using nitrocellulose-bound receptors Inhibition was achieved in the presence of excess 6C9 antibody and partial inhibition with antibody 4G1B4

APPENDIX I

ABBREVIATIONS USED

Apo, apolipoprotein

HDL, high density lipoprotein(s)

LDL, low density lipoprotein(s)

IDL, intermediate density lipoprotein(s)

VLDL, very low density lipoprotein(s)

LCAT, lecithin cholesterol acyl transferanse

LPL, lipoprotein lipase

HMGCoA, 3-hydroxy-3-methylglutaryl coenzyme A

FH familial hypercholesterolaemia

d, density

Ig, immunoglobulin

ELISA, emzyme-linked immunosorbent assay

Fab, fragment antigen binding

RIA, radioimmunoassay

BSA, bovine serum albumin

CEA, carcinoembryonic antigen

DEAE, diethylaminoethyl

EDTA, ethylenediaminetetraacetic acid

FCS, foetal calf serum

HAT, hypoxanthine aminopterin thymidine

LPDS, lipoprotein-deficient serum

Mr, relative molecular mass

PBS, phosphate buffered saline

PMSF, phenylmethylsulphonyl fluoride

SDS, sodium dodecyl sulphate

TEMED, N,N,N^{1},N^{1} tetramethylethylene diamine

Tris, tris(hydroxymethyl)aminomethane.

1 1 Overview monoclonal antibodies and lipoproteins

Monoclonal antibodies are ideally suited to the study of a heterogeneous population of particles such as plasma lipoproteins, and in the last few years their application to lipoprotein research has mushroomed They have the ability to pick out one antigen out of a thousand on a cell or protein surface and they will bind to that determinant wherever it is presented By their binding to the antigenic determinants of apolipoproteins, which are distributed among the various lipoprotein classes, they help to define the metabolic pathways of the lipoproteins Because of the antibody specificity, variant forms of an apolipoprotein molecule can be distinguished and in some instances perhaps, such a variation can be correlated with abnormalities in lipid transport

With regard to quantitation of apolipoproteins and lipoprotein classes monoclonal antibodies are advantageous over polyclonal antibodies The complex immunological properties of some of the lipoproteins results in much variability in polyclonal antisera raised against them The biochemical and immunological properties of apolipoproteins B and E have been studied extensively with monoclonal antibodies, by many workers With apoB, other biochemical techniques would have been of limited use, because it is extremely insoluble in aqueous buffers and is very susceptible to oxidative cleavage Monoclonal antibodies have helped to overcome these obstacles Apolipoproteins B and E are important in lipoprotein metabolism in that they bind cell membrane receptors, thus promoting the cellular uptake of these lipoprotein classes The emphasis on research involving monoclonal

antibodies against these apolipoproteins has been in the characterisation of this important domain of the protein

1 2 Lipoprotein apoproteins

The circulating lipoproteins are a heterogeneous group of macromolecular complexes which are classified according to their hydrated densities The characteristic which sets them apart from other plasma proteins is that the associated lipid renders them able to flotate on ultracentrifugation

The characteristics of the lipoprotein density classes are shown in the table

Table 1 2 Characteristics of the lipoprotein classes in humans

Lipoprotein class	Major core lipids	Major Apoproteins	Density g/ml	Diameter A	Electrophoretic mobility
Chylomicrons	Dietary Triglycerides	A-1,A-11,B-48 C-1,C-11,C-111	<1 006	800-5000	remain at origin
VLDL	Endogeneous Triglycerides	B-100 C-1,C-11,C-111	<1 006	300-800	pre-beta
Remnants	Cholesteryl esters, triglycerides	E, B-100 C-111	1 019	250-350	slow pre-beta
LDL	Cholesteryl Esters	B-100	1 019-1 063	180-280	beta
HDL	Cholesteryl Esters	A-1, A-11	HDL_2= 1 063-1 125 HDL_3= 1 121-1 21	50-120	alpha

From Harrison's Principles of Internal Medicine, 10th ed

Brown et al, 1981, have reviewed lipid transport in humans with particular emphasis on cholesterol and triglyceride transport

While studies of lipoprotein metabolism and associated abnormalities had, up until the 1970's, centred around conventional ultracentrifugal and electrophoretic techniques, the description of the apoprotein components provided a new approach to the study of lipoproteins It became clear by the end of the 1960's that in order to look for a cause of some of the lipoprotein disorders one had to study the biochemical characteristics of apoproteins, in both healthy and diseased subjects For example, while type III hyperlipoproteinaemia had been defined electrophoretically by Fredrickson et al, (1967), the recognition that a mutant form of apolipoprotein E was responsible for what lay behind the abnormality (Utermann et al,1975) was made because the higher resolution technique of isoelectric focussing was the chosen approach for apoprotein analysis The biochemical characterisation of apoproteins has thus yielded new insight into the underlying causes of lipoprotein disorders Apoproteins are known to function in the secretion, transport and metabolism of lipoproteins as well as maintaining the lipoprotein as a stable structure in the plasma Several have now been isolated and characterised and in addition to the elucidation of their amino acid sequence, many apoprotein genes have now been cloned and mapped in the human genome Mutations have also been identified, some of which have adverse affects on lipoprotein metabolism

Apoprotein A-1

ApoA-1 is the major protein component of HDL constituting about 70% of its HDL proteins (Herbert et al., 1982) Its concentration in plasma is 1 0-1 2 mg/ml (Breslow, 1985) and it has a molecular weight of 28,500 daltons The amino acid sequence of apoA-1 has been elucidated by protein sequencing methods (Brewer et al, 1978) and also by DNA sequencing of apoA-1 cDNA clones (For references, see Breslow, 1985)

Residues 99-230 are composed of six tandem 22 amino acid repeats and five of the six repeats begin with proline The secondary structure specified by the amino acids is that of an alpha-helix with a nonpolar side facing the lipid core and a polar side which faces the plasma environment In addition to its ability to bind lipids and confer stability on a lipoprotein particle in plasma, ApoA-1 has been shown to activate LCAT, (Fielding et al, 1972, Soutar et al., 1975) thus promoting cholesterol esterification

Polymorphic forms of apoprotein A-1 have been described Many of them involve amino acid substitutions which alter the charge of the molecule by one unit $ApoA\text{-}1_{Milano}$ (Franceschini et al, 1980) contains a cysteine for arginine substitution at residue 173, which allows the molecule to dimerise Other apoA-1 variants include $ApoA\text{-}1_{Warburg}$, $ApoA\text{-}1_{Giessen}$ (Utermann et al, 1982) and $ApoA\text{-}1_{Munster}$ (1-3) (Menzel et al, 1982), all of which

have amino acid substitutions or deletions

ApoA-II

The next most abundant protein in HDL comprising 20% of its protein, is apoA-II Plasma concentrations of this apoprotein are 0 3-0 5 mg/ml (Breslow, 1985) and it exists as a dimer of molecular weight 17,000 daltons, which can be reduced (at a disulphide bond) to 2 identical subunits of molecular weight 8,500 daltons The amino acid sequence has been elucidated (Brewer et al, 1972) and each sub-unit has 77 amino acids The precise physiological role of apoA-II is still undetermined although it has been found by Fielding et al, (1972), to inhibit LCAT, while Soutar et al, (1975), also found it to inhibit the activation of LCAT
No mutations or abnormalities of human apoA-II have been described

ApoA-IV

Apolipoprotein A-IV in humans occurs in the circulation primarily unassociated with lipoproteins although it is synthesized by the enterocytes of the small intestine during fat absorbtion and is thereafter incorporated onto the surface of nascent chylomicrons that are then secreted into lymph It is an acidic polypeptide of molecular weight 46,000 daltons (Weinberg and Scanu, 1983) Its

carbohydrate content is 6% by weight

A variant form of apoA-IV has been described following a two-step electrophoretic procedure with human plasma (Utermann et al., 1982) The mutant protein, designated apoA-$IV_{Marburg}$, with a more alkaline pI than the normal form, was detected in a normal individual ApoA-IV is of a similar secondary structure to apoA-I and is also genetically linked (on chromosome 11) (Breslow, 1985).

ApoB

Apolipoprotein B is the major protein of low density lipoprotein, but is also found in chylomicrons and VLDL (Herbert et al, 1978) Its concentration in plasma is 0 7-1 0 mg/ml ApoB is essential for secretion into plasma of triglyceride-rich lipoproteins, as evidenced by the fact that in patients with abetalipoproteinaemia, no triglyceride is secreted into the circulation, although it is synthesised excessively within the cell (Herbert et al, 1982)

Apoprotein B is a glycoprotein, which occurs in two forms ApoB-100 has a molecular weight of 500,000 daltons and is synthesised in the liver, whereas B-48, which has about half the molecular weight of B-100 is produced in the small intestine and found on chylomicrons (Kane et al, 1980) Other species of apoB found in LDL are apoB-74 and apoB-26 The biochemical characterisation of apoprotein B has proved difficult because, like a membrane protein, it

is very insoluble in aqueous buffers and it is also very susceptible to oxidation after delipidation This led to the application of the monoclonal antibody technique to characterise the apoprotein immunochemically and consequently more information on the structure of apoB has been derived using this method than for any other apolipoprotein ApoB functions in the delivery of cholesteryl ester-rich lipoprotein particles to cells, a step which is brought about through its recognition of a membrane-bound receptor on the cell surface (Goldstein and Brown, 1983) Apolipoprotein B-100 has recently been sequenced and examined by Knott et al., (1986) The mature protein has 4,536 amino acids and a calculated molecular weight of 514,000 daltons In vitro it is specifically cleaved by thrombin and Kallkrien at two sites, resulting in 3 peptides T2, T3 and T4, (Cardin et al, 1984) ApoB-100 has extensive alpha and beta structure which renders it capable of interacting with lipid 25 cysteine residues are found on the protein, 15 of those in the amino-terminal T4 region and 12 of those in the first 500 amino acids 6 out of every 7 cysteine residues are involved in intramolecular disulphide linkage The T4 segment is highly cross-linked within itself and more globular in structure than the rest of the molecule The cysteine-rich domain may be necessary for transport of the nascent molecule from endoplasmic reticulum to the Golgi apparatus

Using restriction fragments of apoB-100 cDna which were

sub-cloned, monoclonal antibodies binding to apoB-100 fusion proteins were studied, enabling the authors to construct an epitope map on the protein sequence Antibodies 1D1, 2D8, 4G3, 3F5, 3A10 and 5E11 of Marcel et al, (1982) and antibody B47 of Young et al., (1986) bind native LDL Antibodies 2,7,15,16,20 and 22 were raised against apoB-100 fragments and do not recognise whole LDL Seven fusion proteins were generated, encompassing residues 2,488-4,525 Antibodies 4G3 and 3F5, which inhibit LDL binding to the LDL receptor, bind a fusion protein containing residues 3,029-3,132 Antibody B47, which also inhibits LDL receptor binding, strongly binds the fusion protein containing residues 3,350-3,306 Thus the region of the protein near the T3/T2 junction (residue 3249) may be involved in LDL-receptor binding Two sequences near this junction, 3,147-3,157 and 3,359-3,367, contain basic amino acids and the second of the two is similar to the receptor-binding domain of apolipoprotein E The cysteine residues at positions 3,167 and 3,297 are cross-linked so that the epitopes of the receptor-blocking antibodies, which are as far as 218 amino acids apart in primary structure, may be very close in tertiary structure

Yang et al., (1986), also sequenced apoB-100 and found that a synthetic fragment containing residues 3,345-3,381 could suppress HMGCoI7 reductase activity in cultured human fibroblasts This sequence contains one of the two basic regions described by Knott et al, (1986) (residues

3,359-3,367) and it is the one which is similar to the receptor-binding domain of apoE The sequence is (Arg-Leu-Thr-Arg-Lys-Arg-Gly-Leu-Lys)

C-apoproteins

Three distinct polypeptides are present in triglyceride-rich lipoproteins, and in HDL, and have an important role in lipoprotein metabolism ApoC-I constitutes 10% of VLDL protein and 2% of HDL protein (Breslow, 1985) It has a molecular weight of 6,300 daltons and plasma concentration of between 0 04-0 06 mg/ml It has been reported to activate LCAT (Soutar et al., 1975), though not as efficiently as apoA-I.
ApoC-II constitutes 10% of VLDL protein and 1% of HDL protein Its plasma concentration is in the range of 0 03-0 05 mg/ml ApoC-II displays co-factor activity for lipoprotein lipase (La Rosa et al., 1970), the physiological importance of which is demonstrated in patients with inherited apoC-II deficiency Their clinical symptoms are similar to those produced by lipoprotein lipase deficiency (Nikkila, 1983)
A polymorphic form of apoC-II has been described by Havel et al., (1979) The variant is one charge unit more acidic due to a Gln for Lys substitution
ApoC-III comprises about 30% of VLDL protein and 2% of HDL protein Its molecular weight is 8,800 daltons and its concentration in plasma is in the range of 0 12-0 14

mg/ml ApoC-III can be resolved by isoelectric focussing into three forms, designated ApoC-III-0, C-III-1 and C-III-2 respectively, which contain either 0,1 or 2 molecules of sialic acid (Brewer et al, 1974)
The physiological role of apoC-III may lie in the regulation of the metabolism of triglyceride-rich lipoproteins, because it has been shown in vitro to inhibit lipoprotein lipase activity (Brown et al, 1972, Krauss et al, 1973)

ApoD

ApoD, formerly known as "thin-line polypeptide" is found in HDL_3 and has a molecular weight of 22,100 dalton, 18% of which is made up carbohydrate (McConathy and Alavpovic, 1976) This makes it the most glycosylated of the apolipoproteins

ApoE

ApoE is found on VLDL, where it comprises about 10-20% of VLDL protein and on HDL (1-2% of HDL protein), where it comprises most of the protein component of a particular sub-fraction of HDL, known as HDL_C This fraction, at least in dogs, is induced by cholesterol feeding, (Mahley et al., 1975) It has a molecular weight of 36,000 daltons and a plasma concentration range of 0 025-0 050 mg/ml This apoprotein has generated great interest because it is one of two apolipoproteins (apoB-100 being the other), which interacts with cellular lipoprotein receptors (Bersot et al., 1976) HDL_C was shown to bind both LDL receptors and other distinct, apoE-specific receptors on hepatic membranes (Hui et al., 1981)
A mutant form of apoprotein E has been associated with type III hyperlipoproteinaemia (Utermann et al., 1975, Schneider et al., 1981) Since then it has been shown that human apoE occurs in three major forms, which can be separated on isoelectric focussing gels (Weisgraber et al.,

1981, 1982) These isoforms are the result of the presence of multiple alleles of a single gene (Zannis and Breslow, 1981) and are designated E2, E3 and E4 Rall et al.,(1982) have shown that E2 differs from the parent E2 isoform by a Arg 158 - Cys substitution while E4 differs from E3 by a Cys 122 - Arg substitution at residue 112 The discovery of these isoforms paved the way to understanding the underlying defect in familial dysbetalipoproteinaemia, a disorder in which chylomicron remnants accumulate in plasma, because it was found that, while apoE/phospholipid complexes containing E3 or E4 could successfully bind to low density lipoprotein receptors, complexes containing the mutant form, E2, could not, and patients with familial dysbetalipoproteinaemia are almost invariably homozygous for the E2 isoform Other mutant or variant forms of apolipoprotein E have since been found by several authors and have been described by Dargel, (1985)

1 3 Abnormalities in lipid transport

A variety of apoproteins, enzymes and receptors are known to be required for lipoprotein metabolism Mutations in the genes encoding these proteins give rise to polymorphic forms, some of which have no grossly apparent clinical consequences, although they may be insidiously life-threatening, and some which give rise, through an aberrant protein, to either a reduction or an elevation in plasma levels of one or more than one of the different lipoprotein classes These abnormalities are described and divided up into those caused by i) mutant receptors, ii) enzyme deficiencies and iii) apolipoprotein abnormalities

1 3 1 Familial Hypercholesterolaemia

Familial hypercholesterolaemia (FH) is a disorder characterised by premature onset of atherosclerosis Xanthomas are also prevalent in afflicted individuals In heterozygotes, the onset of atherosclerosis and coronary heart disease is later than for homozygotes
It has been known since the late 1930's that familial hypercholesterolaemia is inherited as an autosomal dominant trait (Muller, 1938 and 1939, Thannhauser et al., 1938) In most countries 1 in 500 persons carries a copy of the mutant gene Khachadurian, (1964), showed conclusively the genetic basis of the disorder and

distinguished homozygotes and heterozygotes clinically While it was known that FH individuals had elevated serum levels of cholesterol (350-500 mg/100 ml in heterozygotes, 600-1000 mg/100 ml in homozygotes) Gofman et al., (1954), showed by ultracentrifugal analysis that the increase in cholesterol resulted from elevated low density lipoprotein levels, and only LDL, of the lipoproteins, was raised The reason for the elevated LDL levels in FH individuals was shown to be due to delayed clearance as opposed to increased synthesis by Langer et al, (1972) However, Goldstein and Brown (1973) subsequently demonstrated that the rate of cholesterol synthesis in cultured human fibroblasts is controlled by the content of low density lipoprotein in the culture medium and that this control comes about through LDL suppressing cholesterol synthesis by inhibiting HMG-CoA reductase Goldstein and Brown, then found that in order to produce this inhibition, LDL must bind to a high affinity receptor on the cell membrane In the fibroblasts of individuals with the homozygous form of familial hypercholesterolaemia, this high affinity binding is deficient, (Brown and Goldstein, 1974) As a consequence, there was no inhibition of HMG-CoA reductase and cholesterol is overproduced. The defect in the LDL binding process, caused by a mutation in the gene coding for the LDL-specific membrane receptor, was thus shown to represent the primary genetic abnormality in familial hypercholesterolaemia The LDL in these individuals is degraded more slowly and by less

efficient receptor independent pathways In addition, a net increase in LDL synthesis will come about due to the fact that LDL molecules, unable to be taken up by hepatic receptors, will be converted to LDL (In normal subjects some IDL particles are cleared from the circulation by liver uptake)

The structure of the bovine LDL receptor has been studied with the help of its purification from bovine adrenal cortex (Schneider et al, 1982) and from the prediction of its amino acid sequence following the cloning of human LDL receptor cDNA (Yamamoto et al, 1984) The receptor is synthesised as a precursor of molecular weight 120,000 daltons on SDS gels (Tolleshaug et al., 1982) It can be divided into five domains (Yamamoto et al., 1984) The NH_2-terminal domain contains 322 amino acids including 47 cysteine residues, many of which are in disulphide linkage, making the molecule tightly folded and extremely stable The domain contains 8 repeating sequences, each with 40 amino acids, and at the COOH terminal end of seven of the eight repeat sequences there is a variant of the sequence Asp-Cys-X-Asp-Gly-Ser-Asp-Glu As the receptor-binding site on apolipoproteins B and E contain positively-charged amino acids it has been speculated that these negatively charged residues in the cysteine-rich domain of the LDL receptor interact with the apoprotein ligands

The second domain of the molecule contains approximately 350 amino acid residues This region shows homology to

the amino acid sequence of the polyprotein precursor for mouse epidermal growth factor The third domain contains 48 residues of which 18 are serine or threonine which have carbohydrate chains attached in O-glycosidic linkage The fourth region, containing 22 non-charged amino acids, spans the membrane and the fifth, consisting of 50 amino acids at the COOH-terminal end of the receptor, projects into the cytoplasm This region may function in binding to clathrin, thus allowing the receptor to be incorporated into clathrin-coated pits for internalisation

Several mutations in the gene encoding the LDL receptor, have been distinguished, (Tolleshaug et al,1983, Goldstein and Brown, 1983) All are ultimately manifested in the clinical symptoms of homozygous and heterozygous familial hypercholesterolaemia The mutations can broadly be divided into four classes The first represents null alleles, in which the gene fails to specify synthesis of any detectable receptor protein In the second group synthesised receptors are not transported to the Golgi apparatus and consequently do not undergo carbohydrate processing (Tolleshaug et al, 1982) As a result the receptors do not appear on the cell surface Class 3 mutations encode receptors that are synthesised and processed normally but they fail to bind LDL The receptors of the fourth class of mutation bind LDL, but cannot internalize it, probably because the receptor fails to cluster in coated pits

Yamamoto et al.,(1984) have suggested that genetic

mutations result from unequal crossing over with homologous recombination between DNA sequences encoding the multiple repeats in the cysteine rich NH_2-terminal domain or in the centre of the protein Many of the mutant forms of the receptor are of abnormal length. Those which encode proteins of normal length which fail to bind LDL may have a mutation in one of the codons specifying a cysteine residue A free sulphydryl group is thus left free in this region, which could disrupt the way the molecule is normally meant to be folded
The clinical consequence of the elevated LDL in individuals with familial hypercholesterolaemia is premature atherosclerosis, which comes about through uptake of LDL by scavenger cells or macrophages of the reticuloendothelial system, (Goldstein and Brown, 1977) These cells, overloaded with cholesteryl esters are converted to "foam cells", the classic components of atherosclerotic plaques

1 3 2 Apolipoprotein abnormalities

1 3 2 1 Apolipoprotein A-1 deficiencies

Utermann (1984) has coined the term apolipoprotein opathies for disorders in which a deleterious effect on lipid metabolism has as its underlying cause a structural defect in a lipoprotein apoprotein Apolipoprotein defects have been reviewed by Dargel, (1985)

An apolipoprotein A-1 absence, together with an absence of apoC-111 has been described in two sisters with precocious coronary artery disease by Norum et al,(1982) A second disorder in which ApoA-1 and C-111 are absent (known as ApoA-1,C-111 deficiency variant 11, and previously termed apoA-1 absence) has been described by Schaefer et al., (1985) In both cases, HDL is severely deficient and clinical symptoms include corneal clouding and precocious atherosclerosis One apparent difference between variants 1 and 11 is the presence of skin xanthomas in variant 1 While ApoA-1, C-111 deficiency is associated with premature coronary artery disease, Tangier disease, or the presence of the ApoA-1_{Milano} variant, are not Tangier disease is characterised by storage of cholesteryl esters in certain cells such as macrophages and Schwann cells, (Herbert et al, 1983) Splenomegaly and enlarged tonsils are also apparent. The apoA-1 of Tangier disease is mostly disassociated from HDL and found in the d>1 21 g/ml plasma protein fraction Apoprotein A-1 had been shown to migrate under isoelectric focussing conditions as a series of bands (Zannis et al., 1980) it therefore exists in plasma in multiple isoforms The apoA-1 of Tangier disease also occurs as electrophoretically separable isoforms, (Zannis et al, 1982) However, while the isoprotein patterns were indistinguishable for newly synthesised apoA-1 from Tangier cells and from normal individuals the relative distributions of the isoforms was altered One isoprotein - isoprotein 2, (which represents

the precursor form of apoA-1, called proApoA-1) was relatively increased compared to normal, suggesting a faulty conversion of the precursor to the mature apoprotein (represented by isoprotein - 4 in isoelectric focussing) Rosseneu et al, (1984) studied the lipid-binding properties of isoproteins 2 and 4 of Tangier apoA-1 and found that isoprotein 2 showed only a limited association with lipids compared to isoprotein 4 and to isoproteins 2 and 4 of normal apoA-1 with HDL The basis for the altered isoprotein 2 structure in Tangier ApoA-1 is still unclear

1 3 2 2 Apolipoprotein B deficiencies

ApoB deficiency exists in at least three different forms all of which result from an inborn error of metabolism affecting the synthesis or secretion of this protein The first type - abetalipoproteinaemia, is an autosomal recessive disorder characterised by the absence of chylomicrons, VLDL or LDL in the plasma of affected individuals (Herbert et al, 1978) Clinical symptoms of this condition include fat malabsorbtion, progressive ataxic neuropathy and acanthocytes (spiky red blood cells)

Hypobetalipoproteinaemia is very similar to abetalipoproteinaemia, except that in this case the mode of inheritance is autosomal dominant Hence, heterozygotes have marked lipoprotein deficiencies,

whereas heterozygotes for abetalipoproteinaemia are normal

Normotriglyceridaemic abetalipoproteinaemia is a very rare disorder which only affects apoB-100, so chylomicrons (which carry apoB-48) are synthesised normally, and plasma VLDL of these individuals contains B-48 only Fat absorbtion is normal in these cases

It had been expected that LDL receptor-binding activity and HMG-CoA reductase activity would be completely uncontrolled in these disorders, because of the absence of B-containing lipoproteins to repress receptor synthesis and inhibit the enzyme High rates of cellular cholesterol biosynthesis should result as a consequence However, Myant et al,(1978) found that this was not the case In fact, cholesterol metabolism is regulated as it would be in normal individuals Blum et al, (1982) showed that a subfraction of HDL, which contained apoE was relatively increased in patients and this lipoprotein could successfully compete with ^{125}I-LDL for binding to cultured human fibroblasts to the extent that they could suppress cholesterol synthesis and LDL receptor activity The apoE-rich lipoproteins in patients thus functionally substitute for LDL in the regulation of cholesterol metabolism.

1 3 2 3 Apolipoprotein E deficiencies

A mutant form of apoE has been implicated in familial dysbetalipoproteinaemia, a disorder characterised clinically by premature coronary atherosclerosis and xanthomas, and clinically, by increased intermediate density lipoproteins in plasma, causing hypertriglyceridaemia and hypercholesterolaemia and by abnormal VLDL (known as beta-VLDL) which is relatively enriched in cholesterol, (Goldstein & Brown, 1983) Normally, such particles are rapidly cleared by the liver through apoliprotein E-mediated uptake by receptors on hepatic cells People with familial dysbetalipoproteinaemia, however, are almost invariably homozygous for a particular polymorph of apoE, (Utermann et al, 1979) which is distinguishable by isoelectric focussing Schneider et al, (1981), found that when the apoE from patients was isolated, recombined with lipid complexes and tested for its ability to compete with ^{125}I-LDL for binding to LDL receptors it showed reduced binding activities The abnormal apoE provides an underlying cause for familial dysbetalipoproteinaemia but that is not a full explanation, because the incidence of the disorder (1 1,000,000) is much lower than the incidence of the polymorphism (1 1000) Also Rall et al., (1983), have demonstrated receptor binding defects in the mutant apoE identified from homozygotes who are apparently healthy Other factors are clearly involved in

precipitating the onset of the disease in individuals who are already genetically predisposed, e g obesity, diabetes, age, hypothyroidism or, as Utermann et al, (1979) and Hazzard et al, (1981) have suggested, the complication of a second hyperlipidaemia, such as familial combined hyperlipidaemia

Other apoE mutants have been discovered, some of which display abnormal binding to receptors $ApoE_{Leiden}$, Havekas et al., (1984), for instance, has the same electrophoretic mobility as apoE3 but exhibits decreased binding to human fibroblasts It also has no cysteine residues

Type V hyperlipoproteinaemia, characterised by increased plasma chylomicrons and VLDL, has been associated with an increased prevalence of apoprotein E4, (Ghiselli et al., 1982) In 30 type V individuals the three commonest isoforms were distributed as follows E2, 15%, E3, 33 3% and E4, 51 7% This differed from the distribution in normal individuals of E2, 9 5%, E3, 77 0% and E4, 13 5% The precise molecular defect, however, still remains to be elucidated, particularly in view of the fact that apoE4 does not display any reduced receptor binding activities (For a review, see Dargel, 1985)

1 3 3 Enzyme defects in lipoprotein metabolism

A defect in the enzyme lecithin cholesterol acyl transferase was first described by Norum and Gjone, (1967) LCAT catalyses transfer of fatty acid fron

lecithin to cholesterol, (Glomset, 1968) thus esterifying the latter as it is transported on HDL ApoA-1 acts as a co-factor for the enzyme In patients, cholesteryl ester levels are decreased and free cholesterol and lecithin correspondingly increased due to the lack of enzyme activity The structures of all the lipoprotein particles are altered and within each density group, there is more heterogeneity of size and lipid composition than the fractions of corresponding density from normal plasma HDL's, in particular are heterogeneous and contain disc shaped particles composed of cholesterol, phospholipid and apoproteins A-1 and E These are thought to represent newly-synthesised HDL which would otherwise be converted to the mature form in the presence of LCAT

Another enzyme defect leading to abnormal lipoprotein metabolism is lipoprotein lipase deficiency Affected individuals have the type 1 hyperlipoproteinaemia of Fredrickson's classification Triglyceride removal from blood is impaired and consequently chylomicrons accumulate massively, with lowered levels of other lipoprotein fractions, including HDL (Fredrickson et al, 1978) The disorder appears to be inherited as an autosomal recessive

There may be several causes of the enzyme deficiency, including lack of an enzyme, synthesis of an altered enzyme, or an enzyme capable of catalysing hydrolysis but unable to bind to endothelial cells The enzyme requires apoprotein C-11 as a co-factor and, in fact, a mutant

apoC-II will produce the same symptoms as the enzyme deficiency, even though enzyme activity from these individuals is normal, (Breckenridge et al., 1978, Yamamura et al., 1979)

Brunzell et al., (1983), have also described a familial chylomicronaemia which was due to a circulating inhibitor of lipoprotein lipase activity, which was non-dialysable, heat-stable and sensitive to repeated freezing and thawing This inhibitor appeared to be inherited as an autosomal dominant disorder

1 4 Monoclonal antibodies and lipoproteins

As monoclonal antibodies had proven useful in study of protein structure and in the identification of functional domains of proteins, they were an obvious tool for the study of apolipoproteins and particularly apoB which, because of its unusual physical and chemical properties, is difficult to characterise by other biochemical techniques The application of monoclonal antibodies to lipoproteins has yielded new information which could not have been obtained otherwise
This section reviews the insight which has been gained into apolipoprotein structure, in particular apolipoprotein B, through the use of monoclonal antibodies

1 4 1 Antibodies against apoB

1 4 1 1 The influence of lipid on antigenicity

A panel of monoclonal antibodies against human LDL were raised by Milne et al., (1983), with the aim of identifying and characterising the region of the apoB molecule recognised by the LDL receptor It was because apoprotein B, when delipidated, is so insoluble in aqueous buffers, and therefore difficult to characterise that the monoclonal antibody technique was the chosen approach In addition, the authors wished to distinguish

ımmunochemıcally, the two forms of apoB, whıch are the lıver apoB-100 and apoB-48, whıch ıs ıntestınal ın orıgın, ın order to ascertaın how the two proteıns are related Of the seven antıbodıes produced, fıve of them - 5E11, 3A8, 3A10, 4G3 and 3F5 - recognısed a cluster of determınants localızed around the same regıon of the molecule, whıle 1D1 and 2D8 bound to epıtopes whıch were dıstant from each other and from that bound by the other fıve These fındıngs came from co-tıtratıon experıments, ın whıch two antıbodıes together are reacted wıth a lımıtıng amount of antıgen ın a solıd phase RIA to determıne whether or not the antıbody bındıng ıs addıtıve 1D1 and 2D8 were also shown to be unable to prevent LDL receptor bındıng or cellular cholesterol bıosynthesıs when theır Fab fragments were tested However, the other fıve dıd block bındıng of LDL and cholesterol synthesıs and were therefore presumed to be reactıng wıth the receptor recognıtıon sıte on the ApoB molecule The authors used tryptıc fragments of apo B, separated by SDS electrophoresıs and transferred to nıtrocellulose paper, to explore further the spatıal relatıonshıp between the determınants recognısed by the monoclonal antıbodıes, (Theolıs et al, 1984) The varıous specıes of apoB, namely B-100, B-48 and B-74 and B-26, were also separated on SDS gels, blotted to nıtrocellulose and reacted wıth the antıbodıes (Marcel et al., 1982)

From the results of these varıous experıments the authors were able to construct a theoretıcal map of the

determinants recognised by the antibodies, as they would appear in LDL (Marcel et al., 1985) Their scheme which represents a theoretical linear map showing the spatial relationship of the antigenic determinants is shown here, for both the various apoB species (including a protein whose molecular weight can allow one to call it B-50), and the tryptic fragments

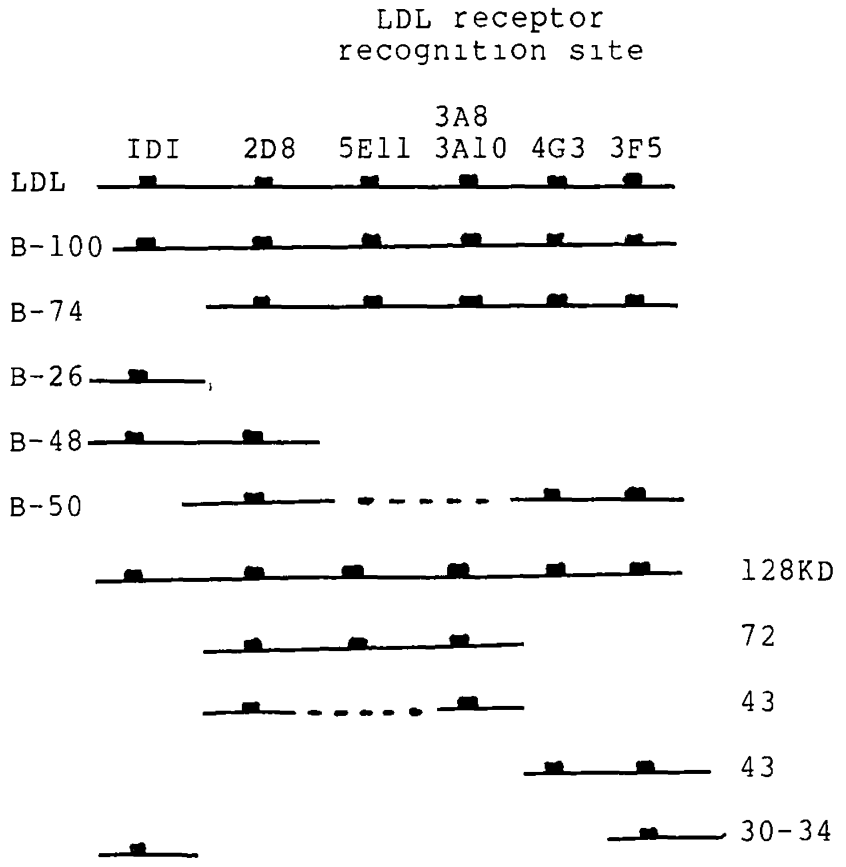


Fig 1 4 1 Schematic map of antigenic determinants of ApoB
From Marcel et al, (1985), (Scheme 4b)

The dotted lines contained in the B-50 and 43,000 dalton fragments indicate that the authors found negative reaction with antibodies 5E11, 3A8 and 3A10, but they hypothesise that the determinants may be present in those fragments, but not expressed

One of the antibodies, 1D1, reacted with apoB-100 B-26 and B-48, but not with apoB-74, on Western blots, implying that the two species of apoB, B-26 and B-74, represented complementary proteolytic fragments of each other It also meant that B-100 and B-48 had sequences in common The antibodies which can block the LDL receptor pathway were not reactive with apoB-48 which suggested that their receptor binding domain may not be present on that protein
The smallest tryptic fragment of apoB-100 which reacted with all the antibodies had an apparent molecular weight of 124,000 on SDS gels The determinants recognised by 3F5 and 4G3 were found together on a 43,000 dalton fragment while those recognised by 2D8 and 3A8 appeared together on a second 43,000 dalton fragment ApoB has been shown to have two main carbohydrate chains, (Swaminathan and Aladjem, 1976), but the pattern of reactivity of the monoclonal antibodies with the tryptic fragments was different from that seen with two lectins specific for the particular carbohydrate chains present, suggesting that the sites recognised by the antibodies are not constituted by the carbohydrate moieties Curtiss & Edgington, (1982) raised a panel of 11 monoclonal antibodies using VLDL and IDL as the immunogens While 9 of the antibodies were capable of binding to apoprotein B after separation on SDS gels and transfer to nitrocellulose, the two remaining antibodies appeared to bind complex epitopes which were abolished by delipidation and electrophoresis It was clear from binding studies with chylomicrons, VLDL and LDL that the various apoprotein B species were immunologically related Tsao, Curtiss and Edgington (1982) used the same panel of antibodies to examine the epitope expression of apolipoprotein B on native lipoproteins Three patterns of

epitope expression became apparent from fluid phase radioimmunoassays Five antibodies identified type 1 pattern of epitope expression, in which they had equal affinity for epitopes on VLDL, IDL and LDL Three antibodies bound IDL and VLDL epitopes with similar affinity but with a modified affinity to LDL and the remaining three antibodies bound only LDL and IDL in a similar manner, and in a different manner to VLDL These findings indicated that apoB possesses some antibody binding sites which are specified by an epitope that is expressed equally on all lipoprotein fractions, while other epitopes are only expressed in equal fashion on IDL and LDL and a third group are expressed equally only on VLDL and IDL Another example of heterogeneity in immunoreactivity among the different lipoprotein fractions is provided by antibody IDI, which is one of the antibodies raised by Marcel's group The authors delipidated and solubilized ApoB in the absence of denaturing agents or detergents according to a method described by Cardin et al., (1982), because it was their aim to study the role of lipids in the antigenicity of apoB Only the determinant recognised by antibody IDI was significantly expressed on the solubilized apoB and was also equally expressed on LDL. However, while IDI reacts with chylomicrons, it requires partial delipidation for antigenic expression The same is true for VLDL

Tikkanen et al., (1984) examined the epitope expression on apoB according as VLDL was treated with lipoprotein lipase They also isolated various density fractions of VLDL by zonal ultracentrifugation and designated the fastest floating fraction as $VLDL_1$, (S_f120-400), the next fastest as $VLDL_2$(S_f60-120) and the next fastest as $VLDL_3$(S_f20-6) These represented VLDL fractions of decreasing size and had

proportionally more protein to lipid The binding of antibodies to the VLDL subfractions and LDL was assessed by allowing them compete with ^{125}I-LDL in a RIA For two of their antibodies the immunoreactivities of VLDL and LDL increased in the order of decreasing flotation rate LDL > $VLDL_3$ > $VLDL_2$ > $VLDL_1$ The effects of lipoprotein lipase on VLDL, was to increase the percentage protein and decrease the percentage of triglyceride The lipolysed particle displayed greater immunoreactivity to the antibodies than the nonlipolysed $VLDL_1$ The data indicated that, in general the affinities of binding increased as VLDL size decreased, although some of the antibodies bound with lower affinity to the lipolysed $VLDL_1$ than to nonlipolysed $VLDL_1$

Patton et al., (1982), have reported similar effects with their anti-LDL monoclonal antibodies After removal of lipids from immobilized LDL by organic solvent extraction a 50% loss of immunoreactivity resulted suggesting that the lipids played an important role in maintaining the antigen structure Immunoreactivity was also lowered following removal of VLDL triglycende by lipoprotein lipase

Marcel et al, (1985), have described experiments using solubilized apoB that was reconstituted into either SDS micelles or cholesterol-lecithin liposomes or else into microemulsions consisting of a cholesteryl oleate core stabilized by a phosphatidycholine layer The antigenicity of the determinant for antibody 2D8 (which was lost on the solubilized apoB) was partly restored on SDS micelles or cholesterol lecithin liposomes, but only incorporation of the apoB into microemulsions, following treatment with SDS, could restore the antigenic determinant for antibodies 3F5, 4G3 and 5E11. These are the sites that are close to the receptor

recognition domain

In conclusion, the antibodies raised by the various research groups were, for the most part, reactive to determinants which were found on the polypeptide region Lipids do not therefore appear to be directly implicated in the antigenic determinant structure but they affect antigenicity in two ways they can "mask" the determinant which then becomes more accessible to the antibody with lipolysis or according as VLDL is converted to LDL The receptor binding site on apoB-100 comes into this category Alternately lipids affect antigenicity by maintaining the protein in the correct conformation for antibody recognition Some of the determinants recognised by the antibodies of Patton's group come into this category A third group of determinants appear to be unaffected by the presence or absence of lipid and are universally expressed on all apoB-containing lipoproteins

With regard to the receptor recognition domain, Marcel's group, (1985) have found that it requires a very precise conformation, conferred by the presence of cholesteryl oleate, for receptor or antibody recognition Tikkanen's group have also shown it to become more accessible to antibodies according as VLDL is converted to LDL, since two of their antibodies, 2a and 2b, which are known to bind a determinant at or near the receptor binding site, bind with higher affinities to LDL than to VLDL subfractions This region of apoB is therefore probably masked by triglyceride molecules on VLDL and less accessible to both antibody and receptor molecules This would suggest that receptor uptake of VLDL remnants would be mediated through apoE binding rather than apoB-100 and Hui et al., (1984) and Krul et al., (1985) have provided evidence that this is the case

1 4.1 2 Monoclonal antibodies and LDL polymorphism

Polymorphism of low density lipoprotein in its immunoreactivity to polyclonal antisera was shown to exist over two decades ago by Blumberg and Colleagues, (1962), who used antisera from patients receiving multiple transfusions Genetic polymorphism of apoprotein B was also reported in other animals and it has been of much interest, particularly in view of the fact that, in swine, aortic intimal lipidosis has been related to a certain genetically determined antigenic type of LDL, (Rapacz et al., 1972) The immunoreactivities of LDL samples from a variety of subjects was analysed by comparing their capacities to compete with ^{125}I-LDL for binding to a selection of monoclonal anti-LDL antibodies in solid-phase assays by Tikkanen et al., (1983) Their objective was the large scale screening of LDL samples in the hope that monoclonal antibodies would detect apoB variants and possibly relate them to individuals at risk for coronary artery disease

The immunoreactivities of LDL from different individuals differed from each other, when their abilities to displace ^{125}I-LDL binding to different antibodies was compared With some antibodies the number of immunoreactivities ranged from 30-400% of the LDL standard

The different LDL preparations were assayed for phospholipid and triglyceride content and it was found that the immunoreactivities for four of the antibodies

correlated positively with percentage phospholipid, while for one antibody, immunoreactivity correlated inversely with percentage triglyceride and positively with percentage cholesterol

Patton et al., (1983), also reported heterogeneity in immunoreactivity among LDL from different individuals with four of their monoclonal anti-LDL antibodies These findings meant that it would not be possible to use monoclonal antibodies to quantitatively determine plasma apoB levels in plasma of different individuals It is clear that LDL differs from one person to another in terms of its antigenicity This is possibly brought about through alterations in lipid content, which might affect the epitope expression on the particle, although immunochemical heterogeneity through differences in protein structure could also be the case

Nonetheless, one of the antibodies of Patton's group detected a significant plasma apoB increase in patients with angiographically documented coronary artery disease, which led them to the conclusion that this antibody was binding to an antigenic determinant which was more commonly expressed in the apoB of these individuals Some of their antibodies also bound isolated LDL, but not LDL in plasma, indicating that LDL preparation alters its structure as an immunogen

Genetic polymorphisms in apolipoproteins A-1, A-IV, and E have been documented by several authors, (see section 1 2) Such techniques were not possible with apoprotein

B, due to its unusual physical and chemical properties which rendered it insoluble in aqueous buffers Schumaker et al., (1984), reported the use of monoclonal antibodies in the detection of phenotypic variants in human LDL A panel of 11 antiapoprotein B antibodies, raised and described by Curtiss and her colleagues (Curtiss and Edgington, (1982), Tsao et al., (1982)), were used in the study

The method of data analysis which was used to screen LDL from different donors for immunochemical variation was referred to as the "binding ratio profile" and expressed as the amount of antibody bound to a sample LDL divided by that bound to a standard LDL (quantitation of antibody bound was by solid phase radioimmunoassay) For each sample of LDL the binding ratios for all 11 antibodies were measured and the binding ratio profile plotted with ratio along the Y-axis and antibodies A to K along the X-axis If the epitope for a particular antibody was expressed equally on the sample LDL as well as the standard LDL, then the binding ratio was approximately 1 0 Three antibodies, however, B, E and F, exhibited reduced binding ratios on some of the donors LDL, awhile for other LDL samples tested every antibody bound with a binding ratio of approximately 1

Three distinct phenotypes emerged from over 100 persons studied with regard to the binding ratios for the three antibodies - those for which the ratios were approximately 1.0 (type A), those for which the ratios were

approximately 0 6 (type B), and those for which the ratios were approximately 0 4 (type C) The authors concluded that these 3 antibodies, which are known from competitive binding experiments to be directed against the same or adjacent epitopes on apoB, recognise and bind a region of apoB which exhibits immunochemical variance Family studies indicate that the polymorphism is genetic and inherited as a co-dominant allele Type B individuals are heterozygous for both forms of the protein LDL, whether isolated from plasma or serum, and whether used freshly or stored frozen beforehand, produced reproducible results The authors described experiments which showed that the most likely possibility for the variation was a single amino acid change which confers on the protein an altered conformation, affecting its antigenicity for those 3 antibodies It would not have been possible to detect such a small structural rearrangement in the molecule with polyclonal antibodies A genetic polymorphism in human LDL was also discovered by Young et al., (1986,a), using a monoclonal antibody - MB19 - which bound to LDL from different individuals in one of three ways - strong, intermediate or weak Family studies revealed that the three MB19 binding patterns resulted from codominant transmission of two common apoB alleles each coding for an allotype with different affinity for MB19 The antigenic determinant did not appear to involve carbohydrate residues, since periodate treatment of the LDL did not alter antibdy binding. The epitope for MB19

is common to both B-100 and B-48 species of the apoprotein Young et al, (1986,b), detected the polymorphism in both apoB-48 and B-100 on Western blots of the delipidated apolipoproteins, and by quantitating the binding ratio for the radioactively labelled antibody bound in both bands, they were able to show that the polymorphism was expressed in parallel in both proteins This provides strong evidence that both apoB-100 and apoB-48 are products of the same gene, although the regulatory processes which lead to the expression of the two different protein products, the one intestinal in origin and the other from the liver, remain to be fully elucidated

1 4 1 3 Monoclonal antibodies and apolipoprotein B quantitation

The heterogeneity of apolipoprotein B, coupled with its known insolubility in aqueous buffers has made its accurate determination in plasma difficult to achieve immunochemically Epidemiological studies, however, have shown a direct relationship with its plasma concentration and the incidence of ischaemic heart disease, so the accurate determination of this protein has become increasingly important

When monoclonal antibodies are used to assay apoB immunochemically, the technique is not without its problems, not least because apoB is heterogeneous and distributed among very low density as well as low density

lipoproteins, so that if, for instance, one wanted to quantitate LDL apoB the antibody should not bind VLDL or chylomicron apoB The antibody should also display similar immunoreactivity to plasma LDL, as it does to LDL after isolation from plasma, and it should not display any differences in immunoreactivity to LDL from different individuals

Monoclonal anti-LDL antibodies produced by Patton et al., (1983) and by Tikkanen et al., (1983), did not show such properties and in fact the range in immunoreactivities of the antibodies of Tikkanen's group for LDL from different individuals ranged from 30-400% of a chosen LDL standard Patton's group raised four monoclonal anti-LDL antibodies, which had heterogeneous immunoreactivity with LDL from different individuals, indicating that they would not be suitable for radioimmunoassays, unless the same LDL standard was always used, because the apparent apoB concentrations detected would vary depending on the immunoreactivity of the given LDL In addition, one of their antibodies bound plasma apoB poorly compared to apoB from isolated LDL Nevertheless, the authors were able to use another of their antibodies in a radioimmunoassay and show that it detected a significant increase in plasma apoB of patients with angiographically documented coronary artery disease They concluded that these patients had an increase in the form of apoB that is specifically recognised by that monoclonal antibody

In contrast, Slater et al., (1985), raised five different

monoclonal antibodies against apoB, from which one displayed a relative specificity for LDL apoB compared to that from VLDL The antibody bound with similar immunoreactivity to LDL from 30 different individuals and was reactive to both native LDL and that isolated from plasma The authors used their antibody called PICI, in an immunoradiometric assay for LDL, which was a more simple and less time-consuming procedure than that proposed by Patton and co-workers The apoB levels given correlated well with LDL cholesterol measurements made from the same samples The authors concluded that the assay, using this antibody, offered a precise and rapid method of measuring LDL in untreated plasma samples

1 4 1 4 Monoclonal antibodies and apolipoprotein B isolation

The use of monoclonal antibodies in the isolation of apolipoprotein-B-containing lipoproteins has been explored by Koren et al., (1986) They have reported the isolation of a high affinity monoclonal antibody which reacted with apolipoprotein B and showed identical binding affinities for all major apoB-containing density classes as well as B-100 and B-48 molecular forms of the protein The antibody allowed complete removal of all apolipoprotein B from plasma of normolipidaemic or hyperlipidaemic patients The comparable binding characteristics of the antibody to apoB on nitrocellulose blots with that of polyclonal antibody against apoB led the authors to the conclusion that the epitope was uniformly expressed on all

apoB forms and they designated their antibody as "pan B" antibody The antibody, D_6, also binds to a domain which is unaffected by delipidation of the protein and which does not exhibit polymorphism among individuals, as evidenced by its identical binding to plasma samples from 25 different, unrelated people LDL and VLDL separated from plasma by the use of an immunoaffinity column with the "pan B" antibody were closely similar in lipid and apolipoprotein composition, to those isolated by ultracentrifugation or with polyclonal antibodies. The lipoproteins isolated by the antibody column were also comparable in size and shape, as judged by electron microscopy, to those obtained by ultracentrifugation or with polyclonal antibodies The monoclonal antibody offered major advantages as an immunosorbent over polyclonal antibodies

1 4 2 Antibodies against apolipoprotein E

A panel of 5 monoclonal antibodies against apolipoprotein E was raised by Milne et al., (1981), and they were found to correlate excellently with mouse polyclonal anti-apoE serum in radioimmunoassays to measure plasma apoE levels Weisgraber et al.,(1983), have used the antibodies to identify and characterise the receptor-binding domain of the protein One antibody, 1D7, inhibits apoE dimyristoyl phosphatidyl choline complexes from binding to LDL receptors on cultured human fibroblasts Thrombolytic fragments of apoE were generated, as were cyanogen bromide cleavage products, and receptor binding studies with these

artificial apoE fragments narrowed the location of the epitope for 1D7 to residues 129-169, and most likely, to the immediate vicinity of residue 140-150 Amino acid substitutions in these regions have also been implicated in the loss of receptor binding activity of apoE

1 4 3 Antibodies against apolipoprotein A-1

Four hypridomas secreting monoclonal antibody against apolipoprotein A-1 have been generated by Weech et al., (1985) The apoprotein used for immunisation was made by preparative isoelectric focussing of HDL_3 The antibodies react with all the charge polymorphisms of apoA-1, indicating that all isoforms of apoA-1 have antigenic determinants in common Two of the four antibodies were found to be reacting with the same or adjacent antigenic determinants

The position of the antigenic determinants was further defined by reacting the antibodies to CNBr fragments of apoA-1, which were separated on Western blots The antibodies reacted with different fragments

The immunoreactive properties of apoA-1 from normal as well as Tangier disease plasma were compared, (Weech et al., 1985 (b)) No abnormalities in the molecular weight of Tangier apoA-1 was found The electrophoretic patterns from isoelectric focussing experiments were also similar for both types of apoA-1

Antibodies reacted with CNBr-cleaved proteins from both normal and Tangier plasma in a similar fashion

1 5 Screening techniques

This section describes some of the procedures which workers in the field of monoclonal antibody technology have devised to rapidly and successfully screen for monoclonal antibodies against antigens of their particular interest

Among the problems encountered with the monoclonal antibody technique is that of knowing which of the positive hybridomas will be of potential interest after the initial screening Monoclonal antibodies have the ability to pick out one antigenic determinant out of thousands, for instance on a cell surface, where many different antigens are present or the binding site of a receptor protein, or the active site of an enzyme Sometimes monoclonal antibodies against one particular protein from a protein mixture are required With such complex substances as immunogens each fusion has the potential for many antibodies of different specificities and only a small percent of these antibodies will be directed against the desired antigencic determinant If the sample used for the screening is of the same degree of purity as it was for the immunisation it will not be possible to know which of the resultant positive hybridomas to grow up At this stage of the fusion experiment time becomes important, because antibody-secreting hybridomas may become overgrown by non-producing variants Also, the constant threat to the

cells of bacterial or fungal contamination, no matter how careful the operator, makes it essential to have the hybridomas frozen as soon as possible Overgrowth of hybridomas by non-producing variants is avoided by cloning, but as this increases the workload exponentially it is of advantage to know which hybridomas to clone beforehand

In the case of fusions where antibodies against one particular protein from a mixture are required, there are two solutions to the problem The first is to use a purified antigen for immunisation, to ensure the production of antibodies directed against the desired determinant The second solution is to develop a screening procedure which is at a level of sophistication to rapidly select only the monoclonal antibodies of potential interest

Tracy et al., (1983), raised a panel of monoclonal antibodies against a protein extract of whole human urine and found that most of the antibodies were directed against albumin, when they had hoped to produce antibodies with a wide range of specificities They developed a method of separating urine proteins on 2-dimensional gel electrophoresis before cutting out the Coomassie bluestained protein spots for homogenisation and immunisation Stained protein spots were also prepared for screening by homogenisation in guanidinium chloride and then labelled with ^{125}I The techniques allowed the production of monoclonal antibodies against a specific

protein component of a complex mixture, even when this protein was in the presence of other, more immuno dominant proteins

Another possible remedy to the problem of coping with the large number of hybridomas which may be present after the initial screening is the technique of directly freezing the fused cells as a means of workload distribution Harwell et al., (1984), found this method helpful, and the hybridomas were recovered successfully on thawing The technique enables the operator to perform one fusion with a large number of cells After fusion the cells are distributed into several groups and frozen Each vial can be thawed individually and diluted to a large volume to achieve early monoclonality of the cells One also has time to modify the screening technique, if desired, before the next thawing

A novel method of generating high affinity monoclonal antibodies of a desired specificity was proposed by Lo et al., (1984) They introduced an electrically-induced fusion technique, which made use of the fact that B-cells express, on their surface, an antigen receptor immunoglobulin of the same antigenic specificity as the secreted antibody The authors coupled antigen to avidin before allowing it to react with B cells from mice previously immunised with the antigen Myeloma cells were allowed to react with a N-hydroxysuccinimide derivative of D-biotin in order to couple the biotin to the cells When the myeloma and B-cells were incubated together at 4^{o}C the

biotinylated mycloma cells adhered to those B-cells which had the antigen-avidin complexes bound to their surface immunoglobulins The cells were then suspended in sucrose and exposed to a transient electric field generated by a high voltage pulse generator, which allowed fusion of cells Fused cells were then plated and cultured in medium containing aminopterin Wells containing growing hybrids were screened for antibody production The two main advantages of the procedure were that very small amounts of antigen could be used and the specificity of the fusion technique ensured that no hybridomas against irrelevant antigens were produced

Many people, including Locker et al, (1983) and Holmberg et al., (1983), have put forward rapid new techniques for the initial screening of hybridoma supernatant for antibody That described by Holmberg et al. involved binding of culture supernatants to small square areas of nitrocellulose filters followed by detection with radiolabelled antigen This method was simple, rapid and versatile to various detection systems and illustrated the use of nitrocellulose membranes in a screening assay There are many other aspects in simplifying and improving the fusion procedure , including the use of intra-splenic immunisations This will shorten the time involved, require less antigen and produce antibodies of high affinity and specificity

1 6 The application of monoclonal antibodies to the characterisation of epitope structure

The characterisation of determinants for a panel of monoclonal antibodies against a given antigen has been approached by workers in various ways When determining whether two antibodies react with the same or different antigenic determinants on an antigenic surface, one common method is to have one antibody labelled and allowed to bind to its antigen at a fixed concentration in competition with varying amounts of unlabelled antibody Wagener et al, (1983), raised a panel of monoclonal antibodies against carcinoembryonic antigen (CEA) and determined their epitope specificities by labelling purified monoclonal IgG with ^{121}I and adding a constant amount of labelled antibody to doubling dilutions of unlabelled antibody before allowing this mixture to bind CEA antigen which was previously coated on a 96-well micro-titre plate Inhibition of binding of labelled antibody by unlabelled antibody was expressed as the percent of the binding of labelled antibody in the absence of unlabelled antibody This technique enabled the authors to judge which antibodies bound to different epitopes, and suggested that this approach could be applicable in general in the screening of monoclonal antibodies for epitope specificity

Watt and Watt, (1983), tested the specificity of their monoclonal antibody panel using a similar idea Their

antibodies were raised against low density lipoprotein The difference in their method, however, was that they used an affinity-purified biotinylated antibody and allowed it compete for LDL binding with varying amounts of unlabelled antibodies in an ELISA in which the detection system involved avidin-peroxidase The very high affinity with which biotin binds avidin made the assay sensitive and the procedure was less hazardous because no radioactive label was included Milne et al, (1983), devised a simpler approach to this problem, and adapted a method of Fisher and Brown, (1980), to soluble antigens Their antibodies were raised against human LDL and they were tested in a solid phase RIA under conditions where the LDL used to coat the micro-titre plates was at a low enough concentration to make it the limiting reactant in the assay Two different labelled antibodies were mixed in a one-to-one ratio before being added to the wells Antibodies which reacted with different determinants bound in an additive manner with a maximum radioactivity that was higher than that for each antibody bound individually. Antibodies which reacted with the same determinant bound with a maximum reactivity that was no greater than that for each antibody bound individually The same authors have also examined the antigenic determinant structure on LDL by experiments which involved chemically modifying the protein. Carbamylation, cyclohexanedione treatment and reductive methylation of LDL were all carried out before reaction with the antibodies in order to distinguish any

difference in the antibodies specificities, before and after modification

The chemically modified LDL was allowed to compete with ^{125}I-labelled control LDL in a solid phase RIA Of their seven antibodies tested, 2 showed very little reactivity with cyclohexane dione-treated LDL, suggesting that an arginine residue was present in the antigenic determinants for those antibodies These determinants were also shown to be sensitive to carbamylation, a reaction which mainly involves lysine residues, while the determinants for all the antibodies were sensitive to reductive methylation The Fab fragments of five of the antibodies were shown to inhibit ^{125}I-LDL binding to human fibroblast receptors, suggesting that they reacted with determinants which involved the receptor-binding site of apoprotein B Watt and Watt, (1983), describe experiments in which the immunoreactivities of the antibodies were assessed after delipidation of apolipoprotein B in both SDS (a denaturing detergent) and polyoxyethylene-9-lauryl ether (C12E9), a non-ionic detergent known to maintain the secondary structure of the protein In ELISA's, three of their six antibodies showed partial or complete loss of reactivity with SDS-delipidated apoB while all six were still reactive with the apoprotein after it was prepared in the non-ionic detergent Two of the antibodies bound SDS-delipidated apoB with greater reactivity than with the undelipidated control, suggesting that the reactive sites were sterically blocked in native LDL, and became more

accessible after SDS treatment

The capacity of monoclonal antibodies to distinguish between closely related proteins was investigated by Slaughter et al., (1981), using a panel of antibodies directed against human placental alkaline phosphatase, an enzyme which exhibits allelic variation The variants, which are determined by a series of alleles at a single genetic locus, can be distinguished electrophoretically, and six common phenotypes occur in the human population Thus, a valuable experimental system for testing the ability of monoclonal antibodies to distinguish closely related members of a set of homologous proteins is provided The six antibodies that they raised were tested by comparing their binding with a sample alkaline phosphatase (from placental extract) to that with a standard, which was the purified type 1 variant of alkaline phosphatase The authors demonstrated that the antibodies could successfully discriminate between the products of the three common electrophoretically defined allelic forms of placental alkaline phosphatase In addition, they provided evidence for polymorphism which could not be defined electrophoretically For example, one antibody bound a determinant which was present on the electrophoretically defined type 1 variant in a manner which suggested that two antigenic forms of the determinant existed among the population, one binding the antibody with a slightly higher affinity than the other

1 7 Epitope characterisation and immunoblotting

The publication by Towbin et al, (1979), of a method for the transfer of proteins from gel electrophoretograms to nitrocellulose described a technique which combined the high resolution electrophoretic separation of protein mixtures with the ability to probe such separated proteins, immunochemically or otherwise, in a straight forward manner

The technique, which Burnette, (1981), has termed "Western Blotting", has found extensive use in the epitope characterisation of apolipoprotein antigens with monoclonal antibodies, in particular with apoB It has allowed Marcel et al., (1982), to determine, firstly, which antibodies reacted with which of the apolipoprotein B species and secondly, by using tryptic fragments, the position of the antigenic determinants relative to each other The principle was applied to apolipoprotein A-1 fragments, after its cleavage by CNBr, by the same authors

Thus immunoblotting has, first of all, confirmed findings made through co-titration experiments on the epitope specificity of the monoclonal antibodies and then enhanced those findings by allowing the authors to construct a physical map of the antigenic determinants on the apolipoproteins

The low density lipoprotein receptor has been identified by ligand blotting using biotinylated LDL (Wade et al.,

1985) or ^{125}I-LDL (Kroon et al, 1984), a technique which eliminates the use of specific antibodies The method provides a simple means of detecting and quantitating the LDL receptor in individuals and thus the effects of dietary or drug-induced changes in LDL receptor concentration in cell extracts can be examined

Aims of this Project

The aim of this project was the production and characterisation of monoclonal antibodies against a range of lipoprotein apoproteins with the view to developing them for use in solid phase immunoassays for the quantitation of both single and combined apoproteins The antibodies would also be assessed as to their usefulness in detecting lipoprotein defects

This thesis will critically examine the success and efficiency of the fusion and screening procedures for the production and detection of specific antibodies A novel method of rapidly selecting antibodies of potential interest will be reported Findings from studies in which the molecular nature of the antigenic determinant for the antibodies, in which blotting and immunodetection was the experimental basis, will also be described and the simplicity and versatility of this technique will be assessed

Chapter 2

MATERIALS and METHODS

2 0 Materials

All cell culture media, sera and supplements (except $NaHCO_3$) were supplied by Gibco Europe Ltd , Paisley, Renfrewshire, Scotland

Cell culture flasks, dishes, tubes, 24-well plates and cryotubes were supplied by Nunc Ltd , Denmark

96-well cell culture plates and lids, centrifuge tubes, syringes and needles were supplied by Becton Dickenson, Oxnard, California

10ml pipettes, universal containers and other plastic disposables were supplied by Sterilin Ltd, Feltham, Middlesex, England

The following chemicals were supplied by SIGMA Chemical Co, Poole, Dorset, U K Ethylenediaminetetraacetic acid (EDTA) Bovine serum albumin (BSA), ethidium bromide, acridine orange, polyethylene glycol (PEG), hypoxanthine, thymidine, aminopterin, o-phenylene diamine, thimerosal, 9-amino-3-ethyl carbazole, molecular weight markers (cat no SDS-7), myosin, phosphorylase b, Coomassie brilliant blue, trypsin, beta-galactosidase, pepsin, soybean trypsin inhibitor, type 11 lipase, neuraminidase, type XI protease (proteinase K), guanidine hydrochloride iodoacetamide, phenylmethylsulphonyl fluoride (PMSF), octylglucopyranoside, 4-chloro-1-naphthol and biotin hydrazide

The following were supplied by BDH Chemicals Ltd Poole, England Sodiumbicarbonate, dimethyl sulphoxide, glycine, hydrogen peroxide, citric acid, di-sodium

hydrogenphosphate, sodium di-hydrogenphosphate, sodium acetate, acrylamide, bisacrylamide, sodium dodecyl sulphate, N,N,N^1,N^1 tetramethylethylene diamine (TEMED), ammonium persulphate, sucrose, beta-mercaptoethanol, trichloroacetic acid, glutaraldehyde, ammonia, sodium hydroxide, silver nitrate, formaldehyde, acetic acid, barbitone (diethyl barbituric acid), sulphosalicylic acid, isopropanol, calcium chloride, boric acid, sodium-m-periodate, ammonium sulphate, maleic acid, triton-X 100, glycerol and Tween 20

Reagents for Protein A-agarose chromatography and DEAE Affi-Gel Blue chromatography and apparatus were supplied by BIORAD Laboratories, 2200 Wright Avenue, Richmond, California, U S A

Potassium bromide and hexane were supplied by May and Baker Ltd , Dagenham, England

Phosphate buffered saline tablets (Dulbecco A) were supplied by Oxoid Ltd , England

Pristane (2,6,10,14 tetramethylpentadecane), and sodium borohydride was supplied by Aldrich Chem Co Ltd , Gillingham, Dorset, U K

Nitrocellulose was supplied by Schleicher and Schull Ltd , D-3354 Dassel, W Germany

Methanol and ethanol were supplied by Reidel de Haën, Aktiengesellschaft, Wunstorfer StraBe 40, D-3016 Seelze 1/Hannover, W Germany

Agarose was supplied by Pharmacia (U K) Ltd , Milton Keynes, Bucks, U K

Amido Black and Sudan Black B were supplied by Gurr Ltd , London, England

Peroxidase-conjugated rabbit anti-mouse immunoglobulins was supplied by DAKO Ltd , Skyttegade 7, DK-2200 Copenhagen, N Denmark

Peroxidase-conjugated swine anti goat IgG was supplied by TAGO, Inc 887 Milten Road, Burlingame, California 94011, U S A

Goat anti-human alpha-lipoprotein and goat anti-human beta-lipoprotein was supplied by Miles Laboratories Ltd., Stoke Court, Stoke Pages, Slough, England

Rabbit anti-human apolipoprotein B was supplied by Behring Ltd

Streptavidin-biotinylated-peroxidase complex was supplied by Amersham International, Amersham, Bucks, U K

Collagenase was supplied by Boehringer Mannheim GmbH Biochemica, P O Box 310120, D-6800, Mannheim, W Germany

96-well ELISA plates were supplied by Dynatech Ltd, Alexandria, Va, USA

Freunds adjuvant was supplied by Gibco Europe Ltd, Paisley, Scotland

Reagents were of analytical grade, wherever possible, and pH measurements were made at room temperature ($20^{o}C$)

Reagents for electrophoresis were specially purified for electrophoresis Glycine was chromatographically homogeneous All solutions were prepared in glass distilled water

METHODS

2 1 Preparation of lipoproteins

Blood from two healthy donors was collected into tubes containing 1mg/ml EDTA (pH 7 4) and the cellular components removed by centrifugation at 2000 rpm, at 4°C for 10 minutes The plasma was adjusted to a density of 1 25 g/ml (d=1 25g/ml) by the addition of solid KBr and overlayered with a solution of 0 9% NaCl / 0 1% EDTA / KBr (d= 1 21g/ml) before centrifugation for 2 hours at 4°C at 48,000 rpm in a Sorvall OTD50 ultracentrifuge using a TV865 vertical rotor, or, in some preparations, a OTD65 ultracentrifuge at 65,000 rpm for 70 minutes
The lipoprotein layer was carefully removed by aspiration, adjusted to d=1 25 g/ml with solid KBr and washed by repeating the ultracentrifugation step
Lipoproteins were dialysed extensively against 0 9% NaCl / 0 1% EDTA, aliquoted and stored at -20°C Delipidation was effected by vortexing the lipoprotein fraction in 10 volumes of diethyl ether for 2 minutes, followed by low speed centrifugation The aqueous phase was then prepared for immunization or electrophoresis as required
The protein content of the lipoproteins and of the delipidated fractions was assayed by the method of Lowry et al.,(1951), using bovine serum albumin as the standard
In some experiments VLDL, LDL, HDL_2 and HDL_3, provided by Drs A Johnson and P Collins of the Royal College of Surgeons of Ireland were used

2 2 Cell Culture and Maintainance

A mouse myeloma cell line Sp2-O-Ag14 was maintained as a monolayer in 50 cm^3 tissue culture flasks in RPMI-1640 tissue culture medium, supplemented with penicillin (100 units/ml), streptomycin (100µg/ml), 27mM $NaHCO_3$ 2mM Glutamine and 10% foetal calf serum Cells were kept at 37°C in a humidified incubator containing 5% carbon dioxide

Long term storage of cells was accomplished by freezing in liquid nitrogen The method used was as follows

Cells were pelleted by centrifugation of the spent medium at 2000rpm for 10 minutes 0 5ml of a solution of 10% dimethyl sulphoxide and 90% foetal calf serum was added to 1 8ml cryotubes and the cells then added The tubes were placed in a Union Carbide Freezing apparatus and slowly frozen over the liquid nitrogen vapour phase for 45 minutes at position F' and 35 minutes at position A before submerging In some cases cells were frozen at -70°C overnight before transfer to liquid nitrogen

Thawing was performed by warming a vial of frozen cells to 37°C and immediately adding to 10ml of serum-free medium The cells were pelleted by centrifugation, resuspended in 2ml medium containing 20% foetal calf serum and added to 24-well tissue culture plates When they had grown to confluency (usually after 24 hours), they were diluted to a volume of 5ml and transferred to 50cm^3 flasks

2 3 Production of hybridomas

2 3 1 Immunisation

8-12 week old male BALB/c mice were immunised intraperitoneally with 100µg of lipoprotein or apolipoprotein extract which was emulsified with an equal volume of complete Freund's adjuvant Two boosts, in incomplete adjuvant, were given at weekly intervals Three days after the final boost, the mouse was killed and its spleen removed In some cases, 3-4 boosts were given at two week intervals and, in one instance, 19 days were allowed to elapse between the 1st and 2nd boost

2 3 2 Cell fusion

Sp2 cells were thawed and cultured and maintained in exponential growth prior to the fusion

A spleen cell suspension was prepared by flushing the spleen of the immunised mouse with medium containing 20% foetal calf serum A portion (100µl) of the cell suspension was diluted with an equal volume of ethidium bromide / acridine orange, and mounted on a haemocytometer for examination under a fluorescent microscope A cell count was made and 10^7 cells were washed by centrifugation at 2000 rpm for 10 mins They were then co-centrifuged with 10^7 Sp2 cells at 4000 rpm for 5 minutes 1ml of a solution of 50% polyethylene glycol (approximate molecular weight = 3,350) in culture medium was added to the pellet

over 1 minute at 37°C followed by incubation for a further minute 10 ml of medium was then added slowly 1 ml over 1 minute, a further 1 ml over 1 minute and the remaining 8 ml over 3 minutes. The cells were centrifuged resuspended in medium containing 20% foetal calf serum, and 100µl aliquots were distributed in 96-well tissue culture plates at a density of $2x10^4$ cells per well After incubating overnight at 37°C in 5% carbon dioxide, the plates were fed with culture medium containing i) 20% foetal calf serum ii) hypoxanthine (136 1 mg/ml) iii) thymidine (38 75 mg/100ml) iv) aminopterin (17 6mg/100ml) and v) a suspension of normal mouse peritoneal cells at 2 $x10^5$ cells /ml

Plates were fed in hypoxanthine-aminopterin-thymidine (HAT) medium on the third and fifth day after the fusion and thereafter twice weekly.

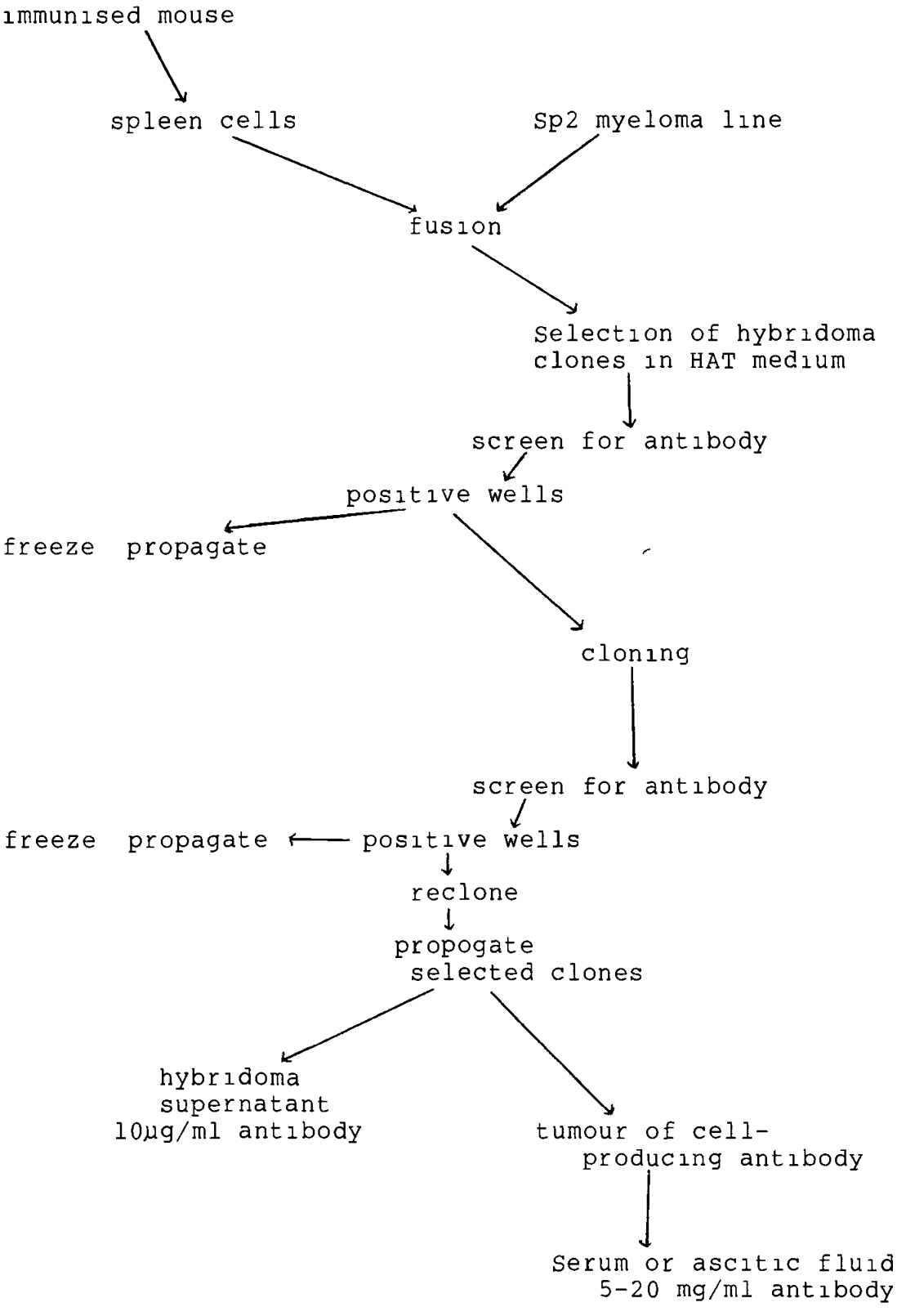


Fig 2 1 Protocol for monoclonal antibody production (from Galfre & Milstein (1981)

2 4 SCREENING ASSAYS FOR ANTIBODY ACTIVITY

2 4 1 ELISA

The presence of anti-apolipoprotein antibodies in the spent medium of the wells containing hybridomas was tested by an enzyme-linked immunosorbent assay (ELISA)
96-well polyvinyl plates were coated with 200µl of a solution of apolipoprotein containing 5µg protein / ml in 5mM glycine, pH 9 2 Control plates (containing glycine only) were set up in parallel After incubation overnight at 4°C the plates were washed four times with bovine serum albumin (5 mg/ml) in phosphate buffered saline (PBS/BSA) The remaining protein binding sites on the wells were blocked by incubating in 200µl of BSA (50mg/ml) in PBS for 3 hours at room temperature
After a further four washes in PBS/BSA, 100µl of hybridoma supernatant was added to test and control plates and incubated at room temperature for 2 hours
All wells were washed 4 times in PBS/BSA and then incubated with 200ul of a 1 1000 dilution of peroxidase-labelled rabbit anti-mouse immunoglobulins for 1 hr at room temperature After a final four washes, 100µl of the substrate solution (40 mg/100ml of o-phenylene diamine plus 0 003% hydrogen peroxide in 0 15M citrate-phosphate buffer) was added 20 minutes after substrate addition, the optical densities of each well were read in an EL-307 ELISA reader (BIO-TEK Ltd) using a 490nm filter

2 4 2 Dot-blotting

In some experiments hybridoma supernatants were tested for antibody in a dot-blotting assay Nitrocellulose sheets were blotted with a non-denaturing electrophoresis gel of apolipoproteins according to the procedures described in chapters 2 6 2 and 2 6 4 Two strips of the blot (5mm wide) were immunodetected with polyclonal anti-alpha-lipoprotein and anti-beta lipoprotein as described in chapter 2 6 4 to visualise the blotted apolipoproteins

Using the detected blot as a guide, 5µl of hybridoma supernatant was carefully spotted on to the undetected portion of the blot at the sites corresponding to the apolipoprotein bands The blot was allowed to dry and ther washed three times for 10 minutes in 0 05MTris/HCl pH 7 4, 0 15M NaCl before incubation in a solution of peroxidase labelled rabbit anti-mouse immunoglobulins, diluted 1 100 in 0 05M Tris / HCl, pH 7 4, 0 15 M NaCl, 0 1% Thimerosal and 2g bovine serum albumin / 100ml, for half an hour at room temperature

After repeating the washing procedure the blots were stained with substrate solution containing 15mg 9-amino-3-ethyl carbazole (solubilised in 4ml dimethyl sulphoxide) + 0 06% hydrogen peroxide in 50ml 0 02M acetate buffer, pH 5 0

2 5 Cloning and propagation of antibody-producing cell lines

2 5 1 Cloning by limit dilution

After recovering the cells from spent medium by centrifugation, a count was made and cells were diluted, in medium plus 10% (v/v) foetal calf serum, to 10, 50 and 500 cells/ml

A preparation of mouse peritoneal cells was made by washing out the fluid contents of the peritoneal cavity of mice with medium plus 10% foetal calf serum 100µl volumes were distributed into 96-well tissue culture plates at a cell density of $2x10^4$/well 100µl of each of the hybridoma cell dilutions were added to the wells, allocating half a plate (48 wells) for each dilution In some cases, the peritoneal cells were omitted Plates were examined microscopically for colony formation after approximately six days

2 5.1 Ascitic tumour growth

BALB/C mice were given an intraperitoneal injection of 0 5ml pristane (2,6,10,14-tetramethylpentadecane) prior to injection of 10^6-10^7 hybridoma cells suspended in 0 2ml medium The number of days allowed to elapse between pristane injection and hybridoma injection was varied from 3 to 21

Tumour growth was evident within 1-2 weeks Ascites fluids were drained from the mice by insertion of a 20G needle into the abdomen Cells were removed from the

collected fluids by low speed centrifugation and, in some cases, were resuspended in fresh medium and injected into a second pristane-primed mouse The fluids were stored at -20°C

2 6 Western blotting and Immunodetection of Apolipoproteins

2 6 1 Electrophoresis under denaturing conditions

The apolipoprotein fractions and, in somecases, delipidated VLDL, LDL, HDL_2 and HDL_3 were subjected to one dimensional electrophoresis according to the method of Laemmli (1970) on gels containing 10% (w/v) polyacrylamide, 0 33% (w/v) bisacrylamide, 0 375M Tris/HCl, ph 8 8, 0 1% (w/v) SDS, 0 1% (w/v) TEMED and 0 1% (w/v) ammonium persulphate The stacking gel contained 0 125M Tris/HCl pH6 8, 0 1% (w/v) SDS, 0 08% Temed (w/v), 0 08% (w/v) ammonium persulphate, 3% (w/v) acrylamide and 0 08% (w/v) bisacrylamide
Samples were prepared in buffer containing 0 123M Tris/HCl, pH 6 8, 2% (w/v) SDS, 10% (w/v) sucrose, 5% (w/v) β-mercaptoethanol and 0 02% (w/v) bromophenol blue and were boiled for 3 minutes
Gels were run in a vertical slab gel apparatus in electrode buffer containing 0 05M Tris, 0 37M Glycine and 0 1% (w/v) SDS at 25mA for 2-3 hours at room temperature
The proteins were stained by soaking the gel in a solution containing 0 1% (w/v) Coomassie brilliant blue, 7 5% (v/v)

acetic acid, 5% (v/v) methanol and 10% (w/v) trichloracetic acid for 1 hour at room temperature Destaining was carried out in 7 5% (v/v) acetic acid +5% (v/v) methanol Gels were also silver stained After fixing the gel in 10% (v/v) glutaraldehyde for 30 minutes, it was washed with distilled water, 3-4 times rapidly and then overnight with shaking at room temperature The gel was incubated in freshly prepared silver solution (33ml of 0.36% (w/v) NaOH, 2.1ml of NH_3, 6ml of 19 4% (w/v) $AgNO_3$, 108 9ml H_2O) for 15 minutes at room temperature on a rocking platform, and then washed rapidly 3-4 times in distilled water The protein bands were visualised by the addition of 0 02% (v/v) formaldehyde 0 005% (w/v) citric acid and the gel was washed with shaking in distilled water for several hours

2 6 2 Electrophoresis under non-denaturing conditions

2 6 2 1 Davis gels

Delipidated lipoproteins of a total lipoprotein preparation, and of lipoproteins of different densities - VLDL, LDL, HDL_2 and HDL_3, were subjected to electrophoresis under non-denaturing conditions using the method of Davis (1964) in running gels containing 0 375M Tris/HCl pH 8 8 4 28% (w/v) acrylamide, 3 125% (w/v) bisacrylamide, 0 0625% (v/v) TEMED and 0 0625% (w/v) ammonium persulphate The stacking gel contained 0 059M Tris/HCl pH 6.9, 3.75% (w/v) Acrylamide, 0.1% (w/v) bisacrylamide, 0 05% (v/v) TEMED, 0 05% (w/v) ammonium

persulphate Samples were prepared in buffer containing 0 0125M Tris, 0 095M glycine, 10% (w/v) sucrose and 0 001% bromophenol blue Electrophoresis was carried out on a slab gel apparatus in electrode buffer containing 0 05M Tris, 0 38M glycine at 25mA for 2-3 hours at room temperature Protein staining was with Coomassie Blue or silver stain, as described for SDS gels

2 6 2 2 Lipoprotein gels

Plasma samples and preparations of LDL, VLDL, HDL_2 and HDL_3 were subjected to polyacrylamide gel electrophoresis under non-denaturing conditions Running gels contained 0 55M Tris/HCl pH 8 9 0 042% (v/v) TEMED, 3 58% (w/v) acrylamide, 0.13% (w/v) bisacrylamide and 0 07% (w/v) ammonium persulphate Stacking gels contained 0 062M Tris/HCl pH 6 7, 0 0575% (v/v) TEMED, 2 5% (w/v) acrylamide, 0 625% (w/v) bisacrylamide, 20% (w/v) sucrose and 0 0005% (w/v) riboflavin and were polymerised with a fluorescent light

Samples were prepared by mixing 2µl with 30µl of unset stacking gel These were then added to the wells and then polymerised

Gels were run in electrode buffer containing 0 005M Tris, 0 05M Glycine pH7 8 for 2 hours at room temperature Gels were stained in 0 11% (w/v) Coomassie brilliant blue, 33% (v/v) Methanol, 3 38% (w/v) sulphosalicylic acid and 10 8% (w/v) trichloroacetic acid for half an hour at 60°C and destained in 30% (v/v) methanol, 5% (v/v) acetic acid

2 6 4 Western blottıng and ımmunodetectıon

2 6 4 1 Western blottıng

Western blottıng was performed wıth gels of apolıpoproteıns followıng electrophoresıs ın SDS gels (Laemmlı, 1970) or non-denaturıng gels accordıng to Davıs (1964) The procedure was based on that descrıbed by Towbın et al,(1979) A nıtrocellulose sheet, whıch was fırst wetted ın blottıng buffer (0 025M Trıs, 0 19M glycıne, 10% (v/v) methanol, pH 8 3) was carefully layered over the gel Two sheets of Whatman 3m fılter paper and 1 BIORAD Scotch Brıte pad were placed on each sıde and the sandwıch was placed ın a Trans-Blot box whıch had been fılled wıth blottıng buffer Electrophoretıc transfer was performed at 252mA overnıght at room temperature wıth the nıtrocellulose to the anode sıde To vısualıse the transferred proteıns nıtrocellulose blots were staıned for total proteın wıth 0 1% (w/v) Amıdo Black ın 45% (v/v) methanol + 10% (v/v) Acetıc acıd Destaınıng was ın 45% (v/v) methanol and 10% (v/v) Acetıc acıd

Alternatıvely, proteıns were staıned wıth Indıa ınk The blot was was washed four tımes for 10 mınutes each tıme ın 0 05M Trıs/HCl, pH 7 4, 0 15M NaCl 0 3% (v/v) Tween 20 It was then ıncubated ın 0 1% (v/v) Indıa ınk (ın the above solutıon) for several hours

For ımmunodetectıon, sheets were ıncubated for 1 hour at room temperature ın blockıng solutıon contaınıng 0 05M Trıs/HCl, pH 7 4, 0 15m NaCl 0 1% (w/v) thımersosal and 2% (w/v) bovıne serum albumın Thıs was followed by

incubation in antibody solution - hybridoma supernatant (undiluted), or polyclonal goat anti-human alpha-lipoprotein (HDL specific) or goat anti-human beta-lipoprotein (VLDL and LDL specific), (diluted 1 200 in blocking solution), for 1 hour at room temperature The blots were washed three times for 10 minutes in 0 05M Tris/HCl, pH 7 4, 0 15m NaCl and then incubated for 30 minutes at room temperature, in peroxidase-conjugated rabbit anti-mouse immunoglobulin (diluted 1 100 in blocking solution), to detect mouse monoclonal antibody, or peroxidase-conjugated swine anti-goat IgG (diluted 1 200), to detect the polyclonal antisera After washing three times for 10 minutes the blots were detected with substrate solution - 15mg 9-amino-3-ethyl carbazole (solubilized in 4ml dimethyl sulphoxide) plus 0 06% (v/v) hydrogen peroxide in 50ml of 0 02M Acetate, pH 5 0 All incubations and washings were performed on a rocking platform Stained blots were washed in tap water

2 6 4 2 Native Blotting

Lipoprotein or plasma samples subjected to 3 58% (w/v) acrylamide gel electrophoresis were capillary blotted The nitrocellulose, pre-soaked in 0 05M Tris/HCl, pH 7 4, 0 15m NaCl, was carefully layered over the gel and pressed with 7 sheets of Whatman 3mm filter paper and wads of tissue under a 1kg weight for 1 hour at room temperature The blot was then immunodetected with antibody following the same procedures as for SDS and non-denaturing gels

2 7 Epitope modification experiments - characterisation of antigenic determinants of apoB

Electrophoresis of delipidated lipoproteins, or in some case delipidated LDL, was performed under non-denaturing conditions as described before in chapter 2 6 2 1 The gels were blotted to nitrocellulose and remaining protein binding sites blocked in all cases Chemical and enzymic modification experiments, performed to characterise the molecular nature of the antigenic determinant, were carried out using the procedures described below

2 7 1 Organic solvent extraction

Blots were cut into 5mm strips and each was incubated in 1ml of hexane / isopropanol (3 2, v/v) at room temperature for 1 hour They were then rinsed rapidly with washing solution before immunodetection with monoclonal and polyclonal antibody as described in Chapter 2 6 4

2 7 2 Enzyme modification

Non-denaturing gels of apolipoproteins were run according to the procedures described in chapter 2 6 4 1 except that the acrylamide and bisacrylamide concentrations were doubled Gel blots, blocked with albumin and cut into 5mm strips, were then incubated for 30 minutes at 37°C in the following enzyme solutions

Blot n°	1	2	3	4
Enzyme added	None	Trypsin 1mg/ml	Trypsin 0 1mg/ml	Trypsin 0 01mg/ml

Blot n°	5	6	7	8
Enzyme added	Collagenase 0 01U/ml	B-galactosidase 0 01U/ml	Pepsin 1mg/ml	Trypsin(0 5mg/ml) +Soybean trypsin inhibitor (5mg/ml)

Table 2 7 2

All the enzyme solutions with the exception of pepsin were diluted in 0 05M Tris/HCl, pH7 4, 0 15M NaCl, 0 01M $CaCl_2$ Pepsin was made up in 0 05M glycine /HCl pH 2 3 The strips were washed in 0 05M Tris/HCl, pH 7 4, 0 15M NaCl, three times for 10 minutes, then blocked and washed again Each set of 8 strips were immunodetected, using the procedure described in section 2 6 4, with the following antibody solutions i) hybridoma supernatant (undiluted) for 1 hour at room temperature ii) peroxidase-conjugated rabbit anti-mouse immunoglobulins (diluted 1 20 in blocking solution) for 1 hour at room temperature and iii)

mouse peroxidase-anti-peroxidase complex (diluted 1 100 in blocking solution) for 30 minutes at room temperature

2 7 3 Trypsin incubation

A non-denaturing gel containing 4 28% (w/v) acrylamide was run blotted and blocked with albumin 5mm strips were incubated in the following solutions

Strip no	1	2	3	4	5	6
Enzyme conc	Control	Trypsin 1mg/ml	Trypsin 0 1mg/ml	Trypsin 0 01mg/ml	Trypsin 0 001mg/ml	Trypsin (0 5mg/ml)+ inhibitor (0 5mg/ml)

The trypsin and inhibitor were diluted in 0 05M Tris/HCl, pH7 4, 0 15M NaCl, 0 01M $CaCl_2$ and incubation was performed at 37°C for 30 minutes The inhibitor solution was mixed with trypsin 5 minutes before addition over the blot At the end of the incubation, the reaction was stopped by adding 1mg/ml soybean trypsin inhibitor over each strip After washing the strips three times for 10 minutes each set of six was immunodetected with undiluted hybridoma supernatant or polyclonal anti-alpha-lipoprotein and anti-beta-lipoprotein, as described previously in chapter 2 6 4

2 7 4 Lipase treatment

Immunodetection was performed on blot strips treated with lipase· solutions containing 0 1M Tris/HCl, pH 8 0, 1mg type II lipase / ml and 10% (w/v) bovine serum albumin were added over the blots for 40 minutes at 37°C

2 7 5 Neuraminidase treatment

Immunodetection was performed on blots treated with 0 01U SIGMA Type VI neuraminidase / ml of 0 05m Tris/HCI, pH7 4, 0 15m NaCl, for 30 minutes at 37°C

2 7 6 Pepsin treatment

Immunodetection was performed on blots treated, for 30 minutes at 37°C, 1mg/ml pepsin in 0 05M glycine/HCl pH 2 3

2 7 7 Proteinase K treatment

Immunodetection was performed on blots treated with 1mg, 0 1mg and 0 01mg/ml Proteinase K in PBS for 30 minutes at 37°C

2 7 8 Reductive methylation

Immunodetection was performed on blots treated with reductive methylating reagents ' Strips were incubated in 0 3M Sodium borate, pH 9 0, for 10 minutes at room temperature before addition of 1mg/ml Sodium borohydride in 0 3m Sodium borate, pH 9 0 1µl at 37% (v/v) formaldehyde was added 0, 6, 12, 18 and 24 minutes after sodium borohydride addition After 30 minutes 1mg sodium borohydride was again added and 1µl formaldehyde additions were continued 30, 36, 42, 48, 54 and 60 minutes after starting the reaction The blots were rinsed several times with 0 15M NaCl, 0 05M Tris/HCl, pH 7 4

2 7 9 Iodoacetamide treatment

Immunodetection was performed on blots treated with iodoacetamide Strips were incubated in 1ml of 0 2M phosphate buffer, pH 7 3, containing 3µl beta-mercaptoethanol for four hours at room temperature 7mg of iodoacetamide were then added over each blot which was incubated in the dark at 0°C for thirty minutes

2 7 10 Periodate oxidation

Immunodetection was performed on blots treated with periodate according to Woodward et al , (1985) Strips were rinsed with 0 05M Na Acetate pH 4 5 before incubating

in the dark for 1 hour at room temperature, in 20mM $NaIO_4$ in 0 05M NaAcetate, pH 4 5 The strips were then incubated in 50mM $NaBH_4$ for 30 minutes

2 8 Monoclonal antibody purification

2 8 1 Ammonium Sulphate precipitation

Monoclonal antibody from hybridoma supernatant was partially purified by ammonium sulphate precipitation Ammonium sulphate was added to 250ml of supernatant to 50% (w/v) saturation and stirred for 30 minutes The precipitate was centrifuged at 2000rpm for 15 minutes The pellet was redissolved in 50 ml PBS and ammonium sulphate added to 50% saturation before re-centrifuging The pellet was exhaustively dialysed against PBS

2 8 2 Protein A Sepharose Chromatography

The ammonium sulphate precipitation fraction of hybridoma supernatant was subjected to immunoaffinity chromatography on a Sepharose-Protein A column Affi-gel protein A (BIORAD) in a volume of 1ml was equilibrated with binding buffer (BIORAD) 7ml of the 50% ammonium sulphate fraction of hybridoma supernatant, mixed with 7ml of binding buffer, was applied to the column which was then washed with several volumes of binding buffer The bound protein material was eluted with 15ml of elution buffer

(BIORAD) and 2ml fractions were collected The column was regenerated by washing with BIORAD regeneration buffer All the column steps were performed at $4^{o}C$

2 8 3 DEAE Affi-Gel Blue Chromatography

DEAE Affi-Gel blue chromatography was used to purify monoclonal antibody from ascites fluid according to the method described by Bruck et al.,(1982) The ammonium sulphate precipitated immunoglobulin fraction of hybridoma supernatants was also further purified by this method 0 6ml of ascites fluids was ultracentrifuged at 100,000g for 30 minutes to remove fibrin clots The supernatant was dialysed against column buffer (0 02M Tris/HCl, pH7 2) overnight and then centrifuged at 10,000g for 15 minutes The fluid was applied to a 6ml column (in a Terumo 20ml plastic syringe) of DEAE Affi-gel blue It was washed with 30ml of column buffer and the bound material step-eluted with increasing concentrations of sodium chloride (25, 50, 60 and 100mM in column buffer) The flow rate was 40ml/hour and fractions of approximately 2 5ml were collected The column was regenerated by washing with 20ml of 6M guanidine hydrochloride, followed by 60ml of 0 5M NaCl, followed by 30ml column buffer All the column steps were performed at $4^{o}C$ The optical densities of the eluted fractions were read in a CECIL spectrophotometer at 280nm Fractions were also assayed by immunodetection on blots of a non-denaturing gel of apolipoproteins and by SDS electrophoresis on 10% gels stained with Coomassie blue

2 9 LDL-receptor binding studies

2 9 1 Electrophoresis of LDL-receptors

Low-density-lipoprotein receptors were partially purified from bovine adrenal cortex membranes by DEAE-cellulose chromatography as described by Schneider et al, (1982) Receptors were subjected to one-dimensional electrophoresis on 7 5% (w/v) polyacrylamide slab gels containing 0 35% (w/v) SDS according to the method of Laemmli (1970) Samples were prepared in buffer containing 10% (v/v) Glycerol and 0 5% (w/v) SDS without boiling Gels were calibrated with SIGMA high molecular weight marker proteins. myosin (205,000); β-galactosidase (116,000), phosphorylase b (97,400), bovine serum albumin (66,000) and ovalbumin (43,000) 0 1% (w/v) bromophenol blue (in sample buffer) was added to the wells at each side of the gel Electrophoresis was performed at 4°C for 3-4 hours at 200V and the gels were blotted to nitrocellulose in 20mM Tris, 150mM glycine, pH8 3 + 20% (v/v) methanol The portion of the blot containing the protein markers was cut off and stained with 0 1% (w/v) Amido Black in 45% (v/v) methanol and 10% (v/v) acetic acid for 5 minutes Destaining was in 10% acetic acid

2 9 2 Ligand Blotting

Human LDL and rabbit beta-VLDL were prepared and biotinylated according to the procedures described by Wade et al.,(1985) The nitrocellulose sheets, preincubated in blocking buffer containing 50mM Tris/HCl, pH 8 0, 2mM $CaCl_2$, 90mM NaCl and 50mg bovine serum albumin / ml, for 1 hour at 37°C, were reacted for one and a half hours with the biotinylated lipoproteins at concentrations of 20ug/ml (biotin LDL) and 10µg/ml (biotin beta-VLDL) in the blocking buffer The sheets were washed, once rapidly, and then twice for 20 minutes, in washing solution containing 50mM Tris/HCl, pH 8 0, 2mM $CaCl_2$ 90mM NaCl and 5mg BSA/ml The sheets were incubated in streptavidin-biotinylated-peroxidase complex (diluted 1 300 in blocking buffer at pH 7 4) for 30 minutes and then washed three times for 10 minutes All the incubations and washings, with the exception of the blocking step, were performed at room temperature on a rocking platform The sheets were developed with substrate solution containing 0 4mg/ml of 4-chloro-1-naphthol+0 03% (w/v) hydrogen peroxide in 50mM Tris/HCl, pH 7 0, 2mM $CaCl_2$ and 90mM NaCl
Ligand blotting was also performed with ^{125}I-LDL 1 4mg of LDL was labelled with 2mCi of ^{125}I according to the iodine monochloride method of McFarlane (1958) and a specific activity of 295cpm/ng was obtained Ligand blotting was performed in exactly the same way as for

biotin-LDL except that 10ug ^{125}I-LDL(m) (= 3x10^{6}cpm) was used and after the washing procedure which followed ^{125}I-LDL incubation the blots were processed for autoradiography using pre-flashed Kodak X-ray film with an intensifier screen

2 9 3 Inhibition experiments with antibodies

2 9 3 1 On nitrocellulose blots

Four antibodies against apolipoprotein B, 5B10, 6C9, 6G10A4 and 4G1B4 and 1 antibody, 3H9, against a HDL_3 apoprotein, previously purified from ascites fluid by DEAE Affi-Gel blue chromatography, were dialysed against 50mM Tris/HCl, pH 8 0, 2mM $CaCl_2$ and 90mM NaCl 1ml of each antibody solution, containing 100µg protein/ml, was incubated with 10ug/ml ^{125}I-LDL overnight at 4^{o}C Similar antibody / lipoprotein solutions were set up with biotin-LDL 1 cm strips of nitrocellulose containing the blotted receptor were blocked and incubated with the antibody / lipoprotein solutions for one and a half hours at room temperature The blots were then washed and processed for autoradiography or detected with streptavidin-biotinylated peroxidase as described above
Similar experiments were performed with biotin-LDL using polyclonal anti-apoB Dilutions of 1 200, 1 100 and 1 50 in combination with 10µg/ml biotin LDL were incubated over the blots, after first allowing LDL and antiserum to combine for 10 minutes in solution.

2 9 3 2 On binding of LDL to skin fibroblasts

Normal human skin fibroblasts were set up at a concentration of $5x10^4$ cells/ml into 60mm plastic Petri dishes containing 3ml Eagles minimal essential medium (with Earle's salts), 25mM $NaHCO_3$, 20mM N-((Trishydroxy Methyl) Methyl) glycine (TRICINE), 100U/ml Penicillin, 100µg/ml Streptomycin and 10% (v/v) foetal calf serum The cells were fed in the same medium 2 days later On the fifth day the dishes were washed in Puck's Saline A and the cells fed in the same medium as above except that FCS was replaced by lipoprotein-deficient serum (at 2 5mg/ml) ^{125}I-LDL (1 76µg/ml)(Specific Activity = 80cpm/ng) was incubated overnight at 4^oC in the presence of a) 500µg/ml antibody 6G10A4 in 50mM Tris/HCl, pH 7 4, 2mM$CaCl_2$ and 90mMNaCl b) 250ug/ml antibody 6C9 in the same buffer and c) buffer alone On the seventh day, the cells were incubated in LPDS medium (containing no $NaHCO_3$) at 4^oC for 45 minutes Each ^{125}I-LDL solution, a) b) and c) were added to duplicate dishes at concentrations of 10, 5, 3, 2, and 1µg/ml In addition, each ^{125}I-LDL solution, together with 400ug unlabelled LDL/ml, was added at concentrations of 10, 5 and 2 µg/ml

After incubation for 2 hours at 4^oC the dishes were washed, 3 times rapidly and then twice for 10 minutes in 0 05M Tris/HCl, pH 7 4, 0 15M NaCl and 0 2% (w/v) BSA After a final quick wash in 0 05mM Tris/HCl, pH 7 4, 0 15M NaCI the cells were solubilised in 2ml of 0 1m NaOH, counted in a gamma spectrophotometer and assayed for protein content by Lowry et al ,(1951) Binding was expressed in terms of ngLDL bound / mg cell protein

CHAPTER 3

FUSION RESULTS

3 1 Recovery of Lipoproteins

Ultracentrifugation was performed under conditions which would allow the extraction of all lipoprotein density classes in a single fraction. The isolated lipoproteins were then partially delipidated by vortexing in 10 volumes of diethyl ether The aqueous phase was subjected to electrophoresis in a 10% polyacrylamide/SDS gel (fig 3 1) Bands with mobilities corresponding to apoB, apoA-1 and ApoE could be identified in this system but other proteins, particularly albumin, were evident also For this reason a second ultracentrifugation step was introduced in subsequent preparations to further purify the isolated lipoproteins

The recovery of the lipoproteins was monitored by determination of cholesterol in the plasma before ultracentrifugation and in the lipoprotein fraction obtained afterwards These assays were performed by the Biochemistry Laboratory in Our Lady's Hospital, Crumlin As expected, the introduction of a second ultracentrifugation step resulted in a reduction in the overall recovery. Proteins were assayed by the method of Lowry et al, (1951). The recovery and protein content for each isolation run is shown below in table 3 1 A second ultracentrifugation step was introduced at run number 4

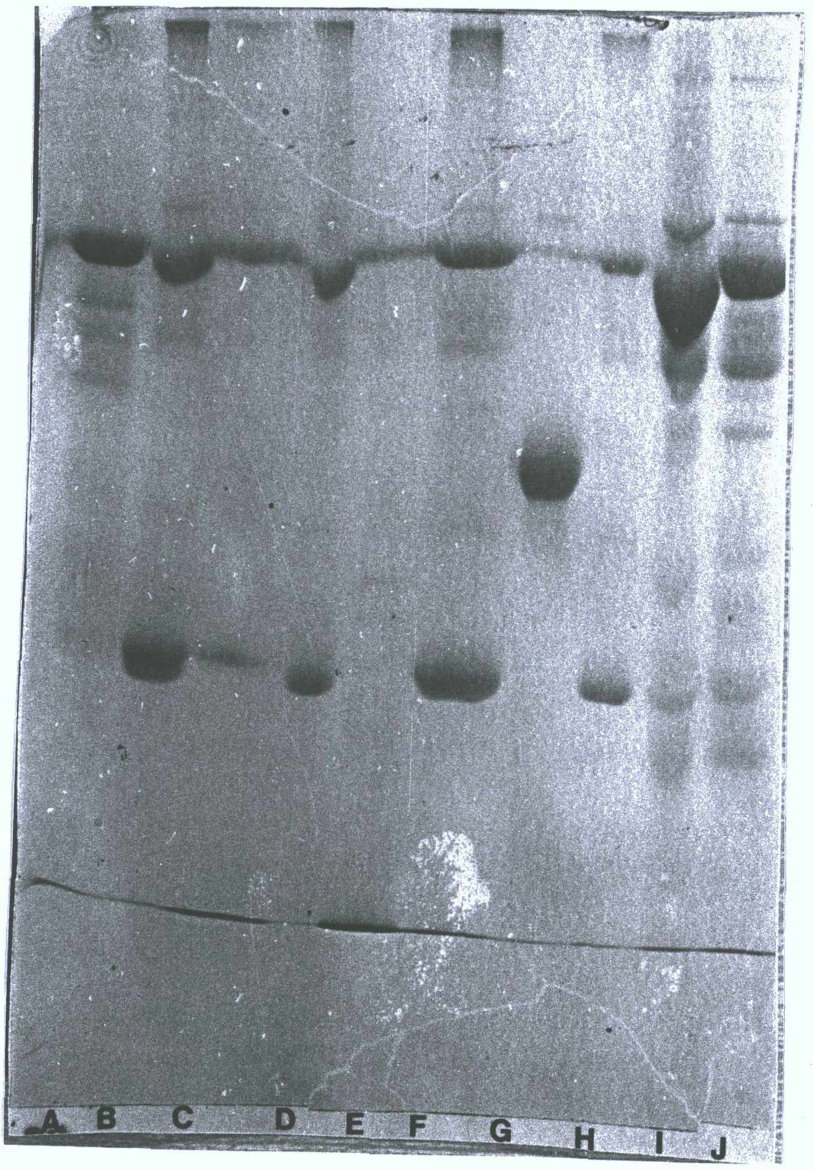

A B C D E F G H I J

Fig 3 1 Coomassie blue-stained gel of delipidated lipoproteins which were run on a 10% polyacrylamide SDS gel along with molecular weight standards A albumin, B and D apolipoprotein (in non-reducing buffer), C Trypsin, E cytochrome C, F and H apolipoprotein (in reducing buffer), G ovalbumin I Whole Serum (non-reduced), J Whole Serum (reduced)

Run No	1	2	3	4	5	6	7	8	9	10
Recovery (%)	193	47	ND	17 1	14 5	15 5	19	26	31	43
Protein Content (mg/100ml)	97 5	137 5	122 5	102 5	80	80	42.5	87 5	52	ND

Table 3 1 Isolation of Lipoproteins

This apolipoprotein preparation was also used for immunisation The schedules for injection varied in earlier experiments but for the large part they followed a scheme of three weekly intraperitoneal injections with the fusion being carried out three days after the final boost

3 2 Fusion Results

16 fusions in all were carried out The numbers of hybridomas generated for each fusion is shown in table 3 2

Fusions 12-14 yielded very little or no hybridomas With successful fusions, colony formation could be seen five to six days after the fusion

For fusions 15 and 16 Sp_2 myeloma cells and materials from Our Lady's Hospital in Crumlin were used and the fusion was carried out there The 16th fusion showed an improvement in hybridoma growth and although a yield of 18% was lower than normal the hybridoma cells grew normally

Fusion No	Immunogen	No wells plated	No cells/well($\times 10^4$)	Hybridoma yield(%)
1	apolipoprotein	464	2	0
2	"	360	2	7 5
3	"	256	2	39
4	"	352	2	37
5	"	960	1	0
6	"	768	1	13
7	"	480	2	100
8	"	608	2	54
9	HDL_3	384	2	56
10	VLDL	384	2	45
11	HDL_3	480	2	14
12	delipidated apoHDL_3	960	2	4
13	"	720	2	0
14	"	480	2	0
15	"	480	2	0
16	"	624	2	18

Table 3 2 Hybridoma yield for fusions 1-16

3 3 Screening Results

Hybridomas were screened by ELISA or, for later experiments, by dotting supernatant to antigen immobilised on nitrocellulose Both methods were successful in detecting anti-apolipoprotein antibodies in the hybridoma supernatants tested Control plates containing no bound apolipoprotein were also set up in the ELISA to detect supernatants which contained irrelevant or "sticky" antibodies which bound non-specifically to the albumin coated wells The screening results for each fusion experiment are shown in table 3 3 with those antibodies that were selected for further study indicated
Plates were read at 492nm in a BIOTEK EL-307 reader following additions of substrate for all ELISAs except those for the first 5 fusions During the first five fusions no reader was available Sera from immunised and non-immunised mice, at a 1 1000 dilution, were used as positive and negative controls respectively In all cases the normal mouse sera gave low positivity to the test wells, while the immune mouse sera gave a stronger reaction and only to the test plates
ELISA experiments for each fusion are described in more detail in the following pages

Fusion No	Number of antibodies produced	Antibody against apolipoprotein	Non-specific antibody
1	0	0	0
2	0	0	0
3	0	0	0
4	3	3	0
5	0	0	0
6	6	3	3
7	23	0	23
8	37	31 inc 5B10, 6C9, 6G10A4	6
9	29	16	9
10	39	31 inc 2B6 3F12,4G1	8
11	2	2	0
12	2	2	0
13	0	0	0
14	0	0	0
15	0	0	0
16	4	3 inc 3H9	1

3 3 1 Screening Results

Fusion 4

Fusion 4 was the first to give positive hybridomas, which were identified visually after the ELISA and named 1A5, 3C2 and 3G3 The cells were cloned by limit dilution, with $2x10^4$ macrophages / well as feeders, at 1,5 and 50 cells / well On screening the cells plated at 5 / well ,which resulted in single colonies, those that gave the strongest reactivity were grown up in 2ml volumes For each antibody there were 2x2ml wells and the hybridomas were named 3C2 (1) and (2), 3G3(1) and (2) and 1H5(1) and (2) The cells were grown up to 10ml volumes and 10^7 of each were injected into Balb/c mice for propagation as an ascitic tumour At this stage, unfortunately the expanded cells were lost to fungal contamination However, the original cloned plates were still being maintained and their cells were rescreened 1H5G11, 3C2F8 and 3C2G10 had the strongest antibody reactivity and were recloned The 3G3 line, however, had become unstable and many of the cells had died Very few wells contained positive hybridomas

3C2F8, 3C2G10 and 1H5G11 were grown as ascitic tumours in mice. An experiment was set up in which the ascites fluids for 3C2F8 and 3C2G10 were then tested over a serial dilution range by an ELISA to determine which dilution would be comparable in binding reactivity to the hybridoma supernatant Serum from the immunised mouse at a dilution of 1 2000 was put up for comparison The fluids were diluted in PBS/BSA over a range extending from 30-fold to 100,000-fold, and reacted with 5ug apolipoprotein bound to

wells of a 96-well plate After substrate addition the absorbances of wells containing neat supernatant, immune mouse serum and the least dilute ascitic fluid were read at 412nm The other wells were examined by visual inspection

The binding of the least dilute ascites fluid was comparable to the supernatant and did not approach the absorbance given by the immune mouse serum This suggested that antigen concentration was limiting the reaction for monoclonal antibody but not for polyclonal antibody which is directed against a wide range of antigenic determinants in the apolipoprotein mixture

Antibody 3C2F8

Dilution factor	30	100	200	400	1000	10,000
Colour Intensity	++	++	++	++	++	+
Abs 412nm	0 254	ND	ND	ND	ND	ND

Dilution factor	50,000	100,000	Hybridoma S/N
Colour Intensity	v faint	v.faint	++
Abs412nm	ND	ND	0 235

Antisera

Serum	Immune mouse serum 1 2000	Normal serum 1 2000	Immune serum (against antigen blank)
Colour Intensity	+++	-	-
Abs 412nm	0 943	-	-

ANTIBODY 3C2G10

Dilution	30	100	200	400	1000	10,000
Colour Intensity	++	++	++	++	++	+
Abs412nm	ND	0 248	ND	ND	ND	ND

Table 3 2 2 Screening ascites fluids of antibodies 3C2F8 and 3C2G10

Fusion 6

A low yield of hybridomas resulted after fusion 6, but when they were screened, 2 hybridomas were strongly positive, and 1 weakly positive, for antibody activity Three other hybridomas secreted antibody which bound to both coated and uncoated wells, (fig 3 3 1) The two strongly-secreting hybridomas, 3B3 and 3E10, and the weak hybridoma, 5D10 were transferred to 2ml volumes and the two strong hybridomas were also cloned After several days, 3E10 and 3B3, but not 5D10, were still positive for antibody activity The cells were grown as ascitic tumours in mice

When the ascitic fluids and cloned cells were screened, the antibodies were found to be binding to both test and control plates, a finding which had not occured previously The reason for this was unknown

Fig 3 3 1 Hybridoma cell supernatants from fusion 6 were screened by ELISA Absorbance readings were taken at 490nm

■ = test wells, ⌐¬ = control wells (containing no bound antigen)

The reactive antibodies from this fusion were 5, 3B3, 6, 3E10, 8, 4A11; 10, 5C9, 11, 5B10, 12 5H11

I = Immunised mouse serum diluted 1 2000

N = non-immunised mouse serum diluted 1 2000

Fusion 7

Five plates of hybridoma supernatant were screened against 2 4ug of apolipoprotein per well 23 wells gave positive binding initially but 21 of these also bound to a greater or lesser extent to uncoated wells (fig 3 3 2)

When supernatants were re-tested some antibodies of potential interest became negative while others reverted to non-specificity 2A6, for instance, stopped secreting antibody while 4F7 bound to an uncoated plate in a subsequent screening The presence of non-specificity among the antibodies was a persistent problem throughout the screening for this fusion, as was the non-reproducibility with respect to the degree of background for any particular antibody from one screening to the next

Fig 3 3 2 23 of the 480 Hybridoma supernatants from fusion 7 that were screened by ELISA

Absorbances were read at 490nm

Continuous lines = test wells, non-continuous lines = control wells Hybrid numbers are named in the table below

I, = immunised mouse serum diluted 1 1000

N, = non-immunised mouse serum diluted 1 1000

Hybrid number	1	2	3	4	5	6	7	8	9	10	11	12
Name	1A10	1B5	1G11	2A6	2A9	2B4	2D4	3H5	4A3	4A5	4E8	4E12

Hybrid number	13	14	15	16	17	18	19	20	21	22	23
Name	4E7	4F9	4G8	4H5	5A11	5A12	5B4	5B5	5B12	5C2	5G4

Fusion 8

A hybridoma yield of 54% was obtained with fusion 8 and this time the ELISA was successful in differentiating antibodies against apolipoprotein from those merely binding non-specifically to uncoated wells 183 hybridomas were screened altogether and 37 were positive. However, 6 of those also bound to uncoated wells The absorbances for the 37 positive hybridomas are shown below in figure 3 3 3 Nine hybridomas were cloned These were 1B10, 1D6, 1F1, 2A6, 3A5, 3D9, 6C9, 6E11 and 6G10 Six hybridomas, 1E2,1F2, 7G1, 1A12, 5E7 and 5B10 were frozen The others became either negative or non-specific on subsequent screening, or the cells died or became contaminated Prior to this second screening, 21 hybridomas were grown up to 10ml volume and the supernatants tested on SDS gel blots of apolipoproteins One antibody, 5B10, bound to a protein band of high molecular weight on the blot (Fig 3 3 4 a+b) None of the other antibodies could be detected in this technique 5B10 was therefore cloned, producing 10 cloned hybridomas When a non-denaturing electrophoresis system was used the supernatants from the 10 cloned hybridomas tested bound faintly to a very thin band at the top of the blot Goat anti-human beta-lipoprotein bound similarly and it was concluded that the antibodies were reactive against apolipoprotein B, and that the determinant for 5B10 was not destroyed by boiling in the presence of detergent

Fig. 3.3.3 37 of the 183 hybridoma supernatants from fusion 8 that were screened by ELISA. Absorbances were read at 490nm. Continuous lines, = test wells, dotted lines, = control wells.

Hybridoma numbers are named in the table.

Hybrid number	1	2	3	4	5	6	7	8	9	10	11	12
Name	1B10	1D6	2A6	2H8	3A5	3D5	3D9	3H11	4C12	4D12	4E11	4G2
Hybrid number	13	14	15	16	17	18	19	20	21	22	23	24
Name	4G12	5B10	5D7	6A7	6C9	6E11	6F11	6G10	7A6	1A12	1E2	1F2
Hybrid number	25	26	27	28	29	30	31	32	33	34	35	36	37
Name	2D12	2E4	2E5	2E9	2F1	3C2	3C5	4D7	5E7	6A8	7G1	3E8	1F1

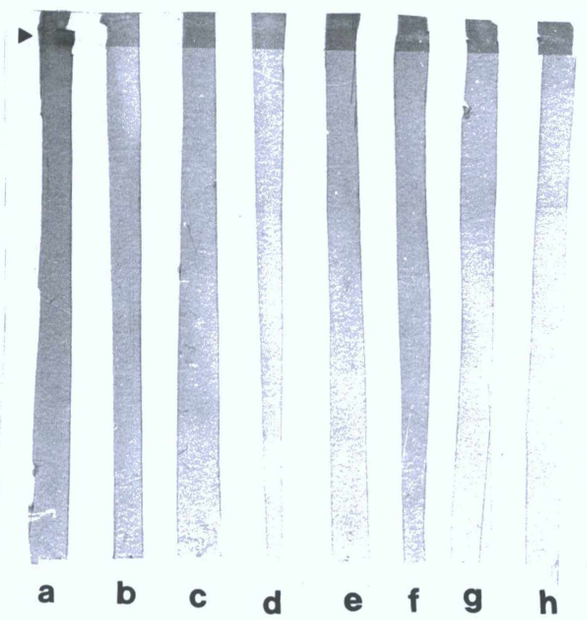
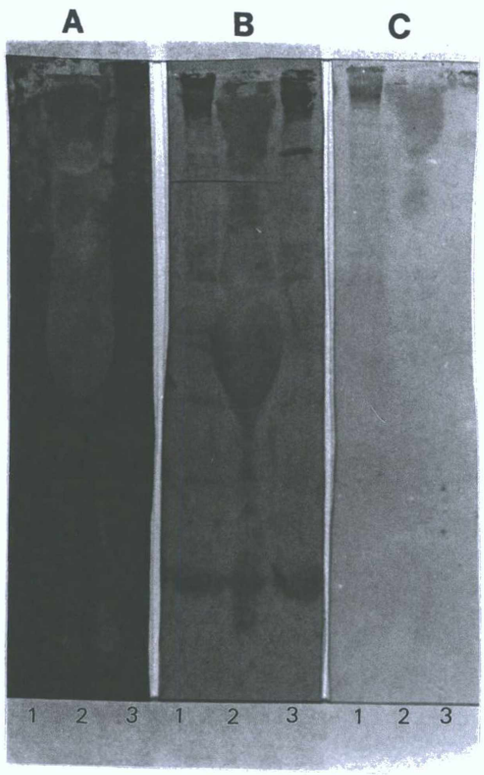

a
b
c
d
e
f
g
h

Fig. 3.3.4a

Eight of the 21 supernatants from fusion 8 which were reacted with nitrocellulose blots of apolipoproteins after 10% polyacrylamide / SDS gel electrophoresis. The blots were detected with 1:500 peroxidase-labelled rabbit-anti-mouse immunoglobulins. a) 5B10, b) 6G10, c) 6E11, d) 6C9, e) 2A6, f) 1F1, g) 1D6, h) 1B10.


A
B
C
1 2 3 1 2 3 1 2 3

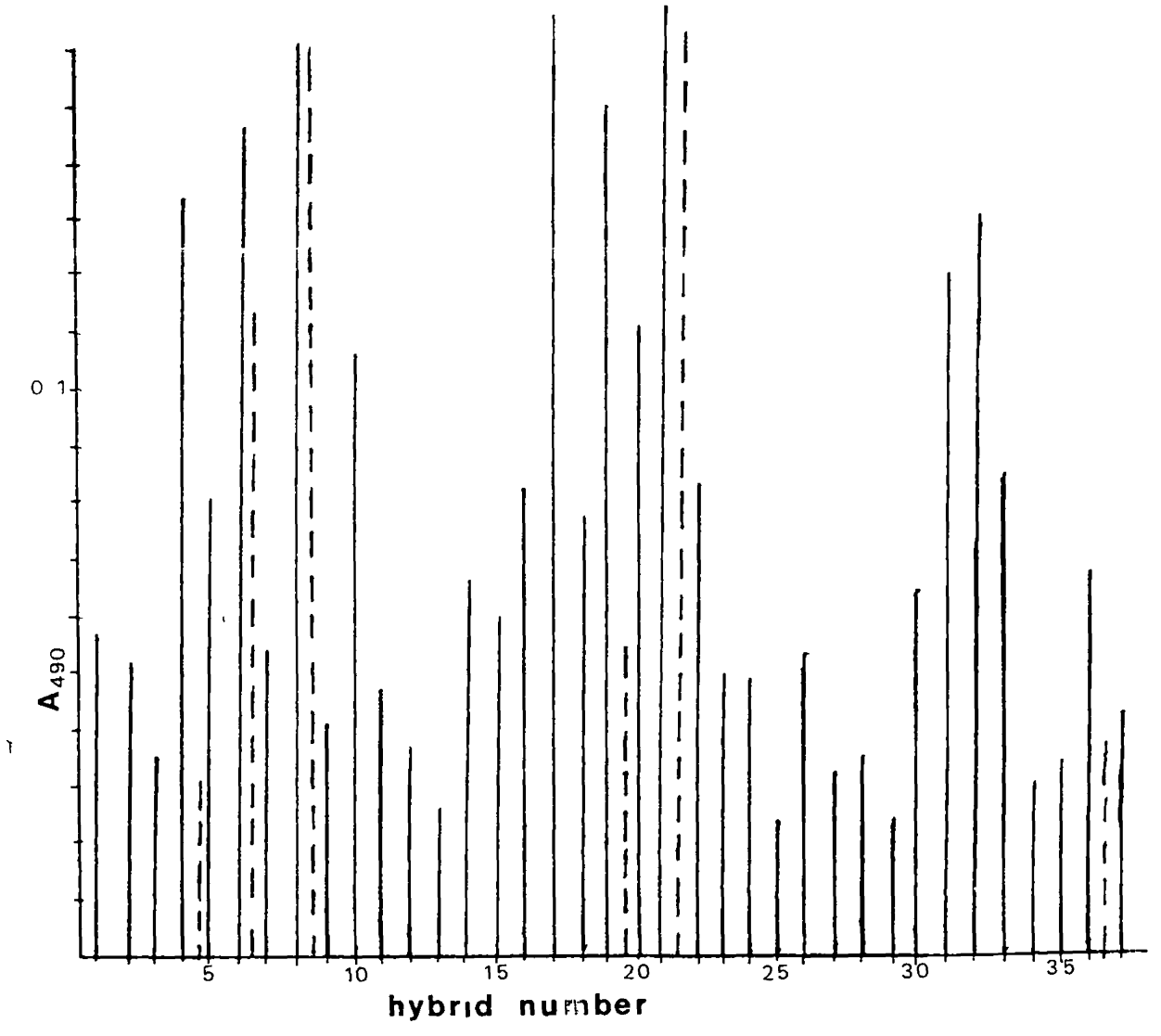

0 1
A_{490}
5
10
15
20
25
30
35
hybrid number

Fig 3 3 4 b

A 10% polyacrylamide /SDS gel of apolipoprotein extract and plasma protein was blotted to nitrocellulose

Lanes 1 and 3 apolipoprotein (2µg)
Lane 2 plasma (diluted 1 100 in PBS)

A Blot treated with goat anti-human alpha-lipoprotein (1 200)
B Blot treated with goat anti-human beta-lipoprotein (1 200)
C Blot treated with monoclonal antibody 5B10 antibody

Blots were detected with peroxidase-labelled swine anti-goat lgG (1 200) (A & B) and peroxidase-labelled rabbit anti-mouse immunoglobulins (C)

Fusions 9 and 10

Figures 3 3 5 and 3 3 6 show the ELISA results for fusions 9 and 10. Eight hybrids from fusion 9 secreted antibodies that were completely free of non-specificity The great number of potentially interesting positive hybridomas, (31 from fusion 10) necessitated a more informative screening procedure, so 5µl volumes of supernatants were dotted to nitrocellulose blots of delipidated lipoproteins at the regions where antigen was bound Fig 3 3 7a is an illustration of a blot dotted with 31 supernatants from fusion 10 Four antibodies, 2B6, 3F12, 3H5 and 4G1 bound the apolipoprotein B band at the top of the blot, and none of the other antibodies could be visualised in this system When these hybridomas were grown up and their supernatants rescreened by immunoblotting 3 antibodies, 2B6, 3F12 and 4G1 gave a strong reaction with apoB (Fig 3 3 8) Twelve supernatants from fusion 9 were also tested by dotting. Only 3 could be visualised on the dot blots and they bound everywhere they were dotted (see Fig 3 3 7b) They were therefore assumed to be non-specific As there was a low hybridoma yield in subsequent fusions, all the cell supernatants were tested by dotting only

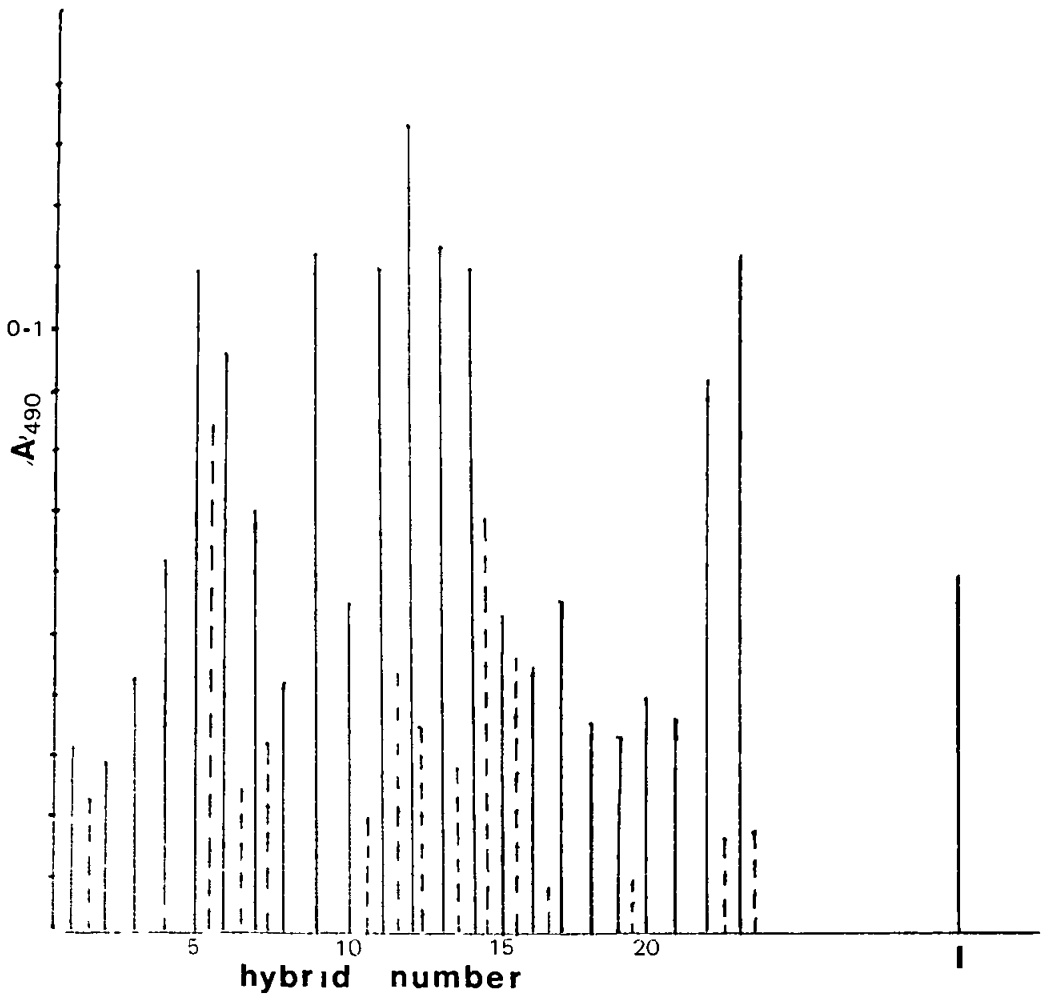

0·1
A_{490}
5
10
15
20
hybrid number

Fig 3 3 5

Hybridoma supernatants from fusion 9 were screened by ELISA Absorbances were read at 490nm Continuous lines = test well Non-continuous lines = control well I = immune mouse serum at 1 1000 dilution

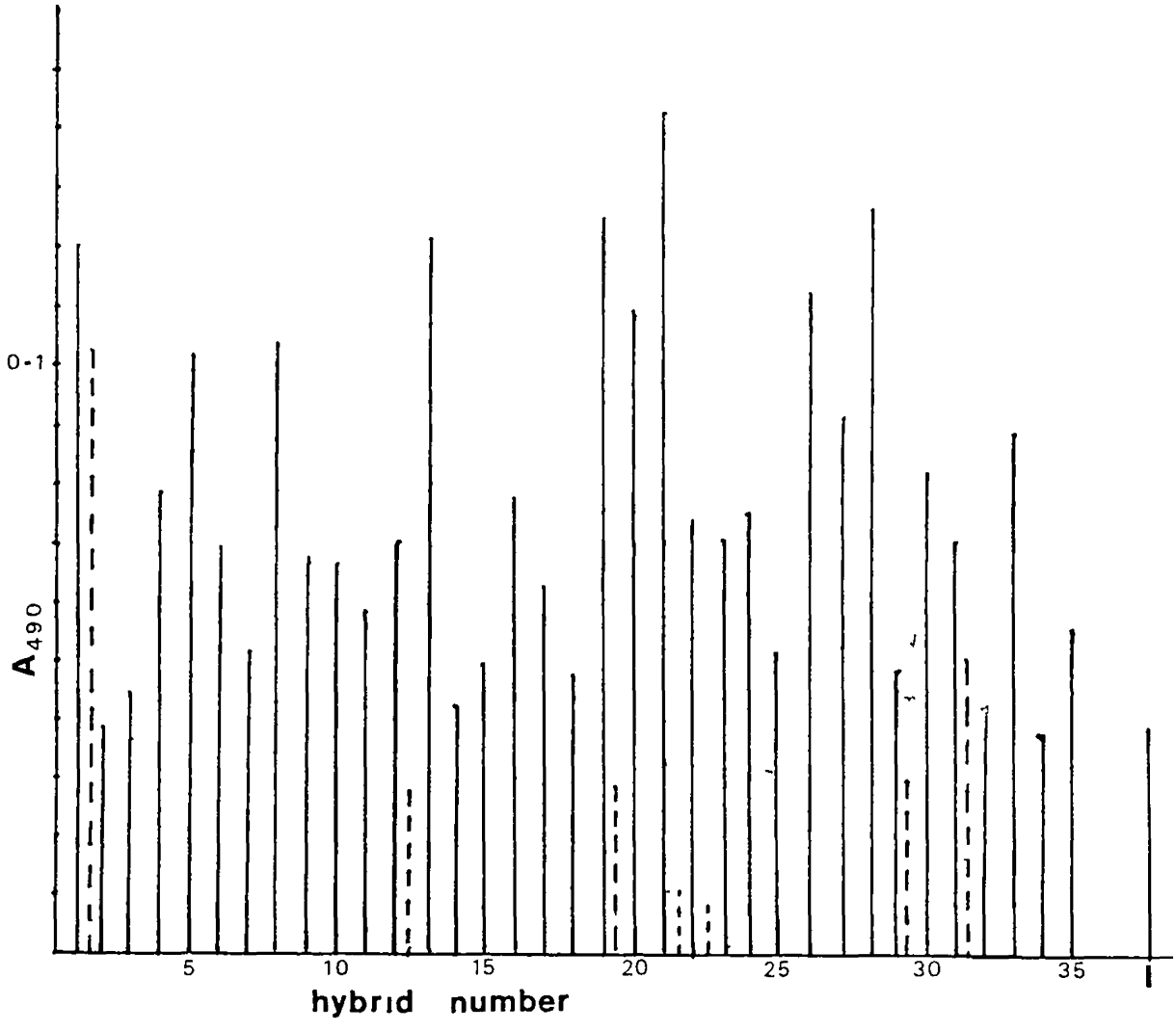

0·1
A_{490}
5
10
15
20
25
30
35
hybrid number

Fig 3 3 6

Hybridoma supernatants from fusion 10 were screened by ELISA Absorbances were read at 490nm Continuous lines = test well. Non-continuous lines = control well I = immune mouse serum at 1 1000 dilution.

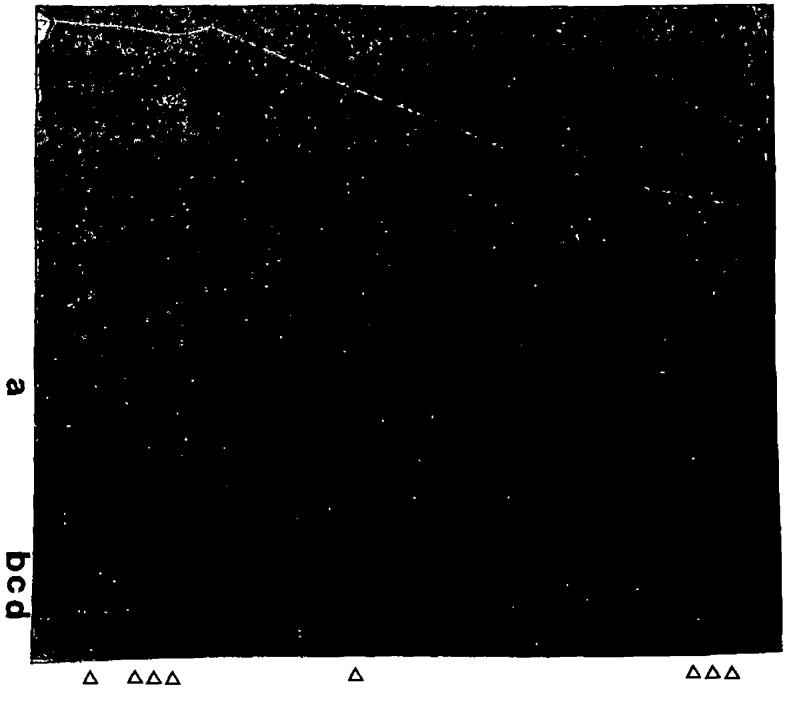

a
bcd

Fig. 3.3 7a.

Thirty one hybridoma supernatants from fusions 9 and 10, which were ELISA-positive, were dotted to nitrocellulose blots of delipidated lipoproteins after electrophoresis in non-denaturing 5% polyacrylamide gels The direction of the gels was from the top of the figure to the bottom 5ul volumes of supernatant were dotted Regions of the blot which were dotted are indicated with arrows and can also be discernable as pale areas on the blot The blot was detected with 1·100 peroxidase-labelled rabbit anti-mouse immunoglobulins. Four antibodies are visible binding apoB at the top of the blot Left to Right. a) 2B6, b) 3F12, c) 3H5, d) 4G1

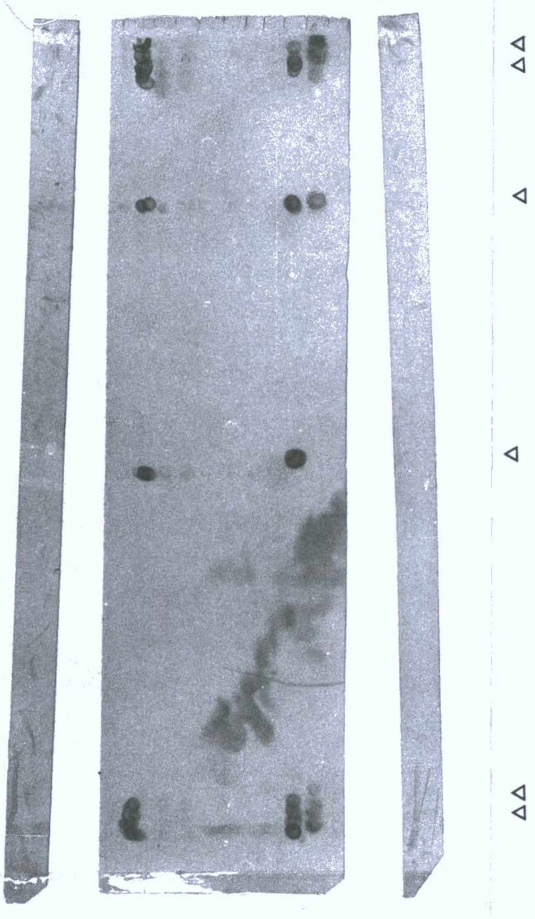

a
bc

Fig 3 3 7b

Twelve hybridoma supernatants from fusion 9 were spotted to nitrocellulose blots of delipidated lipoproteins after electrophoresis in non-denaturing 5% polyacrylamide gels The dotted regions are indicated (◁) The blot was detected with 1·100 peroxidase-labelled rabbit-anti-mouse immunoglobulins. Left to Right· a) antibody 2D6. b) antibody 2E5, c) antibody 2E12

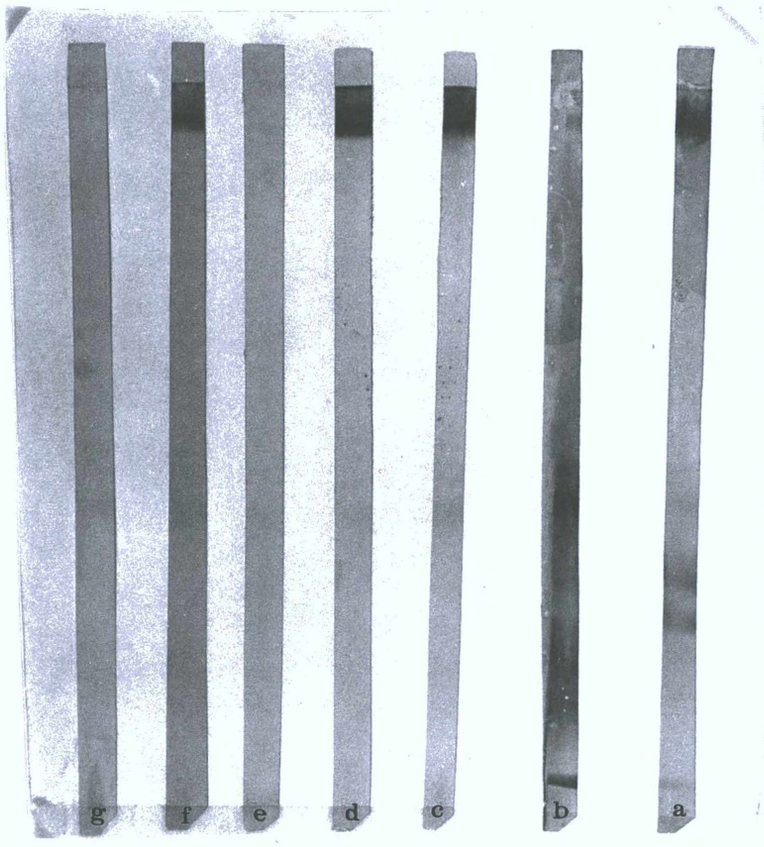

g
f
e
d
c
b
a

Fig 3 3 8

Nitrocellulose blots of apolipoproteins, after electrophoresis in 5% polyacrylamide non-denaturing gels were treated with monoclonal antibodies from fusion 10, which were positive after screening by dotting Polyclonal anti-lipoprotein antibodies were also used

a) 1 200 anti-beta-lipoprotein

b) 1 200 anti-alpha-lipoprotein

c) 2B6

d) 3F12

e) 3H5

f) 4G1

g) 4D5

Bands were detected with 1 500 peroxidase-labelled swine anti-goat IgG (a & b) and 1 20 peroxidase-labelled rabbit-anti-mouse immunoglobulins (c-g)

Fusion 16

Fusion 16 was performed using delipidated HDL_3 as the immunogen, with the view to raising antibodies against apolipoproteins other than apoB. Fig 3 3 9 shows 43 of the 70 hybridoma supernatants that were screened by dotting On the left is an immunoblot of delipidated HDL_3 run on a 5% polyacrylamide non-denaturing gel and detected with anti-alpha-lipoprotein. Under these conditions the proteins have not separated well. Nevertheless the supernatants were dotted to the regions on the undetected blot which corresponded to the protein sites Three supernatants, 1D3, 3H9 and 5F4 bound strongly and with good specificity to a single region on the blot. As they bound with similar pattern, it was concluded that they each recognised the same apolipoprotein 3H9 was grown up to large volumes The supernatant was reacted with nitrocellulose blots of 10% polyacrylamide SDS gel Fig 3 3 10 shows such a blot which was first stained for total protein with amido black, and then immunoblotted with 3H9 supernatant The antibody binds a protein of molecular weight less than 14,100, which runs with the dye front under these conditions

Fig. 3.3.9

Forty three of the 70 hybridomas from fusion 16, which were screened by dotting 5µl of supernatant to nitrocellulose blots of delipidated HDL_3, after non-denaturing electrophoresis in 5% polyacrylamide gels. A portion of the blot, shows on the left of the figure,(●), was reacted with 1:400 anti-alpha-lipoprotein and detected with 1:500 peroxidase-labelled swine anti-goat lgG. Monoclonal antibodies reactive to HDL_3 antigen was detected in the dotted supernatant by treating with 1:100 peroxidase-labelled rabbit anti-mouse lgs.
Positive antibodies: a) 1D3, b) 3H9, c) 5F4.
The direction of electrophoresis was from the top of the figure to the bottom.

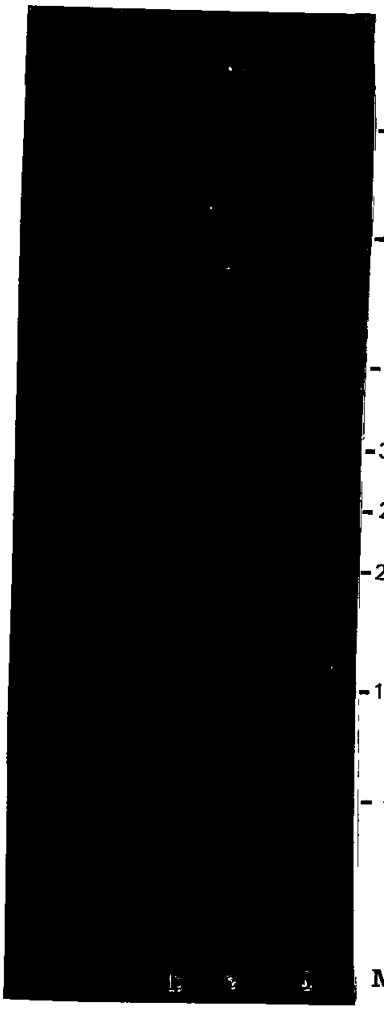

97
66
45
36
29
20
14
◁
$M_r \times 10^{-3}$

Fig 3 3 10

A 10% polyacrylamide / SDS gel of high density lipoprotein was blotted to nitrocellulose The blot was stained with Amido Black B, as described in methods After destaining it was washed 3-4 times in washing buffer before adding supernatant of antibody 3H9 The blot was then detected with 1 100 peroxidase-labelled rabbit-anti-mouse immunoglobulins

a) HDL_3 (reducing buffer), b) molecular weight markers, c) LDL (in reducing buffer), d) HDL_3 (non-reducing buffer) The band detected by 3H9 is indicated with the arrow

3 4 Discussion

3 4 1 Immunisation techniques

Initially 4 bi-weekly injections of delipidated lipoproteins, was chosen because that was what was favoured by Tikkanen et al, (1982), who produced anti-LDL monoclonal antibodies In my experience, however, that process proved lengthy and the fusions resulted in little or no antibody-secreting hybridomas For instance, fusion 4 yielded only 3 hybridomas and although no optical density readings were taken, by visual inspection they were weakly reactive compared to the immune serum

From fusion 5 onwards, the regimen was changed to 3 weekly intraperitoneal injections and it resulted in high yields of productive hybridomas In fusions 8, 9 and 10 many of the monoclonal antibodies bound more strongly than the immune mouse serum, so it appeared that fusions performed after the shorter immunisation procedure were better timed for obtaining rapidly-dividing B-lymphocytes from the spleen

Immunisation schedules vary widely among different workers, (C F Tikkanen et al., (1982), Milne et al.,(1983)) even among groups which have raised antibodies against lipoproteins, so choosing an immunisation schedule that will produce results is very much a matter of experimentation My experience with using a total apolipoprotein fraction as the immunogen was that all the resulting monoclonal antibodies were against

apolipoprotein B even though many other proteins were also present and, judging by the intensity of the stained bands on SDS gels, apoA-1 was present in equal, if not greater, amounts Clearly, apoB is the immunodominant protein under these circumstances When HDL_3 was the immunogen, as in fusions 11 and 12, only a small number of hybridomas resulted, but again, all of them were directed against apoB This protein could not be detected on a stained gel of HDL_3 although a blot of this protein detected with anti-alpha-lipoprotein reveals very faint binding of apoB to the antibody (see figs 4 2 1 and 4 2 3), indicating that trace amounts of apoB are present Only the last fusion resulted in antibodies that were not against apoprotein B and it is noteworthy that the immunisation schedule was altered in this case and, for the first time, no emulsion was used in the last injection

3 4 2 Limitations on screening assays

The ELISA technique that I chose to identify monoclonal antibodies after a fusion was one based on Watt and Watt, (1983), but peroxidase was the choice of enzyme, rather than B-galactosidase as favoured by the authors The specificity and sensitivity of the technique was borne out by the strong reaction of the immune mouse serum and not normal mouse serum, when tested
A visually discernable colour change was a sufficient criterion for positivity, although from fusion 6 on,

optical density readings were taken also When positive antibodies were detected in fusion 4, the hybridoma cells were injected into mice for growth as a ascitic tumour The hybridoma supernatants bound weakly in the ELISA compared with the immune mouse serum and it was hoped that the antibodies in the ascitic fluid would bind more strongly and be suitable for blotting experiments

Ascites fluids from fusion 4, 3C2F8 and 3C2G10 were tested by ELISA at varying dilutions against a fixed amount (5µg/well) of apolipoprotein antigen It was discovered that the amount of antigen bound was limiting the reaction for monoclonal antibody but not polyclonal antibody, so that only enough antibody that would result in an absorbance of 0 25 was allowed to bind (table 3 4) This amount was already present in supernatant and in the 1 1000 dilution of ascites fluid So, for two antibodies from that fusion at least, the antigenic determinant appeared to be present on a minor component of the apolipoprotein mixture, which bound to the ELISA well in very small amounts

It was decided, therefore, to add large amounts of apolipoprotein to SDS gels for blotting in the hope of visualising the antigen A 1·100 dilution of ascites fluid was used Antibody binding was still very faint however Further attempts to visualise monoclonal antibody binding on nitrocellulose blots was unsuccessful until 5B10 from fusion 8 was shown to be reacting with apolipoprotein B on SDS gel blots

For antibodies 3C2F8, 3C2G10 and 1H5G11, the identity of the antigen remained unknown The cells were frozen and further experiments with these antibodies were set aside, while another fusion was carried out in the hope of raising antibodies, which could be more easily detected in SDS gel blots, and therefore identified with regard to their apoprotein antigen The same ELISA method was used as in earlier experiments because of its previous success Unfortunately, at this stage of this project a problem was encountered In fusions 6 and 7, the antibodies bound non-specifically to both antigen-coated and uncoated wells Antibodies raised in fusion 6 were initially apolipoprotein-specific, but just over a month later, the cloned cell lines and the ascites fluids of the same antibodies contained non-specific antibody

In fusion 7, 21 of the 23 antibody-secreting hybridomas that were initially identified were non-specific, with the others reverting to non-specificity or becoming negative before the next screening The reason for this was unknown Fortunately, only 6 non-specific antibodies were identified out of 37, after fusion 8, along with three which became non-specific subsequently

In retrospect, it becomes evident that the percentage of hybridomas secreting non-specific antibody was similar in both fusions 8 and 7 480 hybridomas were screened after fusion 7 and approximately 5% were non-specific, while 183 hybridomas were screened after fusion 8, and only 3-4% were non-specific As 5ug of apolipoprotein were added to

the plates for fusion 8, and only 2 4µg /well for fusion 7, it appears likely that the concentration of the antigen was too low to pick up anything other than non-specific antibody

Immunoblotting was the chosen technique to further characterise the antibodies by identifying their antigen protein However, of 21 supernatants tested, only 5B10 could be visualised reacting with apolipoprotein

Although 6C9 was subsequently shown to be able to bind its SDS-treated antigen after blotting, the proportion of antibodies which still bound after SDS treatment of the antigen was very low One reason for the failure of antibodies to be detected on blots after denaturing gels are used is the irreversible denaturation of the antigenic determinants by the SDS and this phenomenon had been suggested by Burnette, (1981), who stated that it would severely limit screening assays with monoclonal antibodies However, while this appeared to be true in my experience, it was not such a severe problem for other authors who raised anti-apolipoprotein B antibodies Of the seven antibodies of Marcel et al, (1982), four bound strongly to electrophoretic blots of LDL-apoB after SDS gel electrophoresis while under the same conditions the other three bound weakly and were thought to be directed against determinants that were masked by the presence of SDS Curtiss and Edgington, (1982), were able to detect binding of nine of their 11 anti-VLDL or anti-IDL antibodies to apoB, after transfer from SDS gels to

nitrocellulose

One major difference between the method that I used and that of other authors was in the immunisation procedure Also, in my case the immunogen was presented in the partially delipidated form as opposed to using holo-LDL which the other workers used, but it remains unknown whether this had any bearing on the production of monoclonal antibodies which largely recognised antigenic determinants that were sensitive to denaturation, and therefore probably conformational in nature

In any event, electrophoresis in non-denaturing gels followed by nitrocellulose blotting and immunodetection was a successful technique for the characterisation of the monoclonal antibodies of fusion 8 and it enabled the identification of the bound apolipoprotein as apoB

3 4 3 Dot-blotting

While the ELISA technique was successful in detecting anti-apolipoprotein antibodies it was limited in that it could not identify which proteins out of the mixture were specified by the monoclonal antibodies For this, a further screening method had to be devised and while the immunoblotting technique was successful in the case of non-denaturing gels, it required at least 5ml volumes of hybridoma supernatant, which meant that all positive cells identified by the ELISA had to be grown up to larger volumes This obviously took some time, especially with such a large number of hybridomas, and cells could easily become negative for antibody secretion, or succumb to bacterial or fungal contamination at this stage It was also clear that antibodies which initially bound strongly in the ELISA, and were cloned on that basis, were not necessarily the most interesting 5B10 bound less strongly in ELISA's, on both first and second screenings, and was only cloned after it was detected binding to apoB on blots The need for a more rapid and selective screening method became evident A system was needed that would quickly detect positive antibodies and, at the same time, identify the apolipoprotein that was bound by the antibody The method would also determine which antibodies bound strongly to nitrocellulose-immobilised antigens, because the blotting technique was the chosen

approach for monoclonal antibody characterisation and would form the basis of nearly all the ensueing experiments

The dot-blotting technique used after fusions 9 and 10, appeared to fulfill all these requirements Hybridomas were first screened by ELISA and all the positive cell supernatants, (16 from fusion 9 and 31 from fusion 10) were spotted, in 5µl amounts, to the apolipoproteins on nitrocellulose blots of non-denaturing gels Only 4 of the antibodies from fusion 10 were still positive, hence the workload was reduced and potentially interesting antibodies were selected at an early stage

3 4 4 3H9 antibody

A monoclonal antibody against a component of high density lipoprotein was produced after fusion 16 It was one of three antibodies which appeared to be directed against the same apoprotein on blots On 10% SDS gel blots, the antibody, 3H9, binds a low molecular weight protein that runs with the dye front (fig 3 3 10), while on 15% gel blots (not shown) the band stained is very diffuse Low molecular weight proteins of the high density lipoprotein class include apoprotein A-II (Mr = 17,000 (dimeric form)) and the C apoproteins, C-I (Mr = 6500), C-II (Mr = 8800) and C-III (Mr = 8750), which comprise minor components of the HDL fraction Other low molecular weight proteins complexed with HDL include the amyloid related serum protein SAA (Mr = 12,000) (Marhaug et al, 1982) and sterol carrier protein (Mr = 10,000)

The possibility that 3H9 was directed against one of the C-apoproteins appears unlikely because the antibody does not bind VLDL proteins on blots Neither does the protein behave like apoA-II, which would have an apparent molecular weight of 17,000 in non-reducing buffer The identity of the antigen for 3H9 remains unresolved

CHAPTER 4

Immunodetection of apolipoproteins of different density classes

CHAPTER 4

Immunodetection of apolipoproteins of different density classes

Monoclonal antibodies were tested for their reactivity to delipidated lipoproteins, of different density classes, which were separated by electrophoresis under both denaturing and non-denaturing conditions before transfer to nitrocellulose An ELISA was also performed with the lipoprotein fractions, using four monoclonal antibodies from fusion 8 This chapter describes the findings from these experiments

4 1 Immunodetection by ELISA

Preparations of VLDL, LDL, HDL_2, HDL_3 and total lipoprotein were each adjusted to protein concentration of 25ug/ml and 200ul were added to the wells of a 96-well plate

Four monoclonal antibodies from fusion 8, 5B10, 6C9, 6G10A4 and 6E11A5 were allowed to react with the bound lipoproteins in triplicate The absorbance readings are given in table 4 1 1

Antibody	HDL_2	HDL_3	VLDL	LDL	Lipoprotein	Delipidated lipoprotein
5B10	0 064 ±0 018	0 000 ±0 000	0 056 ±0 013	0 122 ±0 007	0 016 ±0 005	0 014 ±0 002
6C9	0 060 ±0 006	0 003 ±0 001	0 024 ±0 009	0 091 ±0 001	0 066 ±0 010	0 016 ±0 002
6G10A4	0 086 ±0 014	0 020 ±0 0005	0 096 ±0 007	0 110 ±0 011	0 057 ±0 015	0 039 ±0 0008
6E11A5	0 035 ±0 002	0 004 ±0 001	0 047 ±0 003	0 088 ±0 002	0 043 ±0 006	0 013 ±0 001
Normal serum	0 002 ±0 001	0 001 ±0 0008	0 001 ±0 000	0 001 ±0 002	0 006 ±0 000	0 004 ±0 0007

Table 4 1 1 ELISA of 4 monoclonal antibodies

Table 4 1 1 Reactivity of monoclonal antibodies with lipoprotein fractions after ELISA 5µg of lipoprotein was added to each well Bound antibody was detected by adding 1.1000 peroxidase-labelled rabbit anti-mouse immunoglobulin The figures are absorbances at 490nm and values represent the mean figure of 3 determinations, ± standard deviation

Normal serum was diluted 1 1000.

Each of the antibodies bound LDL with the greatest reactivity However, they showed differences in their VLDL reactivities Whereas 6G10A4 bound this lipoprotein with almost the same reactivity as it did with LDL, 5B10 and 6E11A5 bound with a reactivity which was approximately half that for LDL, and 6C9 had very low reactivity All the antibodies bound to a delipidated total lipoprotein fraction with very low reactivity and while they bound with low reactivity with HDL_3 they showed intermediate reactivity with HDL_2 This aspect of the results was unexpected

4 2 Immunodetection on nitrocellulose blots of SDS gels of lipoprotein fractions

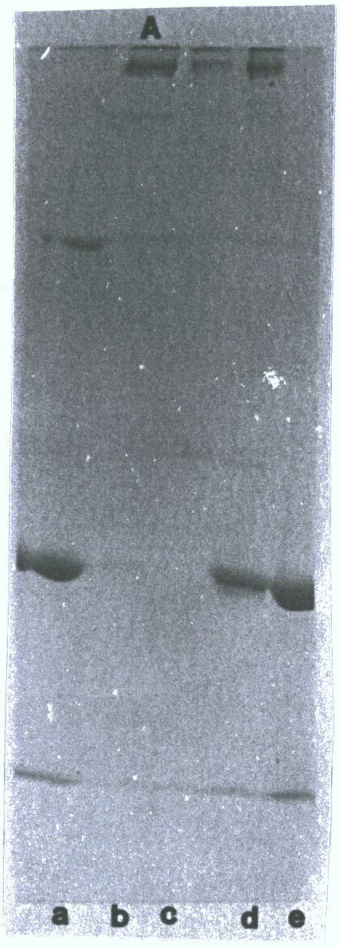
Delipidated lipoproteins of VLDL, LDL, HDL_2, HDL_3 and total lipoprotein were separated on 10% polyacrylamide SDS gels before transfer to nitrocellulose Monoclonal antibodies and polyclonal anti-alpha-lipoprotein and anti-beta-lipoprotein (diluted 1 400 and 1 200 respectively, in blocking solution) were reacted to each of the blotted sets Another blot was stained for total protein with India Ink

Coomassie Blue and silver stained gels are shown in fig 4 2 1 a In the Coomassie stained gel, (fig 4 2 1 a) only bands which correspond in molecular weight to apoprotein

A-1 and apoprotein B can be detected, while the silver stained gels, (figs 4 2 1 B) & C)), reveal several more apolipoprotein bands The total apolipoprotein fraction was prepared without a second ultracentrifugation step and therefore some albumin is visible as a contaminant. The HDL_2 preparation is very severely contaminated with other proteins, while the LDL preparation has some apoA-1 and albumin.

The India Ink-stained blot on fig 4.2 2 shows that the proteins of slower mobility reacted with stain more strongly On the same figure, 4 monoclonal antibodies have been reacted with blots and reveal variations in reactivity to the lipoproteins 5B10 showed the strongest reactivity with the apoprotein B-containing lipoproteins and bound in a different manner to the apoB of the different lipoprotein classes Two other antibodies, 4G1B4 and 3F12D12 bound weakly but with distinct differences in their binding patterns, while 6G10A4 had very little reactivity.

Looking at the apoB as bound by 5B10, it is clear this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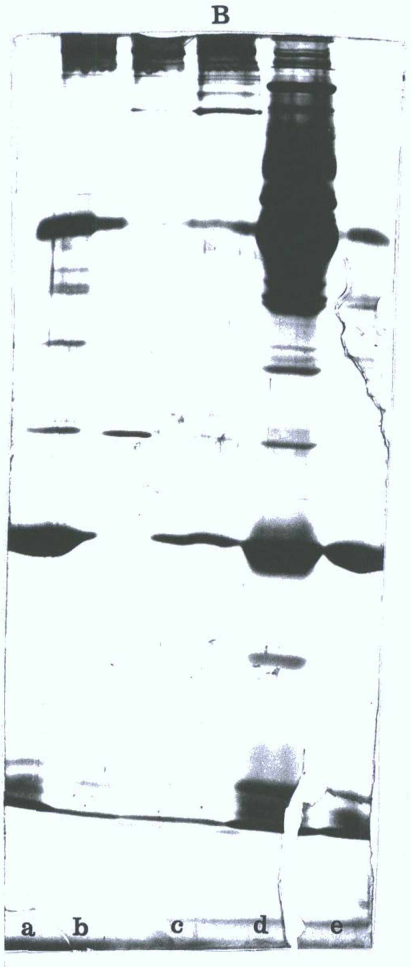
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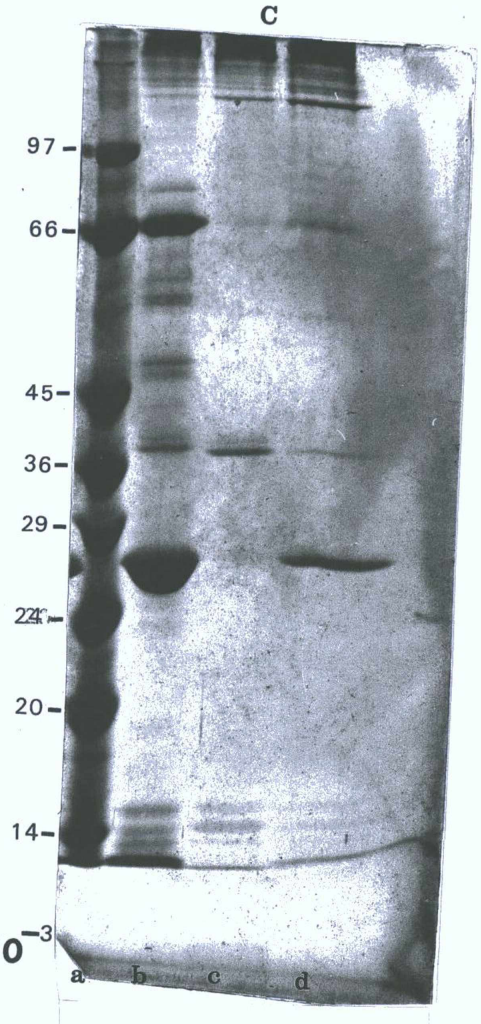

C
97
66
45
36
29
20
14
Mr x 10⁻³
a b c d

Fig 4 2 1

10% polyacrylamide / SDS gels of delipidated lipoprotein fractions were stained with (A) Coomassie Blue, (B & C) silver stain.

Gel A a) HDL_2, b) LDL, c) VLDL, d) total apolipoprotein extract, e) HDL_3.

Gel B Left to right, a) apolipoprotein, b) VLDL, c) LDL, d) HDL_2, e) HDL_3

Gel C Left to right a) Molecular weight standards b) apolipoprotein, c) VLDL, d) LDL


A
B
a b c d e

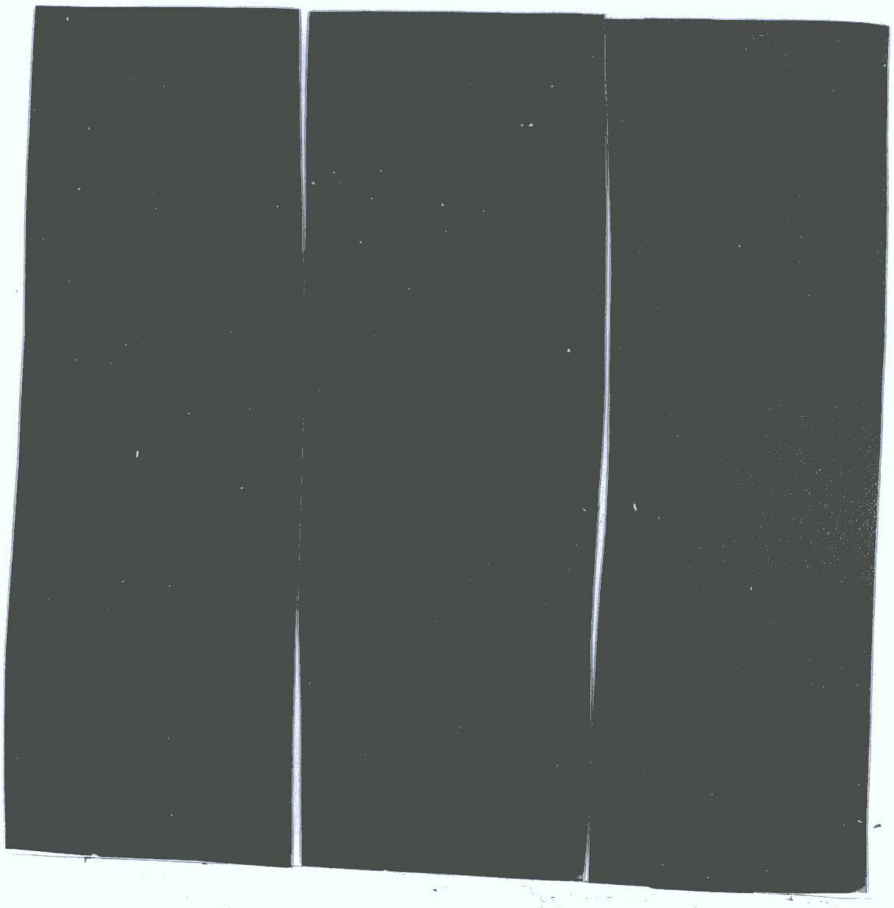


Fig 4 2 2

A SDS / 10% polyacrylamide gel of delipidated lipoprotein fractions was blotted to nitrocellulose A Blot stained with India Ink Left to right a) HDL_3, b) HDL_2, c) LDL, d) VLDL, e) total apolipoprotein Blots B-E were incubated in hybridoma supernatants B) 5B10, C) 6G10A4, D) 4G1B4, E) 3F12D12, and detected with 1 100 peroxidase-labelled rabbit-anti-mouse immunoglobulin a) total apolipoprotein, b) VLDL, c) LDL, d) HDL_2, e) HDL_3

protein migrates under these electrophoretic conditions as a stack of bands which have slightly different patterns in all three lipoprotein fractions. The major difference between the LDL and VLDL lanes and the apolipoprotein lane is the presence of a band of faster mobility in VLDL and LDL which is only barely visible in the apolipoprotein lane This may reflect the difference in isolation procedures for the different lipoproteins or it may be a result of differences in storage times between VLDL and LDL and the apolipoprotein fraction

Within VLDL and LDL the band exhibits a different pattern· there is a weak and strong doublet for LDL while for VLDL a continuous thick band is seen The reason for this is unknown, nor is it possible to say which apoB species is represented by the band

In Fig 4 2 3a polyclonal anti-alpha lipoprotein and anti beta-lipoprotein have been reacted to blots, while in fig 4 2 3b, 7 monoclonal antibodies' reactivities are shown In this case the supernatants were collected from cells after continuously passaging the cells over a period of several months All the collected supernatants for each antibody were then combined and thimerosal was added as preservative to a final concentration of 0 1% (w/v). The pooled supernatants were aliquoted into 25ml volumes and stored at $4^{o}C$ They were still active after several months.

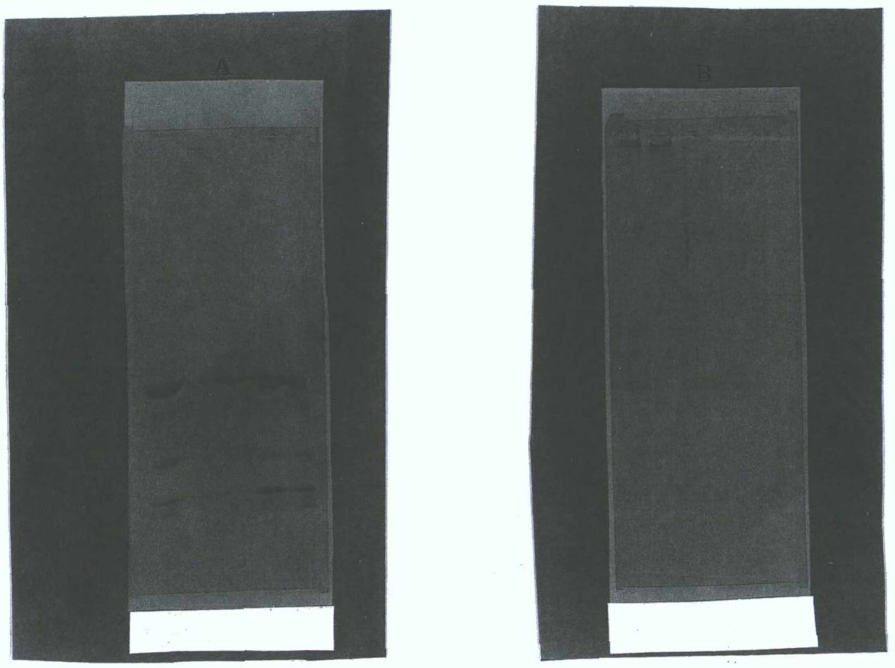

A
B

Fig 4 2 3a.

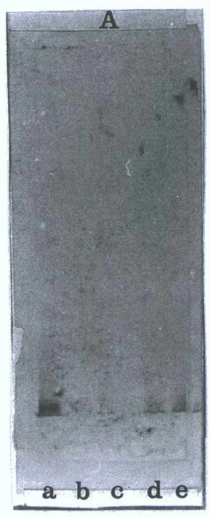
An SDS/10% polyacrylamide gel of delipidated lipoprotein fractions was blotted to nitrocellulose

A Blot incubated in 1:200 anti-alpha-lipoprotein

B Blot incubated in 1·200 anti-beta-lipoprotein

a) total apolipoprotein b) VLDL c) LDL d) HDL_2 e) HDL_3

Bands were detected with 1:500 peroxidase-labelled swine-anti-goat IgG


A
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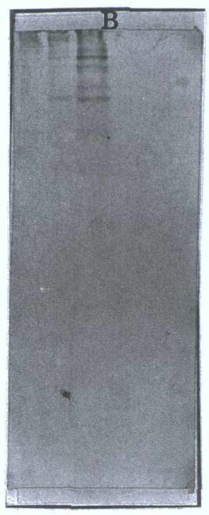
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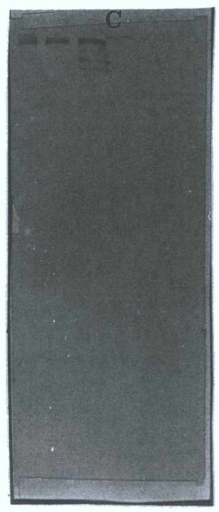
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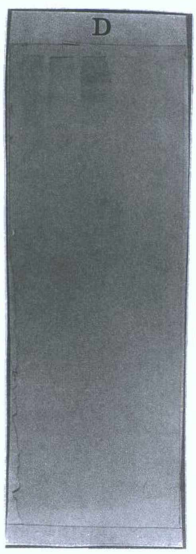
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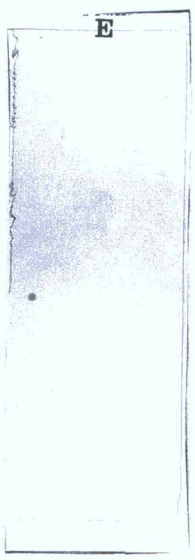

E


F

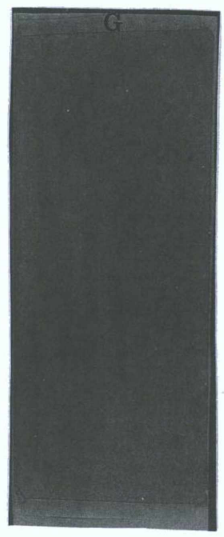

G

Fig 4 2 3b

An SDS/10% polyacrylamide gel of delipidated lipoprotein fractions was blotted to nitrocellulose Blots were incubated in hybridoma supernatants

Blot A, 3H9

Blot B, 5B10

Blot C, 6C9

Blot D, 4G1B4

Blot E, 2B6A12

Blot F, 3F12D12

Blot G, 6G10A4

Left to right a) total apolipoprotein, b) VLDL, c) LDL, d) HDL_2, e) HDL_3

Blots were detected with 1 100 peroxidase labelled rabbit-anti-mouse immunoglobulins

Looking at the polyclonal antibodies first, anti-beta-lipoprotein binds weakly to the upper band of apoB in LDL and apolipoprotein while a thin band of slightly faster mobility can be seen in the LDL lane VLDL was not run on this portion of the gel Background staining of albumin and apoA-1 are also visible HDL_2, HDL_3 and total apolipoprotein is strongly detected by anti alpha-lipoprotein, which also binds other proteins of lower molecular weight These will probably include the C-apoproteins and the monomeric form of apoA-11 though if the sample was incompletely reduced the dimer may also be present

The monoclonal anti-apoB antibodies (fig 4 2 3b) can be divided into two groups 2B6A12, 3F12D12 and 6G10A4 have little or no reactivity at these concentrations of apoprotein while 4G1B4, 5B10 and 6C9 exhibit stronger and more visible binding The binding patterns differ within these three antibodies 6C9 binds the thin band at the top of each lane and the faster LDL band in a manner which is similar to the pattern of the anti-beta-lipoprotein binding, while 5B10 binds this band less strongly and the faster LDL band with greater reactivity Other bands further down the gel are also visible and overall it is the LDL which exhibits the strongest reactivity for this antibody 4G1B4 binds to every band with equal intensity and more faintly than the other two antibodies 3H9 was raised against a delipidated HDL_3 preparation and binds a protein of very low molecular weight in HDL_2, HDL_3 and

more strongly in total apolipoprotein
If a molecular weight of 550,000 is assumed for apoB, then under these electrophoretic conditions apoB-100, and B-74 of LDL, as well as apoB-48 of VLDL are present in the thick band of lowest mobility at the top of the apolipoprotein, VLDL and LDL lanes The molecular weight of the band of faster mobility that is most heavily stained in the LDL lane has been measured at 136,000, which approximates apoB-26 It is not possible under this system to differentiate antibodies against apoB-100 from those against apoB-48.

4.3 Immunodetection on nitrocellulose blots of non-denaturing gels of apolipoproteins

Non-denaturing gels were run according to the method of Davis (1964) with 5% acrylamide and the apolipoprotein fractions added in the same order as for the SDS gels It is clear that this system does not separate apoproteins well, particularly high density lipoprotein apoproteins Fig. 4 3 1 is a silver stained gel of the apolipoprotein fractions, while fig 4 3 1b shows the binding of the polyclonal anti-alpha and anti-beta-lipoprotein to the nitrocellulose replicas.
Little information can be got from the blot treated with anti-alpha-lipoprotein while the anti-beta-lipoprotein reacted with the apoB of VLDL, LDL and apolipoprotein with similar patterns and with equal intensity

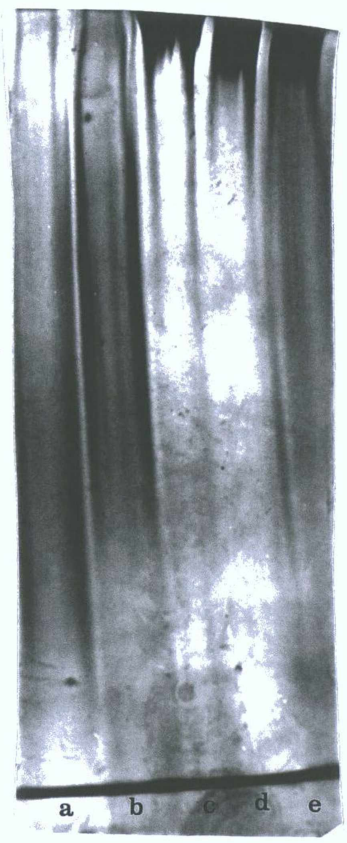

a
b
c
d
e

Fig. 4.3.1a

A 5% polyacrylamide / non-denaturing gel of delipidated lipoprotein fractions was silver stained. Left to right: a) HDL_3, b) HDL_2, c) LDL, d) VLDL e)total apolipoprotein.

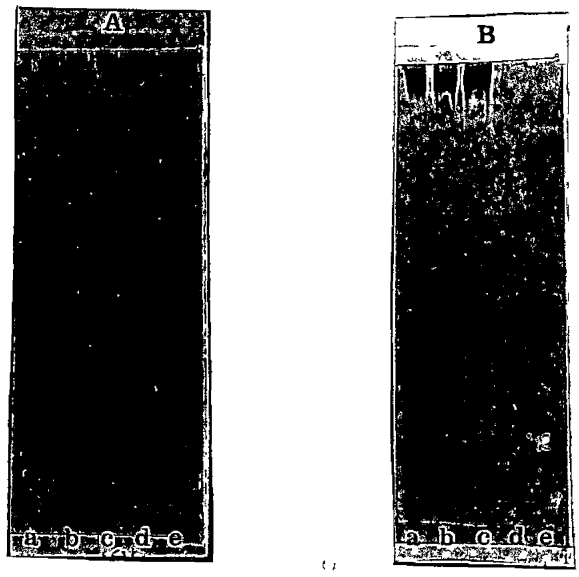
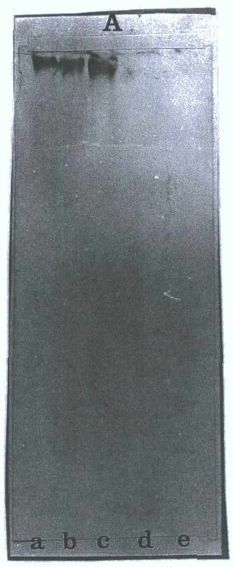

A
a b c d e
B
a b c d e

Fig 4 3 1b

A 5% polyacrylamide / non-denaturing gel of delipidated lipoprotein fractions was blotted to nitrocellulose Blots were incubated in, A 1 200 anti-alpha-lipoprotein B 1 200 anti-beta-lipoprotein and detected with 1 500 peroxidase-labelled swine-anti-goat IgG

Left to right a) total apolipoprotein, b) VLDL, c) LDL, d) HDL_2, e) HDL_3

Fig 4 3.1c illustrates the reaction of the monoclonal antibodies with the replicas. 3H9 is an antibody against a HDL apoprotein and it binds in a pattern which is similar, though more intense, to the anti-alpha-lipoprotein blot The antibody which binds the most strongly to the apoB in this system is 3F12D12 4G1B4 also detects HDL apoprotein as evidenced by its fainter binding to these proteins It also binds to LDL more strongly than VLDL or apolipoprotein On the other hand 6G10A4 appears to bind the apolipoprotein B with slightly more reactivity than it has for the apoB of LDL or VLDL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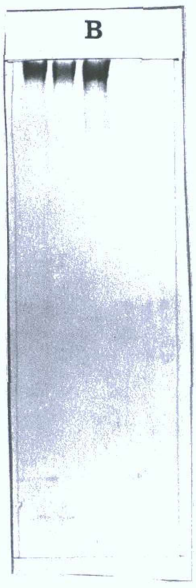
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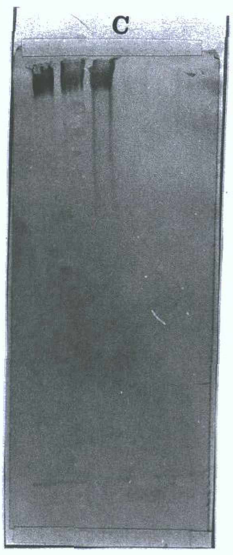
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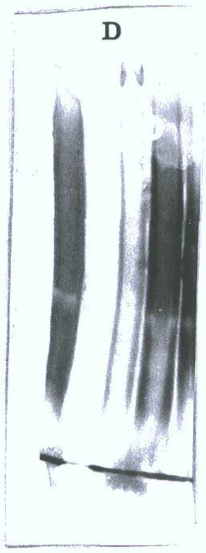
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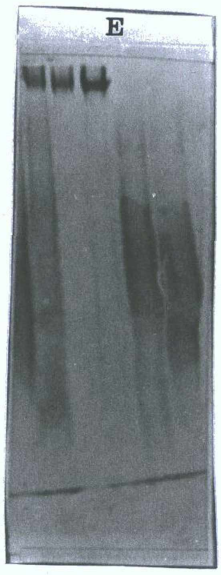
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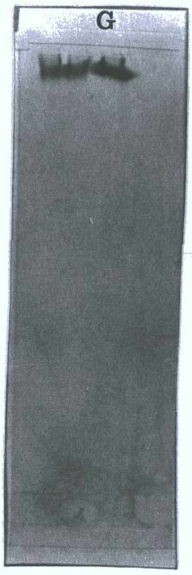

G

Fig 4 3.1c

A non-denaturing / 5% polyacrylamide gel of delipidated lipoprotein fractions was blotted to nitrocellulose.

Blots were incubated in hybridoma supernatant

Blot A. 2B6A12

Blot B 3F12D12.

Blot C 6G10A4

Blot D. 3H9

Blot E 4G1B4

Blot F 5B10

Blot G 6C9

Left to right. a) total apolipoprotein, b) VLDL, c) LDL, d) HDL_2, e) HDL_3

Blots were detected with 1 100 peroxidase-labelled rabbit anti-mouse immunoglobulins

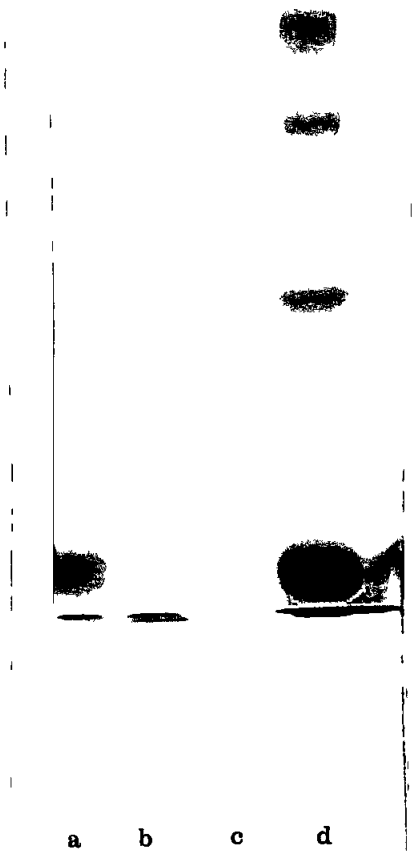

a
b
c
d

Fig 4 4 1a

A 3 58% polyacrylamide / non-denaturing gel of lipoproteins and plasma proteins was stained with Coomassie blue Left to right a) total lipoprotein b) HDL_3 c) LDL d) plasma sample

non-denaturing gels of native lipoproteins.

Figure 4.4.1 shows a photograph of the Coomassie blue stained gel, on which was run freshly obtained plasma, LDL, HDL_3 and the apolipoprotein preparation The albumin band is the thickest and heaviest and alphalipoprotein in this system has the fastest mobility and migrates as a thin, sharp line. It is the only band visible in the HDL_3 lane, while LDL is only barely visible alongside the plasma's beta-lipoprotein band. Anti alpha-lipoprotein (fig 4 4 1b) has stained the alpha-lipoprotein band the most strongly but the other bands in every lane are also plainly visible. Anti-beta lipoprotein has reacted very strongly in this system (fig.4.4 1b) while as for the monoclonal antibodies 3H9 binds very strongly also The anti-apolipoprotein B antibodies can be divided into three groups with regard to their reactivities 2B6A12, 3F12D12 and 6G10A4 bind strongly and beta-liporotein can be distinguished as a band in the plasma lane. 6C9 and 5B10 antibodies react more faintly and bind to the beta-lipoprotein in the plasma lane as a continuous smear 4G1B4 also binds quite faintly but beta-lipoprotein is more distinctly detected by this antibody than by 6C9 and 5B10. Alpha-lipoprotein was also detected by this antibody, which was the case in the Davis gels.
Another point of potential interest is that antibodies 4G1B4, 6C9 and 6G10A4 cross react weakly to the alpha-lipoprotein band The other antibodies do not

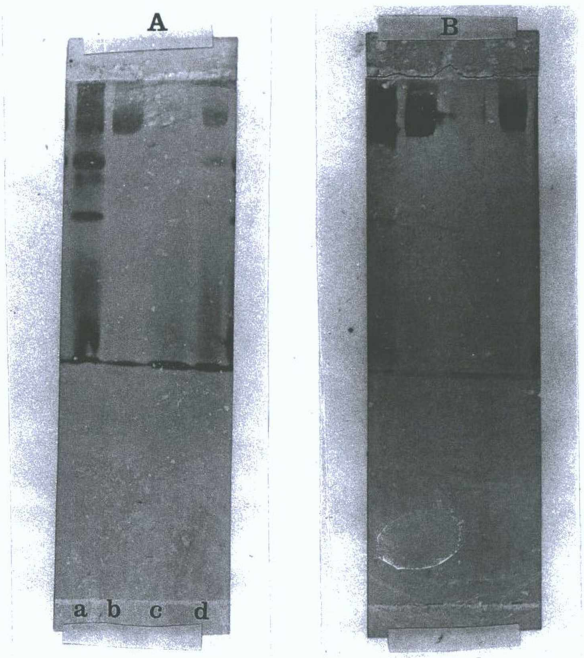

A
B
a b c d

Fig 4.4 1b

A 3 58% polyacrylamide gel of native lipoproteins and plasma was capillary blotted to nitrocellulose. Blots were incubated in A 1·200 Anti-alpha-lipoprotein, B. 1 200 anti-beta-lipoprotein and detected with 1·500 peroxidase-labelled swine-anti-goat lgG

Left to Right a) plasma b) LDL c) HDL_3 d) total lipoprotein

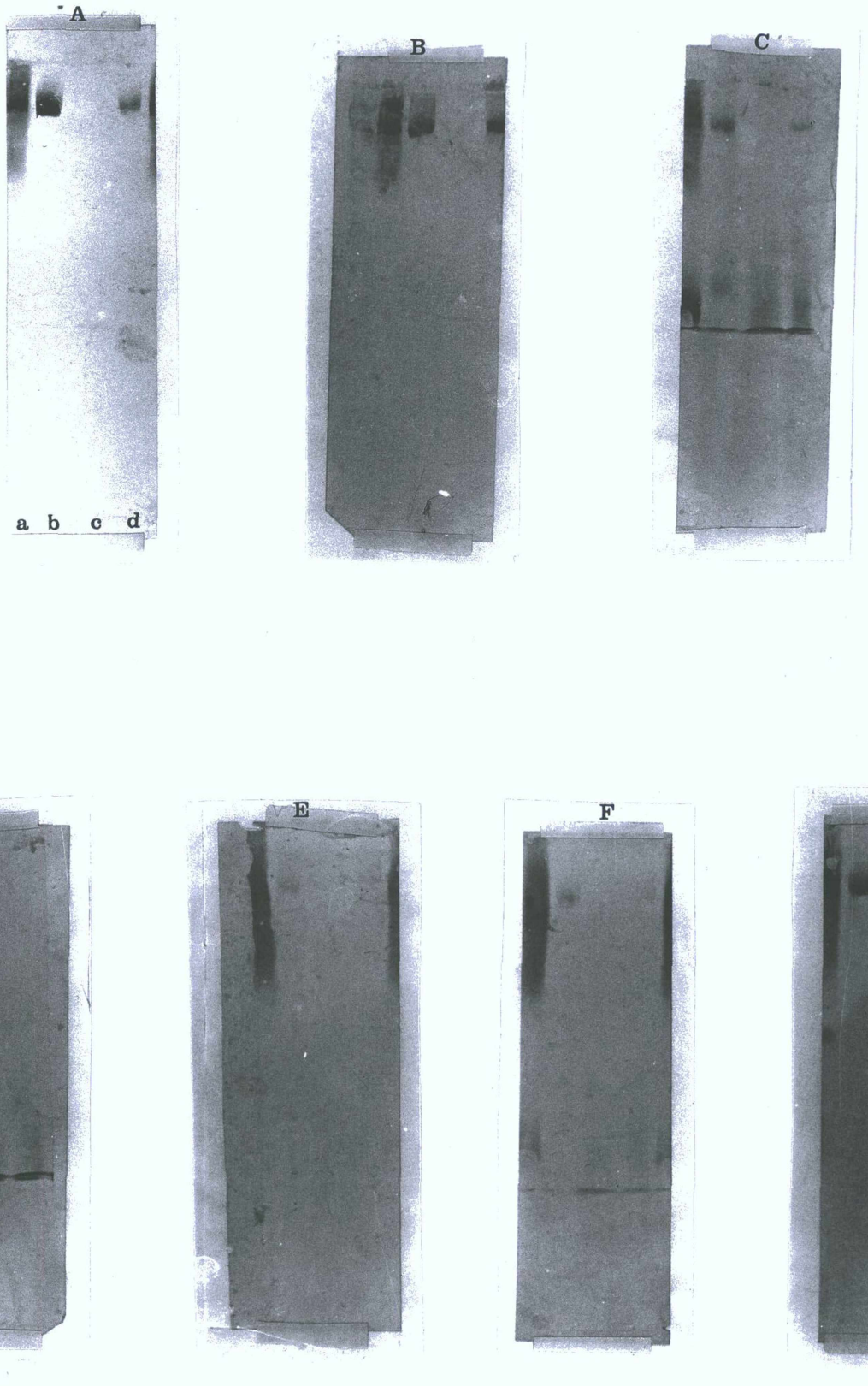

A
a b c d
B
C
D
E
F
G

Fig 4 4 1c

A 3 58% polyacrylamide (non-denaturing gel of lipoproteins and plasma proteins was capillary blotted to nitrocellulose

Blots were incubated in hybridoma supernatants Blot A 2B6A12, Blot B, 3F12D12, Blot C 4G1B4 Blot D 3H9, Blot E 5B10, Blot F 6C9, Blot G 6G10A4 a) plasma, b) LDL, c) HDL_3 d) total apolipoprotein

Blots were detected with 1 100 peroxidase-labelled rabbit anti-mouse immunoglobulins

4 5 Discussion

Immunodetection experiments with monoclonal antibodies were carried out to gain some insight into the properties of the antigenic determinants and to compare their expression, i) among the different lipoprotein classes and ii) in different electrophoretic systems Looking at the reactivities of the polyclonal antisera to begin with, many of the determinants on the apolipoprotein B molecule appear to be removed by boiling in SDS, as shown by the fact that the antiserum binds more faintly to this blot than to the replica of the Davis gel The antibody also bound very strongly on the native gel blot The same is not true for anti-alpha-lipoprotein, which is directed against a range of antigenic determinants distributed over different proteins These are equally antigenic on SDS gel blots as on non-denaturing gel blots

It cannot be ruled out, however, that the apoB molecule in the SDS gel was perhaps less efficiently transferred on to the nitrocellulose than the smaller HDL proteins and than its counterpart on the non-denaturing gels This also would account for the weaker reactivity on SDS gels

The immunodetection experiments performed with SDS gels with all seven monoclonal antibodies and the two polyclonal antisera was a more complete experiment than that performed earlier with only 4 of the monoclonal antibodies The results, however, are more disappointing in that overall the bands stained up more faintly than

previously, particularly for antibody 5B10, and more information on the antibodies specificity could be gained from the earlier experiment

For the monoclonal anti-apoB antibodies, 5B10, 6C9 and 4G1B4 are reactive to determinants that resist boiling in detergent, while for the other three, the determinant is abolished Little difference is seen in the reactivity of the antibodies to apoB on a Davis gel

5B10 and 6C9, however, are less reactive to LDL when it is presented in a native form, as on the 3 58% polyacrylamide gels They perhaps recognise a determinant which becomes more accessible according as LDL is delipidated and subjected to the changes in conformation brought about by boiling in detergent The epitopes for 2B6A12, 3F12D12 and 6G10A4 are sensitive to denaturation, but are presented fully in native lipoproteins indicating that they possibly require the presence of lipid to maintain antigenic structure The partial delipidation in diethylether before electrophoresis in a Davis gel is insufficient to remove this lipid 4G1B4 appears to specify a determinant which is also present on a small portion of alpha lipoprotein and which is stable to detergent treatment The altered patterns of reactivity of 6C9 and 5B10 on SDS gels suggests that the two antibodies are not directed against the same epitope More evidence for this comes from the ELISA While some aspects of the results are unexplained, the differences in the reactivities of the antibodies to VLDL are interesting

in that the determinant for 6C9 is either not present on VLDL apoB or else is inaccessible to the antibody, possibly because lipid molecules obscure the epitope The determinants for 5B10 and 6E11A5 are either present on only half the VLDL molecules presented or else appear on all the molecule but are presented in such a way that the antibodies bind with only half the affinity 6G10A4 recognises a determinant which is almost equally expressed on both VLDL and LDL

Chapter 5

Modification of antigenic determinants on apolipoprotein B

This chapter describes findings from experiments in which apolipoproteins of a total lipoprotein fraction or of low density lipoprotein were separated on non-denaturing (Davis) gels, blotted to nitrocellulose, and then treated with various chemical / enzymic reagents before reaction with six anti-apolipoprotein B monoclonal antibodies The objective was to determine whether such modifications would, through altered immunoreactivities, yield any information on the molecular nature of the epitope, for instance whether the epitope was contained on the peptide, carbohydrate or lipid regions of the molecule

For this reason, lipase and neuraminidase were included among the enzymes, as well as trypsin, pepsin and proteinase K, and blots were also treated with organic solvent before addition of antibody, to assess the lipid dependence of the epitope

When the antigen is immobilised on a solid surface, such as a nitrocellulose blot, the subsequent modification reaction procedure can be performed more rapidly, because at the end of the incubation, unreacted agent can be poured away and the blot washed.

The effects of epitope modification on the subsequent immunoreactivities of polyclonal anti-alpha- and anti-beta-lipoprotein were also studied

5 1 Immunodetection on solvent treated blots

In fig 5 1 1 a blot of apolipoproteins was treated with hexane isopropanol (3 2), before adding monoclonal and polyclonal antibody The aim was to test the sensitivity of the epitope, for each of the monoclonal antibodies, to this treatment

The effect of the solvent on the nitrocellulose was to cause a slight discolouration With regard to the binding of the polyclonal antibodies only a slight reduction in reactivity could be seen, while the monoclonal antibodies display differences in their reactivities in that the solvent has almost completely prevented binding of 5B10, 6C9 and 6G10A4, and slightly lowered the 4G1B4 binding, while 2B6A12 and 3F12D12 binding is unaffected

5 2 Immunodetection on enzyme-treated blots

In fig 5 2 1 1 LDL apoprotein, after non-denaturing gel electrophoresis and blotting to nitrocellulose, was treated with varying dilutions of trypsin before reaction with the monoclonal and polyclonal antibodies At a concentration of 1mg/ml, trypsin removed the epitope for 5B10 and for the polyclonal antibodies, while the epitopes for the remaining five monoclonal antibodies were unaffected.

Several monoclonal antibodies from fusion 8 were also reacted with nitrocellulose-immobilised apo LDL after

electrophoresis in 10% polyacrylamide gels In each case the blot was treated with a range of enzymes, including trypsin at 1mg/ml, 0 1mg/ml and 0 01mg/ml, collagenase, beta-galactosidase and pepsin In fig 5 2 1 2 6C9 and 6G10A4 reactivities to the LDL apoprotein after these treatments are shown It can be seen here that the epitope for 6G10A4 is sensitive to trypsin at an enzyme concentration of 0 1mg/ml, whereas 6C9 binds an epitope which resists trypsinolysis at this concentration 5B10 also bound a trypsin-sensitive epitope (not shown) This conflicts with the findings shown in fig 5 2 1, in which 6G10A4 bound an epitope which was resistant to trypsin treatment On repetition of the experiment described in fig 5 2 1 5B10 was consistent in its reaction with a trypsin-sensitive epitope while 6G10A4 was still binding a trypsin-resistant determinant It was therefore concluded that only 5B10 bound an epitope which was consistently sensitive to trypsin treatment

5 2 2 Pepsin treatment

The effect of pepsin treatment on the immunoreactivities of the determinants for the six monoclonal and two polyclonal antibodies are illustrated in fig 5 2 2 The determinant for 6G10A4 was completely abolished by this treatment, while those for 6C9 and 4G1B4 were partially affected The other three monoclonal antibodies bind with slightly lowered reactivity.

5 2 3 Proteinase K treatment

Fig 5 2 3 shows the effect of Proteinase K treatment on the subsequent immunoreactivities of the monoclonal antibodies All of the antigenic determinants for the range of antibodies tested, were abolished at an enzyme concentration of 1mg/ml Polyclonal anti-beta-lipoprotein was also tested, but it was not inhibited from binding at any of the enzyme concentrations tested

5 2 4 Lipase and neuraminidase treatment

Neither neuraminidase nor lipase treatment of the apolipoprotein antigen affected the immunoreactivities of any of the antibodies (fig 5 2 4 and fig 5 2 5) to any great extent, although slightly reduced binding by antibodies 2B6A12 and 3F12D12 was evident after both enzyme additions
These findings, along with the results from the experiments with the proteolytic enzymes, provide evidence, but not conclusive proof, that the chemical structure of the epitopes for the monoclonal antibodies came from the polypeptide part of the antigen

5 3 Immunodetection on blots treated with chemical agents

5 3 1 Reductive methylation

Blots were reacted with sodium borohydride and formaldehyde in order to reductively methylate the E-amino groups of lysine residues on the apoproteins The

subsequent immunoreactivities of monoclonal and polyclonal antibodies are shown in fig 5 3.1. The effect of this treatment has been to abolish the determinants for antibodies 5B10, 6C9 and 6G10A4, while leaving those for the other antibodies still intact

5 3 2 Alkylation

Alkylation of the immobilised apoprotein B with iodoacetamide was performed prior to incubation with six monoclonal antibodies, but had no effect on any of their determinants (Fig. 5 3 2)

5.3 3 Periodate oxidation.

Periodate oxidation was performed with blots of total apolipoproteins before reaction with monoclonal and polyclonal antibodies The 6C9 supernatant contained no reactive antibody in this instance, since no detection was seen in the control blot The determinants for the rest of the antibodies were unaffected by the treatment, again suggesting that the determinants were not carbohydrate (Fig. 5 3 3)

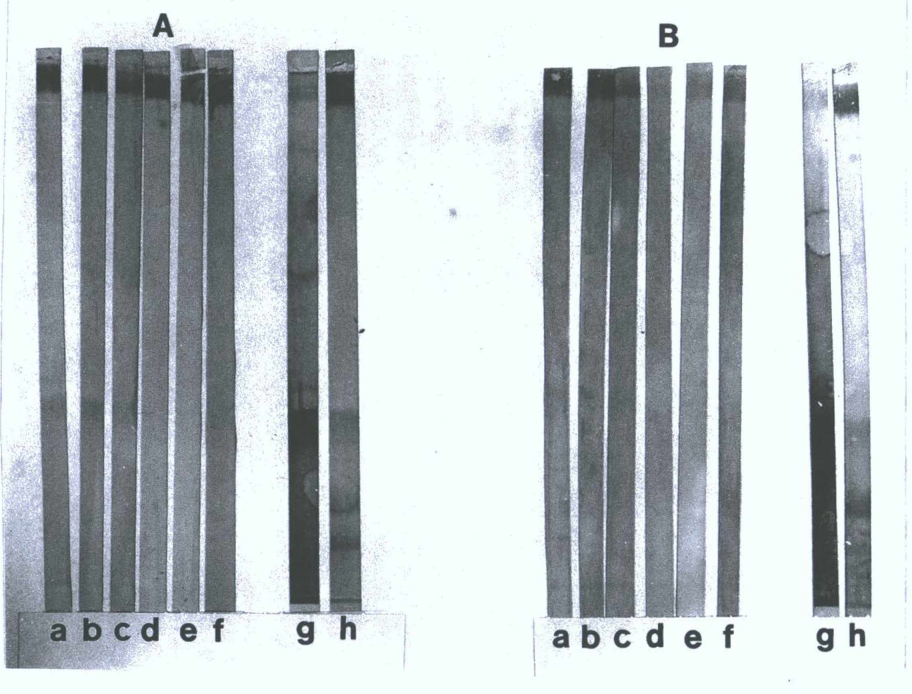

A
B
a b c d e f g h
a b c d e f g h

Fig. 5.1.1

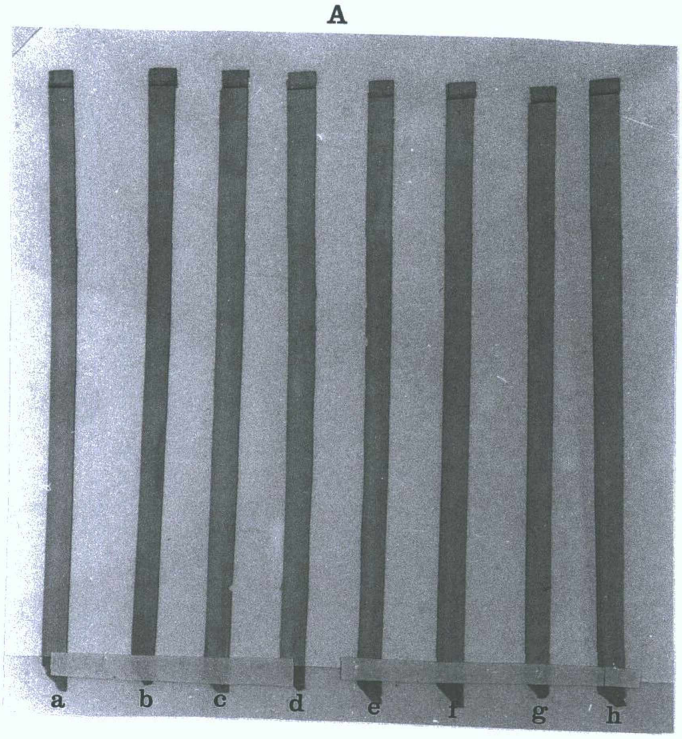
A non-denaturing 15% polyacrylamide gel of apolipoprotein extract was blotted to nitrocellulose. A portion of the blot was incubated in hexane / isopropanol : (3:2; v/v) for 1 hour at RT. Panel A; control strips. Panel B; treated strips. Blots were incubated in : a) 2B6A12, b) 3F12D12, c) 4G1B4, d) 5B10, e) 6C9, f) 6G10A4, g) 1:400 anti-alpha-lipoprotein, h) 1.200 anti-beta-lipoprotein. Bands were detected with 1:100 peroxidase labelled rabbit-anti-mouse immunoglobulins (a-f) and 1:500 peroxidase-labelled swine-anti-goat lgG (g,h).

F
C
G
H

Fig 5 2.1.1

A non-denaturing / 5% polyacrylamide gel of delipidated low density-lipoprotein was blotted to nitrocellulose The blot was cut into 5mm strips and treated with trypsin at different concentrations. a) control strip, b) 1mg/ml, c) 0 1mg/ml, d) 0.01mg/ml, e) 0 001mg/ml, f) 0 5mg/ml plus 0.5mg/ml Soybean Trypsin inhibitor Blots were then incubated in: A. 1 200 anti-alpha-lipoprotein, B. 1 200 anti-beta-lipoprotein, C 2B6A12, D 3F12D12, E 4G1B4, F 5B10, G. 6C9, H 6G10A4 Bands were detected with 1·500 peroxidase-labelled swine-anti-goat IgG (A, B) and 1 100 peroxidase-labelled rabbit-anti-mouse immunoglobulins (C-H)

A



B

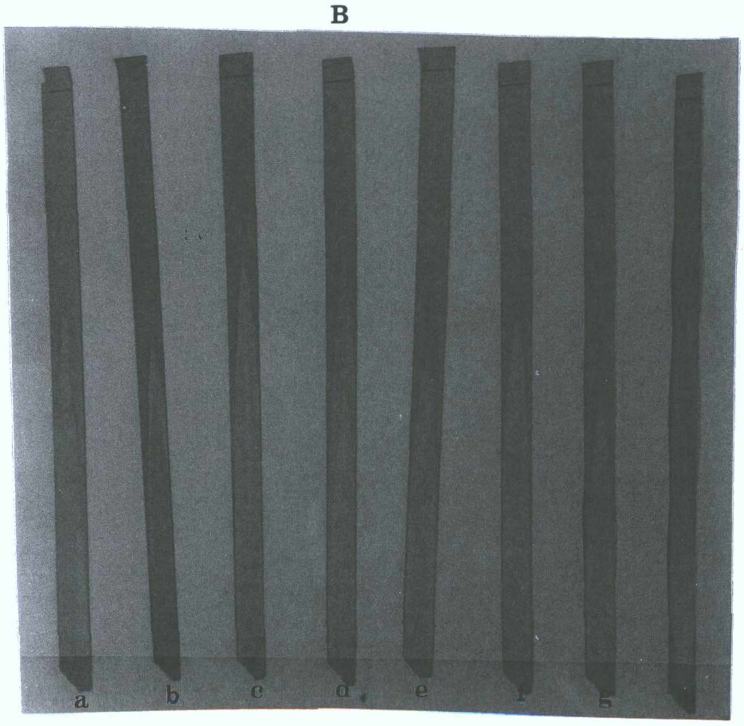


Fig. 5.2.1.2.

A non-denaturing 10% polyacrylamide gel of apolipoproteins was blotted to nitrocellulose. Blots were cut into strips and reacted with a) washing buffer 0.15M NaCl/0.05M Tris/HCl, pH 7.4, b) 1mg/ml trypsin (in washing buffer), c) 0.1mg/ml trypsin d) 0.01mg/ml trypsin e) 10U/ml collagenase f) 1mg/ml β-galacrosidase g) 1mg/ml pepsin in 50mM glycine HCl pH 2.3 h) 1mg/ml trypsin + 10mg/ml soybean trypsin inhibitor. Blots were then reacted with hybridoma supernatant, and detected with 1:20 peroxidase-labelled rabbit-anti mouse immunoglobulin, followed by 1:100 mouse anti-peroxidase / peroxidase complex. A. antibody 6C9, B. Antibody 6G10A4.

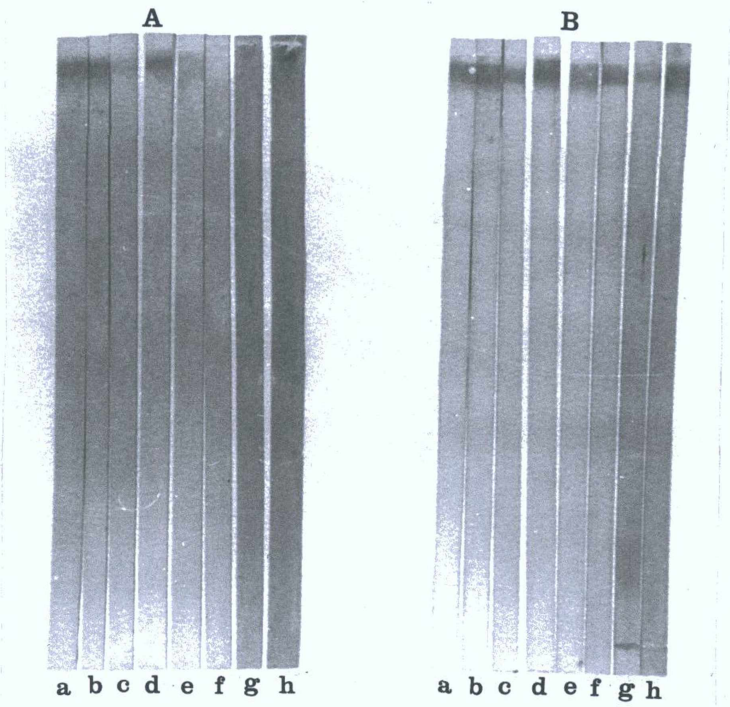

A
B
a b c d e f g h
a b c d e f g h

Fig. 5 2.2

A non-denaturing / 5% polyacrylamide gel of delipidated low density lipoprotein was blotted to nitrocellulose The blot was cut into strips, which were incubated in 1mg/ml Pepsin in Glycine / HCl, pH 2 3 for 30 minutes at 37°C. Panel A pepsin treated strips, panel B, control strips Strips were then incubated in a) 2B6A12, b) 3F12D12, c) 4G1B4, d) 5B10, e) 6C9, f) 6G10A4, g) 1 200 anti-alpha-lipoprotein, h) 1:200 anti-beta-lipoprotein. Bands were detected with 1 100 peroxidase-labelled rabbit-anti-mouse immunoglobulins (a-f) and 1.500 peroxidase-labelled swine-anti-goat IgG (g,h).

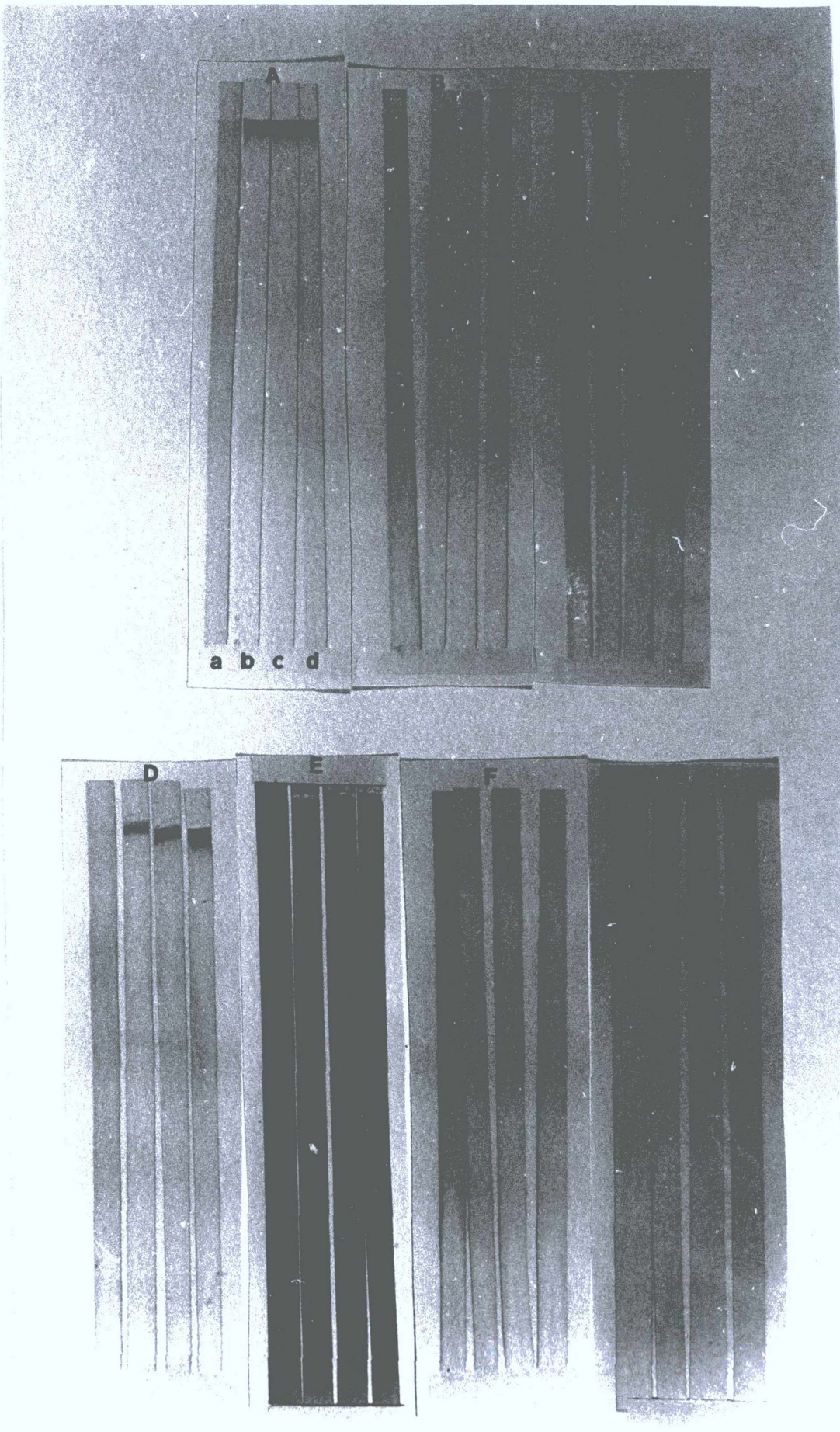
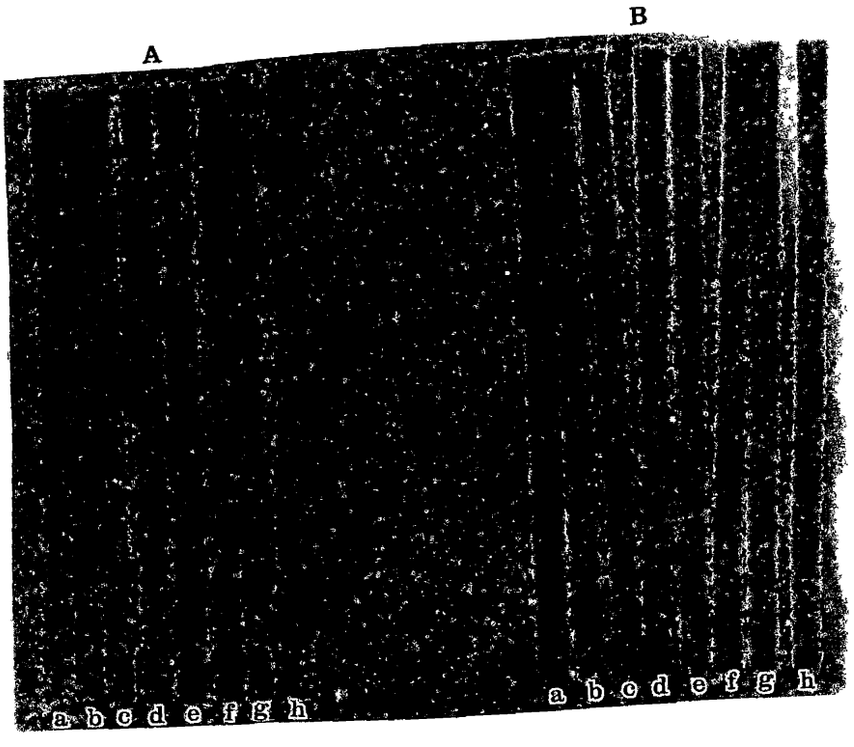

A
B
a b c d
D
E
F

Fig 5.2.3

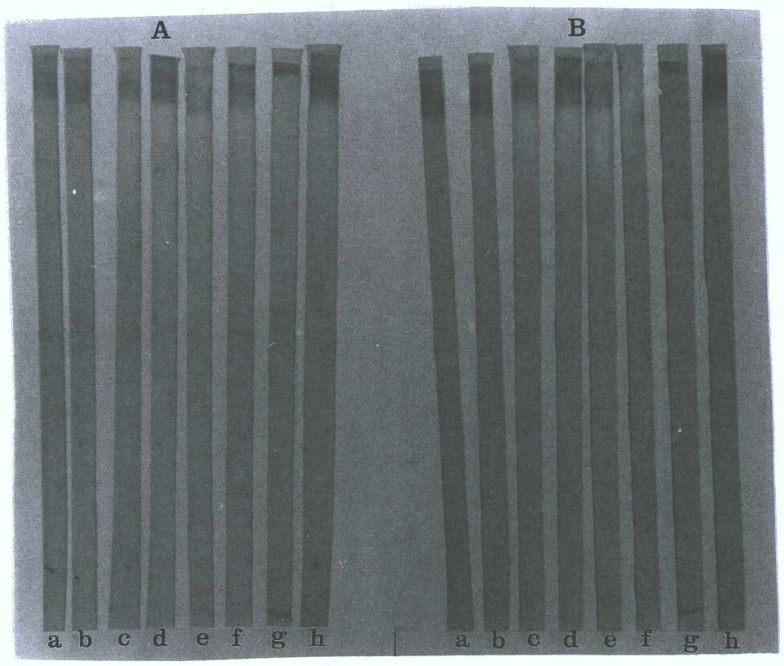
A non-denaturing/5% polyacrylamide gel of delipidated low-density-lipoprotein was blotted to nitrocellulose The blot was cut into strips and incubated with proteinase K at different concentrations· a) lmg/ml, b) 0 lmg/ml, c) 0 0lmg/ml, d) control strip The blots were incubated with A. 2B6A12, B 3F12D12, C. 4G1B4, D. 5B10, E 6C9, F 6G10A4, G. 1 200 anti- -lipoprotein.

Bands were detected with 1 100 peroxidase-labelled rabbit-anti-mouse immunoglobulins (A-F), and 1:500 peroxidase-labelled swine-anti-goat lgG (G, H).


A
B
a b c d e f g h
a b c d e f g h

5 2 4

A non-denaturing / 5% polyacrylamide gel of delipidated low-density-lipoprotein was blotted to nitrocellulose The blot was cut into strips and incubated in neuraminidase as described in methods A Control strips, B treated strips The blots were incubated in a) 2B6A12, b) 3F12D12, c) 4G1B4, d) 5B10, e) 6C9, f) 6G10A4, g) 1 200 anti-alpha-lipoprotein, h) 1 200 anti-beta-lipoprotein Bands were detected with 1·100 peroxidase-labelled rabbit-anti-mouse immunoglobulins (A-F), and 1 500 peroxidase-labelled swine-anti-goat IgG (G, H).


A
B
a b c d e f g h
a b c d e f g h

5.2.5.

A non-denaturing / 5% polyacrylamide gel of delipidated low-density-lipoprotein was blotted to nitrocellulose. The blot was cut into strips and treated with lipase, as described in methods. A. lipase treated strips, B. control strips. The blots were incubated in a) 2B6A12, b) 3F12D12, c) 4G1B4, d) 5B10, e) 6C9, f) 6G10A4, g) 1:200 anti-alpha-lipoprotein, h) 1:200 anti-beta-lipoprotein Bands were detected with 1:100 peroxidase-labelled rabbit-anti-mouse immunoglobulins (A-F), and 1:500 peroxidase-labelled swine-anti-goat IgG (G, H).

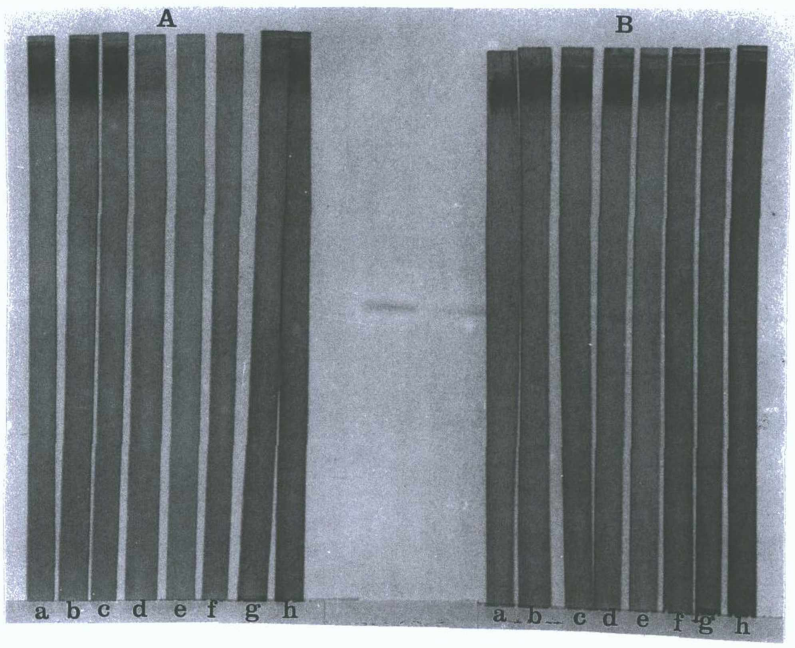

A
B
a b c d e f g h
a b c d e f g h

Fig 5 3.1

A non-denaturing / 5% polyacrylamide gel of delipidated low-density-lipoprotein was blotted to nitrocellulose. The blots were cut into strips and reductively methylated with sodium borohydride / formaldehyde, as described in methods. A treated strips, B. untreated strips The strips were incubated in a) 2B6A12, b) 3F12D12, c) 4G1B4, d) 5B10, e) 6C9, f) 6G10A4, g) 1 200 anti-alpha-lipoprotein, h) 1 200 anti-beta-lipoprotein Bands were detected with 1:100 peroxidase-labelled rabbit-anti-mouse immunoglobulins (a-f), and 1·500 peroxidase-labelled swine-anti-goat IgG (g, h).

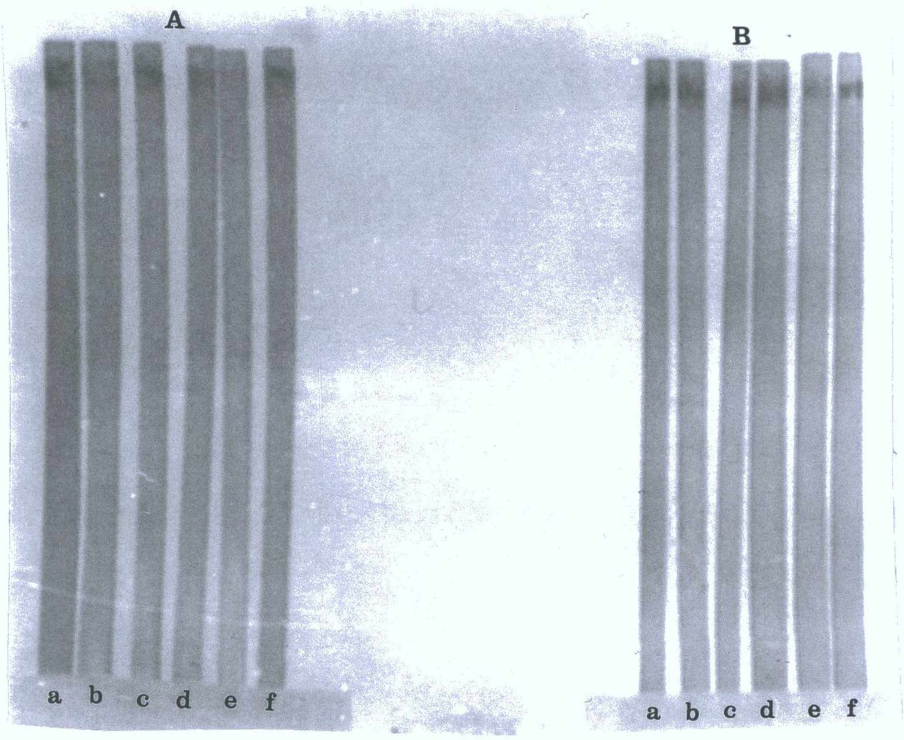

A
B
a b c d e f
a b c d e f

Fig 5 3 2

A non-denaturing / 5% polyacrylamide gel of delipidated low-density-lipoprotein was blotted to nitrocellulose The blots were cut into strips and treated with iodoacetamide as described in methods A control strips, B treated strips The strips were incubated in a) 2B6A12, b) 3F12D12, c) 4G1B4, d) 5B10, e) 6C9, f) 6G10A4 and detected with 1 100 peroxidase-labelled rabbit-anti-mouse immunoglobulins

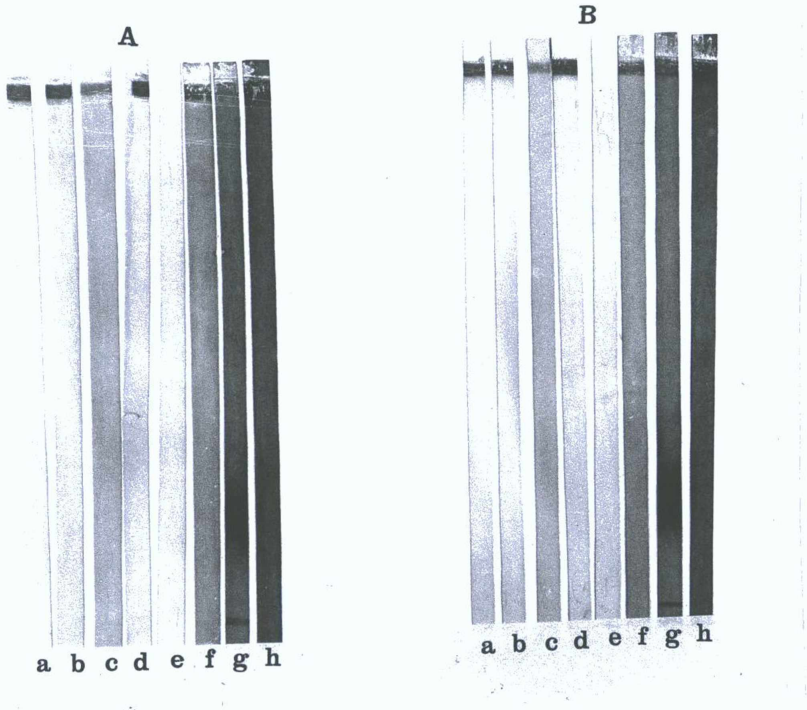

A
B
a b c d e f g h
a b c d e f g h

Fig 5 3 3

A non-denaturing / 5% polyacrylamide gel of delipidated low-density-lipoprotein was blotted to nitrocellulose The blots were cut into strips and oxidised with periodate A Treated strips, B Control strips The strips were incubated in a) 2B6A12, b) 3F12D12, c) 4G1B4, d) 5B10, e) 6C9, f) 6G10A4, g) 1 200 anti-alpha-lipoprotein, h) 1 200 anti-beta-lipoprotein Bands were detected with 1·100 peroxidase-labelled rabbit-anti-mouse immunoglobulins (a-f), and 1 500 peroxidase-labelled swine-anti-goat IgG (g, h).

5 4 Discussion

Table 5 4 1 summarises the effects of the various chemical and enzymic treatments of the nitrocellulose-bound apolipoprotein B on the subsequent immunoreactivities of the antibodies The suggestion that all of the antigenic determinants are located on a polypeptide region of the antigen comes from the effect of proteinase K on the immunoreactivities and the absence of any notable effect after lipase and neuraminidase treatment However, it is possible that the proteinase K treatment cleaved the antigen so extensively that peptide fragments were released off the blot These fragments could have included the epitope which was itself composed of lipid or carbohydrate material Also, lipase and neuraminidase are both specific enzymes whose substrates are, respectively, triacetin and sialic acid, so the absence of an effect does not exclude the possibility of epitopes which include other lipid or carbohydrate moieties

Periodate oxidation treatment of blots, which was reported by Woodward et al., (1985), was a more general, and simple, method of detecting antibodies specific for carbohydrate epitopes The method, which involves periodate oxidation of neighbouring hydroxyl groups on sugars to dialdehydes at acid pH, is easily applicable to antigens immobilised on blots However, no inhibition of binding for any of our antibodies was observed with this technique providing further evidence for the absence of carbohydrate epitopes Patton et al., (1982), reported a 50% loss of immunoreactivity with his panel of monoclonal antibodies, after delipidation of LDL immobilised on micro-titre

plates, by hexane / isopropanol (3 2, v/v) 95% of lipid was removed by this procedure and only 10% of protein

I adapted this procedure to nitrocellulose blots, using LDL which had been partially delipidated by ether treatment and found that binding of 5B10, 6C9 and 6G10A4 was inhibited No loss of immunoreactivity was observed with the polyclonal antibodies, which suggested that the integrity of the nitrocellulose membrane, and the protein structure, was not destroyed by the solvent

If the epitopes for the antibodies are polypeptide in nature, its still possible that lipids play a vital role in stabilising the antigenic determinant and maintaining its correct conformation for antibody recognition This may be the case for the epitopes for antibodies 5B10, 6C9 and 6G10A4 However, in the case of 5B10 and 6C9, such observations are irreconcilable with their abilities, previously shown, to bind to blots of SDS gels, where one would expect complete delipidation to have taken place Further investigation into the precise role played by lipid moieties in the determinants for these two antibodies is clearly required The experiments with trypsin and pepsin yielded more information on the molecular nature of the antigenic determinant The epitope for 5B10 was the only one found to be repeatedly sensitive to trypsinolysis, implying that lysine or arginine residues may be involved, while the inhibition of binding of 6G10A4 and to a lesser extent 4G1B4 and 6C9 after pepsin treatment suggests that their antigenic determinants involve hydrophobic amino acid residues Modification of lysine residues by sodium borohydride and

formaldehyde treatment, which methylates free amino groups of these residues, lowered the immunoreactivities for 5B10, 6C9 and 6G10A4 The mode of inhibition was possibly steric hindrance caused by the introduction of a methyl group

These experiments, taken together, indicate the presence of several distinct epitopes on the apolipoprotein B molecule There is evidence, but no definite proof, that the epitopes are to be found on the polypeptide region of the molecule The experiments which used trypsin, pepsin, lipase, neuraminidase and reductive methylation all gave consistent results on repetition The other experiments were performed once only

Chapter 6

Monoclonal Antibody Purification

6 1.1 Agarose-Protein A chromatography

Monoclonal antibody from 6G10A4 supernatant was extracted by ammonium sulphate precipitation and then subjected to agarose-protein A column chromatography The optical density readings of the eluted fractions are plotted in fig 6 1 1 and it can be seen that large amounts of protein material ran straight through the column, and very little protein was eluted with the glycine/HCl wash Antibody activity was contained in the unbound fraction, as evidenced by reacting both bound and unbound fractions with blots, hence the protein A did not bind the monoclonal antibody

Fig 6 1 1

7ml of ammonium sulphate extract of hybridoma superntant of antibody 6G10A4 was added to 7ml of column buffer before being added to the column Bed volume was 0 7ml Fractions of approximately 2ml were collected The bound material was eluted from the column with elution buffer at fraction 15 Fractions 4, 15, 16 and 17 were tested for antibody on blots of apolipoprotein The results, expressed as + or - , for positive or negative antibody expression, are shown on the figure

6 2 2 Affi-gel Blue Column Chromatography

Affi-Gel Blue chromatography was the next method chosen to purify the monoclonal antibodies because it was hoped that all the antibodies in the panel would be purified successfully with this method, rather than just some of them, which would be the case with the protein A column Both the precipitates of the ammonium sulphate fraction of hybridoma supernatant and ascites fluids were used Five antibodies, 3H9, 5B10, 4G1B4, 6C9 and 6G10A4, were purified Fig 6 2 1, (A and B) shows the elution profile after 3H9 and 5B10 supernatants were put through the column NaCl concentrations of 10mM, 25mM, 50mM and 100mM were used and at the latter three concentrations, protein was eluted off the column Antibody reactivity in each of my eluted fractions, which was measured by immunoblotting with 5% Davis gels of apolipoprotein was very spread out over all the fractions tested, when 5B10 and 3H9 were run (Fig. 6 2 1) When 6C9 and 4G1B4 supernatants were run, reactivity could be detected in all the salt washes and also in the material that did not bind to the column 6G10A4 antibody did not bind to the column its reactivity could only be picked up in the unbound fraction

The same technique for antibody purification was also tried with ascites fluid, because it was felt that it would provide better starting material than hybridoma supernatant, being more concentrated and less heterogeneous

Elution steps of 25mM, 50mM, 60mM and 100mM NaCl were used

and each salt wash eluted protein Both 5B10 and 3H9 immunoreactivities were very strong in the 50mM NaCl wash (fig. 6 2 2) so that, for these two antibodies in any case, the antibody was purified with comparitive success from ascites fluid

The other three antibodies, however, separated less well 6C9 reactivity was mostly contained in the unbound material and in the fractions eluted by the 60mM and 100mM NaCl washes Very little protein was eluted by the 25mM and 50mM washes in this instance

4G1B4 reactivity could not be detected in any fraction It was concluded that there was no antibody present in the ascites fluid because the cell line had become negative just before or during tumour propagation

The elution profile for 6G10A4 purification showed a lot of protein material eluting with 25mM NaCl However antibody reactivity was detected in the 100mM NaCl fraction

The five antibodies therefore behaved very differently in the purification procedure 5B10 and 3H9 could be purified relatively successfully, 6C9 is unusual in that reactivity can be detected in all the eluted fractions tested, as well as in the unbound material, and 6G10A4 did not bind when supernatant was used as starting material, but did partly bind when ascites fluid was used

The purity was also assessed by examining the protein content of the eluted fractions on 10% polyacrylamide SDS gels Very little protein could be visualised in the Coomassie blue-stained gels, (fig 6 2 3), even though strong antibody reactivity was detected in these

fractions The fractions after 3H9 purification are shown in the figure

Purified antibodies were pooled and stored at $4^{o}C$ with sodium azide added as preservative For those antibodies in which only a portion bound to there column, the eluted fractions only were kept because they were deemed to be relatively purer

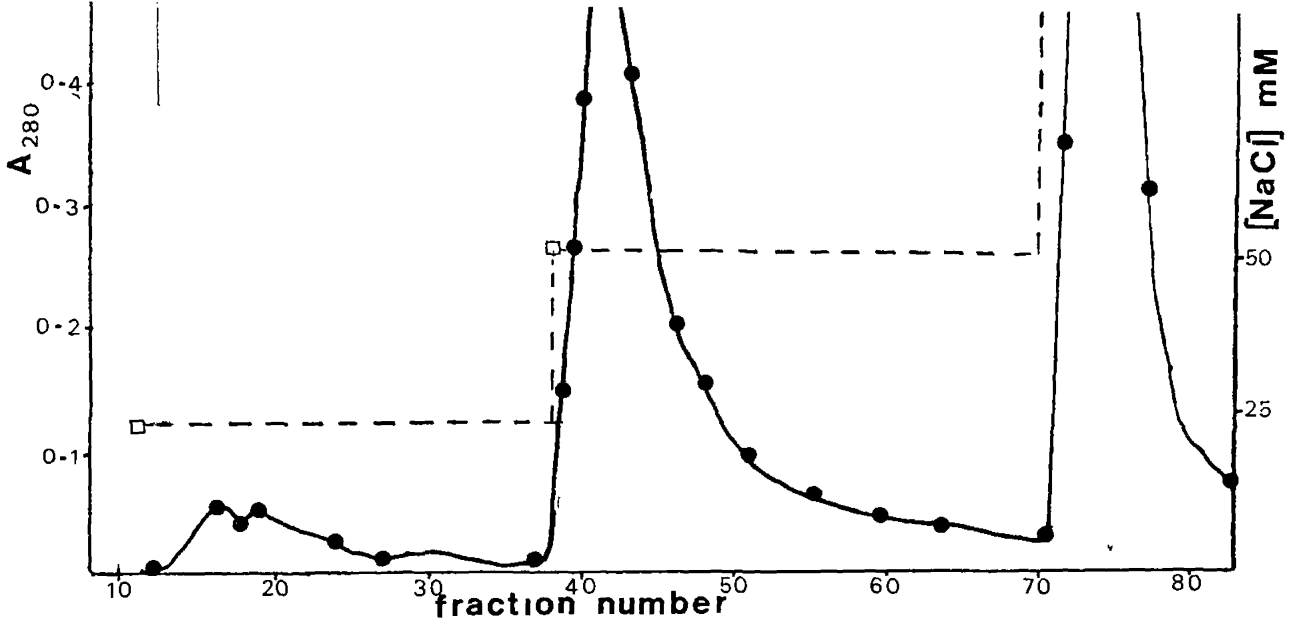
Fig 6 2 1

Purification of, A, 3H9 antibody, B, 5B10 antibody, from the ammonium sulphate extract of hybridoma supernatant, by Affi-Gel Blue column chromatography Fraction volumes of 2 5ml were eluted with 25, 50 and 100mM NaCl washes (represented by the broken line) Absorbancies were read at 280nm in a CECIL spectrophotometer, (●-●) (▼) Fractions that were positive for antibody activity, after immunoblotting with 5% Davis gels of apolipoproteins

C Purification of 6C9 antibody Fractions were screened for antibodies by double diffusion 4µl of each fraction was added to the outer wells The centre wells contained 4µl of rabbit-anti-mouse immunoglobulins (▽), Antibody-containing fractions

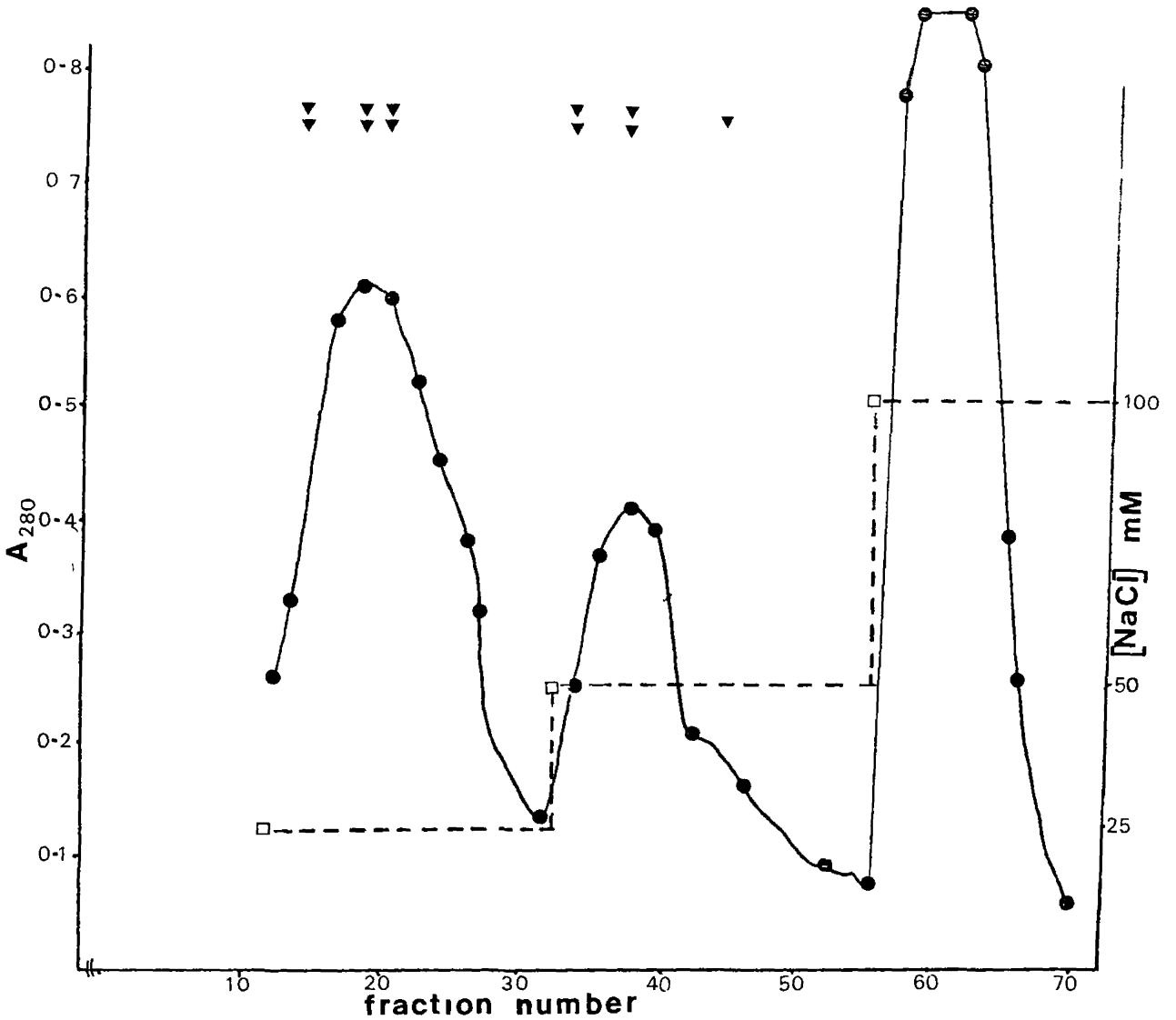
D Purification of 6G10A4 Antibody could only be detected in the unbound fraction

E Purification of 4G1B4 antibody Fractins were tested for antibody by immunoblotting (▼) and by double diffusion (▽)


A280
0·4
0·3
0·2
0·1
[NaCl] mM
50
25
10
20
30
40
50
60
70
80
fraction number

A

0·8
0·7
0·6
0·5
100


0·8
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0·6
0·5
0·4
0·3
0·2
0·1
A280
10
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30
40
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60
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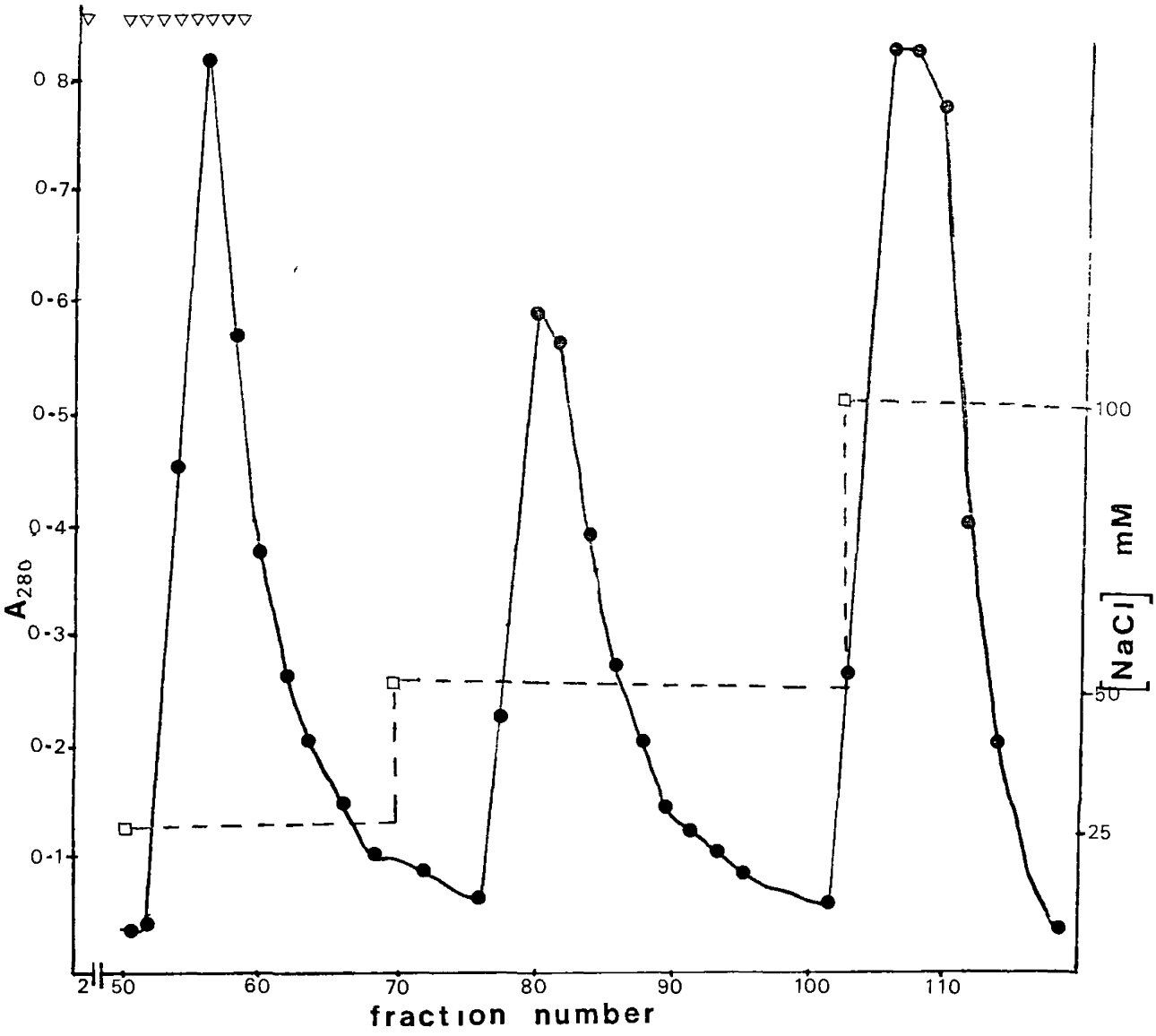
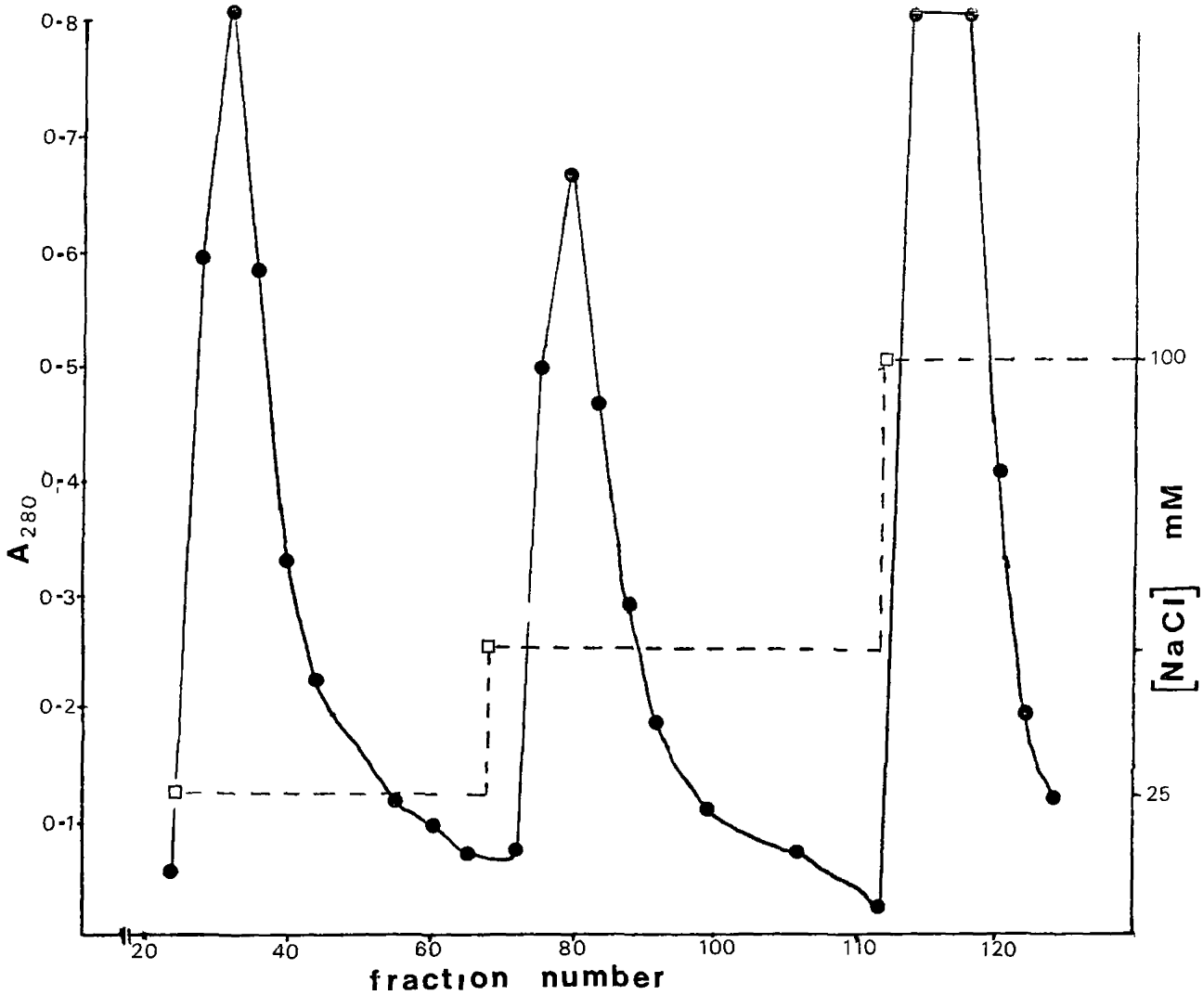
fraction number
100
50
25
[NaCl] mM

B


0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
A_{280}
50
60
70
80
90
100
110
fraction number
100
50
25
[NaCl] mM

C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0·7
0·6
0·5
0·4
0·3
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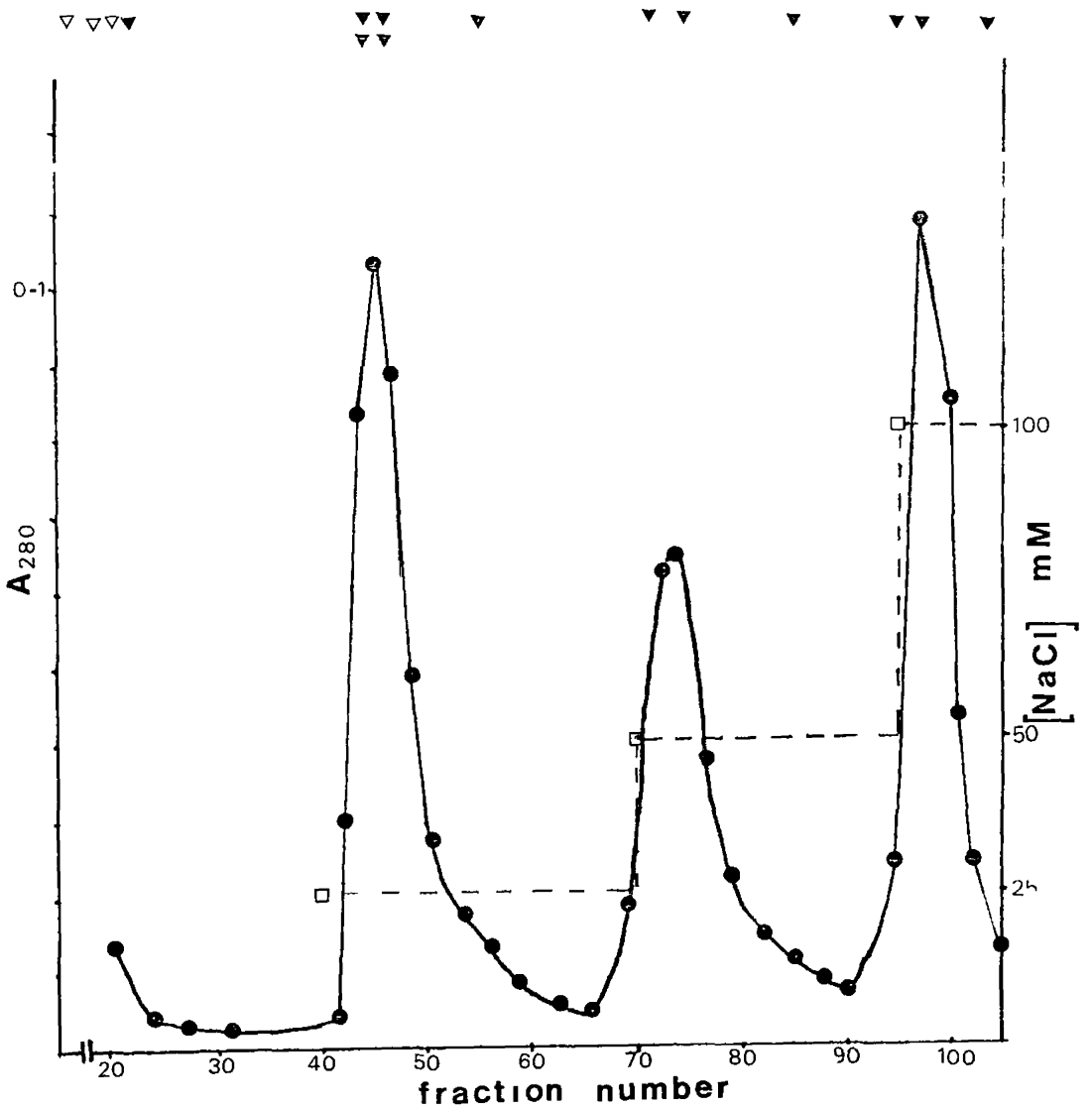
0·1
A280
20
40
60
80
100
110
120
fraction number
100
25
[NaCl] mM

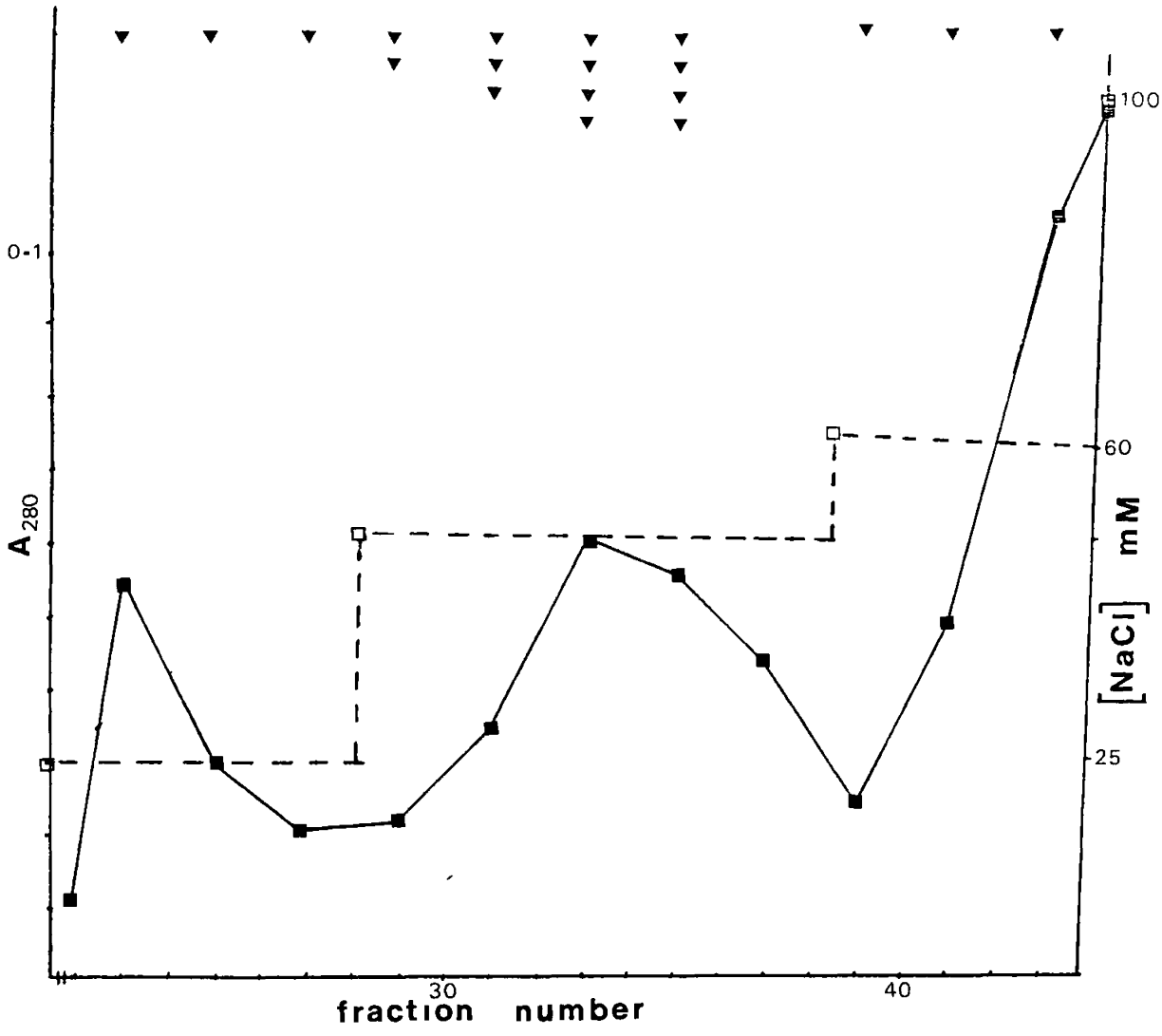
D


0·1
A_{280}
100
50
[NaCl] mM
20
30
40
50
60
70
80
90
100
fraction number

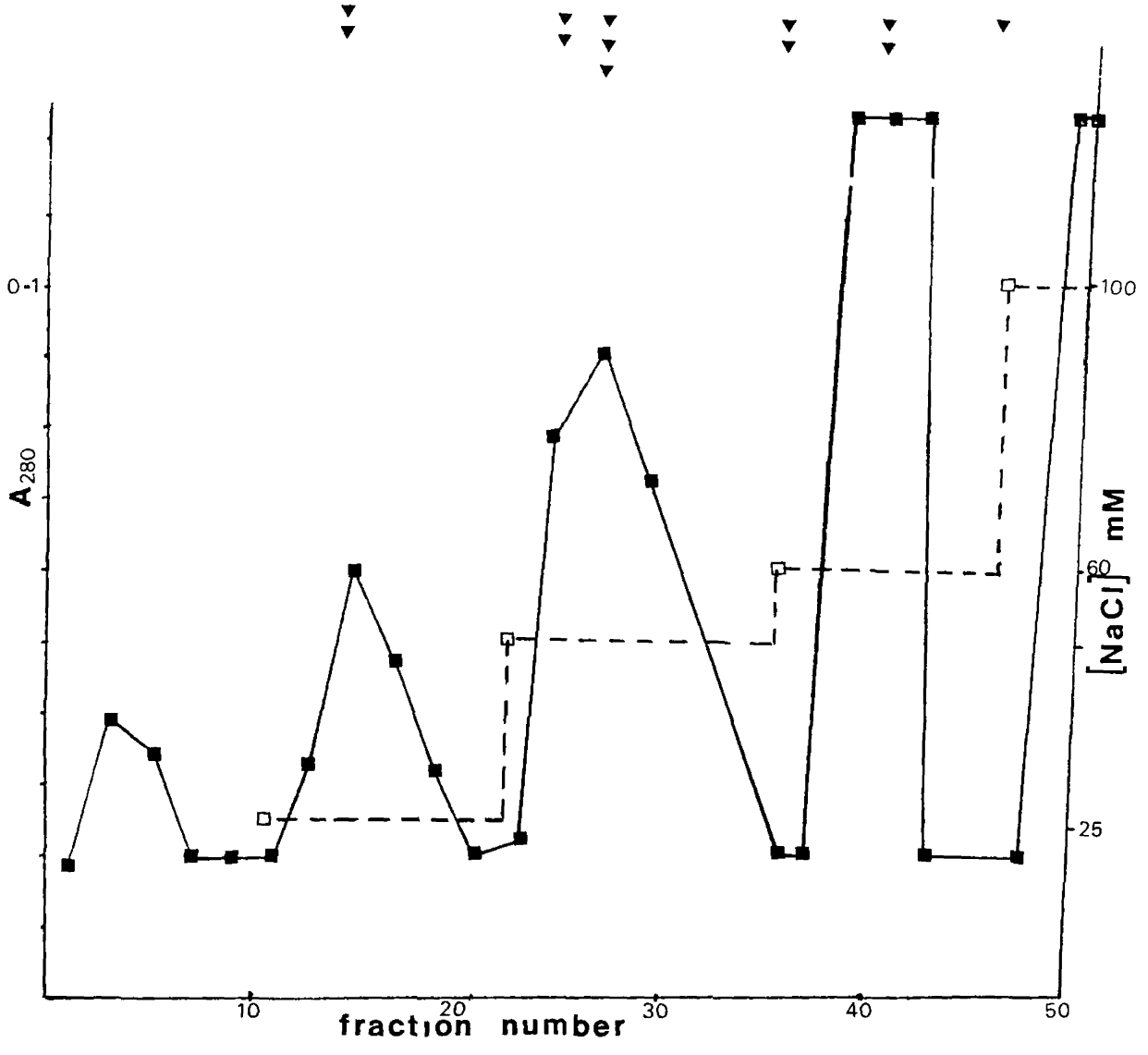
E

Fig 6 2 2

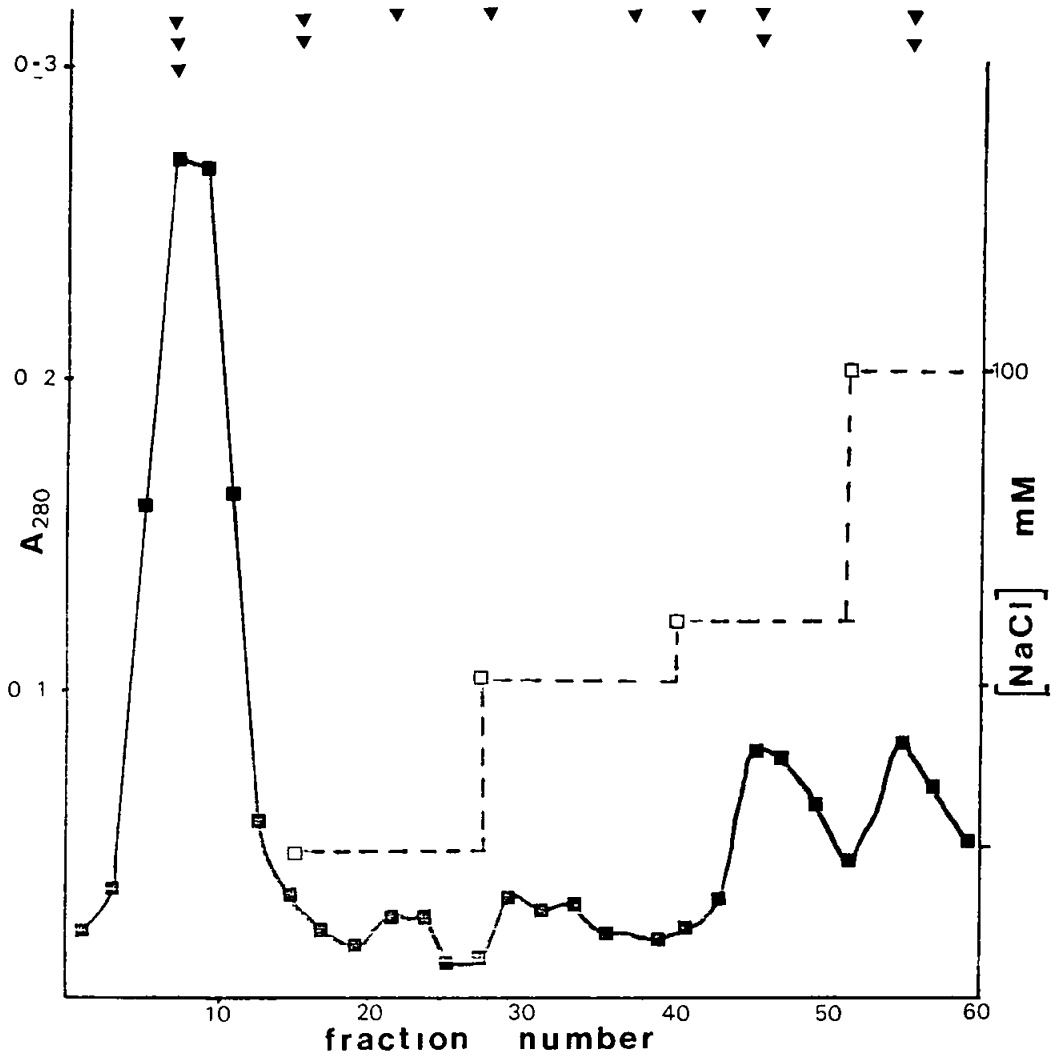
Purification of monoclonal antibody from ascites fluid by Affi-Gel Blue Column Chromatography A 5B10 B, 3H9 C, 6C9 D, 6G10A4 2 5ml fractions were collected and antibody was eluted with 25mM, 50mM, 60mM and 100mM NaCl washes, (represented by the broken line) (▼), fractions that were positive for antibody after immunoblotting with 5% Davis gels of delipidated LDL Absorbances were read at 280nm in a CECIL spectrophotometer,(■-■).



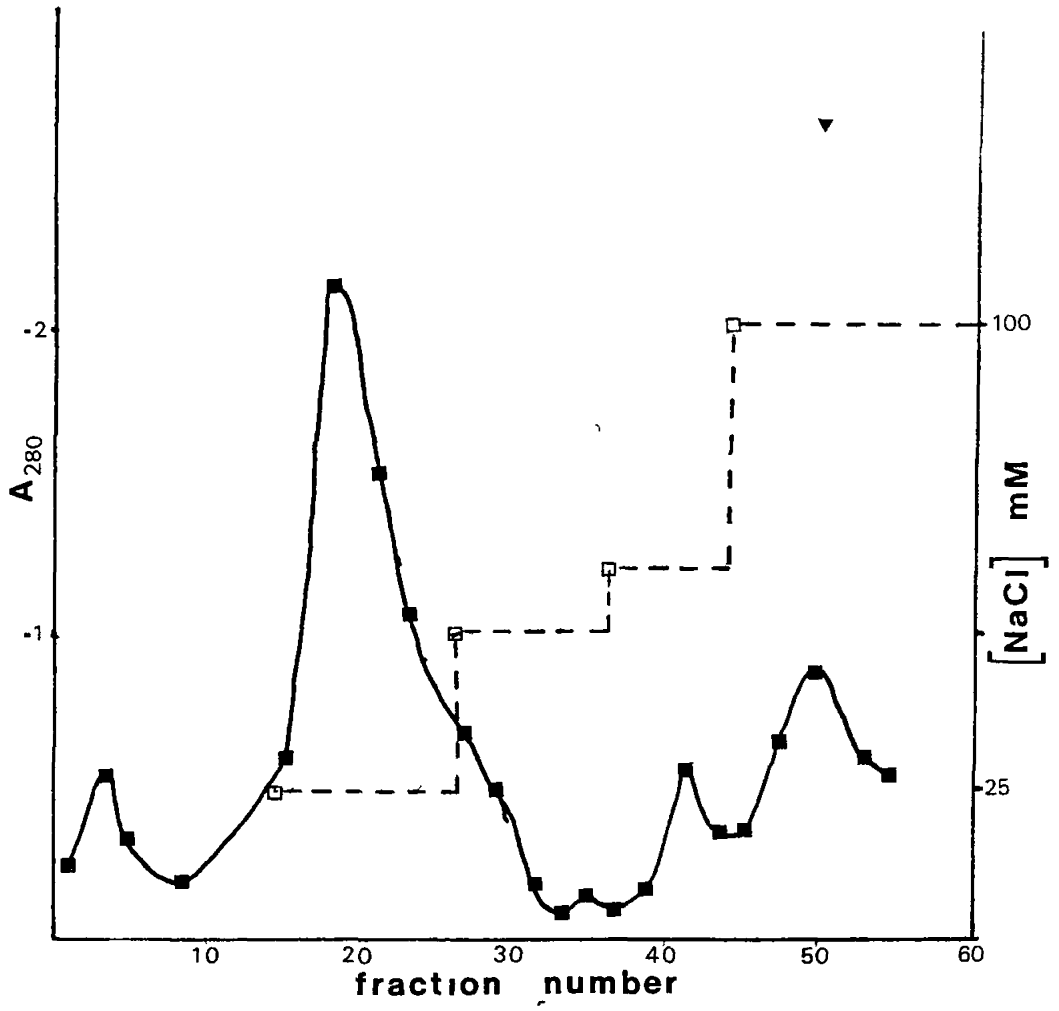
A



B



C



D

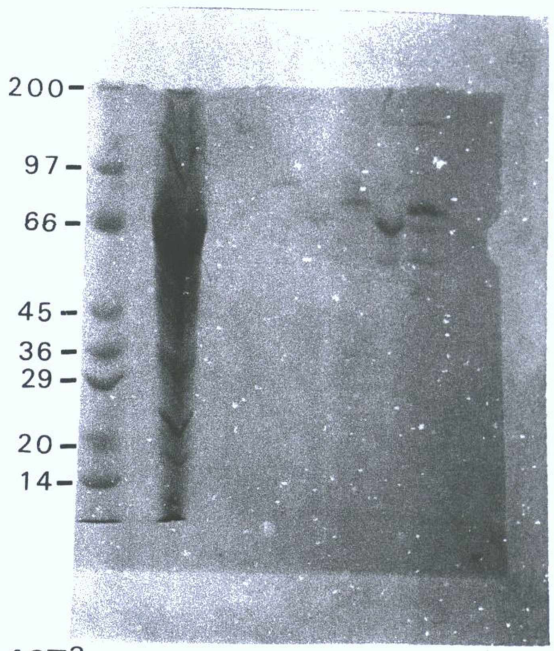

200
97
66
45
36
29
20
14
$M_r \times 10^{-3}$
a b c d e f g h

Fig 6 2 3

Affi-gel blue-purified fractions of mouse ascites fluid containing 3H9 monoclonal antibodies were examined on 10% SDS / polyacrylamide gels

a) molecular weight standards,

b) unpurified ascites fluid (starting material),

c) fraction 15 (protein eluted with 25mM NaCl (not reduced)),

d) fraction 15 (reduced),

e) fraction 27 (eluted with 50mM NaCl)(Not reduced),

f) fraction 27, (reduced),

g) fraction 40, (eluted with 60mM NaCl),(not reduced),

h) fraction 40, (reduced)

Chapter 7

Receptor binding studies

7 1 Inhibition by antibodies of binding

Four anti-apoB antibodies, 4G1B4, 5B10, 6C9 and 6G10A4, and one antibody directed against a HDL apoprotein, 3H9, were partially purified by Affi-Gel Blue Chromatography and incorporated into LDL-receptor binding studies to determine whether the antigenic determinant for the antibodies involved the receptor recognition domain of apoB

The method used was ligand blotting, in which both biotinylated and iodinated LDL were used as ligands LDL was labelled with biotin via carbohydrate residues as described by Wade et al.,(1985) Normally, biotin-labelling of proteins is through the reaction of biotin N-hydroxysuccinimide with free amino groups of lysine residues (Guesdon et al, 1979) However, to do this with LDL would affect the interaction of LDL with the LDL-receptor because Weisgraber et al., (1978), have shown that free lysine amino qroups are required for this Wade et al conjugated biotin to sialic acid residues on the apoprotein and demonstrated that it did not affect lipoprotein receptor binding

In addition to that, my enzymic modification studies with nitrocellulose-bound apoB, together with the periodate oxidatin experiment, led me to believe that biotin-labelling of LDL via carbohydrate residues would not affect monoclonal antibody binding This was confirmed when the antibodies were reacted with biotin-LDL bound to a micro-titre plate and detected with

peroxidase-labelled rabbit anti mouse immunoglobulins, (data not shown)

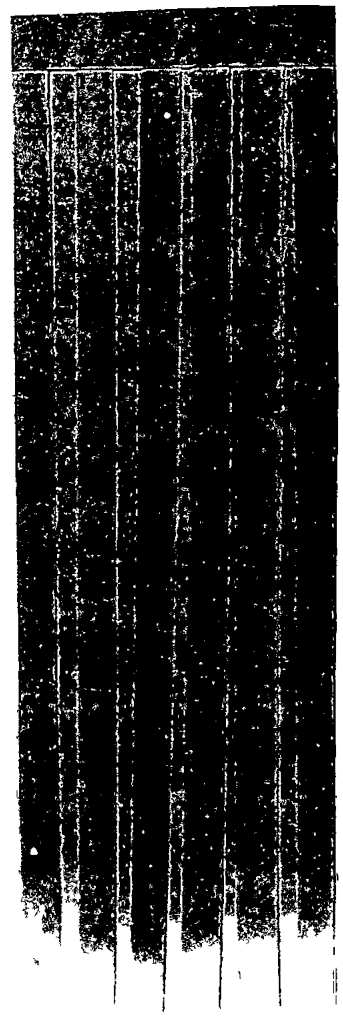
Fig 7 1 1 shows a nitrocellulose strip detected with biotinylated beta-VLDL Studies done previously by Wade et al., (1985) has provided strong evidence that the stained band represented the LDL receptor. On this blot the protein has a molecular weight of approximately 120,000 The strip on the right was stored at -20^{o}C for 11 days implying that the receptor protein is not stable to storage under these conditions

Biotin-LDL combined with anti-apoB polyclonal antibody dilutions were incubated over nitrocellulose blots of receptors. After detecting the strips, the three control biotin-LDL preparations, as well as that present in the two more dilute antibody solutions, had bound to the receptor, while binding was much fainter in the most concentrated antibody solution (fig 7 1.2) implying that LDL-receptor interaction ws being inhibited The fact sheet that accompanied the anti apoB antiserum stated that lipoproteins had been removed so the inhibition was unlikely to be caused by competition from other lipoproteins

The biotin-LDL detected the bound receptor very faintly and was much weaker than the biotin-beta-VLDL (cf Figs 7.1.1 and 7 1 2) 7.1 3 shows a blot which was treated with ^{125}I-LDL and autoradiographed. Clearly, this represents a more sensitive detection system.

Fig. 7 1.1.

Detection of the LDL receptor, after separation on a 7 5% polyacrylamide / SDS gel, and transfer to nitrocellulose, by ligand blotting with biotin-beta-VLDL Strips were incubated in 10µg lipoprotein / ml before detection with streptavidin-biotinylated-peroxidase complex The strip on the right was stored at -20°C for 11 days before being ligand-blotted



f e d c b a

Fig 7 1 2

Detection of the LDL-receptor on nitrocellulose blots, after SDS/7.5% polyacrylamide gel electrophoresis, by ligand blotting with 10µg biotin-LDL / ml together with various dilutions of anti-apoB a)-c) control strip (no antibody), d) 1·200 anti-apoB, e) 1.100 anti-apoB, f) 1·50 anti-apoB Bands were detected with streptavidin-biotinylated-peroxidase complex

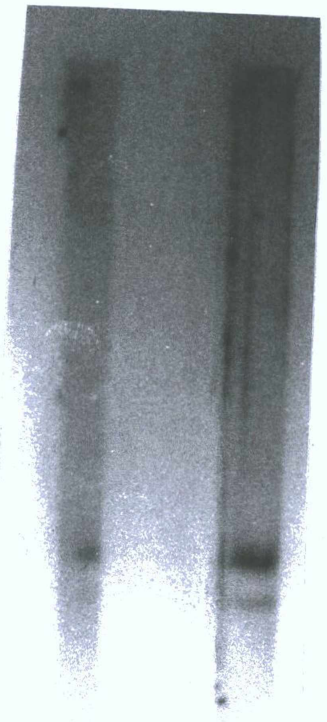


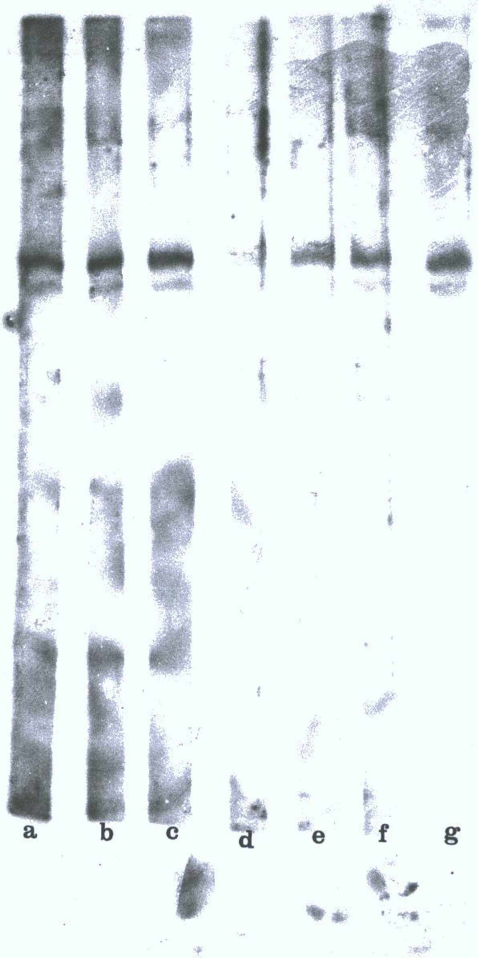
Fig 7.1 3.

Detection of the LDL-receptor, after transfer from 7 5% polyacrylamide / SDS gels to nitrocellulose, with ^{125}I-labelled LDL Blots were incubated with 10ug lipoprotein / ml and then processed for autoradiography using Kodak X-ray film


a
b
c
d
e
f
g
h

Fig. 7.1.4.

Detection of nitrocellulose-bound LDL receptor with ^{125}I-LDL. 10µl lipoprotein/ml was incubated in the presence of 100ug/ml of the Affi-Gel blue purified antibody solutions at 4^{o}C overnight before reacting with the blots. The strips were then processed for autoradiography using Kodak X-ray film. a) - c) control ^{125}l-LDL (no antibody), d) ^{125}I-LDL + 3H9 e) ^{125}I-LDL + 5B10 f) ^{125}I-LDL + 6C9, g) ^{125}I-LDL + 6G10A4, h) ^{125}I-LDL + 4G1B4.


a
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d
e
f
g

Fig 7 1 5.

Detection of nitrocellulose-bound LDL-receptor with ^{125}I-LDL 10µg lipoprotein / ml was incubated overnight at 4°C with Affi-Gel blue purified antibody 6C9 solution at different protein concentrations Blots were incubated with lipoprotein / antibody complexes and detected by autoradiography, as described in methods
Left to right a)-c) control ^{125}I-LDL (no antibody) d) ^{125}I-LDL + 6C9 (100µg protein / ml) e) ^{125}I-LDL + 6C9 (75µg/ml) f) ^{125}I-LDL + 6C9 (50µg/ml), g) ^{125}I-LDL + 6C9 (25µg/ml)

Fig 7 1 6

Binding of ^{125}I-LDL to cultivated human fibroblasts Cell monolayers were prepared in dishes as described in methods 212µg ^{125}I-LDL (Spec Act 80 cpm/ng) were incubated overnight at 4°C in the presence or absence of a) 300µg protein containing 6C9 antibody, b) 600µg protein containing 6G10A4 antibody At the beginning of each experiment the indicated amounts of ^{125}I-LDL were added to the dishes After incubation at 4°C for 2 hours the medium in each dish was removed The cell protein content of each dish was measured and the amount of ^{125}I-LDL bound to the cells was determined Values represent the mean of duplicate determinations (⊙) control ^{125}I-LDL ●) ^{125}I-LDL + 6C9, ■), ^{125}I-LDL + 6G10A4.

Antibodies 3H9, 5B10, 6C9, 6G10A4 and 4G1B4 were tested for their ability to inhibit ^{125}I-LDL binding to nitrocellulose-bound LDL receptors The ^{125}I-LDL was incubated with the antibody solutions at 4^{o}C overnight with a ratio of protein to lipoprotein at 10 to 1 to allow complete formation of antibody-LDL complexes Fig 7 1 4 shows that the antibodies, 6C9 and 4G1B4 caused inhibition of binding When 6C9 was tested again at lower antibody concentrations, LDL receptor binding was restored (fig 7 1 5)

Binding inhibition studies were also performed with cultured human fibroblasts, using antibodies 6C9 and 6G10A4 Antibody / lipoprotein complexes were allowed to form overnight at 4^{o}C before adding to the cells In this case the antibody protein / LDL ratio was 1 4 1 for 6C9 and 2 8 1 for 6G10A4 After a 2 hour incubation at 4^{o}C the binding was measured by determining the amount of radioactive protein in the dissolved cells While 6G10A4 had no effect on receptor-mediated binding of ^{125}I-LDL to fibroblasts, the addition of 6C9 to ^{125}I-LDL solution appeared to slightly enhance cellular binding (fig 7 1 6)

7 2 Discussion

Monoclonal antibodies against apoB which inhibit LDL from binding to LDL receptors are potentially interesting in that they are likely to be directed against an antigenic site at or near the receptor binding domain and can thus

be used in the immunological study of this region Ligand blotting with labelled LDL provides a more rapid and technically simpler method of testing antibodies than binding studies with cultured fibroblasts Biotin-LDL was the chosen ligand at the start of our experiment but it was found to detect the receptor with much less sensitivity than biotin-beta-VLDL This lipoprotein was isolated from WHHL rabbits and interacts with the receptor through an apolipoprotein E-mediated reaction (Hui et al., 1984) Certain ApoE- containing lipoproteins, namely apoE-HDL_C, lDL and remnants bind more preferentially to the LDL receptor than those which contain only apoB-100 (Brown and Goldstein, 1983) so this could explain the greater sensitivity of biotin-beta-VLDL, which also contains apoE

In my experience it was found that ^{125}I-labelled LDL was a more sensitive detector of the bound receptor than its biotinylated counterpart and was therefore the chosen ligand for the monoclonal antibody inhibition studies When biotin-LDL was also tested, the results obtained were the same as for ^{125}I-LDL i e 6C9 and 4G1B4 inhibited LDL binding However the bands were very faint and are not reproduced here

With the exception of 4G1B4, these experiments were carried out using purified antibodies from ascites fluid 4G1B4 was purified from hybridoma supernatant No attempt was made to test untreated hybridoma supernatant over the blots because it was believed that lipoprotein present in foetal calf serum would interfere with the ligand binding by competing with the labelled LDL It is possible,

however, that serum free medium could be used to culture the hybridomas and the spent cell supernatant used in the tests The benefit of this would be the time saved in not having to purify antibody and screening assays could be devised to look for antibodies against the apoB region of interest, i e the receptor binding domain, very rapidly after a fusion.

A large excess (10 1) of antibody solution was required to effect inhibition Milne et al., (1983) also used an antibody excess to LDL in their experiments although they used purified Fab fragments (from ascites fluid)

Tikkanen et al, (1982), achieved 70% inhibition of binding when equal amounts of intact antibody and ^{125}I-LDL (in concentrations of ug/ml) were used

6C9 did not prevent LDL from binding to the receptor in the fibroblast experiments, in which only a slight excess of antibody solution was used to form antibody-LDL complexes If anything, binding was slightly enhanced

It should be remembered that the antibody preparation was not entirely pure after the Affi-Gel blue purification so that the true ratio of antibody to LDL would be, in fact, lower than 1 4 1 here

Young et al., (1986), have also described the screening of monoclonal antibodies against LDL to determine whether or not they inhibited LDL-receptor interaction It is interesting that they used hybridoma supernatant in their assays 0 1ml supernatant was incubated with 0 4ml of ^{125}I-LDL, (final concentration, 2 5µg/ml), in Dulbecco's modified Eagles medium (DMEM) containing 2 5mg/ml lipoprotein-deficient serum for 12h at 4^{o}C Hence,

assuming a antibody concentration of 10µg/ml in supernatant, (Galfre & Milstein, 1981) 1 µg intact antibody was incubated with 1 0ug ^{125}I-LDL Presumably the bovine LDL in the FCS of the hybridoma supernatant was too dilute to competitively inhibit ^{125}I-LDL to any great extent

Chapter 8

General discussion

Six of the 7 monoclonal antibodies selected for further study were directed against apolipoprotein B, so the bulk of my work was devoted to the immunochemical characterisation of this protein, mainly by probing the molecular structure of the determinants specified by the antibodies

On the LDL particle, lipid, carbohydrates and proteins are present as potential antigens and immunoblotting, followed by chemical or enzymic modification of the immobilised antigen provides a useful method to "type" the antigenic determinant into one of the above three categories

In addition, studies with immunoblotting from different electrophoretic systems allowed the comparison of the immunochemical properties of the antigenic determinants under both denaturing and non-denaturing conditions, as well as on native lipoproteins.

My work, therefore, has taken a different line of approach to that of other authors, who concentrated on assigning antigenic determinants to different apolipoprotein B species and comparing their expression among various lipoprotein classes

Looking at each antigenic determinant individually 2B6A12 and 3F12D12 have behaved in identical fashion throughout the experiments and are perhaps directed against the same antigenic determinant Proteinase K treatment of the blot inhibited 2B6A12 and 3F12D12 from binding to the apoprotein B This enzyme, a bacterial protease, inhibited subsequent binding of all the

monoclonal antibodies and may have done so through extensive degradation of the protein Had proteinase K not caused inhibition of monoclonal antibody binding it would have provided strong evidence that the antigenic determinant was not to be found on the polypeptide region of the molecule, but the inhibition, on the other hand, does not prove conclusively that the determinants are polypeptide in nature

The binding site on apoB for antibodies 2B6A12 and 3F12D12 is abolished when the protein is subjected to denaturation in the presence of SDS, so it is most likely conformational i e it may be that the determinant is made up of amino acids which, although distant from each other on the polypeptide chain, are spatially adjacent on the proteins tertiary structure

None of the chemical or enzymic modification experiments were able to determine which amino acids were contained in the determinant for these antibodies

The determinant for 6G10A4 is also conformational and antibody binding was inhibited after treatment of the blot with solvent, suggesting a requirement for lipid molecules to maintain the antigenic structure On native LDL and total lipoprotein, the antibody binds strongly and with a pattern similar to that of 2B6A12 and 3F12D12 This antigen is susceptible to modification of an amino group by reductive methylation treatment which makes it likely that lysine is close to or at the antibody binding site The determinant is also very sensitive to pepsin

treatment, which suggests that a hydrophobic amino acid residue is present

4G1B4 was unlike other antibodies, in that it bound a determinant which was also expressed on high density lipoproteins, as well as on apolipoprotein B Of the modification experiments, only pepsin treatment resulted in a slightly lowered binding reactivity for this antibody so little information on what amino acids make up the antigenic determinant could be gained This determinant was not destroyed by protein denaturation in SDS and was therefore probably sequential

The determinant for antibody 5B10 was the only one to be consistently susceptible to trypsin cleavage, implying that a lysine or arginine residue was contained in the antibody binding site The site was unaffected by pepsin treatment and because the determinant was not destroyed by SDS treatment it, also, is probably sequential Reductive methylation of lysine residues caused inhibition of antibody binding

6C9 was also an antibody which was inhibited by modification of lysine in the antigen This antibody bound to a determinant which was, like the ones for 5B10 and 4G1B4, not destroyed by SDS treatment, and it was also partially sensitive to pepsin cleavage. The reactivity of 6C9 to apoprotein B in VLDL was lower than for LDL in an ELISA suggesting that it is preferentially expressed in the latter protein In the experiments with trypsin, the determinant for 6C9 was originally not susceptible to

cleavage, and in subsequent experiments binding was still visible, at all trypsin concentrations 6C9 was included among the antibodies which were tested for their ability to inhibit LDL from binding to the LDL-receptor and it demonstrated inhibition when LDL-receptor was immobilised on nitrocellulose blots

5B10 and 6C9 also demonstrated similar binding patterns to lipoprotein on native gel blots

Of the five enzymes used in the modification experiments, two, pepsin and proteinase K, were less discriminate in their substrate specificity than trypsin, lipase and neuraminidase, and more of the antibodies tested bound to determinants that were sensitive to hydrolysis by these enzymes The only determinant repeatedly affected by trypsinolysis was that for 5B10, while neuraminidase caused some slight inhibition of polyclonal anti-alpha-lipoprotein binding to a protein band of high mobility in the Davis gels (fig 5 2 5) This band may contain the C-apoproteins, of which apoC-III exhibits varying degrees of sialylation Lipase failed to affect any of the antigenic determinants studied, even though it appeared that lipid played a role in maintaining the antigenic determinant structure for three monoclonal antibodies The lipase used, SIGMA type 1, releases free fatty acids from triacetin substrate and it's possible that apoB was not a suitable substrate for this enzyme The most obvious form of lipase for these studies is lipoprotein lipase and in fact incubations of blots in

post-heparin plasma (as a source of the enzyme) were tried originally Unfortunately the blots had such high background staining after immunodetection, following this treatment, that it was very difficult to tell if binding was reduced or not It was for this reason that I used the alternative, though cruder, method of delipidation through organic solvent treatment, and also tried a different, commercial lipase

It appears that specific enzymes are less successful in obtaining information about the antigenic determinant than chemical reagents It is noteworthy that high enzyme concentrations were needed to effect any inhibition A possible explanation for this may be that, because the substrate is immobilised, breaking the peptide bond (in the case of trypsin) makes little difference to the antigen structure, i e it makes minimal changes to the gross physical structure of the protein and therefore its reactivity with antibody is unchanged On the other hand, reductive methylation, as an example of a chemical modification, caused two methyl groups to be added on to lysine residues, which was probably a greater perturbation of the antigenic structure

Experiments to determine whether any of the monoclonal antibodies could inhibit LDL from binding to its receptor were performed because it had been discovered that three of the antigenic determinants were susceptible to reductive methylation of amino groups Means and Feeney (1968), have demonstrated that the only amino acid

residues affected by this treatment were lysine and the N-terminal amino acid Antibodies 5B10, 6C9 and 6G10A4 were therefore possible reactive to determinants which included lysine residues and therefore possibly the receptor recognition domain of apoB, which Weisgraber et al, (1978), had shown to involve this amino acid
When Milne et al., (1983) modified LDL by reductive methylation, it caused an almost total loss of reactivity with all seven of their anti-LDL antibodies Five of those were later demonstrated to be inhibitory to LDL-receptor binding on cultured human fibroblasts.
Of my five antibodies, only 6C9, of the three which were inhibited by reductive methylation was able to inhibit ^{125}I-LDL-receptor binding 4G1B4 also caused some inhibition, which was an unexpected finding in view of the fact that this antibody binds to a determinant which does not contain lysine. This may be a reflection on the distance of the determinant from the receptor binding domain and from lysyl residues
Formation of antibody / ^{125}I-LDL complexes using intact antibody molecules instead of Fab fragments still allowed LDL to bind to the receptor in the presence of 5B10 and 6G10A4, as well as 3H9, so it is unlikely that 6C9 and 4G1B4 inhibited through precipitation of LDL or steric hindrance
In conclusion, at least five distinct antigenic determinants were specified by the panel of anti-apoB antibodies and some insight into the molecular structure

was gained through the application of the immunoblotting technique It was also possible to use blotting to compare the immunoreactive properties of the determinants in both delipidated and native proteins, as well as in plasma, and to ascertain whether SDS treatment perturbed the determinant or not

As dot-blotting was also introduced as a screening technique the usefulness to my research was three-fold It should be possible to use this panel to screen the apolipoprotein B of individuals for alterations in structure Schumaker et al.,(1984) has already described the discovery of an apoB polymorphism which was detected with 3 monoclonal antibodies of the 11 described originally by Curtiss and Edgington (1982) and Tsao et al., (1982)

The polymorphism was shown to be due to an alteration in protein structure which was most likely caused by an amino acid substitution However, the method they used for screening the LDL was unwieldy and perhaps open to misinterpretation Young et al., (1986), reported a polymorphism in apoB which could be detected by monoclonal antibody MB19, (which was also from Curtiss's panel), using a solid-phase RIA, which was a much simpler method This polymorphism turned out to be the same one as that identified by Schumaker's group, but the improved assay procedure resulted in a re-evaluation of the polymorphism frequency in the population The blotting procedure should provide a simple and successful method of screening

apoB for polymorphism and could be adapted for delipidated and native lipoproteins, as well as SDS-treated samples However, as there is evidence from Tikkanen et al., (1983), that alterations in immunoreactivity can be caused by differences in lipid composition among individuals, one would need to take measures to ensure that any variation in immunoreactivities were not the result of such differences,but were a true reflection of a variant apoB protein

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