STUDIES ON THE PRODUCTION, CHARACTERISATION AND APPLICATIONS OF MONOCLONAL ANTIBODIES AND ANTIBODY-BASED CONJUGATES.

A dissertation submitted for the degree of Ph.D.

by

Dolores J. Cahill.

Under the supervision of

Professor Richard O'Kennedy,

December, 1994.

School of Biological Sciences,

Dublin City University, Dublin 9, Ireland.

ABSTRACT

The work described in this thesis involved the production of antibodies to several defined antigens and the chemical synthesis of bispecific antibodies

Monoclonal antibodies against glial fibrillary acidic protein (anti-GFAP), and against a GFAP-like protein on the human anaplastic astrocytoma cell line, G-CCM (anti-G-CCM) were produced by electrofusion and by fusion using polyethylene glycol, respectively These antibodies were purified with saturated ammonium sulphate (SAS) and protein A These antibodies were fully characterised by ELISA, Isotyping, SDS-PAGE, Immunocytochemistry, Dot Blot, Western immunoblotting, BIAcore, HPLC, Immunofluoresence and FACS An attempt was made to clone and express the variable regions of the anti-G-CCM monoclonal antibody in *Escherichia coli*

Iodinated Bolton-Hunter reagent (IBHR) was conjugated to bovine serum albumin (BSA), ovalbumin and keyhole limpet haemocyanin (KLH) These IBHR-protein conjugates were characterised by the iodide assay and the Bradford protein assay The IBHR-BSA conjugate was used to immunise six mice and a rabbit to produce anti-IBHR antibodies A monoclonal anti-IBHR antibody was not obtained However, a polyclonal anti-IBHR antibody was produced and was purified by SAS, protein A and CNBr-activated sepharose affinity chromatography This antibody was characterised by ELISA, SDS-PAGE and HPLC

Bispecific antibodies were chemically generated using (i) anti-GFAP and anti-IBHR and (ii) anti-G-CCM and anti-IBHR Ricin is a highly cytotoxic compound Bispecific antibodies were made using an acquired anti-Ricin A chain monoclonal antibody together with anti-G-CCM and anti-GFAP These bispecific antibodies were characterised by ELISA, SDS-PAGE, HPLC and FACS and may be used to target radioactive compounds and toxins to astrocytoma cells, *in vivo*

A catalytic monoclonal antibody was generated using a transition state analogue-KLH conjugate as an immunogen in six mice This antibody was precipitated using sodium sulphate and purified by protein G affinity chromatography This antibody catalysed the hydrolysis of the corresponding carbonate and its rate kinetics were determined

DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

DOLORES J. CAHILL.

DEDICATION

To my mother,

and the memory of my father

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Prof Richard O'Kennedy, for his support throughout my Ph D research Special thanks are due to Dr Ken Carroll for his helpful advice and interest during this project and to whom I am grateful

I am especially indebted to Ms Carolyn Wilson for her expert technical assistance in caring for the animals used in this project and for her support through the many difficulties encountered Dr Liz Moran is appreciated for her assistance with Western immunoblotting and for her interest and advice with the problems experienced during the production of monoclonal antibodies

I thank Prof Martin Clynes for the use of the facilities at the National Cell and Tissue Culture Centre

I would like to thank all the members of the Applied Biochemistry Group, past and present, for their friendship, kindness and assistance

I am indebted to Dr Mark Searcy, U C D , for his useful suggestions and discussions regarding the catalytic antibody work I am grateful to Dr Gerry Gallacher, University of Brighton, for supplying me with the chemical compounds and polyclonal antibody used in the catalytic antibody work

I acknowledge Dr Ellen Vitetta, University of Texas Southwestern Medical Centre, for allowing me to visit her laboratory and facilitating my research with ricin Permission to use the anti-ricin A chain antibody was gratefully obtained from Dr Patrick Trown, Xoma Corporation, California and Dr Malcolm V Pimm, University of Nottingham, supplied this antibody The technical expertise of Mrs Kate Cotter, University of Maynooth, during the FACS analysis is appreciated

I would like to thank the members of the John Barry Building, especially Fiona, Geraldine and Margaret, for their assistance with the molecular biology aspects of this research

Special thanks are due to the 'Good Time Club' for ensuring life was never dull D C U rowing club is especially appreciated for lots of happy times at Islandbridge and for many enjoyable weekends away

Finally, I would like to thank John for his unfailing support, encouragement and assistance throughout this Ph D , especially during the writing of this thesis

PUBLICATIONS

Prosser, E , Cahill, D and O'Kennedy, R , (1991)
'Antibody-mediated *in vivo* detection and treatment of disease '
J Biomed Sciences, 1 145-152

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Invited chapter for *The Encyclopedia of Analysis*, in press

Cahill, D , Moran, L M and O'Kennedy, R
'Production and characterisation of a monoclonal antibody against the human astrocytoma cell line G-CCM, which also binds GFAP, an astrocytoma associated antigen '
In preparation

ABBREVIATIONS

A	Absorbance
ADCC	Antibody-dependent cell-mediated
ATP	Adenosine triphosphate
B-Cell	B lymphocyte cell
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indoyl-phosphate
BHR	Bolton-Hunter reagent
	(3-(4-hydroxyphenyl)propionic acid N-hydroxysuccinimide ester)
BSA	Bovine serum albumen
C	Constant
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CEA	Carcinoembryonic antigen
CDC	Complement dependent cytolysis
cDNA	Complementary DNA
CDR	Complementarity-determining region
CFA	Complete Freund's adjuvant
conc	Concentration
CTP	Cytidine triphosphate
cv	Column volume
D	Diversity
DEPC	Diethylpyrocarbonate
dH_2O	Distilled water
ddH_2O	Double distilled water
DMEM	Dulbecco's modification of Eagle's medium
$DMEMS_{10}$	DMEM supplemented with 10% (v/v) FCS
DMF	N,N'-dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNP	2,4,-di-nitro-phenol
dNTP	Deoxyribonucleoside triphosphate
DPX	Distrene, dibuthyl phthalate, xylene
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
F(ab)	F(ab) fragment of immunoglobulin

$F(ab')_2$	$F(ab')_2$ fragment of immunoglobulin
FACS	Fluorescence activated cell sorting
Fc	Antibody constant region
FCS	Foetal calf serum
FITC	Fluorescein-isothiocyanate
GFAP	Glial fibrillary acidic protein
GTP	Guanosine triphosphate
H	Heavy chain
HA	Hypoxanthine 8-azaguanine
HAMA	Human anti-mouse antibody
HAT	Hypoxanthine aminopterin thymidine
H/E	Haematoxylin and Eosin stain
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
HIPO	HEPES, insulin, sodium pyruvate, oxaloacetic acid
HPLC	High pressure liquid chromatography
HRP	Horse radish peroxidase
HT	Hypoxanthine thymidine
^{131}I	Iodine-131
IBHR	Iodinated Bolton-Hunter reagent
IFA	Incomplete Freund's adjuvant
Ig	Immunoglobulin
IL-6	Interleukin-6
IMEM	Iscove's DMEM
^{111}In	Indium-111
i p	Intraperitoneal
i v	Intravenous
J	Joining
κ	Kappa light chain
KLH	Keyhole limpet haemocyanin
L	Light chain
LB	Luria Bertani broth
LMP	Low melting point
mab	Monoclonal antibody
MHV	Mouse hepatitis virus
mRNA	Messenger RNA
MWM	Molecular weight markers
n	Number of replicates
NA	Not applicable
NBT	Nitro blue tetrazolium

ND	Not determined
NRK	Normal rat kidney
OD	Optical density
ONPG	O-nitrophenyl galactopyranoside
OPD	O-phenylenediamine
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline (Dulbecco's A)
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMSF	Phenyl-methyl-sulphonyl fluoride
PVC	Polyvinyl chloride
RES	Reticuloendothelial system
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Reverse transcriptase
RXN	Reaction
SAR	Structure-activity-relationship
SAS	Saturated ammonium sulphate
SD	Standard deviation
SDS	Sodium-dodecyl sulphate
SPDP	N-succinimidyl-3-(2-pyridyldithiol)propionate
SPR	Surface plasmon resonance
Taa	Tumour-associated antigens
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
TBE	Tris-borate-EDTA
TBS	Tris buffered saline
^{99}Tc	Technetium-99
T cell	T lymphocyte
TCM	T-cell-conditioned medium
TE	TRIS-EDTA
TEMED	N,N,N′,N′-tetramethyl-ethylaminediamine
TRIS	Tris(hydroxymethyl)methylamine
TTP	Thymidine triphosphate
upH_2O	Ultrapure water
UV	Ultraviolet
V	Variable
w t	weight

UNITS

g	gravity
hr(s)	hour(s)
kD	kiloDalton
mg	milligram
min	minute(s)
ml	millilitre(s)
M	Molar
mM	millimolar
µg	microgram
µM	micromolar
N	normal
ng	nanogram
nm	nanometre
v/v	volume per volume
w/v	weight per volume

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SECTION 1 INTRODUCTION

1 1 Introduction

Cancer represents a complex group of diseases characterised by a common feature the failure of the malignant cell to respond to the usual homeostatic growth control mechanisms of the host This results in autonomous cancer cell proliferation that is passed on to all daughter cells by their malignant progenitors Thus, cancer may well be perceived, primarily, as a genetic problem Studies of cancer tissue have, however, involved multiple areas of biological research Early studies included investigations of the metabolic properties of tumour cells and, subsequently, a search for constituents, or properties, that might be unique to tumour tissue

Conventional cancer therapies exploit the fact that neoplastic cells and tissues are more rapidly dividing and usually more vascular than normal cells However, other healthy cells such as liver cells, bone marrow and germ cells also divide rapidly and are often killed non-selectively as a side-effect of such therapies (Britton *et al*, 1991) An alternative therapeutic approach is to exploit the differences in the proteins and carbohydrate antigens which are present on the surface of cancerous and healthy cells If there was a way to direct drugs and other molecules *selectively* to target cells, many diseases could be treated without affecting non-diseased tissues (Smyth *et al*, 1993) Such a selective therapy would allow the drug dosage to be reduced, the harmful side effects to be minimised and, thus, the efficacy of the drug could be improved Selective delivery systems would have wide applications, including use as vaccines to treat malaria (Coppel, 1986), against viral infections such as AIDS and hepatitis (Co *et al*, 1991), against autoimmune diseases such as arthritis (Sany, 1990), and would be especially useful in locating and perhaps treating cancer (reviewed by Goldenberg, 1993)

Although enormous advances in our understanding of monoclonal antibodies have occurred in the last twenty years, their strategic applications still constitute a major growth area in biological, biotechnological and clinical science In 1986, Kohler and Milstein were awarded the Nobel Prize in recognition of the importance of their contribution to the development of means for the production of monoclonal antibodies (Kohler and Milstem, 1975) Their true prize must be the realisation that their pioneering work has led to an explosive improvement in our understanding of immunology and has produced new possibilities for the investigation, diagnosis and treatment of many hitherto poorly understood diseases

With these advances in immunology, it was inevitable that the concepts and technology of this rapidly expanding field would be applied to studies in cancer research One such approach would be to use highly specific monoclonal antibodies which would selectively recognise the altered protein or carbohydrate antigens on tumour cells and could therefore, form the basis of selective diagnostic and therapeutic systems (Smyth *et al*, 1993)

Researchers involved in monoclonal antibody technology are currently looking at new ways of generating murine, human and genetically engineered monoclonal antibodies (Roguska *et al*, 1994) (Figure 1 1) Immunoconjugates, which are monoclonal antibodies which are chemically and genetically conjugated to drugs, toxins and isotopes, have being developed Novel ways of treating diseases, such as cancer, with monoclonal antibodies alone and with immunoconjugates are being investigated (reviewed by Hand *et al*, 1994)

The targeting of immunoconjugates to tumour cells has been a major goal in immunology and the various ways in which monoclonal antibodies may be used to achieve this aim is the focus of this review

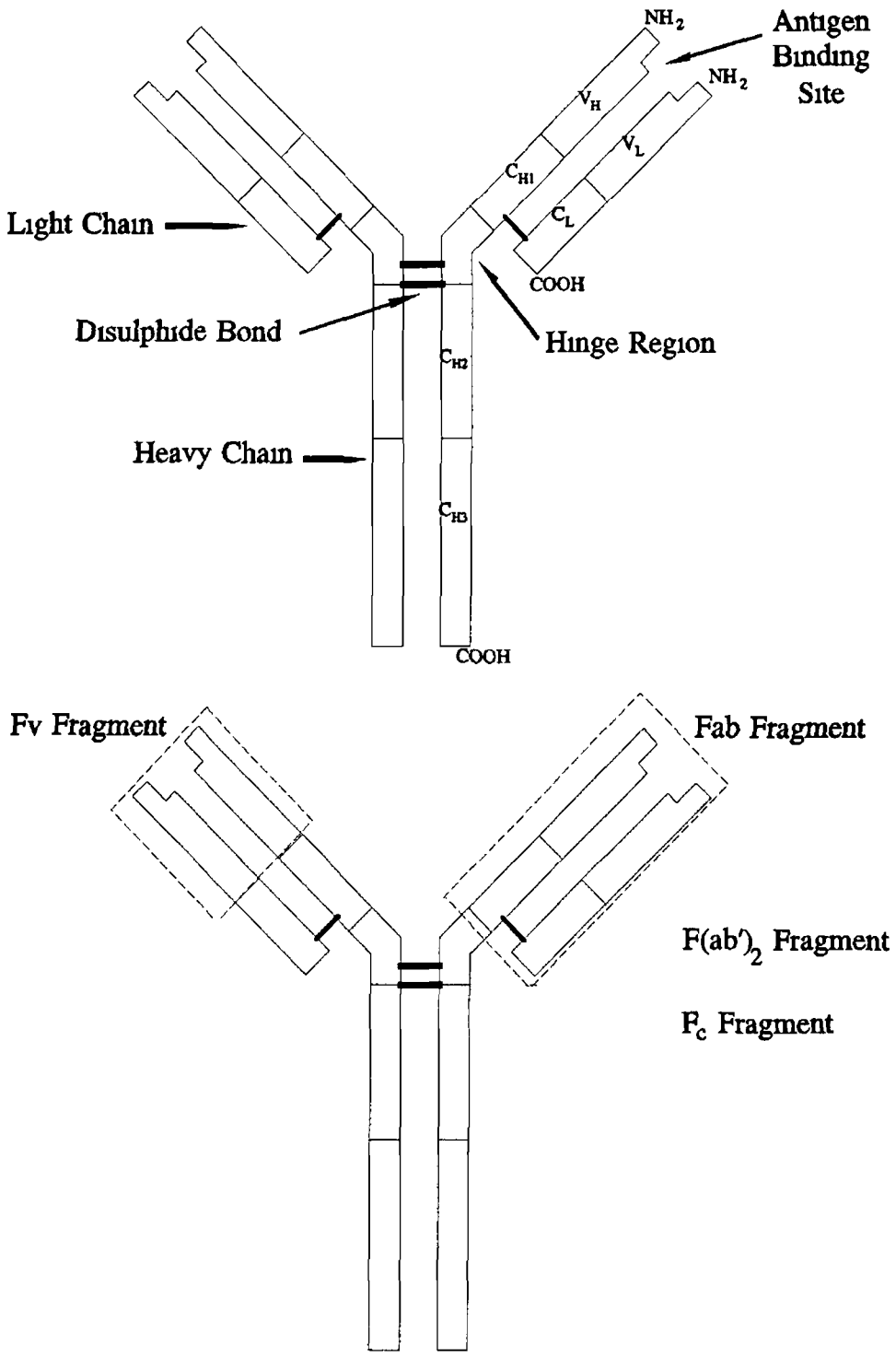

Figure 1.1. Structure of an IgG antibody which consists of heavy (H) or light (L) chains joined together by disulphide linkages The light chains can be either κ or λ and can be further divided into variable (V) or constant (C) regions Various antibody subunits or fragments can be produced, including the Fc fragment which does not bind to antigen, or the $F(ab')_2$, F(ab) and Fv fragments, which can bind to the antigen

1.2. Historical background

The development of target-specific diagnostic or therapeutic agents have been the goal of medicine for over 2000 years, for it was first noted in the medical writings of Grecian scientists, three centuries B C (Baas, 1889)

Attempts to use antibodies made against malignant tissues for cancer treatment can be traced back to the late nineteenth century In 1895, Hericourt and Richet, prepared antisera against a human osteogenic sarcoma in an ass and two dogs and claimed that these sera were effective in reducing two different neoplasms, a fibrosarcoma of the chest wall and a cancer of the stomach

At the turn of the century, the German bacteriologist and immunologist Paul Ehrlich proposed that antibodies might somehow serve to deliver chemically coupled toxic agents to specific cells, coining the term "magic bullet" for such an approach He suggested that the antigenic expression of tumours differed from that of normal cells, encouraging work to raise antibodies which would selectively localise to neoplasms (Ehrlich *et al* , 1904) The development of truly specific antibodies against tumour cells was hampered by the absence of antigens truly specific to tumours (Section 1 9) Ehrlich used his "magic bullet" technique to destroy the leptospira of syphilis without seriously affecting other tissues (Ehrlich *et al* , 1904)

Primary immunisation of isolated lymphoid tissue was first performed in 1912 by Carrel and Ingebritsen In 1929, Witebsky first described the antigenic character of neoplasms The complexity of the antigens and their respective antibodies is augmented through cross-reactivity with other proteins and the fact that tumour-associated antigens can be found in normal tissues It was not until 1948 that Pressman and Keighley successfully labelled antibodies with radioactive isotopes They showed that rabbit antibodies to rat kidney could be labelled with Iodine-131 (^{131}I) without loss of affinity and that, after intra-venous injection, they would localise in the kidneys of living rats Since this important discovery, a series of investigators produced antibodies that appeared to have relative specifity for various tumours (Section 1 9), but these antibodies were later shown to react with fibrin produced in association with rapidly proliferating cancers, and, therefore, were not tumour-specific

In 1957, Pressman and co-workers demonstrated, using a "paired labelling" technique, that anti-tumour antibodies specifically localised in tumours but that radiolabelled rabbit IgG did not Such localisation could be determined by means of radionuclide imaging

(Section 1 13) At this point in time, there were two major limitations to successful diagnostic imaging,

(a) tumour-specific or tumour-associated antigens had not been completely defined and (b) techniques for producing and purifying large quantities of highly specific antibodies to these antigens needed refinement

The first problem was resolved following the immunochemical elucidation of tumour associated antigens, such as carcinoembryonic antigen (CEA) (Gold and Freedman, 1965) and α-fetoprotein, (Section 1 9) Table 1 1 outlines the potential uses for any marker of human tumour growth The second problem was solved following the development of hybridoma technology

In 1965, Gold and Freedman reported the discovery of a component of human gastrointestinal carcinomas, using radiolabelled polyclonal antisera, which was present in the fetal digestive system but was absent from normal gut and cancers of other tissues, this was termed CEA Subsequently CEA has been found to be present in a wide variety of malignant and normal tissues but there is no doubt that it is quantitatively increased in the majority of gastrointestinal cancers (Jaffe, 1990) (Table 1 1) Micheal Potter then induced myelomas in mice which produced large amounts of monoclonal immunoglobulins (Potter and Boyce, 1962) However, it was not possible at this time to induce tumours to synthesise antibodies to an injected antigen Eventually, Kenko Horibata and A W Harris were able to establish a number of lines which they distributed to other laboratories

Table 1 1 Potential uses for human tumour markers

POTENTIAL USES FOR HUMAN TUMOUR MARKERS
Population screening
Adjunct in the diagnosis of cancer
Preoperative indicator of tumour burden
Indicator of therapeutic success
Evidence of postoperative success
Use in tumour localisation

Cesar Milstein subjected a line derived from one of Potter's tumours to intensive study

The problem of propagating a particular clone of B lymphocytes dedicated to the synthesis of a particular antibody was overcome by the development of hybridoma technology by George Kohler and Cesar Milstein in Cambridge Kohler devised a way to fuse B lymphocytes with the related, but malignant, myeloma cells The resulting hybrid cells, or hybridomas, are like B cells in that each produces a single antibody, and like myeloma cells in that they can be grown indefinitely in culture A single hybridoma cell can be grown into a clone of identical cells, which serves as a continuing source of a "monoclonal" antibody against a specific antigen Such a culture can be made to produce any required antibody for as long as it is needed Using Kohler and Milstein's technique, an antigen against which an antibody is required, is injected into a mouse or rat Later, the spleen cells, which are producing the antibody against the antigen are isolated and fused with fast-growing cells in culture The resulting hybridoma (Kohler and Milstein, 1975), properly tended, will go on producing the desired antibody indefinately Such hybridomas are screened for the production of monoclonal antibodies that bind to target cells in culture or to their membranes, but do not bind effectively to a panel of control cells or membranes Murine antibody production is further discussed in Section 2 3 13

The advent of hybridoma technology represented a quantum leap in the field of tumour immunology and since its discovery nearly 20 years ago, many murine monoclonal antibodies to human tumour cells have been made and many antigens have been identified (Section 1 9) Most of these monoclonal antibodies were, however, derived from spleen cells of mice or rats immunised with whole human tumour cells or cell extracts Thus, the results obtained may not directly reflect the intrinsic immunogenicity of human tumour cell antigens in a tumour-bearing host The difficulties which are encountered in producing stable human hybridomas prompted several researchers to 'by-pass' hybridoma technology and, in some instances, animal immunisation altogether (Ward *et al*, 1989) The use of mouse-human chimaeric monoclonal antibodies, humanised monoclonal antibodies and their production in non-myeloma cells, yeast, bacteria and even plants has renewed interest in monoclonal technology (reviewed by Griffiths, 1993) This is discussed in further detail in Sections 1 21 , 1 22 and 1 23

The first publication describing the generation of true human-human hybridomas secreting specific antibody appeared in 1980 (Olsson and Kaplan, 1980) Croce and co-workers (1980) showed that human hybridomas producing monoclonal antibodies could

be produced by fusing human B-cells which secreted the required antibodies, with human myeloma fusion partners However, this technology has taken longer to establish than the corresponding murine technology Hurdles which have limited the development of human monoclonal antibodies include the source of B-lymphocytes, immunisation strategies used, techniques used to immortalise the immunised B-cells, screening methods and problems encountered with large-scale production of human monoclonal antibodies (reviewed by Hand *et al* , 1994)

1.3 Antibody structure and function

The basic structure of an antibody is shown in Figure 1 1 , a more detailed structure is shown in Figure 5 2 1 An antibody is a glycoprotein that consists of two identical heavy (H) and light (L) chains, either kappa (κ) or lambda (λ), which are joined together by disulphide linkages (Roitt *et al* , 1985) Each chain is made up of a variable region (V) and a constant region (C) known as domains The variable region is responsible for antigenic binding and the constant region for effector functions such as complement fixation and antibody dependant cell cytotoxicity (ADCC) There is a hinge region in the heavy chains which facilitates conformational changes in the antibody which occur during circulation in the organism or following binding to antigens The binding site of the antibody is situated in the variable region at the -NH end of both the H and L chains The constant regions of the antibody chain provide a backbone structure The specificity for antigen binding is determined by the three-dimensional structure of the antibody combining site and the precise interaction depends on the amino acid residues that react with the antigen (O'Kennedy and Roben, 1991)

An antibody has a molecular weight of approximately 150,000 - 160,000 daltons The light and heavy chain variable regions (V_L and V_H, respectively) consist of a highly conserved framework structure linked by three hypervariable loops, known as complementarity-determining regions (CDRs), on each of the V_L and V_H chains, which are involved in defining the binding specificity of the antibody The Fc portion of the antibody is involved in binding to surface receptors and contains carbohydrates and has important physiological roles Antibody fragments, e g Fab' and $F(ab')_2$ portions, may be produced by enzymatic cleavage and genetic engineering These have advantages over the whole molecule in certain applications (Cahill *et al* , *in press*)

1 4 The genetics of antibody diversity.

The human body can produce over 200 million antibodies, which is sufficient for every known antigen it may encounter (Roitt *et al*, 1985) The basis of such a vast diversity of antibody molecules is genetic It has been mentioned that antibody light chains can be classified into two main types, κ and λ, based on the amino acid sequence of their constant regions (Section 1 3) There are three λ types and one κ, and any B cell produces only one type of light chain The genes for λ, κ and heavy chains are genetically unlinked However, at each locus, the variable (V) genes are linked to their respective constant (C) genes In humans, the location of the λ and κ light chain genes and heavy chain genes are on chromosomes 2, 22 and 14, respectively The genetic basis of antibody diversity has been reviewed by O'Kennedy and Roben (1991)

1 5 Production of antibodies

When an animal has been immunised with an antigen, on obtaining an immune response, the animal may be bled for polyclonal antiserum or, if a monoclonal is required, a fusion may be carried out (Section 2 3 13) Production of polyclonal antibodies rather than monoclonal antibodies are often more practical They have the advantages that they can be synthesised quickly and relatively cheaply and do not require the same amount of time or level of expertise associated with the production of monoclonal antibodies At present the major production methods for monoclonal antibodies involve the use of ascites fluid generation (Section 2 3 17) and large scale cell culture The production of ascites fluid gives good yields but there are difficulties with the large number of animals required, ethical considerations, high labour costs, the presence of endogenous antibody, and the possibility of contaminating viruses The methods of antibody production, isolation and purification have been reviewed by Cahill *et al* (*in press*)

While large scale cell culture is technologically advanced, the costs are high, mainly due to media requirements This has resulted in research aimed at the generation of antibodies by genetic means using a range of systems including yeast, bacteria and baculovirus expression systems (Section 1 21) and plants (Hiatt and Ma, 1993) Humanising rodent antibodies is an important development which can be achieved through genetic engineering (Section 1 23) and has consequences for human therapy and tumour imaging (Section 1 7)

1 6 Monoclonal antibodies in clinical situations.

It became apparent soon after the discovery of monoclonal antibodies that their selectively and sensitivity would be of medical importance and they have been used to diagnose and treat a variety of diseases Monoclonal antibodies have been used to treat autoimmune disorders such as rheumatoid arthritis (Sany, 1990) They have been used as vaccines to treat malaria (Coppel, 1986), as anti-viral agents (Co *et al* , 1991) and to catalyse the breakdown of cocaine (Landry *et al* , 1993) They have also been used to diagnose non-specific infections and myocardial necrosis (Strauss *et al* , 1991) Particular interest has been expressed, however, in the application of monclonal antibody technology to the detection and treatment of cancer (reviewed by Goldenberg, 1993) The current uses of monoclonal antibodies in oncology are presented in Table 1 2 Several factors influence the type of monoclonal antibody or conjugate which can be used in a particular clinical application (Prosser *et al* , 1991) These factors include tumour cell heterogenity (Section 1 8), the intended usage of the antibody (whether for detection or therapy), the antigen target, the antibody unit used, (whether whole antibody, $F(ab')_2$ or F_v fragment) (Figure 1 1), and the distribution and pharmokinetics of the monoclonal antibody when administered to patients

A more fundamental understanding of cell growth regulation could also provide novel approaches for detecting, preventing and treating different cancers (reviewed by Bast, 1993) Activation of protooncogenes or loss of tumour suppressor genes can have both a prognostic and therapeutic importance Bast, (1993) envisages that ultimately, molecular analysis of individual cancers will guide the application of specific therapies to inhibit activated oncogenes or restore the function of tumour-suppressor genes Circulating growth factors, oncogene products, and tumour-associated antigens may provide markers for earlier detection of some cancers

Table 1 2 Current applications of monoclonal antibodies in oncology

Current applications of monoclonal antibodies in oncology
Diagnosis and detection.
Immunohistopathology for detecting and classifying cell types by the presence or absence of markers in tissues and cell smears
Analysis of body fluids (serum, urine, effusions, etc) for the presence of marker substances by immunoassays
Nuclear imaging with radiolabelled monoclonal antibodies
Intraoperative use of γ–detection probes with radiolabelled monoclonal antibodies
Treatment
Direct cytotoxicity of monoclonal antibodies (complement– or ADCC–mediated)
Inhibition of growth–factor receptors
Immunoconjugates (drugs, toxins, isotopes)
Ex vivo purging of tumour cells from harvested bone marrow
Anti–idiotype monoclonal antibodies vaccines

1 7 Antibody biodistribution and targeting

The use of monoclonal antibodies in cancer detection and therapy in humans requires considerable testing for sterility, pyrogenicity, adventitious viruses of the host cells, and general safety, as prescribed by guidelines from the Food and Drug Administration in the U S A (Hoffman *et al* , 1985)

When an antibody is injected into the bloodstream it passes through a number of campartments including the vascular and extravascular spaces (Table 1 3) as it is metabolised and excreted The journey and potential barriers for an injected antibody which is required to target a tumour partially explain why such a small portion of the injected immunogloublin, between 0 0007% and 0 01% of the injected dose per gram of tissue, targets to tumour (Epenetos *et al* , 1985) After injection into the bloodpool, the initial problem is whether the immunoglobulin binds with circulating antigen or with antigen on blood cells, which would result in antigen–antibody complexes that could reduce the available immunoglobulin and possibly evoke deleterious immune complex disease Initial experience with the injection of anti–CEA immunoglobulin into patients with high blood CEA levels confirmed the formation of CEA–anti–CEA complexes, but

did not show any effect on tumour targeting or adverse reactions (Primus and Goldberg, 1980) Another form of antigen-antibody complexation, involving the sensitisation of patients with the foreign, murine immunogloublin, can have more significant effects on targeting After exposure to whole murine immunogloublin, or repeated injections of immuno-globulin fragments, patients show the formation of human anti-mouse antibodies (HAMA), which increase a patient's risk for serum sickness and anaphylactoid reactions and prevent targeting of the immunoglobulin by complexing it and causing its degradation and excretion The anti-antibodies can be detected in some patients as early as three weeks after injection and in some circumstances can be present for months or years later There is considerable variability in such responses among different patients receiving the same monoclonal antibody Some immunogloublins have shown binding to tumour antigen present in certain normal organs, such as the liver, thus interfering with specific tumour targeting Other factors influencing immunoglobulin targeting are discussed in Section 1 8

1 8 Barriers to targeting of immunoconjugates to tumour cells

Tumour vascularisation and vascular permeability are factors that can affect antibody targeting, and it has been found in tumour model studies that blood flow and vascular permeability can vary between tumour hosts and between tumours in different sites of the same animal The location and density of the antigen being targeted by antibody are also important factors Although current precepts assume that the antigen target needs to be on the cell surface to be accessible, examples of successful targeting when the antigen is present in the external milieu or intracellularly are known (Blumenthal *et al*, 1989) It is generally believed that increased antigen density on the tumour cell results in increased antibody targeting Also of importance is the nature of the antibody's avidity, or binding capacity, for the antigen, which determines the amount and duration of antibody attachment to tumour The normal physiologic and/or anatomic barriers to successful targeting are given in Table 1 3

A major problem in effectively treating cancer is that of tumour cell heterogeneity The cellular heterogeneity of neoplasms has been known since the last century, when historical studies first identified morphological differences, among cells within the same tumour In 1954, Foulds formally documented the existence of distinct morphologies in different areas of a mammary tumour Since then, the use of increasingly sophisticated

methods, and clonal analysis in particular, has revealed extensive heterogeneity in the expression of various phenotypic properties by tumour cells in both primary and metastatic lesions in the same host These include differences in karyotype, function, differentiation, degree of malignancy, antigenicity, immunogenicity, biochemical properties, growth behaviour, susceptibility to destruction by chemotherapeutic drugs and radiation, and the ability to evade the humoral and/or cell-mediated immune reactions mounted by the host

There may be hundreds of subpopulations of cells within a single tumour because of tumour cell heterogeneity which has profound implications for the use of antibodies as targeting ligands (Smyth *et al*, 1993) Indeed, many tumours are composed of only small proportions of malignant cells, the remainder being supportive connective tissue and infiltrative elements, including different blood cells Tumour heterogeneity can be overcome in therapy with radioactive antibodies, since the higher-energy isotopes can kill from a distance of several cell diameters, thus affecting antigen-negative cells within a mixed tumour cell population (Section 1 13)

Finally, tumours at different sites of the body may be amenable to improved targeting when the monoclonal antibodies are administered by the most direct route for tumour accretion The intravenous route has been the most popular means used to target disseminated disease, but when the malignancy is limited to a site or body cavity, intralesioal or intracavitary application, such as intraperitoneal or intrathecal injection, has proven efficacious, both for imaging and therapy

These various barriers and impediments to monoclonal antibody targeting reduce the absolute amount of immunoglobulin accumulated by the tumour, which is a major problem when monoclonal antibodies are used to deliver a therapeutic agent For tumour imaging, however, it is only the *relative* amount of radioactivity deposited in tumour, as compared with that deposed in adjacent tissues, that is responsible for producing the lesion's image The process of cancer imaging with radioactive monoclonal antibodies is gaining in usefulness and acceptability (Goldenberg, 1993)

Table 1.3. Barriers to immunoconjugate targeting *in vivo*.

HOST	TUMOUR HETEROGENEITY
Endothelium and basal lamina	Phenotypic drift/cellular instability
Plasma membrane	Inherent sensitivity to drug
Basement membrane	Development of drug resistance
Blood–brain barrier	Antigenic drift
Epithelial membranes	Membrane transport
Reticuloendothelial system (RES)	Metastatic potential
Immune system (non–RES)	
Inactivating enzymes	
Biodegrading enzymes	

1.9. Cocktails of monoclonal antibodies.

Tumour cell heterogeneity, particularly with regard to antigen expression, can be mitigated by the combined use of a "cocktail" of different antibodies directed against different tumour–associated antigens (Blumenthal *et al.*, 1991).

Hybridoma technology has resulted in the identification of a considerable number of new tumour–associated antigens (reviewed by Goldenberg, 1993) and also in antibodies against different epitopes of the same antigen. In lung cancer, for example, more than 50 monoclonal antibodies have been described (Stein and Goldenberg, 1991). Nevertheless, no truly tumour–specific antibody or antigen has, as yet, been elucidated. Most tumour antigens identified by anti–tumour antibodies appear to be quantitatively increased in neoplasia and frequently react with a wide range of carcinomas so that their antibodies are considered to be pancarcinomic in specificity. Indeed, it is rare to find a monoclonal antibody that is specifically reactive with a single organ or tissue type, whether normal or neoplastic. Despite these limitations, numerous applications of monoclonal antibodies in cancer diagnosis, detection, monitoring, therapy and prognostication have been identified or proposed (Dillman, 1989), as summarized in Table 1.2.

The applicability of monoclonal antibodies in diagnostic histopathology is particularly widespread and can be used for classifying tissues and tumours according to their expression of certain defined markers that reflect differences in cellular genesis,

differentiation and biology (Jaffe, 1990) Immunohistochemical identification of neuroendocrine differentiation in some cases of non-small-cell lung cancers has been positively correlated with response to chemotherapy (Mulshine *et al*, 1991) Of particular interest is the determination of the organ or tissue of origin of undifferentiated metastases Monoclonal antibodies against certain organ-associated antigens, such as prostate-specific antigen, prostatic acid phosphatase, placental alkaline phosphatase, human chorionic gonadotropin, α-fetoprotein, CEA and others, can assist the pathologist in making a diagnosis (Jaffe, 1990) Unfortunately, immunohistology has not enabled a differentiation to be made between malignant and benign lesions, but antibodies can assist in distinguishing primary from metastatic tumours, such as in the lungs (Goldenberg *et al*, 1983) The detection of occult metastases by immunocytologic analysis of bone marrow and other tissue aspirates, as well as lymph nodes and various body fluids, has also proved feasible with selected monoclonal antibodies

The most established application of anti-tumour monoclonal antibodies has been for the measurement of circulating tumour-associated markers, such as CEA, in body fluid immunoassays The lack of tumour specificity of these markers precludes their general use in cancer screening and diagnosis, but they have shown value as diagnostic aids in high-risk groups and to monitor disease absence or recurrence in treated patients (Herberman and Mercer, 1990)

Without the knowledge of markers for all tumour cells, a "typing panel" of receptors will be necessary Avner and co-workers (1989) have shown that it is possible to create therapeutic monoclonal antibodies to an individual patient's tumours within a 6- to 9-month time-frame Their panel consisted of 20 distinct antibodies that bind to different cancer cell surface molecules and epitopes, and antibodies from this panel were used to type patient biopsies These antibodies may be used to create a customised "cocktail" of antibodies, if required, that could be conjugated to chemotherapeutic drugs, toxins or radioactively labelled nucleotides Effective targeting will probably require such cocktails of different antibodies, with different cocktails needed to treat tumours in different patients (Poste, 1986)

A monoclonal antibody cocktail could be composed of different IgG isotopes, such as IgG1 or IgG2 to manipulate different host effector functions IgM and IgG in combination may prove useful, as would a cocktail of high and low affinity antibodies, these would also be designed to manipulate tumour antigens to augment the host's ability

to destroy these cells immunologically Combinations of the above may prove to be more effective The human Fc portion may interact more efficiently with human complement and/or effector cells It is currently undetermined whether IgG1, IgG2, IgG3 and IgG4, either individually or in various combinations, will have any clinical value, or whether whole antibody, $F(ab')_2$, F(ab) or F_v (Figure 1 1) fragments would be more effective The IgM class of antibodies are much larger than the IgGs (Section 3 19) and may not traffic as well, but a combination of an IgM variable region with an IgG heavy chain may circumvent this problem (Glassy and Dillman, 1988) Clinical trials using cocktails of murine monoclonal antibodies are already in progress and those with human monoclonal antibodies will be commencing in the near future (reviewed by McKearn, 1993)

Techniques of molecular biology may be useful to class switch the heavy chains of human monoclonal antibodies (Section 1 21) (Shimizu and Honjo, 1984) which could be of some clinical significance for using monoclonal antibodies alone (Section 1 19) There are differences in the cytotoxic effects resulting from interactions with complement binding and cytotoxicity, IgM is superior to IgG1, which is greater than IgG3, which is greater than IgG2, IgG4 has no apparent activity (Shakib, 1986)

1 10 Targeting of drug immunoconjugates to tumour cells

Most of the drugs used in cancer treatment non-specifically kill all growing cells, resulting in often unacceptable side effects However, considerable ingenuity has been devoted over the past decade to the development of systems for improved drug delivery in cancer treatment These range from efforts to reduce toxicity using (a) rate controlled release preparations and implantable devices, (b) to improving the effectiveness of anti-tumour drugs by using new formulations with improved pharmokinetic and/or pharmacodynamic profiles, (c) to introducing chemical groups for conjugation well separated from the active site which allow covalent linkage of the drug to the antibody without affecting the drug potency or toxicity, (d) to using active groups or carrier molecules such as dextran, polyaminoacids or human serum albumin to minimise reduction in drug potency

Each monoclonal antibody usually has several drug molecules attached to it in order to deliver a potent dose of drug to the selected cell Even when very active drug immunoconjugates are targeted to the most drug-sensitive tumour cells, between 10^6 to

10^7 drug molecules must be accumulated in a tumour area to mediate tumour killing (Reisfeld and Schrappe, 1990) Johnson *et al* (1987) showed that many of the commonly used drugs lose much of their activity following binding to proteins This loss in activity has been attributed to steric hindrance, altered mechanisms of cellular uptake and reduction in the drug half-life following linkage

The target for these drugs is every tumour cell in the patient, since any remaining tumour cells cause recurrence Potential targets for drug targeting to tumour cells include tumour-associated antigens, growth factor receptors and attachment molecules on tumour cell surfaces Other targets include oncogenes, oncogene products and the organ or tissue where the tumour resides

Khawli *et al* (1994) developed seven novel vasoactive immunoconjugates that selectively alter vascular permeability and/or blood volume of tumours *in vivo* in order to enhance monoclonal antibody uptake in tumours These immunoconjugates were composed of IL-1 β, IL-2, tumour necrosis factor-α, physalaemin leukotriene B4, histamine and bradykinin chemically linked to TNT-1, a murine monoclonal antibody that binds necrotic regions in tumours All of these immunoconjugates showed specific enhancement of monoclonal antibody uptake in tumours with no changes seen in normal tissues These studies of Khawli *et al* (1994) indicate that pretreatment with vasoactive immunoconjugates may improve monoclonal antibody uptake in tumours significantly and, thereby, increase the therapeutic index of monoclonal antibody-directed immunotherapy

Seelig *et al* (1994) developed a method for predicting the ability of drugs to cross the blood-brain barrier The tight endothelium of brain capillaries constitutes the permeability barrier for the passive diffusion of substances from the blood stream into the central nervous system To reach the brain, a molecule has first to be absorbed from the blood into the endothelial cell, where it is then released into the brain A prequisite for a substance to diffuse through the blood-brain barrier is therefore a certain degree of lipid solubility They propose an approach which takes advantage of the surface activity of the molecule of interest Three properties of a drug molecule have been suggested to determine its ability to cross the blood-brain barrier, (i) the number of lipophilic groups, (ii) the number of charged groups and their extent of ionisation, and (iii) the molecular size Their study suggests a method of predicting the potential of drugs to cross the blood-brain barrier

Many researchers have abandoned the use of conventional drugs and are beginning to produce new drugs specifically designed for monoclonal antibody conjugation These drugs have been termed structure-activity-relationship (SAR) compounds, and have been designed with both the potency of the drug and the immunoreactivity of the antibody in mind The developments of new SAR drugs such as KS1/4 - DAVLB, a vinblastine derivative (Koppel, 1990), mark the beginning of SAR immunoconjugates

1 11 Targeting of toxin immunoconjugates to tumour cells

Cytotoxic drugs frequently will be less potent than plant or bacterial toxins Toxins were proposed as suitable agents for immunotherapy because of their extreme cytotoxivity A wide array of toxins have been conjugated to monoclonal antibodies for the production of immunotoxins (Vitetta and Thorpe, 1991) Many of the toxins consist of two protein chains a B-chain for cell binding and an A-chain that inhibits protein synthesis Ricin, from the castor bean, is the prototype toxin, whose B-chain is replaced by the monoclonal antibodies to which the A-chain is conjugated Toxins, such as ricin, abrin, pokeweed antiviral protein, gelonin, saporin, diphtheria toxin, and *Pseudomonas* exotoxin, are considerably more toxic on a molecular basis than most chemotherapeutic agents, but most of these toxins are much larger in size For example, ricin A-chain has a molecular weight of 32,000, whereas most chemotherapeutic agents have molecular weights less that 1,000 (e g doxorubicin has a molecular weight of 544) Most natural toxms are themselves immunogenic, thus enhancing the immune response to the immunotoxin These immunotoxins exeit their action in the cytoplasm, where they inhibit protein synthesis After binding to cell surface antigens, they are endocytosed into the cell and incorporated into endosomes (Vitetta and Thorpe, 1991) Some toxin fragments, such as diphtheria toxin, translocate across the membrane of the endosome, whereas others, such as ricin, are transported to the trans-Golgi network, from where they enter the cytoplasm However, most are routed to lysosmes, which degrade the toxins In the cytoplasm, the toxins act on adenosine diphosphate-ribosylate elongation factor 2 (e g , *Pseudomonas* exotoxin) or inactivate the 60S ribosomal subunit, affecting its binding to elongation factor 2 (e g ricin) It has been estimated that less than 10 toxin molecules in the cytoplasm are sufficient to kill a cell (Waldmann, 1991), but considerably more must bind to the cell surface in order for this minimal number to be internalized and translocated

There have been a number of clinical trials in patients with cancer with the initial group of immunotoxins, which were generally unstable *in vivo* Among 127 patients treated in 10 clinical trials with immunotoxins against breast, colorectal, ovarian, and lymphoid malignancies, only 2 complete and 8 partial remissions were observed (Waldmann, 1991) Ricin A-chain conjugated to an IgG2a monoclonal antibody directed against a high-molecular-weight glycoprotein expressed by melanomal cells was given to melanoma patients over a 5-day schedule, and 3 partial responses out of 43 patients treated were reported However, more promising results have been obtained with modified, or second generation, immunotoxins in cancer patients, or when benign diseases were treated with these agents Since one of the problems of using native ricin A-chain, even when bound to monoclonal antibody, is its binding to liver cells via its carbohydrate residues, second-generation deglycosylated A-chain ricin immuno-conjugates are preferably used in clinical trials For example, Vitetta *et al* (1991) recently reported partial responses in 38% of 14 evaluable patients with B-cell lymphomas, and in patients with antigen-positive tumour cells, half of the patients achieved a transient, partial response, lasting between 1 and 4 months At doses of 75mg/m^2 or greater in this phase I trial, three of four patients achieved a partial response Dose-related toxicities included vascular leak syndrome, fever, anorexia, and myalgia, while dose-limiting toxicities involved pulmonary edema and/or effusion, expressive aphasia and rhabdomyolysis with reversible kidney failure There was no liver dysfunction noted, supporting the value of using a deglycosylated ricin A-chain Four of 14 patients (29%), however, made antibody to the immunotoxins, 3 to the ricin, and 1 to both ricin and murine antibody (Vitetta *et al* , 1991)

Even when the ideal antibody and toxin are selected, there still remain the problems of conjugating the toxin to the monoclonal antibody, large-scale production of the immunotoxin, the evocation of human antibodies against the monoclonal antibody and against the toxin, and a dose-limiting capillary leak syndrome, manifested by normal liver function with hypoalbuminemia and edema

Kuzel *et al* (1993) conducted a phase I clinical trial of a diphtheria toxin/IL-2 fusion protein This is a construct in which the native diphtheria toxin-receptor binding-domain has been replaced with human IL-2 This molecule is specifically cytotoxic *in vitro* within 30 minutes for cells that express the high affinity IL-2 receptor The purpose of the study was to investigate the biologic activity of the fusion protein, when

administered three times daily, in terms of toxicity, pharmacokinetics and anti-tumour effects in patients with IL-2 expressing malignancies, such as mycosis fungoides, and other non-Hodgkin's lymphomas Biologic activity was noted in patients with mycosis fungoides, with skin lesions clearing

Siegall *et al* (1994) showed that a single chain immunotoxm fusion protein could cure human breast carcinoma xenografts in athymic mice and rats The immunotoxm was targeted to the tumour cells using an antibody, and was rapidly internalised into these cells

EGF-receptor is amplified or overexpressed in many malignant gliomas and other primary brain tumours, but is low or undetectable in normal brain In a study of Phillips *et al* (1994), this differential expression has been exploited for targeting brain tumours using a transforming growth factor-α-*Pseudomonas* exotoxin, a recombinant toxin Treatment of nude mice bearing glioblastoma or medulloblastoma subcutaneous xenografts with this toxin produced tumour regression and growth delay Their results suggested that the fusion protein was active against primary human brain tumours which had moderate to high EGF-receptor expression For intracranial tumours, they suggest a better method would be direct delivery of the fusion protein into the brain tumours by controlled-release biodegradable polymers or intratumoural implantable catheters, which may represent clinically useful applications of recombinant toxin therapy in tumours with high EGF-receptor expression

Another phase I trial of this diphtheria toxin/IL-2 fusion protein has been reported (Tepler *et al*, 1994) This fusion protein was given as a brief infusion in 15 patients with refractory lymphoid malignancies One complete response was observed in a patient with Hodgkin's disease in relapse and the patient remained free of disease more than two years after completion of therapy The dramatic anti-tumour response seen in one patient and the relative tolerance of the fusion protein in the other patients, indicated the potential utility of this target agent in IL-2-expressing haematologic malignancies

Fishwild *et al* (1994) conjugated gelonin and ricin A-chain, both ribosomal inactivating proteins, to monoclonal antibodies which bind to specific cells and, upon internalisaion, inhibit protein synthesis, ultimately resulting in cell death This study reported that gelonin anti-T cell monoclonal antibodies conjugates were more cytotoxic than their ricin A-chain counterparts when tested against human peripheral blood mononuclear cells Gelonin is more toxic than the ricin A-chain Fishwild *et al* (1994) showed that

a 10 minute exposure to a gelonin immunoconjugate was as effective as a 90 hour exposure to the ricin A-chain immunoconjugate *in vitro* These properties of gelonin might be advantageous for immunoconjugates made with other monoclonal antibodies or receptor-binding molecules

1 12 Adverse effects of monoclonal antibody therapy

Several limitations occur with delivery systems themselves and in order to have an effective immunoconjugate targeting system several criteria must be satisfied (Table 1 4)

Toxic reactions to the administration of monoclonal antibodies are foremost immune reactions, particularly to foreign antibodies, such as murine monoclonal antibodies The nature of the immune reactions is governed by (1) whether it is the monoclonal antibody binding to its antigen target, particularly when it is a circulating antigen and (2) if the administered monoclonal antibody evokes host antibodies (HAMA) (Catane and Longo, 1988) The clinical manifestations of both mechanisms can be quite similar, thus making a differentiation difficult In the first instance, fever, chills, urticaria, dyspnea, hypotension and diarrhoea can occur They are observed fairly soon after monoclonal antibody infusion, are dose-related, and are affected by rate of administration In the case of HAMA, the reactions usually are unrelated to dose and rate of administration and and can be manifested by anaphylaxis, serum sickness, urticaria, fever and hypotension HAMA responses can alter monoclonal antibody pharmcokinetics, impairing targeting and therapy and enhancing monoclonal antibody clearance The HAMA response can involve the production of anti-framework, anti-isotypic and anti-idiotypic antibodies, depending on the particular monoclonal antibody and the dosage schedule Even monoclonal antibodies can evoke anti-allotypic and anti-idiotypic antibody responses

When a toxin is conjugated to a monoclonal antibody, it too can be immunogenic Other adverse clinical manifestations have included flu-like, capillary leak symptoms and hepatic enzyme elevations (Vitetta and Thorpe, 1991) Despite the diverse reactions to unlabelled and conjugated monoclonal antibodies, the clinical experiences to date have indicated that these are relatively safe cancer therapies, and the adverse reactions generally have been easy to control, such as by changing antibody dose, changing infusion rate, administering corticosteroids and antihistamines or possibly treating with

cytoxic immunosuppressants (Goldenberg, 1993)

Another major obstacle to effective cancer chemotherapy is the resistance of tumour cells to cytostatic agents Tumour cells frequently express a multidrug-resistance phenotype Efferth and Volm (1992) have shown that multidrug resistant tumour cells can be effectively killed by monoclonal antibodies, toxins or bispecific antibodies This study has shown that an antibody-directed therapeutic approach to eradication of multidrug-resistant cells might be a promising adjunct to conventional chemotherapy of cancer patients

Table 1 4 Requirements for an immunoconjugate delivery system

REQUIREMENTS FOR AN IMMUNOCONJUGATE DELIVERY SYSTEM
Recognition and interaction of carrier and target
Localisation to target tissue/organ
Delivery of therapeutic concentrations without significant uptake by non-target tissues
Protection of immunoconjugate from premature biodegradation
Protection of immunoconjugate from premature inactivation
Carrier must be able to incorporate a broad spectrum of immunoconjugates without loss of activity
Release of drug/toxin in a controlled predetermined manner once the immuno-conjugate-carrier is at the target
Biodegradable
Biologically inert
Immunogenic, only if required for targeting
Production should be straightforward and reproducible
Clinically cost effective

1 13 Radioimmunoconjugates

Isotopes conjugated to monoclonal antibodies (radioimmunoconjugates) have distinct clinical applications, either in diagnosis by immunoscintigraphy or treatment by radioimmunotherapy (O'Kennedy *et al*, 1993) The aim of radioimmunotherapy is to irradiate and kill antigen-bearing tumour deposits throughout the body with minimal

radiation to normal tissues

The limited success of radioimmunotherapy *in vivo* has been attributed to inability to target sufficient doses of isotope to the tumour Britton and colleagues (1991) reported that one day after the administration of a radioimmunoconjugate, approximately 30% of the administrated radioactivity has been excreted They also reported that an average of 1% of the administered activity is actually located to the tumour site, while 69% accumulates in normal tissues (liver, kidneys, bone marrow and with iodinated compounds, the thyroid) This leads to extensive lethal irradiation of non-specific tissues In many cases far less than 1% of the administered radiation may reach the tumour site Therefore, tumour selection and the route of administration are important parameters in effective radioimmunotherapy

Although tumour imaging only requires a high target/nontarget ratio of counts for optimal results radioimmunotherapy depends on a high concentration of tissue reactivity for a long duration In contrast to drug and toxin immunoconjugates, radiolabelled monoclonal antibodies can kill cells that are at a distance from the targeting, depending on the choice of radionuclide, without the monoclonal antibodies conjugate being internalised Therefore radiolabelled monoclonal antibodies can distribute their cytotoxic energy to antigen-negative cells in the neighbourhood of antigen-positive cells The attractive feature of radioimmunotherapy is the prospect that most normal tissues are spared intensive radiation As in the case of other immunoconjugates, the radionuclide should be linked tightly to the monoclonal antibody without affecting targeting Technically, depending on the choice of radionuclide, considerable progress has been made in the chemistry of antibody radiolabelling Some radionuclides, such as the isotopes of iodine, can be conjugated directly to the antibodies, while others are linked by an intermediate group, such as chelating groups for radiometals

The fact that tumours can accumulate globulins and other macromolecules non-specifically was first reported by Duran-Reynolds in 1939 This phenomenon has already been used effectively in brain tumour imaging with ^{131}I labelled human serum albumin (Chou *et al*, 1951) The success of this method of brain tumour visualisation depended on the intact blood-brain barrier excluding the protein from normal surrounding brain The absence of such a barrier in other tissues has made it difficult to image tumours with non-specific proteins elsewhere in the body since tumour concentrations, although high, did not exceed background (Goodwin *et al*, 1988)

Lastoria *et al* (1993) demonstrated that using ^{131}I–metaiodobenzylguanidine scintigraphy was successful in localising the majority of neuroblastoma lesions in children They showed that since the spread of neuroblastoma is unpredictable, this type of imaging was more sensitive than computed tomography or ultrasonography in localising the majority of neuroblastoma lesions

Immunoscintigraphy has been the most successful application of immunoconjugates in oncology It aims to detect tumours *in vivo*, rather than kill these tumours, uses lower levels of radiation (gamma, γ) by using lower energy isotopes such as ^{111}In and ^{99m}Technetium (^{99m}Tc) and smaller doses of radiolabelled monoclonal antibody conjugate than radioimmunotherapy Typically, patients are administered between 0 5 and 2 0mg of radiolabel for diagnostic purposes, but are administered with up to 500mg of radiolabelled monoclonal antibodies for therapeutic purposes (Britton *et al*, 1991) While radioimmunotherapy is more successful on very small and diffuse tumours, immunoscintigraphy has been most effective on large tumours with larger surface areas to bind antibodies

1 14 Immunocytokines

Another method to treat cancer has been to look at the body's natural mechanism for fighting disease and to redirect the natural cellular molecules called biological response modifiers to kill cancer cells (reviewed by Tanneberger and Pannuti, 1993) Biological response modifiers are defined as 'agents or approaches that alter interactions between tumours and host toward therapeutic advantage, primarily through modification of host response to tumours' (Mihich, 1985) Such modifiers include interferons (Weiner *et al*, 1988), interleukins (IL–1, IL–2), colony–stimulating factors, human granulocyte colony–stimulating factor and tumour necrosis factor (Reisfeld and Schrappe, 1990) and all have been conjugated to monoclonal antibodies and used in cancer treatment

Jinno *et al* (1993) used a drug immunoconjugate targeted against a growth factor receptor This targeted therapy showed selective cytotoxicity to cancer cells with expression of the growth factor receptor Moreover, the cytotoxic effect was positively correlated with the expression level of the receptor

Ferrini and colleagues (1990) noted that immunologists are only beginning to unravel the complex reactions of biological response modifiers, cytokines and growth factors They recognised that many of these molecules have pluripotent effects on many cells

of the immune system and, therefore, the efficiency of biological response modifiers–monoclonal antibody conjugates on tumour cells is more difficult to monitor than other immunoconjugates, such as those with drugs, toxins or isotopes Research aimed at directing biological response modifiers to specific cells may help to elucidate these pluripotent effects

1 15 Bispecific antibodies

Today, hybridomas producing antibodies of a variety of specificities exist in almost limitless quantities In recent years, however, advances in hybridoma production techniques, recombinant DNA manipulation and gene transfection technology have made possible the production of entirely novel bispecific antibodies (Williams, 1988) Most naturally occurring antibodies have two antigen combining sites which both recognise the same antigenic determinant (Figure 1 1)

Bifunctional antibodies have potential for many uses including imaging, therapy (Section 1 16 and 1 17) and diagnostic kits Bispecific antibodies have been produced by chemical reassociation, heterogeneous aggregation and cell fusion (reviewed by Nolan and O'Kennedy, 1990) These methods will be briefly discussed As the name suggests, in bispecific antibodies produced by chemical reassociation, monovalent fragments derived from two monoclonal antibodies are chemically recombined There are some technical disadvantages with this method It may be difficult to dissociate the immunoglobulin chains without some protein denaturation and subsequent loss of antibody activity Unorthodox disulphide bonds, within chains, may be created when the antibody heavy chains are allowed to reassociate An alternative method of preparation involves the covalent attachment of whole monovalent antibodies of different specificities using a heterobifunctional cross-linker such as SPDP (N–succinimidyl–3–(2–pyridyldithiol) propionate) Such reagents can be used in the targeting of cytotoxic T cells (Section 1 16 and 5 1) (Perez *et al* , 1986)

A third method for the preparation of bispecific monoclonal antibodies, cell fusion, was described in 1984 by Milstein and Cuello A so-called hybrid hybridoma', obtained by fusion of two hybridoma cells, each producing its own antibody, contains the genetic information necessary for the production of two different antibodies The antibody genes are codominantly expressed (i e light and heavy chains from both antibody types are expressed) These protein chains recombine in the cisternal space to produce parental

and hybrid antibody molecules Although only a small proportion of light and heavy chain associations yield bispecific molecules (Milstein and Cuello, 1984)

DeSutter and Fiers (1993) reported the genetic engineering of a murine human chimaeric antibody, directed against the tumour marker human alkaline phosphatase, in which one antigen-binding arm had been replaced by *Escherichia coli* β-lactamase The mutated β-lactamase gene was fused, in phase, to the cDNA sequence encoding the hinge region, CH2 and CH3 of the human IgG3 heavy chain (Figure 5 2 1) Approximately 200ng/ml of correctly assembled bifunctional antibody-β-lactamase immunoconjugates were detected in culture supernatant This bifunctional immunoconjugate could be of therapeutic value for the activation of cephalosporin-based anti-cancer prodrugs at the tumour site (DeSutter and Fiers, 1993)

In the future, it may also be possible to create tri- and multi-functional antibodies using antibody engineering (Section 1 21) One of the most innovative and far-reaching areas opened up by this technology is that of catalytic antibodies (Section 6)

Morelli *et al* (1994) have modulated drug induced cytotoxicity by a bispecific monoclonal antibody, that recognised the EGF and doxorubicin, which was produced by somatic hybridisation of two hybridomas The bispecific monoclonal antibody obtained was able to retarget doxorubicin cytotoxicity *in vitro* specifically on EGF-receptor positive cells

Progress towards an understanding of the construction and use of bispecific antibodies in the therapy of tumours has been considerable, this area has been reviewed by Fanger *et al* (1993) The importance of accessory adhesion molecules (Section 1 17) as well as the requirement for cell killing and the mechanisms by which cytotoxicity is mediated (Section 1 16) are being are also being clarified As clinical trials have demonstrated little toxicity and, in some instances, promising responses and long-term survivals, it seems likely that bispecific antibodies will be very useful tools for therapy of tumours Bispecific antibodies are further discussed in Sections 1 16 , 1 17 and Section 5 1

1 16 T cell killing

Much work has been done recently on the use of hybrid antibodies to the T-cell receptor complex activating T-cells for target cell killing, raising the hope that T-cell killing mechanisms might be recruited as an additional effector mechanism for antibody-directed killing of tumour cells *in vivo*

Effective cell-cell interactions are known to be required for cell-mediated cytolysis, Lotze and co-workers (1987) detailed possible models of tumour targeting and focusing of lymphoid effectors Human peripheral blood T-cells can be induced to lyse either cultured or fresh tumour cells *in vitro*, by coating them with heteroaggregates containing anti-T3 cross-linked to anti-tumour antibody Such heteroaggregates bridge the effector cells to the tumour target cells and activate the T-cell lytic mechanism In order to trigger lysis, the anti-T cell portion of the heteroaggregate must be directed against an activation molecule on the T-cell surface such as a component of the T cell receptor complex - T3 or Ti or CD2 (in man) and Ly6 (in mouse) to redirect the specificity of cytotoxic T cells (Segal *et al*, 1988) Lysis mediated by targeted T- and K-cells is enhanced by incubating the effector cells with IL-2, whereas killing by monocytes and neutrophils is boosted by interferon-λ By contrast, the anti-target cell antibody can be directed against any cell surface epitope on the tumour cell, as long as it is expressed at a sufficient density for conjugate formation and T-cell triggering *In vitro*, the cytotoxic activity of targeted T cells resides in the $T8^+$ subset and is rapidly augumented after exposure to IL-2 (Segal *et al*, 1988) *In vivo*, targeted T- and K-cells can also block tumour growth Specific target cell lysis has been achieved by retargeting T-cells with the use of chemically produced antibody heteroaggregates with dual specificity for target and T-cell receptor complexes (Starez *et al*, 1985)

In a study by Duke-Cohan *et al* (1993), they used a bispecific antibody-toxin conjugate directed against CD4 and CD29 antigens simultaneously, as binding through both these antigens would predispose toward endocytosis and delivery of the toxin to the cell interior They demonstrated that the potency of the immunotoxin is not only a function of its affinity but also of its propensity to internalise, and that this property is influenced by the degree of bivalent binding These results have opened up the possibility of engineering bispecific antibody-toxin conjugates for use as therapeutic immunotoxins for selective removal of restricted T cell subsets

Weiner *et al* (1994) developed an anti-CD3 x anti-tumour bispecific antibody that can retarget T cell-mediated lysis in a major histocompatability fashion and can prevent tumour growth in a mouse model They also studied the use of bifunctional constructs which differed in the presence or absence of Fc They showed that the use of bifunctional constructs that lack functional Fc allows for separate manipulation of T cell retargeting and T cell activation, allowing a new method of potential immunotherapy for

malignancy

1 17 Effector cell retargeting

Heteroconjugates of monoclonal antibodies, as well as hybrid monoclonal antibodies were found to induce T cells to act against a target site Hybrid monoclonal antibodies consist of one of the antibody component binding sites (first specificity) being directed against a tumour-associated antigen and the other component binding site (second specificity) is directed against an activation molecule on the T cell surface, (e g T3) (Perez *et al*, 1986) In addition, there have been reports of hybrid hybridomas that secrete bispecific antibodies that can also mediate what is termed "effector cell retargeting" To optimise the potential *in vivo* applicability of effector cell retargeting, the hybrid antibodies should promote high levels of specific cell killing of the desired target with minimium killing of the effector T cells (Clarke and Waldmann, 1987)

Beun *et al* (1993) investigated the ability of bispecific anti-T cell receptor x anti-tumour antibodies, destined for the study of T cell retargeting in a rat colon carcinoma model They found by cocultivating with recombinant IL-2, at concentrations as low as 1U/ml, tumour neutralisation by retargeted effector cells was promoted This non-toxic regimen of IL-2 administration strongly enhanced natural killer-like activity as well as retargeted anti-tumour activity and enabled retargeted effector cells to prevent tumour growth in the majority of animals in this study

1.18 Immunophotodynamic therapy

Chemoimmunoconjugates (Section 1 10), immunotoxins (Section 1 11) and radio-immunoconjugates (Section 1 13) have all been of limited success *in vivo*, mainly due to non-specific uptake of conjugate by non-tumour tissue To overcome this problem, researchers use a two step system by administering the conjugate to the patient and allowing the clearance of the monoclonal before activation of the drug or isotope Novel therapy systems such as immunophotodynamic therapy may be a better solution to this problem

The use of photochemicals in the diagnosis and treatment of certain cancers has received a considerable amount of attention (Mew *et al*, 1985) Tumour phototherapy, or photodynamic therapy, is when light is used in combination with a group of photoactive drugs called photosensitisers to destroy tumour tissue and normal tissue is left relatively

unharmed All forms of photochemical therapy rely on chemical reactions triggered by light The photosensitisers produce singlet oxygen when activated by light By administering photosensitisers such as hematoporphyrin, hematoporphyrin derivatives, chlorines and benzoporphyrins (reviewed by Nelson *et al*, 1988) and then irradiating those areas of their body with tumour masses using light focused from a laser light or other source, significant reductions in the mass of these tumours have been observed This area has been reviewed by Yarmush *et al* (1993)

1 19 Unconjugated monoclonal antibodies in therapy

Unconjugated monoclonal antibodies can play a vital role *in vivo* by stimulating and manipulating the immune system by mimicking a patient's own antibodies This role is often forgotten in the race to find effective immunoconjugates Unconjugated monoclonal antibodies can achieve an anti-tumour response by the following mechanisms (reviewed by Mellstedt, 1990), (i) complement-dependent cytotoxicity, (ii) antibody-dependent cell-mediated cytotoxicity, (iii) anti-idiotypic antibodies, (iv) non-specific-effector cell activation and (v) interaction with receptors on malignant cells

1 20 Catalytic antibodies

The discovery that antibodies *can* catalyse a range of reactions such as acyl-transfer and peptide-bond formations has led to renewed interest in catalytic antibody technology (Harris, 1991) Green and Tawfik (1989) outlined several roles for catalytic antibodies They can act as catalysts in organic, synthetic and sterospecific synthesis Catalytic antibodies may also be used as biosensors and as cleavage systems in protein engineering In addition, catalytic antibodies have several potential clinical applications (Landy *et al*, 1993) These applications include, (i) catalysis of breakdown of bacterial toxins – it was suggested that catalytic antibodies with proteolytic activity would act more speedily and with lower overall dose than unconjugated antibodies, (ii) similarly, catalytic antibody vaccines' may passively immunise against toxins, bacteria, virus and potentially cancer cells, (iii) catalytic antibodies may be used to replace enzymes which are deficient in a patient and (iv) these antibodies may replace monoclonal antibody-enzyme conjugates in prodrug activation

The applications of catalytic antibodies in the field of drug targeting has, as yet, not been pursued However, some of the applications of catalytic antibodies include

esterolytic antibodies, which have already been put to work in the production of a biosensor (Blackburn *et al*, 1990) These catalytic antibodies are proving to be an important new class of biomolecule in which the binding sites are continually regenerated by the catalytic reaction of the substrate In this study, Blackburn *et al* (1990) introduced the use of catalytic antibodies as the molecular recognition element in a reversible potentiometric immunobiosensor Catalytic antibodies have several major advantages over natural enzymes in biosensor applications

The importance of the catalytic activity of antibodies in the body was demonstrated by an anti-vasoactive intestinal peptide antibody found naturally This antibody hydrolysed the vasoactive intestinal peptide, a bronchorelaxant In a study by Ollerenshaw *et al* (1989), they demonstrated that both healthy people and asthma sufferers were shown to have similar levels of the anti-vasoactive intestinal peptide antibody However, they found that the asthma patients had a 50-fold higher affinity for the peptide and thus had less vasoactive intestinal peptide In light of these results, it has been suggested that these antibodies have a role in the airway hyper-responsiveness of asthma (Ollerenshaw *et al*, 1989)

Hirschmann *et al* (1994) have developed a monoclonal antibody, which when induced with a phosphonate diester hapten, catalysed the coupling of p-nitrophenyl esters of *N*-acetyl valine, leucine and phenylalanine with tryptophan amide to form the corresponding dipeptides These results represent a first step toward generating antibodies capable of coupling unprotected amino acids and peptide fragments

Addiction to cocaine afflicts Western populations in epidemic proportions, and the exceptional reinforcing effect of cocaine renders abuse of this stimulant resistant to treatment Landy *et al* (1993) have developed one of the most promising approaches in decades to treat this dependency In their study, Landy *et al* (1993) immunised mice with a phosphate monoester transition-state analogue of cocaine and obtained monoclonal antibodies capable of catalysing the hydrolysis of the cocaine benzoyl ester group Their monoclonal antibody can bind specifically to cocaine and then break it into two inert byproducts, ecgonine methyl ester and benzoic acid They proposed that passive immunisation with such an artifical enzyme could provide a treatment for cocaine dependence by blunting withdrawal symptoms Richard Lerner, president of the Scripps Research Institute has said of Landry's work, that it has the potential to "move catalytic antibodies into the treatment of human problems for the first time"

The development of catalytic antibodies of defined specificity promises to be of considerable value to biology, chemistry and medicine Catalytic antibodies may find a use as therapeutic agents to selectively hydrolyse the protein or carbohydrate coat of viruses, cancer cells or other physiological targets It may also be possible to selectively cleave or ligate complex biomolecules such as polynucleotides, carbohydrates and proteins, thereby facilitating structural/functional studies or allowing the synthesis of new biomolecules with novel properties The ready availability of large quantities of monoclonal antibodies may allow for their use as synthetic tools for the production of pharmaceuticals or new materials The ability to generate antibody combining sites with specific catalytic groups and/or microenvironments should also serve to test fundamental notions of enzymatic catalysis Catalytic antibodies and their applications are further discussed in Section 6

1 21 Antibody engineering

The first advance from hybridoma technology was through the somatic mutation of the cells to produce mutants that could be selected (Rudikoff *et al* , 1982), allowing their functional properties to be changed by switching the heavy chain constant regions (Radbruch, 1986) Gene technology expanded the scope of engineering, and has allowed the synthesis of new antibodies almost to order It may soon be possible to bypass hybridomas entirely with the direct gene transfection from lymphocytes to bacteria (Ward *et al* , 1989)

An antibody is an ideal agent for protein engineering It can be broken into fragments which can be used alone or can be transplanted and swapped from one antibody to another (Jones *et al* , 1986) to make the most suitable antibody For example, in humans the constant regions determine the lysing capacity of the antibody, γ1 isotype is highly effective in both complement-dependent cytolysis (CDC) and ADCC and, as such, would be useful in therapy (Reichmann *et al* , 1988) while the γ4 isotype is inactive so could be used in diagnostic imaging (Van der Zee *et al* , 1986)

Antibody engineering has been used to convert mouse monoclonal antibodies into "human" ones by joining the entire immunoglobulin V domains from the mouse antibody to human C domains (Morrison *et al* , 1984) or by transplanting the complementarity-determining regions (CDRs) of the mouse antibodies into human myeloma proteins (Jones *et al* , 1986) Thus, the rearranged immunoglobulin V genes

provide the raw material for engineering and have been derived by cloning from genomic DNA (Oi *et al*, 1983) or cDNA (Neuberger *et al*, 1984)

The advent of PCR technology has aided in this field (Saiki *et al*, 1985) PCR has been used for genomic (Scharf *et al*, 1986) and cDNA cloning (Saiki *et al*, 1988) It involves repeated rounds of extension from two primers specific for regions at each end of the gene The primer sequence need not match the gene sequence exactly, and when restriction sites are incorporated within the primers, DNA can be cloned directly for expression in mammalian cells (Orlandi *et al*, 1989) or bacteria (Ward *et al*, 1989)

Lu *et al* (1994) demonstrated the improved quantitative analysis of PCR amplified products using an automated system that combines capillary-gel electrophoresis for highly efficient separation and laser-inducer fluorescence for highly sensitive detection which will further expand the applications of PCR

1 22 Clinical applications of antibody engineering

An example of the use of chimaeric antibodies is a chimaeric anti-colorectal cancer monoclonal antibody (Steplewski *et al*, 1988) The murine V_H was combined with a human constant region, while V_L was joined to C_κ In a bid to optimize effector function, the V_H was joined to each IgG isotype in turn and IgG1 was found to be the most clinically effective

Liu *et al* (1987) demonstrated the ability to select monoclonal antibodies with desired specificity and engineered antibody molecules with desirable biologic functions In this study, a mouse-human chimaeric monoclonal was made by substituting the mouse constant domains of the mouse monoclonal antibody with the human γ1 and κ domains which bound to the cluster of designation 20 (CD20) antigen of normal and neoplastic B cells The mouse variable domains were retained This new antibody has the same binding specificities as the original mouse monoclonal but is highly effective in mediating ADCC with human effector cells and CDC with human complement The CD20 antigen of normal and neoplastic B cells may be a suitable candidate for targeting of B cell lymphomas as hemopoietic stem cells do not express CD20 and, also, as CD20 does not internalise after monoclonal antibody binding

DeSutter and Fiers (1993) reported the genetic engineering of a murine human chimaeric antibody, directed against the tumour marker human alkaline phosphatase (Section 1 9)

Ten patients were tested in clinical trials of a murine-human chimaeric antibody specific to a gastrointestinal-tumour antigen (LoBuglio *et al* , 1989) Four patients were given single infusions while the other six received three infusions, one every fortnight Although the plasma half-life of the antibody was on average six weeks longer than a non-chimaeric one and only one person had an antibody response, no tumour regression was reported and all patients' tumours showed signs of progression within a three month period (LoBuglio *et al* , 1989) The clinical applications of antibody engineering has been reviewed by Hand *et al* (1994)

1 23 Humanised antibodies

Humanisation or "reshaping" of murine antibodies is an attempt to transfer the full antigen specificity and binding avidity of murine monoclonal antibodies to a human antibody by grafting the murine CDRs onto a human variable region framework (Reichmann *et al* , 1988) Using Campath-1, a rat antibody capable of recognising an antigen on human lymphocytes, the humanised antibody acted in a similar manner to the parent mouse monoclonal antibody Two patients with non-Hodgkins lymphoma were treated with this humanized Campath-1 (1-20 mg/day for up to 43 days) Neither showed an immune response and the tumours showed signs of regression (Hale *et al* , 1988)

Other humanized antibodies are showing equally positive signs Celltech, the British biotechnology company, are conducting research on antibodies against septic shock, heart disease (Newell, 1989, Queen *et al* , 1989) and on anti-Tac antibody, which blocks binding of interleukin-2 to its receptor (Queen *et al* , 1989) However, to preserve the binding affinity, the majority of the CDR-grafted antibodies require additional amino acid changes in the framework region, because such ammo acids are conformationally important or are in direct contact with the antigen (Foote and Winter, 1992) Such necessary framework changes may introduce new antigentic epitopes and, if many changes are needed, the advantages of CDR grafting over chimaeric antibody constructs would be lost

A solution to this problem was proposed by Roguska *et al* (1994), by maintaining the CDRs and the core of the murine variable region framework but replacing the surface residues of the framework region with those from a human variable region by a process they called "resurfacing" A premise to this approach is that the immunogenicity of

murine antibody variable regions originates with the surface residues and, hence, that the surface will carry most, if not all, of the antigenic potential of the protein

Masat *et al* (1994) isolated a murine antibody that is highly specific for a molecule expressed by human cells of the melanocytic lineage This antibody consists only of a kappa light chain and only in the monomeric form does this kappa light chain specifically bind to its cognate antigen Masat suggests that there may be numerous other examples of monomeric light chain antibodies among the hundreds of thousands of hybridomas generated since the fusion method was described (Kohler and Milstein, 1975) Thus, it might be feasible to use conventional techniques to generate naturally occurring monomeric light chain antibodies for many antigenic specificities

For serotherapy, human monoclonal antibodies are preferred There are, however, considerable difficulties in making human monoclonal antibodies of the required specificity by hybridoma technology (Carson and Freimark, 1986) One way of minimizing these undesirable responses in therapy was provided by antibody engineering with the creation of chimaeric and 'humanised' antibodies (Figure 5 2 1)

Pedersen *et al* (1994) have made a systematic analysis of the known antibody structures to determine the relevant solvent accessibility distributions of amino acid residues in murine and human antibody variable regions and have shown that the sequence alignment positions of surface amino acids for human and murine variable region heavy (V_H) and (V_L) chains are conserved with 98% fidelity across species While the amino acid usage at the surface positions creates surface residue patterns that are conserved within species, there are no identical patterns across species However, Roguska *et al* (1994) have shown that surprisingly few amino acid changes need to be made to convert a murine variable region surface pattern to that characteristic of a human surface They have "resurfaced" two murine monoclonal antibodies which maintained their parental affinities for their cell surface ligands This experiment provides evidence that, despite the differences in the surfaces of mouse and human variable regions, it is possible to substitute one for the other while retaining full antigen binding activity

1 24 Second generation recombinant monoclonal antibodies

Mallender and Voss (1994) reported the design, construction and expression of a bivalent, bispecific, single-chain antibody protein in *Escherichia coli*, which was based on two previously constructed monovalent, single-chain antibodies possessing distinct

specificities Construction of such a model bivalent, bispecific molecule provides a foundation for future assembly of similar molecules designed to identify parameters involved in enhanced binding of antibodies due to avidity and dual specificity

Hand *et al* (1994) reviewed the development of novel immunoglobulin forms They proposed that the genetic modifications may result in more useful diagnostic reagents and in the production of more stable immunoconjugates with the characteristic of more efficient tumour cell killing

1 25 Future developments

During the past decade, the marriage of cancer research and molecular biology has produced a much better understanding of the many biochemical changes underlying cancer development Only recently, however, have researchers begun to apply that growing body of knowledge to the search for better therapies for cancer Two reports have brought one potential treatment aimed at correcting a biochemical alteration of cancer cells a step closer to reality (Kohl *et al* , 1993, James *et al* , 1993)

A so-called oncogene, *ras*, may contribute to as many as one-fifth of all human cancers, including more than half of such common ones as colon cancer A great deal of work has shown that the proteins encoded by *ras* genes occupy a central position in the signaling pathway used by cells to respond to growth factors What apparently happens in cancerous tumours is that as a result of mutations in the genes, Ras proteins are always turned on, so that they constantly promote cell division, even in the absence of stimulation by growth factors However, before either the normal or mutated Ras proteins can function, they have to undergo a series of modifications that enable them to settle on the cell membrane, from which they relay growth signals to the cell interior These two reports (James *et al* , 1993, Kohl *et al* , 1993) have described compounds which interfere with the modification of the Ras protein which might be able to keep the proteins from attaching to the membrane and, thus, might be able to keep the growth of the cell switched off and prevent uncontrolled cell division In theory, drugs designed on this principle might prove more powerful and have fewer side effects than conventional chemotherapeutic drugs, which attack rapidly dividing cells, whether they are cancerous or not

Another hopeful development was made recently (Marasco *et al* , 1993) A team of researchers, using a combination of antibody engineering and gene therapy, have

directed an antibody to a specific location *inside* a cell The antibody Marasco and his colleagues put into cells was able to interfere with the assembly of the AIDS virus, thereby reducing both its cell-killing potential and its infectivity This achievement raises the possibility of using antibodies within cells to block the construction of viruses or harmful proteins, such as the oncoproteins whose activity contributes to the uncontrolled growth of cancer cells If this proves correct, then antibodies may prove as important inside cells as outside

1 26 Thesis outline

The work described in this thesis was designed to exploit the potential of antibodies to perform analytical, targeting and catalytical functions This involved the production of several antibodies to defined antigens and the chemical synthesis of bispecific antibodies In the introduction, the major areas of monoclonal antibody technology for the detection and treatment of cancer have been discussed (Figure 1 2) The areas in which experimental work was undertaken during this thesis are boxed in Figure 1 2

In this thesis, the following aspects of antibody technology were explored,

(i) a monoclonal antibody to a protein, Glial Fibrillary Acidic Protein (GFAP), which is associated with human astrocytomas (Section 3) was produced

(ii) a monoclonal to a human astrocytoma cell line, called G-CCM, which also reacts with commercial GFAP (Section 3) was produced

(iii) monoclonal and polyclonal antibodies to Iodinated Bolton-Hunter Reagent (IBHR) (Section 4) were developed

(iv) bispecific antibodies were produced between the anti-GFAP and anti-G-CCM and anti-Ricin-A chain and the polyclonal anti-IBHR (Section 5 1)

(vi) a method to genetically clone and express the variable regions of the anti-GFAP antibody in *Escherichia coli* (Section 5 2) was examined

(v) a monoclonal antibody was produced which catalysed a chemical reaction, e g to catalyse a prodrug -> drug reaction (Section 6)

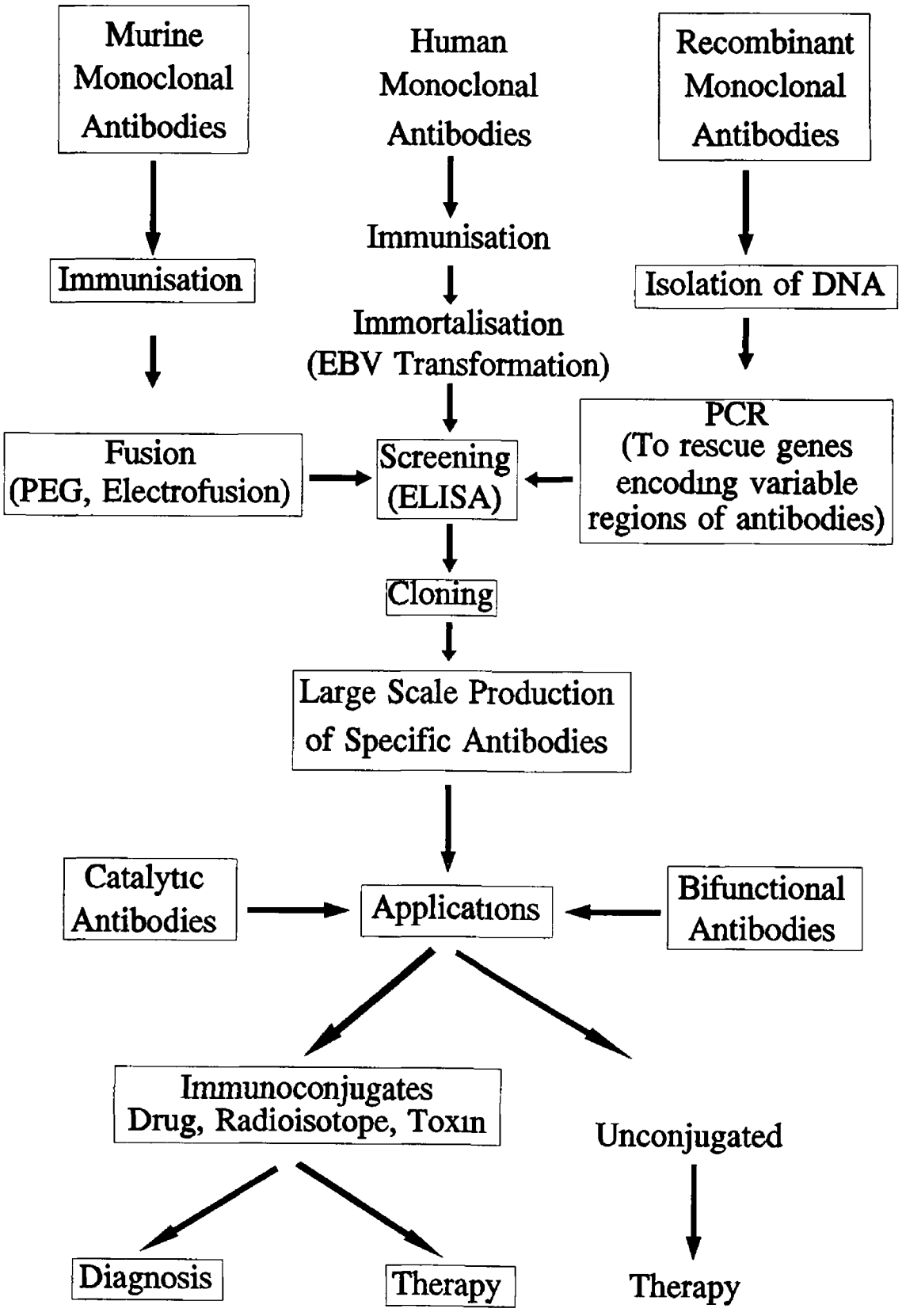

Figure 1.2. Summary of the major areas of research in the production and application of monoclonal antibodies for the detection and treatment of cancer The boxed headings indicate areas in which experimental work was undertaken

SECTION 2 : MATERIALS AND METHODS

2.1. Materials.

Reagents used in experimental work were of analytical grade and were obtained from Sigma Chemical Co., Poole, Dorset, England; BDH Chemicals Ltd., Poole, Dorset, England and Riedel de Häen, AG, Seelze, Hannover, Germany. Cell culture media, supplements, sera and disposable plasticware were supplied by Flow Laboratories, Woodcock Hill, Harefield Road, Hertz, WD3 1PQ, England, ICN Biomedicals Ltd., Irvine, Ayrshire KA12 8NB, England; Greiner, The Science Centre Ltd., 159 Lower Rathmines Road, Dublin 6 and Medical Supply Company, Unit 9, Santry Hall Industrial Estate, Santry, Dublin 9. Stains used for cytogenetic and histological analysis were obtained from BDH Chemicals Ltd., Poole, Dorset, England. Genetic reagents were obtained from Pharmacia, Unitech, United Drug House, Belgard Road, Tallaght, Dublin 24. Restriction enzymes were obtained from New England Biolabs, 32 Tozer Road, Beverly, MA 01915, U.S.A. and from Promega Corporation, 2800 Wood Hollow Road, Madison, WI 53711-5399, U.S.A.

A number of specific reagents, which were obtained from various suppliers are listed.

Balb/c mice were obtained initially from Trinity College, Dublin 2 and subsequently from Harlan Olac Limited, Shaw's Farm, Blackthorn, Bicester OX6 OTP, England.

Bolton-Hunter reagent (3-(4-hydroxyphenyl)propionic acid N-hydroxysuccinimide ester) and dimethylsulphoxide (Sure Seal) were obtained from Aldrich Chemical Company, Gillingham, Dorset, England.

Borosilicate glass tubes (16x100mm) were obtained from Corning Glass Works, Corning, New York 14831, U.S.A.

Bradford reagent was obtained from Bio-Rad Laboratories Ltd., Richmond, California, U.S.A.

BriClone was obtained from Dr. Kenneth Carroll, National Cell and Tissue Culture Centre (NCTCC), Dublin City University.

Dulbecco's Modification of Eagle's Medium (DMEM) with 3.70g/L of sodium bicarbonate without glutamate was obtained from Flow/ICN Flow laboratories, Irvine, Scotland KA12 8NB.

DNA Primers were obtained from the Department of Genetics, Trinity College, Dublin.

Ethanol was obtained from Merck, Darmstadt, Germany.

Millex-GV and Millex-HA 0.22μm single use filter units were obtained from Millipore, France through Medical Supply Company Ltd. NUNC Immunoplates Maxisorp-96 well

non-sterile (certified) were also obtained from the Medical Supply Company.

Hoechst 33258 was obtained from Calbiochem, Bering Diagnostics, La Jolla, CA 92037.

Hybond-C-Super supported pure nitrocellulose paper (0.45μm, 30cmx3m) was supplied by Amersham International, Amersham, Buckinghamshire, HP7 9NA, England.

Liquid Carbon Dioxide was obtained from Irish Industrial Gases, Bluebell, Dublin 12.

Liquid Nitrogen was obtained from Cooper Cryoservice Limited, 6 Chalet Gardens, Lucan, Co. Dublin.

New Zealand White Rabbits were obtained from Ballina Laboratories, Carrentrila, Ballina, Co. Mayo.

NuSieve^{R} GTG Agarose was obtained from FMC BioProducts, 191 Thomaston Street, Rockland, ME 04841, U.S.A.

Phosphate buffered saline (Dulbecco A) (PBS) tablets were obtained from Oxoid Limited, England.

Polyethylene Glycol 1500 was obtained from Boehringer Mannhiem, Hannover, Germany.

PAGEL^{R} precast polyacrylamide gels for electrophoresis were obtained from ATTO Corporation, 2-3 Hongo 7-Chrome, Bunkyo-ku, Tokyo, Japan.

Protein A columns and BCA Reagent were obtained from Pierce and Warriner (U.K.) Limited, 44 Upper Northgate Street, Chester, Cheshire, CH1 4EF, England.

Protein G-Sepharose 4 Fast Flow column (5ml) and Sephadex G-25 Medium were obtained from Pharmacia, address as above.

Rapi-Diff II, a triple stain pack for rapid Romanowsky staining was obtained from DiaCHEM, Diagnostic Developments, Units 2-4, 50 Everton Road, Birkdale, Southport, Merseyside PR8 4BT, England.

Sterile Needles (Microlance 2) were obtained from Becton Dickinson and Company Limited, Dun Laoghaire, Co. Dublin.

Trypsin/EDTA (0.05%, v/v /0.02%, w/v) in PBS (without Ca^{++} or Mg^{++}), Penicillin/Streptomycin (10,000units/ml and 10,000μg/ml, respectively) and Gentamicin (20,000μg/ml) were purchased from Seromed, Biochrom KG, Leonorenstraβe 2-6, D-1000 Berlin 46, Germany.

Ultrogel AcA44 was obtained from IBF Biotechnics, Villeneuve-La-Garenne, France.

Several commercial kits were used for analytical purposes; these are given in Table 2.1.

Table 2.1. Summary of commercial kits used.

Name	Source	Address
QuickPrep™ mRNA Purification Kit SEROTEC Isotyping Kit	Pharmacia	Unitech Limited, United Drug House, Tallaght, Dublin 24
Bicinchonic Acid (BCA) Protein Assay Kit	Pierce Chemical Co	Rockford, IL 61105, U S A
CEL-PRIME *IN VITRO* Immunization for High IgG Yield	Immune Systems Ltd	P O BOX 99, Westbury on Trym, Bristol BS10 5NU, England
Sigma Mouse Monoclonal Antibody Isotyping Reagents	Sigma Immuno-chemicals	P O BOX 14508, St Louis, MO 63178, U S A

The animal cell lines used are summarised in Table 2 2 The antigens used in the enzyme-linked immunosorbant assays (ELISA) are detailed in Table 2 3 , while the antibodies used in immunocytochemistry, ELISA and fluorescence activated cell sorting (FACS) analysis are listed in Table 2 4

Table 2.2. Animal cell cultures used *in vitro* and *in vivo*.

Cell Line	Cell Type	Catalog No.	Supplier
MCF-7	Human, breast adenocarcinoma	HTB 22	ATCC, 12301 Parklawn Dr , Rockville, Maryland 20852, U S A
LOVO	Human, colon adenocarcinoma	CCL 229	
NRK	Rat, normal kidney	CRL 6509	
HL-60	Human, promyelocytic leukaemia	-	
A549	Human, lung carcinoma	-	
EJ-138	Human, bladder carcinoma	8511412	ECACC, PHLS Porton Down, Salisbury, Wiltshire SP4 0JG, U K
NSO/1	Mouse, balb/c myeloma	84112004	
G-CCM	Human, astrocytoma	86022702	
G-UVW	Human, astrocytoma	86022703	
$IJK_{(t)}$	Human, astrocytoma	86022704	
T-24	Human, bladder	-	Dr J Tager, Amsterdam University, 1105AZ, Netherlands
H5683	Human, brain	-	Dr Alice Redmond, Dr Geraldine Grant, NCTCC, D C U
B21	Human, brain		
K562	Human, erythroleukaemia	-	Laboratory Stocks, Dublin City University
SP2/0-Ag14	Mouse balb/c myeloma		

Table 2.3. Antigens used in ELISAs.

Antigen	Supplier
GFAP, (code 77-106) from bovine spinal cord	ICN Immunobiologicals, P O Box 1200, Lisle, IL 60532
IBHR-BSA, IBHR-KLH, IBHR-oval	Produced during thesis research
PHOS-KLH	Dr Gerry Gallacher, Division of Chemistry, University of Brighton, Brighton BN2 4GJ
Ricin-A chain	Dr Denis Thatcher, Zeneca Corporation, England
Anti-recombinant ricin-A chain monoclonal antibody (608/7)	Dr Malcolm V Pimm, University of Nottingham, University Park, Nottingham NG7 2RD
Cell lines	See Table 2 2

Permission to use anti-ricin-A chain antibody was gratefully obtained from Dr Partick Trown, Xoma Corporation, 2910 Seventh Street, Berkeley, California 94710, U S A

Table 2 4. Monoclonal antibodies used in immunocytochemistry, ELISA and FACS analysis. (alk. ph. = alkaline phosphatase).

Antibodies used	Label	Dilution	Code	Supplier
Goat anti-mouse IgG (whole molecule)	No label	10μg/ml	M-8642	Sigma Immunochemical Co , P O Box 14508, St Louis, MO 63178, U S A
	alk ph	1/9500	A-5153	
	peroxidase	1/1000	A-4416	
Fab specific	FITC	1/32	F-4018	
Monoclonal anti-GFAP, clone G-A-5	No label	1/200	69-110	ICN Immunobiologicals, P O Box 1200, Lisle, IL 60532, U S A
Goat anti-mouse Ig	No label	1/500	083-67	ATAB, P O Box 60, Scarborough, MG 04074, U S A
Monoclonal alk ph anti-alk ph (APA-AP)	alk ph	Neat	A-2806	Sigma, as above
Goat anti-mouse IgM	FITC	1/32	F-2031	Sigma, as above
Goat anti-mouse IgM (μ chain)	alk ph	1/1000	A-7784	Sigma, as above
Mouse IgMκ			M-2770	Sigma, as above
Mouse IgG				Sigma, as above
Anti-Ricin-A chain		1/100	608/7	Prof Malcolm V Pimm, Univ of Nottingham

Table 2.5. Subtrates used in immunocytochemisty, ELISA and FACS.

Substrate	Reagents	Conc.	Supplier Code
5-bromo-4-chloro-3-indoyl phosphate (BCIP), Disodium salt	0 1M Amino-methyl-propanol (5mM MgCl, 0 01% v/v Triton X-405, 0 01% w/v NaN_3), pH 10 25	1μg/ml	Sigma, B-6149
o-phenylene-diamine [50μl 20% (v/v) H_2SO_4 used to stop reaction]	0 1M Citrate buffer, pH5 0, 50μl 1% (v/v) H_2O_2	2mg/ml	Sigma, P-9029
Fast red TR/ Naphthol AS-MX phosphate (when 1 tablet is dissolved in 10ml H_2O)	Fast red TR Naphthol AS-MX Levamisole 0 1M Tris/HCl buffer, pH 8 2	1 0mg/ml 0 4mg/ml 0 15mg/ml	Sigma, F-0900
p-Nitrophenyl Phosphate	10% (v/v) diethanolamine, 0 5mM $MgCl_2$, pH 9 8	1mg/ml	Sigma, P-2765

2.2. Equipment.

Cells in culture were examined using a Nikon type ELWD 0 3 inverted phase contrast microscope A Nikon Optiphot microscope was used for fluorescence and phase contrast microscopy

A Holten LaminAir HB 2448 K laminar flow was used for all cell culture work

The temperature and carbon dioxide controlled incubators used for cell culture were Jouan EG115 IR and Heraeus Instruments B 5060EK/CO_2

Cell counts were performed on a Neubauer Hemocytometer slide

A Heraeus Christ Labofuge 600 and Labofuge GC were used to centrifuge universal tubes (1 - 20ml) A Heraeus Biofuge A was used for smaller volumes (0 - 1 5ml) The higher speed centrifuges and rotors used were Sorvall^R RC-5B Refrigerated Superspeed Centrifuge with Sorvall^R GSA (13000rpm max) and Sorvall^R SS-34 (20500rpm max) rotors A Beckman J2-21 centrifuge with a Beckman JA 20 rotor were used in the extraction of mRNA A Beckman L8-70M Ultracentrifuge with Ty 70-1 TI rotor was used in the purification of cell membranes for Western blotting

Slides of cells in suspension were prepared using a Heraeus Sepatech Labofuge Ae with disposable inserts used for each slide prepared

A Biohit Proline^R Electronic 250 automatic octopipette was used in cloning work and with ELISAs

A Kika-Werk, Ultra-Turrax homogenizer was used to prepare stable adjuvant-antigen suspensions prior to injection

Electrofusion for hybridoma production was performed using a Biojet CF electrofusion apparatus supplied by B Braun, Amsterdam, Holland

A Hetosicc CD-52 freeze drier was used to lyophilise IBHR conjugates and was supplied by Heto Laboratory Equipment, Leopardstown Road, Foxrock, Dublin 18 A Vitris Consol 4 5 from The Vitris Company Incorporated, Gardiner, N Y 12525, U S A was used to lyophilise cell membranes for Western blotting analysis and to lyophilise DNA primers

Absorbances in 96 well plates (e g from ELISAs) were read using the Titertek TwinPlus Microplate Reader and the Titertek Multiscan PLUS MK11

A Shimadzu UV-160A UV-Visible Recording Spectrophotometer was used to measure the absorbance of solutions

The HPLC system used was Beckmann System Gold supplied by B M Browne,

Sandyford Industrial Estate, Foxrock, Dublin 18 and serviced by Brennan and Co , 61 Stillorgan Industrial Park, Stillorgan, Co Dublin The column used was a 10μm Protein Pak SW 300 column with a mobile phase of 0 1M phosphate buffer, pH 7 0

A LKB Bromma 2050 Midget Electrophoresis Unit was used in the preparation of SDS-PAGE gels and a Bio Rad Trans-Blot SD semi-dry transfer cell was used in Western immunoblotting PAGEL^{R} precast polyacrylamide gels for electrophoresis were also used These gels were run using the ATTO model AE-6100 mini gel apparatus using an ATTO Crosspower 3500 power supply

Electroporation of *E coli* cells was carried out on a ProGenetor Pulse Controller from Hoefer Scientific Instruments, which was fitted with a Gene Pulse Cuvette Unit (Bio-Rad)

The PCR machines used were LEP Scientific Prem^{TM} and Hybaid Omni Gene

Photographs for immunocytochemistry and immunofluorescence were taken using a Nikon FX-35 WA camera An NCB10 filter was used with colour film and an ND2 filter was used with black and white film The automatic timed exposure apparatus used was a Nikon Model HFX-IIA The colour film used was Kodak Gold II ISO/ASA 100 colour film and Ilford HP5 Plus400 black and white film ISO/ASA 400

Photographs for genetics work were taken using an Olympus OM-20 camera using T-max 100 film (Kodak) and an A003 red filter (Cokin, France) which blocks UV light Vivitar close-up lenses were used when required

2.3. Techniques involved in the production, purification and characterisation of monoclonal antibodies.

2.3.1. Routine culture of cells in suspension.

All tissue culture was performed in a laminar flow All materials used were sterilised by either autoclaving, filter sterilisation (using a 0 22μm filters) or were purchased sterile The cell lines used were maintained in DMEM-S_{10} [Dulbecco's Modification of Eagle's Medium containing 7 5% (v/v) sodium bicarbonate, supplemented with L-glutamine (2mM), Hepes (1mM) and FCS 10%, v/v] Media for the MCF-7 (and MCF-7 brain variant) breast adenocarcinoma cell line was further supplemented with sodium pyruvate (1mM) Cultures were initiated at cell densities of 2x10^{5} cells/ml using 7ml of medium per 25ml culture flask or 12ml of medium per 75cm^{2} culture flask Cells

were harvested, when approximately 75% confluent, by flushing them from culture surfaces using a sterile pasteur pipette All animal cells in culture were incubated in a humid, 5% CO_2 atmosphere at 37°C

2.3.2. Routine cell culture of adherent cell lines.

The G-CCM, G-UVW, $IJK_{(t)}$, T-24, MCF-7, A549, HL-60, H5683, B21 and EJ-138 cell lines are strongly adherent and required trypsinization to release them during harvesting and subculturing procedures All of the culture medium was decanted and 4ml of a trypsin EDTA solution [0 05%, v/v 0 0004%, w/v in sterile 0 01M PBS, pH 7 3] was added to the flasks After 1 minute this trypsin was removed and a further 4-5ml of trypsin EDTA solution was added and incubated at 37°C for 10 minutes The cell suspension was decanted into 5-10ml of complete culture medium to inactivate the trypsin and centrifuged at 1,200rpm for 10 minutes in universal tubes Cells were resuspended in 36ml of culture medium and divided into 3x75cm^2 sterile flasks The MCF-7 and $IJK_{(t)}$ cell lines do not adhere to plastic as strongly as the above cell lines and require a more dilute trypsin solution (0 05%, w/v) trypsin,0 02%, w/v, EDTA in 0 01M PBS, pH 7 3) to minimise surface antigen losses (Sasaki, *et al* , 1981)

Sterile 0 01M PBS was prepared by dissolving 8 0g NaCl, 0 2g KCl, 1 44g Na_2HPO_4 and 0 24g KH_2PO_4 in 800ml of distilled water The pH was adjusted to 7 3 and the volume of water was adjusted to 1 litre This solution was dispensed in convenient volumes, sterilised by autoclaving and stored at room temperature Phosphate buffered saline (PBS), 0 01M, pH 7 3 was routinely prepared by dissolving one PBS tablet in 100ml ddH_2O

2.3.3. Cell counts and viability staining.

Cell counts were performed on a Neubauer Hemocytometer slide Acridine orange/ethidium bromide and/or trypan blue were routinely used to determine cell viabilities (Mishell *et al* , 1980)

The acridine orange/ethidium bromide stain solution contained 0 1mg acridine orange and 0 1mg ethidium bromide in 100ml 0 01M PBS, pH 7 3 An equal volume of stain was added to a known volume of cells and examined by UV fluorescence microscopy Live cells stain green while dead cells stain orange

For trypan blue staining, a known volume of cells was added to an equal volume of

stain (0 2%, w/v), left for one minute and were then examined by light microscopy Live cells excluded the dye and remained white while dead cells stained blue Longer incubation periods with stains leads to non-specific uptake of dye and inaccurate results The total number of cells (T) in the cell suspension was calculated using the number of cells counted on the grid (C), as follows,

$$T = C \times D \times 10^4 \times V$$

where D is the dilution factor, in the case of a 1 1 dilution, D=2, V is the volume of cell suspension available and 10^4 is the conversion factor used

2.3.4. Long term storage and recovery of animal cells.

Cryopreservation methods described by Reid and Cour (1985) and Price (1985) were used Stocks of cells were maintained in liquid-nitrogen freezers Washed cells were resuspended to the desired concentration (usually $1x10^6$/ml) in FCS supplemented with 5% (v/v) dimethylsulphoxide (DMSO, cryoprotectorant) Aliquots of 1ml were placed in sterile cryotubes (ICN-Flow) and frozen at a rate of approximately -1°C per minute to -70°C on a freezing tray (Union Carbide) before being immersed in liquid nitrogen Recovery of cells from liquid nitrogen was achieved by thawing the cells rapidly to 37°C (Care must be taken to wear a face mask due the possibility of a vial exploding) The cells were then added to 10ml of DMEM at 37°C, pelleted by centrifugation at 500g for 10min and resuspended in 10ml fresh $DMEMS_{10}$ culture medium in $25cm^2$ culture flasks Cells recovered from liquid nitrogen were approximately 90% viable

2 3.4.1. Antibiotics used in culturing cells.

A sterile penicillin/streptomycin stock solution (100x) of 10,000units/ml and 10,000 μg/ml, respectively, was purchased (Section 2 1) and was used to give protection against bacterial infection This solution was aliquoted asceptically to single use volumes and stored at -20°C The usual working concentration used was 100umts/ml penicillin and 100μg/ml streptomycin

Gentamicin was occasionally used when a broader spectrum antibiotic than penicillin/streptomycin was required This was bought as a 100x sterile stock solution (20,000μg/ml) (Section 2 1) which was aliquoted aseptically to single use volumes and stored at 4°C The stock solution is very stable The usual working concentration used was 200μg/ml

2.3.5. Mycoplasma detection using the Hoechst 33258 fluorescence assay.

Since mycoplasma contamination affects the growth and viability of cell cultures, all cell lines used were routinely checked using the DNA-intercalating agent bisbenzimid (Hoechst 33258) which detected the presence of mycoplasma in the cellular cytoplasm The detection method used was further described by Carroll and O'Kennedy, (1988) Cultures of mycoplasma-free normal rat kidney (NRK) cells were established on glass coverslips using 5 x10^3 cells in 1ml of DMEM containing 6% (v/v) FCS and cultured overnight Supernatants from cultures to be tested were then added to the NRK cells, which were reincubated for a further 3 to 4 days or until the cells were approximately 70% confluent Duplicate NRK cultures were fed with fresh media and used as a negative control Positive controls, previously prepared and stored at 4°C were included The coverslips were then washed three times in 0 01M PBS, pH 7 3, (taking care to keep the cells on the uppermost surface at all times) and fixed for exactly six minutes in 1 1 methanol acetone solution at -20°C The coverslip preparations were then rinsed three times in 0 01M PBS, pH 7 3, and stained for 10 minutes in Hoechst 33258 (0 05μg/ml in 0 01M PBS, pH 7 3)(toxic) The stain was washed from the cells using 0 01M PBS, pH 7 3, and the coverslips air-dried and mounted on clean slides Slides were stored in the dark until viewed under UV light on a Nikon fluorescence microscope at 100X magnification with a B2 combination filter (Nikon) The membranes of contaminated NRK cells had areas of fluorescent DNA which could be associated with mycoplasma while no such fluorescence was found in negative and control slides

2.3.6. Use of adjuvants to stimulate an immune response.

Adjuvants are non-specific stimulators of the immune response Used properly, they allow a much stronger immune response to be generated (Cahill *et al* , in press) The most commonly used adjuvants are Freund's complete and incomplete adjuvants (CFA and IFA, respectively) Both CFA and IFA consist of nonbiodegradable mineral oils CFA also contains killed *Mycobacterium tuberculosum* and is only used for the initial immunisation, all subsequent immunisations are given with IFA The effect of the adjuvant is to cause a local irritation leading to an inflamation response which attracts large numbers of immune effector cells to the site of injection The antigen, which has been emulsified in the oil using a Kika-Werk Ultra-Turrax homogeniser, is released

slowly to give a prolonged exposure to the immune system *M tuberculosum* contains muramyl dipeptide in its extracellular matrix which is a potent stimulator of immune cell function during primary immunisations It stimulates the release of various lymphokines which play a crucial role in the development of the immune response Freund's adjuvant was always given intraperitioneally and must **never** be given intravenously

2.3.6.1. Types, doses and preparation of antigens for injection with and without adjuvant.

Soluble proteins, such as glial fibrillary acidic protein (GFAP), iodinated Bolton-Hunter reagent-BSA conjugate (IBH-BSA) or phosphogen-KLH (PHOS-KLH) when used to immunise mice, were always given in the presence of an adjuvant (Section 2 3 6) If the antigen is in short supply, such as GFAP, it can be given at doses of 20μg to 60μg per mouse Where a large amount of the antigen is available, 50μg to 200μg per mouse may be given It is seldom worthwhile to administer larger amounts

To reconstitute GFAP (250μg), 100μl of sterile ultrapure H_2O was added to dissolve the protein, then 145μl of $DMEMS_{10}$ was added to prevent the protein adhering to the glass when reconstituted, lastly 5μl of gentamycin was added to eliminate contamination

To prepare for administration the antigen is made up in a bijou at a concentration of 50μg in 100μl of 0 01M PBS, pH 7 3 per mouse 200μl of the appropriate Freund's adjuvant per mouse is added to the antigen solution and the mixture is emulsified using an homogenisor Emulsification is achieved, when the two solutions do not separate if left standing at room temperature 300μl of this solution is administered intraperitionally per mouse using a 1ml sterile syringe and a 26xG3/8 gauge needle Particulate antigens include whole cells, such as G-CCM, which make good antigens, because they are readily phagocytosed by the antigen-processing cells of the immune system Live cells are given at 10^5 to 10^7 cells per injection, intraperitoneally (i p) in 300μl of 0 01M PBS, pH 7 3, which had been warmed to 37°C

2.3.6.2. Immunisation protocol of mice for the production of monoclonal antibodies.

Male balb/c mice were used for the production of monoclonal antibodies Mice bred in

the animal house facility at Dublin City University were used, mitally However, subsequently, because of the problem of mouse hepatitis virus (MHV) contamination and IgM production, certified mice were obtained from Harlan Olac Limited Normally five mice were immunised at a time

Four types of immunogens were used

(i) a human anaplastic astrocytoma cell line, G-CCM, in order to produce anti-G-CCM monoclonal antibodies (Section 3)

(ii) Glial Fibrillary Acidic Protein (GFAP), a commercially produced pure protein, in order to obtain anti-GFAP monoclonal antibodies (Section 3)

(iii) an IBHR-BSA conjugate to obtain monoclonal and polyclonal anti-IBHR antibodies (Section 4), and

(iv) a Phosphogen-KLH conjugate in order to obtain monoclonal antibodies which would react with the phosphogen and hydrolyse the corresponding amide (Section 6)

All injections were given i p , except for the final GFAP injection (50μg), which was given i v in 100μl of warmed sterile 0 01M PBS, pH 7 3, through the pre-warmed tail vein of a mouse The following immunisation protocol was used for the production of monoclonal antibodies,

Day 1 (i) The protein antigen-adjuvant emulsion was prepared (Section 2 3 6) in CFA using 50μg of antigen per 200μl of emulsion or (ii) $5x10^6$ G-CCM cells were resuspended in 500μl of 0 01M PBS, pH 7 3

Day 14 Reimmunise as in (i), except IFA is used in place of CFA for this, and succeeding immunisations

Day 28 Repeat immunisation as on Day 14

Day 42 Repeat immunisation as on Day 14

Day 49 Each mouse that had been immunised was tail bled (Section 2 3 8) and the specific antibody titre checked by ELISA (Section 2 3 16) against the appropriate antigen or alternative conjugate for specific antibody production

Day 56 If the titre obtained from day 49 was low, the mice were boosted as on Day 14

The mice with the strongest immune response to the antigen were either,

(i) selected and reimmunised by an i p injection or,

(ii) as was the case with the protein antigens, boosted with an i v injection or,

(iii) the mouse was sacrificed and a splenectomy performed (Section 2 3 9) in order to perform a fusion (Section 2 3 13) or,

(iv) the mouse was sacrificed three days prior to a fusion and a splenectomy carried out (Section 2 3 9) in order to perpare for an *in vitro* immunisation (Section 2 3 11) Routinely three fusions were performed separately on the same day

2.3.6.3. Specific immunisation protocol for mice immunised with G-CCM cells.

Care was taken when preparing G-CCM cells for immunisation These cells are adherent and 0 05% (w/v) trypsin in 0 01M PBS (Section 2 3 2) was used to detach the cells from the tissue culture flask The cells were incubated in this trypsin solution for as short a time as possible as, otherwise, surface antigens may be removed by the trypsin The immunisation protocol is detailed in Section 2 3 6 3 Five mice were immunised with G-CCM cells in 0 5ml of PBS, per mouse, as outlined in Table 2 6

Table 2.6. Immunisation schedule of balb/c mice immunised with G-CCM cells in 0.01M PBS, pH 7.3.

Day	NUMBER OF CELLS (x 10^6) injected
1	1
16	4
31	1
48	2
65	1

On day 56, the mice were tail bled and their sera were tested by ELISA on G-CCM- and GFAP-coated plates The results obtained are shown in Table 3 1 and Figure 3 1

2.3.7. Procedures for the production of anti-G-CCM monoclonal antibodies.

From the results of the screening of the sera obtained from G-CCM immunised mice (Table 3 1), the mouse which gave the best result was sacrificed and the spleen cells

used in electrofusion as described in Section 2 3 13 2 $8x10^6$ spleen cells were harvested (Section 2 3 9) and 1 $7x10^7$ SP2/0 cells were obtained from culture (Section 2 3 1) The spleen and myeloma cells were mixed in a 1 1 ratio

Following the electrofusion, the cells were seeded at $1x10^4$ cells per well in sterile 96 well culture plates Three electrofusions were performed and the resulting hybridomas were cared for as outlined in Section 2 3 14 with the fused cells were selected using hypozantine 8-azaguanine (HA) The positives were selected by ELISA (Section 2 3 16) against an anti-mouse IgG antibody in order to select for clones which were secreting antibody (Section 2 3 16 5) The supernatant from these antibody-producing clones were further screened by ELISA against G-CCM (Section 2 3 16 1) and, finally, against GFAP (Section 2 3 16 3) This was to find an antibody that would react with a GFAP-like antigen on G-CCM cells

The anti-G-CCM antibodies were then isotyped by the various methods outlined in Section 2 3 18 All of the monoclonal antibodies produced isotyped as IgM This, at first, appeared to be an extraordinary result as the mice had been immunised four times over a period of two months and had been given an *in vivo* or *in vitro* boost in some cases

However, on examination of the immunisation records of these mice, it was noticed that a number of the immunised mice, of all ages, died between January and Feburary 1991 These were the first of such immunised mice to die In July and August of 1991, when, on isotyping, only IgM monoclonal antibodies were obtained and on discussing with other colleagues attempting to produce monoclonal antibodies using mice from our animal house, five co-workers had produced an IgM antibody in the previous 6 months, and no IgG monoclonal antibodies were produced within this period

As suspecion grew of some viral contaminant of the mice colony in D C U , the mice were screened for immunodeficiency viruses which would either kill these mice, as had happened,or would render them incapable of mounting an IgG response to an injected antigen and would only allow them to produce an IgM response

Two of the ten mice screened came up positive for the mouse hepatitis virus (MHV) These mice tested negative for Sendai, PVM and MVM viruses Although this was only a small sample, it demonstrated that our balb/c colony was contaminated with the MHV virus From the animal house records, it was shown that the supplier of our balb/c mice

had changed in December 1990, and from that time mice were obtained from a source which was known by the supplier to be MHV contaminated, but the supplier did not inform our animal house personnel until questioned when the screening results were obtained

The assistance and support of Ms Carolyn Wilson is acknowledged and appreciated during this period

When the monoclonal antibodies obtained from these fusions isotyped as IgM, attempts were made to repeat immunisations in isolation facilities in Trinity College, Dublin, along with immunising mice there to produce catalytic antibodies (Section 6) Certified disease- and virus-free mice were imported from Harlan Olac Ltd (Section 2 1) and were brought directly to isolators in Trinity Such attempts were without success as there was a shortage of trained technicians to care for these mice in the isolators in Trinity College Due to the inability to keep these mice in the isolators for the required length of time for a sufficient immunisation schedule, the mice were returned to D C U without being immunised

2.3.7.1. Procedures for the production of anti-GFAP monoclonal antibodies.

The GFAP used (Table 2 3) was determined to be 98% pure by SDS-PAGE Its source was from bovine spinal cord and its molecular weight (M_r) was 52,000 Due to the expense of GFAP, only three mice were immunised with GFAP as outlined in Section 2 3 6 The immunisation schedule is given in Table 2 7

Table 2.7. Immunisation schedule of balb/c mice immunised with GFAP.

Day	GFAP (μg/ml) injected
1	50
21	50
40	50
58	50

On day 48, the mice were tail bled and their sera were tested by ELISA on GFAP and G-CCM coated plates The results obtained are shown in Table 3 2 and Figure 3 2 One mouse was given an i v injection, on day 58, of filter sterilised GFAP in PBS maintained at 37°C This solution was filter sterilised using the low protein-binding 0 22μm filters from Becton Dickinson Only 15μg were administered i v and the remaining 35μg were administered i p The mouse was bled and sacrificed on day 61 The sera obtained was used as a positive control for ELISAs and throughout the experiment A splenectomy was performed (Section 2 3 9), 1 24x10^8 spleen cells were harvested, washed with red cell lysis buffer (Section 2 3 11 4) and resusdended in 8ml $DMEMS_0$ 2ml (i e 3 1x10^7 cells) of this solution was used in the immunisation as follows,

5ml $DMEMS_0$
5ml sTCM
100μl 100x 2-Mercaptoethanol
50μl Gentomycin (5μl/ml)
12μl GFAP (1μg/μl)

This solution was dispensed at 5x10^6 cells per well of a 6 well sterile tissue culture plate and was incubated for 3 days in 5% CO_2 at 37°C The spleen cells were then resuspended, pooled and counted Using the PEG method (Section 2 3 13 1), 1x10^8 spleen cells were fused with 3x10^7 SP2/0 cells (i e a 3 33 1 ratio of spleen cells to myeloma cells) The fused cells were plated onto 15 plates which were monitored for growth of clones after 10 days These clones were screened for anti-GFAP antibodies by ELISA (Section 2 3 16) and the antibody-producing hybridomas were expanded according to Section 2 3 14

Employing the *in vitro* immunisation method again, a second mouse was used 6 72x10^7 cells were incubated with GFAP as above and according to Section 2 3 11 5 After 3 days, 6x10^6 cells were electrofused (Section 2 3 13 2) with 6x10^6 SP2/0 cells The parameters used in the electrofusion are detailed in Section 2 3 13 2

2.3.8. Tail bleeding of mice.

The serum of each immunised mouse was tested in order to monitor the response to the injected antigen This was done by placing the mouse under an infrared lamp for 5 minutes to warm it up and increase the blood flow to its tail veins making them easier

to visualise The lower tail vein was swabbed with 70% (v/v) alcohol, nicked close to the tip of the tail using a sterile scalpel blade and the drops of blood were collected The blood was then placed at 37°C for 1 hour and the clot was released by tapping the tube against the bench The tube was placed at 4°C overnight and then centrifuged at 1000g for 10 minutes The serum was collected using a pipette and the pellet discarded The serum was then tested by ELISA to determine the titre of antigen-specific antibody present (Section 2 3 16) 0 02% (w/v) sodium azide was added to the serum and stored in 50μl lots at -20°C

2.3.9. Isolation of immunised splenocytes for cell fusion.

The mouse was sacrificed by cervical dislocation A scissors, forceps and tissue dissociation sieve were autoclaved and when in use they were immersed in 70% (v/v) alcohol The sacrificed mouse was immersed in a beaker of 70% (v/v) alcohol, and then transferred onto a sterile dissection board in a laminar flow All subsequent steps were carried out under aseptic conditions The mouse was pinned down on its right hand side and using sterile instruments, the skin was cut away from the abdomen to expose the peritoneal membrane which was sprayed with 70% (v/v) alcohol The peritoneal membrane was dissected and the spleen removed carefully, ensuring that the spleen was not torn and the gut, or any other tissue, was not pierced

The spleen was placed in a sterile petri dish containing 20ml of warmed DMEM-S_{10} A sterile tissue dissociation sieve (Sigma) was placed in a second petri dish and 10ml of medium was poured into it The spleen was transferred to the sieve and, using a sterile pestle, the spleen was pressed through the sieve into the medium to produce a cell suspension The medium can be collected using a 20ml syringe and passed through the sieve a number of times to ensure a uniform cell suspension This medium was collected into a 50ml centrifuge tube and the large clumps were allowed to settle The supernatant was then transferred to a fresh tube and the cells washed once in DMEM-S_{10} The red blood cells were lysed using filter-sterilised red cell lysis buffer (Section 2 3 11 4) The cells were washed in DMEM-S_{10}, stained using trypan blue (Section 2 3 3) and counted The viability should be greater than 95% The cells were then washed twice in DMEM-S_0 It is important for the success of the fusion that no protein is present in the medium at this stage as it would interfere with the polyethylene glycol (PEG) The cells were left at 37°C and used as soon as possible

2.3.10. Preparation of myeloma cells for fusion.

Both NSO/1 (NSO) and SP2/0-Ag14 (SP2/0) cells were used in cell fusions, NSO were used initially, but it was subsequently found that using SP2/0 gave a 10-fold, or greater, number of clones NSO and SP2/0 cells were maintained in the exponential phase of growth for 7 days prior to fusion, by dividing a suspension of the cells every two days and the day before the fusion was to be undertaken Both NSO and SP2/0 cell lines were tested for the presence of mycoplasma (Section 2 3 5) These cell lines were also checked for the presence of any type of contamination by culturing them in antibiotic-free medium for 7 days prior to fusion The NSO cell line was pretested for HAT sensitivity by confirming their inability to grow in DMEM-S_{10} containing HAT (100μM hypoxanthane, 1M aminopterin and 60μM thymidine) Similarly, the SP2/0 cells were pretested for their inability to grow in DMEM-S_{10} containing HA (100μM hypoxanthane and 2μg/ml 8-azaguanine)

2.3.11. *In vitro* immunisation.

In vitro immunisation is used where an antigen is toxic or is very expensive, as with GFAP, or where it was available in only minute quantities, as was the case with Phos-KLH This method is an alternative to *in vivo* immunisations (Section 2 3 6), where isolated lymphocytes can be sensitised to antigens *in vitro* These cells can then be used in cell fusions for monoclonal antibody production (Section 2 3 13)

In vitro immunisations offer the following distinct advantages,

(i) only very small amounts of antigen are needed, i e μg instead of the mg quantities required *in vivo*

(ii) the normal controls of the immune system are absent *in vitro* allowing responses against self antigens to be generated and

(iii) the methods are simple and rapid Immunisations are complete in 3 to 5 days as opposed to several weeks *in vivo*

The major disadvantage associated with the *in vitro* response is that most of the responses obtained are primary responses consisting mainly of IgM class antibodies Secondary responses are not normally seen unless the donor lymphocytes have had some prior exposure to the relevant antigen *in vivo*, or special kits are used such as the CEL-PRIME *in vitro* immunisation kit for high IgG yield from Immune Systems Ltd (Table 2 1) It has been demonstrated, however, that a secondary response is obtained

if the animals used as a source of lymphocytes are given an *in vivo* immunisation 28 days prior to the *in vitro* immunisation (DeBoer *et al* , 1988)

The methods described here are essentially those of Ossendorp *et al* (1986) in which isolated murine lymphocytes are immunised *in vitro* in a serum-free medium called LINO This medium was first described by Yssel *et al* (1984) and was designed for the culture of lymphocytes For successful *in vitro* immunisations a rich source of B-cells is required, which usually come from a mouse spleen and a source of T-cell derived growth factors These factors were supplied in the form of a T-cell-conditioned medium (sTCM) Mouse thymocytes were cultured for 48 hours in LINO during which time they secrete a variety of lymphokines and other growth factors (Ossendorp *et al* , 1986) These factors play a crucial role in the development of the immune response

2.3.11.1. Preparation of LINO.

The basal medium used was Iscove's DMEM (IMEM) (Flow laboratories) The order of addition of the components of LINO is very important The proteins must be fully dissolved before adding any lipids

1 litre of LINO was prepared as follows, although this may be scaled down for smaller volumes 2 5g of BSA was added to 50ml of IMEM and allowed to fully dissolve 5mg of bovine insulin (25units/mg) was dissolved in 2ml of 0 01N HCl and added to the medium 26mg of human transferrin was dissolved in 2ml of 0 01M PBS, pH 7 3, and added A 1 1000 dilution (v/v) of 100% ethanolamine in 0 01M PBS, pH 7 3 was prepared and 2ml of this solution was then added 10ml of penicillin (10,000units/ml) streptamycin (10,000μg/ml) solution was added to the medium 5mg of linoleic acid was dissolved in 1ml of absolute alcohol and 0 2ml of this was added 5mg of palmitic acid was dissolved in 1ml of absolute alcohol and 0 2ml of this solution was added to the medium 5mg of oleic acid was dissolved in 1ml of absolute alcohol and 0 2ml was added to the medium The medium was then filter sterilized using a 0 22μm filter and adjusted to 1 litre with IMEM, and it was then dispensed into 50ml lots and stored at -20°C 1% L-glutamine (v/v) and 50μM 2-mercaptoethanol (Section 2 3 11 2) were added to the medium just before use

2.3.11.2. Preparation of a stock solution of 2-mercaptoethanol.

0 5ml of 2-mercaptoethanol was added to 6 6ml of ddH_2O and 5ml of this solution was

added to 95ml of ddH_2O This was filter sterilized, aliquoted and stored at -20°C The stock concentration was $5x10^{-2}M$ and this was used at a dilution of 1 1000 Once thawed, it should not be refrozen

2.3.11.3. Preparation of sTCM in LINO.

Scissors, forceps and a tissue dissociation sieve were autoclaved and placed in 70% alcohol in a laminar flow 30ml of DMEM was placed in a sterile 50ml tube and kept at 37°C Five unimimumsed female balb/c mice, 5 to 6 weeks old, were sacrificed by cervical dislocation and placed in alcohol One of these mice was then pinned onto a dissection board The skin was cut away from the abdomen and a wide flap from the abdomen to the shoulders was cut to expose the rib cage The entire mouse was sprayed with alcohol The rib cage was cut from the zyphoid process to the axillary region and the flap was pulled up over the head The thymus, which is a small, white, bi-lobed organ just anterior to the heart is then visible The thymus was then carefully lifted out and placed in the tube containing DMEM This procedure was performed on each of the mice and their thymuses were pooled These were then transferred to a sterile tissue dissociation sieve in a petri dish and 25ml of DMEM was added The thymuses were pressed through the sieve to get a uniform cell suspension using a pestle The remaining tissue in the sieve was discarded The cells were washed twice in DMEM and once in LINO and the cells were resuspended at $5x10^6$ cells per ml in LINO The medium was dispensed in 25ml lots into $75cm^2$ culture flasks and incubated at 37°C in 5% CO_2 for 48 hours The culture supernatants were harvested and centrifuged at 300rpm for 10 minutes The final supernatant was filter sterilized and stored in 10ml lots at -80°C

2.3.11.4. Preparation and use of red cell lysis buffer.

0 874g of NH_4Cl, 0 1g $KHCO_3$ and 3 67mg of EDTA were dissolved in a final volume of 100ml of ddH_2O The pH was adjusted to 7 4 with 1N NaOH, filter sterilized and stored at room temperature The cell pellet, for example splenocytes, was resuspended in 15ml of lysis buffer Lysis should be complete in 2 to 3 minutes and the cells were then washed in 15ml of DMEM-S_{10} If lysis was incomplete, (i e the pellet was still red), the pellet can be re-washed in DMEM-S_{10}

2.3.11.5. *In vitro* immunisation of isolated murine splenocytes.

The *in vitro* immunisation medium was prepared as follows, 50ml of LINO (Section 2 3 11 1) was thawed by warming to 37°C, 2-mercaptoethanol (Section 2 3 11 2) was added to a final concentration of $5x10^{-5}$M and L-glutamine was added at a concentration of 1% (v/v) 25ml of sTCM (Section 2 3 11 3) was thawed by warming to 37°C and added to an equal volume of LINO This is the immunisation medium All media were kept at 37°C while not in use A balb/c mouse was sacrificed, the spleen was removed and a cell suspension prepared (Section 2 3 9) The red blood cells were lysed (Section 2 3 11 4) and the final cell preparation was washed in LINO The cells were resuspended to $5x10^6$ cells per ml in the immunisation medium The antigen was prepared (Section 2 3 6 1) and added at concentrations from 0 1 to 5μg/ml If this is the first immunisation with an antigen, it is recommended that a range of antigen concentrations from 1ng/ml to 10μg/ml be used Generally, most antigens will give a good response at around 1μg/ml, but this is very dependent on the antigen in question 2ml lots were then dispensed aseptically into the wells of 6 well culture trays, wrapped in parafilm and incubated at 37°C in 5% CO_2 These cells can be harvested after 3 days for use in cell fusions (Section 2 3 13) Blasts of activated B-cells are seen after 4 to 5 days of culture

2.3.11.6. *In vitro* immunisation using the Immune Systems' Cel-Prime 'High IgG Yield' kit.

The instructions accompanying the kit were followed throughout this procedure Sterile antigen was prepared as described in Section 2 3 6 1 The vial of support cells and support cell medium provided were thawed and the contents of the vial were resuspended in 5ml of the support cell medium and aseptically transferred to a 25cm^2 flask These cells are incubated at 37°C with 5% CO_2 for 24 hours The medium was then discarded and replaced with 5ml of fresh support medium with 30μg of sterile antigen (Section 2 3 6 1) added and incubated for 48 hours The bottles of immunisation medium and splenocyte medium provided were thawed at room temperature and these media were heated to 37°C The spleen was removed from a 10-12 week balb/c mouse (Section 2 3 9) and placed in a petri dish with 5ml of splenocyte medium and the spleen treated to produce a single cell suspension These cells were centrifuged for 5min and the splenocyte cell pellet was resuspended in 5ml

temperature and these media were heated to 37°C. The spleen was removed from a 10-12 week balb/c mouse (Section 2.3.9.) and placed in a petri dish with 5ml of splenocyte medium and the spleen treated to produce a single cell suspension. These cells were centrifuged for 5min and the splenocyte cell pellet was resuspended in 5ml immunisation medium. The medium from the support cells was discarded and the splenocyte suspension transferred to this flask. The remaining immunisation medium was added to this flask, along with 30μg of fresh, sterile antigen. The lid of the vented flask was firmly tightened and the flask was placed horizontally on the incubator and left undisturbed for 3-4 days at 37°C with 5% CO_2. After this time, the primed splenocyte culture is ready for fusion to myeloma cells (Section 2.3.13.1).

2.3.12. ELISA-spot assay: assessment of *in vitro* immunisation.

To assess the efficacy of an *in vitro* immunisation experiment, one must be able to enumerate the numbers of specific antigen-reactive B-cells generated during the immunisation. A measure of the success of the immunisation is the number of antigen-reactive hybridomas obtained in subsequent cell fusions. However, it can take several weeks and a lot of work before such information is available. A simpler and more rapid approach is to use an ELISA-spot assay. The principles of this assay are based on standard ELISA technology. Two major differences in this assay, however, are that (i) cells are incubated with fixed antigen and (ii) the substrate solution is provided in a solid-phase rather than an aqueous phase (Sedgewick and Holt, 1983; Czerkinksy *et al.*, 1983).

The antigen to which the antibodies had been raised, was coated onto 6 well trays in the normal fashion. An aliquot of the cells from the *in vitro* immunisation (Section 2.3.11.5.) were incubated with the antigen. During the incubation period, the cells secrete their antibody onto the antigen-coated surface. If the cells were producing specific anti-antigen antibodies, then this antibody would bind to the antigen in the area around the cell. Non-specific antibodies do not bind and will disperse into the surrounding medium.

The cells were then washed from the plates leaving the antigen-coated well with small areas of specifically bound antibody which correspond to the antigen reactive cells. An anti-mouse antibody linked to alkaline phosphatase (Table 2.4.) was then added which binds the first antibody. The excess antibody is removed by washing and the enzyme

substrate is added in a solid-phase of agarose Colour development was only obtained in those areas which correspond to the locations of the original antigen reactive cells because both the antibody and the substrate are fixed in position A measure of the number of antigen reactive cells generated in the *in vitro* immunisation can be obtained by counting the number of colour spots

2.3.12.1. Preparation of reagents for the ELISA-spot assay.

To prepare carbonate buffer, 0 15g of sodium carbonate and 0 293g sodium bicarbonate were dissolved in 100ml of ddH_2O and was brought to pH 9 6 using conc HCl and 5M NaOH, as appropriate Phosphate buffered saline (PBS), 0 01M, pH 7 3 (Section 2 3 3) was routinely prepared by dissolving one PBS tablet in 100ml ddH_2O The wash buffer was prepared by adding 0 1% (v/v) Tween-20 to PBS Amino-methyl-propanol buffer (0 1M) was prepared by dissolving 150mg magnesium chloride (5mM), 0 1ml Triton X-405 (0 01%, v/v) and 1 0g sodium azide (0 1%, w/v) (highly toxic) was dissolved in 30ml of ddH_2O 95 8ml of amino-methyl-propanol was added with stirring The pH was adjusted to 10 25 with conc HCl This solution was left overnight at room temperature and then the pH was readjusted to 10 25 The final volume was brought to 1 litre and stored at 4°C To prepare BCIP substrate, 0 05g of 5-Bromo-3-chloro-indoyl-phosphate (BCIP, 2 3mM) was dissolved in 50ml of amino-methyl-propanol buffer (0 1%, w/v) and left for 1 hour at room temperature This was then filtered through 0 45μm filters to remove any undissolved material and warmed to 40°C 3% (w/v) of 36°C gelling agarose was prepared in ddH_2O, boiled to dissolve the agarose and subsequently cooled to 60°C 12 5ml of the 3% (w/v) warmed agarose (final concentration is 0 6%, w/v) was added to the BCIP solution and was kept at 40°C until required

2.3.12.2. ELISA-spot assay.

100 to 200μg/ml of antigen in 1 5ml of carbonate buffer, pH 9 6, was added to each well of a 6 well tissue culture plate and incubated overnight at 4°C The wells were washed twice with wash buffer, PBS-Tween This was prepared by adding 0 05% (v/v) Tween-20 to 0 01M PBS, pH 7 3 Tween-20 is polyoxethylenesorbitan monolaurate (Section 2 3 16 2) 2 5ml of 1% (w/v) BSA in 0 01M PBS, pH 7 3, was added to each well to block unbound sites and incubated for 1 hour at 37°C The plate was washed as before Previously washed *in vitro*-immunised lymphocytes (Section 2 3 11 5) were

before Previously washed *in vitro*-immunised lymphocytes (Section 2 3 11 5) were prepared at concentrations of $1x10^4$, $1x10^5$ and $1x10^6$ cells/ml in $DMEMS_{10}$ 1ml of these cell solutions was added to duplicate wells of the 6 well plate which was then incubated on a level surface for 2 hours at 37°C in a vibration-free incubator The plates were washed twice in 0 01M PBS, pH 7 3 Then 2 0ml/well of cold 0 01M PBS, pH 7 3, with 10mM EDTA was added and left at room temperature for 10 minutes The plate was washed four times in wash buffer 6ml of a 1 7000 dilution of anti-mouse Ig-alkaline phosphatase-conjugated antibody (Table 2 4) was prepared in Tris-buffered saline (TBS) [2M Tris-HCl, pH 9 5, 5M NaCl and 1M $MgCl_2$, made up to 1L in ultrapure H_2O] 1ml/well of this solution was added to the plate and incubated at 37°C for 2 hours or overnight at 4°C The plates were four washed times in TBS buffer and rinsed once in amino-methyl-propanol buffer 1 5ml of BCIP substrate in 0 6% agarose (w/v) was added on a level surface and the agarose allowed to solidify The plates were transferred to a 37°C incubator Blue spots began to appear after 60 to 90 minutes and were counted with the 10x objective of an inverted microscope

2.3.13. Fusion protocols used in the production of monoclonal antibodies.

The fusions performed were based on the technique described by Kohler and Milstein (1975)

2.3.13.1. Hybridoma cell production by PEG-mediated cell fusion.

The media and solutions used throughout this procedure were sterile and were kept at 37°C The mouse spleen and myeloma cells, NS0/1 or SP2/0, were counted (Section 2 3 3) and the cells were combined in a 50ml centrifuge tube in a 5 1 spleen myeloma ratio However, ratios of 10 1 and 2 1 have also been used successfully This cell mixture was then washed twice in serum-free DMEM (DMEM-S_0) Cells were spun at 4,000rpm for 10 minutes and the supernatant was fully decanted The cells were fused in PEG-1500 as follows, one ml of the PEG solution was added to the pelleted cells over one minute with gentle mixing, ensuring the temperature was kept constant at 37°C by keeping the tube in a 37°C water bath in the laminar flow throughout the procedure This was left for a further minute while the tube was mixed with a continuous gentle swirling motion The PEG was then slowly diluted by the dropwise addition of 3ml of DMEM-S_0 over three minutes, followed by 10ml of medium over a further 10 minutes

The cells were incubated at 37°C for 30 minutes to allow the hybrid cell membranes to form The cells were then pelleted and resuspended at a concentration of $2x10^5$ cells/ml of DMEM-S_{10} supplemented with either 1x hypoxanthine, aminopterin and thymidine (HAT) if fused with NSO or hypoxanthine 8-azaguanine (HA) if SP2/0 cells were used, plus 5% (v/v) BRIclone, and 1x HIPO HIPO supports the growth of hybridomas and consists of HEPES (1M), insulin (20iu/ml), sodium pyruvate (5mg/ml) and oxaloacetic acid (15mg/ml)

200μl of this solution was added to 11 rows of a sterile 96 well culture plate The parent myeloma cells in selective media were added to the 12th row in order to confirm that these cells were sensitive to the selective media The myeloma cells died out in 7-8 days The spleen cells die in 3-4 days Therefore, any cells growing after 10 days are hybrid cells or hybridomas The cells were left undisturbed for seven days and subsequently fed with DMEM-S_{10} containing HAT or HA Plates were checked for the presence of clones after 14 days and the supernatant was checked for specific antibody production by the sandwich ELISA (Section 2 3 16 4), "supernatant" ELISA (Section 2 3 16 5), cell fixation ELISA (Section 2 3 16 1) or antigen ELISA (2 3 16 3), as appropriate, when the cells in the wells were 70% confluent The hybridomas grown in HAT were weaned off this medium after 2 weeks in culture by replacing it with 1x hypoxanthine and thymidine (HT) medium After 4 weeks all hybridomas were cultured and expanded in DMEM-S_{10} without HT or HA

2.3.13.2. Generation of hybridomas by electrofusion.

The basis of this technique is discussed in Section 2 3 13 2 Immunised splenocytes were collected from either an immunised mouse (Section 2 3 9) or from *in vitro* immunised splenocytes (Section 2 3 11) The SP2/0 myeloma cells were harvested from a culture in log-phase of growth (Section 2 3 10) The spleen and myeloma cells were prepared as described (Sections 2 3 9 and 2 3 10) counted, and the viability assessed using trypan blue (Section 2 3 3) The cells were mixed in a 1 1 ratio to give $6x10^6$ cells/ml total and then pelleted If SP2/0 cells were used, the cells were resuspended in pronase solution (0 05mg/ml pronase in DMEM-S_0) for 3 mins This reduces the surface charges for the cells, thus aiding and increasing cell fusion One ml of the pronase solution was used per $1x10^6$ cells 1ml of FCS was added to stop the action of the pronase If NS0/1 cells were used the pronase step was omitted as it

caused these cells to clump The cells were then washed three times in 10ml of fusion medium (0 225M sucrose, 0 2mM calcium chloride and 0 2mM magnesium chloride) at room temperature The cells were then resuspended at $6x10^6$ cells/ml in fusion medium and transferred into a sterile eppendorf or bijou 250μl of this cell suspension was drawn into the sterile cell fusion chamber and the electric field was applied to the chamber for inducing dielectrophoresis, followed by high voltage pulses to induce fusion Dielectrophoresis is induced by a field strength of 250V/cm and amplitude of 1MHz Fusion of the myeloma and spleen cells was induced by 3 consecutive pulses of 3kV/cm and 15μsec duration The pulse interval was 1 second The electrical conditions for an electrode gap width were set as follows,

(i) Dielectrophoresis

Frequency	1 5MHz
Amplitude 1	0V
Amplitude 2	5V
Duration (delay 1)	0V
Duration (ramp)	0sec
Duration (delay 2)	30sec

(ii) Fusion Conditions

Pulse strength	40V
Pulse duration	15μsec
Pulse number	3
Pulse interval	1sec
Alignment-off time	10msec

(iii) Post-Alignment Conditions

Frequency	1 5MHz
Alignment time	30sec
Alignment time (constant/ramp)	constant

The cells were then removed from the chamber and pipetted into 5ml of warmed DMEM-S_{10} and incubated at 37°C for 30 minutes From this point the procedure is the same as for PEG fusions (Section 2 3 13 1)

2.3.14. Expansion of the antibody-producing hybridomas.

The clones were initially screened for specific antibody production by sandwich and

supernatant ELISA (Section 2 3 16 5) when they became 70% confluent in the 96 well plate Those cells that test positive were expanded into 24 well plates and were re-screened to ensure that they continue to produce and secrete the relevant antibody using the antigen-specific ELISA (Section 2 3 16 3) Positive clones from this second screening were transferred to 6 well plates when they reached 80% confluency Samples were also frozen in liquid nitrogen as back-ups in case of contamination (Section 2 3 4) The remaining cells were expanded to 25cm^2 flasks and, subsequently, used in the cloning procedure (Section 2 3 15)

2.3.15. Cloning of suspension cells by limited dilution.

Hybridoma cultures, in exponential growth phase, were cloned by limiting dilution in 96 well plates in order to ensure monoclonality of cells The cloning medium was prepared prior to cell cloning, and consisted of culture medium supplemented with 10% (v/v) FCS, 5% (v/v) BRIclone and 1% (v/v) HIPO Cells were removed from culture flasks, 24 well plates or 6 well plates, and a very accurate count of the viable cells was obtained (Section 2 3 3) The cells were diluted to a stock concentration of 50 cells/ml in cloning medium and 200μl/well were plated over 6 rows of a 96 well plate (i e 10 hybridoma cells per well) This stock medium was further diluted to 5 cells/ml and 2 5 cells/ml to give a final concentration of 1 and 0 5 hybridomas per well when plated out at 200μl/well The 96 well plates were incubated for 5-6 days and then examined microscopically for clone formation Wells containing one clone per well should represent monoclonal cultures of hybridomas and were allowed to grow to 70% confluency, expanded and checked for maintenance of antibody production by antigen-specific ELISA This cloning procedure was repeated to ensure monoclonality Eventually, following sequential cloning by limited dilution, the cells were tested for mycoplasma contamination, expanded into 25 cm^2 flasks and frozen as back-up stocks in liquid nitrogen (Section 2 3 4)

2.3.16. Enzyme-Linked Immunosorbent Assay (ELISA).

ELISAs were used to determine if the various monoclonal and polyclonal antibodies produced, bound to their respective antigens The antigens used in the various ELISAs performed are outlined in Table 2 3 and the secondary antibodies used are given in Table 2 4

2.3.16.1 Cell fixation for use in ELISA.

Two methods are described for the setting up and performance of a solid-phase enzyme-linked immunosorbant assay (ELISA) for use with fixed cells as antigens

Method 1.

In this method, adherent cells to be fixed onto 96 well plates were obtained from actively growing cultures This procedure was used with the following adherent cell lines, G-CCM, G-UVW, $IJK_{(t)}$, A549, H5683, MCF-7, EJ-138, LOVO and T-24 Flat bottomed flexible (NUNC) plates were washed 3 times in 0 01M PBS, pH 7 3, followed by the addition of 100μl/well of 0 1% (w/v) poly-L-lysine in 0 01M PBS, pH 7 3

After incubating for 1 5 hours at 37°C, the poly-L-lysine was removed This solution may be re-used up to 10 times The plates were re-washed three times in 0 01M PBS, pH 7 3 The cells to be fixed to the plates were obtained by trypsinisation (Section 2 3 2) They were washed in 0 01M PBS, pH 7 3 and 100μl of a suspension of $5x10^5$-$5x10^6$ cells per ml in 0 01M PBS, pH 7 3, was added to each well After 1 5 hours at 4°C, 150μl of 0 1% (v/v) glutaraldehyde in 0 01M PBS, pH 7 3, was added to each well to give a final concentration of 0 075% (v/v) glutaraldehyde (Carroll *et al* , 1990) It is important that the cell layer was not disturbed and that no air bubbles were present in any of the wells, during the addition of glutaraldehyde After a maximium of 10 minutes at 4°C, the plates were washed three times in 0 01M PBS, pH 7 3 100μl of 100mM glycine in 0 01M PBS, pH 7 3, was added to the wells to block any remaining unreacted glutaraldehyde Following incubation at 37°C for 30 minutes, the plates were again re-washed three times in 0 01M PBS, pH 7 3 150μl of storage/blocking buffer, [1% (w/v) BSA in 0 01M PBS, pH 7 3, containing 0 1% (w/v) sodium azide as preservative], was then added to each well The plates were thoroughly sealed using Parafilm and tin foil to prevent evaporation of the storage/blocking buffer and then stored at 4°C Under these conditions it was possible to store the plates for up to six months with no apparent loss of activity The plates were checked regularly for evaporation of buffer The remaining steps are as described for an ELISA (Section 2 3 16 3) after the plates have been blocked

Method 2.

A second method of fixation was used mainly for non-adherent cell lines such as K562

and HL-60 and may also be used for adherent cell lines which grow quickly in culture, such as EJ-138, H5683 and T-24 Pelleted cells were resuspended in complete culture medium at a concentration of $5x10^4$ cells/ml Each well of a 96 well plate received 100μl of this suspension The plates were sealed and placed in an incubator (5%, v/v, CO_2 at 37°C) and examined daily They were grown to approximately 75% confluency and this time interval depended on the growth rate of the cell line Cells were usually ready for fixing after 2-3 days The medium was then removed and the wells were washed three times with 0 01M PBS, pH 7 3 150μl of 0 1% (v/v) glutaraldehyde in 0 01M PBS, pH 7 3, were added to each well to give a working concentration of 0 075% (v/v) glutaraldehyde (Carroll *et al* , 1990) The remaining steps in this procedure were exactly as those of method 1

2.3.16.2. Preparation of reagents for an ELISA.

All solutions were stored at 4°C, unless otherwise stated

(i) Carbonate buffer was made by dissolving 1 06g of Na_2CO_3 and 2 94g $NaHCO_3$ in dH_2O, adjusting the pH to 9 6 with conc HCl or 5M NaOH, as appropriate, and adjusting the volume to 1 litre with dH_2O

(ii) 0 01M Phosphate buffered saline, pH 9 0, was prepared by dissolving 1 42g Na_2HPO_4 and 8 76g NaCl in 900ml dH_2O The pH was adjusted to pH 9 0 with 0 1M NaOH and made up to 1 litre

(iii) Washing solutions were prepared as follows, (a) 0 01M Phosphate Buffered Saline (Dulbecco's A), pH 7 3 (PBS) was prepared by dissolving 1 PBS tablet into 100ml distilled (dH_20) or ultrapure water, routinely ultrapure H_20 was used PBS-Tween was prepared by adding 0 05% (v/v) Tween-20 to the 0 01M PBS, pH 7 3, solution with stirring

(iv) Blocking solutions were prepared as follows, (a) 0 05% (w/v) porcine gelatin in 0 01M PBS, pH 9 0, was heated to 40°C while stirring This solution was allowed to cool at 4°C and was then checked to ensure it did not solidify at this temperature and was fully dissolved If it was not fully dissolved, it was heated again (b) 1% (w/v) BSA was made up in 0 01M PBS, pH 9 0, and dissolved by gentle stirring

(v) Amino-methyl-propanol buffer was prepared by dissolving 150mg magnesium chloride (5mM), 0 1ml Triton X-405 (0 01%, v/v), 1 0g sodium azide (0 1%, w/v) in 30ml dH_20 95 8ml of amino-methyl-propanol (0 1M) was added with stirring The pH

was adjusted to 10 25 with conc HCl This was left overnight at room temperature, and the pH was readjusted to 10 25 and the final volume brought to 1 litre with dH_20

(vi) The various secondary antibodies used and their dilutions are outlined in Table 2 4 The secondary antibodies used were alkaline phosphatase-conjugated and the manufacturers' instructions were routinely followed

(vi) The substrates used to develop the colour of the labelled secondary antibodies in ELISAs are outlined in Table 2 5 (a) The substrate used routinely for ELISA was 5-bromo-4-chloro-3-indol-phosphate, disodium salt (BCIP) made up at 1mg/ml (w/v) in amino-methyl-propanol buffer The substrate was prepared just before use and was dissolved by gentle stirring in the dark Both the secondary antibody and the substrate were checked for activity by mixing in a 1 1 ratio in the dark, where a deep blue colour developed after approximately 5 minutes (b) O-phenylenediamine (OPD) was also used and was freshly prepared just before use, as follows, 20mg OPD was added to 10ml 0 1M sodium citrate buffer, pH 5, and stirred gently This is light sensitive and was kept in the dark Directly before use 2 0μl of 30% (v/v) H_2O_2 was added and the absorbances determined at 492nm

2.3.16.3. ELISA for the detection of antibodies to protein or hapten-protein antigens.

In this indirect, non-competitive ELISA system the antigen is coated onto a microtitre plate and the antibody binding is detected using a labelled secondary antibody The antigen solution is made up in carbonate buffer (0 05M Na_2CO_3, pH 9 5) (Section 2 3 16 2) at a concentration range of 1-10μg/ml A concentration of 10μg/ml at 100μl/well was generally used (Table 2 3) Following addition of the antigen , the plates were left at 37°C for 2 hours or at 4°C overnight Following the incubation period, the antigen solution was decanted and the plates were then washed using 0 01M PBS, pH 7 3 The antigen solution may be used up to 8 times if stored with 0 005% (w/v) sodium azide After pouring the wash solution onto the plate, the plates were tapped, then inverted and tapped onto tissue paper to remove any remaining liquid This procedure was repeated four times Non-specific binding sites were blocked with 1% (w/v) BSA in 0 01M PBS, pH 7 3, or 0 25% (w/v) gelatin in 0 01M PBS, pH 7 3, (200-250μl/well) for one hour at 37°C or overnight at 4°C If sodium azide is added to the blocking solution, the plates, if tightly sealed, may be stored at 4°C for a number

of weeks at this stage Otherwise, the blocking solution was decanted and the plates were then washed four times with PBS-Tween and once with PBS, as already described 100μl/well of the samples to be tested were added to the wells on the plate in duplicate or triplicate Examples of the samples added include appropriately diluted ascitic fluid, culture supernatant, dilutions of test sera, sera from pre-immunised mice as a negative control and sera from immunised mice as a positive control The plates were left at 37°C for 2 hours The samples were decanted and the plates were washed four times with PBS-Tween and once with PBS, as before The appropriate secondary antibody (usually alkaline phosphatase-conjugated secondary antibody) (Table 2 4) diluted in (TBS) [2M Tris-HCl, pH 9 5, 5M NaCl and 1M $MgCl_2$, made up to 1L in ultrapure H_2O] was added to the plates (100μl/well) and incubated at 37°C for one hour After a thorough washing of the plates to remove unbound conjugate, 100μl of substrate (BCIP in amino-methyl-propanol buffer) was added to each well When sufficient colour had developed, (which took up to an hour,) the plate was 'blanked' against one of the negative controls (a well which had 0 01M PBS, pH 7 3, added instead of a sample) and the absorbance was recorded at 620nm on a Titertek TwinPlus Microplate Reader (ICN-Flow) Table 2 3 gives an outline of the many different ELISAs performed

2 3.16.4. Sandwich ELISA.

The sandwich ELISA was used in the initial screening stage of fusions where a large number of clones or 96 well plates had to be screened for antibody production Here the 96 well ELISA plate was coated with 10μg/ml anti-mouse IgG (whole molecule) (Table 2 4) as the antigen The plate was blocked as previously described (Section 2 3 16 3) and supernatant from wells containing clones were added at 100μl/well, the remainder of the ELISA is as described in Section 2 3 16 3 where the alkaline phosphatase-conjugated antibody, in TBS, and BCIP were subsequently added

2 3 16.5. 'Supernatant' ELISA.

This ELISA was developed to minimise use of expensive or scarce antigen and to confirm that hybridomas continued to produceantibody having been initially characterised by ELISA (Section 2 3 16 1) This involved coating the wells with supernatant at 100μl/well, the wells were then blocked and secondary antibody and substrate added as described in Section 2 3.16 3.

2.3.17. Generation of monoclonal antibody-containing ascitic fluid from mice.

Once the monoclonal antibody has been initially characterised, large quantities can be produced as ascitic fluid in mice by injection of the hybridoma cells into the peritoneum of the mouse

Before injection of the hybridoma cells, the mice were primed This was done by injecting 0 5ml of FIA or pristane 7 days prior to injecting $1x10^6$ hybridoma cells i p in 0 01M PBS, pH 7 3, at 37°C The ascitic fluid was recovered by sacrificing the mouse when the abdomen became enlarged The skin covering the abdominal area was removed, the peritoneum was punctured and the ascitic fluid removed The monoclonal antibodies were recovered by centrifuging the ascitic fluid at 1200rpm and collecting the supernatant This was aliquoted and stored at -20°C This method may also be used to produce large numbers of hybridoma cells In this instance, the removal of the ascitic fluid was performed under aseptic conditions and on spinning down the fluid the pellet is also retained This pellet contains the hybridoma cells, which may be resuspended in culture medium and grown in culture (Section 2 3 1) This procedure also has the advantage of removing any mycoplasma contamination from the hybridoma cells (Carroll and O'Kennedy, 1988)

Table 2.8. Outline of the different ELISAs to characterise the various monoclonal, polyclonal and bispecific antibodies produced, (alk. ph. = alkaline phosphatase).

Antigen	Conc.	Desired Antibody	2° Antibody
IBHR-BSA **IBHR-KLH** **IBHR-OVAL**	10μg/ml IBHR	Monoclonal and Polyclonal Anti-IBHR	Anti-Mouse and -Rabbit IgG alk ph
PHOS-KLH **PHOS-BSA**	10μg/ml	Monoclonal Anti-Phos	Anti-Mouse/ IgG alk ph
GFAP **G-CCM** **G-UVW**	10μg/ml $5x10^5$ cells/ml	Monoclonal Anti- GFAP and Anti- GFAP/G-CCM	Anti-Mouse IgG/IgM alk ph
T-24, HL-60 **MCF-7, EJ-138** **H5683, A549** **LOVO, K562**	$5x10^5$ cells/ml	Monoclonal Anti-GFAP and Anti-GFAP/G-CCM	Anti-Mouse IgG/IgM alk ph
RICIN-A **CHAIN**	10μg/ml	Monoclonal Anti- Ricin-A chain	Anti-Mouse IgG alk ph
KLH **BSA** **OVALBUMIN**	10μg/ml	Monoclonal Anti-Phos Monoclonal or Polyclonal Anti-IBHR	Anti-Mouse/ Sheep IgG alk ph

2.3.18. Isotyping of the monoclonal antibodies.

The isotype of the antibodies produced was performed using two kits, one from Sigma ImmunoChemicals and one from SEROTEC (Table 2.1.).

2.3.18.1. Procedure for antigen-mediated ELISA for isotyping.

The Sigma Immunochemicals' kit was used for this procedure. 0.1ml of appropriately diluted antigen was used to coat each of 12 wells of a microtitre plate which was then incubated for 1 hour at 37°C. The coating solution was removed and the plate washed 3 times using 0.01M PBS, pH 7.3, containing 0.05% (v/v) Tween-20 (washing buffer). The coating solution may be used again up to 5 times, if stored in borosilicate glass tubes at -20°C. If plastic tubes were used for storage of the antigen, a precentage of the protein adheres to the sides of the tubes and would be lost. This does not happen when borosilicate glass tubes were used. This was important in the case of GFAP, as it was particularly expensive. The monoclonal antibody to be tested was applied to a 96 well ELISA plate, at an appropriate dilution, in 0.01M PBS, pH 7.3. 0.1ml of undiluted cultured supernatant was added to each of 12 wells. The plate was incubated for 2 hours at room temperature and was washed 3 times with washing buffer. 0.2ml of a 1:1000 dilution of the isotype specific reagents supplied with the kit, in 0.01M PBS, pH 7.3, were required for each sample being tested. 0.1ml of each of the diluted isotype specific reagents were added in duplicate to the coated wells. The plate was incubated at room temperature for 30 minutes and washed as before. 0.1ml of a peroxidase-labelled rabbit anti-goat IgG, at a 1:5000 dilution in washing buffer, was added to each well and this was incubated at room temperature for 15mins and then washed as before. The o-phenylenediamine substrate was freshly prepared as outlined in Table 2.5. 0.1ml was added to each well. After 30mins, development of a yellow colour indicated a positive result and the reaction was stopped using 50μl of 20% (v/v) H_2SO_4.

2.3.18.2. Procedure for indirect ELISA for the determination of antibody isotype.

The Sigma Immunochemicals' kit was also used in this procedure. The antibody to be tested was diluted in 0.01M PBS, pH 7.3. A 1:5000 dilution was used to isotype ascitic fluid and 1:1000 dilution was used for a purified antibody. 0.1ml of undiluted culture supernatant was added to each of 12 wells. The covered plate was incubated for 1 hour at 37°C and the coating solution was removed. The plate was washed three times with

washing buffer (0 01M PBS, pH 7 3, containing 0 05%, v/v Tween 20) The following steps were as those outlined in Section 2 3 18 1 from where the diluted isotype specific reagents, in PBS, were added to duplicate wells

2.3.18.3. Procedure for isotyping monoclonal antibodies using isotyping strips.

These strips were obtained from Sigma and each strip was placed in a 30ml sterile universal 10ml of supernatant, from the hybridoma to be isotyped, was added to the universal and left shaking for 20min The strip was washed in 0 01M PBS, pH 7 3, blocked as before, re-washed and 10ml of the provided secondary antibody, diluted in 0 01M PBS, pH 7 3, was added and left shaking at room temperature for 20min The strip in the universal was washed with PBS, as before, for 5min and the substrate provided was added A dark brown band appeared over the isotype of the antibody tested after 10min The reaction was stopped by placing the strip in ultrapure H_2O and then allowing it to air dry

2 3 18.4. Procedure for isotyping monoclonal antibodies using the SEROTEC kit.

The anti-mouse monoclonal isotyping kit from Serotec has been specifically designed to identify the class and subclass of monoclonal antibodies in tissue culture supernatants The principle of this test system is based on red cell agglutination A positive, agglutinated result is produced when highly specific antibody, which is coupled onto sheep red blood cells, recognises and binds to the particular isotype to which it is directed This binding forms a lattice on the bottom of the microtitre plate well, termed agglutination The sensitivity of the reagent cells in detecting mouse immunoglobulins has been developed to optimally detect antibody concentrations within the normal range found in tissue culture supernatants, i e 1-20μg/ml

To reconstitute the freeze dried reagents supplied in the kit, 1ml of reconstitution diluent was added to each reagent vial to be used The vials were then recapped and after overnight storage at 4°C, the reagents were fully reconstituted The vials were stored at 4°C and are stable for 6 months In this procedure, only sterile tissue culture supernatants with no visible contamination should be used The test procedure should be performed on a vibration free bench at room temperature

A 1 50 dilution of the supernatant to be tested was made and was mixed by inversion If it was suspected that the antibody concentration in the supernatant was low, it was

used at a 1:10 dilution. 30μl of the diluted supernatant to be tested was placed into each of eight wells across the microtitre plate provided. The reagent bottles containing the resuspended cells were inverted gently and 30μl of each specific isotyping reagent was added to one of the wells containing the supernatant to be tested. This was done for IgG1, IgG2a, IgG2b, IgG3, IgA, IgM and for positive and negative controls. The plate was covered with the lid provided and holding the lid firmly the plate was tapped gently at the side to mix the reagents. The plate was left on a cool flat surface, with as little vibration as possible, for one hour for the results to develop. A partial or full carpet of agglutination is a positive result and a small, red button is a negative result. The final result should show one agglutinated well for the positive control and only one other agglutinated well, thereby indicating the isotype of the mouse monoclonal antibody tested.

2.3.19. Purification of the monoclonal antibodies.

The ascitic fluid was purified by saturated ammonium sulphate (SAS) precipitation according to Knutson *et al.* (1991). In this procedure, the thawed ascitic fliud was clarified by centrifugation at 10,000g for 5mins at 4°C. The protein concentration was then adjusted to 10-12mg/ml with 20mM Tris, pH 8.0. A saturated ammonium sulphate solution, which had been adjusted to pH 7.4 with ammonium hydroxide and was at 4°C, was slowly added to the diluted ascitic fluid with stirring to achieve 45% saturation of ammonium sulphate. After 1 hour at 4°C, the suspension was centrifuged at 10,000g for 5mins at 4°C. The pellet was resuspended to the same volume as the original diluted ascitic fluid with 100mM Tris, 150mM NaCl at 4°C and exhaustively dialysed against the same buffer. Following dialysis, the sample was subjected to centrifugation at 10,000g for 15mins at 4°C, to remove precipitated proteins. The SAS-treated ascitic fluid was further purified by protein A affinity chromatography or protein G purification. These methods are described in Section 2.4.13. and Section 2.6.3., respectively.

2.3.20. Characterisation of monoclonal antibodies.

2.3.20.1. Preparation of reagents for High Pressure Liquid Chromatography (HPLC).

The column used was a 10μm Protein Pak SW 300 column with a mobile phase of

0.1M phosphate buffer, pH 7.0 with a flow rate of 0.5ml/min. This column was a size exclusion column where molecules of greater molecular weight are eluted and detected before molecules of lower molecular weight. 0.1M sodium phosphate buffer was prepared by adding 13.8g NaH_2PO_4 and 14.2g Na_2PO_4 to 900ml ultrapure H_2O, adjusting the pH to 7.0 and the volume to 1 litre with ultrapure H_2O. All buffers were filtered and sonicated for 30mins before use. The protein standards and samples were made up at 1mg/ml in 0.1M phosphate bufffer, pH 7.0. Absorbance due to protein was detected at 280nm. After use, the column was washed and stored in 0.1M phosphate buffer, pH 7.0 containing 0.05% (w/v) sodium azide.

2.3.20.2. Cytospin preparation of cells.

Cytospins were prepared by adding 100μl of a $5x10^4$ cells/ml in 0.01M PBS, pH 7.3., to the cytospin chambers placed over slides. The cells were centrifuged onto these slides at 1000rpm for 2mins at room temperature. The cytospin preparations were air dried and stored at -20°C in tin foil in a slide holder.

These slides were thawed by removing them from the freezer and leaving to stand at room temperature, wrapped in tin foil, for 20mins. Thereafter the slides were not allowed to dry when being used in APAAP or FITC-conjugate labelling. If required, slides were fixed by immersion for 30sec in a solution of acetone:absolute alcohol:formaldehyde in a 19:19:2 ratio, respectively.

2.3.20.3. Staining of cytospin preparations.

Rapi-DiffII, a triple stain pack for Romanowsky staining from DiaCHEM (Section 2.1.) was used to examine the cellular and nuclear morphology of myeloma cells. The kit consists of three solutions: solution A was the fixing solution, thiazine dye in methanol; solution B was the acid dye, eosin Y in phosphate buffer and solution C was the basic dye, methylene blue (polychromed) in phosphate buffer. Optimal results were obtained when the slides were immersed in solution A for 30secs, solution B for 35secs and then solution C for 45secs. The slides were rinsed briefly in buffered water, pH 6.8, allowed to dry and were then viewed under a microscope.

2.3.20.4. The alkaline phosphatase anti-alkaline phosphatase (APAAP) detection system.

Using the APAAP system, the slides were treated like an ELISA, the primary antibody,

blocking solution (Goat/FCS/Tris buffer), anti-mouse IgG, APAAP and substrate were each incubated for 30mins, with four washes in Tris buffer between each step The substrate used was a BCIP and nitro blue tetrazolium (NBT) which generates an intense black-purple precipitate at the site of enzyme binding The reaction proceeds at a steady rate, which allows accurate control of the development of the reaction by the length of incubation

The following three stock solutions were prepared prior to developing the cell staining (1) NBT was prepared by dissolving 0 5g of NBT in 10ml 70% (v/v) dimethyl-formamide (2) BCIP was prepared by dissolving 0 5g of BCIP (disodium salt) in 10ml of 100% dimethylformamide (3) When using the APAAP system for the detection of antibody binding on slides the following recipe was used to make 10ml of the alkaline phosphatase buffer solution, 0 5ml of 2M Tris-HCl, pH 9 5, 0 2ml of 5M NaCl, 50μl of 1M $MgCl_2$ and 9 25ml of dH_2O All stocks are stable at 4°C for at least one year Immediately before use, the substrate was prepared by adding 66μl NBT stock to 10ml of alkaline phosphatase buffer This was mixed well and 33μl of BCIP stock was then added and used within 1 hour 100μl of this solution was added to each slide which was developed at room temperature in the dark A typical incubation was approximately 20 - 30min The reaction was stopped by rinsing the slide with 0 01M PBS, pH 7 3, containing 20mM EDTA The slides were counterstained and mounted in DPX (Sections 2 3 20 5 and 2 3 20 6 , respectively)

2.3.20.5. Methyl green counterstaining of alkaline phosphatase/BCIP-stained slides.

The slides were counterstained with 2 0% (w/v) methyl green for 1min and were then washed in 0 01M PBS, pH 7 3 The slides were either mounted in DPX (Section 2 3 20 6) or were dehydrated in 95% (v/v) alcohol followed by 100% absolute ethanol and cleared in xylene before mounting in DPX

2.3.20.6. Mountants for slides.

DPX is named for its components, 10g of distrene 80, 5ml of dibutyl phthalate and 35ml of xylene DPX mountant was used to mount slides by allowing the slides to dry, but not overdry, adding a drop of DPX mountant and then quickly placing a coverslip on top, avoiding air bubbles

Slides can also be mounted using glycerol jelly, which is an aqueous mountant It

consisted of 10g of gelatine power dissolved in H_2O by warming and then 70ml of glycerol was added One drop of phenol or sodium merthiolate was added as an antibacterial agent This was stored at 4°C and must be melted and freed of air bubbles before use which is conveniently done in a vacuum-embedding chamber Because of the low refractive index (1 42) many unstained structures remain visible in this medium

2.3.20.7. Detection of antibody binding using the 'eppendorph' ELISA.

This was used primarily with FITC-labelled primary antibodies (Carroll *et al* , 1990) The eppendorphs were coated with 2% (w/v) BSA in 0 01M PBS, pH 9 0 for 2hrs at room temperature or at 4°C overnight This solution was then discarded and $1x10^6$ cells in 0 01M PBS, pH 7 3, containing 3% (v/v) FCS and 0 01% (w/v) sodium azide From this step, everything was kept at 4°C in the dark The cells were centrifuged and 0 1ml of the FITC-conjugated primary antibody at a suitable dilution was added, as was the negative control, PBS These were incubated for 30min and the cells were washed three times in ice cold 0 01M PBS, pH 7 3, containing 3% (v/v) FCS and 0 01% (w/v) sodium azide The slides were kept in the dark and photographed immediately as the fluorescence fades

2.3.20.8. Fluorescence Activated Cell Sorting (FACS) analysis to determine antibody specificity.

The interest and assistance of Mrs Kate Cotter, University of Maynooth, in the FACS analysis is gratefully acknowledged and appreciated

The cells were removed from culture (Section 2 3 1 , 2 3 2) and brought to a cell count of $1x10^6$ (Section 2 3 3) The eppendorphs to be used were coated with 2% (w/v) BSA in 0 01M PBS, pH 9 0, for 2hrs at room temperature or at 4°C overnight This solution was then discarded and $1x10^6$ cells in 0 01M PBS, pH 7 3, containing 3% (v/v) FCS and 0 01% (w/v) sodium azide From this step, everything was kept at 4°C in the dark The cells were centrifuged and 0 1ml of the primary antibody, at a suitable dilution, was added, as was the negative control, 0 01M PBS, pH 7 3 These were incubated for 30min and the cells were washed three times in ice cold 0 01M PBS, pH 7 1, containing 3% (v/v) FCS and 0 01% (w/v) sodium azide After this step, the cells were blocked with 2% (v/v) goat serum (Sigma) in PBS/FCS/azide to block any Fc receptors on cells The appropriate anti-mouse FITC-conjugated antibody (Table 2 4)

was then added. The cells were pelleted by centrifugation at 1080rpm for 5min and resuspended in the PBS/FCS/azide solution. 1ml of 70% paraformaldehyde (Section 2.3.20.9.) was added dropwise, to fix the cells and was left at room temperature for 30mins. The cells were pelleted again at 1080rpm for 5mins and washed twice in the PBS/FCS/azide solution. The cells were finally resuspended in this solution, stored in the dark at 4°C and read in a FACS Scan IV machine from Becton Dickinson as soon as possible. The cells were resuspended in FACS fluid before being analysed.

2.3.20.9. Preparation of paraformaldehyde for use in FACS.

Paraformaldehyde (70%, w/v) was prepared by adding 25ml 0.01% sodium azide/0.01M PBS, pH 7.3, to 3.5g paraformaldehyde. This was heated to 60°C. Four drops of 1M NaOH was added and the solution should clarify. The volume was made up to 50ml with 0.01% sodium azide/0.01M PBS, pH 7.3. The solution was filtered and stored at 4°C.

2.3.21. Dot immunoblotting.

Care was taken to use gloves and forceps when handling the Hybond-C-Super nitrocellulose membrane used in this procedure. A $5cm^2$ grid was scored on the membrane using a fine pencil, leaving a margin around the edges to handle the membrane.

The membrane was soaked in 0.01M PBS, pH 7.3, for 5min in a small container ensuring the entire membrane was wet. The PBS solution was poured off and the membrane was allowed to air-dry. 0.1mg/ml of antigen in 0.01M PBS, pH 7.3, was prepared and 1-2μl of this solution was applied to duplicate squares.

Negative and positive controls were prepared in a similar manner. A positive control was a square coated with GFAP where the primary antibody was commercial anti-GFAP diluted in 0.01M PBS, pH 7.3. A negative control was a square without antigen and was incubated with the same dilution of the primary antibody, for example anti-GFAP in 0.01M PBS, pH 7.3.

The antigen was left to bind for 30min at room temperature. The wash buffer used was 10mM Tris, 150mM NaCl and 0.5% Tween-20, w/v, this solution was adjusted to pH 7.5 and brought to 2.5L with ultrapure H_2O. The blot was washed twice in wash buffer and the sites not bound by antigen were blocked with 20ml of 1% (w/v) BSA in 0.01M

PBS, pH 7 3, ensuring the whole membrane was completely immersed The blot was immersed in the blocking solution for 30min at room temperature and was gently shaken at intervals The blocking solution was poured off and the membrane was washed three times with wash buffer and twice with PBS, as before

1-2μl of sample, e g supernatant or ascitic fluid diluted in 0 01M PBS, pH 7 3, with 2% (w/v) BSA and 0 1% (v/v) Tween-20, was added to the blot and left at room temperature for 2 hours The plate was washed, as before, and 20ml of a 0 1μg/ml of anti-mouse IgG (whole molecule) alkaline phosphatase-conjugated secondary antibody (Table 2 4), diluted in TBS with 2% (w/v) BSA and 0 1% (v/v) Tween-20 was then added to the blot

The blot was left shaking for 30min at room temperature and was then washed three times, for 5min each, in 0 01M PBS, pH 7 3, containing 0 1% (v/v) Tween-20 and three times in 0 01M PBS, pH 7 3 10 3ml of the BCIP/NBT substrate solution (Section 2 3 20 4) was made up and the substrate solution was added to the dot blot container and left to develop for 5-10min When the colour had developed, the reaction was stopped by pouring off the substrate and washing the membrane in distilled water This was then poured off and the membrane left to air dry The blots were stored in the dark at room temperature to prevent fading and photographed as soon as possible

The dot blots in Figure 3 4 1 were performed as follows, 2μl of a 10μg/ml solution of GFAP from lot 16 (GFAP "A") was used as the antigen on the upper portion of each dot blot and the same quantity of GFAP from lot 17 (GFAP "B") was used on the bottom portion In Figure 3 4 1 (a) was treated with 10ml of a 1 1000 dilution of commercially available anti-GFAP in 0 01M PBS, pH 7 3, (b) the negative control for commercially available anti-GFAP, 0 01M PBS, pH 7 3, (c) examined with 2DH5 clone A10 supernatant (1 5 dilution in 0 01M PBS, pH 7 3) and (d) the negative control the negative control which had $DMEMS_{10}$, added instead of hybridoma supernatant These results are discussed in Section 3

2.3.22. Preparation of cell membranes for Western immunoblotting.

This procedure was performed according to Barrand and Twentyman (1992) Lysis buffer was prepared by adding 10mM KCl, 1 5mM $MgCl_2$ and 10mM Tris-HCl to 1L of ultrapure H_2O and the pH was brought to 7 4 using conc HCl or 5M NaOH, as appropriate The lysis buffer was kept on ice Immediately before use, 2mM of a

protease inhibitor, phenyl-methyl-sulphonal fluoride (PMSF) (toxic), was dissolved in a minimum volume of acetone and added to 40ml of boiling lysis buffer When dissolved, this 40ml was added to the remainder of the lysis buffer and kept on ice Cell membranes were prepared for Western immunoblotting by first washing 10^8 cells obtained from cell culture (Sections 2 3 1 and 2 3 2) with 0 01M PBS, pH 7 3, three times at 2000rpm for 5min The cells were resuspended in 5ml of ice cold lysis buffer and sonicating using a Braun Labsonic U sonicator with a repeating duty cycle of 0 5 and low speed The cell membranes were kept on ice throughout the procedure These membranes were then checked at intervals during the sonication to ensure the correct amount of cell fragmentation was obtained by microscopic examination The cell membranes were transferred to centrifuge tubes and centrifuged in a Sorvall^R RC-5B refrigerated Superspeed Centrifuge using a Sorvall^R GSA rotor at 7,800rpm for 15min at 4°C The plasma membrane enriched-supernatant was retained and centrifuged again using a Beckman Optima XL-80 Ultracentrifuge at 38,500rpm for 1hr 15min at 4°C The pellet was resuspended in 500μl of ice-cold lysis buffer, sonicated for 2-3 seconds and was kept at 4°C 3x100μl of this solution was divided into eppendorphs and 50μl of this solution was retained to perform a BCA protein assay (Section 2 4 9 3) Parafilm was placed over the eppendorphs which were kept on ice throughout These eppendorphs were frozen quickly by placing in a -70°C freezer for 15min They were then placed in a Vitris Consol 4 5 freeze dryer overnight and were stored at -20°C until required for use in Western blots (Section 2 3 22)

2 3 22.1. Procedure for Western immunoblotting.

The electrophoresis mini-gels used for the Western blots were made-up according to Table 2 9 The molecular weight markers used are given in Table 2 10 15μl of a 1ng/ml sample was prepared in reducing Laemmli loading buffer This buffer consisted of 2 5ml of 1 25M Tris-HCl pH 6 8, 0 5ml 10% (w/v) SDS, 2 9ml mercaptoethanol, 8 7% (v/v) glycerol, adjusted to 50ml with ultrapure H_2O The cell membrane samples (Section 2 3 22) were mixed by vortexing and 3-5μl of each sample was taken for use in a dot blot (Section 2 3 21) 0 1mg of bromophenol blue (BDH chemicals Ltd) was added to the remaining 10-12μl of each cell membrane sample Laemmli running buffer was placed between the gel and the container and 10μl of each sample (not boiled) and of the boiled molecular weight markers were loaded per well When the gel was loaded,

more running buffer was added Gels were run on a LKB 2050 Midget electrophoresis unit at 40 milliamperes (250 volts) until the first bromophenol blue band was near the bottom of the gel

A square of Hybond-C-Super nitrocellulose paper the size of the mini-gel was carefully cut out, taking care not to touch the paper and to wear gloves This paper was moistened by placing gently on blotting buffer (25mM Tris, 192mM glycine, pH 8 3 in 500ml ultrapure H_2O - no methanol) and was allowed to wet by capillary action from the bottom and left to soak for 10min

The Bio Rad Trans-Blot Semi-dry transfer cell system was used to transfer the proteins from the gel to the nitrocellulose paper, as proteins go toward the anode in this system This system was set up by placing four sheets of filter paper pre-soaked in blotting buffer on the anode Any air bubbles were removed using a glass pasteur pipette The moistened nitrocellulose paper was positioned on top of the filter paper The gel was placed on top of the nitrocellulose paper and four sheets of moistened filter paper were placed on top of the gel

The dryer was set at 15 volts maximum and the limit for the current was 0 340 amperes Transfer took 30min On transfer, the membrane was treated in a similar manner to the dot blot, from the stage of the dot blot protocol where the antigen was added to the membrane (Section 2 3 21) The variations in this procedure are briefly outlined All the antibodies were diluted in TBS [0 1% (w/v) Tween-20 in 2M Tris-HCl, pH 9 5, 5M NaCl and 1M $MgCl_2$, made up to 1L in ultrapure H_2O] The washing buffer was 0 5% (v/v) Tween in TBS The blots were blocked at room temperature for 1 5 hours with 5% (w/v) Blotto in TBS The primary antibodies were left at 37°C for 1 5 hours The secondary antibodies at a 1 1000 dilution of alkaline phosphatase-conjugated anti-mouse IgG or IgM in TBS, as appropriate, were incubated for 1 hour at 37°C (Table 2 4) BCIP/NBT in TBS was added until colour developed This was approximately 5-10mins

Table 2.9. Composition of resolving and stacking electrophoresis gels for Western blots.

Gel constituents	7.5% Resolving mini-gel	Stacking Gel
29 1% (w/v) Acrylamide - 0 9% (w/v) Bisacrylamide Stock	3 8ml	0 8ml
Tris-HCl	3 0ml (1 875M, pH 8 8)	0 5ml (1 25M, pH 6 8)
Ultra Pure Water	8 0ml	3 6ml
Ammonium Persulphate (added last)	50μl	20μl
SDS (10%, w/v)	150μl	50μl
TEMED	7 5μl	5μl

Table 2.10. Molecular weight markers used in Western immunoblotting.

Molecular weight marker	Molecular weight
Myosin	205,000
β-galactosidase	116,000
Phosphorylase	97,400
BSA	66,000
Ovalbumin	45,000
Carbonic anhydrase	29,000

2.3.23. Conjugation of fluorescein isothiocyanate (FITC) to proteins and antibodies.

Fluorescein isothiocyanate (Isomer I) was conjugated to (i) the proteins IBHR-BSA and IBHR-KLH and (ii) antibodies using a modification of Goding (1976). FITC was conjugated to IBHR-BSA and IBHR-KLH to facilitate the detection of bispecific antibodies containing the anti-IBHR polyclonal antibody (Section 5.1.) by FACS analysis. The anti-G-CCM and anti-GFAP antibodies were directly labelled to facilitate the detection of these antibodies by FACS and immunofluorescence.

Antibody concentrations of equal to or greater than 10mg/ml relative to fluorochrome concentrations of 10-20μg/mg of antibody, favour conjugation to antibody (up to 70%). Satisfactory conjugation of FITC to antibody solutions of lower concentrations, for example 1mg/ml, can be obtained if the fluorochrome concentration is correspondingly increased (100μg/mg antibody). Protein concentrations of the purified ascitic fluid or protein solution used were determined using the BCA assay (Section 2.4.9.3.)

The protein and purified antibody preparation from ascitic fluid samples were dialysed overnight against freshly prepared 0.1M sodium carbonate buffer, pH 9.5. This dialysis helps to remove extraneous nucleophiles such as Tris, amino acids, ammonium ions or azide that might inhibit conjugation. At pH 9.5, a large fraction of the ϵ-amino groups on lysine are unprotonated enabling thiourea bonds to form with the thiocyanate groups of the fluorochrome.

A stock solution of FITC (1mg/ml) was dissolved in pure DMSO to prevent hydrolysis. The appropriate volume of the fluorochrome solution to add to the protein or ascitic fluid solution was calculated as follows;

(i) when the protein or ascitic fluid solution had a concentration of greater than or equal to 10mg/ml, 10μg of the fluorochrome solution was added per mg of protein or antibody solution used, (ii) when the concentration of protein or antibody solution was less than 10mg/ml, 100μg of the fluorochrome solution was added per mg of protein or antibody.

The fluorochrome solution was added in 20μl amounts to the protein or antibody solution with gentle stirring. This was left in the dark for 3-4 hours at room temperature. The free fluorochrome was separated from the FITC-protein conjugate on a Sephadex G-50 column in 0.01M PBS, pH 7.0, with 0.01% (w/v) sodium azide. The size of the column was 32cm x 3.2cm and the exclusion limit was between 20,000 and 50,000. According to Goding (1976), the first coloured band to be eluted is the

conjugated antibody This was stored at 4°C in the dark

2.3.23.1. Calculation of the fluorescein:protein (F:P) ratio.

The fluorescein coupling i e the ratio of fluorescein to protein, can be estimated by measuring the absorbance at 495nm and 280nm The difference in absorbance (A) for fluorescein (495nm-280nm) should be between 0 3 and 1 0, all the FITC-protein conjugates and the FITC-labelled antibody solutions prepared during this project were in this range

Ratios below these yield low signals, while higher ratios show high backgrounds (Harlow and Lane, 1988) If the ratios are too low, the conjugation would have to be repeated using lower levels of antibody and higher levels of label If higher levels were found the labelling procedure would have to be repeated with appropriate changes or the labelled protein could be further purified on a DEAE column

The F P ratio for the fluorescein conjugates can be calculated using a simple formula proposed by The and Feltkamp (1970a, b) They included a correction for the contribution of FITC to the total absorbance at 280nm Mouse [IgG] represents the mouse IgG concentration

$$\text{F P ratio} = \frac{2\ 87 \times A_{495}}{A_{280} - 0\ 35 \times A_{495}}$$

$$\text{Mouse [Ig G]} = \frac{A_{280} - 0\ 35 \times A_{495}}{0\ 69} \text{ mg/ml}$$

Using either of these methods the ratio of FITC conjugated to a protein or antibody may be calculated

2.3.23.2. Indirect immunofluorescence and its use in antibody characterisation.

In this system, binding of test antibody to the antigen is detected using a second antibody which is fluorescently labelled When immunofluorescence is carried out on live cells only cell surface components are recognised If immunofluorescence is performed on fixed cells or on frozen tissue sections, intracellular antigens are also detectable

The cells to be tested were adjusted to a concentration of $1x10^6$/ml in DMEM-S_{10} and 100μl of the cell suspension was pipetted into each eppendorph tube 100μl of the test antibody, either ascitic fluid diluted 1 100 in DMEM-S_{10}, purified antibody preparation from ascitic fluid, supernatant or positive and negative controls was added to each tube The negative control in each case was DMEM-S_{10} and the positive control was serum from immunised mice or commercially available anti-GFAP antibody

The tubes were mixed gently and incubated for 30 minutes at 4°C The cells were then washed three times in 0 01M PBS, pH 7 3, containing 0 1% (w/v) sodium azide (PBS-azide) The cells were incubated for 30 minutes at 4°C with 100μl of a dilution of fluorescein-isothiocyanate (FITC)-linked anti-mouse IgG or IgM at appropriate dilutions (1 32 and 1 28, respectively) with DMEM-S_{10} The cells were then washed three times in PBS-azide and the supernatant aspirated from the cell pellet Each cell pellet was resuspended in a minimum amount of PBS-azide 10μl of this cell suspension was placed on a microscope slide, covered and observed for fluorescence Samples may be stored (if required) away from the light at 4°C

2.3.24. Characterising antibody affinity using BIAcore.

In the BIAcore system the optical phenomenon used for detection of antibody-antigen binding is surface plasmon resonance, (SPR) This is discussed in Section 3 15

2.3.24.1. Preparation of samples for BIAcore.

Antigen samples should be resuspended in 0 01M PBS, pH 7 3, H_2O or other physiological buffer It is important that the antigen be pure and at a concentration of 50μg/ml or greater Only 300μl-500μl is needed normally, but in cases where the antigen immobilises poorly, more may be needed If the antigen is a conjugate, the molecular weight of the protein and hapten is required along with how many haptens are conjugated per protein molecule BIAcore works better with BSA-conjugates than some antigens alone as sometimes when using unconjugated antigens the epitope on the antigen may be concealed

In the case of the antibody, it must also be pure and at as high a concentration as possible, e g 0 5ml of 500μg/ml would be sufficient This should also be in a low salt buffer, like 0 01M PBS, pH 7 3

2.3.30. Molecular biology techniques.

2.3.30.1. Treatment of plastics for use with mRNA and DNA.

All plasticware was treated by washing with chloroform (toxic) in a fume hood and autoclaving. Plastic tips were only used if the container had been previously unopened. These were also chloroform washed and autoclaved. Gloves were worn at all times when handling these tips, even before the chloroform wash.

2.3.30.2. DEPC-treatment of solutions for use with mRNA and DNA.

Diethylpyrocarbonate (DEPC, toxic) was used to inhibit RNases in the preparation of solutions for RNA manipulation. DEPC (0.2%, v/v) was added to the solution which was left at room temperature for at least 20 minutes and then autoclaved to inactivate the DEPC. DEPC was not added directly to solutions containing Tris as it is highly unstable in these solutions decomposing rapidly into ethanol and carbon dioxide (Sambrook *et al.*, 1989). Gloves were worn at all times, and changed frequently, when preparing solutions for the manipulation of RNA to avoid the introduction of RNases.

2.3.30.3. Purification of mRNA from cultured cells.

Gloves were worn at all times when isolating, purifying or handling mRNA as it can be easily degraded by RNases.

(a) Extraction of cultured cells.

Polyadenylated RNA was extracted from the exponentially growing anti-G-CCM hybridoma, 3BH2 clone A3 cells, using the QuickPrep™ mRNA purification kit (Table 2.1.). Thirty minutes before use, the kit was removed from storage at 4°C and left to equilibrate to room temperature. The extraction buffer was placed at 37°C at this time. The extraction buffer was shaken periodically and when all the crystalline material had dissolved, it was cooled to room temperature. mRNA was extracted from the 3BH2A3 hybridoma and these cells, which grow in suspension, were stained using trypan blue exclusion, counted and their viability determined (Section 2.3.3.). The cells were pelleted by centrifugation and the supernatant was decanted and discarded. 1.5ml of extraction buffer was added to the pelleted cells. The cells were disrupted using a homogenizer (Kika-Werk, Ultra-Turrax), to ensure a homogeneous extract.

3ml of elution buffer was added to the cell extract to dilute the sample followed by thorough mixing The sample was homogenised briefly and then transferred into a sterile polypropylene centrifuge tube The remaining elution buffer was placed at 65°C until needed Having ensured each tube is counter-balanced, the diluted extract was centrifuged for 10min at 12,000xg using a Beckman JA20 rotor in a Beckman J2-21 centrifuge at room temperature

(b) Isolation of mRNA

The Oligio(dT)-Cellulose Spun Column provided with the QuickPrep™ mRNA purification kit (Table 2 1) was inverted several times to resuspend the matrix The top and bottom closures were removed and the column was placed in a 15ml centrifuge tube and balanced against a counterweight This was centrifuged at 350xg for 2min (1,400rpm in a Heraeus Christ Labofuge 600 centrifuge) The column was removed from the centrifuge tube and the liquid in the tube was discarded The bottom closure on the drained column was put back on and the column was placed upright in a rack Using a sterile pipette, 4ml of the supernatant from the final step in the mRNA extraction procedure was transferred onto the surface of the column During this transfer, disturbing the pelleted cellular material was avoided The top closure of the column was put back on and the column was inverted several times to resuspend the resin The column was gently mixed for 10min by inverting the column manually The column was placed in a 15ml centrifuge tube and balanced against a counterweight leaving both top and bottom closures securely on This was centrifuged at 350g for 2min, to separate the resin from the suspension The top closure was removed, the supernatant was decanted and discarded while taking care to avoid disturbing the resin 3ml of high salt buffer provided was applied to the top of the resin and the top closure was replaced on the column The matrix was resuspended by gentle tapping of the bottom of the column The column was placed in a 15ml tube and centrifuged at 350g for 2mins The top closure was again removed, the supernatant was decanted and discarded The wash, using the high salt buffer, was repeated twice, exactly as described above 3ml of the low salt buffer was applied to the column and the top closure was replaced The matrix was resuspended by gentle tapping of the bottom of the column The column was placed in a 15ml centrifuge tube and centrifuged at 350g for 2min. The top closure was removed, the supernatant was decanted and discarded

The bottom closure was removed, 3ml of low salt buffer was applied to the top of the resin and centrifuged at 350g for 2min A sterile 1 5ml screw-top microcentrifuge tube was placed inside a 15ml centrifuge tube so that the tip of the column was inside the opening of the screw-top microcentrifuge tube ("the collection tube")

The bound poly(A)$^+$ RNA was eluted as follows, using three successive washes with the elution buffer provided which had been *prewarmed* to 65°C For each wash, 0 25ml of buffer was pipetted onto the top of the column, the column in its collection tube, was then centrifuged at 350g for 2min The collection tube was not changed between washes in order that the entire 0 75ml eluate was collected in the same sterile tube The screw-top microcentrifuge tube was removed from the 15ml centrifuge tube using a sterile (autoclaved) forceps, which was flamed before use The collected sample and the remaining elution buffer was placed on ice

(c) Quantitation and precipitation of mRNA

The concentration of RNA in the final eluate can be estimated by ethidium bromide fluorescence (Sambrook *et al* , 1989) or by spectrophotometry, which was the method used and is described below However, the disadvantage with the spectrophotometric procedure is that it consumes one-third of the sample

To protect the RNA sample from degradation by RNases, the 0 5ml quartz glass cuvettes used were pretreated by soaking in concentrated HCl methanol (1 1) for 1 hour and then rinsing several times in DEPC-treated water DEPC-treated water was made by preparing a 0 1% (v/v) solution of DEPC in ultrapure H_2O Sterile glassware was used throughout the procedure This was allowed to stand at room temperature overnight and was then autoclaved

0 25ml of the column eluate was placed in a sterile microcentrifuge tube and an equal volume of elution buffer was added The absorbance of the diluted sample was read at 260nm (A_{260}) in a Shimadzu UV-160A UV-Visible recording spectrophotometer using elution buffer as a blank This reading must be between 0 05 and 2 0 to reflect the RNA concentration accurately, if the absorbance was above 2, the sample would have been diluted further to determine the RNA concentration This diluted sample was discarded

Knowing that for RNA, 1 A_{260} unit = 40μg/ml, the concentration of the RNA present ([RNA]) was calculated using the following formula

[RNA] = A_{260} x D x 40μg/ml

where D = final dilution factor In the simplest case above, this would be 2

If the absorbance of the diluted sample was greater than or equal to 0 5, the sample may be used directly for complementary DNA (cDNA) synthesis (Section 2 3 30 9) If the absorbance was less than 0 5, the sample must be precipitated and redissolved at a higher concentration to ensure efficient cDNA synthesis (Pharmacia, 1991)

2 3.30.4. Rules for generating primers.

The following rules were adhered to when designing primers

There should be 100% homology at the 5' end of the primer for the first 4 to 5 bases The primer should ideally be no more than 24 bases long The primer should not contain inverted repeats as this would form a secondary structure within the template DNA The G-C and A-T ratio should be equal and constant throughout the primer sequence The G-C content determines the melting temperature in the PCR reaction and this can be calculated according to Section 2 3 30 10 There can be no complementarity between the 3' end of the forward primer and the 5' end of the backward primer as this would result in primer dimers and stop the PCR reaction The primer must be absolutely specific for the region of the genome required The homology of a chosen region can be checked against DNA of that species stored in the GENBANK, which contains over 200,000,000 bases and 150,000 sequences The primer should also contain the required restriction enzyme sites for forced cloning, which should be in frame with the promoter in the vector and should not contain a stop codon, such as TAG, in frame If the primer sequence has to be modified to incorporate a specific site, only 2 or 3 bases in the middle of the primer should be changed and, if possible, the change should only be in the second or third base of a codon It would be better to check if there is another restriction enzyme available which does not require changing the sequence This can be checked easily and quickly using the SeqAid™ II, version 3 0 or later, software analysis package which is currently available

2.3.30.5. Analysis of the DNA regions to be amplified by polymerase chain reaction (PCR) primers.

The first and most important step in engineering antibodies is to generate PCR primers which will only amplify the DNA region of interest The protein regions of a mature

antibody corresponding to the DNA regions amplified by the primers selected are shown in Figure 5 1 In order to do this, the target sequences of each of the primer pairs were compared with each other using the computer package SeqAid™, version 3 0 and the following results were obtained,

The region which would be amplified by VKFOR and VKCBAC2 showed no alignment greater than 2 bases Therefore, a primer anywhere along this sequence could be used The region amplified by 1HEAVY and HCH1BAC2 was then analysed and alignment between position 30 of 1HEAVY and position 21 in HCH1BAC2 was observed It was important that the primers be specific for the region of the first 30 nucleotides in 1HEAVY and the first 21 bases in HCH1BAC2

The region amplified by the postulated 1HEAVY and HCH2 primers was compared and an alignment between position 24 of 1HEAVY and position 6 in HCH1BAC2 was observed, implying that it was important that the primers be specific for the region of the first 23 nucleotides in 1HEAVY and that the HCH2 primer be made from position 1 In all cases the beginning and ends of the sequences were chosen as regions for primer sequences The target regions were also checked to ensure they did not form regions of hairpin loops, i e they were not complementary to themselves

2.3.30.6. Design of amplification primers to be used in PCR.

The murine hybridoma used was that producing the anti-G-CCM monoclonal antibody, 3BH2A3 (Section 3), and isotyped as IgM with kappa (*k*) light chains

In order to apply the PCR to the cloning and expression of these immunoglobulin genes, the conserved regions at each end of the nucleotide sequences encoding V domains of mouse immunoglobulin heavy (V_H) and *k* light chain were identified Primers were then designed for the PCR amplification, which incorporated restriction sites for forced cloning into a suitable vector The five primers used in this project amplify up the nucleotide regions corresponding to the antibody protein segments shown in Figure 5 1 and were used in the combinations given in Table 5 1 1 The rules for the generation of primers (Section 2 3 30 4) were adhered to in order to generate these primers

In order to find the conserved sequences for both the heavy and light chains, the database of Kabat *et al* (1987) was consulted The beginning of the nucleotide sequences correspond to the mature N terminus of the protein As expected, the

nucleotide sequences encoding the protein CDRs are variable and those corresponding to the J region segments are conserved However, there are several other conserved regions, in particular, those encoding the mature N terminus of both V_H and V_k domains

In order to find if the sequences obtained in Kabat *et al* (1987) were representative in light of the expanded database in the intervening years, the GENBANK database of known DNA sequences was searched Not all of the database was searched, only the list of sequences in the database which had the keywords 'mouse' and 'immunoglobulin' in the title of the paper or, if unpublished, in the keywords for the sequence The number of sequences in this subset was 3,555

When the primer regions were chosen, they were analysed using SeqAid to search for restriction enzyme sites in the primers If a necessary change in the DNA sequence was required, the amino acid change was analysed to ensure that, where possible, the change resulted in a silent mutation

The primers pairs were also checked to see if there was any inverted repeats in the primer sequence or if the primer sequence of corresponding primers were inversions of each other as if this were the case they would form primer dimers This procedure was followed for each of the five regions of interest Figure 5 2 shows the antibody protein segments that these five primers would amplify corresponding to the regions of the DNA sequence Each of these primers are detailed below

Primer sequence of Variable region, kappa light chain

This sequence was taken from sequence numbers 75 and 76 (Clark *et al* , 1985) obtained in Kabat *et al* (1987) This primer sequence was called **VKFOR** (Figure 5 1) Sequence number 76 was used to generate the database search

The sequence chosen as **VKFOR** had one base pair change (in bold type), to introduce a *Sac* I restriction site GAGCT↓C, underlined in the primer sequence below *Sac* I cuts at position 1119 of the pComb3 vector system for antibody expression (Section 5)

VKFOR GAC ATT GAG CTC ACC CAA TCT CCA

Primer sequence of Variable region, heavy chain

This sequence was taken from sequence numbers 148 and 153 (Boersch-Supan *et al* ,

1985) obtained in Kabat *et al* (1987) These sequences were both generated from hybridomas derived from balb/c mice Sequence number 153 was used to generate the database search The primer sequence obtained was called **1HEAVY**

The sequence chosen as **1HEAVY** required no base pair changes to introduce a *Pst* I restriction site CTGCA↓G, underlined below *Pst* I cuts at position 157 of the pComb3 vector system

1HEAVY GAG GTT CAG CTG CAG CAG TCT

These two primers are for the forward sequence and correspond to the 5'→3' reading of the genetic code To generate the primers for the complementary DNA strand, the sequence must be reversed in polarity (i e from 3'→5' to 5'→3') and the complementary strand made (i e substituting A for T, T for A, C for G and G for C)

Primer sequence of Constant region, kappa light chain

This sequence was taken from sequence numbers 3 (Hieter *et al* , 1980), 4 (Max *et al* , 1979), and 7 (Hamlyn *et al* , 1981) obtained in Kabat *et al* (1987) The primer sequence obtained was called **VKCBAC2**, as these sequences were identical, sequence number 3 was used to generate the database search The sequence chosen as **VKCBAC2** had two base pair changes (in bold type and underlined) to introduce a *Xba* I restriction site T↓CTAGA, underlined below *Xba* I cuts at position 1131 of the pComb3 vector system

VKCBAC2 CTC ATT CCT TCT AGA GCT CTT GAC complementary strand

Primer sequence of Heavy Constant chain, CH1 region

This sequence was taken from sequence numbers 18 (Kawakami *et al* , 1980), and 19 (Auffray and Rougeon, 1980) obtained in Kabat *et al* , (1987) Both of these were of IgM sequences of newborn mouse genomic DNA This primer sequence was called **HCH1BAC2**, as these sequences were identical, sequence number 18 was used to generate the database search The sequence was chosen although **HCH1BAC2** had three base pair changes (in bold type) to introduce a *Spe* I restriction site **A**↓CTAGT, underlined below *Spe* I cuts at position 175 of the pComb3 vector system

HCH1BAC2 ATG CAG ATC ACT AGT TTT GCC TCC complementary strand

Primer sequence of Heavy Constant chain, CH2 region

This sequence was again taken from sequence numbers 18 (Kawakami *et al* , 1980), and 19 (Auffray and Rougeon, 1980) obtained in Kabat *et al* (1987) Both of these were of IgM sequences of newborn mouse genomic DNA However, the CH2 region was analysed to generate this primer This primer sequence was called **HCH2**, as these sequences were identical, sequence number 18 was used to generate the database search The sequence was chosen although **HCH2** had three base pair changes (in bold type) to introduce a *Spe* I restriction site A↓CTAGT, underlined below As above, *Spe* I cuts at position 175 of the pComb3 vector system

HCH2 TAC ATT GGG ACT AGT CTC TGC GAC complementary strand

Amplification primers VKFOR and 1HEAVY were designed to be complementary to the mRNA in the beginning of the FR1 regions of V_L and V_H, respectively The primers VKCBAC2, HCH1BAC2 and HCH2 were designed to be complementary to the first-strand cDNA encoding the more conserved heavy chain constant regions, C_L, CH_1 and CH_2 (Figure 5 1) The restriction sites for forced cloning were incorporated in the middle or towards the 5' end (the least conserved part) of the primers, and mismatches were minimised near the 3' end The degree of complementarity of the 3' end of each primer to each of the entries in the Kabat *et al* (1987) data base was checked with SeqAid For most entries in Kabat *et al* (1987) and GENBANK, the primer regions proved to be perfectly homologous or had only one or two mismatches The restriction sites at each end of the mature antibody domains would allow the DNA segment to be excised and the amplified cDNA to be inserted into the pComb vector

2.3.30.7. Extraction of oligonucleotides from their synthesis columns.

As this procedure may be hazardous, care was taken to wear eye protection and to take all necessary precautions In a fume cupboard, 1ml of ammonium solution or $[(NH_4)_2OH]$ in a 1ml syringe was passed through the column into another 1ml syringe attached to the other side of the column This was repeated 3-4 times and the 1ml of solution was then left in the column for at least 15min The 1ml was then eluted into a sterile 4ml bijou bottle The 1ml elution step was repeated twice more, and the elutate was put into the bijou after each 15min in the column The bijou bottle was sealed tightly with parafilm and incubated at 55°C for at least 8hrs or overnight The

ammonium solution containing the oligonucleotide was freeze dried and resuspended in 200μl of sterile Tris-EDTA, pH 7 5, or water The oligonucleotide was ethanol precipitated overnight by the addition of 1/10 the volume of 3M sodium acetate and twice the volume of ice-cold ethanol, at -20°C

The pellet was washed twice with ice-cold 70% ethanol by gently pouring the ethanol on and off the pellet, it is crucial at this stage that the pellet is not disturbed The pellet was resuspended in 300μl of sterile water and stored in aliquots at -20°C The primers were quantified as outlined in Section 2 3 30 8

2.3.30.8. Quantification of PCR primers.

To quantitate the primers, an aliquot was removed from -20°C and thawed The eppendorphs were spun at top speed for 30min and the pellet was resuspended in 200μl of 70% ice-cold ethanol and spun for 10min This eliminates contaminating salts, etc The pellet was then resuspended in 500μl of sterile water A 1 50 dilution (D) in water was then prepared (20μl primer solution 980μl sterile water) and the absorbance (A) was measured at 620nm The DNA concentration (μg/ml) = A x D x 20, where a reading of 400μg/ml is expected The molarity of the DNA was calculated by multiplying the concentration by the weight of an average base (325) and then by the length of the primer All the primers were used from a stock concentration of 50pmol/μl

The quantification of the VKFOR primer, 24 nucleotides in length, is calculated as an example Since the approximate molecular weight of the VKFOR primer = 24 x 325g = 7800g, then a 1M solution would be 7800g/L

1M = 7800g/L $\Rightarrow$ 1mM = 7 80g/L $\Rightarrow$ 1pM = 7 80x10^{-9}g/L

$\Rightarrow$ 1pM/μl = 7 80x10^{-9}g/μl

$\Rightarrow$ 50pM/μl = 3 90x10^{-7}g/μl

= 3 90x10^{-5}g/μl total DNA, if the primer is resuspended in 100μl of sterile H_2O

20ng of primer DNA is required per PCR reaction The amount of DNA (ng/μl) is calculated as follows,

Total DNA = 39 0x10^{-6}g/μl $\Rightarrow$ 39 0μg DNA per μl This can be then simply diluted to 20ng/ul and 1μl per PCR reaction can be used

2.3.30.9. Synthesis of cDNA.

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2.3.30.9. Synthesis of cDNA.

Best results for the synthesis of cDNA were obtained when the cDNA was prepared

immediately from the mRNA The primer pairs for the three cDNA, and subsequent PCR, reactions are shown in Table 5 1 1 cDNA was prepared by using 1 0μg RNA (Section 2 3 30 9) dissolved in 3μl of elution buffer from the mRNA isolation kit and 2μl of a 10ng/μl solution of the appropriate primer pairs was added (i e 20ng of DNA was used) This was heated to 70°C for 10mm and was then chilled quickly with ice water Then 5μl (20 units) of placental RNase inhibitor (RNase in), 5μl of 10x reverse transcriptase (RT) buffer, 1μl of a solution of dNTPs (dGTPs, dATPs, dTTPs, dCTPs) each at a concentration of 20mM, 5μl of 0 1M DTT and 0 5μl of a 200units/μl solution of single stranded murine reverse transcriptase was brought up to a total volume of 25μl with DEPC-treated sterile water This solution was mixed by gentle vortexing Any resulting bubbles may be eliminated by brief centrifugation in a microcentrifuge Incubation at room temperature for 20min and at 42°C for 5min followed The reaction was then incubated at 90°C for 5min and left on ice for 10min 1μl of RNase H was added and left at 37°C for 20min This mRNA-cDNA hybrid gave best results if used in PCR immediately Otherwise it was stored at -20°C

2.3.30.10. Calculating the melting temperatures of primers.

The equation used to calculate the melting temperature (T_m) of the primers is as follows,

$$T_m = 81\ 5 - 16\ 6(\log_{10}[Na^+]) + 0\ 41(\%\ G+C) - (600/N),$$

where N = chain length (Sambrook *et al* , 1989) This equation predicts accurately the melting temperatures for oligonucleotides as short as 14 nucleotides and as long as 60-70 nucleotides

2.3.30.11. PCR protocol.

All PCR reactions were set up in a laminar flow under sterile conditions and the PCR ingredients were added in the order shown below, the template DNA was added last to each vial to prevent cross contamination The oligionucleotides were kept on ice at all times A typical PCR reaction was set up as follows,

78 5μl sterile water,

8 0μl dNTP mix (20mM each)

10 0μl of 10x Taq buffer

0 5μl 5' primer (50pmol)

0 5μl 3' primer (50pmol)

0 5μl *Thermus aquaticus* (0 5 units/μl) (Taq)

2 0μl of template DNA (100ng from Section 2 3 30 9)

The total volume was 100μl, care was taken to keep the Taq polymerase on ice at all times and to minimise its time out of -20°C The reaction was mixed and centrifuged briefly to remove any bubbles, this was then layered over with 100μl mineral oil, to prevent evaporation of the small volume of liquid during the high temperatures involved in the PCR cycle The PCR cycle conditions were optimised for each individual experiment The product of a PCR reaction should be a fragment or fragments of DNA of defined length Following the PCR reaction, a 10μl sample with 2μl of 6x sample buffer (Section 2 3 30 25) with appropriate molecular weight marker were examined on an agarose gel of between 0 8%-4 0%, stained with ethidium bromide and view under UV light The PCR machine used was PREM™ LEP Scientific, supplied by the Medical Supply Company

2.3.30.12. Electrophoresis of nucleic acids.

Agarose (0 7%, w/v) in Tris-acetate-EDTA (TAE) was routinely used for the analysis of plasmid and chromosomal DNA Mini gels were run at 100V in TAE buffer on a Horizon 58 gel box system for approximately 1 hour, (while total DNA digests were run 40-50V on a Pharmacia GN-200 or BRL H4 gel box for 14-16 hours) 4% (w/v) Nusieve^R GTG (FMC) agarose or 1 5% (w/v) agarose in TAE were used to separate PCR products and primers These gels were run at 75V in the Horizon 58 or Horizon 11 14 Midi gel systems for 1-2 hours Nusieve^R GTG agarose is a low gelling (< 30°C) and low melting (< 65°C) temperature agarose It finely resolves nucleic acid fragments less than 1000bp and can distinguish fragments as small as 8bp At a given Nusieve^R GTG agarose concentration, DNA electrophoresed in gels in the presence of tris-borate-EDTA (TBE) buffer runs more slowly than DNA electrophoresed in TAE buffer In 4% gels using TAE buffer, bromophenol blue migrates similarily to a 70bp fragment, while in TBE buffer this dye migrates similarily to 30bp (FMS Bioproducts, Denmark)

The following is the range of separation of DNA on agarose gels,

Agarose %	**Optimal range of separation of linear DNA (kb)**
0 3 (run at 4°C)	60 0 - 5 0
0 6 (run at 4°C)	20 0 - 1 0

0 9	7 0 - 0 8
1 2	6 0 - 0 4
1 5	4 0 - 0 2
2 0	3 0 - 0 1

The molecular weight marker used in these gels was ϕX174, restricted with *Hind* III which gave distinct bands at the following molecular weights,

1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118 and 72

2.3.30.13. Preparation of ethidium bromide to stain DNA.

One gram of ethidium bromide **(highly mutagenic)** was added to 100ml H_20 and stirred for several hours to ensure the dye was dissolved, resulting in a stock concentration of 10mg/ml This was used at a final concentration of 1μg/ml in dH_20 The container was wrapped in tin foil and stored at 4°C Gloves were worn at all times when handling solutions or gels containing ethidium bromide Ethidium bromide waste was collected, treated with activated charcoal and filtered through 3MM Whatman filter paper The clear liquid was disposed normally and the solids contained on the filter paper were incinerated

2.3.30.14. Visualisation of nucleic acids.

All nucleic acid gels were stained in 1μg/ml ethidium bromide for 15min, washed in dH_2O for 15min and then visualised on a UV transilluminator Photographs were taken on an Olympus OM-20 camera using T-max 100 film (Kodak) and an A003 red filter (Cokin, France) which blocks UV light Vivitar close-up lenses were used as required

2.3.30.15. Development of black and white negatives.

The black and white photographic film used (Section 2 2) was loaded onto the spool of a small tank developer, in complete darkness, and 250ml of D76 developer was added Air bubbles were removed by tapping a few times off the bench After the required development time, the developer was removed and may be re-used The developer tank was rinsed twice with water, then 250ml of fixer was added, and left for an hour or overnight It was then removed and the negative was left to soak in water until all the pink colour was removed The negative was then dried and printed

2.3.30.16. Rapid purification of DNA fragments.

This was carried out using the Magic PCR Preps™ Purification System from Promega,

according to the instructions Briefly, one magic column was prepared for each PCR product purification required The column was prepared by attaching the syringe barrel from a 3ml disposable tube to the leur-lock extension of each mini-column 100μl of direct purification buffer (50mM KCl, 10mM Tris-HCl, pH 8 8, at 25°C, 1 5mM $MgCl_2$, 0 1% Triton X-100) was dispensed into a 12x75mm polyproplene tube 100μl of each PCR reaction was added to the polyproplene tube and mixed 1ml of the magic PCR resin was added to the tube and vortexed briefly 3 times over 1min The resin containing the bound DNA was pipetted into the syringe barrel The syringe plunger was inserted slowly and the slurry was gently pushed into the mini-column using the syringe plunger The mini-column was then washed with 2ml of magic PCR preps column wash solution (80% isopropanol) The syringe barrel was removed and the mini-column was transferred to a 1 5ml microcentrifuge tube The mini-column was spun for 20sec at 14,000g in a microcentrifuge to dry the resin The mini-column containing the resin-bound DNA was set at room temperature for 15min to allow the evaporation of any isopropanol present The mim-column was transferred to a new microcentrifuge tube and 50μl of Tris-EDTA buffer (10mM Tris-HCl pH 7 5, 10mM EDTA) was applied to the column After 1min the bound DNA fragment was eluted by spinning the microcentrifuge tube containing the mini-column for 20sec at 14,000g The mini-column was removed and discarded The purified DNA was stored in the microcentrifuge at 4°C or at -20°C

2.3.30.17. Phenol extraction of DNA.

DNA was extracted from a PCR sample using phenol The PCR sample was removed from under the layer of mineral oil and an equal volume of the phenol chloroform isopropanol (25 24 1) mixture was added It was mixed by vortexing and then centrifuged at 14,000rpm in a microcentrifuge for 1min Two layers were formed The upper layer contains the DNA and was removed without disturbing the phenol layer underneath

2.3.30.18. Preparation of plasmid for ligation.

The restriction enzymes used were stored at -20°C and diluted according to the manufacturers' instructions The PCR fragments were digested with their appropriate restriction enzymes and ligated to their respective plasmids The purified DNA from

the heavy chain PCR reactions were ligated to the pTACO1H plasmid, which carries the carbenicillin resistance gene and the light chain DNA was ligated to pTCO1 which carries the chloramphenicol resistance gene The plasmids were prepared for ligation by being cut with the appropriate restriction enzymes in a volume of 300μl at 37°C for 2hrs The reaction mix was extracted with phenol/chloroform (Section 2 3 30 17) and ethanol precipitated Subsequently, this was treated with calf intestine alkaline phosphatase, which catalyses the removal of 5'-phosphate residues from single or double stranded DNA and RNA This enzyme was used to prevent vector DNA from re-ligating on itself by adding 0 5μl of calf intestine alkaline phosphatase (0 5 units) to 12μl of vector DNA and incubating it at 37°C for 20min To stop the reaction the following were added and incubated at 65°C for 10min,

10xSTE (TNE)	10μl
10mM Na_2EDTA	10μl
20% SDS	2 5μl
Tris-EDTA, pH 7 5	100μl
H_2O	60μl

Following extraction of DNA from the reaction mixture (Section 2 3 30 17), the vector DNA was purified on a 0 8% agarose gel and the vector DNA band was excised and purified (Section 2 3 30 16)

2 3.30.19. Ligation of vector and insert DNA.

The enzyme T4 DNA ligase catalyses the formation of a phosphodiester bond between adjacent 3'-OH and 5'-phosphate termini in double stranded DNA This enzyme was used in the ligation of cohesive ended fragments to vector DNA Ligation reactions were carried out overnight at 16°C and were set up as follows,

insert DNA in H_2O	15 4μl
vector DNA	1 0μl
10x ligation buffer	2 0μl
0 5M DTT	0 6μl
T4 DNA ligase	1 0μl

The total reaction volume was 20μl

2.3.30.20. Preparation of electrocompetent *E coli* cells.

Electroporation was the method used to introduce DNA into competent *E coli* TG1 cells and these cells were prepared by inoculating one litre of LB with 1% (v/v) of an *E coli* culture, which had been grown overnight at 37°C This culture was shaken vigorously at 37°C to an A_{600nm} of 0 5-0 8 The flask was chilled on ice for 15-30min and the cells were centrifuged at $4000g_{max}$, 4°C for 15min The cell pellets were resuspended slowly, in a shaking ice water bath, in one litre of ice-cold distilled water Centrifugation and resuspension was repeated, as previously, in 0 5L of ice-cold distilled water Following resupension, the cells were centrifuged as before and resuspended in 20ml of 10% (v/v) ice-cold glycerol The cells were again centrifuged and resuspended finally in 2-3ml of 10% (v/v) glycerol The competent cells were dispensed into 100μl aliquots and stored at -80°C for up to 6 months

2.3.30.21. Electroporation of *E. coli* cells.

Electroporation of electrocompetent cells was carried out using a BioRad Gene pulser™, set at 25μFaradays capacitance, 2 5kV and 200 Ohms to deliver a single exponentially decaying pulse The electrocompetent cells were thawed on ice and 1-5μl of plasmid DNA was added The DNA must be free of excess protein and salts to prevent arcing The mixture of cells and DNA was transferred to an ice-cold 0 2mm electroporation cuvette This was then quickly dried and tapped sharply to dislodge bubbles which can also cause arcing Immediately following the pulse, 1ml of SOC medium (Section 2 3 30 25 5), at room temperature, was added to the cuvette and the cell suspension was placed at 37°C in a microfuge tube Following 30min recovery time, dilutions of the cells were plated onto selective medium and incubated at 37°C for 24 hours

2.3.30.22. Small scale plasmid preparation from *E coli* cells.

This method was described by Birnboim and Doly (1979) 1 5ml of overnight broth culture was pelleted at 14,000rpm and resuspended by vortexing in 100μl of solution 1 (Section 2 3 30 25 3) and was incubated at room temperature for 5min 200μl of solution 2 (Section 2 3 30 25 3) was added and the samples were left on ice for a further 5min 150μl of solution 3 (Section 2 3 30 25 3) was added, the samples were placed on ice for a further 10min and then centrifuged at 14,000 for 5min The supernatant was removed and 400μl of Kirby mix added After thorough mixing, the

samples were spun at 14,000rpm and the aqueous layer removed and precipitated with 0 8ml of ice-cold ethanol Following 10min at room temperature, the plasmid DNA was pelleted by centrifugation at 14,000rpm The pellet was washed twice in 70% (v/v) ethanol, dessicated and resuspended in 50μl of TE

2.3.30.23. Antibiotics used in molecular biology techniques.

All antibiotics were purchased from Sigma and stock solutions were stored at 4°C and -20°C Carbenicillin stock solution (10mg/ml) was made up in 50% (v/v) ethanol, stored in the dark and used with *E coli* at a concentration of 100μg/ml Chloramphenicol was dissolved in 98% (v/v) ethanol and used at a working concentration of 20μg/ml with *E coli*

2 3.30.24. Growth conditions and storage of strains

E coli strains were routinely grown shaking in LB at 37°C Long term storage of *E coli* strains was in 50% (v/v) glycerol at -20°C TG1 cells were kept on M9 minimal medium to prevent the loss of the *lac*Z phenotype

2.3.30.25. Preparation of buffers and solutions used in molecular biology.

All buffers were dispensed and stored at -20°C, unless otherwise stated

TE buffer (50x)

Tris-HCl	50mM
Na_2-EDTA	1mM

This solution was adjusted to pH 8 0 using conc HCl, sterilised by filtration (0 22μm filter) or autoclaved and stored in aliquots at 4°C

3M Sodium acetate, pH 5 2

Sodium acetate $3H_2O$ (408 1g) was dissolved in 800ml H_2O, the pH was adjusted to 5 2 with glacial acetic acid and the volume brought up to 1 litre This solution can also be filter sterilised or autoclaved and stored at 4°C

0 5M EDTA (pH 8 0)

Disodium ethylene diamine tetraacetate $2H_2O$ (181 6g) was added to 800ml of H_2O and stirred vigorously The pH was adjusted to 8 0 with NaOH

10x PCR buffer

500mM KCl

100mMTris-HCl (pH 8 3)

15mM $MgCl_2$

1% (w/v) gelatin

2.3.30.25.1. Solutions used in electrophoresis.

10x Tris Borate EDTA electrophoresis buffer (TBE)

Tris-HCl	108 0g
Na_2-EDTA	9 3g
boric acid	55 0g
H_2O to	1 0L

This solution was adjusted to pH 8 0 with conc HCl

50x Tris Acetic Acid EDTA electrophoresis buffer (TAE)

Tris-HCl	242 0g
0 5M Na_2-EDTA (pH 8 0)	100 0ml
H_2O to	1 0L

This solution was adjusted to pH 8 0 with conc HCl

6x Bromophenol blue

bromophenol blue	0 25% (w/v)
sucrose in H_2O	40 00% (w/v)

This was stored at 4°C DNA samples were mixed in a 5 1 ratio with bromophenol blue prior to loading on agarose gels

2.3.30.25.2. Preparation of Sucrose, Triton X, EDTA, Tris (STET) buffer for lysis of *E coli* transformants.

8% (w/v) sucrose

5% (w/v) Triton X 100

50mM Na_2 EDTA

50mM Tris-HCl (pH 8 0)

2.3.30.25.3. Solutions for gene clean procedure.

Solution 1

0 5M glucose	1 0ml
0 1M Na_2EDTA	1 0ml
1 0M Tris-HCl (pH8 0)	0 25ml
H_2O	7 75ml

Solution 2

1N NaOH	2 0ml
10% (w/v) SDS	1 0ml
H_2O	7 0ml

Solution 3

5M potassium acetate	30 0ml
glacial acetic acid	5 75ml
H_2O	1 425ml
pH	4 8ml

5M potassium acetate

To prepare this solution, 60ml of 5M potassium acetate, 11 5ml of glacial acetic acid and 28 5ml H_2O were mixed This solution was 3M with respect to potassium and 5M with respect to acetate This solution was used in the preparation of solution 3

Phenol/Chloroform (Kirby mix)

100g of AnalaR grade phenol was dissolved in 100ml chloroform 4ml of isoamyl-alcohol and 0 8g of 8-hydroxyquinoline was then added and the mixture was stored under 100mM Tris-HCl, pH 7 5 in a dark bottle at 4°C

2.3.30.25.4. Solutions used in ligations.

10x STE/TNE

10mM Tris-HCl (pH 8 0)

1M NaCl

10mM Na_2EDTA This was used in the calf intestinal alkaline phosphatase reaction

10x Nick translation buffer/Klenow reaction buffer

0 5M Tris-HCl (pH 7 2)

0 1M $MgSO_4$

1mM dithiothreitol

500μg/ml BSA (fraction V, Sigma)

10x Ligation buffer

200mM Tris-HCl (pH 7 6)

100mM $MgCl_2$

100mMdithiothreitol

6mM ATP

This was used for cohesive ended ligations

Dithiothreitol (1M DTT)

DTT (3 09g) was dissolved in 0 01M Sodium acetate (pH 5 2) It was filter sterilised, dispensed and stored at -20°C

ATP (0 1M)

ATP (60mg) was dissolved in 800μl of sterile H_2O and the pH adjusted to 7 0 with 0 1M NaOH The volume was adjusted to 1ml with H_2O, dispensed and stored at -20°C

2.3.30.25.5. SOC medium for electroporation of *E coli*

Tryptone	2 0% (w/v)
Yeast extract	0 5% (w/v)
NaCl	10 0mM
KCl	2 5mM
$MgCl_2$	10 0mM
$MgSO_4$	10 0mM
Glucose	20 0mM

2.4. Techniques involved in the production, purification and characterisation of iodinated Bolton-Hunter reagent (IBHR), and of polyclonal and monoclonal antibodies against IBHR.

The problems associated with direct iodination and radiolabelling of proteins can be minimised using a second molecule to carry the radioiodine (Section 4) One such molecule used was developed in 1973 by Bolton and Hunter The molecule they used was 3-(4-hydroxyphenyl) propionic acid N-hyroxysuccinimide ester, now known as Bolton-Hunter reagent (BHR) (Bolton and Hunter, 1973)

In order to produce antibodies to the BHR molecule or to iodinated-BHR (IBHR), as is too small to elicit an immune response on its own, it must be conjugated to a carrier protein, BSA (Section 2 4 5) To produce antibodies to IBHR, it is important, however, to allow for a large enough spacer arm between the IBHR molecule and protein in the conjugate to prevent any interference between these molecules The iodide in the IBHR-protein conjugate is detected using an iodide assay based on the Sandell-Kolthoff reaction, (Sandell and Kolthoff, 1934) The reaction involves the oxidation of arsenic (III) by cerium (IV) which is catalysed by trace amounts of iodide Sandell and Kolthoff (1934) first reported that a minute quantity of iodide could be detected by measuring its catalytic effect on the slow cerium(IV) - arsenic(III) reaction

$$2\ Ce^{4+} + As^{3+} \rightarrow 2\ Ce^{3+} + As^{5+}$$

A microassay was developed and later optimised (O'Kennedy *et al* , 1989, O'Kennedy and Keating, 1993) (Section 2 4 9 2) Two methods were used to determine the concentration of protein in a sample, (a) a miniaturized Bradford assay (Bradford, 1976) and (b) a miniaturized Bicinchoninic Acid (BCA), protein assay (Smith *et al* , 1985) (Section 2 4 9 3) The IBHR molecule was chosen to work with in this project as in the radiolabelled form, iodine has the highest specific activity available commercially Monoclonal and polyclonal antibodies against IBHR were produced, purified and characterised (Section 4)

2.4.1. Preparation of IBHR.

A 0 1M solution of Bolton-Hunter reagent (BHR) and a 2M solution of iodine monochloride (ICl) (toxic) were made up in ethyl acetate ICl was added to BHR at a 2 1 molar ratio and allowed to react overnight at 4°C The resultant mixture, a white precipitate, was recovered by centrifugation and the pellet was then washed in ice cold

ethyl acetate After recentrifugation, the IBHR was dried under nitrogen and stored at -70°C The purity of the preparation was tested by thin-layer chromatography on fluorescent silica gel plates developed in ethyl acetate toluene (1 1, v/v) and compared to two previous iodinations of the same BHR IBHR is very sensitive to moisture When thawing from -20°C it should not be opened until it has reached room temperature Before using the IBHR for standards or conjugation, 4mg of IBHR was dissolved in 40μl N,N'-dimethylformamide (DMF), 960μl benzene was then added to bring the total volume to 1ml The solution was aliquoted at 1mg per vial and dried under nitrogen in a fume cupboard 1mg of IBHR was redissolved in a minimum volume (0 2ml) of DMF, ultrapure H_2O was added to bring the concentration to 1mg/ml and this was used directly for conjugation or as standards Further dilutions were made up in 0 01M PBS, pH 7 3

2.4.2. Preparation of IBHR standards.

1mg of IBHR was dissolved in 0 2ml DMF before being diluted to give a final concentration of 1μl/ml with ultrapure H_2O Standards were prepared in the range 0-1μl/ml and these were used in the iodide assay to quantify the IBHR concentration of the IBHR-proteins conjugate produced

2.4.3. Preparation of protein standards to determine the protein concentration of the IBHR-protein conjugates produced

BSA, KLH, ovalbumin and IgG used in Bradford assays were made up in 0 01M PBS, pH 7 3, to a final concentration of 1mg/ml Further dilutions of these standards were also made up in 0 01M PBS, pH 7 3

BSA, KLH ovalbumin and IgG standards used in the ELISA were made up to a concentration of 10μg/ml using sodium carbonate buffer, pH 9 0 BSA, KLH ovalbumin and Rabbit IgG standards used for electrophoresis, absorbtion spectrum analysis and HPLC analysis were made up in 0 01M PBS, pH 7 3, buffer up to a final concentration of 1mg/ml

2.4.4. Preparation of 0.1M borate buffer for use in the conjugation of IBHR to protein.

9 54g of disodium tetraborate was dissolved in 250ml dH_2O, and 1 5g of boric acid was

dissolved in 250ml dH_2O In order to bring the boric acid solution to pH 8 5, 54ml of the disodium tetraborate solution was added

2.4.4.1. Preparation of 20x 0.01M PBS, pH 9.0

34 8g of potassium hydrogen phosphate was dissolved in 500ml dH_2O 27 6g sodium dihydrogen phosphate 2-hydrate was dissolved in 500ml dH_2O 10ml of the potassium hydrogen phosphate solution was added to 400ml of the sodium dihydrogen phosphate 2-hydrate solution The pH was adjusted to 9 using concentrated sodium hydroxide 9 5g Sodium chloride was added to the solution to give 0 01M 20x PBS, pH 9 0

2.4.5 Conjugation of IBHR to BSA, KLH, and ovalbumin.

4mg of IBHR was dissolved in DMF, washed in benzene and then dried under nitrogen The IBHR was again dissolved in 800μl DMF and used for protein conjugation 4ml of a 1mg/ml solution of BSA (grade V), KLH (type VIII) or ovalbumin (grade V), (Sigma) in 0 1ml borate buffer, pH 8 5, (Section 2 4 4) was added to the IBHR solution with thorough mixing The reaction was allowed to proceed on ice for two hours, with mixing The solution was then centrifuged at 13,000g for 2 minutes to remove any precipitate formed The unconjugated IBHR was removed from the conjugated IBHR initially using chromatography on Ultrogel AcA44 (Section 2 4 6) and subsequently, with dialysis against a large volume of 0 01M PBS, pH 7 3, overnight at 4°C, with mixing

2.4 6 Gel filtration using Ultrogel AcA44.

A 16mm internal diameter x 360mm column of Ultrogel AcA44 column was prepared by resuspending 100ml of Ultrogel in 50ml of 0 01M PBS, pH 9 0 The Ultrogel has a linear fractionation range of approximately 10,000-130,000 daltons and an exclusion limit of approximately 200,000 daltons The gel suspension was mixed gently with a glass rod and connected to a vacuum pump for 30min to remove air bubbles The column was poured and then washed with 3 column volumes (cv) (1cv = 80ml) of 0 01M PBS, pH 9 0, at a flow rate of 1ml/min The conjugated sample (Section 2 4 5) was added and 80 one ml fractions were collected at a flow rate of 1ml/min The protein content of the fractions obtained was determined using the Bio-Rad micro-Bradford assay using a protein standard curve in the range 0-100μg/ml protein, read at

a wavelength of 595nm (Section 2 4 9 3) The IBHR incorporation was determined using the iodide microassay using IBHR standards in the range 0 1-1 0μg/ml (Section 2 4 9 2) Before the column was stored at 4°C, 0 02% (w/v) sodium azide was added to the wash buffer ensuring that at least 1cv of this was run through the column

2.4.7. Preparation of dialysis tubing.

Gloves were worn at all times when handling dialysis tubing Dialysis tubing (Sigma) was prepared by boiling it in 500ml of ultrapure H_2O in a glass beaker for 20min after addition of 50mg of ethylene diamine tetraacetic acid (EDTA)

2.4.8. Concentration of dialysed samples.

After dialysis the conjugated sample, still in the tubing, was immersed in a trough containing crystalline sucrose at 4°C The sucrose removed the water in the sample by reverse osmosis The osmosis was continued until the desired sample volume was reached In order to remove any sucrose that may have entered the sample, the concentrate was again dialysed against PBS at 4°C for 1 hour

2.4 9. Characterisation of the IBHR-protein conjugates produced.

The conjugates were characterised according to O'Kennedy *et al* (1989)

2.4.9.1. Preparation of ceric ammonium sulphate and arsenious acid for the iodide assay.

Ceric ammonium sulphate (0 1M) was made up in 2 5M H_2SO_4 Dilutions of this stock solution were made up fresh daily in 10% (v/v) H_2SO_4 to a final concentration of 0 028M ceric ammonium sulphate Arsenious acid (0 075M) (toxic) was prepared by placing 14 84g of arsenious trioxide (toxic, potential carcinogen) in 700ml of distilled water containing 28ml of concentrated sulphuric acid The mixture was heated carefully to near boiling with constant stirring until most of the arsenic trioxide had dissolved Some precipitation of arsenious oxide may occur The solution was then allowed to cool to room temperature before being filtered The resulting solution was made up to 1L with distilled water

2.4.9.2. Iodide microassay procedure.

The Bolton-Hunter reagent used in this project was labelled with non-radioactive iodine

which was detected using the Sandell-Kolthoff reaction (Sandell and Kolthoff, 1934) These standards, in duplicate or triplicate, were added to the wells of NUNC Immunosorb Maxisorb microtitre plates to a final volume of 100μl Samples for analysis, in duplicate, were added to remaining wells with a final volume of 100μl Arsenious acid (60μl) was added to each well using a multichannel pipette Ceric ammonium sulphate solution (25μl) was added to all wells except to column 1 The plate was mixed and blanked against column 1 Absorbance readings were taken at precisely timed 1 minute intervals Wells incubated with 0 01M PBS, pH 7 3, served as zero controls These wells will have the highest absorbance values as no iodide is present to catalyse the Sandell-Kolthoff reaction The absorbance of each of the standards and samples were read every minute for five minutes at 414nm on a Titertek Twin Reader Plus

2.4.9.3. Protein concentration determination.

The two methods used to determine the concentration of protein in a sample are discussed The reagents for both assay systems were obtained in kit form from Pierce Chemical Company (Table 2 1)

(a) Bradford Coomassie protein reagent kit procedure.

A miniaturized version of the assay kit was developed in order to minimise the amount of sample required The assay had the the advantage of providing a rapid indication of the protein level This is based on the fact that Coomassie Brilliant Blue G-250 exists in two different colour forms, red and blue When the dye binds to the protein, the red form is converted to the blue form which can be detected by monitoring the absorbance at 595nm In order for the dye to bind, the molecule must possess a macromolecular form and an active aromatic functional group This makes the assay suitable for protein determination, but BHR also satisfies this criteria This means that BHR interferes with the assay This assay was used for protein determination as iodine interferes to a greater extent with the BCA assay Aliquots of 10μl of diluted standard (e g 0 01-0 1mg BSA, KLH, mouse IgG/IgM, as appropriate) or unknown protein samples were pipetted into a 96 well plate (NUNC), to which 200μl of Pierce Protein assay reagent was added The plate was mixed and the absorbance read after 5mins at room temperature, at 595nm, on a Titertek Twin Reader Plus

(b) BCA protein assay reagent kit procedure.

In this assay Cu^{++} combines with protein under alkaline conditions to form Cu^{+}, which reacts with BCA to give a coloured product Two separate reagents consisting of an alkaline bicarbonate buffer Reagent A and Reagent B, 4% (w/v) copper sulphate solution are supplied with the kit The BCA protein assay working reagent was made up by mixing 49 parts of Reagent A with 1 part of Reagent B, and was stable for one week at room temperature Two different miniaturized protocols were followed One used incubation at 37°C for 30 minutes for protein concentrations in the range of 0 02-1 5 mg/ml The second method used incubation at 60°C for 30 minutes for protein concentrations in the range of 0 001 - 0 25 mg/ml 10μl of each standard or unknown protein sample and 200μl of BCA working reagent was added to each well of a 96 well plate For control wells, 10μl of diluent was used The plates were incubated at the selected temperature and time Absorbance readings were taken at 562nm on a Titertek^{R} TwinPlus Reader All samples and standards were prepared in duplicate or triplicate The BCA gave more accurate protein readings than the Bradford Assay for compounds which did not contain iodine, as less interference was obtained from detergents and chelating agents However, the assay system required incubation at preset temperatures and results were not as quickly obtained when the Micro-Bradford assay was used

2 4.9.4. Investigation of the interference of IBHR with the Bradford assay.

Two sets of BSA standards were prepared (Section 2 4 9 5) in the range 0-20μl/ml in triplicate One of these sets was spiked with a 2μg/ml concentration of IBHR The Bradford assay was continued as in Section 2 4 9 3 and the absorbances read at 595nm

2.4.10. Production of monoclonal anti-IBHR antibodies.

An attempt was made to produce monoclonal antibodies to IBHR Five balb/c mice were immunised with the IBHR-BSA conjugate as outlined in section 2 3 6 The immunisation schedule was as follows each mouse was immunised with 50μg IBHR-BSA on days 1, 14 and 28 These mice were tail bled on day 35 and the results obtained are shown in Table 4 2 An *in vitro* immunisation was performed (Section 2 3) using 20μg IBHR-BSA (1μg/μl) in 0 01M PBS, pH 7 3

Following *in vitro* immunisation, the spleen cells were electrofused with NSO cells The

clones obtained were screened for anti-IBHR antibodies by ELISA (Section 2 3 16 3) against the following, IBH-BSA, IBH-KLH, IBH-Ovalbumin, BSA, KLH, Ovalbumin (Table 2 3) The results are presented and discussed in Section 4 The antibody-producing hybridomas were expanded according to Section 2 3 14

2.4.11. Production of polyclonal anti-IBHR antibodies.

As the production of a monoclonal anti-IBHR antibody was unsuccessful, the polyclonal approach was undertaken

Serum was obtained from a four month old male New Zealand white rabbit 7 days prior to immunisation, to determine the background response of the rabbit to the antigen The results are shown in Table 4 3 This rabbit was considered suitable for use in the production of polyclonal antibodies against IBHR as the non-specific binding of the serum to IBHR was low This serum was used as a negative control in ELISAs during all stages of the purification of the anti-IBHR polyclonal antibody

The rabbit was immunised subcutaneously with IBHR-BSA along the back The initial injection was of IBHR-BSA suspended in FCA (Section 2 3 6 1), all subsequent injections were intramuscular using FIA, as outlined in Section 2 3 6 The immunisation schedule is given in Table 2 11

The injections administered after day 80 (Table 2 11), used 0 4mg IBHR-BSA in a total volume of 0 4ml Test bleeds were performed 11 days after the third and subsequent injections using the marginal ear vein of the rabbit The sera obtained were assayed for anti-IBHR polyclonal antibodies (Table 4 4) The rabbit was immunised over an 8 month period, bled at intervals and the serum obtained (Section 2 4 1 1) ELISAs were performed to obtain the specific antibody titre (Table 4 4)

Over the entire immunisation schedule the recommended amount of antigen to use for a rabbit is between 0 5 - 4 0mg of protein (Mayer and Walker, 1987) For polyspecific antisera, 4 0mg of antigen is preferable Antibodies are produced against the carrier protein and the hapten when a conjugate is used as an immunogen In the case of the IBHR-BSA conjugate to separate the anti-BSA from the anti-IBHR antibodies, two different separation methods were undertaken and compared The methods used in producing, purifying and characterising the anti-IBHR polyclonal antibody are outlined

Table 2.11. Immunisation schedule of a rabbit immunised with IBHR-BSA in order to produce anti-IBHR polyclonal antibodies.

Day	IBHR-BSA (mg) injected
1	0 2
30	0 3
50	0 4
80	0 4

2.4.11.1. Isolation of rabbit serum.

Eleven days after each immunisation, when the anti-IBHR antibody titre was greater than 1 10,000, the rabbit was bled from the marginal ear vein, having first used a lamp to warm the ear thereby stimulating the blood flow Serum was obtained by allowing the blood from the rabbit to stand at room temperature for 20min in order that the blood would coagulate These clots were then gently broken up using a pipette tip and centrifuged at 3000rpm for 20mins The resulting serum was aliquoted and stored at -20°C This serum was precipitated with ammonium sulphate and further purified by either protein A or by an IBHR-KLH-CNBr affinity chromatography column The reactivity of the purified samples was also tested by ELISA against IBHR-KLH, KLH, IBHR-ovalbumin, ovalbumin, IBHR-BSA and BSA (Tables 4 8 1 - 4 8 3)

2.4.11.2. Preparation of saturated ammonium sulphate.

1M saturated ammonium sulphate (SAS) was prepared by adding 761 0g ammonium sulphate to 1L of ultra pure water (4 1M solution at room temperature) with constant stirring at room temperature The solution may be warmed to 35°C to aid dissolution Once dissolved, the solution was left at room temperature overnight to allow precipitation of excess salt The solution was then poured into a separate container, and deemed to be saturated A 50% (w/v) solution of the SAS was prepared by making a 1 1 dilution of the 100% (w/v) saturated solution with ultrapure H_2O

2.4.11.3. Ammonium sulphate precipitation of rabbit serum or ascitic fluid from mice.

This method was described by Hudson and Hay (1980) The rabbit serum (or ascitic fluid from mice) was cooled to 4°C and the pH was adjusted to 7 2 with conc HCl or 5M NaOH as appropriate An equal volume of 100% (w/v) SAS was added dropwise to the serum at 4°C, while stirring This was stirred for one hour at 4°C before being centrifuged at 10,000rpm for 20mins at 4°C The supernatant was discarded and the pellet was resuspended in a volume equivalent to that of the original sample with ice cold 0 01M PBS, pH 7 3 An equal volume of ice cold 50% (w/v) SAS was added dropwise with stirring and again centrifuged as before This step was repeated using 45% (w/v) SAS, with the final pellet being resuspended in a volume of 0 01M PBS, pH 7 3, corresponding to half the volume of the original sample The resulting antibody solution was dialysed against several changes of ultrapure water and finally 0 01M PBS, pH 7 3 Following dialysis, the antibody solution was removed from the tubing and recovered by centrifugation at 1,000g for 5mins at 4°C, in order to remove any debris A small sample was removed to determine the protein concentration and to check the purity of the antibody 0 02% (w/v) sodium azide was added to the final supernatant which was dispensed and stored at -20°C

2.4.12. CNBr-sepharose affinity chromatography of SAS-treated serum.

(a) Preparation of CNBr-sepharose affinity chromatography buffers.

Preparation of coupling buffer, 100ml of a 0 1M sodium bicarbonate ($NaHCO_3$) solution was adjusted to pH 8 3 using 0 5M NaCl

Preparation of blocking buffer, 0 2M glycine was adjusted to a final pH of 8 3 using coupling buffer

Preparation of washing and regeneration buffers, 0 1M sodium acetate was brought to pH 4 5 using 0 5M NaOH 0 1M Tris was brought to pH 8 5 using 0 5M NaOH 0 01M PBS, pH 7 3, was prepared by dissolving one Dulbecco's A tablet in 100ml of distilled water

Preparation of dissociation buffer, 0 1M glycine was adjusted to pH 2 5 using conc HCl

Preparation of pH-correction buffer, 12 11g of trizma base (tris[hydroxymethyl]amino-methane) was suspended in a minimum volume of distilled water before its pH was

adjusted to pH 10 5 using conc HCl This solution was then made up to 100ml and allowed to dissolve properly, (as trisma base is difficult to dissolve in water at neutral pH)

(b) Preparation of immunosorbant for CNBr-sepharose affinity chromatography.

0 4g of CNBr-activated sepharose was suspended in 3ml of 0 001M HCl before being transferred to a sintered glass funnel to be washed for 15mins with 80ml of HCl 2ml of coupling buffer was then added to the activated sepharose 600μl of a 10mg/ml solution of anti-rabbit antibody was diluted in coupling buffer to a final volume of 2 8ml The activated sepharose was then added to the antibody and allowed to bind for 2 hours on a shaking water bath at room temperature The conjugate was then washed with 5ml of coupling buffer in a sintered glass funnel before being blocked with 3ml blocking buffer for one hour at room temperature The resulting gel was washed with three alternating cycles of pH, 5ml of 0 1M acetate buffer, pH 4 5 followed by 0 1M Tris buffer, pH 8 5, before being resuspended in PBS, pH 7 3

A 1ml syringe barrel was used as the column support in which a small piece of glass wool was inserted in the base to prevent gel leakage The CNBr-activated sepharose was carefully poured into the syringe barrel until the immunosorbant level reached the 1ml mark A little 0 01M PBS, pH 7 3, was added on top of the column to prevent the gel from drying out The column was covered with several layers of Parafilm and stored at room temperature

(c) Use of Immunosorbant.

After storage, the Parafilm was removed and the column was washed with 10ml of 0 01M PBS, pH 7 3, in order to remove any protein that leaked during storage 2ml fractions were collected and each of these were tested for the presence of protein using the BCA reagent (Section 2 4 9 3) Half of the purified antibody sample from the SAS-treated serum (Section 2 4 11 3) was applied to the column with a very slow flow rate (> 1ml/hr from this column) 1ml fractions were collected and again tested for protein After application of the sample, the column was washed with 0 01M PBS, pH 7 3, until no protein was detected in the fractions The flow rate was increased using a peristaltic pump to wash the column and dissociating buffer was then added After 1ml was collected, the column was again sealed and left for 15mins to allow the rabbit antibody

to dissociate from the anti-rabbit antibody in the gel The column was again opened and 1ml fractions were collected Each of these fractions was at a very low pH (dissociating buffer had a pH of 2 5) and hence 125μl of 1M Tris buffer, pH of 8 5 was added in order to prevent antibody denaturation The fractions were also tested for the presence of protein (see (d) below) Those fractions which contained protein were pooled The column was regenerated by washing the column with 5ml of 0 1M Tris-HCl buffer, pH 8 5, followed by 5ml of sodium acetate buffer, pH 4 5, and finally 5ml of 0 01M PBS, pH 7 3 The column was sealed with several layers of Parafilm and stored at 4°C for further use

(d) Testing Fractions for Protein.

A NUNC 96 well microtitre plate was used in this test 200μl of Bradford reagent (Section 2 4 9 3) (diluted 1/3 with water) was added to each of the 96 wells 10μl of the fraction to be tested was added to a well If a blue colour resulted after 5mins, protein was deemed to be present The absorbances were also read at 595nm

2.4.13. Preparation of protein A affinity chromatography separation buffers.

Preparation of start buffer, 0 6057g of Tris was suspended in a minimum volume of distilled water before its pH was corrected to 8 6 using conc HCl This was then adjusted to a volume of 100ml

Preparation of elution buffer, 21g of citric acid was dissolved in 1L of dH_2O Solid disodium hydrogen phosphate was added until a final pH of 4 5 was obtained This gave a 0 15M solution of citrate buffer This was diluted 2 3 to obtain a 0 1M solution

Preparation of pH-correction buffer, 12 11g of Tris was suspended in a minimum volume of distilled water before its pH was adjusted to 9 0 using conc HCl The volume was brought to 100ml with dH_2O and the Tris was allowed to dissolve

Carbonate buffer, this buffer was prepared by making up 100mls of stock 0 01M solution of sodium carbonate and 0 035M solution of sodium hydrogen carbonate in ultrapure H_2O These solutions were mixed in a 1 1 ratio and the pH was adjusted to 9 6 using conc HCl or 5M NaOH as appropriate

2.4.13.1. Separation of SAS-treated serum by protein A affinity chromatography.

Prepacked protein A affinity chromatography columns from Pierce were used Each 1ml

column contains protein A covalently linked to agarose beads which has a binding capacity of approximately 20mg IgG This system has a high salt buffer system which allows a better than usual yield of IgG1 to be obtained The top stopper of the protein A column was removed and storage buffer was poured off The bottom stopper was removed and the column was equilibrated by running 5cv of the Immunopure IgG binding buffer provided, through the column This buffer was prepared by adding 19 88g sodium phosphate (dibasic) to 800ml ultrapure H_20, the pH was adjusted to 8 0 with 80% phosphoric acid and brought to 1 litre 2ml samples were collected and tested to ensure that protein was not leaking from the column (Section 2 4 9 3) Half of the sample from the ammonium sulphate cut was diluted in Immunopure IgG binding buffer and was applied to the column with a slow flow rate (6ml/hr) This was allowed to run completely into the column which stops flowing automatically when the buffer level reaches the top frit The column was washed down with 15cv of the Immunopure IgG binding buffer 1ml fractions were collected and tested for protein by monitoring the absorbance at 280nm, where an absorbance of 1 0 = 0 8mg/ml, or the protein concentration can be measured using the BCA assay Those samples with protein were pooled and were dialysed against 0 01M PBS, pH 7 3, overnight The column was regenerated by washing with 4cv of 0 1M citric acid adjusted to pH 2 3 with 6 Normal (N) NaOH Before storage, the column was washed with a further 2cv of ultrapure H_2O containing 0 02% (w/v) sodium azide The column was stored at 4°C in the upright position, and is stable for up to 1 year Each column may be regenerated up to 10 times

2.4.14. Preparation of porcine gelatin.

The preparation of this solution has been described in Section 2 3 16 2 The solution was used as the blocking solution in the following anti-IBHR antibody ELISAs

2.4.15. ELISA for the determination of the titre of anti-IBHR rabbit serum.

ELISAs were carried out as detailed in Section 2 3 Details relevant to the ELISAs used to screen the anti-IBHR antibody are described

24 wells of a microtitre plate were coated with 10μl/ml of IBHR-ovalbumin conjugate made up with carbonate buffer (Section 2 3 12 1), while the other 72 wells were coated with ovalbumin, IBH-KLH and KLH (Table 2 3) This was accomplished by addition

of 100μl/well of each preparation to a section of the plate, this was then stored at 4°C overnight The plate was then washed four times with 0 01M PBS, pH 7 3, before being blocked with porcine gelatin, (1μg/ml, in 0 01M PBS, pH 7 3) at 200μl/well for 2 hours at 37°C

The plate was then washed four times with PBS/Tween-20 (0 1%, w/v) and once with 0 01M PBS, pH 7 3 Suitably diluted rabbit serum samples and positive and negative controls (100μl) were added to wells of the plate, as appropriate, and left to incubate for 2 hours at 37°C or at 4°C overnight The wells were washed four times with PBS/ Tween-20 (0 1%, w/v) and once with 0 01M PBS, pH 7 3, as before and a 1 7000 dilution of alkaline phosphatase-conjugated secondary antibody in TBS (i e goat-anti-rabbit IgG) (Table 2 4) was added (100μl/well) and allowed to incubate for an hour at 37°C

The wells were washed again as before and then the substrate, (BCIP in amino-methyl-propanol buffer, Table 2 5), at 100μl/well, was added The reaction was allowed to proceed at 37°C in the dark until a suitable colour had developed, which took approximately 45mins The absorbance was read at 620nm

2.4.15.1. Competitive ELISA to determine the anti-IBHR antibody specificity

A competitive ELISA was performed using the SAS-precipitated, protein A and CNBr-sepharose affinity chromatography-purified antibodies by coating the wells of a 96 well microtitre NUNC plate with 10μg/ml of the antigen in carbonate buffer, pH 9 6, incubated overnight at 4°C The plate was washed four times with 0 01M PBS, pH 7 3, and then blocked with 250μl of blocking solution [1% (w/v) BSA in 0 01M PBS, pH 9 6] When IBHR was used the blocking solution was 2% (v/v) gelatin (Section 2 3 16 2) in 0 01M PBS, pH 9 6 The plate was incubated at 37°C for two hours or overnight at 4°C The plate was washed four times with PBS-Tween and once with 0 01M PBS, pH 7 3 25μl of the antigen, at 10μl/ml, and 75μl of a serial dilution of the test antibody, in blocking solution, was added to the test wells and incubated at 37°C for two hours The secondary antibody and substrate were added, as detailed in Section 2 3 16 3 , and the absorbance was read at 620nm

2.4.16. Stability study of the purified antibodies.

A stability study of the protein A and CNBr-sepharose affinity-chromatography purified

antibodies (10μg/ml) diluted in 0 01M PBS, pH 7 3, was established at 4°C and 25°C An aliquot of antibody, stored at each temperature, was taken regularly and its stability determined by direct ELISA (Section 2 3 16 3) The stability was estimated as a percentage of a control antibody (aliquoted and stored at -20°C)

2.4.17. SDS-polyacrylamide gel electrophoresis (PAGE) of purified antibodies.

Monoclonal, polyclonal and bispecific antibodies and their fragments were subjected to sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) using modifications of the method of Laemmli (1970) Proteins were separated on a resolving gel containing 7 5% (w/v) acrylamide (neurotoxin), 0 33% (w/v) bis-acrylamide, 0 75M Tris, 0 2% (w/v) SDS, 0 1% (v/v) TEMED and 0 1% (w/v) ammonium persulphate, pH 8 8 A stacking gel containing 3 5% (w/v) acrylamide and 0 08% (w/v) fresh ammonium persulphate, pH 6 8, was used The electrode buffer contained 0 025M Tris, 0 192M glycine, 0 1% (w/v) SDS in distilled water, pH 6 8 Samples for electrophoresis were resuspended in a buffer containing 0 062M Tris, 2 0% (w/v) SDS, 10% (v/v) glycerol and 0 001% (w/v) bromophenol blue 0 5% (v/v) 2-mercaptoethanol was included only when reducing conditions were required The samples were prepared at a concentration of 0 1mg/ml in the loading buffer and boiled for 2 minutes before being applied to the gel (10-20μl volumes were used) Electrophoresis was carried out at a constant current of 40mA for about 30 minutes at 4°C using an LKB 2050 Midget Electrophoresis Unit Following electrophoresis, the gels were fixed by immersion in a methanol distilled water (1 2) solution containing 11 4% (w/v) trichloroacetic acid and 3 4% (w/v) sulphosalicylic acid for one hour They were then stained with 0 25% (w/v) Coomassie Brilliant blue R-250 in a 5 1 1 solution of methanol, acetic acid and distilled water for one hour Destaining was carried out overnight in a solution of ethanol, acetic acid and distilled water (1 3 6)

When premade Atto gradient gels were used, they were run at a constant current of 75mAmps until the marker dye reached the bottom of the gel (approximately 2hrs)

2.4.17.1. Preparation of electrophoresis reagents.

The acrylamide stock solution was a 30% (w/v) solution of acrylamide added to a 0 8% (w/v) solution of N,N-methylenebisacrylamide and then made up to 100ml with ultrapure H_2O

The resolving gel buffer was made up by dissolving 9 085g of Tris in ultrapure H_2O and, using conc HCl, was brought to a pH of 8 8 The volume was then made up to 100ml with ultrapure H_2O to give a final concentration of 0 75M The stacking gel buffer was made up by dissolving 3 0285g of Tris in conc HCl until a final pH of 6 8 was reached The volume was then made up to 100ml with ultrapure H_2O to give a final concentration of 0 25M

Ammonium persulphate had to be freshly prepared when needed This was done by making up 10ml of a 10% (w/v) solution of ammonium persulphate in ultrapure H_2O 1g of sodium dodecyl sulphate (SDS) was dissolved in 10ml of ultrapure H_2O to give a 10% (w/v) SDS solution

The sample buffer was prepared by dissolving 2g of SDS, 0 969g of Tris and 50mg of bromophenol blue in 10ml of glycerol, adding 5ml of β-mercaptoethanol and making up to 100ml with ultrapure H_2O

The destain solution was made up by mixing together acetic acid, methanol and water with a ratio of 1 4 5 This was done by adding 100ml of acetic acid, 400ml of methanol and 500ml of ultrapure water Care must be taken when handling these reagents

The staining solution consisted of 1g of Coomassie Brilliant Blue R-250 dissolved in 200ml of destain solution

The running buffer was prepared by dissolving 0 303g of Tris in 500ml ultra pure water and 0 144g of glycine in 500ml of ultrapure water The two solutions were mixed and the pH adjusted to 8 3 using conc HCl SDS was then added to a final concentration of 0 1% (w/v)

Table 2.12. Composition of resolving and stacking electrophoresis gels.

Gel Constituents	Resolving Gel	Stacking Gel
Acrylamide Stock	14 0ml	2 5ml
Tris-HCl	8 4ml (pH 8 8)	1 2ml (pH 6 8)
Ultra Pure Water	18 8ml	7 5ml
Ammonium Persulphate	200μl	80μl
SDS	240μl	100μl
TEMED	40μl	100μl

2 4.17.2. Electrophoresis of the purified polyclonal antibodies.

(a) Pouring the Gels.

There were two types of gels used in the separation of the bands, a resolving gel and a stacking gel The resolving gel is the one that actually separates the different bands from each other The stacking gel is used to allow the sample to be applied

The composition of these gels are outlined in Table 2 12 The TEMED (N,N,N'N'-tetramethylethylenediamine) acts as a catalyst for the polymerisation reaction and thus gels the solution It is for this reason that the TEMED was the last component to be added, just before the gels were poured The glass plates used to hold the gel were washed with H_2O and acetone was used to wipe them down prior to pouring to remove any residual contaminants A small amount of Vaseline was smeared over the edges of the plates to about 0 5cm inside them This was to allow a water tight seal to form between the plates and the spacer The spacer was also lightly smeared with vaseline for the same reason

The spacer was carefully placed between the two plates Care was taken not to allow any Vaseline to enter the gel area The apparatus was then screwed tightly in place The area on which the plates stand was also lightly smeared with Vaseline before they were placed on it The apparatus was again screwed tightly down until a seal formed In order to test the seals, the space between the plates was filled with water If no water leaked, the seals were deemed to be water tight

The resolving gel was poured first The TEMED was added to the rest of the constituents and the solution was carefully poured into the inter-plate space until it reached a level just before the end of the comb space A thin layer of water was added on top of this to aid in the polymerisation reaction and to form an even uniform line at the top of the gel The resolving gel was allowed to set at room temperature for approximately 30min before the stacking gel was poured

The stacking gel was poured in the same manner as the resolving gel The spacer comb was then inserted This allowed the formation of wells at the top of the gel Care must be taken to ensure no air bubbles were trapped The stacking gel was allowed to set for approximately 15min at room temperature

(b) Preparation and application of the samples.

200μl of sample buffer was added to 200μl of each of the samples to be run on the gel

They were then placed in boiling water for 1min to allow the proteins to denature After boiling, 40μl of each sample was carefully applied into each separate well in the gel using a Hamilton Syringe

(c) Running the gel.

The gel was removed from the stand and placed in the electrophoresis apparatus and screwed in This apparatus was an LKB Bromma 2050 Midget electrophoresis unit with a 2197 power supply The running buffer was carefully added on top of the gel, ensuring that the samples were not disturbed, until it had gone over the level of the conducting wire The system was run at 30mA per gel until the bromophenol blue in the sample buffer reached the end of the gel, which took approximately 2 hours PAGEL^{R} precast polyacrylamide gels for electrophoresis were also used These were gradient gels with gel concentrations from 5% (w/v) to 20% (w/v) and were supplied by ATTO Corporation (Section 2 1) These gels were run using the ATTO model AE-6100 mini gel apparatus using an ATTO Crosspower 3500 power supply at 20mA per gel

(d) Staining and destaining the gel.

After electrophoresis, the gel was carefully removed from the plates Wet latex gloves were used by the operator during this procedure Wetting the gloves prevents the gel from adhering to them Care must be taken not to disrupt, break or tear the gel The gel was then completely immersed in the stain solution for 15-20min The staining solution was completely poured off and destaining solution added as before The destain was changed when it had become very blue The gel could be left with tin foil covering it for 3-4 days until it was photographed

2.4.17.3. Molecular weight markers used in electrophoresis

The prestained molecular weight markers (Sigma) used in SDS electrophoresis are detailed in Table 2 13

Table 2.13. Molecular weight markers used in SDS electrophoresis. The apparent molecular weight is the weight of the molecule when it is conjugated to the dye

Molecular weight marker	Native molecular weight	Apparent molecular weight
β-galactosidase	116,000	125,000
Fructose-6-phosphokin-ase	84,000	86,000
Pyruvate kinase	58,000	65,000
Ovalbumin	45,000	48,000
Lactic dehydrogenase	36,500	38,000
Triose phosphate isomerase	26,600	33,500

2.4.18. Examination of the absorption spectrum of purified antibodies and FITC-labelling of IBHR-protein conjugates.

A Shimadzu UV-160A UV-Visible recording spectrophotometer was used for this procedure The absorbance spectrum was monitored over the range 200-800nm for purified antibodies and over the range 200-560nm for FITC labelled IBHR-protein conjugates The appropriate buffers were used as blanks, i e , citrate buffer for the Protein A affinity chromatography samples, glycine for the CNBr-sepharose affinity sample and 0 01M PBS, pH 7 3, for the BSA, KLH, ovalbumin proteins and their conjugates, and for IgG and IgM standards and samples

2.5. Techniques used in the production and characterisation of heteroconjugate antibodies.

2.5.1. Preparation of heteroconjugate antibodies.

The preparation of heteroconjugate antibodies (Section 5 1) involves the linking of two complete antibodies via a heterobifunctional cross-linker such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) This is a very quick method and the procedure used was that of Bush and Winkler, (1989)

Each of the antibodies used was dissolved in 0 01M PBS, pH 7 3, at a final concentration of 2 0mg/ml $9x10^{-5}$mM of SPDP, a thiolating agent, in ethanol was added for every 1mg of antibody The final ethanol concentration should be less than 10% (v/v) The addition of 80μl of a 2 25mM SPDP solution, with constant stirring, to 1ml of antibody solution (2mg/ml) satisfied this criterion After 30mins at room temperature, each of the antibody solutions was desalted on a Sephadex G-25 column (32cm x 3 2cm) to remove the excess reagent and products of low molecular The column was equilibrated with 0 1M sodium phosphate, 5mM EDTA, 0 15M NaCl, pH 6 0 In each case the fractions containing the antibody, as determined by BCA (Section 2 4 9 3), were pooled One of the parent antibodies was then reduced with 100μl of 1 5mM dithiothreitol (DTT, toxic) dissolved in the 0 1M sodium phosphate, 5mM EDTA, 0 15M NaCl, pH 6 0 for 15min at room temperature This reduced sample was then desalted using the Sephadex G-25 column (32cm x 3 2cm) equilabrated, as before, to remove excess reducing agent and any 2-thiopyridone formed The heterodimers were produced by mixing the reduced parent antibody (following DTT treatment) with the non-reduced SPDP-treated parent antibody and incubating them together at 37°C for 1 hour

Antibody 2mg/ml in 0 01M PBS, pH 7 3

↓

9×10^{-5}mM SPDP in ethanol was added per 1mg of antibody

↓

Left for 30mins at room temperature

↓

Each antibody solution was desalted in a Sephadex G-25 column (32cm x 3 2cm)

↓

One of the antibodies was reduced with 100μl of 1 5mM DTT

for 15mins at room temperature

↓

This antibody was desalted using a Sephadex G-25 column (32cm x 3 2cm), as before

↓

The antibody treated with DTT was mixed with the SPDP-treated parent antibody

and incubated at 37°C for 1 hour

2.5.2. Characterisation of the bispecific heteroconjugate antibodies produced.

ELISAs were performed to assess the activity of the parent antibodies and the hetero-conjugate as outlined in Section 2 3 16 3 The ELISAs chosen depended on the species of the antibodies (mouse or rabbit) and their target antigen These heteroconjugates were also examined by HPLC, FACS, SDS-PAGE and immunofluorescence The methods are given in Section 2 3 and the results are given in Section 5 1

2.6. Techniques utilised in the production and characterisation of catalytic antibodies.

In order to detect catalysis of the monoclonal antibodies produced during this project, it was first necessary to repeat the procedure outlined in Gallacher *et al* (1991) for the determination of the kinetics of the hydrolysis of the carbonate ester (Section 6) In order to determine the kinetics, the crude normal sheep serum and the serum from the immunised sheep 271 were isolated by sodium sulphate precipation and chromatography on Protein G-Sepharose (Gallacher *et al* , 1991)

2.6 1. Purification of IgG from sheep 271 and from non-immunised sheep.

This procedure was from Gallacher *et al* (1991) 180mg of anhydrous sodium sulphate ($NaSO_4$) was added to 1ml of serum, with vortex-mixing, (i) from sheep 271 and (ii) to provide a negative control from a sheep that had not been immunised, i e normal sheep serum In each case, the resulting suspension was gently mixed for 30min and was then centrifuged at 3000g for 10min at 25°C The supernatant was removed The precipitated IgG was then washed twice using 2ml of 18%, w/v, sodium sulphate The final pellet was re-dissolved in 2ml of 50mM sodium phosphate buffer, pH 7 5 The

resulting solution (2ml) was subjected to chromatography on a Protein G-Sepharose 4 Fast Flow column (5ml) (Section 2 6 3) and the absorbance (280nm) was monitored The adsorbed IgG was eluted with 0 1M glycine/HCl buffer, pH 2 7, and 2ml fractions of the eluate were collected in tubes containing 1M $NaHCO_3$ The fractions containing IgG were pooled and the buffer was exchanged with 50mM sodium phosphate buffer, pH 8 0, by chromatography on a Sephadex G-25 column (32cm x 3 2cm) (Section 2 6 2)

2.6.2. Preparation of Sephadex G-25 column

Sephadex G-25 medium was swollen in ultrapure H_2O overnight at 4°C, where 1g of dry weight powder gives 4-6ml of swollen gel The size of the column prepared was 32cm x 3 2cm column It was equilibrated with 50mM sodium phosphate buffer, pH 8 0

2.6.3. Purification of antibodies using Protein G.

Separating IgG from sodium sulphate purified sheep serum was carried out simply and effectively in a single step using Fast Flow protein G Sepharose 4 This is a valuable tool in the separation of monoclonal antibodies from ascitic fluid because of its binding characteristics Protein G from Pharmacia LKB is a recombinant Streptococcal F_c receptor from which the albumin-binding region of the native molecule has been deleted genetically Compared with Protein A affinity chromatography, Protein G binds more strongly to polyclonal IgG from, for example, cow, sheep or horse Protein G binds to the F_c region of IgG from many species without affecting their antigen-binding sites Fast Flow Protein G Sepharose 4 was supplied preswollen in 20% (v/v) ethanol To pack the gel, the ethanol solution was first removed by washing with ultrapure H_2O (pH 7) on a sintered glass filter While the gel was still on the filter, it was resuspended in starting buffer, 20mM phosphate buffer, pH 7, and transferred to the column The column was packed and equilibrated with two cv (column bed volumes) of starting buffer and the column was then ready for use

The samples were brought to pH 7 0 to be the same pH as the starting buffer

The IgG was eluted using 0 1M glycine-HCl buffer, pH 2 7, since the binding between the immobilised protein G and the IgG is strong In order to preserve the activity of the acid-labile IgG, a few drops of 1M Tris-HCl, pH 9 0, were added to those tubes which

would contain the fractions containing IgG, so that the final pH of the fraction would be approximately neutral.

After elution, the column was re-equilibrated with starting buffer at 4°C, if it was to be used again in a short period. For longer periods of storage, the gel was stored at 4°C in 20% (v/v) ethanol to prevent microbial contamination.

2.6.4. Kinetics of the hydrolysis of the carbonate ester.

The release of 4-nitrophenolate from the carbonate ester was monitored in 50mM sodium phosphate buffer, pH 8.0, containing 0.67% (v/v) acetonitrile at 25°C by recording the increase in the absorbance at 400nm (A_{400}) with the use of a Shimadzu UV-160A UV-Visible recording spectrophotometer and quantified by using $\epsilon_{400} = 1.65 \times 10^4 M^{-1} cm^{-1}$. Stock solutions of the substrate were prepared in acetonitrile. The concentration of the stock solutions was adjusted in order that 20μl of this solution in a final reaction volume of 3ml gave a final concentration in the range 0.2-16μM. In some cases reaction mixtures also contained 0.2μM IgG prepared from sheep 271 or 0.2μM IgG prepared from the non-immunised sheep, hybridoma serum or from mouse ascitic fluid, controls or sample. The concentration of IgG in the eluate from the Sephadex G-25 (32cm x 3.2cm) column was determined using $\epsilon_{280} = 2.0 \times 10^5 M^{-1} cm^{-1}$ and was usually 3μM.

Initial rates (υ) were determined from slopes of linear progression curves (Section 6.). Those for the reactions carried out in the absence of IgG, carboxylesterase, $DMEMS_0$, control ascitic fluid, whole sera or in the presence of IgG prepared from non-immunised sheep increased linearily with increase in substrate concentration [S] and were closely similar at a given [S]. The initial rates obtained in the presence of IgG from non-immunised sheep were subtracted from those obtained at the same values of [S] in the presence of the IgG prepared from sheep number 271 to provide the rates of the polyclonal catalytic antibody-catalysed reaction. Other parameters such as V_{max} and K_m can be calculated (Gallacher *et al.*, 1991). The catalytic activities of the monoclonal antibodies produced were also determined (Section 6.).

2.6.5. Immunisation schedule for the production of monoclonal catalytic antibodies.

Seven days prior to immunisation, blood was obtained from six balb/c mice by tail bleeding (Section 2.3.8.). This was used for negative controls. An amide conjugate

between the carboxy group of the 4-nitrophenyl 4'-carboxy-methylphenyl phosphate and an amino group of KLH was synthesised (Gallacher *et al.*, 1991). The phosphate immunogen (Phos-KLH) (XI) used was produced and supplied by Dr. Gerard Gallacher (Table 2.3). 6 male balb/c mice were immunised with Phos-KLH with the aim of producing catalytic monoclonal antibodies (Table 2.14.). The mice were tail bled (Section 2.3.8.) and their sera tested by ELISA on Phos-BSA and BSA-coated plates. The results obtained are shown in Table 6.1.

Table 2.14. Immunisation schedule of six balb/c mice immunised with the phosphate immunogen (Phos-KLH) (XI) (Gallacher *et al.*, 1991).

Day	Concentration of Phos-KLH (μg/ml) injected
1	100
19	50
32	100

On day 40, the mice were tail bled (Section 2.3.8.) and their sera were tested by ELISA on Phos-BSA and BSA coated plates. The results obtained are shown in Table 6.1.

In order to screen the sera obtained, the NUNC Immunosorb Maxisorb microtitre plates used were coated at a range of Phos-BSA and BSA concentrations (10, 15, 20 and 25μg/ml) to determine the optimal coating concentration. 10μg/ml gave the best result, with the least background and was the coating concentration used.

2.6.6. *In vitro* immunisation of isolated murine splenocytes with Phos-KLH.

In order to produce mouse anti-Phos monoclonal antibodies, an *in vitro* immunisation was performed (Section 2.3.11.5.) using mouse spleen cells which were *in vitro* immunised with Phos-KLH. The immunised spleen cells were fused with SP2/0 cells using the PEG method (Fusion 1) (Section 2.3.13.1.).

1 $1x10^8$ spleen cells were harvested and these were washed with red cell lysis buffer (Section 2 3 11 4) and resuspended in 8ml $DMEMS_0$ 2ml (i e 3 $1x10^7$ cells) of this solution was used in the immunisation as follows,

5ml $DMEMS_0$

5ml sTCM

100μl 2-Mercaptoethanol

50μl Gentamycin (5μl/ml)

20μl Phos-KLH (1μg/μl)

This solution was dispensed at $5x10^6$ cells per well of a 6 well sterile tissue culture plate and was incubated for 3 days in 5% CO_2 at 37°C The success of this *in vitro* immunisation was assessed using the ELISA-spot assay (Section 2 3 12 2) This assay showed that 131 hybridoma cells producing the desired antibody were obtained

The spleen cells were then resuspended, pooled and counted (Section 2 3 3) Using the PEG method (Section 2 3 13 1), ratios of spleen myeloma cells between 2 1 and 10 1 were successful The limiting factor was generally the number of myeloma cells The cells from each of these three fusions were plated onto 5 plates which were monitored for growth of clones after 10 days

The hybridomas were screened by ELISA against Phos-BSA, BSA, Phos-KLH and KLH (Section 2 3 16 3) The plates were washed with PBS-Tween 20 and PBS after each step The plates were blocked with 0 25% (w/v) porcine gelatin in 0 01M PBS, pH 7 3 (Section 2 4 14) 100μl of neat supernatant taken from a 24 well tissue culture plate and controls were added in duplicate to wells of the microtitre plate 100μl/well of a 1 4000 dilution in PBS of goat anti-mouse IgG Fab' specific alkaline phosphatase conjugated antibody (Table 2 4) was added and incubated for 1 hour at 37°C The Fab' specific goat anti-mouse secondary antibody was used in an effort to optimise the number of IgG-secreting hybridomas obtained A 1mg/ml solution of BCIP in amino-methyl-propanol buffer (Section 2 3 16 2) was made up and 100μl/well added to the microtitre plate The plate was incubated at 37°C and read at 620nm after 30mins The antibody-producing hybridomas were expanded according to Section 2 3 14 and the results obtained using these antibodies are outlined in Section 6

SECTION 3 PRODUCTION AND CHARACTERISATION OF ANTI-G-CCM AND ANTI-GFAP MONOCLONAL ANTIBODIES

3 Outline

The main purpose of the research described in this thesis was to develop a monoclonal antibody to a GFAP-like protein on a human anaplastic astrocytoma cell line, G-CCM (anti-G-CCM) and a monoclonal antibody to commercially available glial fibrillary acidic protein (GFAP), known as anti-GFAP These monoclonal antibodies were produced, purified and fully characterised by ELISA, HPLC, isotyping, SDS-PAGE, dot blot, western immunoblotting, BIAcore, immunocytochemistry, immunofluorescence and FACS

3 1 Introduction

Glial fibrillary acidic protein, a constituent protein of intermediate filaments of glial cells of the astrocyte lineage, occurs widely among vertebrates (Ontenıente *et al* , 1983) and invertebrates (Cardone and Roots, 1990) and is also generally present on primary glial brain tumours In general, intermediate filament proteins in the central nervous system (CNS) have been conserved through evolution Intermediate filaments are 8-10nm fibers that form part of the cytoskeleton of virtually all types of eucaryotes On the basis of their tissue restrictive distribution, intermediate filament proteins have been divided into five classes desmin, vimentin, GFAP, neurofilament proteins and keratins (Cardone and Roots, 1990) All intermediate filament proteins have the same structural organisation with a central α-helical rod domain flanked by amino and carboxy termini of variable sizes

Within one cell, chemical components of two intermediate filaments can exist, either as individual filaments, or as co-polymers in the same filament In astrocytes, filament subunits may vary in terms of ratios of component proteins and associated accessory proteins depending on the stage of astrocyte development In immature astrocytes, the dominant filament is vimentin, but with astrocyte maturation vimentin is replaced by GFAP

Brain tumours are classified into designated grades (I-IV), depending on the degree of malignant dedifferentiation present Designation of the higher grade malignancy is called anaplastic The term "anaplasia" describes cellular pleomorphism, increased cellularity, increased amount of mitotic activity, dedifferentiation, abnormal stromal reactions, especially vascular proliferation, and, finally necrosis formation with or without pseudo-palisading of nuclei or presence of metastases

The G-CCM and G-UVW cell lines used in this study are both anaplastic astrocytomas of adult human origin G-CCM had been shown to be entirely GFAP-positive in that it is expressed throughout the cell cycle during all subculturing stages (Frame *et al*, 1984) G-IJK$_{(1)}$ had only a minor component of GFAP-positive cells (Frame *et al*, 1984) In this latter study, it was shown that cell lines derived from histologically similar anaplastic astrocytomas showed considerable heterogeneity of phenotypic expression This reflects the heterogenous nature of cells in malignant astrocytomas with the inevitable selection of cells most suited to growth in culture The cell lines used in the study of Frame *et al* (1984) exhibited a range of morphologies and states of biochemical differentiation from highly differentiated, in the case of G-CCM to undifferentiated, in the case of G-ATA The astrocyte marker protein, GFAP, was present in cultures derived from 12-16 weeks post-conception foetal brain and in the human glioma, G-CCM and the rat glioma, C_6 They demonstrated that highly differentiated astroglial cells are able to grow and divide in culture They showed that GFAP was consistently visualised in only two of the glioma lines G-CCM was entirely GFAP-positive while C_6 was 50-60% positive under standard culture conditions in sub-confluent cultures Of the remaining glioma lines studied, G-IJK$_{(1)}$ had only a minor component of GFAP-positive cells Cell lines derived from normal adult brain in this study were entirely GFAP negative With G-CCM, there was no visually detected increase in the number of GFAP-positive cells with increasing cell density

The chemistry, neurobiology, molecular biology and immunology of GFAP have been reviewed by Eng and Shiurba, (1988) Extensive use of GFAP antibodies in neuro-biology has established GFAP as a prototype brain antigen in CNS immunochemistry and as a standard marker for neuroscience research (Eng and Shiurba, 1988)

3 2 Rational for producing monoclonal antibodies against GFAP and 'GFAP-like' antigens

The production of monoclonal antibodies against antigenic structures makes it possible to tackle problems of direct clinical relevance in a well-defined syngenic system (Prosser *et al*, 1991) In particular, it makes it feasible to investigate (a) the binding capability of conjugated monoclonal antibodies to glioma cells *in vivo*, (b) the fate of labelled monoclonal antibodies *in vivo* as they overcome the blood-brain barrier, and (c) the applicability of drug-antibody complexes aimed at destroying glioma cells *in*

vivo without seriously damaging the host (Stavrou *et al* , 1983)

Monoclonal anti-GFAP antibodies have proven to be a valuable tool for use in immunocytochemical localisation of GFAP in normal CNS tissue and certain tumours and metastases of glial antigen as well as for immunofluorescent labelling of cultured mammalian cells Cell culture has become an established technique in oncology Although cell lines are reasonably stable, it is accepted that cell cultures change during long-term culture and this is especially true for gliomas Westphal *et al* (1990) stated that GFAP expression did not disadvantage the successful culturing of cells but that GFAP expression was an indicator that long-term survival of a cell line would be poor in most cases However, they had one cell line which started with 1% of GFAP positive cells and later developed into a 99% positive cell line

The research in this thesis is based in part on work done by Elizabeth Moran In her Ph D thesis, monoclonal antibodies against G-CCM cells were produced and characterised One hybridoma, BI10H appeared to be particularly interesting (Moran, 1989) as it reacted with a 'GFAP-like' antigen which appeared to be present on the surface of glioma cells It was decided to attempt to produce additional anti-G-CCM and anti-GFAP monoclonal antibodies to further study this phenomenon The following sections report on the production, purification and characterisation of monoclonal antibodies against G-CCM cells and against GFAP

3 3. Immunisation of mice with G-CCM cells

The immunisation protocol is detailed in Section 2 3 6 3 Care was taken when preparing these cells for immunisation to use low concentrations of trypsin (Section 2 3 2), and for a minimum time to reduce damage to surface antigens Sera obtained from five mice immunised with G-CCM were tested by ELISA on G-CCM- and GFAP-coated plates The results obtained are shown in Table 3 1

Table 3 1 Testing of sera (1 100 dilution in 0 01M PBS, pH 7 3) from five mice immunised with G-CCM cells by ELISA against G-CCM- and GFAP-coated plates The absorbance was read at 620nm (A_{620}) The results for the positive control (commercial anti-GFAP, 1 400 dilution in 0.01M PBS, pH 7 3) and the negative controls (0 01M PBS, pH 7 3), normal mouse serum (1 100 dilution in 0 01M PBS, pH 7 3) and NSO supernatant are also given The data represents the mean values of triplicate determinations (n=3) and the SD are in brackets

G-CCM immunised mouse number	G-CCM-coated plates	GFAP-coated plates
1	0 395 (0 005)	0 294 (0 004)
2	0 401 (0 008)	0 551 (0 001)
3	0 656 (0 001)	0 561 (0 002)
4	0 469 (0 010)	0 534 (0 009)
5	0 517 (0 000)	0 510 (0 004)
Commercial anti-GFAP (1 400 dilution)	0 062 (0 01)	0 153 (0 014)
PBS (0 01M, pH 7 3)	0 032 (0 032)	0 030 (0 022)
Normal mouse serum (1 100 dilution)	0 034 (0 012)	0 029 (0 018)
NSO supernatant	0 029 (0 021)	0 033 (0 028)

3 4 Production of an anti-G-CCM monoclonal antibody

Mouse 3 was sacrificed, and a splenectomy and an electrofusion were performed (Section 2 3 7) The spleens from mice 4 and 5 were fused to NS0 myeloma cells using PEG (Section 2 3 13 1) A scheme of the results of this procedure is outlined below,

3 FUSIONS (PEG or electrofusion)

↓

15 plates made, 129 wells contained clones

↓

115 wells were expanded into 24 well plates, all screened by ELISA using anti-mouse IgG as antigen

↓

48 clones gave positive ELISA values against anti-mouse IgG (> 0 150), these were expanded and screened against G-CCM

↓

21 clones were found to react positivly with G-CCM, these were expanded to 24-well plates, and screened against GFAP

↓

1 clone became negative, 4 died out and the 9 hybridomas which reacted most strongly were 1BD5, 1BD11, 2AG5, 2BC11, 2BF11, 2BF12, 2DG7, 2ED10 and 3BH2

↓

These were screened again against G-CCM and GFAP by ELISA and following this screening, five anti-G-CCM clones were considered for further characterisation, 1BD11, 2AG5, 2DG7, 2ED10 and 3BH2 These were screened for mycoplasma contamination (Section 2 3 5) and were negative

↓

These five hybridomas were subcloned twice by limiting dilution (Section 2 3 15), screened for anti-G-CCM and anti-GFAP activity by ELISA (Section 2 3 18) and isotyped (Section 2 3 18) 3BH2 was selected for further study as it reacts strongly with G-CCM and GFAP and also binds to the astrocytoma cell line G-UVW The remaining hybridomas were stored in liquid nitrogen but were not further characterised

↓

3BH2 was grown up as ascites (Section 2 3 17) in balb/c mice The ascitic fluid was purified and characterised and this monoclonal antibody will be referred to as the anti-G-CCM monoclonal antibody

3.5. Immunisation of mice with GFAP.

Three mice were immunised with comercially available GFAP (Table 2.3.) in 0.5ml 0.01M PBS, pH 7.3. (Section 2.3.7.1.). Following four immunisations, the mice were tail bled and their sera were tested by ELISA on GFAP and G-CCM-coated plates. The results obtained are shown in Table 3.2.

Table 3.2. Testing of sera (1:100 dilution in 0.01M PBS, pH 7.3) from three mice immunised with GFAP by ELISA against GFAP- and G-CCM-coated plates. The absorbance was read at 620nm (A_{620}). The results for the positive control (commercial anti-GFAP, 1:400 dilution in 0.01M PBS, pH 7.3) and the negative controls (0.01M PBS, pH 7.3, normal mouse serum [1:100 dilution in 0.01M PBS, pH 7.3], NSO supernatant and $DMEMS_{10}$ medium, are also given. The data represents the mean values of triplicate determinations (n=3) and the standard deviations are in brackets.

GFAP immunised Mouse number	G-CCM-coated plates	GFAP-coated plates
20	0.075 (0.006)	0.312 (0.001)
34	0.062 (0.003)	0.275 (0.006)
37	0.050 (0.001)	0.304 (0.004)
Commercial anti-GFAP (1:400 dilution)	0.034 (0.012)	0.107 (0.007)
PBS (0.01M, pH 7.3)	0.003 (0.001)	0.008 (0.016)
Normal mouse serum (1:100 dilution)	0.030 (0.008)	0.037 (0.010)
NSO supernatant	0.024 (0.012)	0.035 (0.020)
$DMEMS_{10}$ medium (1:400 dilution)	0.029 (0.009)	0.035 (0.018)

On day 58, one mouse was given an i v injection of filter-sterilised GFAP in 0 01M PBS, pH7 3, which was maintained at 37°C (Section 2 3 7 1) The mouse was bled and sacrificed on day 61 The serum obtained was used as a positive control for ELISAs and throughout the antibody purification stages of the experiment An *in vitro* immunisation (Section 2 3 11) was performed and the details of this experiment are given in Section 2 3 7 1

The remaining two mice were also *in vitro* immunised After 3 days, these spleen cells were electrofused (Section 2 3 13 2) with SP2/0 cells, as described in Section 2 3 7 1 The resulting hybridomas were maintained as outlined in Section 2 14 and the fused cells were selected (Section 2 3 14) The positive clones were selected by ELISA (Section 2 3 16) against an anti-mouse IgG antibody, G-CCM and finally GFAP in order to obtain an antibody which would react with a GFAP-like antigen on G-CCM cells

3 6 Production of the anti-GFAP monoclonal antibody

The results of the *in vitro* immunisations, fusion, screening and cloning of the mice immunised with commercially available GFAP are summarised, as follows,

IN VITRO IMMUNISATION

↓

3 FUSIONS (PEG or electrofusion)

↓

15 plates were made, 110 wells contained clones

↓

96 clones were expanded into 24 well plates, each of these clones were screened by ELISA using anti-mouse IgG as antigen

↓

40 clones gave positive ELISA values (> 0 150), these were expanded and screened against GFAP

↓

10 clones were found to react positively with against GFAP, these were expanded to 24-well plates

↓

4 clones became negative, 2 died out and 4 continued to produce The four hybridomas

which reacted most strongly with GFAP were 1BC6, 2BC7, 2DH5 and 3EG7 These were subcloned twice by limiting dilution (Section 2 3 15), were screened for anti-GFAP antibody production by ELISA (Section 2 3 16) and isotyped (Section 2 3 18) These hybridomas were screened for mycoplasma contamination (Section 2 3 5) and were negative

↓

The monoclonal antibody 2DH5 was chosen for further study as it reacted with the greatest specificity to GFAP, was the most stable to freeze-thawing and was the best producer of antibody It was grown as ascites in balb/c mice (Section 2 3 16) 1BC6, 2BC7 and 3EG7 were stored in liquid nitrogen and were not further characterised The 2DH5 monoclonal antibody was chosen for further study and will be referred to as the anti-GFAP monoclonal antibody

Initially the hybridomas from the GFAP immunised mice were screened with GFAP from lot 19 and very few positives were obtained It was then decided to screen these hybridomas using GFAP from lot 16 which was used as the immunogen 10 times as many positives were subsequently found and a lot variation in GFAP was noted which was confirmed by dot blot (Figure 3 4 1) The supplier (ICN Immunobiologicals, Table 2 3) confirmed a change of source of GFAP between lots 16 and 17

3 7 Screening of the 3BH2 and 2DH5 monoclonal antibodies against G-CCM, G-UVW and GFAP by ELISA

The 3BH2 and 2DH5 monoclonal antibodies were grown as ascites in balb/c mice Titres were determined by serial dilution of the ascitic fluid (1 10 - 1 10^6) These dilutions were screened by ELISA (Section 2 3 16) and a suitable working dilution was obtained for each antibody (Table 3 3) After titres and working dilutions had been determined against the immunogens, the monoclonal antibodies were screened against G-CCM, G-UVW and GFAP (Table 3 4) Following this initial characterisation, these two antibodies were screened by ELISA against other cell lines (Table 3 5) (Section 2 3 16 1)

It should be noted that the cell lines G-CCM and G-UVW and the other adherent cell lines used (Section 2 3 2) had been treated with trypsin to detach them from the culture flasks and they still retained antigenic reactivity Absorbance readings less than 0 1 were

considered negative

Commercially available anti-GFAP antibody was tested by ELISA against GFAP and G-CCM, in order to compare the results with those obtained when 2DH5 was similarly screened The results obtained are shown in Table 3 4 The commercially available anti-GFAP antibody acted as a positive control throughout the characterisation of 2DH5, the 'anti-GFAP' antibody

The cell line $IJK_{(t)}$ was used in each of these assays However, it was subsequently established that this cell line was contaminated with a rat glioma cell line C_6 (PHLS, *personal communication*) For this reason, the results obtained using this cell line are not presented

The titre of the 3BH2 crude ascitic fluid against G-CCM was determined to be 1 50,000 from Table 3 3 and the working dilution was 1 10,000 The working dilution of the SAS-treated 3BH2 ascitic fluid was 1 5,000 The titre of the 2DH5 crude ascitic fluid against GFAP was determined to be 1 50,000 (Table 3 4) and the working dilution was 1 10,000 The working dilution of the SAS-treated 2DH5 ascitic fluid was 1 7,500

3 8 Screening the anti-G-CCM antibody by ELISA against other cell lines.

The following cell lines G-CCM, G-UVW, MCF-7, HL-60, LOVO, K562, T-24, EJ-138, A549, H5683, HL-60 and B21 (Table 2 2) were grown in culture and used to coat 96-well plates for use in ELISAs (Section 2 3 16 1) These cell lines were also used in Western immunoblotting (Section 2 3 22 1), immunocytochemistry (Section 2 3 20 3) and FACS (Section 2 3 20 8) The results of the ELISAs using these cell lines are given in Table 3 5 These antibodies showed no cross-reactivity by ELISA with any other tumour cell line available in the laboratory The anti-G-CCM antibody binds strongly to the astrocytoma cell lines, G-CCM and G-UVW, as would be expected The anti-GFAP antibody binds to a lesser extent to these two astrocytoma cell lines

Table 3 3 Investigation of the binding activity of the 3BH2 monoclonal antibody. Crude, SAS-precipitated and protein G affinity chromatography-treated ascitic fluid were screened by ELISA against G-CCM, G-UVW and pure, lyophilised GFAP, respectively The results for each antibody fraction were determined by serial dilution of the ascitic fluid (1 10 - 1 500,000) in 0 01M PBS, pH 7 3 A suitable working dilution and titre was obtained for each antibody. The absorbance was read at 620nm (A_{620nm}) and n=1

Test Fraction	*Crude ascitic fluid*	*SAS-treated*	*Protein G-treated*	*SAS-treated*	*SAS-treated*
Antibody dilution	**G-CCM coated**			**G-UVW coated**	**GFAP coated**
1 10	0 583	0 465	0 139	0 364	0 554
1 50	0 522	0 417	0 149	0 336	0 349
1 100	0 471	0 389	0 136	0 284	0 306
1 500	0 364	0 312	0 083	0 259	0 228
1 1000	0 284	0 317	0 022	0 249	0 201
1 5000	0 177	0 238	0 000	0 151	0 116
1 10000	0 149	0 162	0 000	0 108	0 110
1 50000	0 115	0 077	0 000	0 000	0 085
1 100000	0 104	0 013	0 000	0 000	0 000
1 500000	0 096	0 000	0 000	0 000	0 000

Negative controls for this assay (n=3) gave readings of < 0 05 and included 0 01M PBS, pH 7 3 and $DMEMS_{10}$, normal mouse serum (1 100 dilution) and SP2/0 supernatant The positive control was commercially available anti-GFAP (1 400) against GFAP and was 0 126 (0 004), n=3

Table 3 4 Investigation of the binding activity of the 2DH5 monoclonal antibody Crude, SAS-treated and protein G affinity chromatography-treated ascitic fluid were screened against G-CCM, G-UVW and pure, lyophilised GFAP, respectively The results for each antibody were determined by serial dilution of the ascitic fluid (1 10 - 1 100,000) in 0 01M PBS, pH 7 3 A suitable working dilution and titre was obtained for each antibody The absorbance was read at 620nm (A_{620nm}) and n=1

Test Fraction	*Crude ascitic fluid*	*SAS-treated*	*Protein G-treated*	*SAS-treated*	*SAS-treated*
Antibody dilution	**GFAP coated**			**G-UVW coated**	**G-CCM coated**
1 10	0 654	0 333	0 347	0 243	0 248
1 50	0 533	0 328	0 366	0 262	0 255
1 100	0 440	0 278	0 287	0 240	0 192
1 500	0 341	0 242	0 224	0 179	0 165
1 1000	0 242	0 197	0 149	0 172	0 142
1 5000	0 250	0 205	0 157	0 149	0 111
1 10000	0 197	0 140	0 124	0 053	0 035
1 50000	0 127	0 108	0 074	0 000	0 000
1 100000	0 060	0 011	0 014	0 000	0 000

Negative controls were as described for Table 3 3

Table 3.5. Examination of the binding activity of the 3BH2 and 2DH5 monoclonal antibodies to the following cell lines, G-CCM, G-UVW, MCF-7, HL-60, LOVO, K562, T-24, EJ-138, A549 and H5683. A dilution of 1:100 for each antibody in 0.01M PBS, pH 7.3, was used in each case. The absorbance was read at 620nm (A_{620nm}) and n=1.

Cell line	3BH2	2DH5
G-CCM	0 471	0 144
G-UVW	0 284	0 140
MCF-7	0 021	0 024
HL-60	0 028	0 059
LOVO	0 011	0 033
K562	0 089	0 094
T-24	0 021	0 034
EJ-138	0 005	0 021
A549	0 034	0 050
H5683	0 018	0 029

Negative controls were as described for Table 3 3

3.9. Isotyping of the monoclonal antibodies obtained.

The monoclonal antibodies obtained were isotyped according to Section 2 3 18 Supernatant and ascitic fluid were used in the determination of class, subclass, where appropriate, and light chain determination The determination of the subclass of the light chain was done using the indirect ELISA (Section 2 13 18 2) and was found to be kappa for all the antibodies isotyped The results obtained using supernatant from the anti-G-CCM and anti-GFAP antibodies with isotyping strips from Sigma (Section 2 3 18 3) are shown in Figure 3 1 Using this method, 3BH2 isotyped as IgG1 and IgM 2DH5 isotyped as IgM The Serotec isotyping kit (Section 2 3 18 4) gave an

isotype of IgG1 and IgM for anti-G-CCM and IgG1 and IgM for anti-GFAP monoclonal antibodies Each of the nearly 100 antibody-producing positives obtained for the 6 fusions performed during this project was isotyped by various methods (Section 2 3 18) All isotyped as IgM This was an extraordinary result, considering the mice had been hyperimmumsed (Section 2 3 6 3 and Section 2 3 7 1 , respectively) and considering an anti-IgG assay (Section 2 3 18 2) had been used for screening However, other researchers in D C U involved in monoclonal antibody production were obtaining similar results at the time The probability of infection of the balb/c colony with mouse hepatitis virus (MHV) was suspected and confirmed This is discussed in Section 3 19

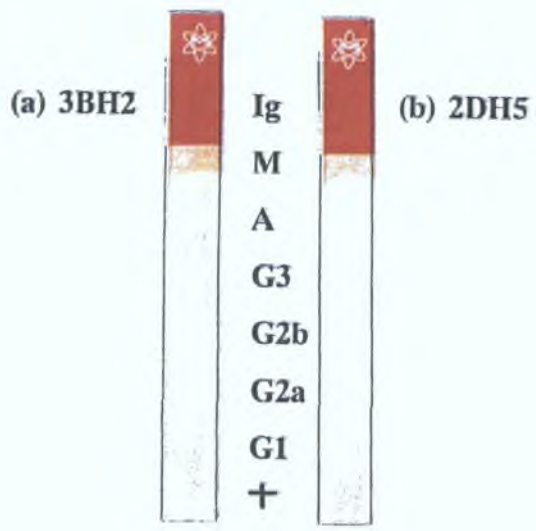


Figure 3.1.

Results obtained using isotyping strips from Sigma of neat supernatant from (a) 3BH2 clone A3 and (b) 2DH5 clone A10. 3BH2A3 isotyped as IgG1 and IgM. 2DH5 isotyped as IgM.

3 10 Purification of ascitic fluid from the anti-G-CCM and anti-GFAP monoclonal antibodies

The 3BH2 and 2DH5 antibodies were purified by a series of methods, each with a view to increasing the purity and specificity of the resulting immunoglobulins The protein concentration was determined following each stage of the purification procedure

The ascitic fluid was treated with saturated ammonium sulphate (SAS) (Knutson *et al* , 1991) (Section 2 3 19) SAS precipitation was used to isolate all immunoglobulins, but this sample may contain some contaminating proteins from the ascitic fluid Protein G Sepharose Fast Flow (Section 2 6 3) affinity chromatography was used to purify the sample further With this method of purification only antibodies are eluted from the column The remaining contaminating proteins are washed through the column The results were compared by ELISA (Tables 3 3 and 3 4)

Initially it was thought that many problems would be encountered in purifying ascitic fluid of the IgM isotype and purification using a Bio-Sil SEC 400 column from Bio-Rad (Shen *et al* , 1989) was considered However, on examining even crude ascitic fluid on a Protein Pak HPLC column it was seen that the antibody had a retention time of 16 21min with very little contaminating proteins (Figure 3 2 1) Possible alternative methods of IgM antibody purification are discussed in Section 3 19

The concentration of the ascitic fluid and the purified antibody fractions were found using the Bradford assay (Section 2 4 9 3) with mouse IgG or IgM as the protein standard The results are given in Table 3 6

A study of the crude and SAS-treated ascitic fluid from 3BH2 and 2DH5 showed that at -20°C, the antibody activity of both antibodies decreased over a period of 6 months, but the purified, SAS-treated sample was less stable The activity of the crude ascitic fluid, as determined by ELISA, decreased by 22% and the SAS-treated sample decreased by 60%, in the same period

Table 3 6 Concentrations of crude and purified 3BH2 and 2DH5 ascitic fluid as determined by the Bradford assay The absorbance was read at 595nm

Antibody fraction	Concentration (mg/ml)	
	Anti-G-CCM (3BH2)	Anti-GFAP (2DH5)
Crude ascitic fluid	31 5	20 0
SAS-treated	6 0	7 9
Protein-G treated	2 2	1 7

3 11 HPLC analysis of crude and purified ascitic fluid

HPLC chromatograms were preformed using a 10μm Protein Pak SW 300 column with a mobile phase of 0 1M phosphate buffer, pH 7 0, and a flow rate of 0 5ml/min Absorbance was detected at 280nm

The anti-G-CCM and anti-GFAP monoclonal antibodies, 3BH2 and 2DH5, were grown as ascitic fluid in mice (Section 2 3 17) and were analysed on HPLC, along with commercial mouse IgG and IgM as standards for comparison In this system, mouse IgG standard gave a peak at 16 21min (Figure 3 2 1) Mouse IgM standard gave a major peak at 10 01min, which represents protein eluting outside the resolution range of the column, and a minor peak at 18 97min, which may depict IgM subunits (Figure 3 2 2)

When fresh, crude 3BH2A3 ascitic fluid was examined, a minor peak at 10 16min was seen This corresponds to protein eluting in the void volume of the column and may be IgM However, a major peak at 16 36 was also observed, which corresponds to the retention time of 16 21min of mouse IgG in this system The protein G affinity chromatography-purified sample gave one peak at 16 55min The presence of only one peak in this sample indicated a high level of purity The results are given in Figures 3 3 Fresh, crude 2DH5 ascitic fluid gave peaks at 9 73min and 16 69min The first peak was protein eluting in the void volume and the second peak corresponds to the elution time of IgG The SAS-treated sample gave a major peak at 16 86 and a minor peak at 9 85 The protein G affinity chromatography-purified sample gave one peak at 16 59min These results are presented in Figures 3 3 (1 -5) and discussed in Section 3 19

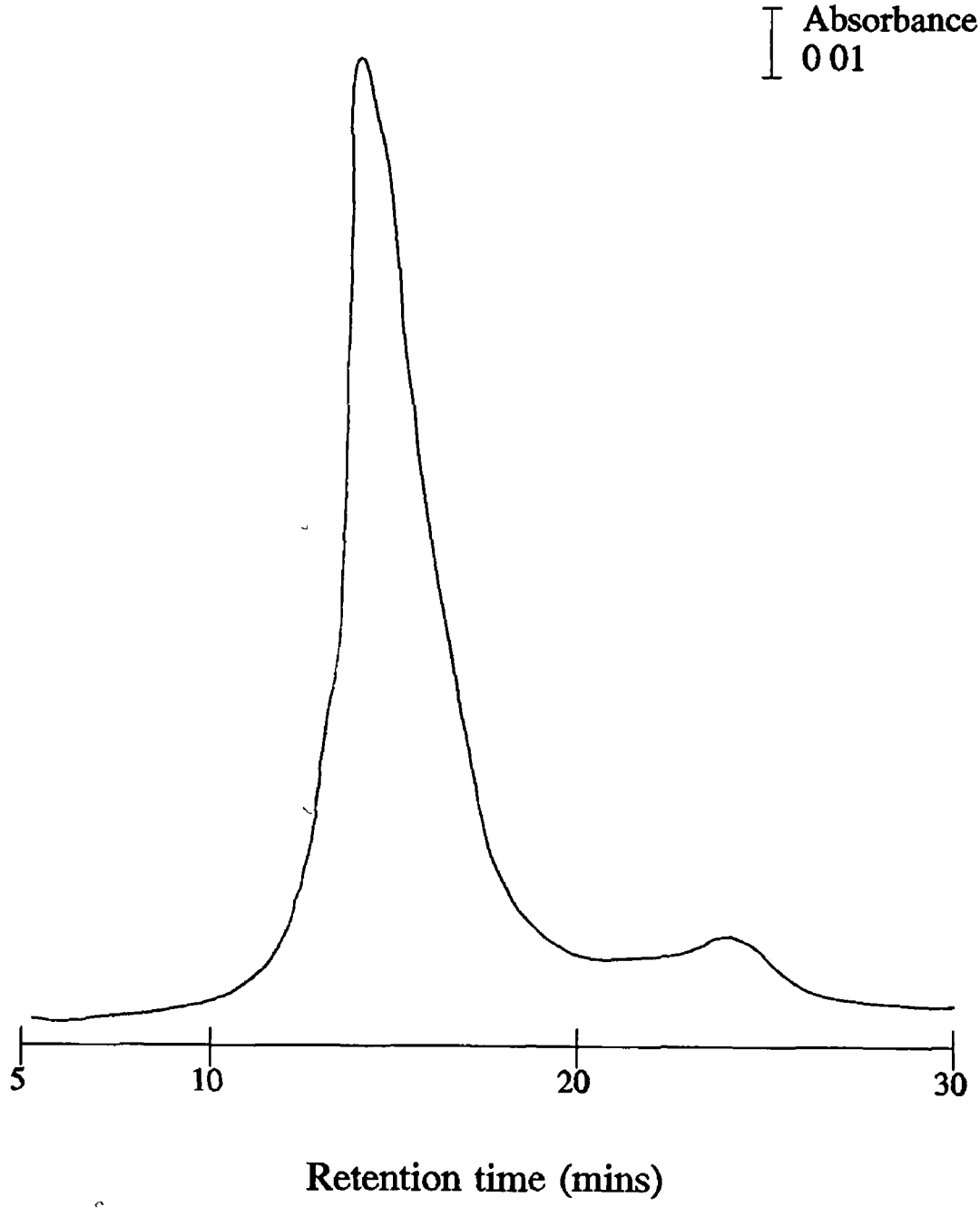


Figure 3.2.1.

HPLC chromatogram of commercial mouse IgG (2mg/ml) The column used was a 10μm Protein Pak SW 300 column with a mobile phase of 0 1M phosphate buffer, pH 7 0, and a flow rate of 0 5ml/min Absorbance was detected at 280nm One major peak was detected with a retention time of 16 21mins This is representative of IgG (150kD)

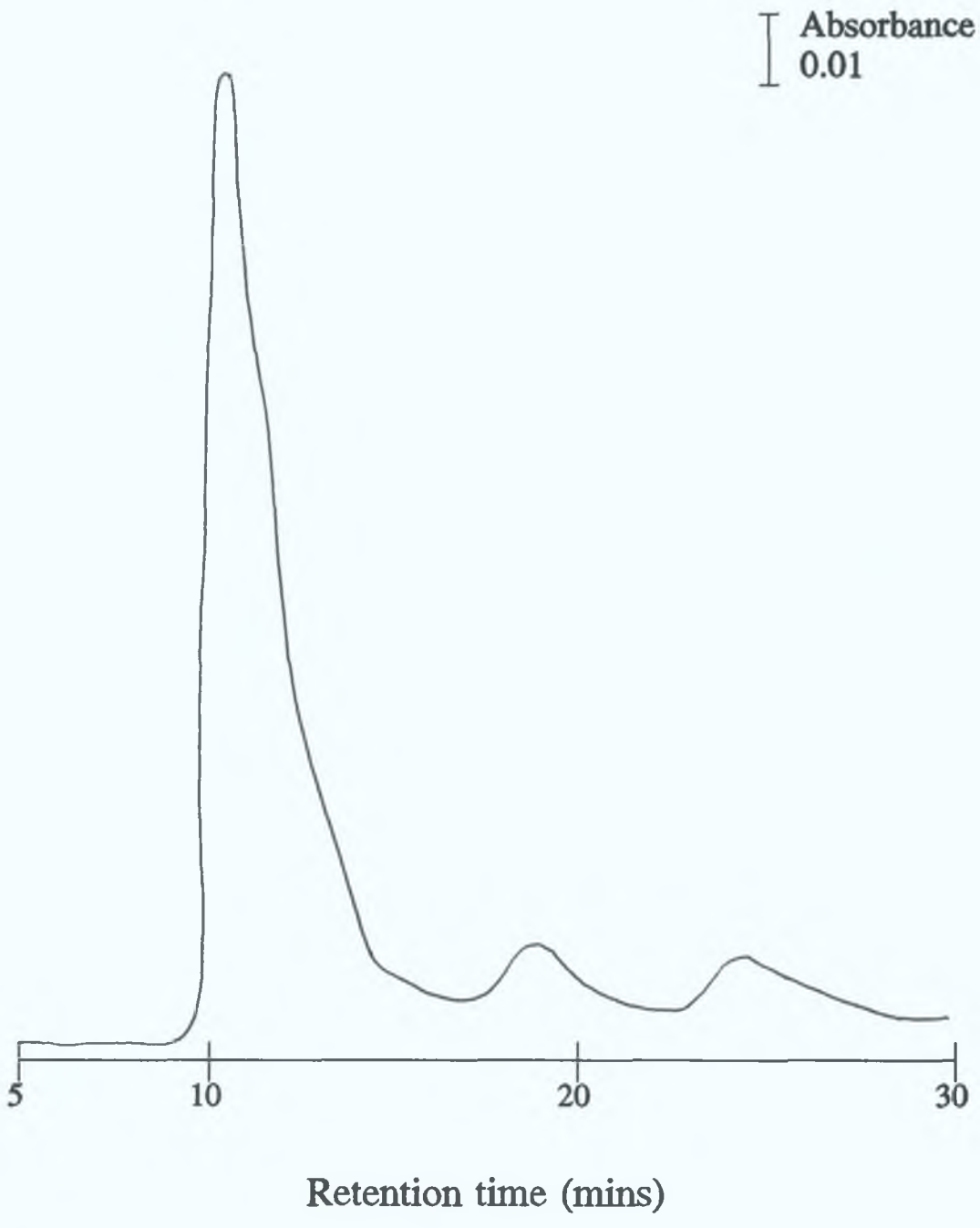


Figure 3.2.2.

HPLC chromatogram of commercial mouse IgM (2mg/ml). The column used was a 10μm Protein Pak SW 300 column with a mobile phase of 0.1M phosphate buffer, pH 7.0, and a flow rate of 0.5ml/min. Absorbance was detected at 280nm. One major peak was detected with a retention time of 10.01mins. This is representative of IgM (approximately 900kD) which eluted outside the resolution range of the column. A minor peak was detected at 18.97min which may represent IgM subunits.

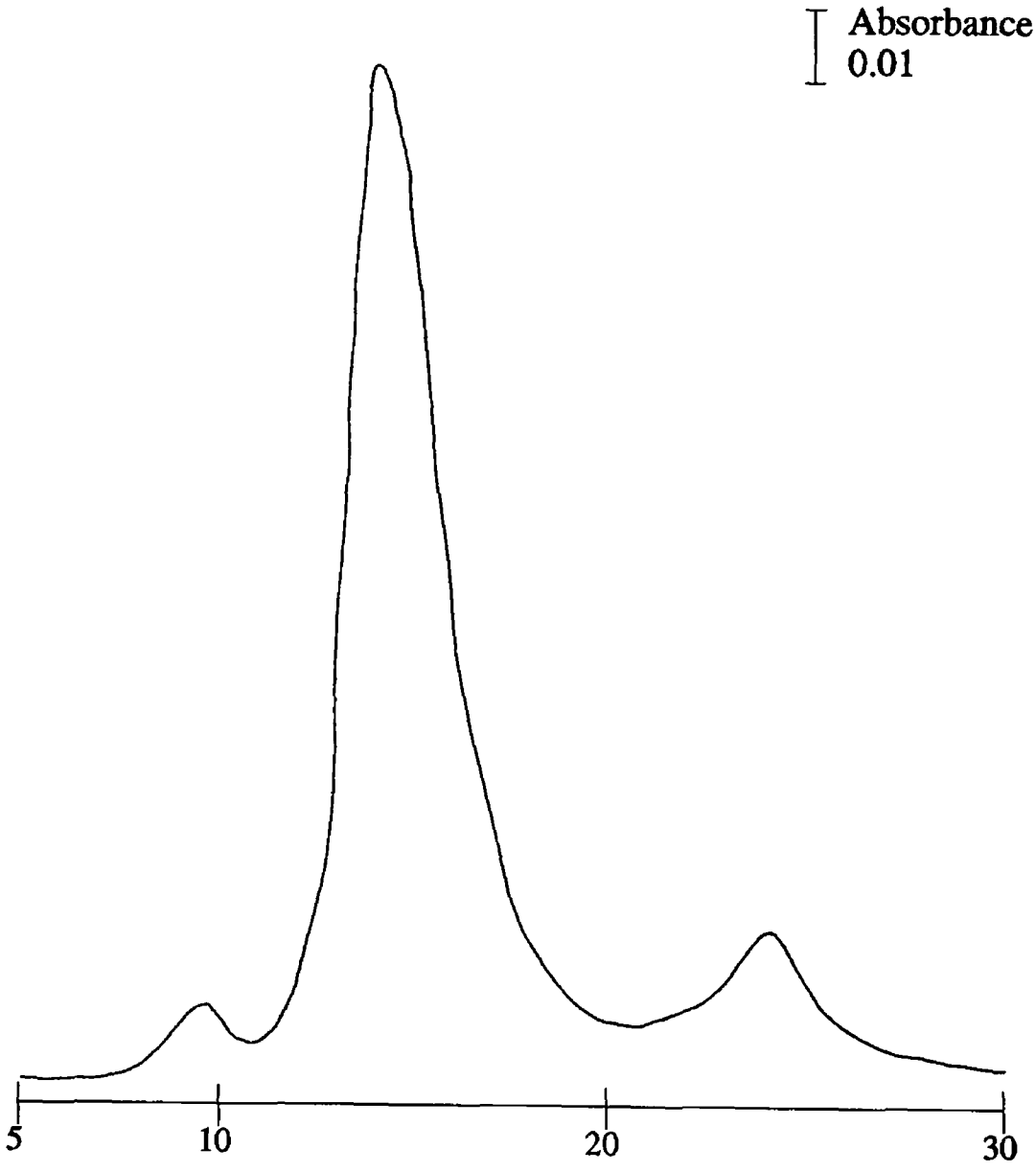


Figure 3.3 1.

HPLC chromatogram of fresh, crude 3BH2A3 ascitic fluid (2mg/ml) The column used was a 10μm Protein Pak SW 300 column with a mobile phase of 0 1M phosphate buffer, pH 7 0, and a flow rate of 0 5ml/min Absorbance was detected at 280nm One major peak was detected with a retention time of 16 36mins This is representative of IgG (180kD) The minor peak at 10 16min was outside the resolution range of the column and may have been IgM antibody The minor peak at 24 72min possibly identifies denatured protein

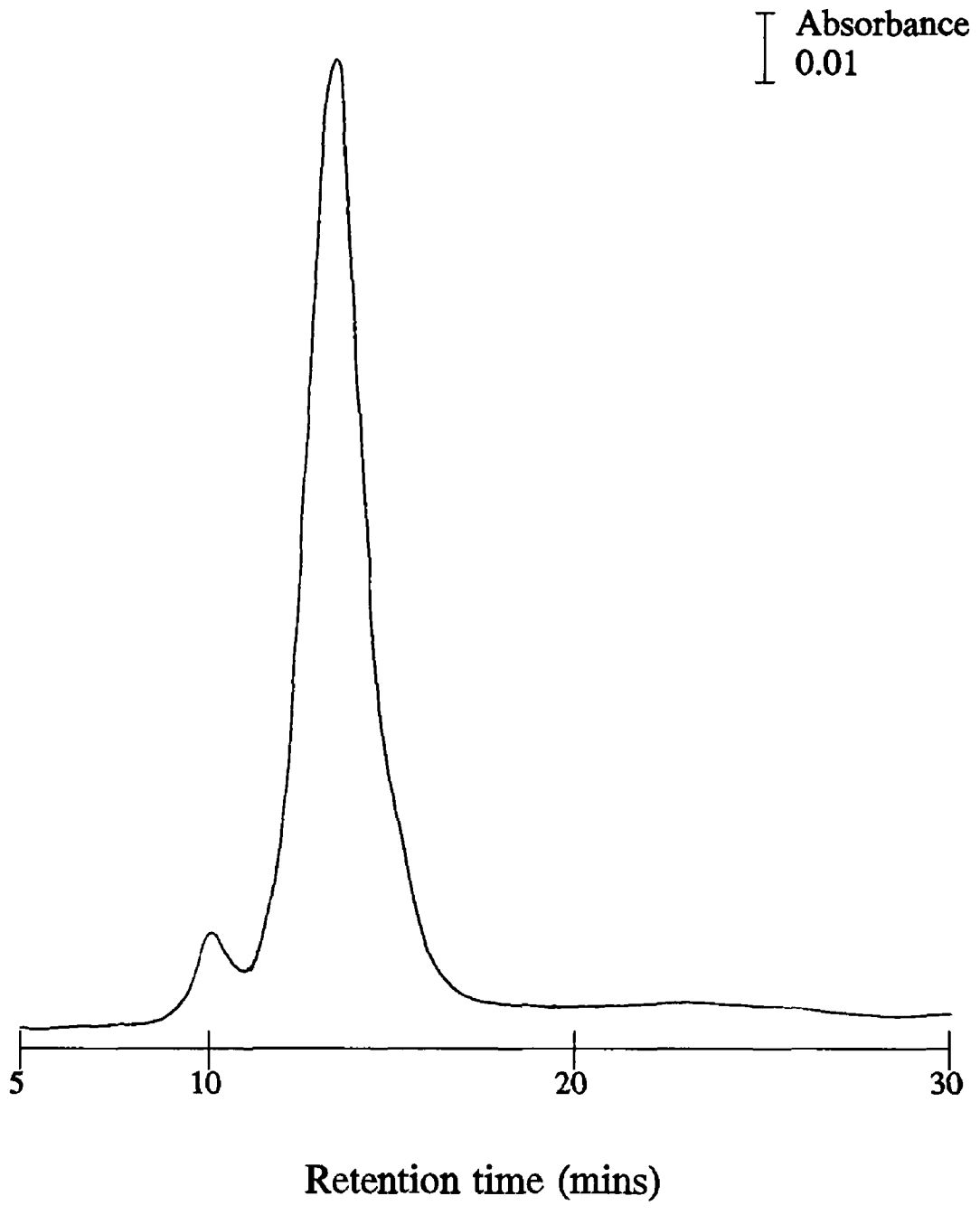


Figure 3.3.2.

HPLC chromatogram of SAS-treated 3BH2A3 ascitic fluid (2mg/ml) The column used was a 10μm Protein Pak SW 300 column with a mobile phase of 0 1M phosphate buffer, pH 7 0, and a flow rate of 0 5ml/min Absorbance was detected at 280nm One major peak was detected with a retention time of 16 42mins This is representative of IgG (180kD) The protein in the minor peak at 10 01min eluted outside the resolution range of the column

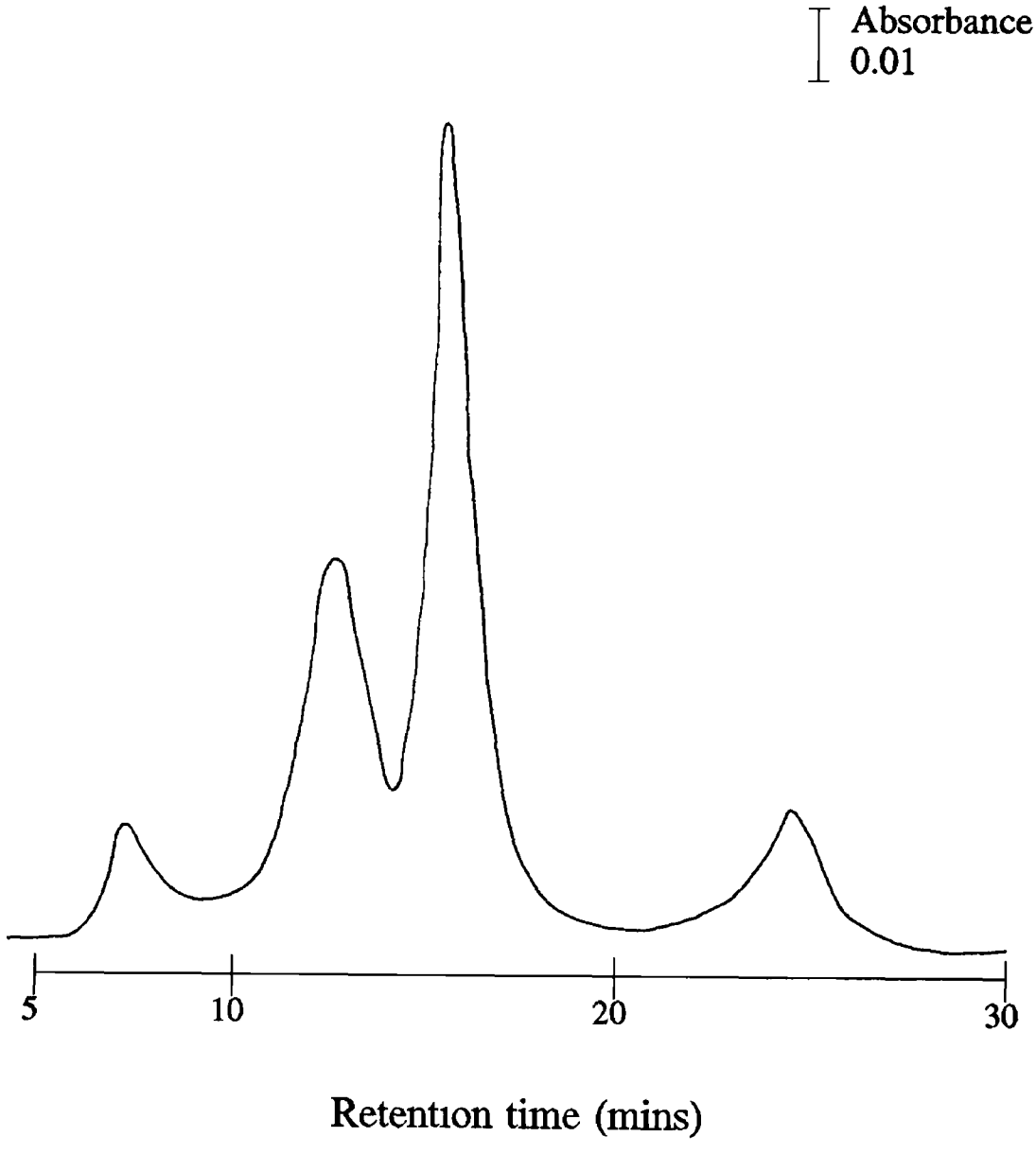


Figure 3.3.3.

HPLC chromatogram of SAS-treated 3BH2A3 ascitic fluid (2mg/ml) incubated with GFAP (20μg/ml) for 30min at 37°C The column used was a 10μm Protein Pak SW 300 column with a mobile phase of 0 1M phosphate buffer, pH 7 0, and a flow rate of 0 5ml/min Absorbance was detected at 280nm One major peak was detected with a retention time of 17 00mins This represents IgG (180kD) An extra peak was observed at 14 02min which distinguishes the GFAP-3BH2A3 complex which has a molecular weight of approximately 232kD (IgG=180, GFAP=52kD) The minor peaks at 9 86min eluted in the void volume of the column and may be IgM antibody The peak at 24 6min possibly identifies denatured or contaminating protein

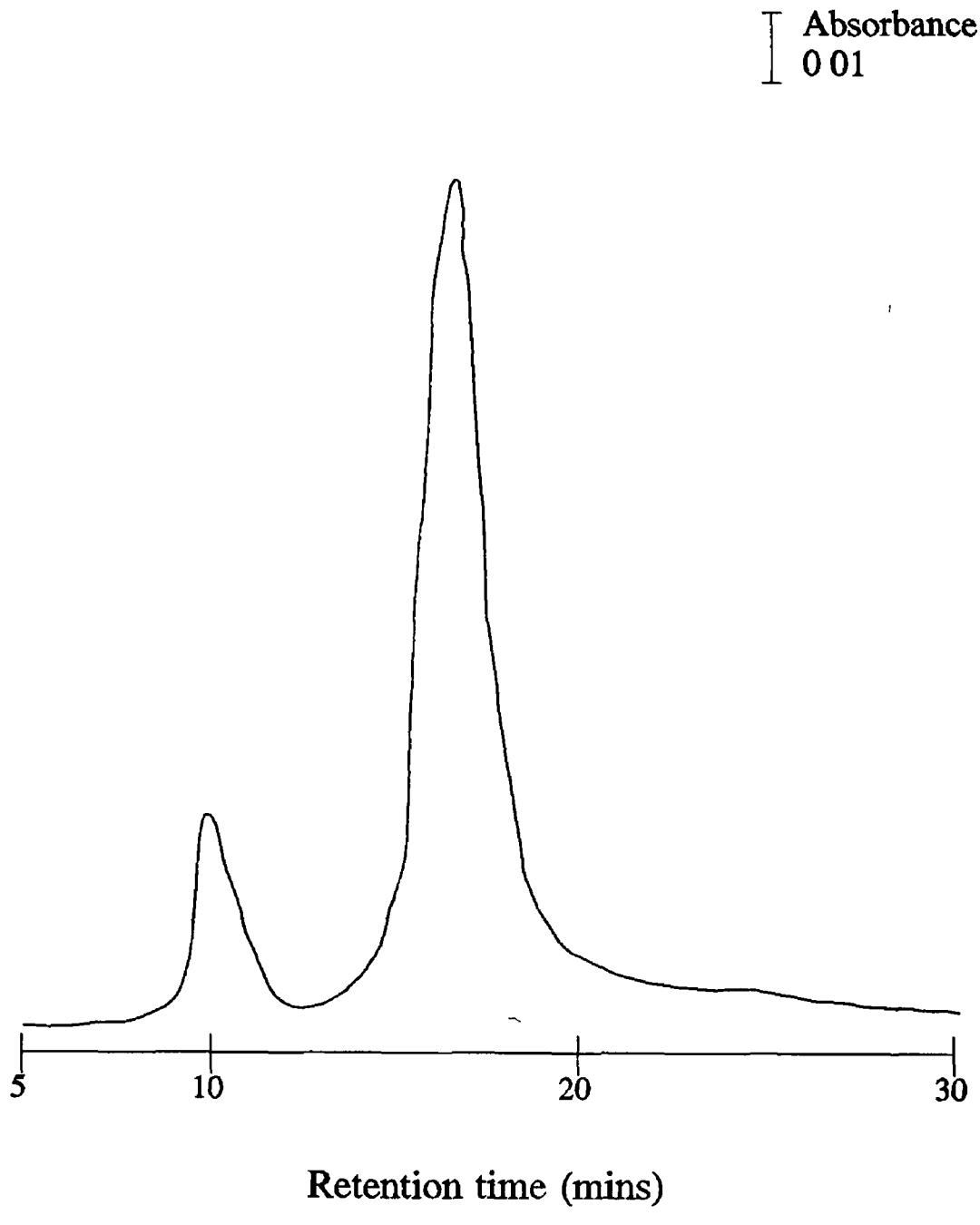


Figure 3.3.4.

HPLC chromatogram of SAS-treated 2DH5 ascitic fluid The column used was a 10μm Protein Pak SW 300 column with a mobile phase of 0 1M phosphate buffer, pH 7 0 and a flow rate of 0 5ml/min Absorbance was detected at 280nm One major peak was detected with a retention time of 16 86mins which is representative of IgG (180kD) The minor peak at 9 85min was outside the resolving range of the column

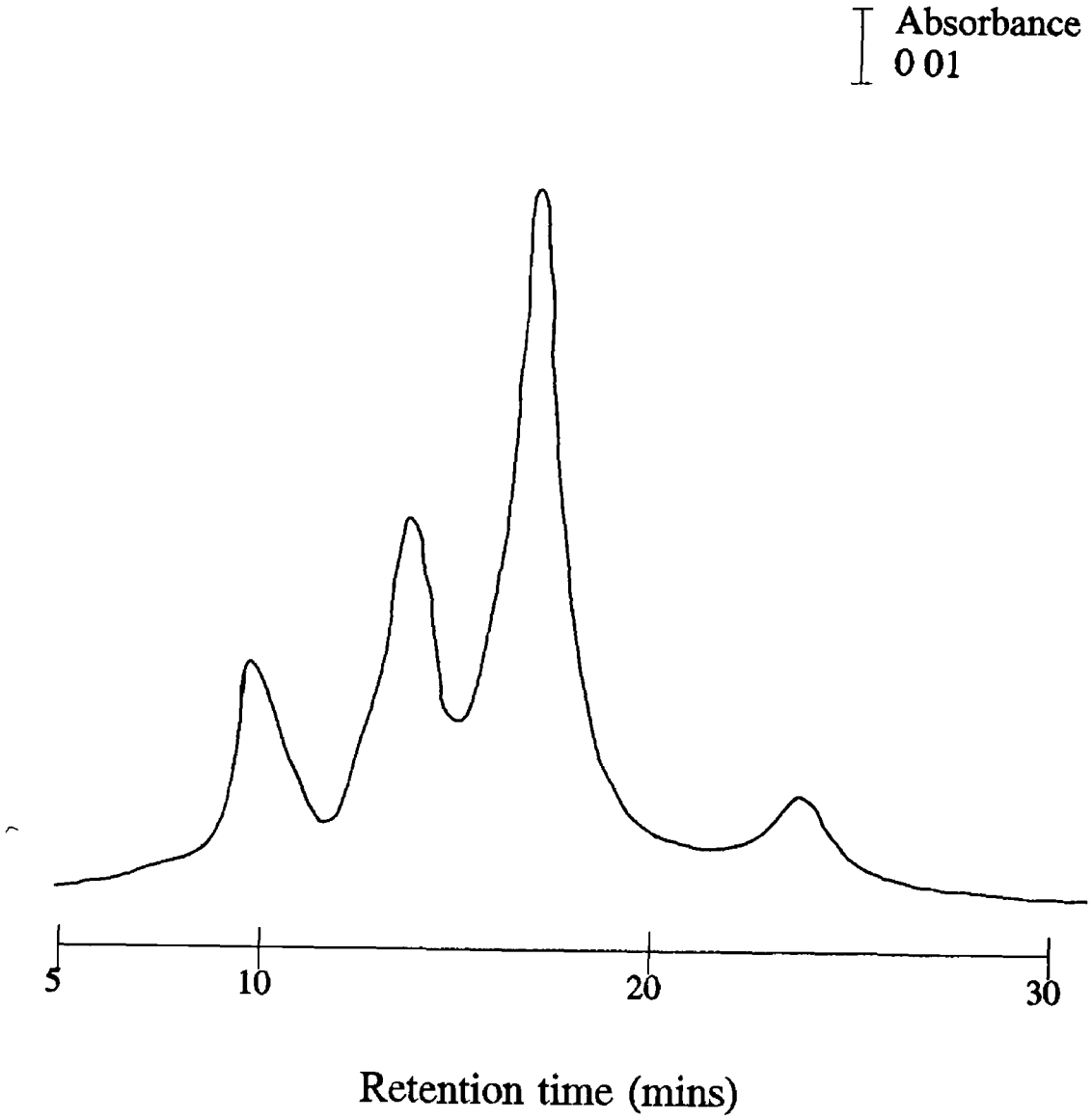


Figure 3.3.5.

HPLC chromatogram of SAS-treated 2DH5 ascitic fluid (2mg/ml) incubated with GFAP (20μg/ml) for 30min at 37°C The column used was a 10μm Protein Pak SW 300 column with a mobile phase of 0 1M phosphate buffer, pH 7 0, and a flow rate of 0 5ml/min Absorbance was detected at 280nm The major peak at 16 94mins represents IgG (180kD) An extra peak was observed at 14 99min which distinguishes the GFAP-2DH5 complex which has an approximate molecular weight of 232kD (IgG=180kD, GFAP=52kD) The minor peaks at 9 88min was outside the resolution range of the column and the peak at 23 95min possibly identifies denatured protein GFAP had a retention time of 22 91min

3 12 Dot immunobots used for initial screening of the anti-G-CCM and anti-GFAP antibodies

Dot immunoblotting was a relatively quick method for determining if the antigen of interest was present and if the test antibody reacted with this antigen GFAP was supplied by ICN Immunobiologicals (Table 2 3) Dot immunoblotting was performed prior to carrying out electrophoresis and Western immunoblotting A lot variation was noticed between the GFAP used to initially immunise and screen the anti-GFAP monoclonal antibodies, called GFAP "A", up to and including lot 16 and lots of GFAP obtained subsequently, were called GFAP "B" This variation was first noticed by ELISA (Section 3 6) and was confirmed using a dot blot (Section 2 3 21) The results are shown in Figure 3 4 1

It was found that the monoclonal antibodies 1BC6, 2BC7 and 3EG7 gave a better response with GFAP "A" than 2DH5, which reacted with GFAP "B", which was used for the latter immunisations ICN Immunobiologicals, California, confirmed a change in source after lot 16

The positive control used throughout there experiments was the mouse monoclonal anti-GFAP, clone G-A-5, also from ICN Immunobiologicals (Table 2 3) This product was immunoglobulin ascitic fluid containing a mouse IgG1 antibody produced by mice immunised with purified GFAP from porcine spinal cord It had also been tested for immunocytochemial localisation of GFAP from man, pig and rat The information sheet supplied with this antibody preparation states that in indirect immunofluorescence labelling on alcohol fixed paraffin embedded sections or frozen sections this antibody stains astrocytes and Bergmann glia cells This antibody also localises GFAP in the immunoblot technique, as can be seen in Figure 3 4 1

The dot blots shown in Figure 3 4 1 were treated as follows, (i) with 10ml of a 1 400 dilution of commercially available anti-GFAP which reacts with GFAP "B" only, (ii) the negative control, where PBS, pH 7 3, is substituted for the commercially available antibody, (iii) 10ml of a 1 5 dilution of 2DH5 clone A10 supernatant which can be seen to react with GFAP This reaction was not seen in (iv) the negative control which had $DMEMS_{10}$, added instead of hybridoma supernatant

Fresh membrane preparations were prepared for use in dot-immunoblotting and Western immunoblotting A serine protease inhibitor, phenyl-methyl-sulphonal fluoride, PMSF (Section 2 3 22), was added to the preparations (Barrand and Twentyman, 1992) Protein

concentrations were determined using the BCA assay (Section 2 4 9 3)

These dot blots were performed to confirm if the membrane preparations had been prepared correctly and to verify that 3BH2 clone A3 recognises the cell lines G-CCM and G-UVW and does not react with the cell lines EJ-138, K562, A549 and T-24 The results are shown in Figure 3 4 2 A positive result for $IJK_{(t)}$ can be seen on **all** blots $IJK_{(t)}$ was subsequently shown to be cross contaminated with C_6 rat glioma cells, either before or during its deposition in the European Collection of Animal and Cell Cultures (Table 2 2), and non-specific binding of the secondary antibody to these cells would explain this result

3.13. SDS-PAGE was performed on crude and purified antibody from ascitic fluid

SDS-PAGE was carried out according to Section 2 4 17 and the results are shown in Figure 3 5 These results show that an antibody band was observed at 150,000 daltons for commercial IgG and a band was observed in the protein G-purified ascites This shows that the concentration of the antibody in this sample was low, confirming the results obtained by ELISA

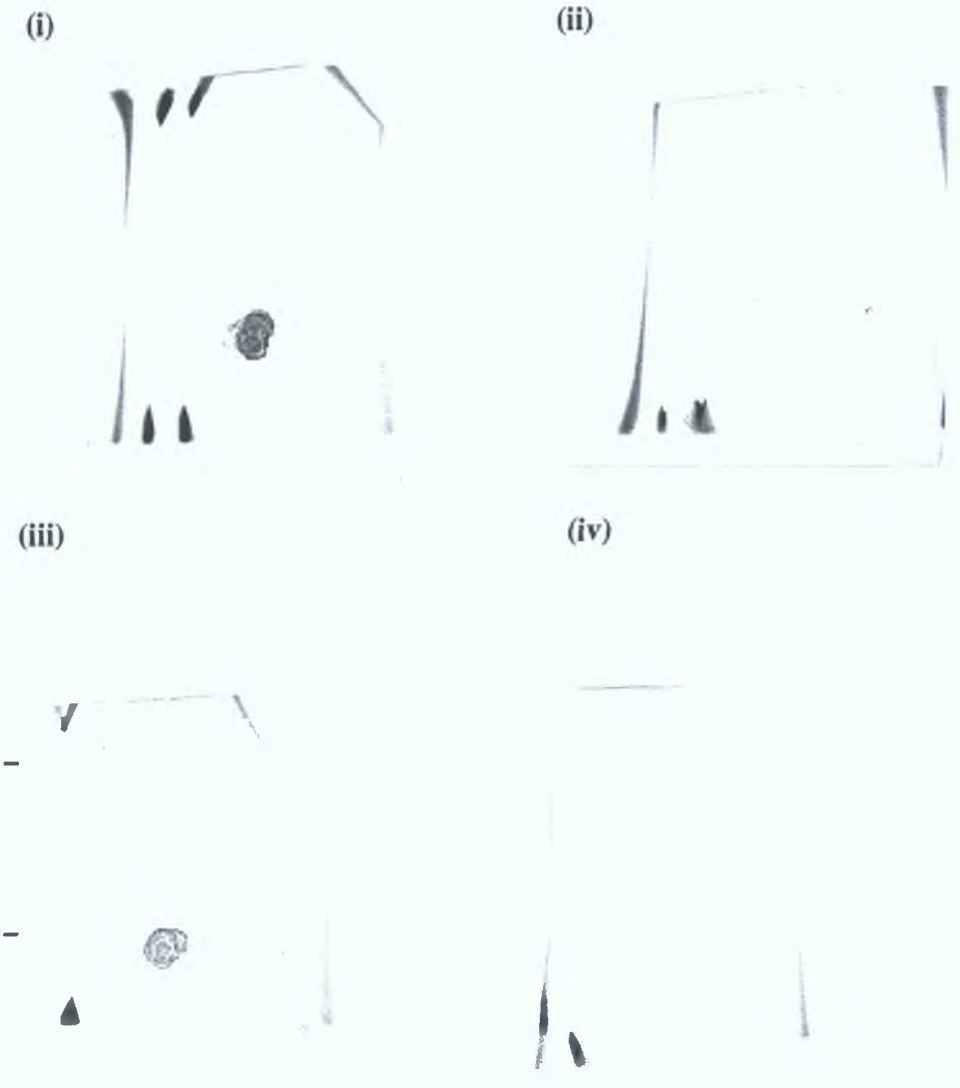


Figure 3.4.1.

Dot blots were performed to show that 2DH5 clone A10 recognises the GFAP "B". GFAP "A" was used as the antigen on the upper portion of **each** dot blot and the same quantity of GFAP "B" was used on the bottom portion. The dot blots were treated as follows; (i) with 10ml of a 1:400 dilution of commercially available anti-GFAP in 0.01M PBS, pH 7.3. It can be seen that this reacts only with GFAP "B", (ii) the negative control, using 0.01M PBS, pH 7.3, (iii) with 2DH5 clone A10 supernatant (1:5 dilution in 0.01M PBS, pH 7.3), this reacted with the GFAP "B" also and (iv) the negative control, with $DMEMS_{10}$ added instead of antibody.

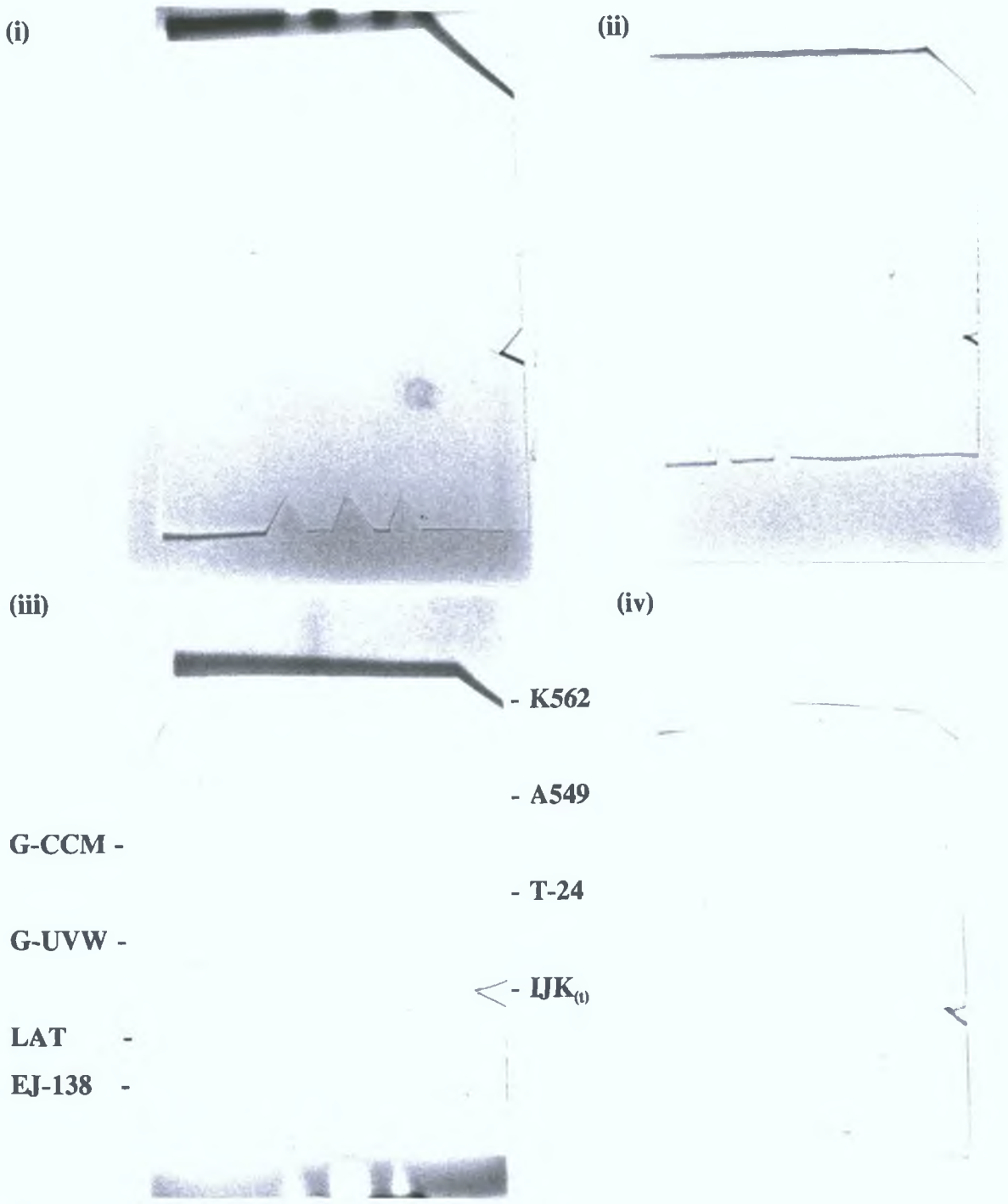


Figure 3.4.2.

Dot blots performed to show that 3BH2 clone A3 recognises the G-CCM and G-UVW cell lines. The following cell lines were included on each dot blot; G-CCM, G-UVW, LAT, EJ-138, K562, A549, T-24 and $IJK_{(t)}$. The dot blots were treated as follows: (i) the negative control, with $DMEMS_{10}$ added instead of antibody, (ii) the negative control, with 0.01M PBS, pH 7.3, (iii) with a 1:20 dilution of 3BH2 clone A3 supernatent diluted in 0.01M PBS, pH 7.3, and (iv) with a 1:400 dilution of commercially available anti-GFAP antibody in 0.01M PBS, pH 7.3.

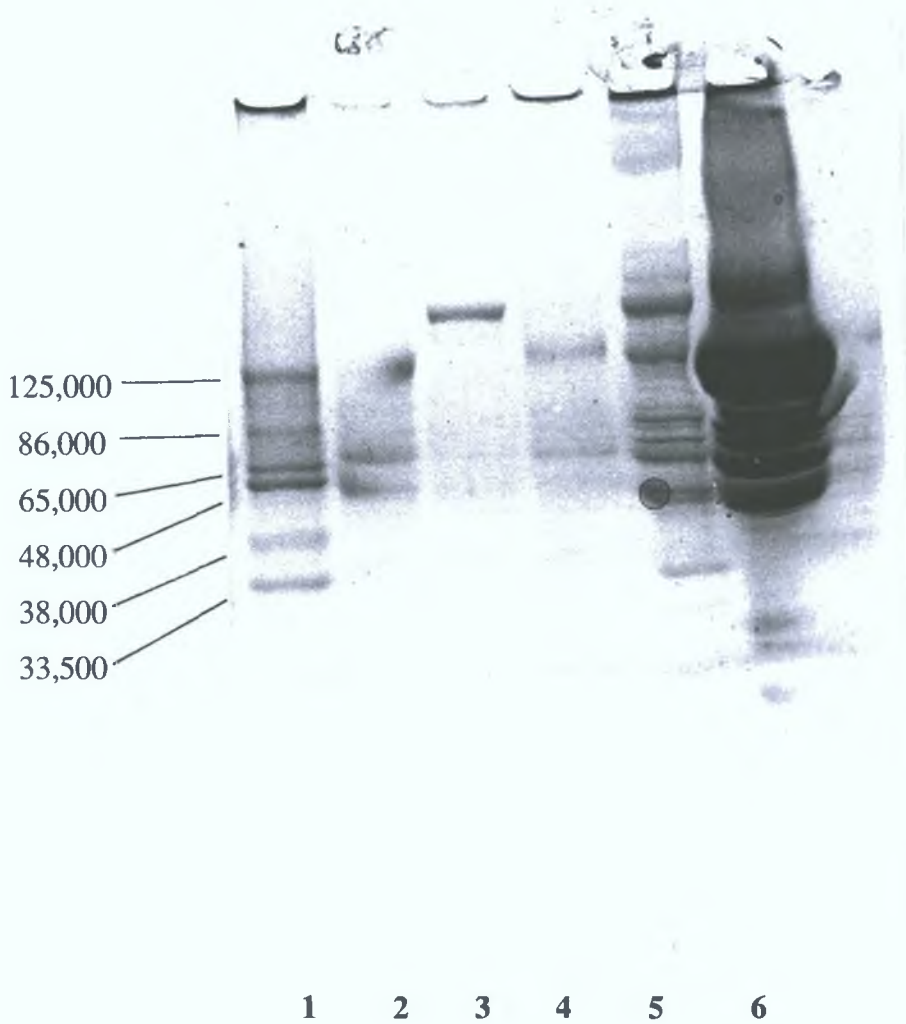


Figure 3.5.

A 5-20% (w/v) gradient PAGEL^{R} precast polyacrylamide gel from ATTO was loaded as follows; Lane (1) molecular weight markers,in daltons, (2) [anti-Ricin-A chain, SAS-treated], (3) mouse IgG standard, 2mg/ml, (4) anti-G-CCM (3BH2) ascitic fluid, protein G-treated, (5) anti-G-CCM, ascitic fluid SAS-treated, (6) anti-G-CCM, ascitic fluid (1:10 dilution in 0.01M PBS, pH 7.3). The gel was run at 20mAmps per gel.

3.14. Western blotting to determine the specificity of the anti-G-CCM and anti-GFAP antibodies.

SDS-PAGE gels containing commercial GFAP were transferred onto nitrocellulose in the same manner as preparations from cell membranes (Section 2 3 22 1) These blots were probed with supernatant from 3BH2 clone A3 and 2DH5 clone A10 (Figure 3 6 1) or ascitic fluid from these monoclonal antibodies (Figure 3 6 2) A positive control, commercially available anti-GFAP monoclonal antibody, was also used In Figure 3 6 1 , blot A was treated with a 1 100 dilution of commercially available anti-GFAP in 2% (w/v) BSA, 0 01% (v/v) Tween-20 in 0 01M PBS, pH 7 3 Blot B was tested with a 1 5 dilution of 3BH2 clone A3 supernatant

Commercially available anti-GFAP reacts strongly with an antigen of approximately 45kD, which is, presumably, GFAP Other bands were seen at molecular weights of approximately 145kD and 295kD These bands may be due to the antibody reacting with contaminating protein in the GFAP preparation or they may be due to, perhaps, the antibody reacting with multimers (trimer or hexamer aggregates) of GFAP The commercially available anti-GFAP antibody did not react with G-CCM or G-UVW 3BH2 clone A3 gave a faint band at the same molecular weight for G-CCM, G-UVW and GFAP Faint bands can be seen at molecular weights of 145kD and 295kD also This Western blot was repeated with a dilution of ascitic fluid and a clearer result was obtained in Figure 3 6 2 The anti-G-CCM blot was tested with a 1 100 dilution of SAS-treated anti-G-CCM (3BH2A3) ascitic fluid in 0 01% (v/v) Tween-20 in TBS The anti-GFAP (D C) blot was examined with a 1 100 dilution of 2DH5A10 ascitic fluid in 0 01% (v/v) Tween-20 in TBS The anti-GFAP blot was investigated with a 1 300 dilution of commercially available anti-GFAP in 0 01% (v/v) Tween-20 in TBS The negative (-ve) control blot was analysed with 0 01% (v/v) Tween-20 in TBS instead of primary antibody and shows no non-specific binding of the secondary antibodies used On each of the test blots [anti-G-CCM, anti-GFAP (D C) and anti-GFAP], the lane which was loaded with commercially available GFAP shows a large band in the 40kD to 50kD range A faint band was also seen with all three of these antibodies at approximately 100kD A band was seen at approximately 150kD in the GFAP lane of this Western blot which was treated with the anti-G-CCM antibody Each of the three antibodies also pick up a band at approximately 50kD on the lanes loaded with G-UVW and G-CCM From the result of this Western blot, it was deduced that the 2DH5

antibody reacts with GFAP and the 3BH2 antibody reacts with GFAP and to a GFAP-like antigen on G-CCM and G-UVW cells at a molecular weight of 50kD With anti-G-CCM and anti-GFAP (D C), in Figure 3 6 2 , faint bands are also seen at the top of each lane This may have been due to either (i) these antibodies reacting with aggregates of the cell membrane preparations of G-CCM and G-UVW which were not properly separated by sonication and, therefore, were of a much larger molecular weight, or (ii) GFAP is also found as multimers in these cell lines and the antibodies are reacting with these multimers

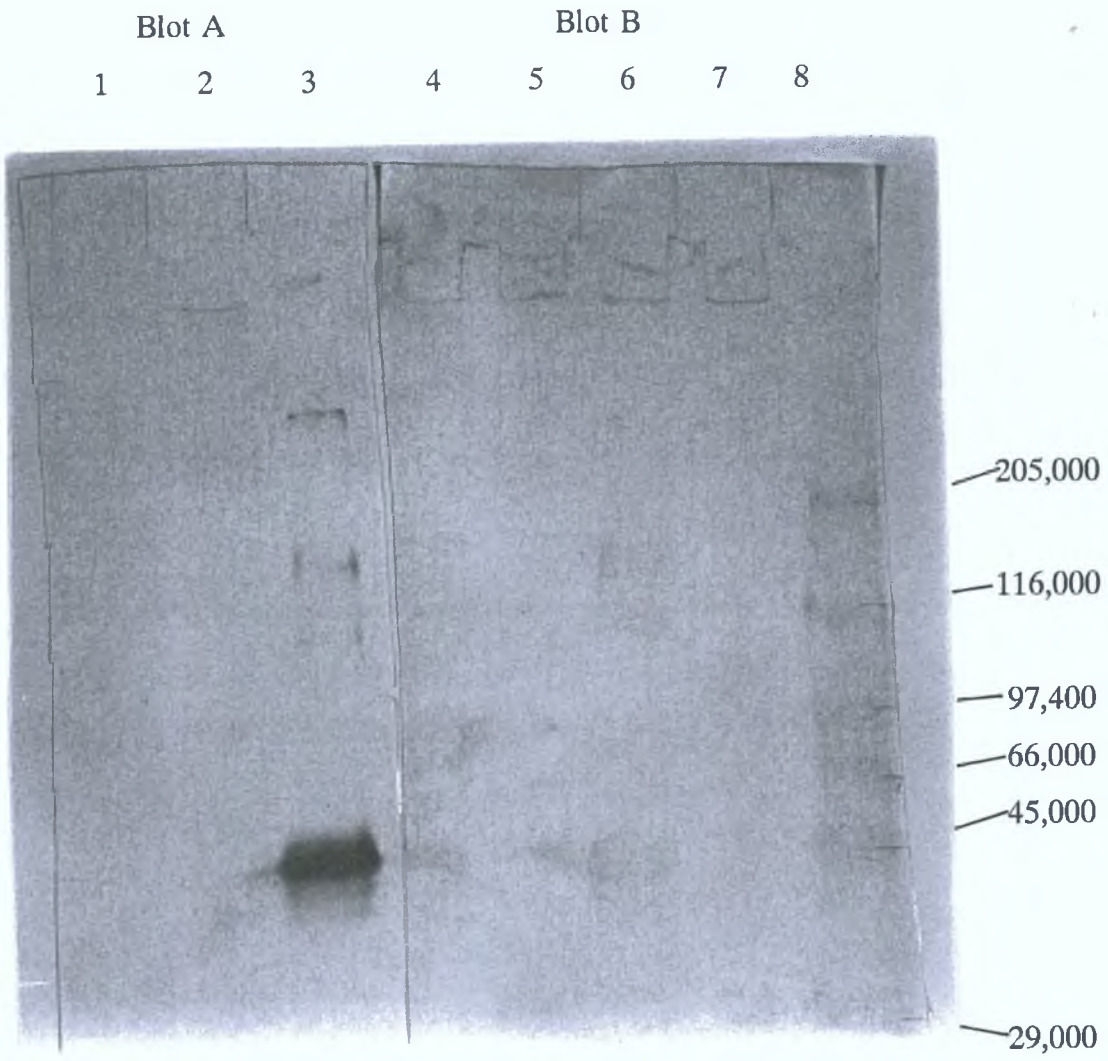


Figure 3.6.1.

Westerns immunoblots using cell membranes and GFAP, as follows; Blot A was treated with a 1:100 dilution of commercially obtained anti-GFAP in 2% (w/v) BSA, 0.01% (v/v) Tween-20 in 0.01M PBS, pH 7.3. Blot B was examined with a 1:5 dilution of 3BH2 clone A3 supernatant. The lanes were loaded as follows;

Blot A : (1) G-UVW, (2) G-CCM and (3) GFAP. Blot B : (4) G-CCM (5) G-UVW (6) GFAP (7) $IJK_{(t)}$ and (8) MWM, in daltons (Table 2.10.).

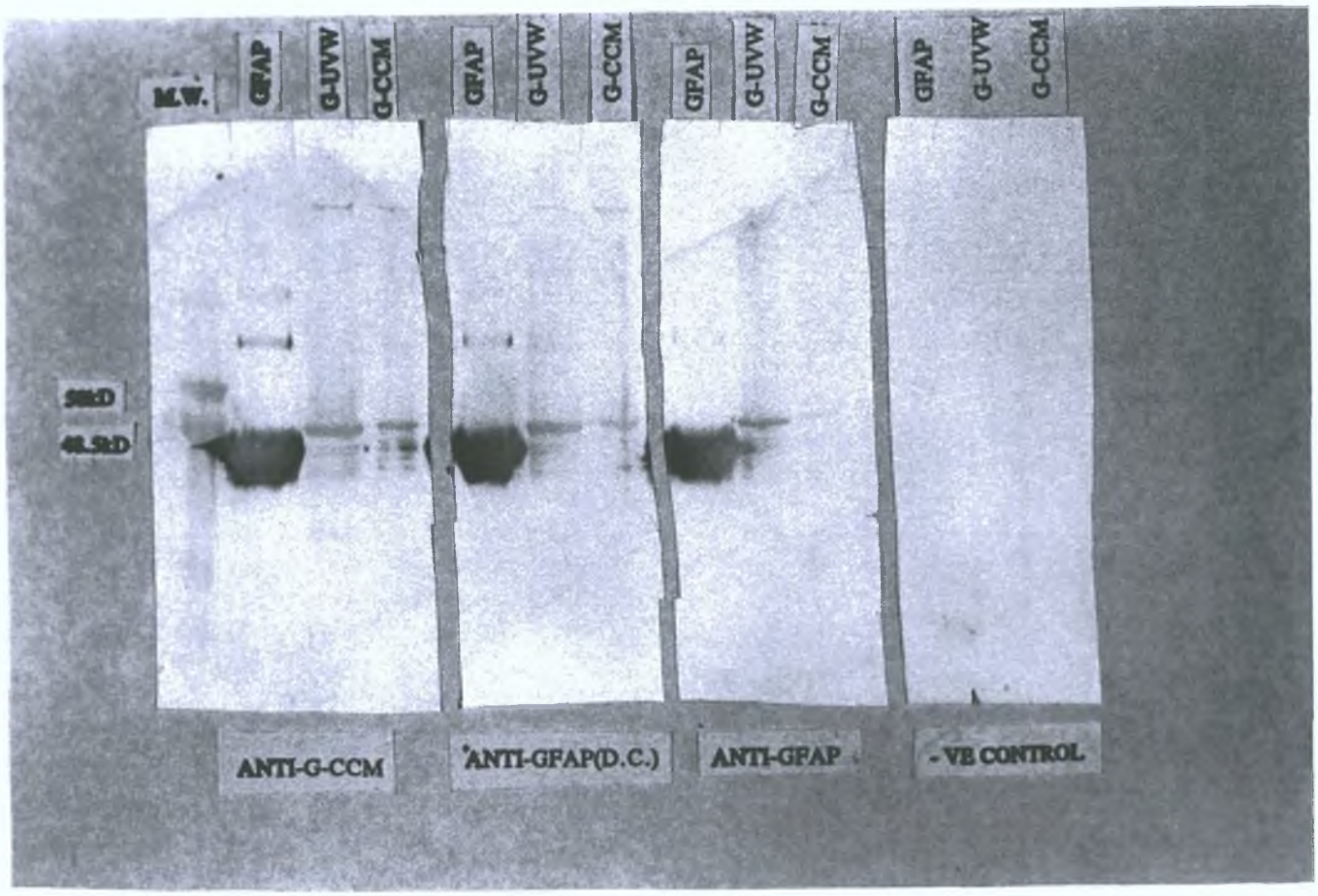


Figure 3.6.2.

Westerns blots of purified GFAP and membrane proteins from G–CCM and G–UVW cell lines. The anti–G–CCM blot was examined with a 1:100 dilution of SAS–treated anti–G–CCM (3BH2A3) ascitic fluid in 0.01% (v/v) Tween–20 in TBS. The anti–GFAP (D.C.) blot was tested with a 1:100 dilution of 2DH5 ascitic fluid in 0.01% (v/v) Tween–20 in TBS. The anti–GFAP blot was tested with a 1:300 dilution of commercially available anti–GFAP in 0.01% (v/v) Tween–20 in TBS. The negative (–ve) control blot was treated with 0.01% (v/v) Tween–20 in TBS instead of primary antibody and shows no non–specific binding of the secondary antibodies used.

3 15 Analysis of the affinity constant of 3BH2A3 for GFAP using BIAcore

With BIAcore, the optical phenomenon used for detection is surface plasmon resonance (SPR) The SPR response is correlated to changes in refractive index at a sensor chip surface The difference in refractive index is caused by mass changes on this surface, for example when the analyte binds to the immobilised ligand The SPR signal is monitored continuously so that chemical interactions between biomolecules can be studied in real time (Borrebaeck *et al* , 1992, VanCott *et al* , 1992)

The 3BH2A3 sample used in the BIAcore was SAS-treated ascitic fluid diluted 1 1 in 0 01M PBS, pH 7 3, with 0 05% sodium azide The GFAP sample was 100μg of unreconstituted, commercially available GFAP from ICN Immunobiologicals (Table 2 3) The samples were prepared (Section 2 3 24 1) The results were obtained by Paul Roben while he was at the Scripps Institute in La Jolla, California and his assistance is gratefully acknowledged

The association/dissociation rate constants and affinity constants for (a) the 3BH2A3 antibody and (b) the commercially obtained anti-GFAP antibody against GFAP were analysed using a BIAcore machine (Section 2 3 24)

The equilibrium constant K_A is the ratio of the association k_{ass} and dissociation rate constants k_{diss}

$$K_A = k_{ass}/k_{diss}$$

To measure association, a low flow rate of antibody passing over a high concentration of bound antigen is used, to ensure that each antigen will be bound by an antibody To measure the rate of association the concentration of antibody bound is plotted against time From this graph the relative responses at different concentrations can be calculated If this is plotted against time, the slopes obtained can be plotted against concentration to obtain k_{ass}

In Figure 3 7 1 , a sensogram shows the real time binding of the anti-G-CCM (3BH2A3) monoclonal antibody to surface GFAP, in BIAcore Response Units (RU) An average slope, read every 5 seconds, was calculated at each of the time points (crosses) along the binding curve To measure the rate of association, the concentration of antibody was plotted against time, from this graph the relative responses of the 3BH2A3 antibody at different concentrations can be calculated (Figure 3 7 2) These results were plotted against time and the slopes obtained were plotted against concentration to obtain the association constant, k_{ass}, which was found to be $2\ 95 \times 10^7 M^{-1}$

To measure dissociation, the antigen was coated at a low concentration to ensure that all the antigens present are bound to the surface of the sensor chip The low concentration of coating antigen also ensures that if the antibody was washed off, there will not be other antigens available for it to bind to At high antibody concentrations and high flow rate, all the antigens present will be bound by antibody When the sample pulse passed the sensor surface and was replaced by buffer, the concentration of free analyte (antibody) dropped suddenly to zero The derivative of the response curve then reflected the dissociation rate,

$$\frac{dR}{dt} = -k_{diss}R_t$$

This was only valid under the assumption that re-association of released analyte was negligible, i e analyte was efficiently removed by the buffer flow Integrating with respect to time gives,

$$\frac{\ln R_{t1}}{R_{tn}} = k_{diss(tn-t1)}$$

where R_{tn} is the response at time n and R_{t1} is the response at an arbitary starting time of 1 The dissociation rate constant k_{diss} can be obtained by plotting the log of the drop in response against the time interval

The affinity constants were calculated automatically by the BIAcore system using the results given in Figures 3 7 The affinity constant for commercially available anti-GFAP against GFAP was found to be $9\,6x10^{8}mol^{-1}$ and the affinity constant for 3BH2A3 against GFAP was $2\,95x10^{7}mol^{-1}$ The affinity constant for the commercial antibody was high, as expected The affinity of the 3BH2A3 antibody for GFAP was also satisfactory, considering the immunogen was the anaplastic astrocytoma cell line, G-CCM The 2DH5A10 antibody was not determined due to insufficient time being available

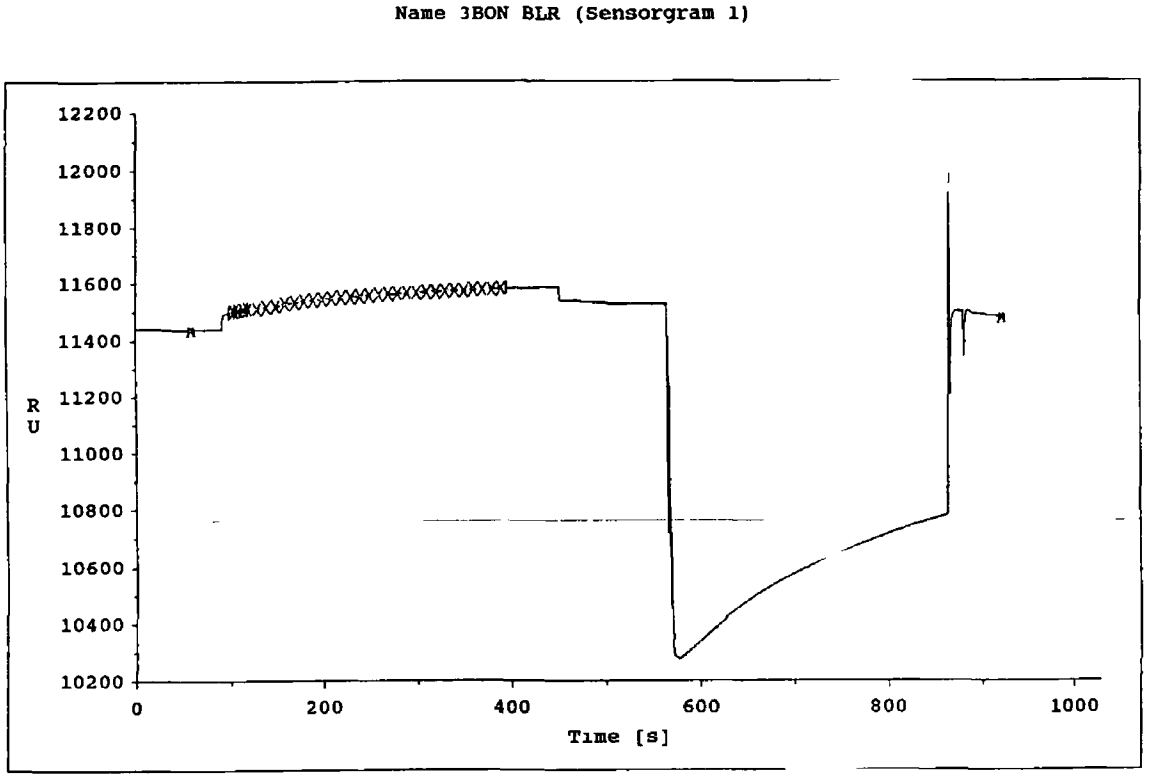


Figure 3 7 1

An example of a sensogram showing real time binding of the anti-G-CCM (3BH2A3) monoclonal antibody to surface GFAP, in BIAcore Response Units (RU) An average slope, read every 5 seconds, was calculated at each of the time points (crosses) along the binding curve

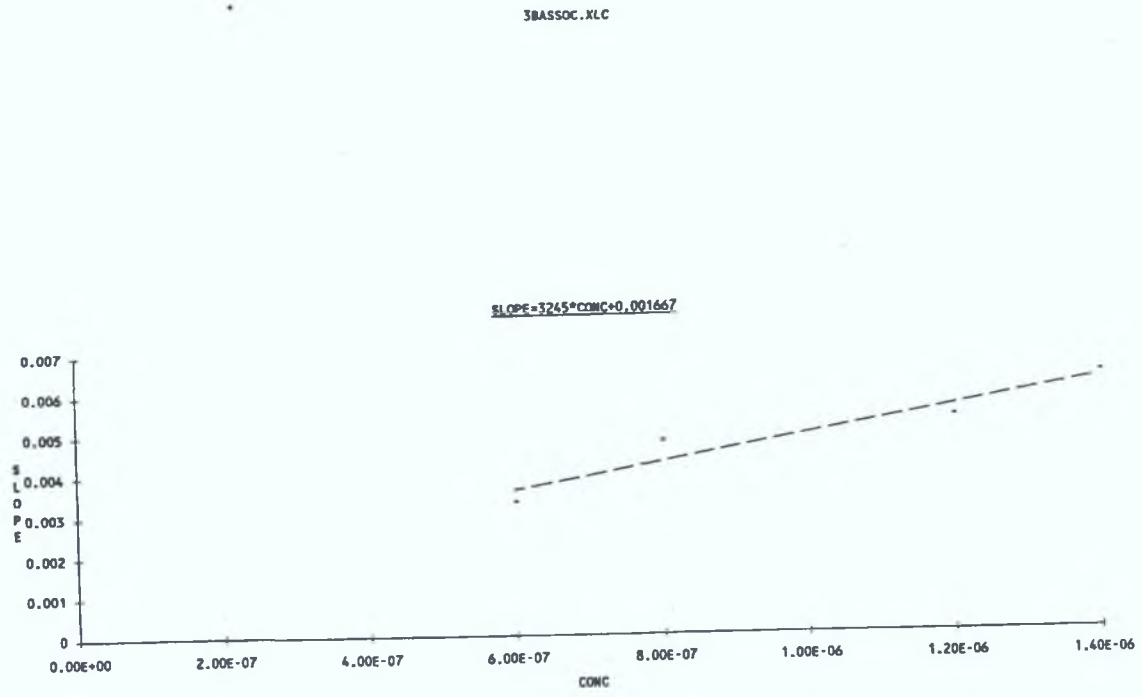


Figure 3.7.2.

The results from Figure 3.7.1. were plotted against time and the slopes obtained were plotted against concentration to obtain the association constant, k_{ass}, which was found to be $2.95 \times 10^{7} M^{-1}$. The results are discussed in Section 3.15.

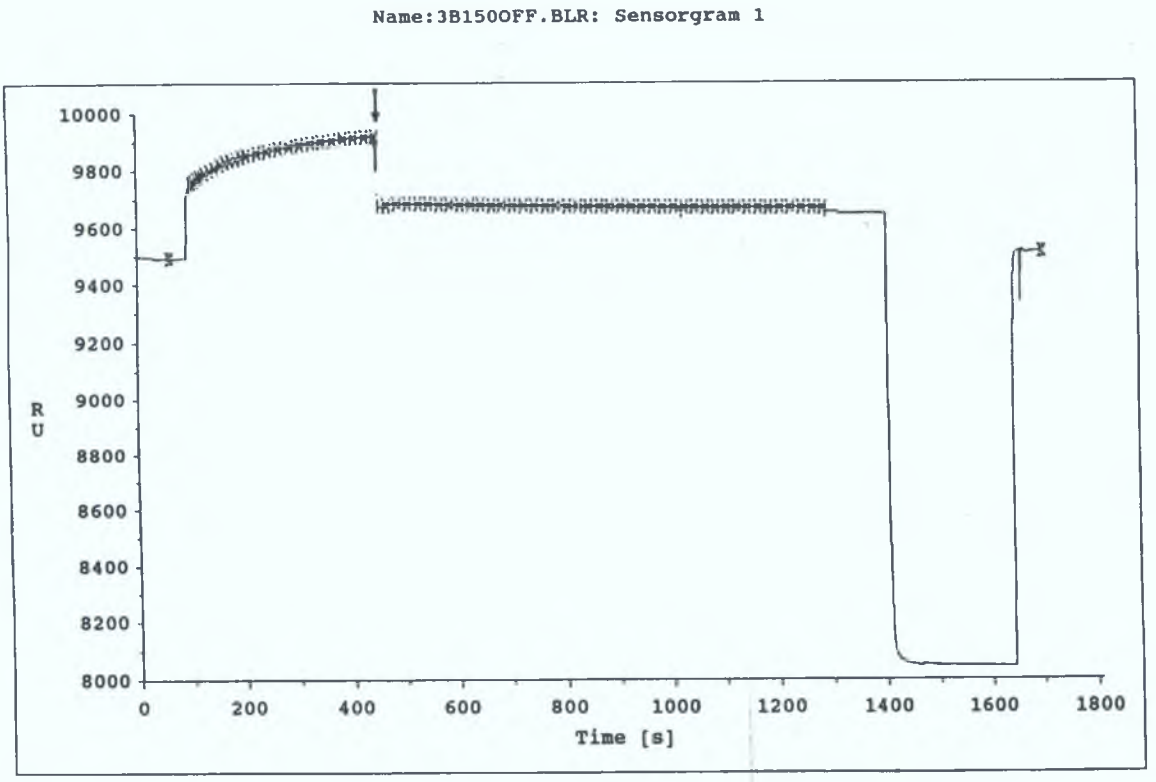


Figure 3.7.3.

To measure antigen–antibody dissociation, the antigen was coated at a low concentration to ensure that at the high antibody concentrations and high flow rate used, all the antigens present are bound. When the sample pulse has passed the sensor surface and is replaced by buffer, the concentration of free analyte drops suddenly to zero (arrow). The derivative of the response curve then reflects the dissociation rate.

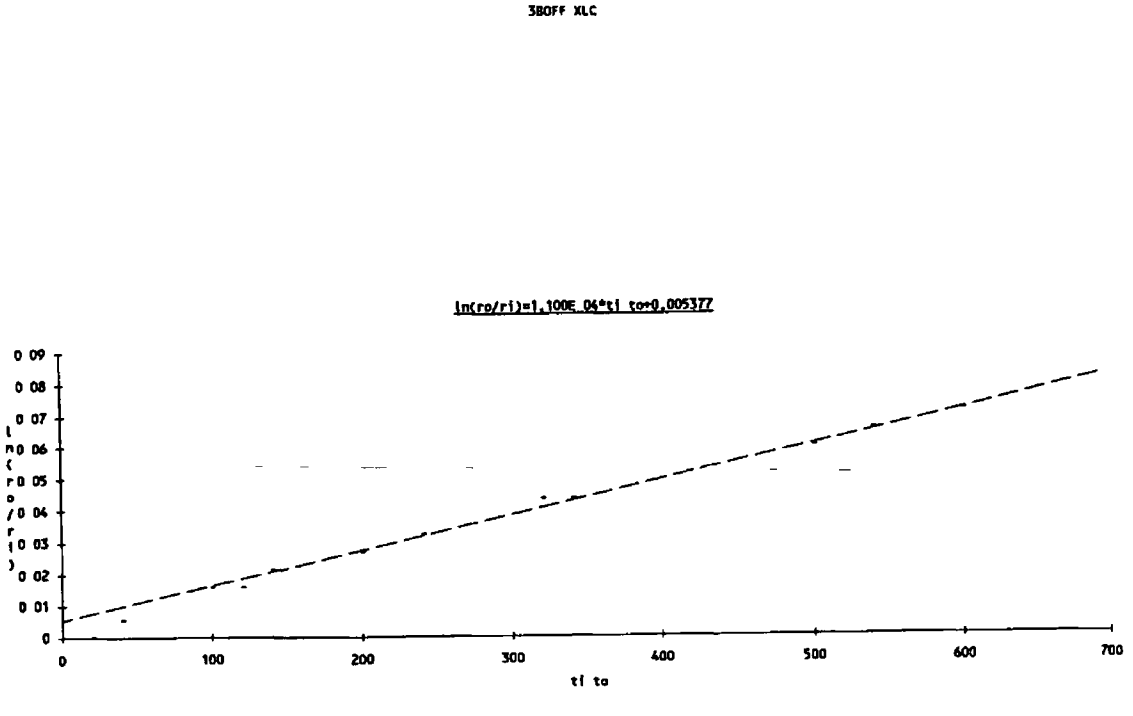


Figure 3 7 4

The dissociation rate constant, K_{diss}, can be obtained by plotting the log of the response curve (Figure 3 7 3) against the time interval (VanCott *et al* , 1992) This is discussed in Section 3 15

3 16 Characterisation of the anti-G-CCM and anti-GFAP antibodies by immunocytochemistry

A photograph of the G-CCM cells in culture is shown in Figure 3 8 1 The staining of G-CCM cells by Romanowsky staining, as described in Section 2 3 20 3 , is seen in Figure 3 8 2

Immunocytochemistry was used to determine the specificities of the anti-G-CCM and anti-GFAP antibodies with respect to G-CCM and G-UVW cell lines The SAS-treated anti-G-CCM and anti-GFAP ascitic fluid samples were used in the immunocyto-chemical studies The optimum dilution of the antibodies was determined by initially examining a series of dilutions A stock of 2mg/ml of each purified antibody was used at a dilution of 1 10 to 1 100 The preparation of cytospin samples using cell lines is outlined in Section 2 3 20 2 The staining of the cytospin preparations employing the APAAP detection system (2 3 30 4) was determined using (i) a 1 100 dilution of SAS-treated 3BH2A3 ascitic fluid in 0 01M PBS on G-CCM and G-UVW cells (Figures 3 8 3 and 3 8 4 , respectively), and (ii) SAS-treated 2DH5 ascitic fluid, similarly diluted, was tested against G-CCM and G-UVW (Figures 3 8 5 and 3 8 6) Strong staining with 3BH2A3 and 2DH5 on the cell membrane was observed and, in some cells, cytoplasmic staining and staining of astrocytic processes were also seen (Figure 3 8 7) Negative samples were prepared by substituting the primary antibody with 0 01M PBS, pH 7 3 (Figure 3 8 8) Normal tissues were not studied due to the difficulty of obtaining such samples

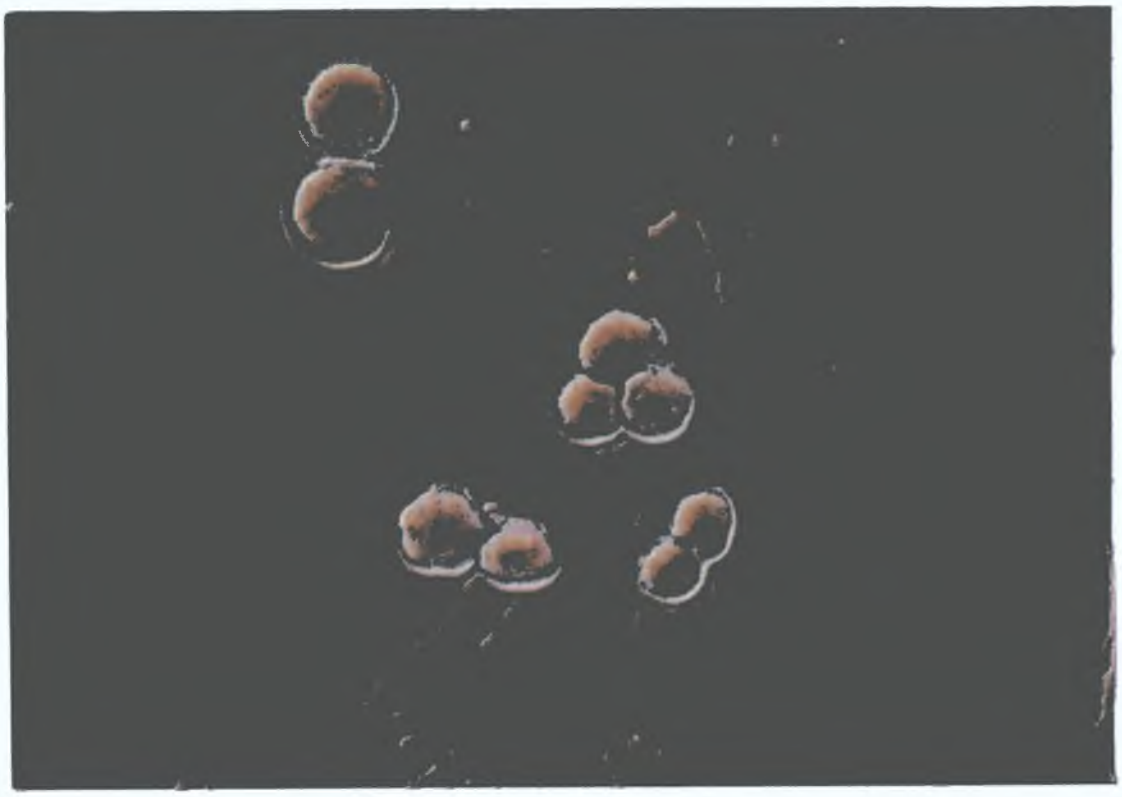


Figure 3.8.1.

G-CCM cells in culture at 40x magnification, photographed using a Nikon type ELWD 0.3 inverted phase contrast microscope.

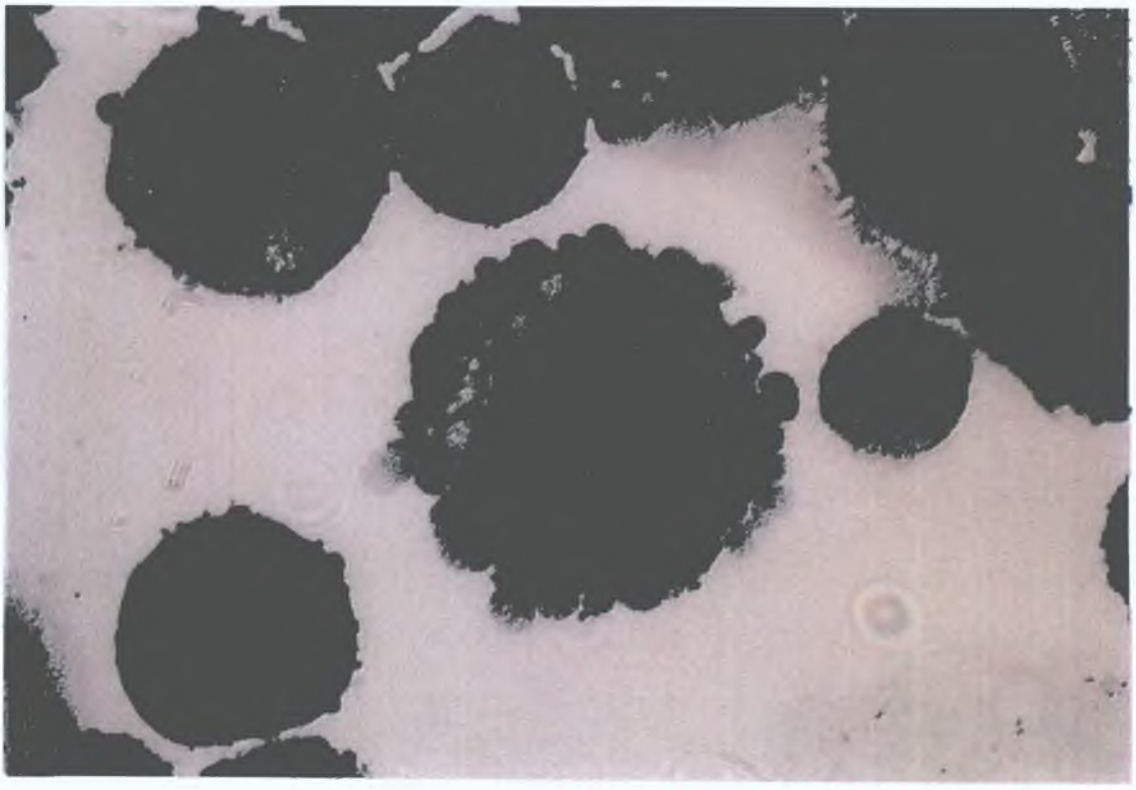


Figure 3.8.2.

G-CCM cells stained by Romanowsky staining (Section 2.3.20.3.) at 400x magnification, photographed as in Figure 3.8.1.

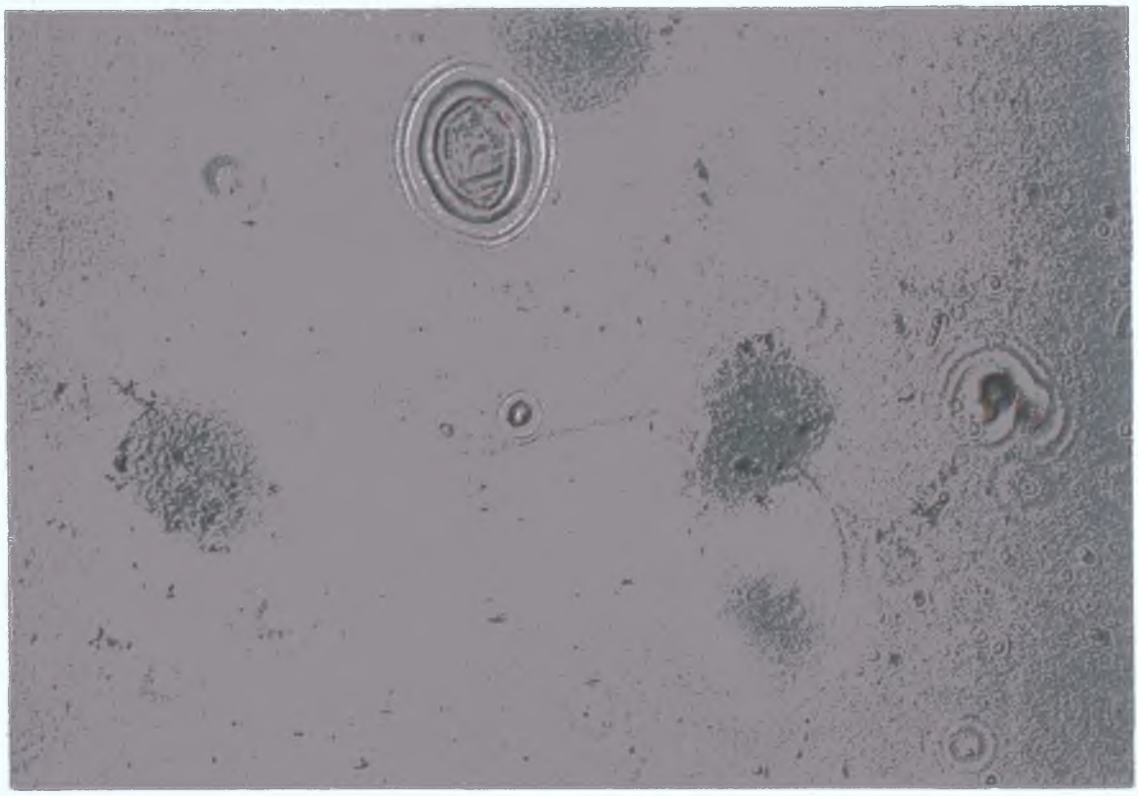


Figure 3.8.7. 3BH2A3 (1:100 dilution) was incubated with a cytospin preparation of G–CCM cells which were then detected using the APAAP system (100x magnification), showing staining of the astrocytic proccesses. The cells were counterstained with methyl green.

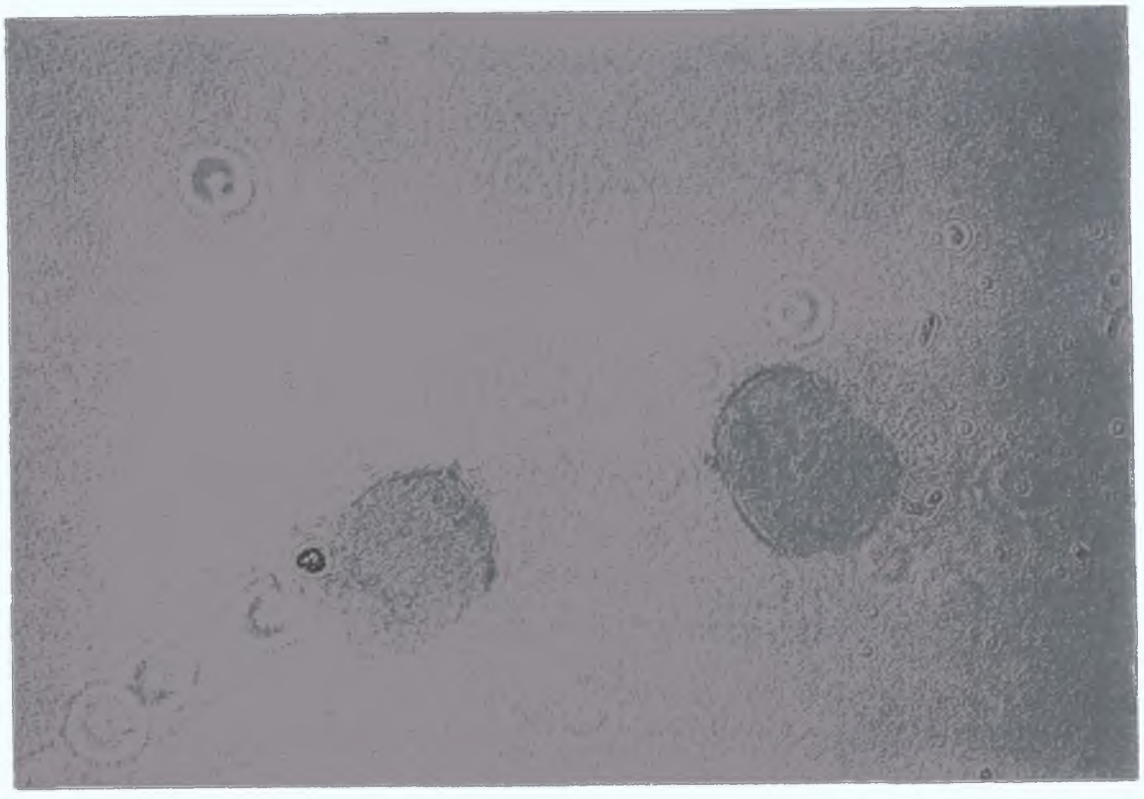


Figure 3.8.8.

Negative samples were prepared by substituting the primary antibody with 0.01M PBS, pH 7.3. The cells were counterstained with methyl green.

Figure 3.8.5. 2DH5 (1:100 dilution) was incubated with a cytospin preparation of G–CCM cells which were then detected using the APAAP system (100x magnification). The cells were counterstained with methyl green.

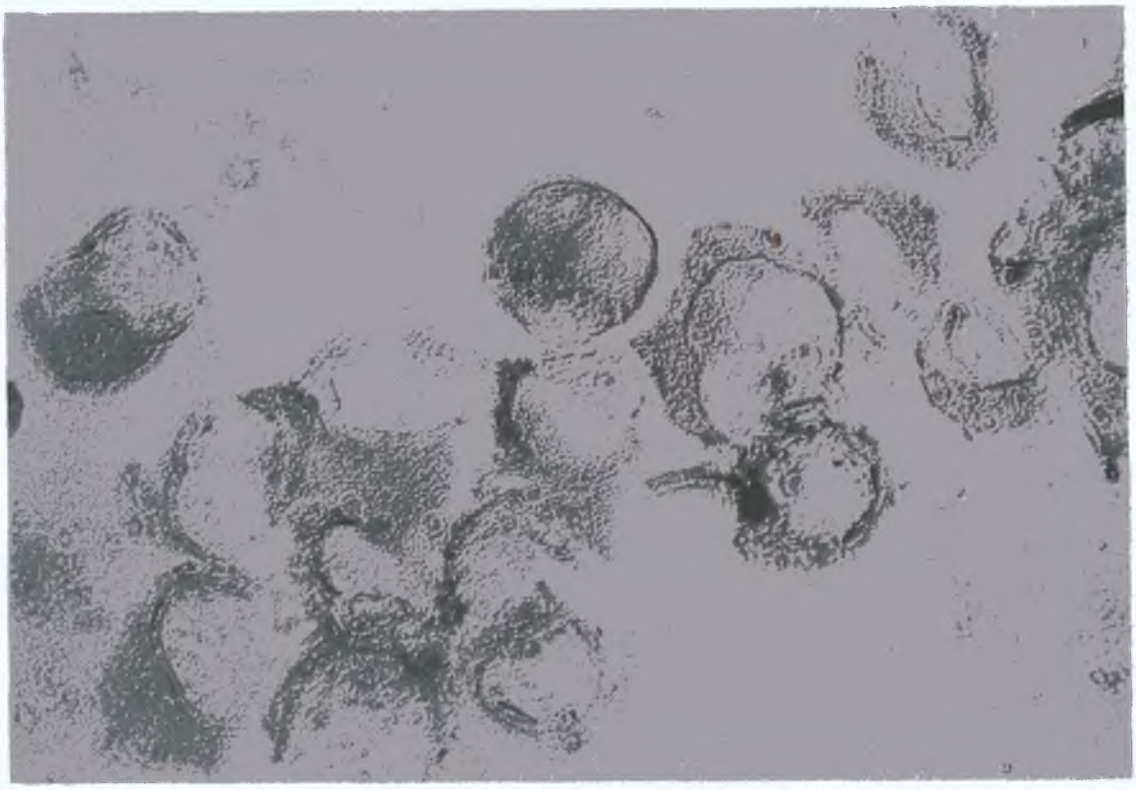


Figure 3.8.6. 2DH5 (1:100 dilution) was incubated with a cytospin preparation of G–UVW cells which were then detected using the APAAP system (100x magnification). The cells were counterstained with methyl green.

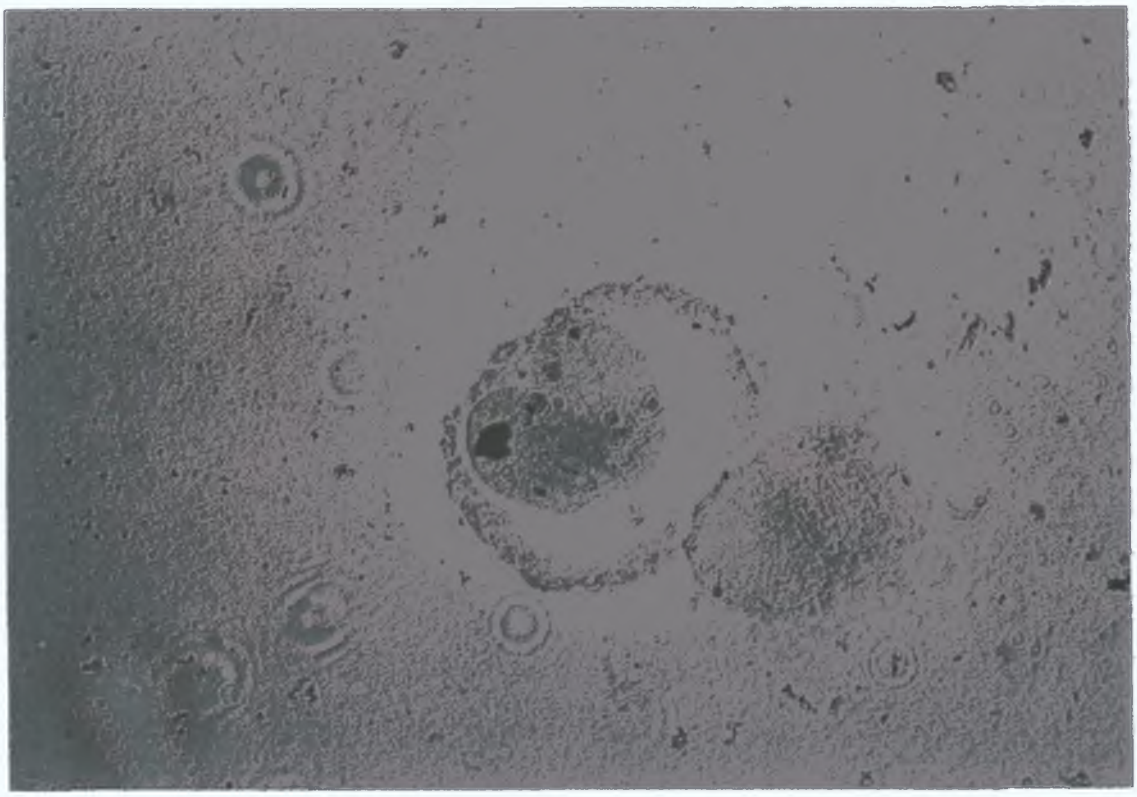


Figure 3.8.3. 3BH2A3 (1:100 dilution) was incubated with a cytospin preparation of G–CCM cells which were then detected using the APAAP system (100x magnification). The cells were counterstained with methyl green.

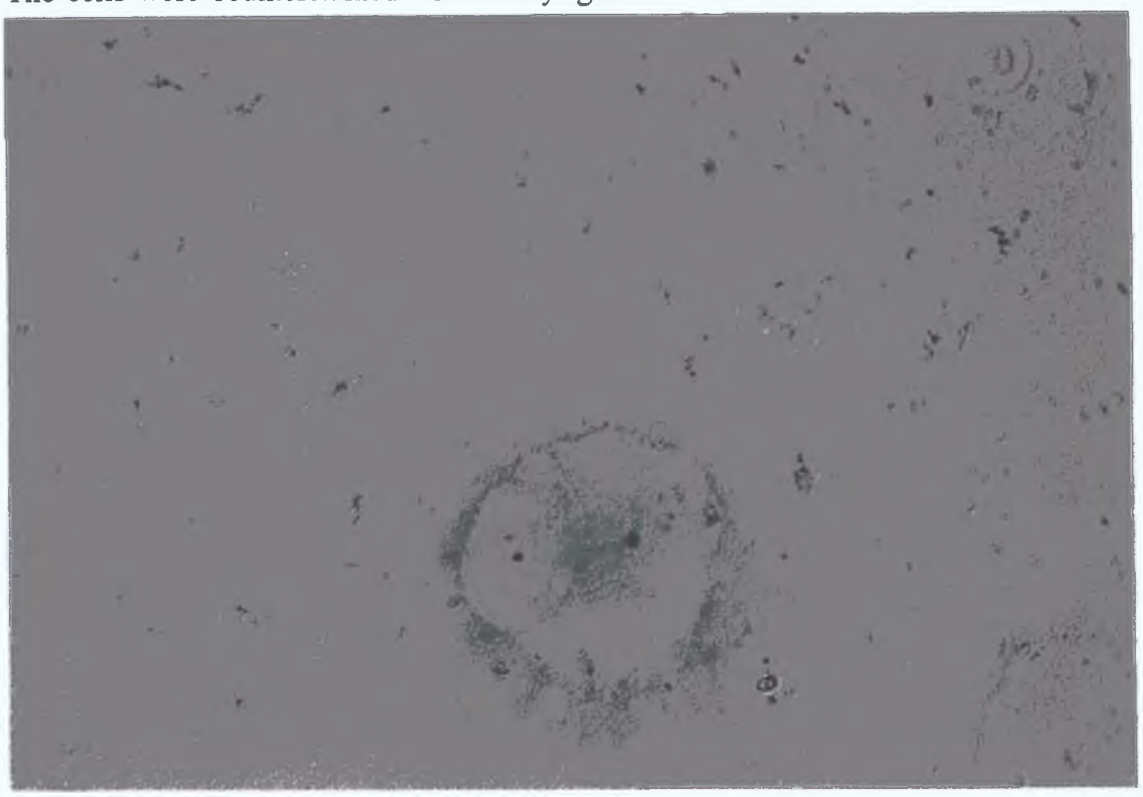


Figure 3.8.4. 3BH2A3 (1:100 dilution) was incubated with a cytospin preparation of G–UVW cells which were then detected using the APAAP system (100x magnification). The cells were counterstained with methyl green.

3 17 Characterisation of the anti-G-CCM antibody by immunofluorescence.

Indirect immunofluorescence enables the detection of surface antigens on live cells This method may be performed on single-cell suspensions or on cells attached to a culture dish (Section 2 3 23 2) When immunofluorescence is carried out on live and viable cells, only cell surface components are recognised (Schachner, 1982) Using this method, the first specific antibody is applied, followed by treatment with a second antibody carrying fluorescein as the detectable label The second antibody reacts with the first, thereby visualising indirectly the antigen of study

When the G-CCM cells were treated by direct immunofluorescence using 3BH2, as the primary antibody, a green-yellow ring around the cell surface was clearly seen when photographed under ultraviolet light (Figure 3 9 1) This is indicative of cell surface localisation of the antigen recognised (Schachner, 1982) When the G-CCM cells were tested with the 2DH5 monoclonal antibody a fainter fluorescent ring was also seen On testing G-CCM cells with commercially obtained anti-GFAP monoclonal antibody by this method, no fluorescent ring was visible around the cell surface This result was expected as GFAP is intracellular in location and therefore would not be detected in live cells by this method These results show that the 2DH5 monoclonal antibody is immunologically similar to the commercially available anti-GFAP monoclonal antibody, but they are not identical in their reactivities

This result indicate that the antigen recognised by these antibodies is located on the cell membrane Cytoplasmic staining of some cells would indicate that the antigen recognised by these two antibodies may also be intracellular in location It was not possible to detect intracellular binding on live cells by immunofluorescence, but it was possible to detect intracellular binding on fixed, cytospin preparations

Figure 3.9.1. 3BH2A3 incubated with live G–CCM cells and treated with an FITC–labelled secondary antibody, at 400x magnification, photographed under ultraviolet light using a Nikon Optiphot microscope.

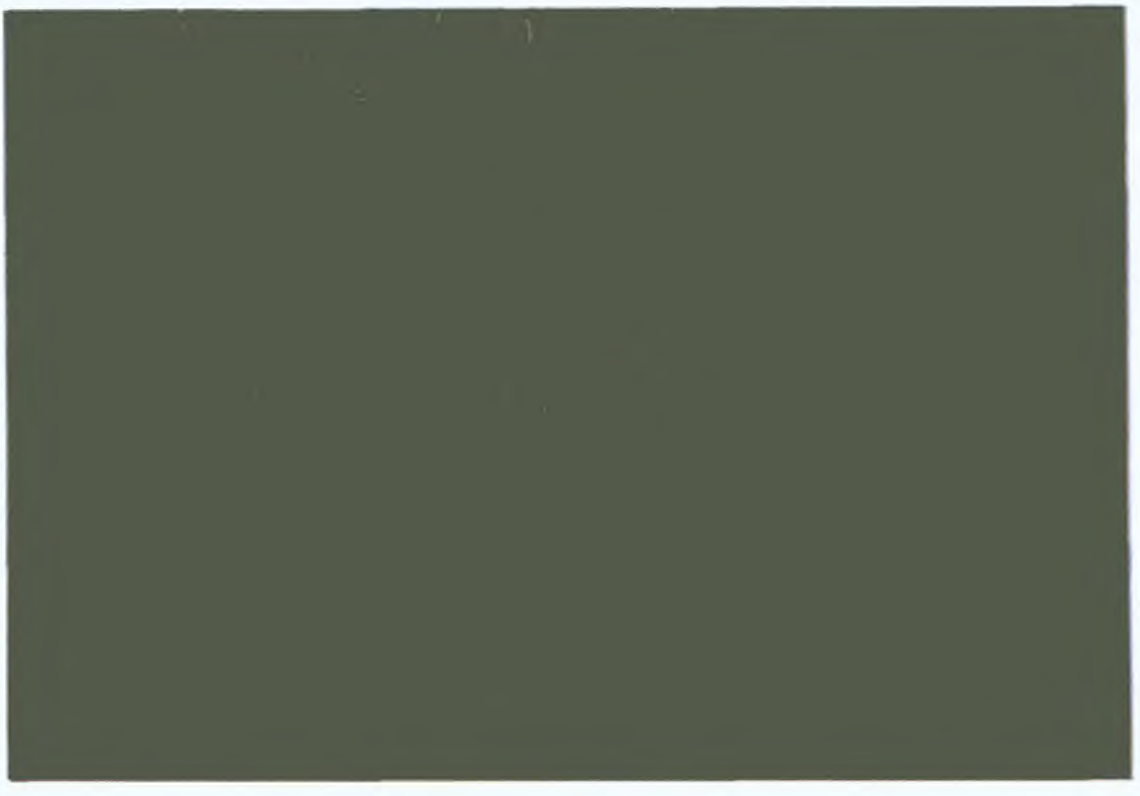


Figure 3.9.2. 3BH2A3 antibody incubated with live G–CCM cells and treated as in Figure 3.9.1, photographed under visible light using a Nikon Optiphot microscope, at 400x magnification.

3 18 Characterisation of the anti-G-CCM and anti-GFAP antibodies by FACS

For FACS analysis, the availability of single-cell suspensions with a high degree of viability is required to permit the recognition of cell surface components only

The FACS procedure was carried out as described in Section 2 3 20 8 and the results are shown in Figures 3 10 (1 -7) All samples in each experiment are measured at the same gain settings Arbitrary thresholds were established, and the relative frequencies of cells with the same or higher fluorescent intensities are computed In this way, direct comparisons can be made between populations of interest to determine the relative frequency of cells with the same fluorescent intensities in different samples The selection of the arbitrary threshold is determined on the basis of the shape of the frequency distribution

Two negative controls were used for each experiment, (i) using PBS instead of the primary antibody, labelled as "FITC ONLY" and (ii) using PBS instead of both the primary antibody and the FITC-labelled secondary antibody, labelled as "NO FITC" If the cells have been prepared and blocked properly, these two peaks should be identical (Figure 3 10 1) The results of the binding of a 1 10 dilution of the SAS-treated 3BH2 and 2DH5 ascitic fluid incubated with G-CCM cells is shown in Figures 3 10 1 and 3 10 2 The results of the binding of a 1 10 dilution of the SAS-treated 3BH2 and 2DH5 ascitic fluid incubated with G-UVW cells is shown in Figures 3 10 3 The positive control was a dilution of G12 ascitic fluid which reacts with the K562 human erythroleukemic cell line (Figure 3 10 4) This was kindly obtained from Orla Nolan, D C U Positive binding activity of 3BH2 and 2DH5 against the K562 erythroleukaemia cell line was not considered unusual as the expression of common surface antigens by the brain and lymphoid cells and tissues has been reported (Wilkstrand and Bigner, 1985) The profiles obtained when using the 3BH2 and 2DH5 antibodies with various cell lines (Table 2 2) are given (Figures 3 10 5 1 and 3 10 5 2) The results of the FACS analysis using crude antibody supernatant from the 3BH2 and 2DH5 hybridomas are shown in Figures 3 10 6 and 3 10 7

The FACS analysis confirmed the western and immunofluorescence results, that the 3BH2 and 2DH5 antibodies appear to be directed to a common antigen which is immunologically similar to GFAP, but appears to be a cell membrane antigen These two antibodies gave similar FACS results throughout

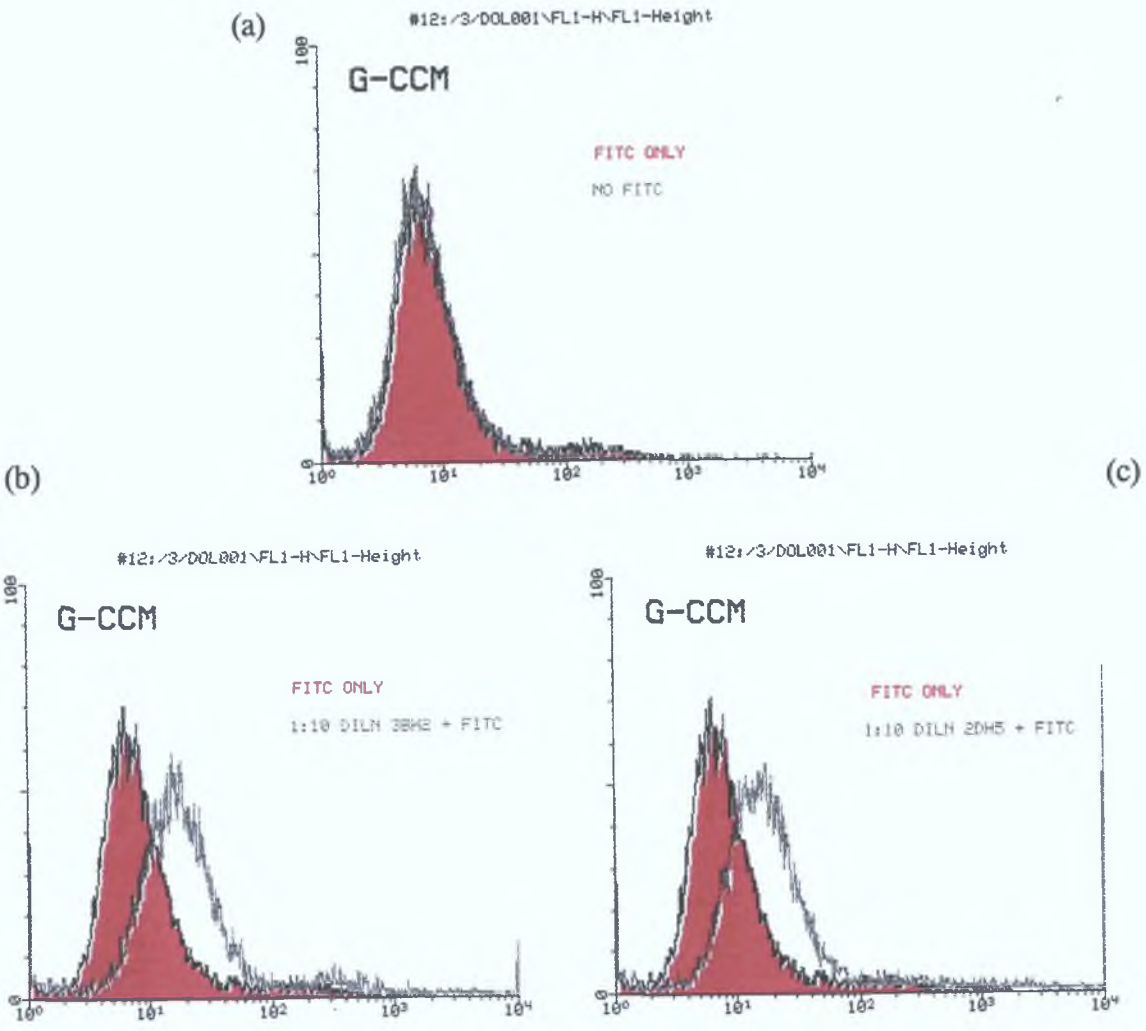


Figure 3.10.1. The FACS analysis of the binding of SAS-treated 3BH2 and 2DH5 ascitic fuid (1:10 dilution) incubated with G-CCM cells is shown in (b) and (c) respectively. Also shown, in (a), are the two negative controls used for each experiment; (i) using PBS instead of the primary antibody, labelled as "FITC ONLY" and (ii) using PBS instead of both the primary antibody and the FITC-labelled secondary antibody, labelled as "NO FITC". These two peaks are identical showing that the cells have been prepared and blocked properly and that there is no non-specific binding of the FITC-labelled antibody.

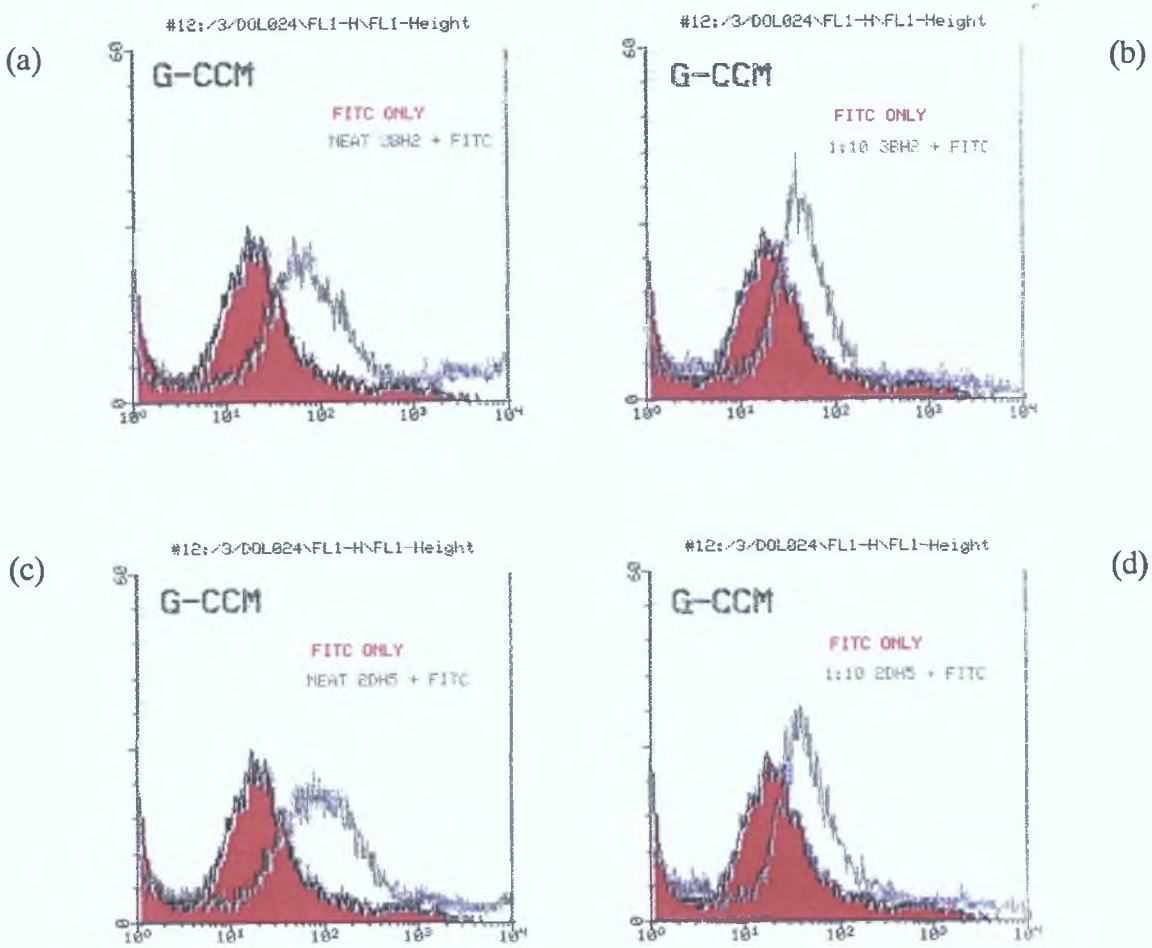


Figure 3.10.2. The FACS analysis of the binding of (a) neat SAS–treated 3BH2 ascitic fluid, (b) a 1:10 dilution of SAS–treated 3BH2 ascitic fluid, (c) neat SAS–treated 2DH5 ascitic fluid and (d) 1:10 dilution of SAS–treated 2DH5 ascitic fluid incubated with G–CCM cells is shown.

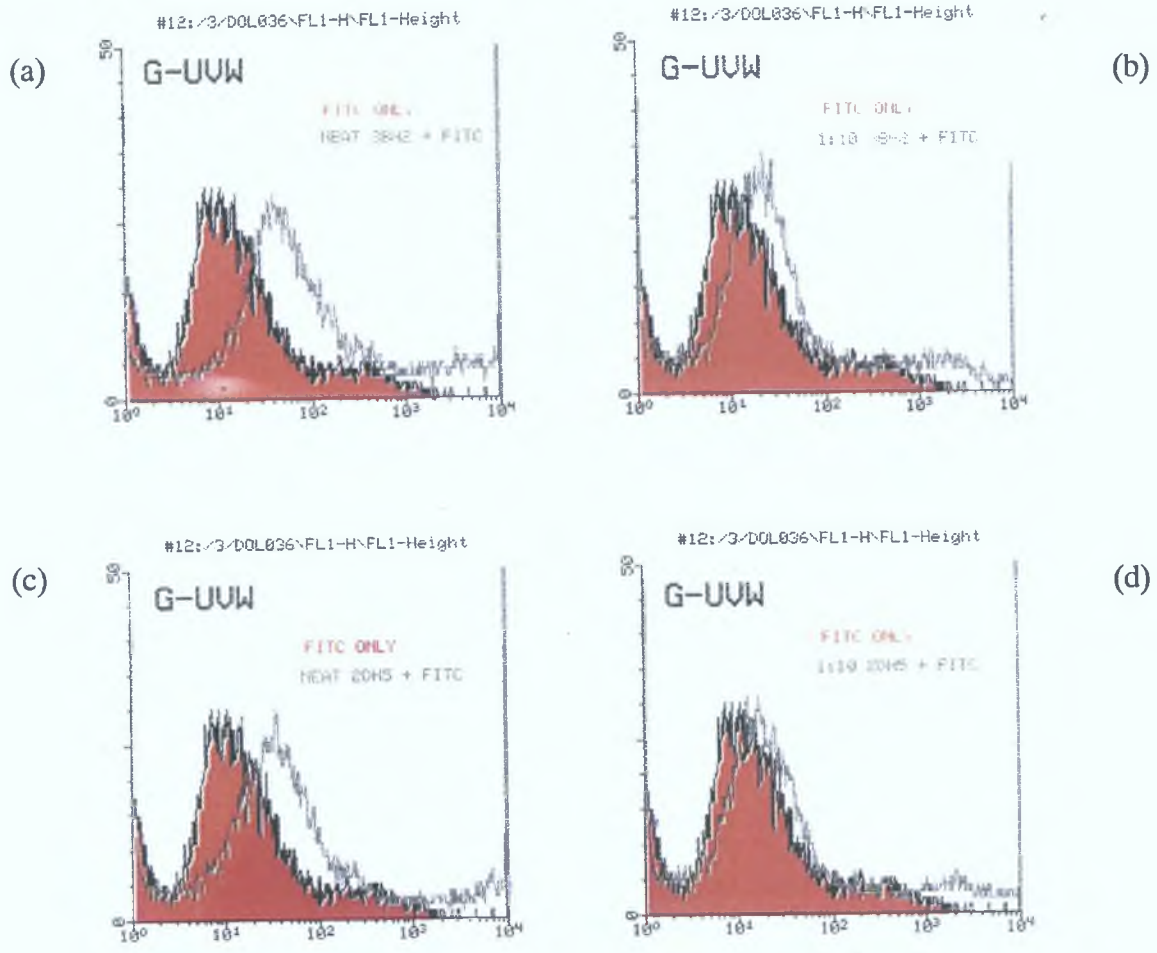


Figure 3.10.3. The FACS analysis of the binding of (a) neat SAS-treated 3BH2 ascitic fluid, (b) a 1:10 dilution of SAS-treated 3BH2 ascitic fluid, (c) neat SAS-treated 2DH5 ascitic fluid and (d) 1:10 dilution of SAS-treated 2DH5 ascitic fluid incubated with G-UVW cells is shown.

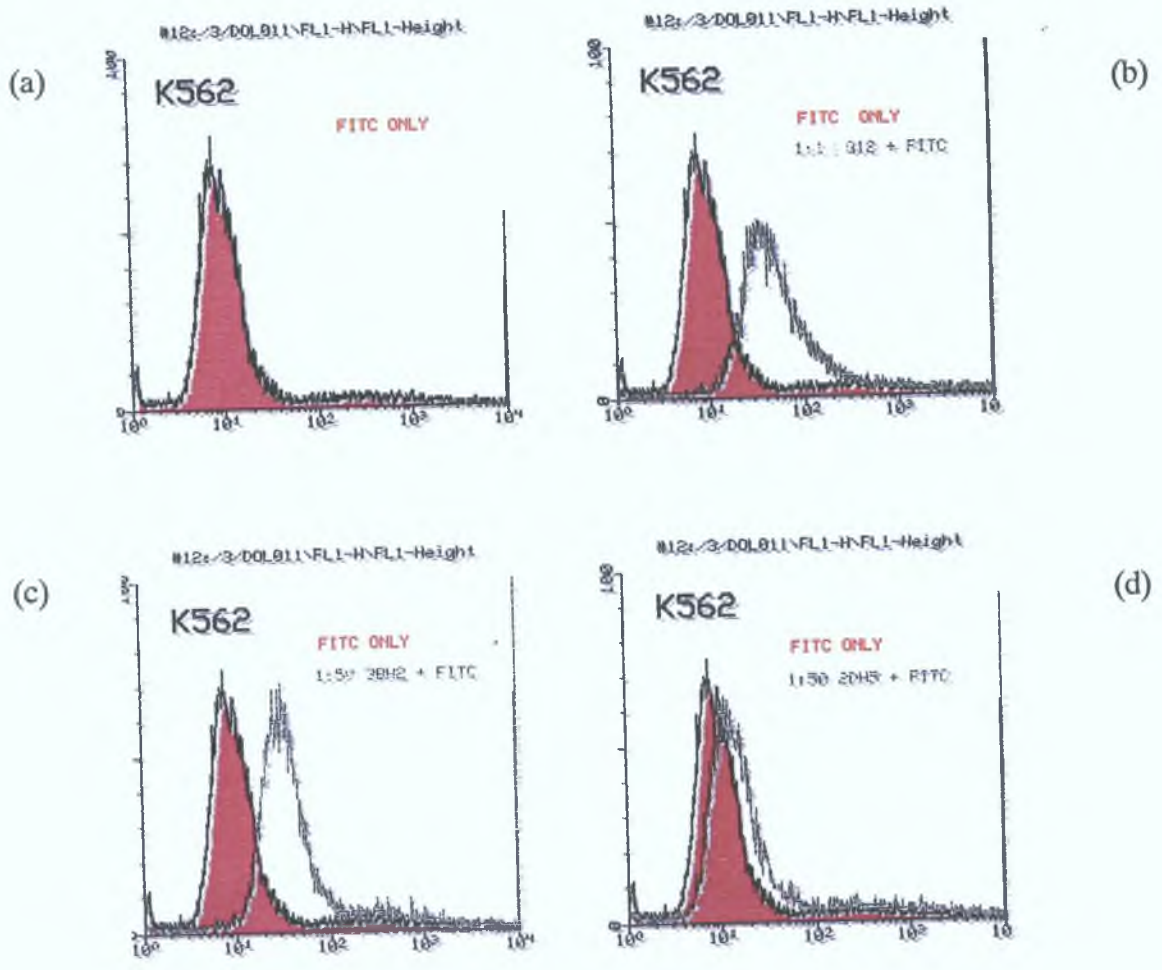


Figure 3.10.4. The FACS analysis of (a) the negative control sample (0.01M PBS, PH 7.3, instead of primary antibody), (b) the positive control sample, a 1:10 dilution of G12 ascitic fluid which reacts with the K562 human erythroleukemic cell line, (c) a 1:50 dilution of SAS-treated 2DH5 ascitic fluid incubated with the K562 cell line.

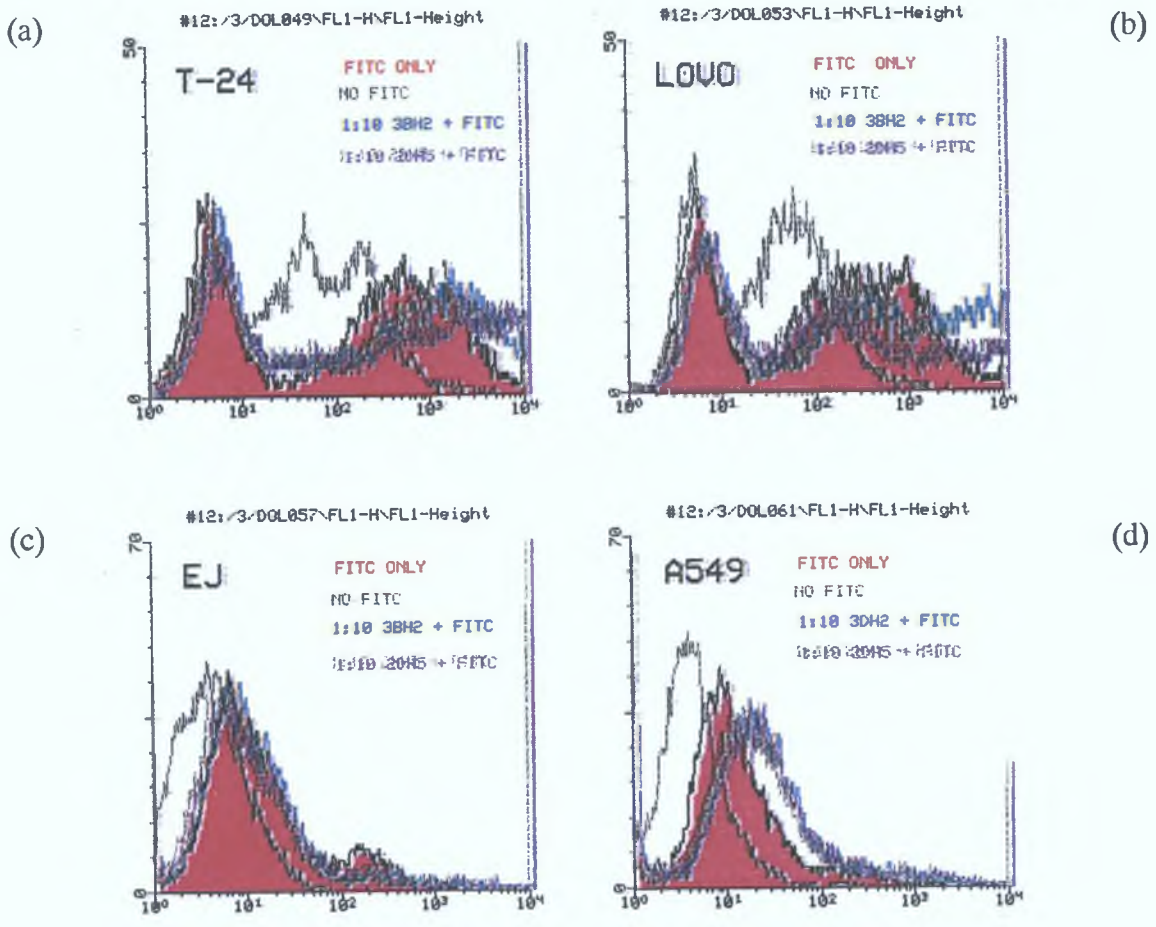


Figure 3.10.5.1. The FACS analysis of a 1:10 dilution of SAS–treated 3BH2 and 2DH5 ascitic fluid with the (a) T–24, (b) LOVO, (c) EJ–138 and (d) A549 cell lines (Table 2.2.) are given, which show that there is no specific binding between the 3BH2 or 2DH5 antibodies and these cell lines.

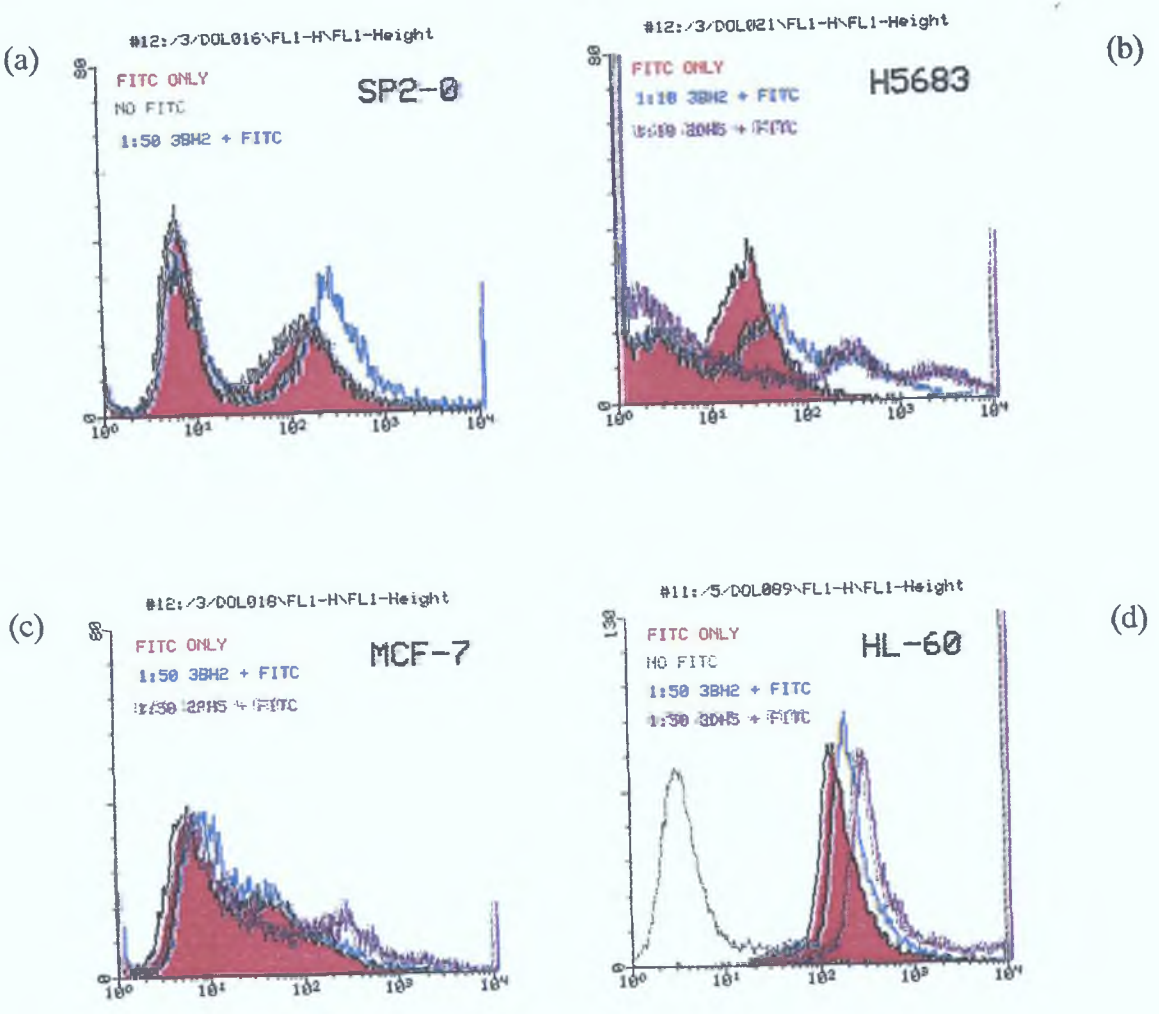
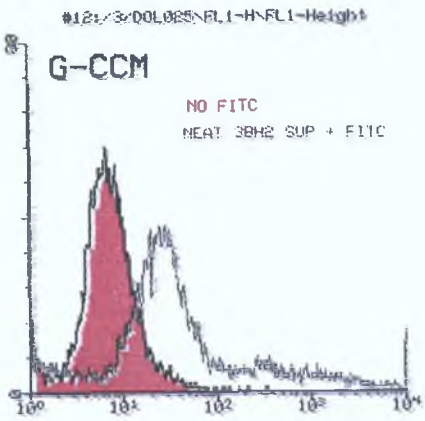
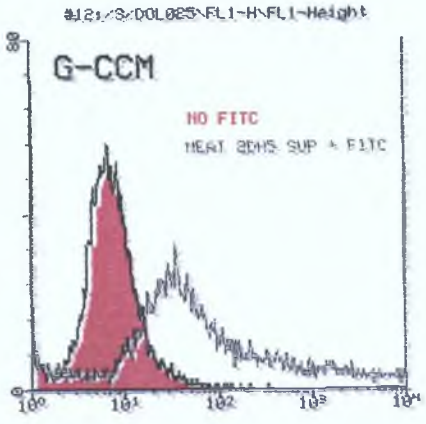


Figure 3.10.5.2. The FACS analysis of a 1:10 dilution of 3BH2 and 2DH5 SAS-treated ascitic fluid with the (a) SP2/0, (b) H5683, (c) MCF-7 and (d) HL-60 cell lines (Table 2.2.) are given, showing that there is no specific binding between the 3BH2 or 2DH5 antibodies and these cell lines.

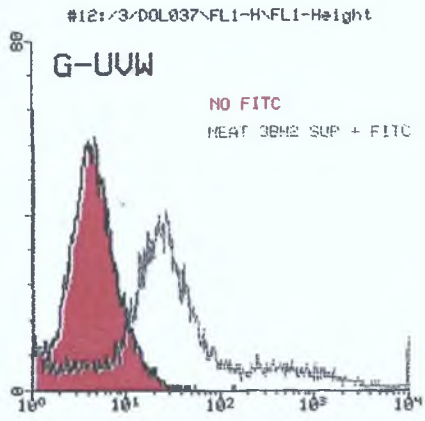
(a)



(b)



(c)



(d)

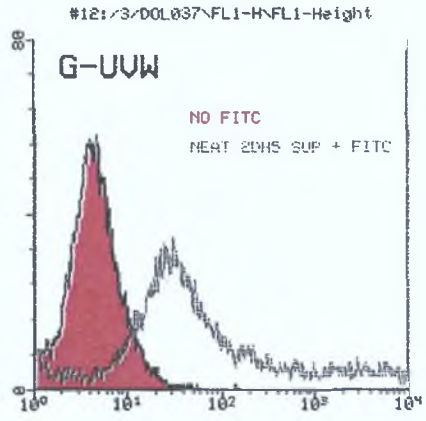


Figure 3.10.6. The results of the FACS analysis using neat antibody supernatant from; (a) 3BH2 and (b) 2DH5 hybridomas against G-CCM cells and (c) 3BH2 and (d) 2DH5 hybridomas against G-UVW cells.

3 19 Discussion.

The aims of this research were achieved, as monoclonal antibodies against the anaplastic astrocytoma cell line, G-CCM, and against commercially available GFAP were produced, purified and characterised

An anti-GFAP antibody was produced as GFAP is one of the well characterised nervous system markers Others include the three protein subunits of neurofilaments, neuro-specific enolase, myelin basic protein and S-100 protein The most important and reliable of these nervous system markers is GFAP which is a well characterised marker for the immunohistochemical diagnosis and classification of brain tumours of the glial group

This protein was isolated from fibrous astrocyte-rich multiple sclerosis plaques by Eng *et al* (1971) GFAP is apparently composed of 1-7 peptides in the range of 40,500 to 54,000 daltons and it is a definitive marker of astrocytes and their processes GFAP is a cell-specific intermediate filament protein and is the major protein constituent of astroglial filaments It plays a major role in defining and maintaining astrocyte shape under normal conditions as well as during astrocyte response to CNS injury (Noetzel, 1990)

Human GFAP is 52,000 daltons and it has been cloned by Bongcam-Rudloff *et al*, 1991) The complementary DNA (cDNA) of a human GFAP has been cloned and sequenced and the amino acid sequence deduced This sequence permitted the prediction of the complete amino acid sequence of the GFAP monomer subunit and showed its sequence homology with vimentin (67%) and desmin (65%) The chromosomal location of the GFAP gene has been established (17q21) by Southern blot hybrids and by *in situ* hybridisation (Bongcam-Rudloff *et al*, 1991)

GFAP shows species-specific amino acid sequence heterogenity, and its molecular weight ranges from 48kD for mouse, 49kD - 52kD for human, 50kD for bovine and 51kD for rat Comparison of the nucleic acid sequences between human and mouse cDNA revealed near 90% identity overall Except for small interspecies polypeptide sequence variations, GFAP appears to be a conserved protein, as its phenotypic expression is unaffected by central nervous system (CNS) mutations (Jimpy mouse) or oncologic events (human glioma) (Eng and Shiurba, 1988) Brenner *et al* (1994) studied the transcriptional regulation of the GFAP gene to gain insight into astrocyte function and also to develop an astrocyte-specific expression system They have produced

transgenic mice carrying the bacterial lacZ reporter gene linked to a 2 2kb 5'-flanking sequence derived from the human GFAP gene that previously had been shown to direct astrocyte-specific transcription in cultures In this study, they reported that this promoter also directs expression to astrocytes in the CNS This specific expression of the GFAP-lacZ transgene means that it is now possible to target expression of other heterologous genes to astrocytes *in vivo*, and to study the mechanisms of reactive gliosis at the DNA level

GFAP immunoreactivity in the mature CNS is restricted to glial filaments within protoplasmic astrocytes in grey matter and fibrous astrocytes in white matter At the surface of the brain, GFAP immunoreactivity is especially concentrated in astrocytes which form the outer limiting membrane, the glia limitans GFAP has a slower rate of turnover than any of the neurofilament proteins and this is consistent with a possible structural role for glial filaments (Smith *et al* , 1984)

In this project, fusions were performed in the hope of obtaining a monoclonal antibody which would react specifically against GFAP A second set of fusions were performed to produce a monoclonal antibody which would bind an antigen on a human astrocytoma cell line These antibodies could be used to aid in the classification of brain tumours and/or to target immunoconjugates to tumour cells to assist in the localisation and treatment of brain tumours (Section 1 7) The tumour cell line used, G-CCM, was diagnosed as grade IV malignant glioma and as such was probably relatively strongly antigenic Eng (1982) stated that GFAP is very antigenic, since samples heavily contaminated with tubulin elicited antibodies primarily to GFAP and not to tubulin Hybridomas secreting antibody which reacted strongly with G-CCM were produced from fusions following immunisation with this cell line The antibody 3BH2 was selected on the basis of this initial screening and results of its purification, binding activity to a range of cell types, class and characterisation were presented The monoclonal antibody designated 2DH5 was produced following immunisation with the commercially available GFAP, both *in vivo* and *in vitro*, and was chosen for further study for the corresponding reasons as 3BH2 Both monoclonal antibodies reacted strongly to their antigens, were stable to freeze-thawing, produced large amounts of antibody and grew well in culture

Since G-CCM proved to be a GFAP-positive cell line, all the antibodies produced from the fusions involving G-CCM immunised spleen cells were screened against GFAP for

binding activity by the ELISA method to determine if any of the hybridomas obtained, produced an antibody which reacted with G-CCM and GFAP As whole cells were used for immunisation purposes, it was not expected that antibodies to an intracellular component, such as GFAP, would be produced However, since the 3BH2 antibody recognised epitopes similar to GFAP, the possibility was investigated These monoclonal antibodies were initially screened against G-CCM, G-UVW and GFAP by ELISA and the results are given in Tables 3 3 and 3 4

The titres and working dilutions of the 3BH2 and 2DH5 antibodies were outlined in Section 3 7 These two monoclonal antibodies were screened against other cell lines available in the laboratory (Table 3 5) The only cell line which showed some cross-reactivity with the anti-G-CCM antibody was K562

As part of the initial screening, the supernatants from the positive hybridomas produced during this project were isotyped All of the antibodies produced were IgM However, using the isotyping strips from Sigma (Figure 3 1), 3BH2 isotyped as IgG and IgM, with kappa light chains The Serotec isotyping kit (Table 2 1) consistently gave an isotype of IgG1 and IgM for both 3BH2A3 and 2DH5A10

When these monoclonal antibodies isotyped as IgM, it was, initially, considered a surprising result as the mice used to produce these monoclonal antibodies had been hyperimmunised However, at this time, other researchers in D C U attempting to produce monoclonal antibodies were obtaining similar results The possibility of infection of the balb/c colony with the mouse hepatitis virus (MHV) was suspected Ten serum samples from mice from the D C U mouse colony were tested by the Microbiology Laboratories, 56 Northumberland Road, Middlesex HA2 7RE, and two mice tested positive, by ELISA, for MHV

MHV is a single-stranded RNA virus, family Coronaviridae, genus *Coronavirus* MHV is described as being extremely contagious It is one of the most ubiquitous infections of laboratory mice worldwide, with reported prevalence rates often exceeding 80% (Committee on infectious diseases of mice and rats, 1991) MHV is a frequent contaminant of transplantable tumours and cell lines It has been reported to alter many experimental results including alterations in immune function In immunocompetent mice MHV infection causes, among a range of symptoms, immunosuppression or immunostimulation during acute infection and chronic immunodepression in persistent infection (Committee on infectious diseases of mice and rats, 1991) There is widespread belief

that coronaviruses can cause asyptomatic persistent infection with virus shedding over a period of months In an MHV epidemic in a mouse colony, the spectrum of diseases range from inapparent infection to overwhelming infection and death (Holmes *et al*, 1986) MHV epidemics in laboratory animal colonies have been implicated as a possible cause of variability in diverse experimental protocols

MHV can infect ascitic fluid and lymphoid cells Viruses remaining in a cell, without overtly damaging or destroying it, can sometimes alter its specialised functions These features enable certain viruses to invade the very tissues and cells in which the immune responses are generated, causing alterations in immune function B cells can also be rendered immunologically ineffective by exposure to large amounts of virus (Mims, 1986) The extensive use of mice for the production of hybridoma antibodies in ascitic fluid may present an opportunity for dissemination of MHV in animal colonies One strain of MHV (MHV-A59) can readily infect hybridoma cells generated by fusion of murine splenic lymphocytes with myeloma cells (Holmes *et al*, 1986) It is therefore possible that hybridomas may become infected when, during the preparation of hyperimmune ascitic fluid, they are passaged in the peritoneal cavity of mice with inapparent MHV infections The contaminated hybridoma cells might then transmit MHV to other mice, thus becoming a source for spread of infection within a mouse colony This illustrates the importance of eliminating MHV from mouse colonies in order to prevent compromise of research data There is currently a commercially available MHV antibody test ELISA system from Organon Teknika (Science Park, Milton Road, Cambridge CB4 4BH) which could be used to routinely test for the presence of MHV

An alternative to using mice for the production of ascitic fluid could be systems like Technomouse This is a recent *in vitro* cell culture system from Northumbria Biologicals that is designed to provide an economical and automated alternative for the production of large amounts of monoclonal antibodies The system consists of five bioreactor cassettes, each equivalent in production terms to 20 laboratory mice (Product review, 1992)

It was thought initially when these antibodies isotyped as IgM that problems would be experienced storing and purifying the ascitic fluid obtained using these monoclonal antibodies However, few problems were experienced in purifying and using the ascitic fluid obtained from the 3BH2 and 2DH5 hybridomas In fact, when SAS-treated ascitic

fluid from these monoclonal antibodies was examined by HPLC only one major peak was obtained, at 16 42mins for anti-G-CCM (Figure 3 3 2) and 16 86 for anti-GFAP (Figure 3 3 4) This corresponded to mouse IgG This result was confirmed by SDS-PAGE (Figure 3 5)

If required, however, IgM monoclonal antibodies could have been purified by a method based on the low solubility of IgM in dilute salt solutions and on the ability of gel-permeation matrices to desalt protein solutions (Bouvet and Pirès, 1991) Knutson *et al* (1991) described a method for the purification of murine monoclonal antibodies of the IgM class, where ascitic fluid was subjected to ammonium sulphate precipitation, gel filtration chromatography on Ultrogel AcA22 and finally ion exchange chromatography on DEAE-sepharose They found that although the antibody was greater than 85% pure after gel filtration and greater than 95% pure after ion exchange chromatography, only 50% of the antibody activity was recovered after the gel filtration purification and only 10% of the initial antibody activity was recovered after the ion exchange chromatography Therefore, since the recovery of *immunoreactive* IgM antibody was the goal in this project, and similar findings were obtained using the protein G-purified sample in this study, the ammonium sulphate-treated ascitic fluid was used for applications with these antibodies (Knutson *et al* , 1991) Only small amounts of ascitic fluid were used for purification purposes as, on storage, crude ascitic fluid was found to be more stable at -20°C than purified antibody Crude ascitic fluid was probably more stable due to the higher protein concentration

It is also possible to purify IgM, and IgG, ascitic fluid using a hydroxylapatite HPLC under very mild conditions (Josic *et al* , 1991) In their study, both types of monoclonal antibody were purified, the problem of precipitation of IgM antibodies at low salt concentrations at the beginning of a chromatographic run was avoided by adding sodium chloride to the separation buffers It could have also been possible to attempt to isolate IgG1-secreting switch variants from IgM hybridomas by a two-site ELISA (Mercken *et al* , 1991) or by subcloning (Faguet and Agee, 1993)

The binding activity of the antibodies was initially checked by dot immunoblotting (Figures 3 4 1 and 3 4 2) A change in the commercial GFAP purchased from ICN Immunobiologicals (Section 3 12) was suspected by ELISA and confirmed by dot blotting Figure 3 4 1 showed that 2DH5 reacted with GFAP from lot 17 Dot blotting confirmed that 3BH2 reacted specifically with G-CCM A positive result was also

observed for the cell line $IJK_{(1)}$ on all blots, including the negative control (Figure 3 4 2) $IJK_{(1)}$ was subsequently shown to be cross-contaminated with the C_6 rat glioma cells (Section 3 12) and this apparently positive reaction was probably due to non-specific binding to these cells of the secondary antibody used

The initial Western blot used a 1 5 dilution of the 3BH2 monoclonal antibody supernatant and only a faint, single band was obtained when this antibody was screened against the G-CCM and G-UVW cell lines This band corresponds to the band obtained when GFAP was probed with the commercially available anti-GFAP monoclonal antibody (Figure 3 6 1)

Another Western blot was prepared (Figure 3 6 2) using (i) SAS-treated 3BH2 and (ii) 2DH5 ascitic fluid and a single band was obtained when each antibody was screened against cell membranes prepared from G-CCM and G-UVW A large band at approximately 50kD was seen in all cases when these two antibodies, and the commercially available anti-GFAP antibody, were tested against GFAP This large band was due to denaturation of this protein on storage or the loading of too much protein on the gel This result suggested that the antigen(s) recognised by these two antibodies was similar in nature

The affinity constants for (a) the 3BH2A3 antibody and (b) the commercially available anti-GFAP, showing that both antibodies reacted with GFAP, were obtained using the BIAcore sensor system (Borrebaeck *et al*, 1992, VanCott *et al*, 1992) (Section 3 15) The affinity constant for commercially available anti-GFAP against GFAP was found to be $9\ 6x10^{8}mol^{-1}$ The affinity constant for 3BH2A3 against GFAP was $2\ 95x10^{7}mol^{-1}$ Using immunocytochemistry (Section 3 16), it was seen that the antigen recognised by 3BH2A3 was found on the cell surface and in the cytoplasm of dead cells In Figure 3 8 7, a cytospin preparation of G-CCM cells, incubated with the 3BH2A3 monoclonal antibody and stained using the APAAP detection system, showed staining of the astrocytic processes of G-CCM cells Preliminary *in situ* hybridisation experiments performed on mouse cultured cells with biotinylated cDNA probes indicated that specific GFAP mRNAs are present along astrocytic processes, suggesting a subcellular sorting of the GFAP (Rataboul *et al*, 1988) Iwa *et al* (1988) showed that in astrocytomas, using an avidin-biotin-peroxidase complex method with a monoclonal antibody against GFAP, the cell bodies and processes were positive with delicate fibrillary patterns, in anaplastic astrocytoma, the cytoplasm and the processes were intensively stained

From immunofluorescence studies on live cells, it was seen that the antigen recognised by 3BH2A3 was on the cell surface (Figure 3 9 1) When the G-CCM cells were tested by indirect immunofluorescence using 3BH2, as the primary antibody, a green-yellow ring around the cell surface was clearly seen (Figure 3 9) This is indicative of cell surface localisation of the antigen recognised (Schachner, 1982) When the G-CCM cells were tested with the 2DH5 monoclonal antibody a fluorescent ring was also seen On testing G-CCM cells with commercially obtained anti-GFAP monoclonal antibody by this method, no fluorescent ring was visible around the cell surface This result was expected as GFAP is intracellular in location and, therefore, would not be detected in live cells by this method These results show that the reactivity of the 3BH2 monoclonal antibody is immunologically similar to the anti-GFAP monoclonal antibody, but is not identical

Cytoplasmic staining of some cells would indicate that the antigen recognised by these two antibodies may also be intracellular in location It was not possible to detect intracellular binding on live cells by immunofluorescence, but it was possible to detect intracellular binding on fixed, cytospin preparations

From the FACS analysis it was seen that the two antibodies recognised antigens which were similar but not identical The results obtained when 3BH2A3 and 2DH5A10 were screened against G-CCM and G-UVW were very similar However, differences were noticed when these antibodies were screened against other cell line Although the antibody concentrations were the same, the anti-G-CCM antibody reacted more strongly to the K562 cell line than did the anti-GFAP monoclonal antibody (Figure 3 4 10) In Figure 3 10 5 2 , the anti-GFAP antibody reacted with the cell line HL-60 (Table 2 2) whereas the anti G-CCM antibody did not

These results indicate that the antigen, or antigens, recognised by the 3BH2A3 monoclonal antibody on the G-CCM and G-UVW is located on the cell surface This conclusion is substantiated by (i) the positive results obtained by Western blotting of cell membrane preparations, (ii) the result obtained by indirect immunofluoresence on live cells, (iii) FACS analysis of these two antibodies In the case of the antigen recognised by 3BH2A3, it is difficult to definately confirm the exact nature of the antigen

In a study by De Muralt *et al* (1987), among the primary cultures analysed, cells from 21/41 malignant astrocytomas were found to contain GFAP The synthesis of this protein was generally observed up to the fifth passage After more than ten passages only five

lines out of 21 which were originally positive still retained the capacity to produce GFAP The reactivity spectrum of the three anti-glioma monoclonal antibodies used in the study of DeMuralt and co-workers demonstrated that none of them were directed against a true tumour-specific antigen expressed only by gliomas and not by other tumours or normal tissue The two monoclonal antibodies described in this study appeared to be directed against two antigenic determinants on the same polypeptide chain with an apparent molecular weight of 48kD, with similar phenotypes to GFAP Immunocytochemical analysis, however, showed that two of the antibodies immunoprecipitated the same polypeptide chain with an apparent molecular weight of 48kD From these results, it was concluded that the two monoclonal antibodies recognise two different antigenic determinants present on the same molecule Experiments using immunoperoxidase staining of frozen tissue sections in the study of DeMuralt *et al* (1987) showed that the recognised antigen is expressed not only on the cell membrane but also in the cytoplasm of tumour astrocytes It is possible the monoclonal antibodies produced in this thesis recognise the same or a similar antigen to that of DeMuralt *et al* (1987) Further work needs to be performed to investigate the antigen or epitope on the astrocytoma cell lines used in this thesis which are recognised by 3BH2A3 and 2DH5A10 study

Krauss *et al* (1994) produced a polyclonal against the high molecular weight neurofilament polypeptide obtained from cytoskeletal extracts of bovine spinal cord This antibody reacted with the immunogen, with the middle molecular weight neurofilament polypeptide and with a 51kD protein, GFAP, but not with the low molecular weight neurofilament polypeptide When this antibody was affinity purified with GFAP, it reacted with both purified GFAP and high molecular weight neurofilament polypeptide They reported that digestion with alpha-chemotrypsin was used to determine the recognition site of the affinity purified antibodies and only fragments of the tail domain of the high molecular weight neurofilament polypeptide reacted with the antibody They proposed that common epitopes exist between the variable C-terminal domains of this neurofilament polypeptide and GFAP

The majority of primary CNS neoplasms are anaplastic astrocytomas and supratentorial glioblastoma multiforme Even though these tumours generally do not metastasize outside of the cranial vault, they carry a grave prognosis The difficulty in discerning

the margin between the growing tumour edge and normal brain parenchyma and the restricted permeability of the blood-brain barrier to imaging and chemotherapeutic agents contribute to the difficulty in managing these diseases (Section 1 8) Better visualisation techniques could potentially aid the neurosurgeon in (a) making a decision to attempt surgical resection, (b) visualising and removing classically described finger-like extensions of the glioblastoma, and (c) minimising the removal of normal brain parenchyma (Kornguth *et al* , 1989) The improved definition of the tumour border and of the finger-like extensions of glioblastomas may also permit more effective stereotactic interstitial irradiation of the tumour

Malignant gliomas are invariably fatal Various therapeutic regimens have been developed as adjuvant therapy The preparation of biocompatible materials, which bind tumour cells to a greater extent than they bind to vascular endothelial cells, may permit the development of targeting agents for delivery of molecules that are effective in imaging and chemotherapy (Section 1 6) At best, however, these regimens have only been modestly successful at extending survival

It is now generally agreed that the GFAP antiserum stains only astrocytes of the CNS GFAP is proving to be of particular diagnostic importance in the following problems (1) the demonstration of astrocytic cellular differentiation in either primitative or highly anaplastic CNS tumours, (2) the study of mixed tumours of the CNS, (3) the diagnosis of gliomas invading or metastasising to the meninges and in extra neural sites, and (4) the exclusion of non-glial tumours that may superficially resemble astrocytomas (Eng, 1982) The immunohistochemical technique of Eng and Rubinstein (1978) has become an essential tool for the more precise identification of diagnostically difficult tumours

The following lines of evidence indicate that GFAP is the principal protein constituent of the glial filament (1) GFAP can be isolated from nervous tissue preferentially enriched in fibrous astrocytes containing glial filaments (e g multiple sclerosis plaques), (2) antibody specific for GFAP immunocytochemically labels only astrocytes at the light-microscopic level and glial filaments at the electron-microscopic level, (3) intermediate filaments isolated from normal bovine brain and spinal cord have chemical properties similar to GFAP from multiple sclerosis plaques

Since the initial reports describing the use of GFAP antiserum for tumour diagnosis (Deck *et al* , 1976), polyclonal and monoclonal antibodies to GFAP are used routinely in medical centers throughout the world to assist in the diagnosis of human neoplasms

For instance, an inverse relationship between the amount of GFAP and the degree of anaplasia has been found for astrocyte neoplasms including glioblastomas (Eng and Rubinstein, 1978) The presence of GFAP continues to be the main test for the degree of differentiation, the degree of anaplasia (dedifferentiation), and aberrant differentiation for those investigators studying molecular aspects of glial neoplasms

Distinct antigenic heterogenity is evident among and within brain tumours, and patterns of antigenic expression can change continuously (Section 1 8) Monoclonal antibodies that recognise these antigens facilitate the objective typing of intracranial tumours It is possible that antibodies, including the anti-G-CCM and anti-GFAP monoclonal antibodies developed during this project, could serve as carriers for radionuclides and cytostatic or cytotoxic agents The principal strategy for immunologic management of tumours is the development of corresponding complex immunologic probes for diagnosis and treatment Instead of using a single probe or carrier immune complex, panels of monoclonal antibodies may serve as carriers for agents to detect and destroy tumours (Section 1 9)

In the past, a frequent problem with anti-GFAP polyclonal antibodies was the question of GFAP specifity With the development of GFAP-specific monoclonal antibodies, these questions have been largely resolved and those monoclonal antibodies with a high degree of specificity can be used in diagnosis of neurosurgical tumour biopsies and in experimental neurooncology A retrospective immunochemical survey of 71 intracranial and intraspinal neoplasms with a "cocktail" of three monoclonal antibodies to GFAP yielded immunostaining results, with one exception, identical to those obtained with a highly specific polyvalent GFAP antiserum (McLendon *et al*, 1986) These three monoclonal antibodies represent a continuous supply of well-defined, monospecific reagents It is hoped that the antibodies produced and characterised during this project could contribute to the application of monoclonal anti-GFAP antibody "cocktails" which may provide a standard reagent for large, multi-institutional studies of human brain tumours

In summary, GFAP has become an immunohistochemical marker for determining glial origin of many human brain tumours In this project, monoclonal antibodies against GFAP and the brain tumour cell line, G-CCM, were produced, purified and characterised The long range experimental goals of this work are to use operationally specific anti-glioma monoclonal antibodies for *in vivo* imaging and therapeutic carrier

studies in experimental animals prior to clinical investigation The future of immunotherapy in the management of patients with anaplastic gliomas remains to be defined, however, the multi-modality approach, combining radiotherapy, chemotherapy and immunotherapy has a strong appeal

SECTION 4 PRODUCTION OF ANTI-IODINATED BOLTON HUNTER REAGENT (IBHR) ANTIBODIES

4 Outine

The aim of this project was to produce antibodies against the iodinated Bolton-Hunter reagent (IBHR) This was carried out as iodine's radioisotopes have the highest specific activity of any radioisotope If applied to the clinical situation, as an antibody against radiolabelled IBHR, it could be used in the detection and localisation of tumours (reviewed by O'Kennedy *et al* , 1993)

In order to immunise animals to produce an anti-IBHR antibody, Bolton-Hunter reagent (BHR) was iodinated and this IBHR was conjugated to BSA, ovalbumin (IBHR-oval) and KLH The protein and iodide content of these conjugates were determined The IBHR-BSA conjugate was then used as an immunogen to try to produce monoclonal antibodies against the IBHR hapten in five balb/c mice and one New Zealand White rabbit The IBHR-ovalbumin and IBHR-KLH conjugates were used to screen for these anti-IBHR antibodies The production of monoclonal anti-IBHR antibodies was not successful and the polyclonal approach was chosen This rabbit polyclonal antibody was purified and subsequently characterised by ELISA, HPLC and SDS-PAGE It was also used to produce heteroconjugates between this anti-IBHR polyclonal antibody and (i) the monoclonal anti-G-CCM, (ii) monoclonal anti-GFAP and (iii) monoclonal anti-r-Ricin A (Section 5) In order to detect the presence of the heteroconjugate, by a non-radioactive method, the IBHR-BSA and IBHR-KLH conjugates were further labelled with FITC and detected by immunofluorescence and FACS

4 1 Introduction

Isotopes conjugated to antibodies have distinct clinical applications, either in diagnosis by immunoscintigraphy or treatment by radioimmunotherapy (O'Kennedy *et al* , 1993) Radioactive iodine (^{125}I) could have been used during this project as it can be detected with great sensitivity by simple methods that are virtually unaffected by the reaction milieu and independent of other environmental factors Proteins radiolabelled with (^{125}I) have the advantage that they can be easily detected (Section 1 13), however they have the disadvantage that they tend to lose activity upon storage faster than dictated by the half-life of the isotope (Johnstone and Thorpe, 1982) This can be explained due to a number of factors, (i) Radiation Destruction the products of the radioactive decay can damage the protein (ii) Loss of Iodine the protein-iodine molecule can break down to release free radioiodine and protein (iii) Protein Deterioration some proteins tend to

aggregate, with subsequent loss of activity upon iodination Furthermore, radiolabels have drawbacks associated with health risks and in the disposal of radioactive waste For these reasons, non-radioactively labelled iodide was used in this project The use of cold iodine as a label eliminated the problems associated with the use of radioactivity and with the stability of the isotope A molecule, which could be readily iodinated, was developed in 1973 by Bolton and Hunter The molecule they used was 3-(4-hydroxyphenyl) propionic acid N-hyroxysuccinimide ester, now known as Bolton-Hunter reagent (BHR) The BHR was developed to carry iodine which may or may not be radioactively labelled In this project, the IBHR was not detected using radioactivity but by measuring the catalytic effect of iodide on the reduction of ceric ammonium sulphate by arsenious acid which was first described by Sandell and Kolthoff (1934) O'Kennedy *et al* (1989) described a microassay system for the determination of iodide and iodine-containing compounds based on the Sandell-Kolthoff reaction This was later optimised and applied (Keating *et al* , 1991, O'Kennedy and Keating, 1993)

4 2 Iodination of the BHR and testing the purity of the IBHR produced

There are many techniques to iodinate proteins, for example MacFarlane (1958) introduced the use of iodine monochloride (ICl) This method can be used to iodinate the BHR since it is a phenol and can be iodinated under the same conditions as the tyrosine in proteins (Section 2 4 1) The important advantages of this method is that the total amount of iodine incorporated into the iodinated material is known and can be controlled simply by limiting the amount of ICl available for iodination (Doran and Spar, 1980) and that the protein to be conjugated is not exposed to harsh oxidising and reducing agents

The purity of the IBHR preparation was tested by thin-layer chromatography on fluorescent silica gel plates developed in ethyl acetate toluene (1 1, v/v), where,

$$R_f = \frac{\text{Average spot migration}}{\text{Average solvent front}} = \frac{3\ 833\text{cm}}{8\ 3\text{cm}} = 0\ 46$$

This was compared on the same thin-layer chromatography plate with a previous iodination of the same BHR, this gave an R_f of 0 46 The IBHR used in O'Kennedy *et al* (1989) was run simultaneously and also gave an R_f of 0 46 The difference in the reported R_f values was due to the use of different plates

4 3 Conjugation of IBHR to proteins and purification of the IBHR-protein product

BSA, ovalbumin and KLH were labelled with IBHR The free IBHR was separated from bound IBHR by gel filtration according to O'Kennedy *et al* (1989) (Section 2 4 6) This was done using an Ultrogel AcA44 column and 1ml fractions were collected The fractions which contained the free IBHR and the IBHR-protein conjugate were determined using the Bio-Rad micro-Bradford assay (Section 2 4 9 3) The absorbance of every sample of the IBHR-protein conjugates was taken and the readings of every fifth fraction is shown in Figure 4 1 The first peak on this graph represents iodinated protein No protein was found in the second peak by the Bio-Rad micro-Bradford assay This peak represents unconjugated IBHR as detected using the iodide assay (Section 2 4 9 2)

In the case of IBHR-BSA, samples 18 to 30 were pooled, for the IBHR-ovalbumin conjugate, samples 19 to 30 were pooled and with IBH-KLH, samples 16 to 28 were pooled, to provide the conjugate sample

4 4 Determination of the amount of iodine in the IBHR-protein conjugates produced

The iodine in the conjugate was detected using an iodine assay based on the Sandell-Kolthoff reaction, (Sandell and Kolthoff, 1934), who first reported that a minute quantity of iodide could be detected by measuring its catalytic effect on the slow cerium(IV) to arsenic(III) reaction,

$$2Ce^{4+} + As^{3+} \text{ ------> } 2Ce^{3+} + As^{5+}$$

The cerium (IV) is yellow in colour but the resulting product is colourless The reaction can be followed spectrophotometrically by measuring the intensity of the cerium yellow colour after a defined time period This means that the rate of catalysis, and thus the amount of iodine, can be calculated by the degree of colour present which is read at 414nm A microassay was developed and later optimised by O'Kennedy *et al* (1989) The absorbances for this iodide microassay were read each minute for 5 minutes in order to correlate the best data for the assay The results obtained are shown in Figure 4 2

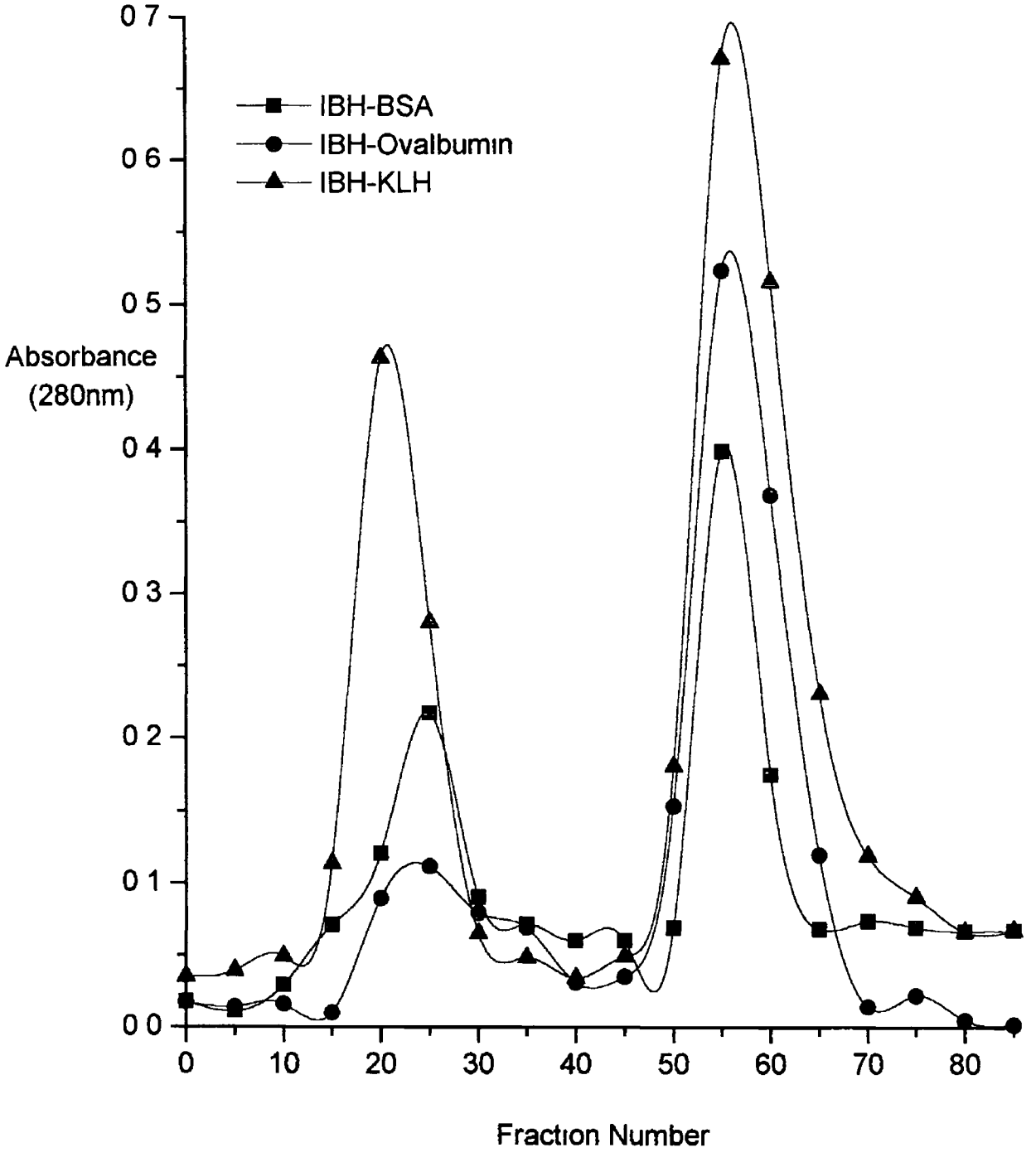


Figure 4.1. Isolation of IBHR labelled BSA, ovalbumin and KLH conjugates by chromatography on Ultrogel AcA44 (16mm internal diameter x 360mm) The eluant was 0 01M PBS, pH 9 0, the flow rate was 1ml/min and 1ml fractions were collected The absorbance reading of every sample was monitored at 280nm (A_{280}) and the absorbance of every fifth sample is given The first peak (fractions 16-30) represents the iodinated protein No protein was found in the second peak (fractions 50-70) which represents unconjugated IBHR

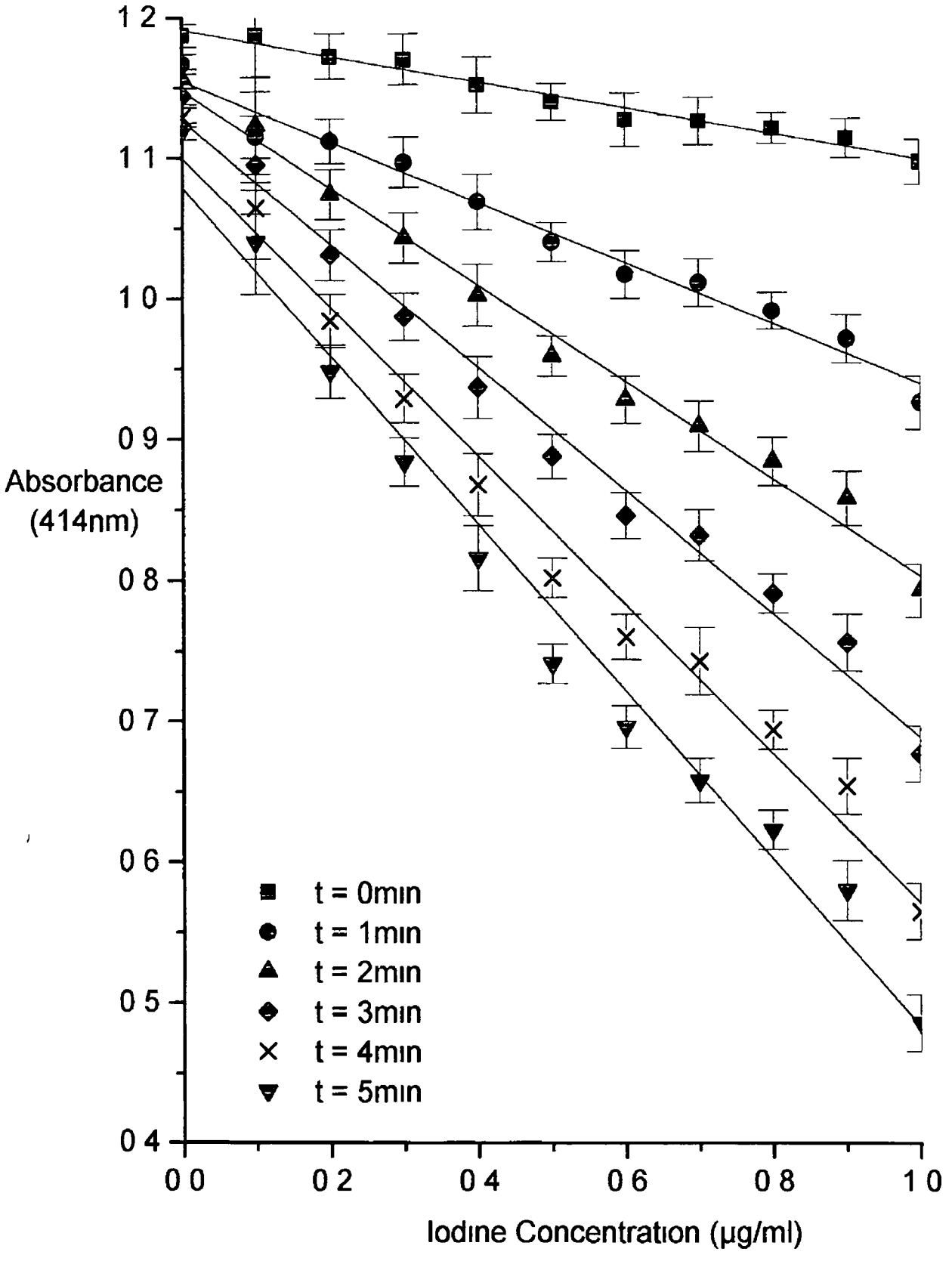


Figure 4.2. Calculation of Iodide concentrations using the Iodide assay Absorbance readings taken every minute for 5min measured at 414nm (A_{414}) from the Iodide assay The number of determinations (n) was 3

4.5 Determination of the amount of protein in the IBHR-protein conjugates produced

Since IBHR hapten conjugation introduces groups into the protein (Knight and Welch, 1978) that absorb at 280nm, the use of absorbance at 280nm as a measure of protein concentration is no longer accurate The protein in the conjugate may be detected using a variety of protein assays IBHR was found to interfere with the BCA protein assay (Section 2 4 9 3) (Smith *et al* , 1985)

The assay chosen to determine protein concentration was the Bio-Rad micro-Bradford assay, (Section 2 4 9 3) (Bradford, 1976) BHR interferes with the Bio-Rad micro-Bradford assay To investigate this interference a standard curve was set up with two sets of BSA standards, one being spiked with a known concentration of IBHR The results of the extent of this interference are shown in Figure 4 4

In order to quantify the effect of the IBHR on the results of the Bio-Rad micro-Bradford assay, determinations of protein concentration were made using an IBHR concentration equivalent to that determined in the IBHR-BSA conjugate as a zero control in the assay, the protein concentration could then be directly measured

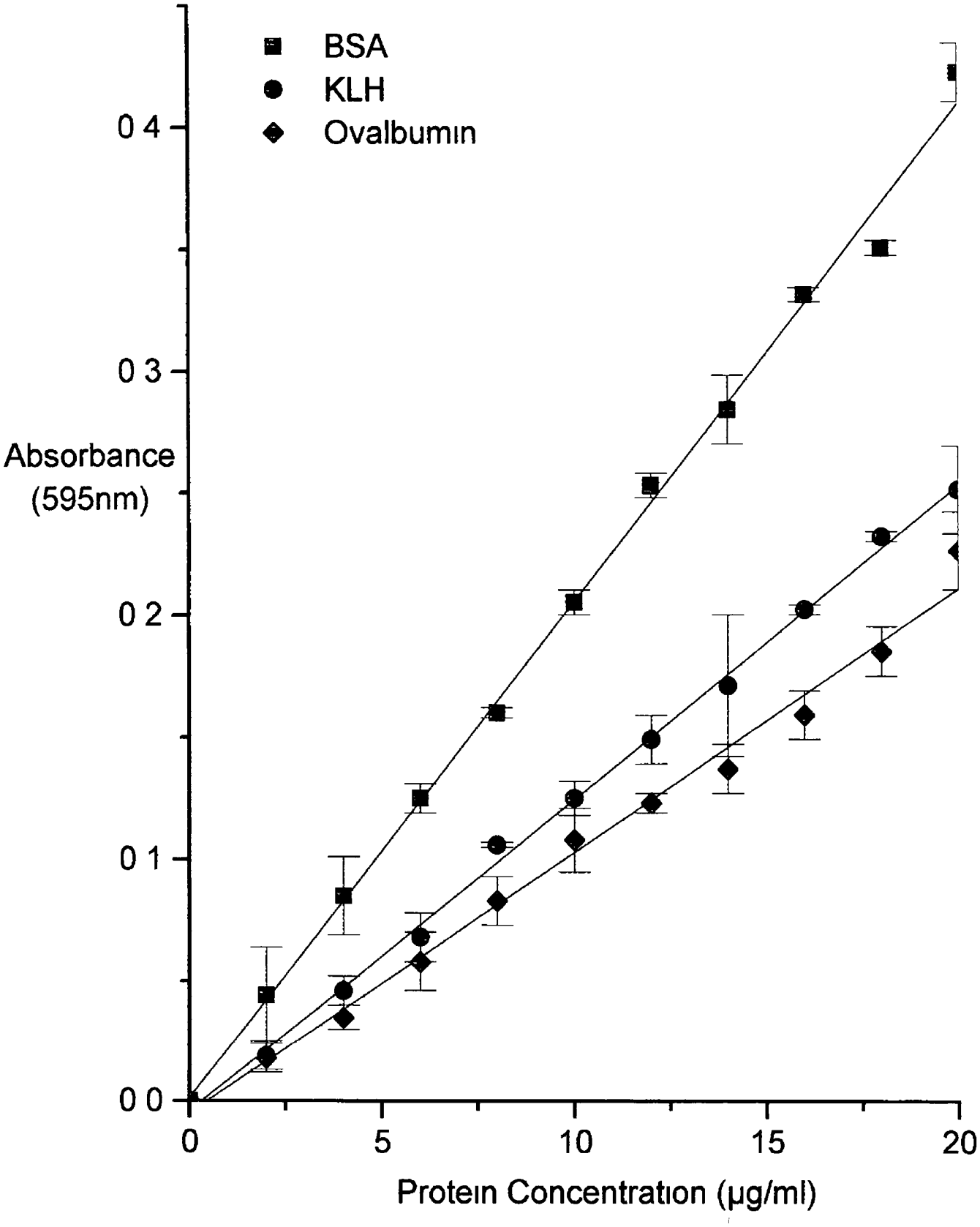


Figure 4.3. Absorbances read at 595nm (A_{595}) from the Bradford microassay procedure to determine protein concentrations in the range 0-20μg/ml for BSA, KLH and ovalbumin, the number of determinations was 3 (n=3)

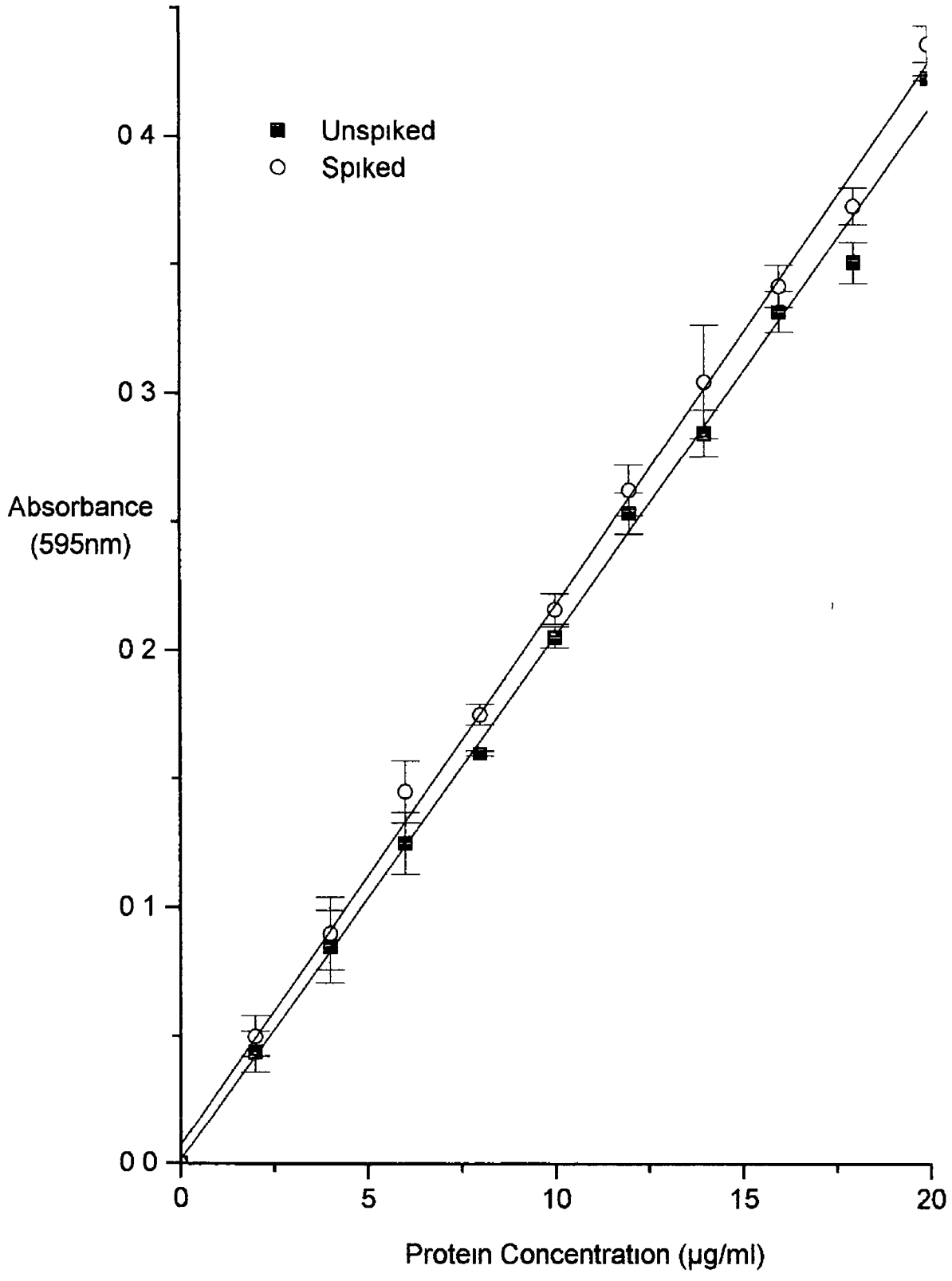


Figure 4.4. Results from the Bradford microassay procedure to investigate the interference of IBHR with this assay Duplicate BSA standards (0-20μg/ml) were prepared and one set was spiked with 2μg/ml IBHR The absorbance was read at 595nm (A_{595}) and the number of determinations was 3

4 6 Determination of the concentration ratios of iodide and protein in each conjugate.

The molar ratios of iodine and protein in each conjugate were calculated based on the following molecular weights, BHR=263 25D, I=126 92D, BSA=68kD, KLH=3,000kD and ovalbumin=45kD

The protein concentrations were found to be in the same range BSA was 7 0mg/ml, ovalbumin was 12 0mg/ml and KLH gave a reading of 8 0mg/ml The iodide concentrations were also similar, with IBHR-BSA being 161 6mg/ml, IBHR-ovalbumin was 136 0 and IBHR-KLH was 144 8mg/ml The IBHR to protein ratios (μg mg) show that BSA has the largest number of IBHR haptens per mg of protein (BSA has 59 lysines of which 30-35 are available for coupling) and was, therefore, chosen as the immunogen in the production of anti-IBHR antibodies as it had the largest IBHR concentration per mg of protein The results are shown in Table 4 1

Table 4 1 The concentration of iodide and protein in the conjugates produced were determined.

Conjugate	Iodide Conc (μg/ml)	Protein Conc (mg/ml)	IBHR Protein μgIBHR/mg protein	IBHR Protein Molar Ratio
IBHR-BSA	161 6	7 0	23 09 1	12 4 1
IBHR-oval	136 0	12 0	11 33 1	4 1 1
IBHR-KLH	144 8	8 0	18 10 1	427 9 1

4 7 Immunisation schedule for balb/c mice immunised with IBHR-BSA conjugate

The immunisation of the five balb/c mice with the IBHR-BSA conjugate was carried out as outlined in Section 2 3 6 These mice were tail bled seven days after the third immunisation and the absorbances obtained are shown in Table 4 2

Table 4 2 Screening of sera from mice immunised with the IBHR-BSA conjugate Sera (1 100 dilution) from five mice immunised with IBHR-BSA were tested by ELISA against IBHR-ovalbumin and ovalbumin-coated wells on the same plate The absorbance was read at 620nm The absorbance readings obtained from the ovalbumin-coated wells were subtracted from the absorbance readings obtained from the IBHR-ovalbumin-coated wells in order to determine the amount of antibody against IBHR present in the serum The data represents the mean values of triplicate determinations, with the standard deviation in brackets

IBHR-BSA-immunised mouse number	IBHR-oval coated plates	Ovalbumin coated plates	IBHR-oval - oval
1	0 241 (0 023)	0 167 (0 009)	0 074
2	0 368 (0 02)	0 391 (0 009)	0 000
3	0 165 (0 006)	0 085 (0 005)	0 080
4	0 450 (0 03)	0 362 (0 013)	0 088
5	0 378 (0 062)	0 092 (0 017)	0 286

The controls (n=3) used for this ELISA, with standard deviations in brackets, included,

0 01M Phosphate Buffered Saline (PBS), pH 7 3, 0 004 (0 004)

normal mouse serum, diluted 1 100 with 0 01M PBS, pH 7 3 0 018 (0 006)

and SP2/0 supernatent 0 019 (0 009)

4 8 Production of monoclonal anti-IBHR antibodies in balb/c mice

Mouse number 5 was sacrificed and a splenectomy performed (Section 2 3 9) An *in vitro* immunisation (Section 2 3 11) was carried out (Section 2 4 10) The electrofusion was performed under the conditions in Section 2 3 13 2

4 9 Summary of anti-IBHR fusion, screening and cloning

IN VITRO IMMUNISATION

↓

ELECTROFUSION

↓

15 plates made, 111 wells contained clones which produced mouse antibodies 102 clones reacted strongly with IBHR-BSA, 9 clones did not react with IBHR-BSA

↓

These 102 clones were screened against BSA, 17 clones appeared initially to react with IBHR-BSA and not to cross-react with BSA These clones were expanded into 24-well plates, 4 of these clones subsequently reacted with BSA

↓

The 13 remaining clones chosen for further study were the following, A1B7, A6A9, A6B9, A6C9, A6D8, A7A12, A7B12, C1B10, C2D5, C2E10, C5A2, C5F2, C8E9 These were expanded and screened against ELISA plates coated with anti-mouse IgG, IBHR-BSA, BSA, IBHR-KLH, KLH, IBHR-ovalbumin, ovalbumin, poly-L-lysine and porcine thyroglobulin

The positive control used in these ELISAs was sera obtained from these mice by tail bleeding (Section 2 3 8) before they were sacrificed Negative controls included pre-immumsation sera and 0 01M PBS, pH 7 3, used instead of the primary antibody in ELISA

↓

On screening by ELISA, none of the antibody-secreting clones produced an antibody that reacted with the IBHR hapten on IBHR-BSA conjugates and which did not react with the protein carrier, BSA No clones were found to react exclusively with the IBHR hapten on ovalbumin or KLH conjugates that did not cross-react with the carrier protein

4 10 Production of polyclonal anti-IBHR antibodies

As the production of a monoclonal anti-IBHR antibody was unsuccessful, the polyclonal approach was undertaken Seven days prior to immunisation of the four month old male New Zealand White rabbit, serum was obtained and was assayed for non-specific

antibodies (Table 4 3) Since the maximum titre of non-specific antibodies was only 1 400, this rabbit was considered suitable for use in the production of polyclonal antibodies against the IBHR This pre-immunisation serum was used as a negative control in ELISAs during all stages of the experiment

4 11 Immunisation of the IBHR-BSA conjugate in a New Zealand White rabbit. The rabbit was immunised (Section 2 4 11) over an 8 month period The rabbit was bled at intervals, its serum was obtained (Section 2 4 11 1) and ELISAs were performed to obtain the titre of specific antibody (Table 4 4)

Table 4 3 Dilutions of normal rabbit serum, taken from a New Zealand White male rabbit before immunisation with IBHR-BSA This was screened by ELISA against plates coated with IBHR-BSA, BSA, IBHR-KLH, KLH, IBHR-ovalbumin, ovalbumin The absorbance readings obtained from the unconjugated protein-coated wells (e g BSA) were subtracted from the absorbance readings obtained from the IBHR-protein-coated wells (e g IBHR-BSA) in order to determine the amount of antibody against IBHR present in the serum The absorbance of one set of determinations was read at 620nm (A_{620})

Dilution	IBHR-BSA	IBHR-oval	IBHR-KLH
1 25	0 014	0 295	0 000
1 50	0 146	0 251	0 086
1 75	0 141	0 241	0 148
1 100	0 124	0 136	0 160
1 200	0 001	0 021	0 092
1 400	0 016	0 008	0 011

The controls used for this ELISA were as described in Table 4 2

Table 4 4 Dilutions of sera taken from a New Zealand White rabbit over a period of 8 months after immunisation with IBHR-BSA screened by ELISA against plates coated with IBHR-BSA, BSA, IBHR-ovalbumin, ovalbumin, IBHR-KLH and KLH The absorbance readings obtained from the unconjugated protein-coated wells (e g BSA) were subtracted from the absorbance readings obtained from the IBHR-protein-coated wells (e g IBHR-BSA) in order to determine the amount of antibody against IBHR present in the serum The absorbance of one set of determinations was read at 620nm (A_{620}) and the reading taken in each instance is of a 1 1000 dilution (arbitrarily chosen)

Day	IBHR-BSA	IBHR-oval	IBHR-KLH
1	0 071	0 100	0 063
30	0 165	0 130	0 079
50	0 206	0 179	0 097
80	0 183	0 153	0 091
120	0 330	0 515	0 478

The controls used for this ELISA were as described in Table 4 2

Table 4 5 Dilutions of the final serum taken from a New Zealand White rabbit after immunisation with IBHR-BSA The serum was screened by ELISA against plates coated with IBHR-BSA, BSA, IBHR-ovalbumin, ovalbumin, IBHR-KLH and KLH The absorbance readings obtained from the unconjugated protein-coated wells (e g BSA) were subtracted from the absorbance readings obtained from the IBHR-protein-coated wells (e g IBHR-BSA) in order to determine the amount of antibody against IBHR present in the serum The absorbance of one set of determinations was read at 620nm (A_{620})

Dilution	IBHR-BSA	IBHR-oval	IBHR-KLH
1 100	0 075	0 276	0 299
1 250	0 139	0 370	0 305
1 500	0 113	0 321	0 324
1 750	0 133	0 335	0 307
1 1000	0 159	0 278	0 345
1 2500	0 188	0 282	0 321
1 5000	0 167	0 296	0 313
1 7500	0 261	0 244	0 262
1 10000	0 257	0 179	0 107
1 25000	0 179	0 109	0 105
1 50000	0 181	0 069	0 101
1 75000	0 084	0 000	0 032
1 100000	0 073	0 000	0 000

4 12 Determination of the polyclonal anti-IBHR antibody titre

By immunising with a conjugate, there are antibodies produced against the carrier protein and the IBHR The increase in titre was monitored by test bleeds during the period of the immunisation schedule (Table 4 4) The rabbit was bled 11 days after each immunisation The titre of the anti-IBHR antibody from this serum was taken as 1 50,000 as it gave a positive response with IBHR-containing conjugates (Table 4 5) As can be seen from the table, the final serum, obtained when the rabbit died, gave the highest response to IBHR and as a large volume of serum was recovered (50ml) This batch of serum was used for further purification and characterisation

4 13 Further purification and characterisation of the anti-IBHR polyclonal antibody

The antibody was purified by a series of methods, each with a view to increasing the purity and specificity of the resulting immunoglobulins The protein concentration was determined following each stage of the purification procedure (Table 4 6)

The serum was initially treated with saturated ammonium sulphate (SAS) (Section 2 4 11 3) (Table 4 7) and subsequently this SAS-purified serum was further purified by either CNBr-activated sepharose affinity chromatography (Section 2 4 12) or by Protein A affinity chromatography (Section 2 4 13) and the results compared by ELISA (Tables 4 8 1, 4 8 2 and 4 8 3)

SAS precipitation was used to isolate all immunoglobulins, but the SAS-precipitated sample may contain some contaminating proteins from serum Protein A affinity chromatography was used to purify 1ml of the SAS-precipitated sample (protein conc = 27 5mg/ml) further, as only IgG antibodies are eluted from this column The remaining contaminating proteins were washed through the column

CNBr-activated sepharose coupled to IBHR-BSA was used to further purify the anti-IBHR-BSA antibodies using another 1ml of the SAS-treated sample (Table 4 6) The anti-IBHR antibodies were eluted from the column (Section 2 4 12)

After CNBr-activated sepharose affinity chromatography, protein was found in fractions 7, 8, 9 and 10 Following protein A affinity chromatography, protein was found in fractions 4, 5 and 6 The protein concentration of the crude serum and the samples from the various stages of anti-IBHR antibody purificationwas found using the Bradford assay with rabbit IgG as standard and the results are given in Table 4 6

Table 4 6 Determination of the protein concentration of the anti-IBHR polyclonal antibody at different stages of purification

Stages of purification	Concentration (mg/ml)
Crude serum	6 0
SAS-purified serum	27 5
CNBr-sepharose affinity chromatography-purified	0 4
Protein A affinity chromatography-purified	1 96

The results of the titering of the SAS-treated sera are presented in Table 4 7 The results of the titering of the CNBr-activated affinity chromatography and protein A affinity chromatography samples against (i) IBHR-BSA and BSA are given in Table 4 8 1, (ii) IBHR-ovalbumin and ovalbumin are given in Table 4 8 2 and (iii) IBHR-KLH and KLH are given in Table 4 8 3

4 14 Analysis of ELISAs performed on purified anti-IBHR sera

As can be seen from Table 4 7 , some of the dilutions of the SAS-treated anti-IBHR serum gave an excellent response to the IBHR-BSA conjugate at a titre of 1 100,000 A difference in absorbance (at 620nm) of 0 100 between the IBHR-protein reading and the reading obtained from the unconjugated protein alone was used as the cut-off absorbance It was expected that a high response would be obtained against BSA since that was the carrier protein of the immunogen However, the antibody response obtained for the SAS-treated anti-IBHR serum screened by ELISA against IBHR-ovalbumin and IBHR-KLH were also 1 100,000

The maximum non-specific response to ovalbumin and KLH was negligible at a dilution of 1 7,500, and the difference between these and their corresponding IBHR-conjugates absorbance readings was 0 300 approximately

The antibody-containing serum was further purified by either CNBr-sepharose affinity chromatography or protein A affinity chromatography As is shown in Tables 4 8 1,

4 8 2 and 4 8 3, the maximum titre of the affinity sample was only 1 250, again taking 0 100 as the cut-off absorbance On protein A affinity chromatography purification, the titres obtained were higher, being 1 2500 for IBHR-BSA and only 1 100 for the other conjugates

Due to the higher antibody titre of the SAS-purified serum, the SAS-treated anti-IBHR serum was used in further experiments requiring this antibody and in the production of heteroconjugates

4 15 Competitive ELISA to test specificity and stability of the SAS-treated anti-IBHR antibody

The direct ELISA was routinely used to screen for the presence of anti-IBHR antibodies However, a competitive checkerboard ELISA was performed, with IBHR-KLH and KLH as the coating antigen at 10μg/ml The anti-IBHR antibody was diluted in a range from 1 100 to 1 10,000 with a free IBHR concentration of 10μg/ml to 40μg/ml of IBHR These results are shown in Table 4 8 4

The stability of the SAS-precipitated anti-IBHR antibody was determined at 4°C and 25°C in PBS, pH7 2, to determine the stability of the antibody during storage at these temperatures The absorbance was compared to a similar dilution of antibody (1 1000) stored at -20°C, tested simultaneously The result for each temperature was calculated as a percentage of the control and is shown in Table 4 9 This table indicated that for long term storage -20°C give the best results If it is not possible to store the antibody sample at -20°C, then the antibodies should be stored at 4°C Storage at 25°C gave a significant deterioration in antibody activity

The purity of the antibodies were determined by HPLC and SDS-PAGE, under non-reducing conditions, (Sections 4 16 and 4 17, respectively)

Table 4 7 Dilutions of SAS-treated anti-IBHR polyclonal serum (27 5mg/ml) were screened by ELISA against plates coated with IBHR-BSA, BSA, IBHR-ovalbumin, ovalbumin, IBHR-KLH and KLH The absorbance was read at 620nm (A_{620}).

Dilution	IBHR-BSA	IBHR-oval	IBHR-KLH
1 100	0 085	0 197	0 285
1 250	0 090	0 153	0 219
1 500	0 117	0 129	0 167
1 750	0 100	0 118	0 219
1 1000	0 132	0 152	0 257
1 2500	0 122	0 151	0 282
1 5000	0 130	0 164	0 342
1 7500	0 256	0 190	0 258
1 10000	0 271	0 173	0 293
1 25000	0 213	0 199	0 208
1 50000	0 218	0 085	0 123
1 75000	0 207	0 132	0 133
1 100000	0 162	0 112	0 116

The controls used in this ELISA was as described in Table 4 2

Table 4 8 1 Anti-IBHR serum (after SAS precipitation) was further purified by either CNBr-activated sepharose affinity chromatography or protein A affinity chromatography The results were compared by diluting the purified sample in 0 01M PBS, pH 7.3, and screening by ELISA against plates coated with IBHR-BSA and BSA The results shown were acquired by subtracting the absorbance obtained from BSA coated wells from the corresponding IBHR-BSA coated wells The absorbance of one set of determinations was read at 620nm (A_{620})

Coating	IBHR-BSA		BSA	
Dilution	CNBr sepharose affinity sample	Protein A sample	CNBr sepharose affinity sample	Protein A sample
1 100	0 241	0 583	0 074	0 354
1 250	0 127	0 462	0 071	0 282
1 500	0 092	0 365	0 044	0 174
1 750	0 075	0 293	0 031	0 135
1 1000	0 068	0 240	0 000	0 119
1 2500	0 064	0 207	0 000	0 108
1 5000	0 048	0 116	0 000	0 077
1 7500	0 000	0 088	0 000	0 000
1 10000	0 000	0 000	0 000	0 000

The controls used in this ELISA were as described in Table 4 2

Table 4 8 2 Anti-IBHR serum (after SAS precipitation) was further purified by either CNBr-activated sepharose affinity chromatography or protein A affinity chromatography The results were compared by diluting the purified sample and screening by ELISA against plates coated with IBHR-ovalbumin and ovalbumin. The absorbance was read at 620nm (A_{620})

Coating	IBH-ovalbumin		Ovalbumin	
Dilution	CNBr sepharose affinity sample	Protein A sample	CNBr sepharose affinity sample	Protein A sample
1 100	0 138	0 156	0 041	0 059
1 250	0 056	0 132	0 000	0 020
1 500	0 000	0 063	0 000	0 000
1 750	0 000	0 000	0 000	0 000

The controls used in this ELISA were as described in Table 4 2

Table 4 8 3 Anti-IBHR serum (after SAS precipitation) was further purified by either CNBr-sepharose affinity chromatography or protein A affinity chromatography The results were compared by diluting the purified sample and screening by ELISA against plates coated with IBHR-KLH and KLH The absorbance was read at 620nm (A_{620})

Coating	IBHR-KLH		KLH	
Dilution	CNBr sepharose affinity sample	Protein A sample	CNBr sepharose affinity sample	Protein A sample
1 100	0 103	0 169	0 033	0 040
1 250	0 041	0 077	0 000	0 000
1 500	0 000	0 000	0 000	0 000

The controls used in this ELISA were as described in Table 4 2

Table 4 8 4 Competitive ELISA used to determine the specificity of anti-IBHR serum (after SAS precipitation) to the IBHR antigen Plates were coated with 10μg/ml of IBHR-KLH and KLH, the results shown are the subtraction of these readings (IBHR-KLH - KLH) A range of dilutions of the anti-IBHR antibody (1 100 to 1 1000) with a range of dilutions of IBHR also added (10μg/ml to 40μg/ml) The absorbance was read at 620nm (A_{620}), n=2

Concentration of competing IBHR antigen				
Antibody dilution	40μg/ml IBHR	30μg/ml IBHR	20μg/ml IBHR	10μg/ml IBHR
1 100	0 340	0 352	0 397	0 413
1 250	0 288	0 294	0 335	0 365
1 500	0 258	0 266	0 299	0 291
1 1000	0 212	0 243	0 248	0 267

The controls used in this ELISA were as described in Table 4 2

Table 4 9 Stability study of the SAS-precipitated anti-IBHR serum determined at 4°C and at 25°C The results were compared to a stock dilution of antibody (1·1000 dilution stored at -20°C) These results were obtained by ELISA on plates coated with IBHR-KLH and KLH These absorbances were subtracted from each other and calculated as a precentage of the control antibody, treated similarly The absorbance was read at 620nm (A_{620}), n=3 and the SD are in brackets.

Number of days	4°C, % of control	25°C, % of control
20	83 2 (0 091)	76 7 (0 107)
40	78 9 (0 088)	69 3 (0 059)
60	74 3 (0 026)	65 0 (0 070)

4 16 HPLC analysis of the purified anti-IBHR serum

The neat serum and each of the purified samples (i e SAS-precipitated, CNBr - activated sepharose affinity chromatography- and protein A affinity chromatography-treated samples), along with commercial rabbit IgG as a standard for comparison, underwent HPLC analysis using a 10µm Protein Pak SW 300 column with a mobile phase of 0 1M phosphate buffer, pH 7 0 and a flow rate of 0 5ml/min The resulting chromatograms are shown in Figures 4 5 1 to 4 5 3

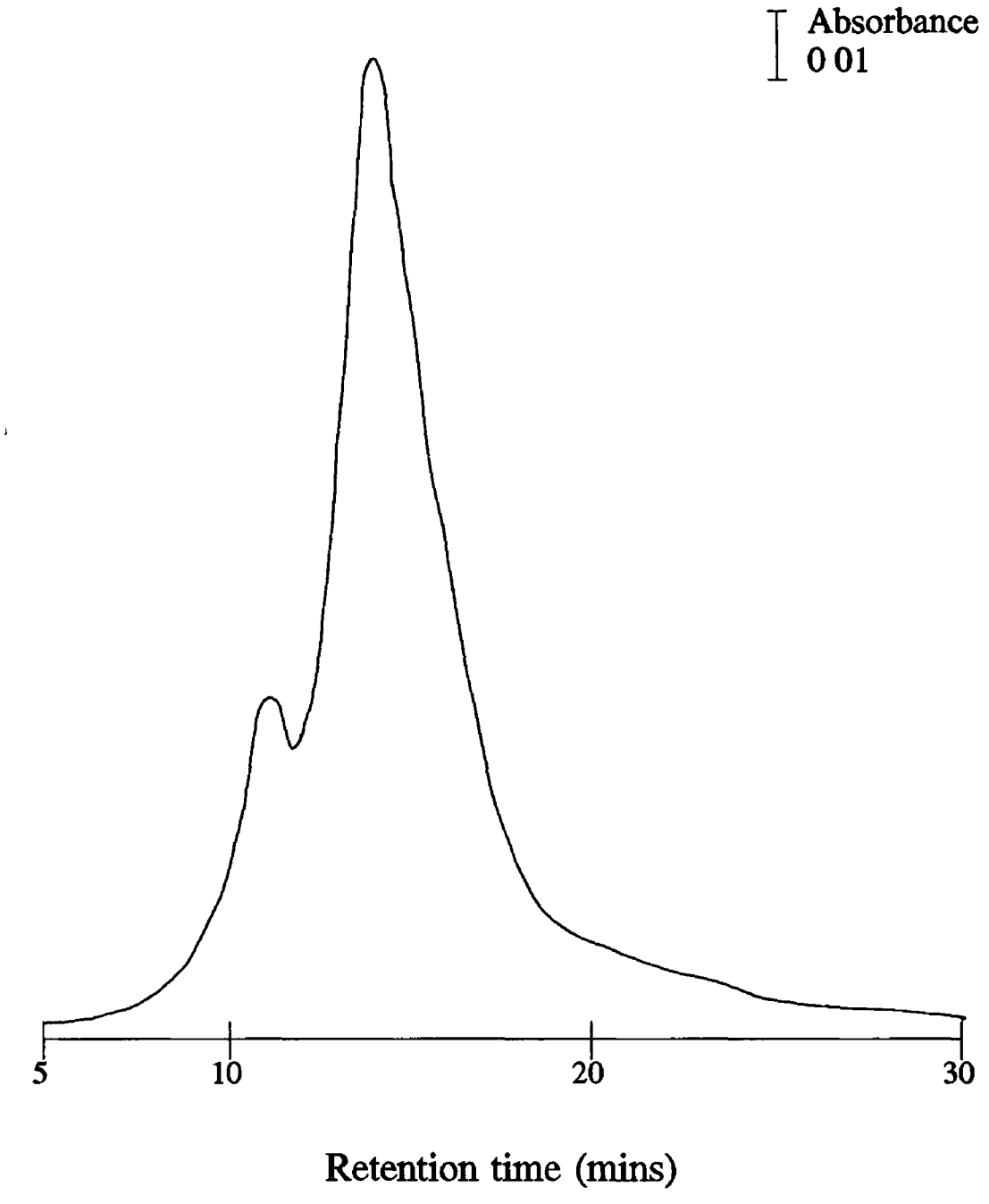


Figure 4.5.1

HPLC chromatogram of commercial rabbit IgG The column used was a 10μm Protein Pak SW 300 column with a mobile phase of 0 1M phosphate buffer, pH 7 0 and a flow rate of 0 5ml/min Absorbance was detected at 280nm One major peak was detected with a retention time of 16 39mins This is representative of rabbit IgG (150kD) The minor peak at 13 93min may depict contaminating protein, perhaps used to stabilise this antibody

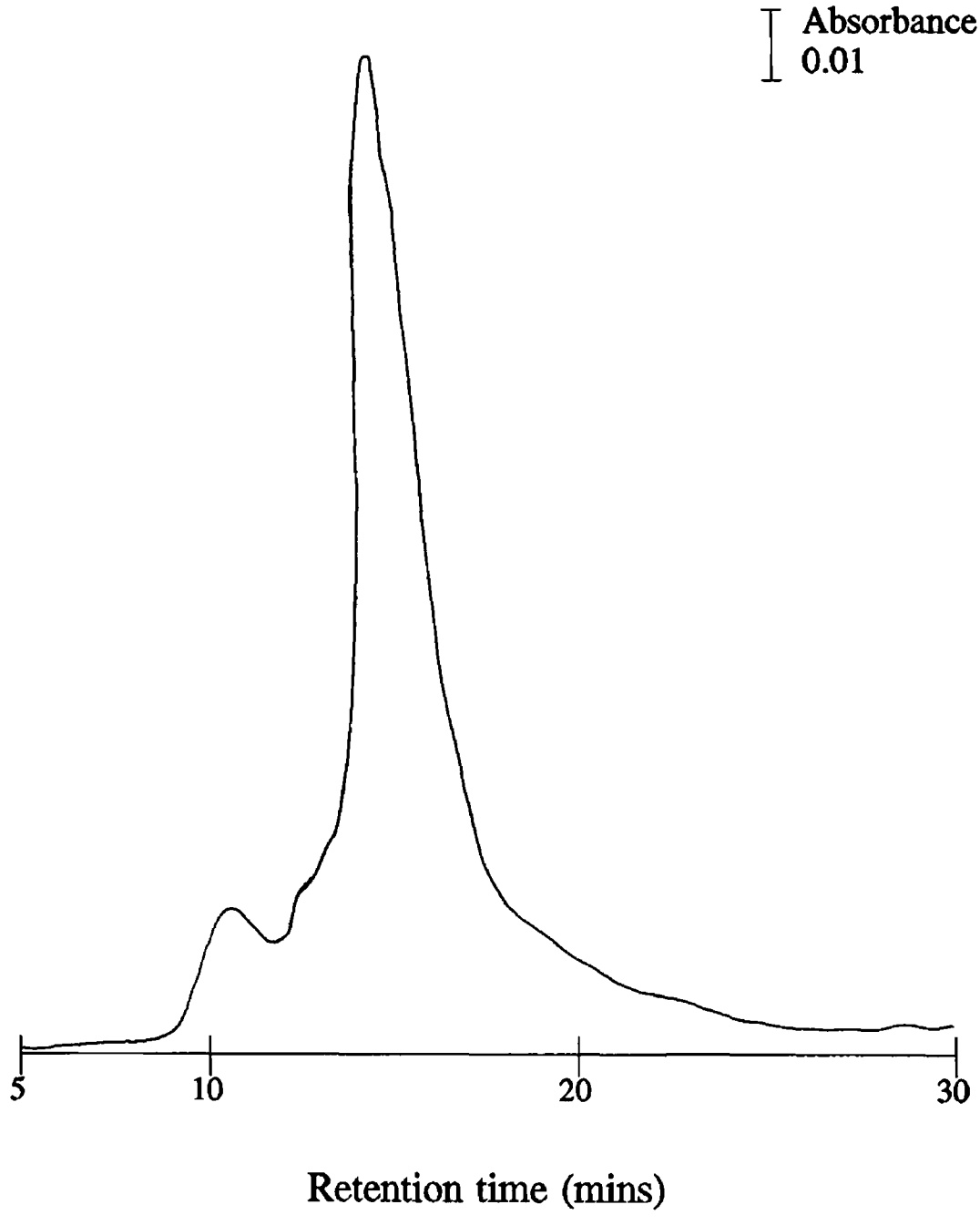


Figure 4.5.2.

HPLC chromatogram of SAS-treated anti-IBHR serum The column used was a 10μm Protein Pak SW 300 column with a mobile phase of 0 1M phosphate buffer, pH 7 0 and a flow rate of 0 5ml/min Absorbance was detected at 280nm One major peak was detected with a retention time of 16 35mins This corresponds to rabbit IgG The minor peak at 10 27min corresponds to protein eluting in the void volume of the column, which may be due to contaminating proteins

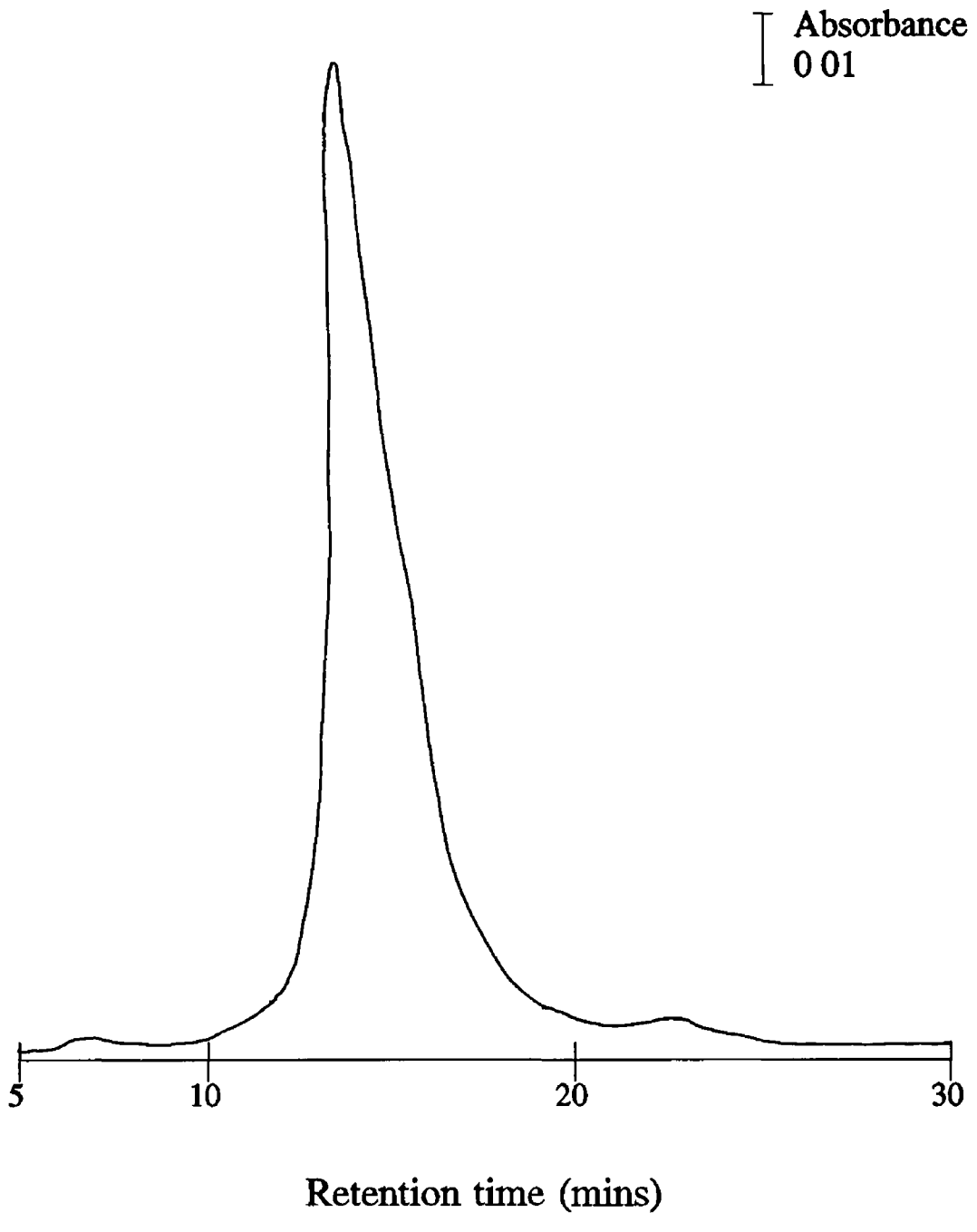


Figure 4.5.3.

HPLC chromatogram of protein A affinity chromatography-purified anti-IBHR serum The column used was a 10μm Protein Pak SW 300 column with a mobile phase of 0 1M phosphate buffer, pH 7 0 and a flow rate of 0 5ml/min Absorbance was detected at 280nm One major peak was detected with a retention time of 16 39mins which represents rabbit IgG

4 17 Electrophoresis of the anti-IBHR neat and purified antibody samples

A 5-20% (w/v) SDS-PAGE non-reducing gel was used for the detection of the neat and purified anti-IBHR antibody samples (Section 2 4 17) Molecular weight markers were also included which would allow for the determination of molecular weights of the various bands seen on the gel With the knowledge that an IgG molecule is approximately 150kD, the molecular weights of the bands produced from the purified samples can be calculated and compared with the true weights to identify the presence of antibodies

Figure 4 6 1 shows the stages of the purification of the rabbit polyclonal antisera, with results as would be expected There were a lot of proteins in the pre-immunised serum and in the crude serum The SAS-treated serum contains less contaminating proteins and, it appeared some large molecular weight protein, which might possibly be IgM, did not enter the gel The sample obtained following purification by protein A affinity chromatography was a pure IgG band as was expected from the HPLC result (Figure 4 6 3) The CNBr-sepharose affinity chromatography-purified sample did not show up on the gel, which may be due to the very low protein concentration in this sample

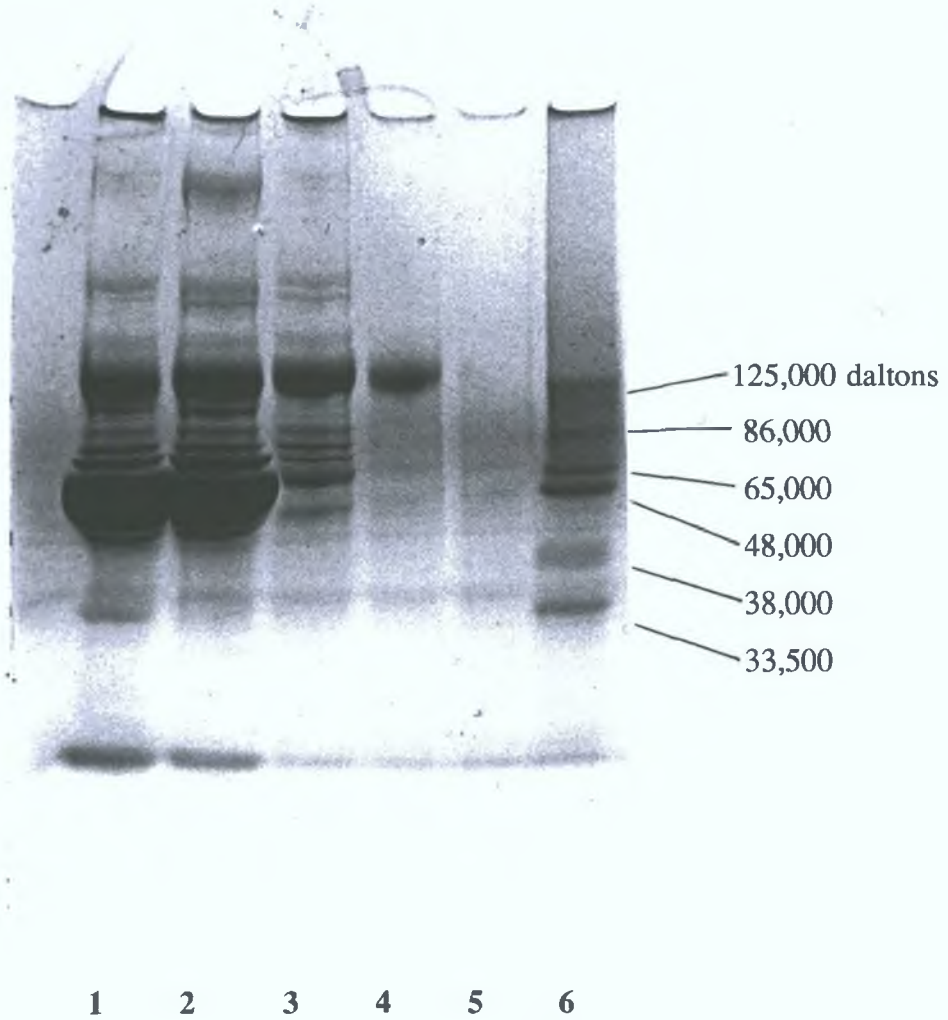


Figure 4.6. A 5-20% (w/v) gradient PAGEL^{R} precast polyacrylamide gel from ATTO was loaded as follows; Lane (1) Pre-immune serum from rabbit, (2) anti-IBHR crude serum, 1:10 dilution, (3) SAS-treated anti-IBHR serum, (4) protein A affinity chromatography-treated anti-IBHR serum, (5) CNBr-activated sepharose affinity chromatography-purified anti-IBHR sample, (6) Molecular weight markers. The gel was run at 20mAmps per gel.

4 18 Fluorescent labelling of IBHR-BSA and IBHR-KLH by FITC.

This procedure was carried out using the IBHR-BSA and IBHR-KLH conjugates (Section 2 3 23) Protein or antibody concentrations of greater than or equal to 10mg/ml relative to fluorochrome concentrations of 10–20μg/mg, favour conjugation to antibody (up to 70%) as discussed in Section 2 3 23 and in Goding (1976) Lower antibody concentrations (1mg/ml) can be used if the fluorochrome concentration is correspondingly increased (100μg/mg of protein)

The FITC protein ratio can be calculated using a simple formula proposed by The and Feltkamp (1970a,b) which included a correction factor for the contribution of FITC to the total absorbance at 280nm (Section 2 3 23) The fractions were collected from a Sephadex G-25 column (internal diameter 32cm × 3 2cm), the absorbances of each sample were read at 495 and 280nm (Harlow and Lane, 1988) The results obtained are shown in Table 4 10 and in Figure 4 8

As can be seen from Table 4 10 , the FITC protein ratios obtained for these conjugates were between an absorbance ($A_{495} - A_{280}$) of 0 3 and 1 0 (Section 2 3 23 1) This result signifies that the FITC protein ratios are in the correct range and may be used directly The FITC-labelled conjugates were used in the detection of bifunctional antibodies (Section 5 1)

Table 4 10 Results of the absorbance of the FITC labelled IBHR-BSA and IBHR-KLH conjugates measured at 495nm (A_{495}) and 280nm (A_{280}) to calculate the FITC protein ratio

Protein	Fraction No	A_{495}	A_{280}	$A_{495} - A_{280}$
FITC-IBHR-BSA	1	0 796	0 289	0 507
FITC-IBHR-BSA	2	1 098	0 346	0 752
FITC-IBHR-KLH	1	1 137	0 528	0 609
FITC-IBHR-KLH	2	1 447	0 539	0 908

The eluate from the column, before the samples were loaded, was used as a blank when obtaining these readings

4 19 Electrophoresis of IBHR conjugates on SDS-PAGE

The following samples were analysed by SDS-PAGE, IBHR-KLH-FITC, IBHR-KLH, KLH, IBHR-ovalbumin, ovalbumin, IBHR-BSA-FITC, IBHR-BSA, and BSA (Figure 4 7) In this gel, the IBHR conjugated samples appear similar Neither of the IBHR-protein-FITC conjugates were detectable, due to the low concentration of protein in these samples which were then analysed by absorbance spectra (Figure 4 8)

4 20 Analysis of absorption spectra of IBHR-protein-FITC conjugates

Spectrum analysis was carried out using a Shimadzu UV-160A UV-Visible recording spectrophotometer over the range 200-580nm to determine the conjugation of the IBHR-protein conjugate to FITC The samples examined were BSA, IBHR-BSA, IBHR-BSA-FITC, KLH, IBHR-KLH and IBHR-KLH-FITC and the results are displayed in Figures 4 8

BSA and KLH gave similar absorption spectra to their IBHR conjugates As can be seen, both FITC-labelled conjugates show distinct absorbance peaks at 492 5nm for IBHR-BSA-FITC and 493 0 for IBHR-KLH-FITC in close agreement with the expected absorption peak of 495nm for FITC These FITC-labelled conjugates were used in the detection of heteroconjugates containing anti-IBHR polyclonal antibodies by immunofluorescence and FACS (Section 5 1)

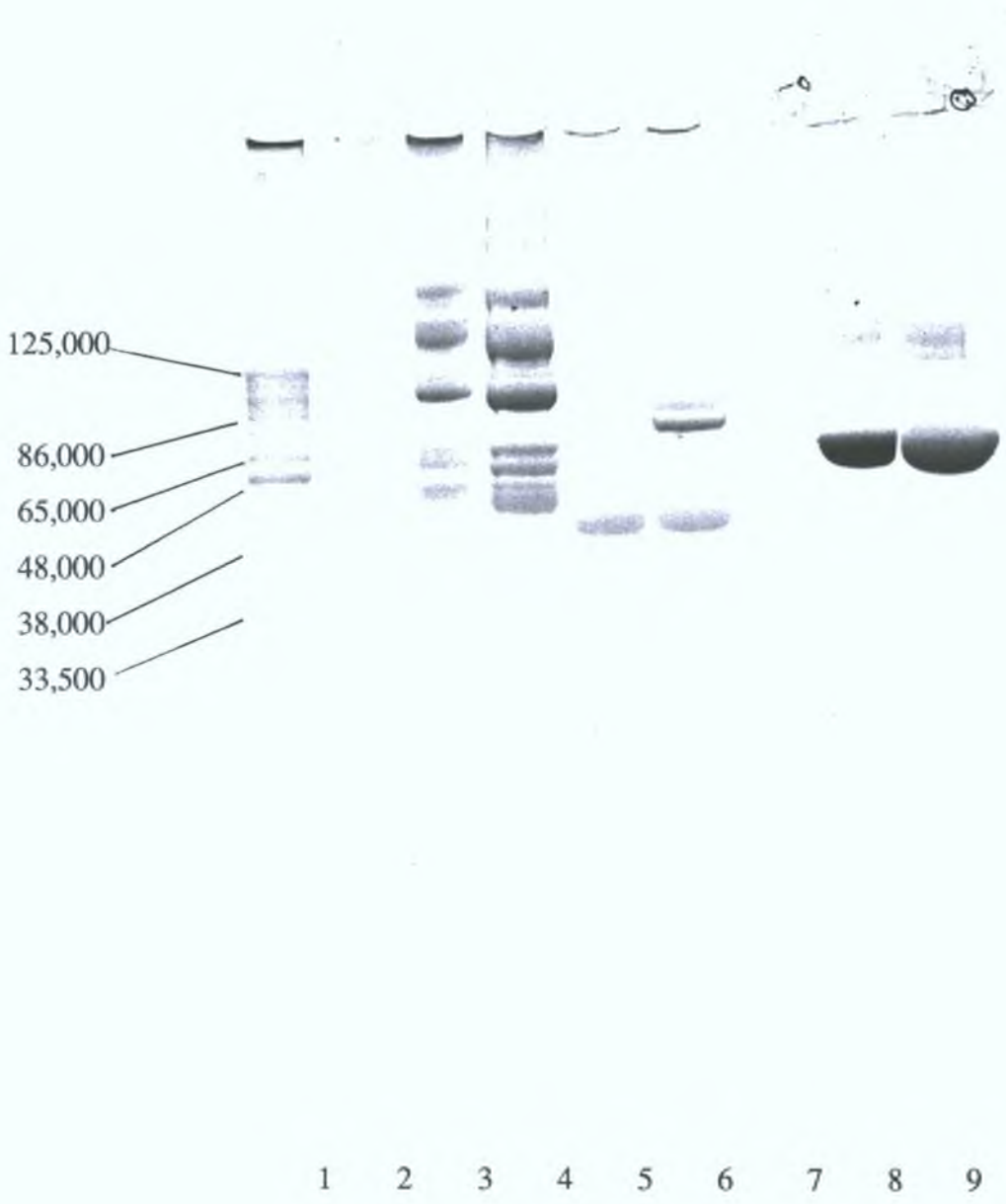


Figure 4.7. A 5-20% (w/v) gradient PAGEL^{R} precast polyacrylamide gel from ATTO was loaded as follows; Lane (1) Molecular weight markers, (2) IBHR-KLH-FITC, (3) IBHR-KLH, (4) KLH, (5) IBHR-ovalbumin, (6) Ovalbumin, (7) IBHR-BSA-FITC, (8) IBHR-BSA and (9) BSA. The gel was run at 20mAmps per gel.

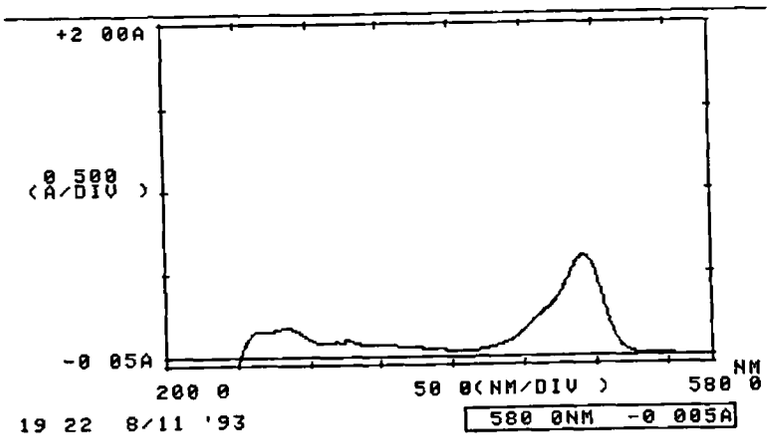


Figure 4 8 1 Absorption spectrum of an IBHR-BSA-FITC sample analysed on a Shimadzu UV-160A UV-Visible recording spectrophotometer in the range 200-580nm

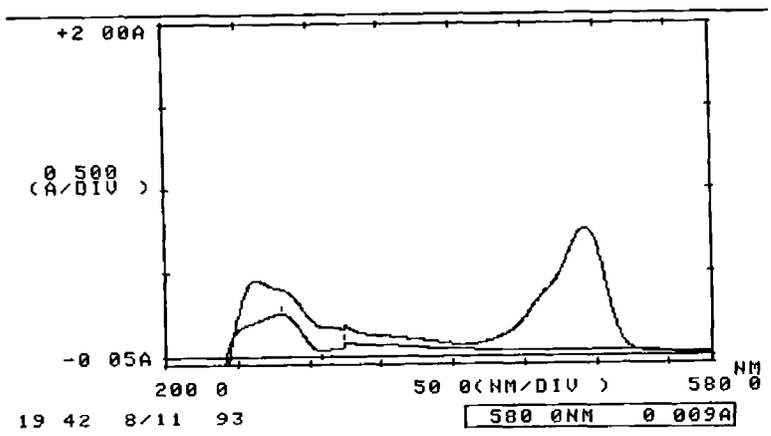


Figure 4 8 2 Absorption spectrum of an IBHR-KLH-FITC sample analysed on a Shimadzu UV-160A UV-Visible recording spectrophotometer in the range 200-580nm The lower line in the figure represents the absorption spectrum of the IBHR-KLH conjugate

4.21 Discussion

The first aim of this project was to iodinate BHR, conjugate the IBHR to the following proteins, BSA, ovalbumin and KLH and to characterise the resulting conjugates

BHR was iodinated using ICl (Doran and Spar, 1980) and was found by thin-layer chromatography to be pure (O'Kennedy *et al*, 1989, Section 4 2) The IBHR molecule was conjugated to BSA, KLH and ovalbumin thus circumventing the problems with direct iodination of proteins (Section 4 1) This alternative method of detecting iodine in the IBHR-protein conjugates has proved invaluable during this project

For conjugation to proteins, the iodinated reagent reacts with the lysyl residues present in most proteins and peptides to form amide bonds (Section 4 3) Knight and Welch (1978) reported that using this method, lysine residues were labelled predominately but iodinated histidine and tyrosine were also produced For the production of monoclonal and polyclonal antibodies, this method also has the advantage that the introduction of the IBHR will occur at the surface lysyl moieties, and in large proteins this should occur randomly Therefore, the IBHR hapten should be easily recognised by the immune system of the injected animal

ELISAs were used extensively for the characterisation of the anti-IBHR antibodies produced (Section 2 4 15) This IBHR was then conjugated to BSA, ovalbumin and KLH (O'Kennedy *et al*, 1989) and the free IBHR was separated from bound IBHR by gel filtration and the absorbance reading monitored The first peak was iodinated protein and the second peak represented unconjugated IBHR (Figure 4 1) These fractions from the first peak were pooled to give the IBHR-protein sample These conjugates were characterised for iodide and protein content The amount of iodide was determined using the Sandell-Kolthoff reaction (Sandell and Kolthoff, 1934) and the iodide microassay (O'Kennedy *et al*, 1989)

The oxidation of arsenic by cerium is catalysed not only by inorganic iodide but also by iodine-containing compounds such as thyroxine, proteins and drugs (reviewed by Keating, 1990, O'Kennedy *et al*, 1989) The Sandell-Kolthoff reaction is very sensitive and is, therefore, often used to determine micro amounts of these species by kinetic methods It has been found that the catalytic activity of iodide in organic compounds is inferior to that of inorganic iodide (Pantel and Wiesz, 1977) This is due to the nature of the side groups attached to the iodine atom on the parent molecule This has resulted in the concept of a relative molar coefficient of catalytic activity', F (Pantel, 1982) This

is a measure of catalytic activity of iodo compounds in comparison to that of inorganic iodide Timotheou-Potamia (1988) introduced the K coefficient which is defined as the 'catalytic activity per iodine atom of the compound', the K value of IBHR is 33%

The readings from this assay at 2 minutes gave the best regression (0 995) (Table 4 2) and were used to determine the IBHR content of the conjugate, assuming the catalytic activity of the iodine atom is not greatly affected by the nature of side groups attached to the benzene ring (Timotheou-Potamia, 1988) Since these side groups do not change appreciably when a protein is conjugated using IBHR, it is expected that the catalytic activity of the iodine atom attached to the protein should be similar to IBHR (O'Kennedy *et al* , 1989)

Since IBHR hapten conjugation introduces groups into the protein (Knight and Welch, 1978) that absorb at 280nm, the use of absorbance at 280nm as a measure of protein concentration was no longer accurate The protein in the conjugate may be detected using a variety of protein assays IBHR was found to interfere with the BCA protein assay (Smith *et al* , 1985), and the one chosen to determine protein concentration was the BioRad micro-Bradford assay, (Figure 4 3) (Bradford, 1976)

In order for the dye to bind, the molecule must possess a macromolecular form and an active aromatic functional group This makes the assay suitable for protein determination, but as BHR also satisfies this criteria, it interferes with this assay To investigate this interference a standard curve was set up with two sets of BSA standards, one being spiked with a known concentration of IBHR, the results of the extent of this interference are shown in Figure 4 4 This interference was found to result in a uniform increase in absorbance of between +0 005 and +0 022 Considering this result, determinations of protein content were made using an IBHR concentration equivalent to that determined in the conjugate as a zero control in the protein assay It would be more accurate if a protein assay which was not subject to IBHR interference was used The relative IBHR protein concentrations were obtained from these results and the IBHR-BSA conjugate was found to contain the highest ratio of iodine to protein (Table 4 5) This was used as immunogen in the production of anti-IBHR antibodies

The second aim of the project was to produce, purify and characterise antibodies against the IBHR hapten 5 balb/c mice were immunised with IBHR-BSA in order to produce monoclonal anti-IBHR antibodies Their anti-IBHR titres were assayed by ELISA, the mouse with the highest titre was selected for monoclonal antibody production An *in*

vitro immunisation was performed on the spleen cells of this mouse, followed by an electrofusion The clones obtained were assayed by ELISA against IBHR-BSA, BSA, IBHR-ovalbumin, ovalbumin, IBHR-KLH and KLH No hybridoma was obtained which was specific for IBHR, all reacted to some extent with the carrier protein This may have been because the IBHR, with a molecular weight of 390D, was too small to elicit an adequate immune response for monoclonal antibody production

The polyclonal antibody approach was, therefore, undertaken and was successful A rabbit was immunised with IBHR-BSA over a period of 8 months The pre-immunisation serum gave a maximun non-specific anti-IBHR titre of 1 400 Following immunisation, the serum was purified using SAS and was then screened by ELISA, a titre of up to 1 100,000 was obtained However, 1 50,000 was considered the titre of this SAS-treated sera since it gave a positive response with IBHR-containing conjugates and did not cross-react with their carrier proteins (Table 4 9)

This serum was further purified by either protein A affinity chromatography or CNBr-activated affinity chromatography, which did not provide improved results Both of these purification techniques resulted in a much lower antibody titre against IBHR

The purity of the antibodies were determined by HPLC and SDS-PAGE, under non-reducing conditions, (Sections 4 16 and 4 17, respectively) The commercial rabbit IgG gave a large peak at 16 39min which corresponds to IgG (150kD) by HPLC (Section 4 16) A minor peak at 13 93min was also observed by HPLC which may correspond to larger proteins introduced to stabilise this antibody The SAS-treated anti-IBHR serum gave a large distinct band at 16 35min, in excellent agreement with the commercial rabbit IgG A smaller peak was seen at 10 27min, this represents proteins eluting in the void volume of the column The protein A affinity chromatography-treated sample produced a HPLC chromatogram with a single peak at 16 32min which corresponds to purified IgG This showed that the sample was pure but, as was observed in the ELISAs (Section 4 14), this sample had lost most of its anti-IBHR antibody activity For these reasons, the SAS-purified sample was used in further applications with this antibody, including the production of heteroconjugates (Section 5 1)

The purification stages of the polyclonal anti-IBHR antibody loaded on an SDS-PAGE gel are shown in Figure 4 6 Some contaminating proteins were seen in the SAS-precipitated sample The protein A-purified sample gave one band at 150,000 daltons which agrees with the results obtained by HPLC However, as previously mentioned, this

sample had lost most of its activity and was not used The CNBr-activated sepharose affinity chromatography sample was too dilute to be seen on the gel

The stability of this antibody was analysed and, on storage at 4°C for two months, its activity was shown to decrease to 74 3% of the activity of a control stock of antibody stored at -20°C At room temperature, its activity decreased to 65% over the same period Therefore, the optimum storage temperature for this antibody is -20°C but for short periods it may be stored at 4°C

The IBHR-BSA and IBHR-KLH conjugates were further labelled by FITC (Goding, 1976) The fluorescein coupling was estimated by measuring the absorbance at 495nm and at 280nm (Harlow and Lane, 1988) (Table 4 10) The fluorescein coupling was confirmed by absorption spectra analysis (Figure 4 8) and by the use of these FITC-labelled conjugates in the detection of the heteroconjugates (Section 5 1)

In summary, some of the aims of this project were achieved BHR was iodinated and the IBHR was conjugated to three proteins These conjugates were characterised and used to produce anti-IBHR antibodies The production of monoclonal anti-IBHR antibodies was, however, unsuccessful A polyclonal antibody was produced against the IBHR hapten, purified and characterised The titre of this anti-IBHR polyclonal antibody was 1 50,000 This purified antibody was used in the construction of heteroconjugates which were detected by immunofluorescence and FACS (Section 5 1)

SECTION 5 BISPECIFIC AND GENETICALLY ENGINEERED ANTIBODIES

5 Outline

This portion is divided into two sections and illustrates the development of monoclonal antibody based reagents and their potential applications

The aim of the first section (Section 5 1) was to produce the following bispecific (or heteroconjugate) antibodies, (i) monoclonal anti-GFAP (2DH5) x polyclonal IBHR, (ii) monoclonal anti-G-CCM (3BH2) x polyclonal IBHR, (iii) monoclonal anti-GFAP x monoclonal anti-rRicin-A chain and (iv) monoclonal anti-G-CCM x monoclonal anti-rRicin-A chain These heteroconjugates were characterised by ELISA, HPLC, SDS-PAGE and FACS

The potential of genetic engineering (Sections 1 21 to 1 24) for solving many of the previous problems associated with the use of murine monoclonals is encouraging In the second section (Section 5 2), an attempt was made to express the anti-G-CCM monoclonal antibody (Section 3) in *E coli* Messenger RNA (mRNA) from the anti-G-CCM hybridoma was isolated and its complementary DNA (cDNA) was produced This was made using specifically developed primers to amplify the regions of interest Following Polymerase Chain Reaction (PCR), the purified DNA was ligated and transformed into *E coli* and screened for inserts in the correct orientation

5 1 Production of bispecific antibodies

5 1 1 Introduction

Since the ability of antibodies to be therapeutically or diagnostically efficient on their own has been limited (Section 1 19), efforts have been made to increase the efficiency of monoclonal antibodies by attaching them to various agents, such as bacterial or plant toxins (Section 1 11), radionuclides (Section 1 13) and cytotoxic drugs (Section 1 7) Research in the past has been concentrated on using reagents derived mainly from the direct conjugation of antibodies to the effector compounds These immunoconjugates have now been used for diagnosis and therapy (Section 1 9 to 1 12) Direct coupling of antibodies to effector compounds has some major disadvantages Chemical manipulation can inactivate antibody binding sites as well as cause crucial alterations in the effector agents, thus decreasing the efficiency of the immunoconjugates (Goldenberg, 1993)

These probems have led to the development of an alternative approach for tumour

targeting in which, instead of coupling effector compounds directly to antibody, a multi-stage delivery system is employed Bispecific antibodies which have two different antigen-specific binding binding sites, one for tumour-associated antigen and the other for the effector compounds, have been developed The precisely designed bispecific antibody is firstly targeted to the tumour site by its tumour specificity After allowing a suitable period of time for the non-specifically bound antibody to be cleared, the effector compound, which is recognised by the second specificity of the targeted antibody, is then injected separately, leading to its specific localisation to the tumour This system may minimise the toxicity in therapy and maximise the quality of tumour imaging These bispecific antibodies also have many potential uses, ranging from immunodiagnosis to targeted delivery of toxic substances to tumours (Kuzel *et al* , 1993, Phillips *et al* , 1994) They have been studied for targeting effector cells (Morelli *et al* , 1994, Weiner *et al* , 1994) or toxins to tumours and initial results look very promising (Tepler *et al* , 1994) Progress towards an understanding of the construction and use of bispecific antibodies in the therapy of tumours has been reviewed by Fanger *et al* (1993)

The production, purification and characterisation of the anti-GFAP and anti-G-CCM monoclonal antibodies was detailed in Section 3 and the description of the anti-IBHR polyclonal antibody can be found in Section 4 A diagram of the anti-G-CCM x anti-IBHR heteroconjugate is shown in Figure 5 1 1 The anti-recombinant Ricin-A chain (rRicin-A) monoclonal antibody (608/7) used in this project was obtained from Dr Malcolm V Pimm (Embleton *et al* , 1991), following permission to use anti-ricin-A chain antibody, by Dr Partick Trown, Xoma Corporation (Table 2 3) The rRicin-A chain was a gift from Dr Denis Thatcher, Zeneca Corporation (Table 2 3)

Initially, it was planned to produce an anti-drug antibody using drugs like methotrexate, daunomycin ar adriamycin which have been previously used to kill cancer cells (Section 1 7) and to use these antibodies in the production of heteroconjugate antibodies with the anti-G-CCM antibody (Section 3) However, in 1992, the laboratory of Dr Ellen Vitetta in the University of Texas SouthWestern Medical Centre at Dallas, was visited with the aim of learning how to handle toxins and how to assay the activity of immunotoxins

Immunotoxins are conjugates of a plant or animal toxin and an antibody which are designed for cell specific killing (Section 1 11) A commonly used toxin is ricin which

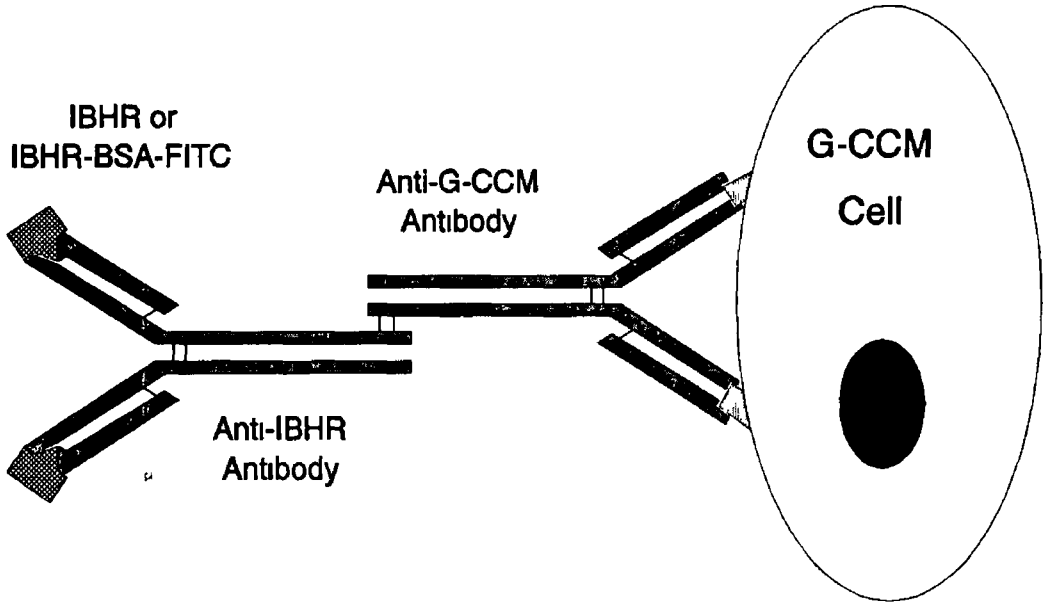


Figure 5.1.1. A diagram of the anti-G-CCM x anti-IBHR heteroconjugate antibody produced

can be purified from seeds of the castor bean *Ricinus communis* These toxins are made up of two chains, A and B The A chain is responsible for the toxin's enzymatic activity and the B chain is responsible for binding the toxin to receptors on the cell surface If the B chain is replaced by an antibody, resulting in an immunotoxin, the A chain alone is not toxic The preparations of immunoconjugates and the method for the indirect immunotoxin assay, which tested the antibody specificity of cultured supernatants was learnt in Dr Vitetta's laboratory, along with how to prepare IgG–A and IgG–B chain conjugates

It was decided that it would not be feasible to accomplish this work in D C U as the preparation of an immunoconjugate is tedious, difficult and expensive with 1g of Ricin–A chain costing $60,000 in 1992 The preparation and characterisation of any A and B chain would have involved testing the lethal dose (LD_{50}) in mice, the LD_{50} A chain should be 1–2mg and the LD_{50} B chain should be 0 5–1mg The immunoconjugates prepared using these chains would have to have been checked by radioimmunoassay or indirect assay In the indirect assay the immunoconjugate is incubated with cells The number of cells killed should be 20% greater than the number of cells killed in the negative control (i e antibody without toxin), to be considered specific It was therefore decided to obtain the anti–rRicin–A chain from Dr M V Pimm and to obtain a small quantity of the rRicin–A chain as a gift from the Zeneca Corporation and to use this in the production and initial characterisation of bifunctional antibodies

Unfortunately, it took over two years to obtain permission from the Xoma Corporation to use the anti–Ricm A chain antibody for research in D C U Owing to this, only limited characterisation of the heteroconjugate antibodies produced was possible

5 1 2 Production of a bispecific antibody

Bispecific antibodies are produced mainly by two methods, fusion of two different hybridoma cell lines, or chemically linking two antibody molecules or their derivatives The production and applications of bispecific antibodies have been reviewed by Nolan and O'Kennedy (1990) (Section 1 15)

In this project, two antibodies were linked using the heterobifunctional cross–linker N–succinimidyl–3–(2–pyridyldithio) propionate (SPDP) This is a quick method and was prepared according to Bush and Winkler (1989) (Section 2 5 1)

5.1.3. Characterisation, by ELISA, of the bispecific heteroconjugate antibodies produced.

ELISAs were performed to characterised the activity of the parent antibodies and the heteroconjugates produced, as outlined in Section 2.3.16.3. The ELISAs procedure chosen depended on the target antigens and on whether the parent antibodies were of mouse or rabbit origin. The results are shown in Tables 5.1.1. to 5.1.4.

Table 5.1.1. Results of ELISAs to demonstrate the binding of the anti-G-CCM x anti-IBHR bispecific heteroconjugate to G-CCM cells, GFAP and IBHR. The reading for IBHR is the result of the subtraction of the absorbance obtained for KLH-coated wells from the absorbance obtained for IBHR-KLH-coated wells. The heteroconjugate was diluted 1:1000, using 0.01M PBS, pH 7.3. The absorbance was read at 620nm (A_{620}) and the number of determinations was two (n=2).

Screening of the anti-G-CCM x anti-IBHR heteroconjugate			
Dilution	**G-CCM-coated**	**GFAP-coated**	**IBHR-coated**
1:1000	0.391	0.204	0.263

Table 5.1.2. Results of ELISAs to demonstrate the binding of the anti-G-CCM x anti-rRicin A chain bispecific heteroconjugate to G-CCM cells, GFAP and rRicin A chain. The parent antibody, at the same dilution gave a reading of 0.378 against rRicin A-chain. The heteroconjugate was diluted 1:1000, using 0.01M PBS, pH 7.3. The absorbance was read at 620nm (A_{620}) and n=2.

Screening of the anti-G-CCM x anti-rRicin A heteroconjugate			
Dilution	**G-CCM-coated**	**GFAP-coated**	**rRicin-coated**
1:1000	0.345 (0.018)	0.172 (0.005)	0.244 (0.026)

Table 5 1 3 Results of ELISAs to demonstrate the binding of the anti-GFAP x anti-IBHR bispecific heteroconjugate to G-CCM cells, GFAP and IBHR The reading for IBHR is the result of the subtraction of the absorbance obtained for KLH-coated wells from the absorbance obtained for IBHR-KLH-coated wells The heteroconjugate was diluted 1 1000, using 0 01M PBS, pH 7 3 The absorbance was read at 620nm (A_{620}), and n=2

Screening of the anti-GFAP x anti-IBHR heteroconjugate			
Dilution	G-CCM-coated	GFAP-coated	IBHR-coated
1 1000	0 140	0 186	0 233

Table 5 1 4 Results of ELISAs to demonstrate the binding of the anti-GFAP x anti-rRicin A chain bispecific heteroconjugate to G-CCM cells, GFAP and rRicin A chain The parent antibody, at the same dilution gave a reading of 0 378 against rRicin A-chain The heteroconjugate was diluted 1 1000, using 0 01M PBS, pH 7 3 The absorbance was read at 620nm (A_{620}), n=2

Screening of the anti-GFAP x anti-rRicin A heteroconjugate			
Dilution	G-CCM-coated	GFAP-coated	rRicin-coated
1 1000	0 121	0 202	0 186

5 1 4 Characterisation, by HPLC, of the bispecific heteroconjugate antibodies produced

The heteroconjugate antibodies were characterised by HPLC following dialysis against the HPLC mobile phase The column used was a 10μm Protein Pak SW 300 column with a mobile phase of 0 1M phosphate buffer, pH 7 0 The result of a typical hetero-conjugate antibody chromatogram, the anti-G-CCM x anti-IBHR, is shown in Figure 5 1 2 The peak at 9 95min was protein eluting in the void volume Any bifunctional antibody would have eluted here The peak at 16 11min was the parental IgG antibodies or breakdown of antibodies which had been in the bispecific antibody

5 1 5 Characterisation, by SDS-PAGE, of the bispecific heteroconjugate antibodies produced

The heteroconjugate antibodies were characterised by SDS-PAGE, the gels used were PAGEL^{R} precast polyacrylamide gels for electrophoresis (Section 2 1) The results are shown in Figures 5 1 2

5 1 6 Characterisation, by FACS, of the bispecific heteroconjugate antibodies produced

The heteroconjugate antibodies were characterised by FACS (2 3 20 8) The results are shown in Figures 5 2 3 The anti-G-CCM x anti-IBHR and anti-GFAP x anti-IBHR heteroconjugate antibodies had previously been incubated with either (i) IBHR-BSA-FITC or (ii) IBHR-KLH-FITC (Section 2 4 18 and 4 18) The negative controls for this experiment are control serum (i e pre-immune serum) pre-incubated with the FITC-labelled IBHR-protein conjugate and the anti-IBHR antibody, pre-incubated with the FITC-labelled IBHR-protein conjugate Both of these controls showed no non-specific binding to the G-CCM or G-UVW cell lines used (Figures 5 2 3 1 and 5 2 3 2) The anti-G-CCM or anti-GFAP arm bound the heteroconjugate to the cell and fluorescence was detected using the FITC-labelled IBHR-protein conjugate, no secondary antibody was required (Figures 5 2 3 3 to 5 2 3 6) The results are discussed in Section 5 2 7

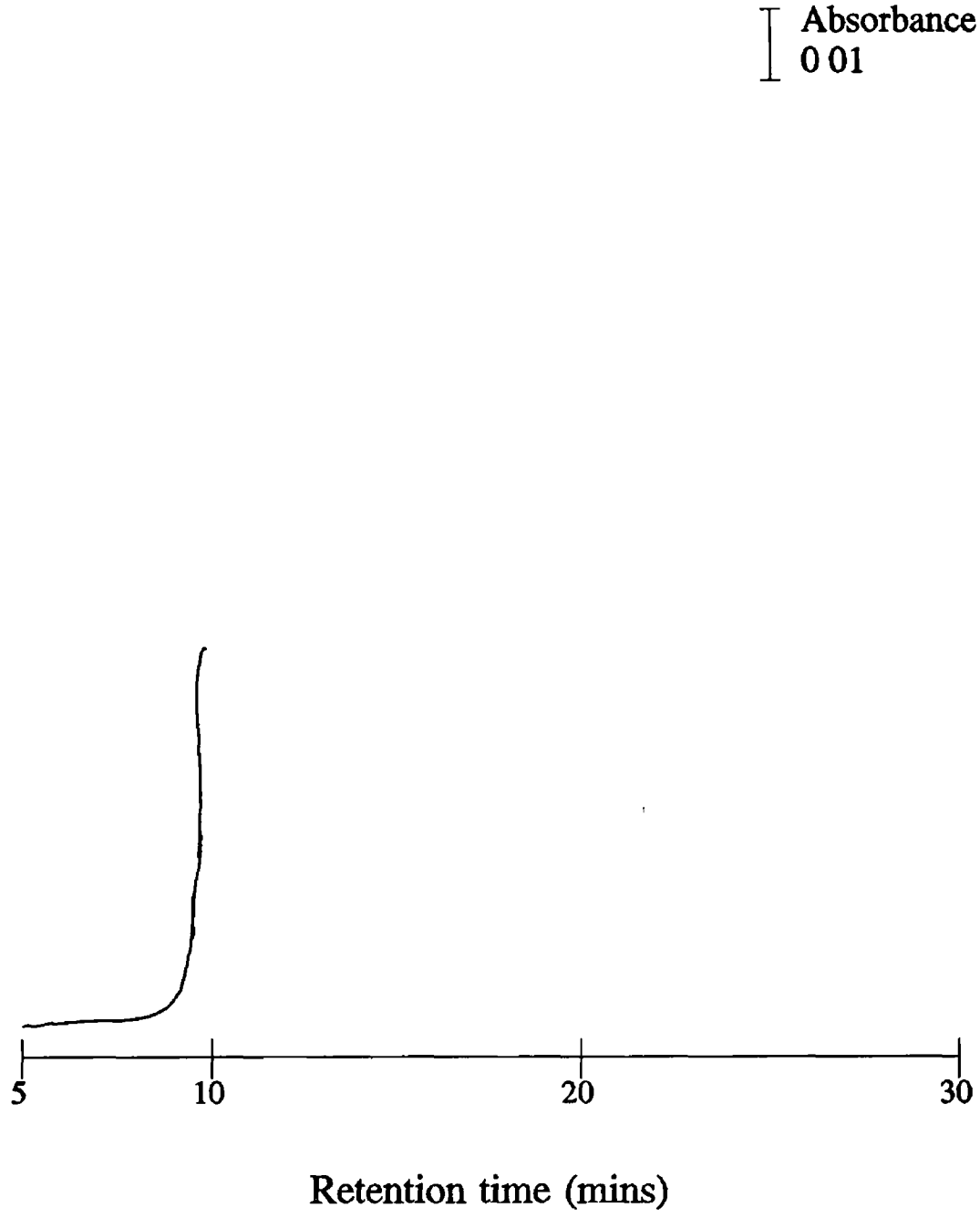
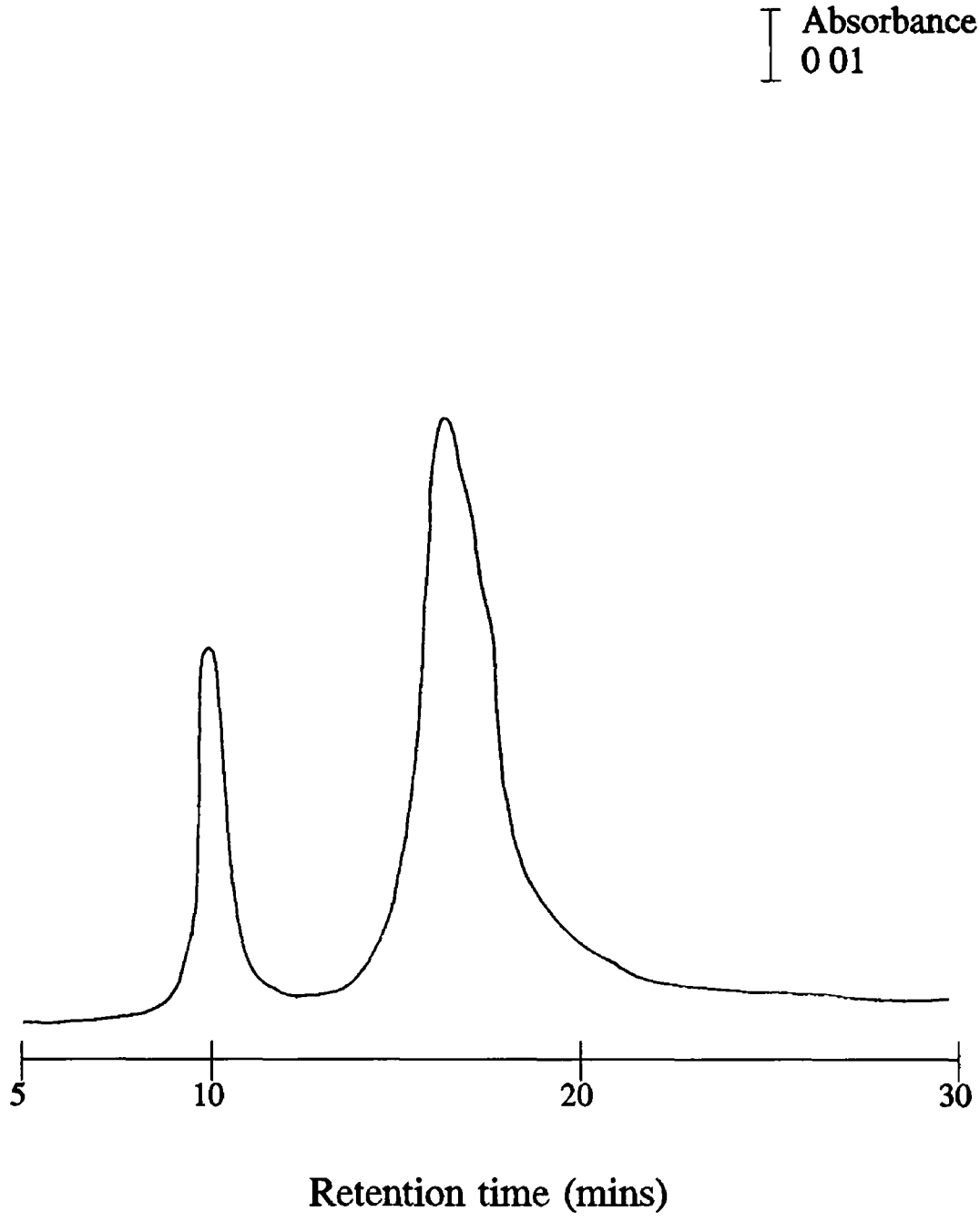


Figure 5.1.2.

HPLC chromatogram of the anti-G-CCM x anti-IBHR heteroconjugate antibody The column used was 10μm Protein Pak SW 300 column with a mobile phase of 0 1M phosphate buffer, pH 7 0 and a flow rate of 0 5ml/min A major peak was detected with a retention time of 9 95min and a second peak was seen at 16 11min The peak at 9 95min represents proteins eluting in the void volume of the column The peak at 16 11min represents the individual unconjugated parental antibodies



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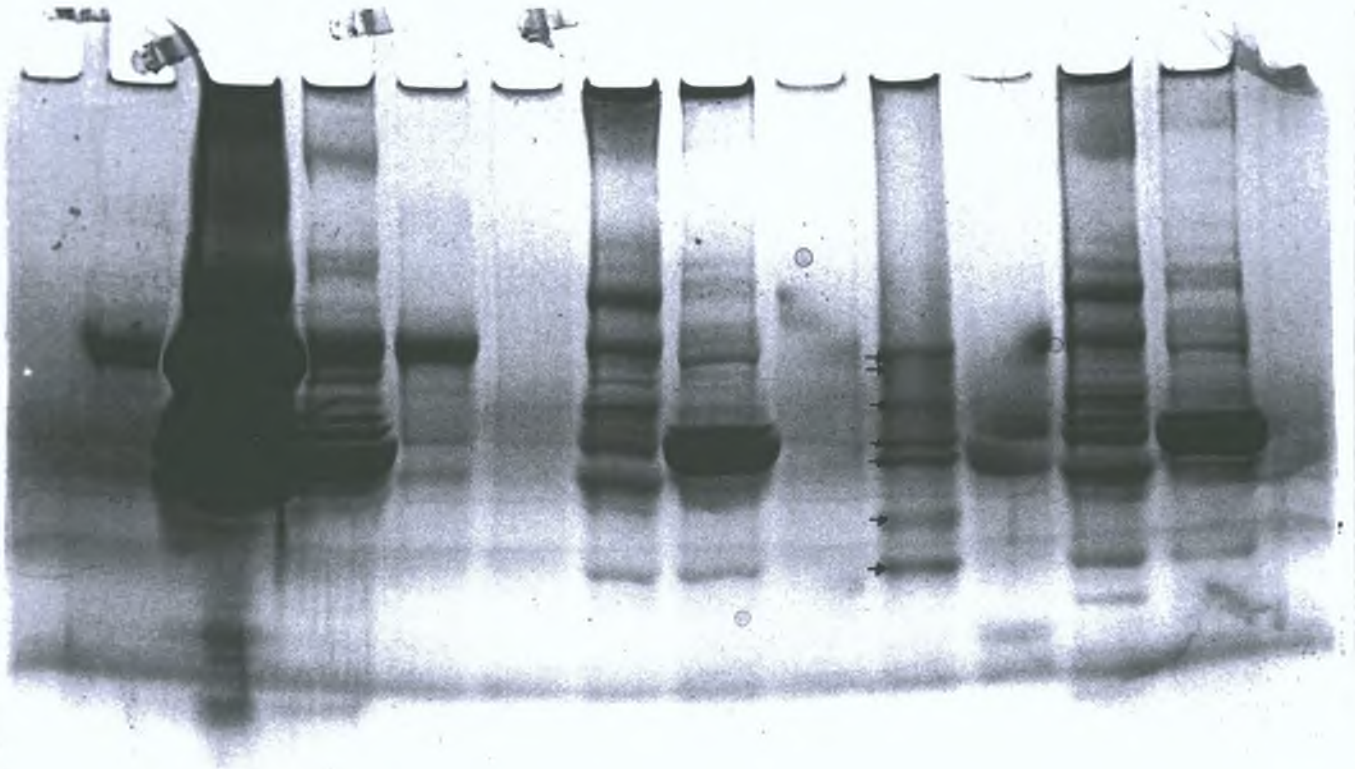


1 2 3 4 5 6 7 8 9 10 11 12

Figure 5.1.2.1. A 5-20% (w/v) gradient PAGEL^{R} precast polyacrylamide gel from ATTO was loaded as follows; Lane (1) SAS-treated anti-IBHR rabbit antibody, (2) SAS-treated anti-G-CCM antibody, (3) anti-G-CCM x anti-IBHR heteroconjugate antibody, (4) mouse IgG standard, (5) rabbit IgG standard, not visible as may have been too dilute, (6) molecular weight markers, arrowed from the top (in kD) 125, 86, 65, 48, 38 and 35.5, (7) SAS-treated anti-GFAP, (8) empty lane, (9) anti-GFAP x anti-IBHR heteroconjugate antibody, (10) SAS-treated anti-rRicin, (11) anti-G-CCM x anti-IBHR heteroconjugate antibody and (12) anti-GFAP x anti-rRicin heteroconjugate antibody. All samples were brought to 2mg/ml. The gel was run at 20mAmps per gel.

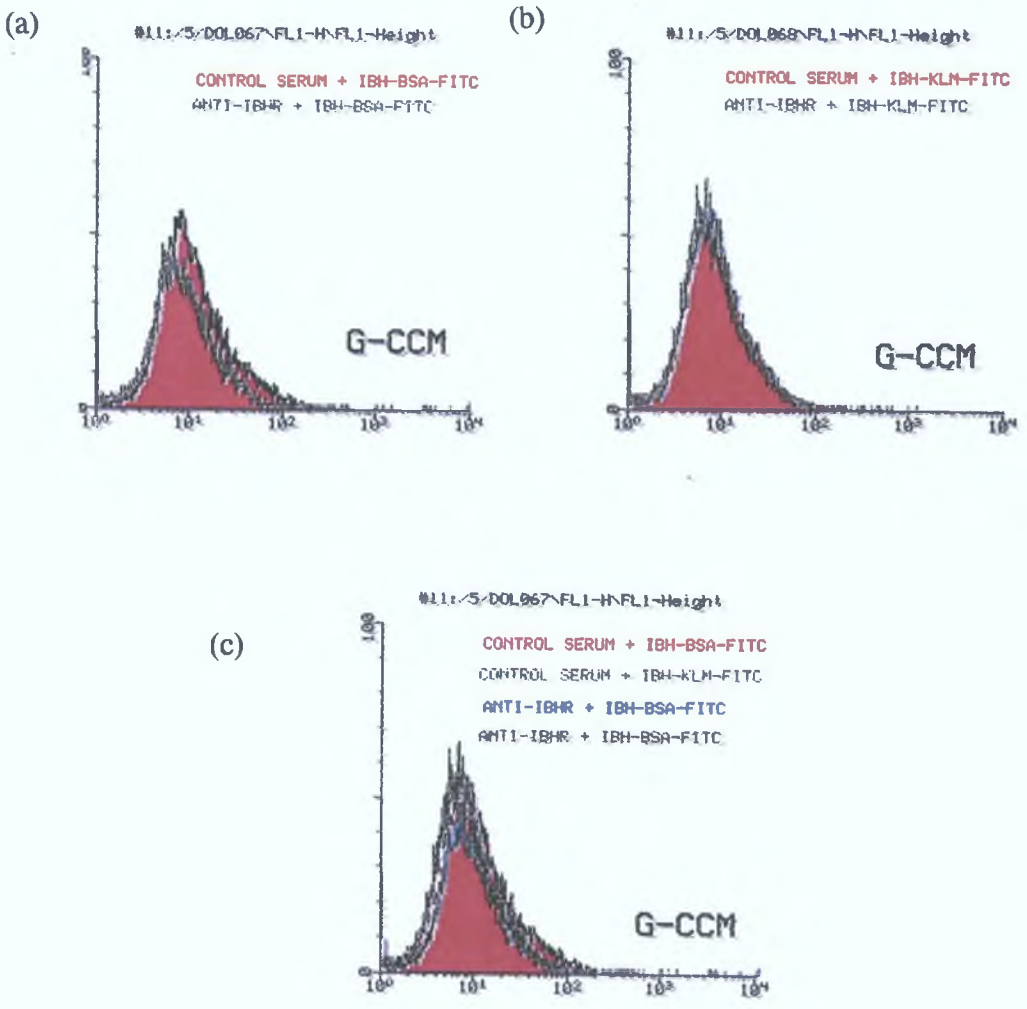


Figure 5.1.3.1. FACS analysis showing the negative controls used in this experiment. These included (i) control serum pre-incubated with the FITC-labelled IBHR-protein conjugate and (ii) the anti-IBHR antibody pre-incubated with the FITC-labelled IBHR-protein conjugate, these were then incubated with G-CCM cells. No non-specific binding of these negative controls were observed.

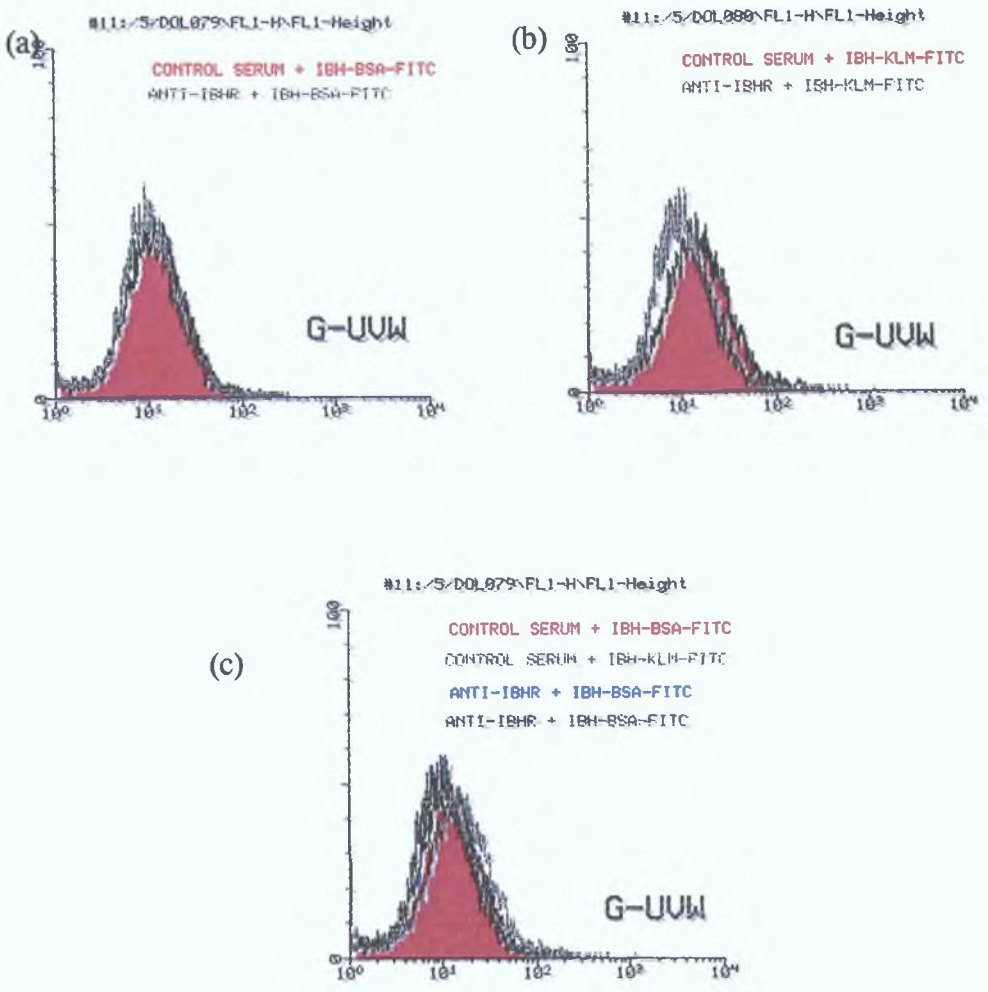
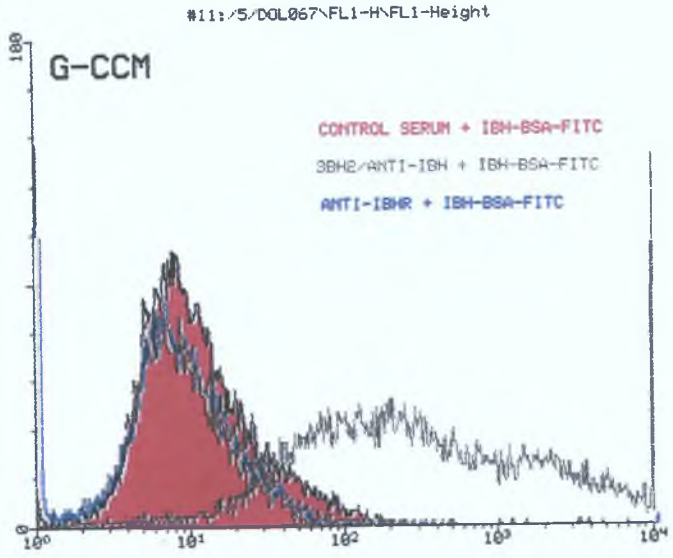


Figure 5.1.3.2. FACS analysis showing the negative controls used in this experiment. These included (i) control serum pre-incubated with the FITC-labelled IBHR-protein conjugate and (ii) the anti-IBHR antibody pre-incubated with the FITC-labelled IBHR-protein conjugate, these were then incubated with G-UVW cells. No non-specific binding of these negative controls were observed.

(a)



(b)

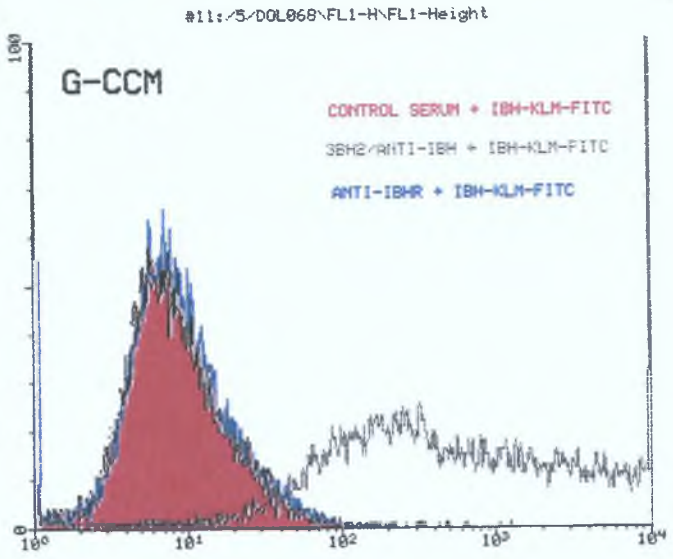
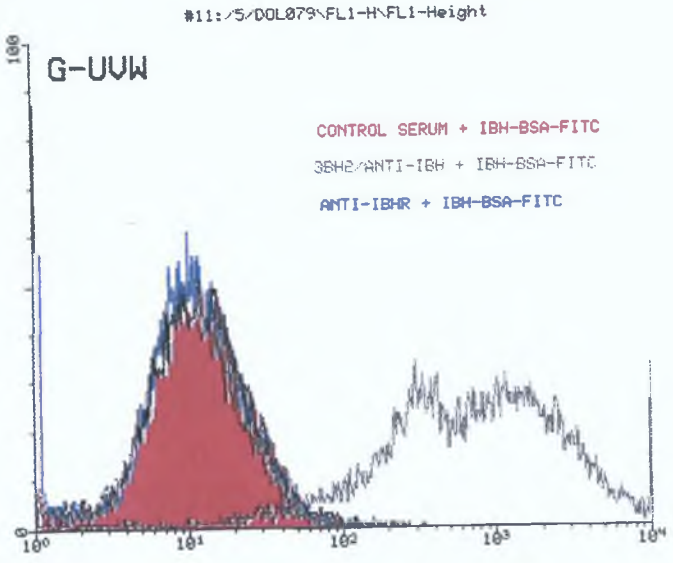


Figure 5.1.3.3. FACS analysis of the binding of the anti-G-CCM (3BH2) x anti-IBHR heteroconjugate antibodies against G-CCM cells. The heteroconjugate had previously been incubated with either IBHR-BSA-FITC or IBHR-KLH-FITC. Fluorescence was detected using the FITC-labelled IBHR-protein conjugate, no secondary antibody was required.

(a)



(b)

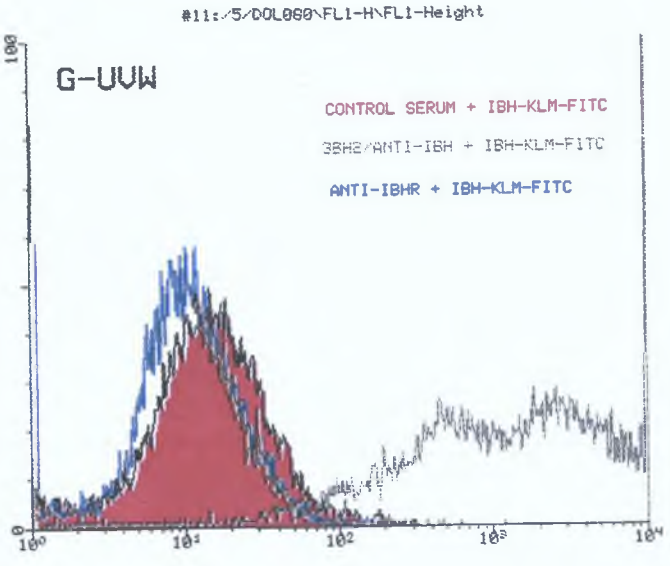
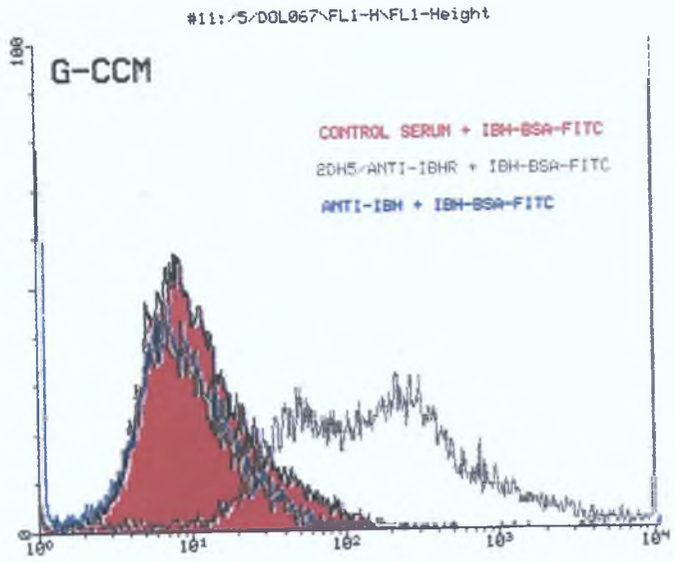


Figure 5.1.3.4. FACS analysis of the binding of the anti-G-CCM (3BH2) x anti-IBHR heteroconjugate antibodies against G-UVW cells. The heteroconjugate had previously been incubated with either IBHR-BSA-FITC or IBHR-KLH-FITC. Fluorescence was detected using the FITC-labelled IBHR-protein conjugate, no secondary antibody was required.

(a)



(b)

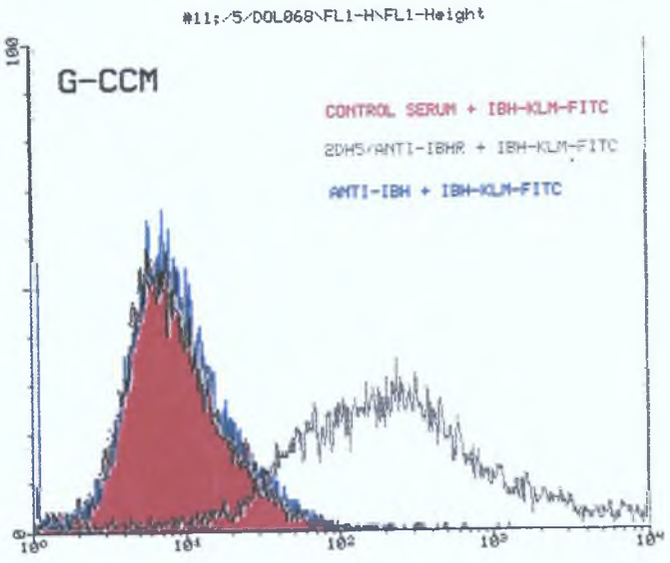
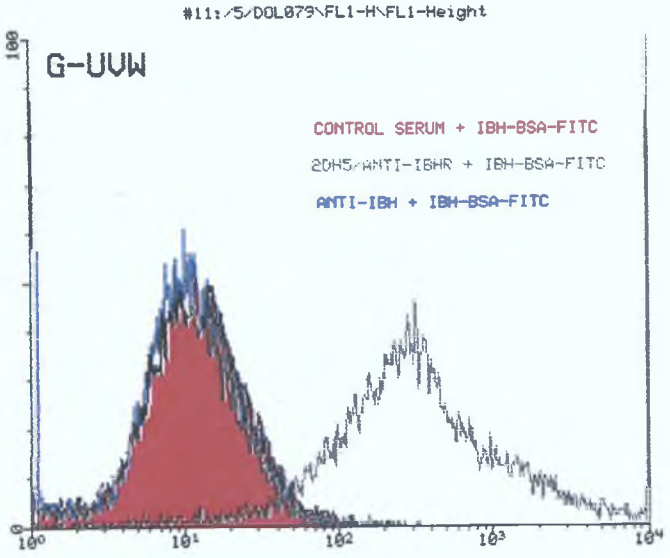


Figure 5.1.3.5. FACS analysis of the binding of the anti-GFAP (2DH5) x anti-IBHR heteroconjugate antibodies against G-CCM cells. The heteroconjugate had previously been incubated with either IBHR-BSA-FITC or IBHR-KLH-FITC. Fluorescence was detected using the FITC-labelled IBHR-protein conjugate, no secondary antibody was required.

(a)



(b)

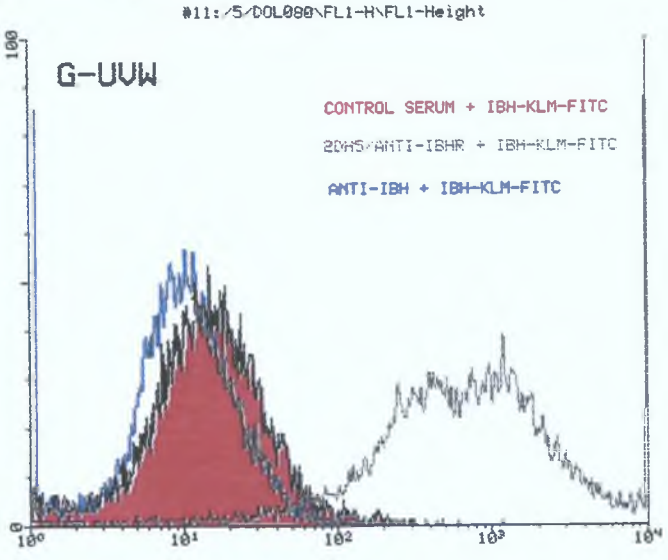


Figure 5.1.3.6. FACS analysis of the binding of the anti-GFAP (2DH5) x anti-IBHR heteroconjugate antibodies against G-UVW cells. The heteroconjugate had previously been incubated with either IBHR-BSA-FITC or IBHR-KLH-FITC. Fluorescence was detected using the FITC-labelled IBHR-protein conjugate, no secondary antibody was required.

5 1 7 Discussion

Targeting of toxic substances to tumour cells has been discussed (Section 1 6) Using the heteroconjugate antibody produced in this section, it would be possible to target ricin A-chain (Section 1 11) or radiolabelled IBHR (Section 1 13) to astrocytoma cells (Section 3) (Figure 5 1 1) Bifunctional antibodies have potential for many uses including diagnostic imaging of tumour cells, therapy and in diagnostic kits, reviewed by Fanger *et al* (1993)

In this section, bispecific heteroconjugate antibodies were made to show the potential application of the monoclonal antibodies produced during this project Heteroconjugate antibodies were generated using anti-G-CCM x anti-IBHR and anti-GFAP x anti-IBHR, as well as anti-G-CCM x anti-rRicin A-chain and anti-GFAP x anti-rRicin A-chain by the method of Bush and Winkler (1989) (Section 2 5 1) These antibodies were initially screened by ELISA (i) to determine if, in fact, heteroconjugate antibodies had been produced and (ii) to determine if the heteroconjugate bound to both parental antigens In all cases (Tables 5 1 1 to 5 1 4), it was seen that heteroconjugate antibodies bound to both antigens The parental, unconjugated antibodies were not separated from the heteroconjugate antibodies in this system, which is a disadvantage as this may result in erroneous results, especially in ELISAs This was particularly a problem where both of the paternal antibodies were of mouse origin, in the case of anti-G-CCM x anti-rRicin A-cham and anti-G-CCM x anti-rRicin A-chain In these cases, it was only possible to show separately that both paternal binding activities were retained in the heteroconjugate

It was simplei to demonstrate that a heteroconjugate antibody had been produced involving the rabbit anti-IBHR antibody and the murine anti-G-CCM and anti-GFAP This was achieved by coating the ELISA plates with G-CCM or GFAP and using alkaline-phosphatase-labelled anti-rabbit antibody as the secondary antibody The results in Tables 5 1 1 to 5 1 4 suggest that heteroconjugate antibodies were produced which retained binding affinities for (i) IBHR and G-CCM and (ii) IBHR and GFAP The unconjugated parent antibodies were also screened against the opposite antigen of the heteroconjugate to ensure there was no non-specific cross-binding In all cases, non-specific binding was seen up to a dilution of 1 200, this was due to the high concentration of antibody No non-specific binding was seen at the 1 1000 dilution used This method (Bush and Winkler, 1989) using the heterobifunctional cross-linker, SPDP,

was fast and reliable A disadvantage of this method is the large size of the resultant heteroconjugate antibodies and, as already mentioned, the heteroconjugate antibodies were not separated from the parental antibodies The bispecific heteroconjugate antibodies were then assessed by HPLC In all cases, following dialysis against the HPLC mobile phase buffer, 0 1M phosphate buffer, pH 7 0, a peak was seen at approximately ten minutes and sixteen minutes It was considered the first peak, which eluted in the void volume, may have been the heteroconjugate antibody and the second peak was unconjugated, parental antibody as it eluted at the same retention time as rabbit and mouse IgG

This assumption was confirmed by SDS-PAGE (Figure 5 1 2) where, on lanes containing heteroconjugate antibody samples, bands were seen corresponding to IgG and protein was seen at the top of the heteroconjugate lanes which may have been the heteroconjugate antibody which was too big, at 300 kD, to enter the gel

The heteroconjugate antibodies were analysed by FACS (Section 2 3 20 8) The negative controls used in this experiment (Figures 5 1 3 1 and 5 1 3 2) were the G-CCM (or G-UVW) cells incubated with rabbit control serum (Section 4) which had previously been incubated with either IBHR-KLH-FITC or IBHR-BSA-FITC It was seen that the peak obtained was exactly the same as the peak obtained when 0 01M PBS, pH7 2, was used A similar result was seen when the anti-IBHR antibody was preincubated with IBHR-BSA-FITC or IBHR-KLH-FITC

In Figures 5 1 3 3 and 5 1 3 4 , the binding of the anti-G-CCM (3BH2) x anti-IBHR heteroconjugate antibody was assessed against the G-CCM and G-UVW cell lines It was seen that a broad peak was obtained, which was quite distinct from the negative control peak This is the type of band obtained with a heteroconjugate antibody (Dr Tom Cotter, St Patrick's College, University of Maynooth, *personal communication*) This result was important as it showed definitively that (i) the anti-G-CCM antibody bound to G-CCM and G-UVW cells, (ii) the anti-IBHR antibody bound to the IBHR present in the IBHR-protein-FITC conjugate, as the FITC-labelled protein was the detection method used by FACS in this experiment, and (iii) that heteroconjugate antibodies involving the anti-G-CCM x anti-IBHR antibodies were produced A similar result was observed when the anti-GFAP (2DH5) x anti-IBHR heteroconjugate antibody, demonstrating that although this antibody was against GFAP, it also bound to a GFAP-like antigen on live G-CCM and G-UVW cells (Section 3 19)

The bispecific antibodies produced in this project could be used to target radiolabelled ^{131}I to astrocytoma cells, if radiolabelled iodine was subsituted for non-radiolabelled iodine in the IBHR A pre-targeting method could be used where the heteroconjugate could be introduced *in vivo* and given time to localise to the tumour and its metastases, if present When the non-specifically bound heteroconjugate was eliminated, the ^{131}IBHR could be administered and localised by tomographic scanning or imaging Such heteroconjugates could be clinically applied in diagnosis by immunoscintigraphy or treatment by radioimmunotherapy

Bispecific antibodies have potential for a wide range of clinical applications (Fanger *et al* , 1993) It is hoped that the potential uses of monoclonal antibodies can be fully exploited, both therapeutically and diagnostically, in the future using heteroconjugate antibodies

In summary, heteroconjugate antibodies were produced during this project using the anti-G-CCM, anti-GFAP, anti-IBHR and anti-Ricin A chain antibodies These heteroconjugate antibodies were characterised by ELISA, SDS-PAGE, HPLC and FACS

5 2 Genetic engineering of monoclonal antibodies

5 2 1 Introduction

The potential of genetic engineering for solving many of the previous problems associated with the use of murine monoclonal antibodies has been discussed in Sections 1 21 to 1 24 This area has been reviewed by Hand *et al* (1994)

Antibodies are humanised (Section 1 23) by grafting the murine CDRs onto a human variable region framework (Reichmann *et al* , 1988) Roguska *et al* (1994) achieved a further advance by "resurfacing" or replacing only the surface residues of the mouse variable framework region with those from a human variable region (Section 1 24) These techniques also allow for the creation of novel antibodies, such as catalytic (Section 6) and bifunctional antibodies and can optimize the performance of existing antibodies (Section 5 1)

5 2 2 Large scale production of antibodies or antibody fragments

The genetics of antibody production have been reviewed by O'Kennedy and Roben (1991), a diagram of the antibody domains is shown in Figure 5 2 1 At present, the major production methods for monoclonal antibodies involve the use of animals for ascites fluid generation and large scale cell culture Although the production of ascites fluid gives good yields difficulties exist, which are discussed in Section 1 5 These difficulties have resulted in research aimed at the generation of antibodies by genetic means using a range of expression systems including yeast, bacteria, baculovirus and plants While these technologies are still to be optimised they offer exciting new methods for antibody production on a large scale (Cahill *et al* , in press)

The use of bacteriophage systems may prove more powerful than bacterial expression systems for the generation of high-titre combinatorial libraries (Cunningham and Harris, 1992) (Figure 5 2 2) The expression of Fv or Fab antibody fragments on the surface of bacteriophage has facilitated efficient screening of large numbers of phage clones ($>10^7$) for antigen-binding activity Phage which binds specifically to antigen-coated plates or columns can then be eluted, propagated and re-screened against antigen Mark *et al* (1991) reported the screening of $2x10^7$ cloned human immunoglobulin genes from an unimmunised human donor by this method, obtaining antigen-specific antibody fragments of moderate affinity The production of human antibodies using bacteriophage enables human antibody fragments of high affinity and specificity to be produced without immunisation This area has been reviewed by Griffiths (1993)

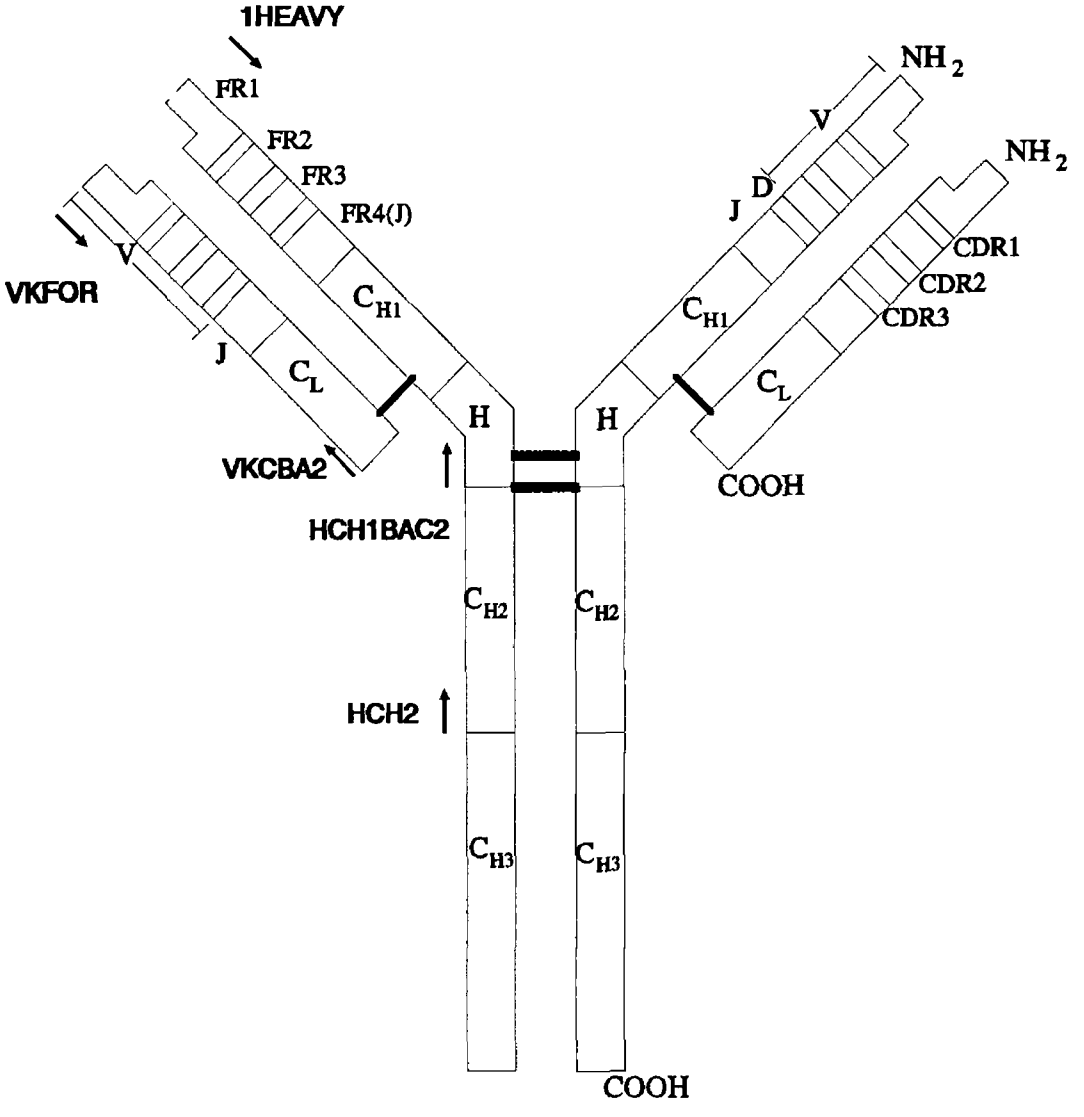


Figure 5 2 1. Diagram of antibody domains The variable and heavy chains of a complete antibody are shown The protein regions of a mature antibody corresponding to the DNA regions amplified by the PCR primers used in this study (Table 5 2 1) are shown with arrows (not to scale) The explaination of symbols are, H = heavy chain, L = light chain, C = constant region, V = variable region, J = joining region, D = diversity region, CDR = complementary determining region and FR = framework region

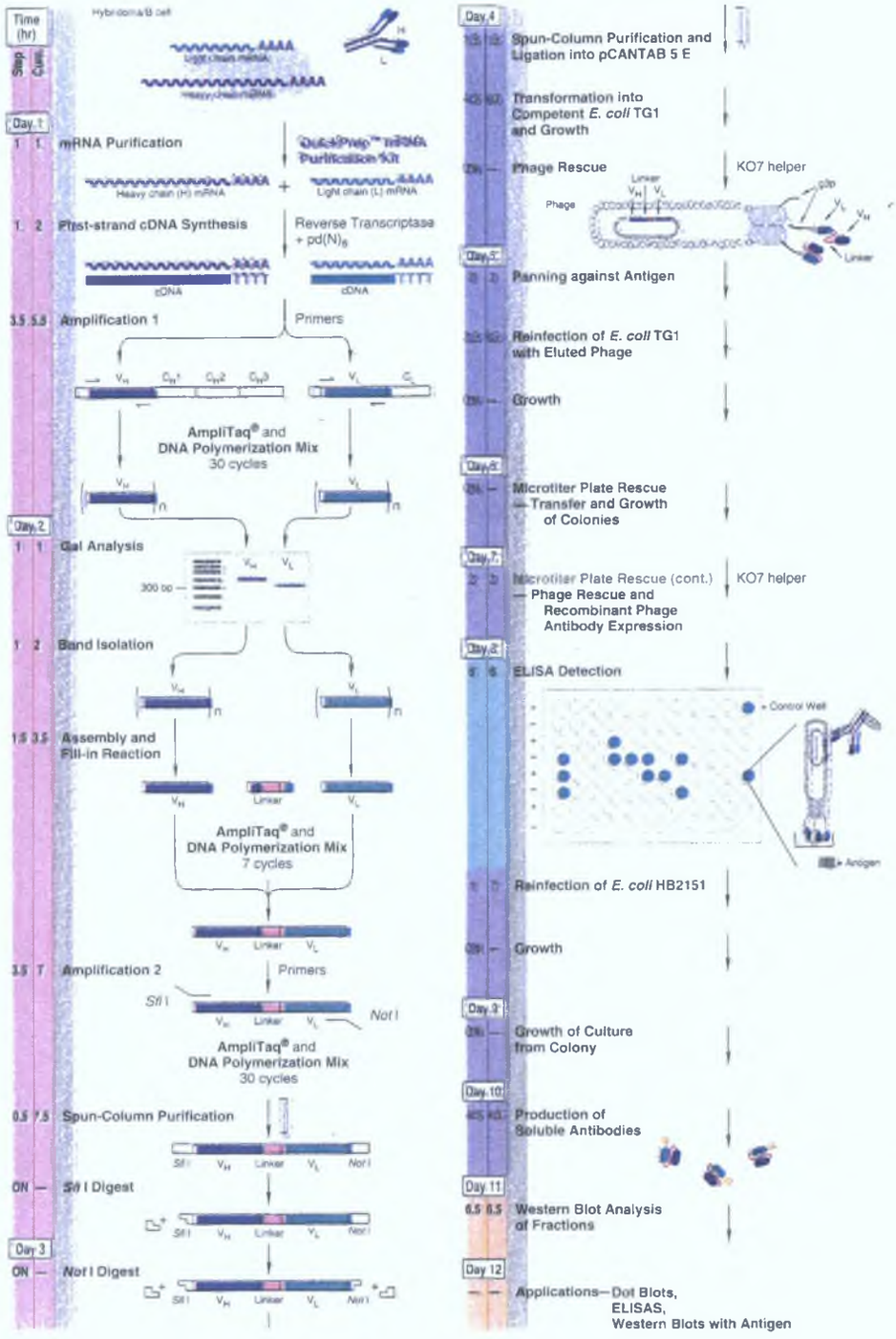


Figure 5.2.2. Overview of the Recombinant Phage Antibody System from Pharmacia (1992).

5 2 3 Vectors and hosts in the cloning of antibody genes

To engineer antibodies, the antibody genes to be used are first cloned into eukaryotic expression vectors The most commonly used are the pSV2 plasmids (Mulligan and Berg, 1981) as they contain (i) a plasmid origin of replication, (ii) a marker for selection in bacteria and (iii) a dominant selection marker for eukaryotic cells A common selection marker for eukaryotic cells is the *E coli* gene encoding the xanthine-guanine phosphoribosyltransferase This allows cells to grow in the presence of mycophenolic acid, provided xanthine is present, which inactivates G418, an antibiotic that prevents eukaryotic cell synthesis (Tan and Morrison, 1988) In this project, the plasmids used for the initial phases were pTACO1H for the heavy chain gene and pTCO1 for the light chain (Figure 5 2 3 (a) and (b)) These could then be excised and re-ligated sequentially into the pComb3 system (Figure 5 2 4) which enables the expression of engineered antibodies in *E coli* This pComb3 vector was constructed to fuse antibody Fd chains (comprising of V_H and C_{H1} domains with the C-terminal domain of cpIII (Figure 5 2 5) The Fd/cpIII fusion and light chain proteins were placed under the control of the *lac* promoter/operator sequences and directed to the periplasmic space by *pelB* leader sequences for functional assembly on the membrane

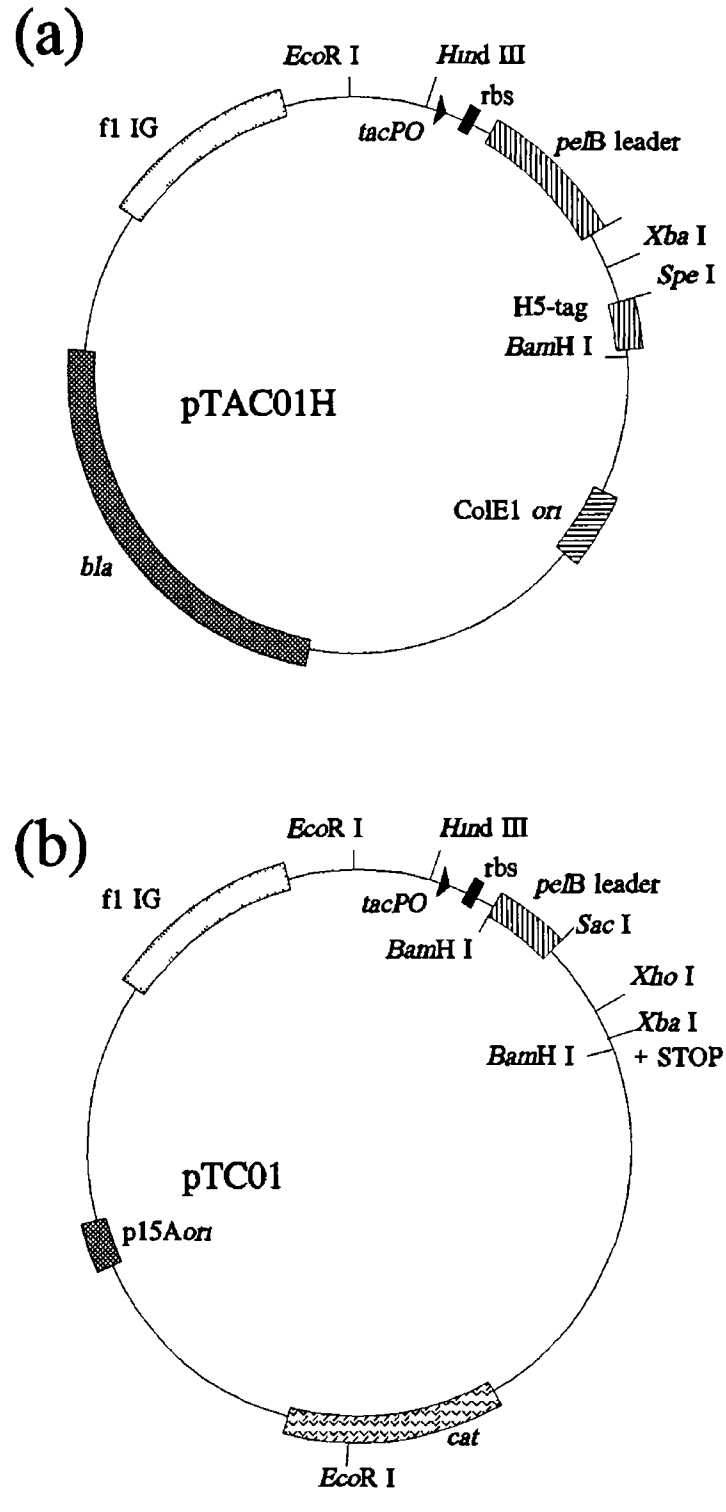


Figure 5.2.3.(a) and (b). The plasmids pTACO1H and pTCO1 The heavy chain PCR fragments were ligated to the pTACO1H plasmid, which carries the carbenicillin resistance gene and the light chain PCR fragments were ligated to pTCO1 which carries the chloramphenicol resistance gene

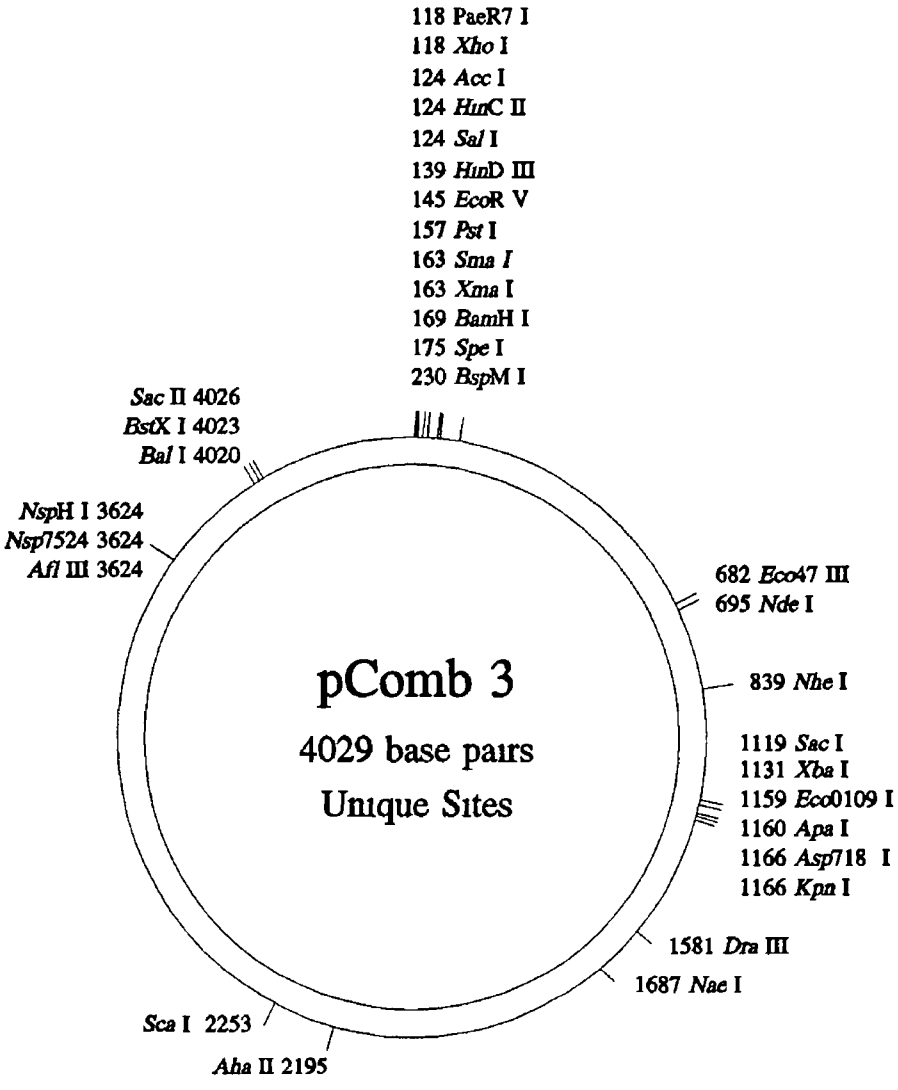
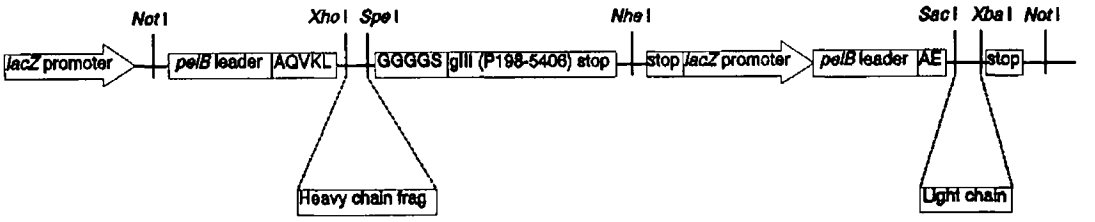


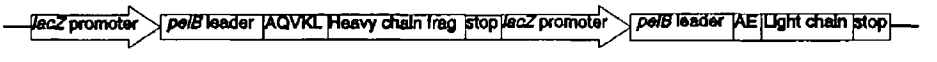
Figure 5.2.4. A detailed diagram of the pComb vector showing the regions where DNA fragments for the antibody heavy and light chain can be inserted when used to secrete antibody fragments



Surface Display Phagemid pComb 3

1) *Nhe* I, *Spe* I digest
-663bp gIII fragment

2) ligate



Soluble Fab Expressing Phagemid

Figure 5.2.5.

The pComb vector showing how it can be used as a phagemid expressing soluble Fab fragments

5 2 4 Overview of expression of murine monoclonal antibodies in *E coli.*

The 3BH2A3 hybridoma cells (Section 3) were grown in culture and the mRNA isolated using oligio-(dT)-cellulose spun columns This mRNA was used as a template for the synthesis of the first cDNA strand using reverse transcriptase This mRNA-cDNA hybrid was used as the template for PCR By developing probes specific for the conserved sequences in antibodies, only the required DNA was amplified and could be subsequently purified by column chromatography or electrophoresis In this way, the specific antibody DNA can be isolated from hybridoma cells If a mouse-human chimaeric antibody was obtained, it could be detected by ELISA (Mixter *et al* , 1986)

5 2 5 Purification and quantification of the PCR primers

The primers were purified from their columns (Section 2 3 30 7) and quantified (Section 2 3 30 8) To confirm these primers were purified correctly from their columns, they were run on a 4% Nusieve agarose gel (Figure 5 2 5)

5 2 6 Results of mRNA isolation

In order to genetically engineer or humanise the mouse monoclonals produced against G-CCM (Section 3), mRNA was isolated from the rapidly growing hybridoma cell line, 3BH2 clone A3, at a cell count of $1\ 59 \times 10^7$ using the QuickPrep mRNA purification kit from Pharmacia, according to Section 2 3 30 3 This kit is designed for the direct isolation of polyadenylated RNA from eukaryotic cells and tissues, bypassing the need for intermediate purification of total RNA

The precautions regarding removal of all RNAses from glassware, plastics and reagents to be employed in the isolation and use of mRNA were strictly followed These are outlined in Section 2 3 30 1

The mRNA was quantitated using the Shimadzu UV-160A UV-Visible recording spectrophotometer Knowing that for RNA, 1 A_{260} unit = 40μg/ml, the concentration of the RNA present, was calculated using the following formula

$[RNA] = A_{260} \times D \times 40\mu g/ml$

where D = final dilution factor, in the simpliest case above, this would be 2

If the absorbance of the diluted sample was greater than or equal to 0 5, the sample may be used directly for cDNA synthesis (Section 2 3 30 9) If the absorbance was less than 0 5, the sample must be precipitated and redissolved at a higher concentration to ensure

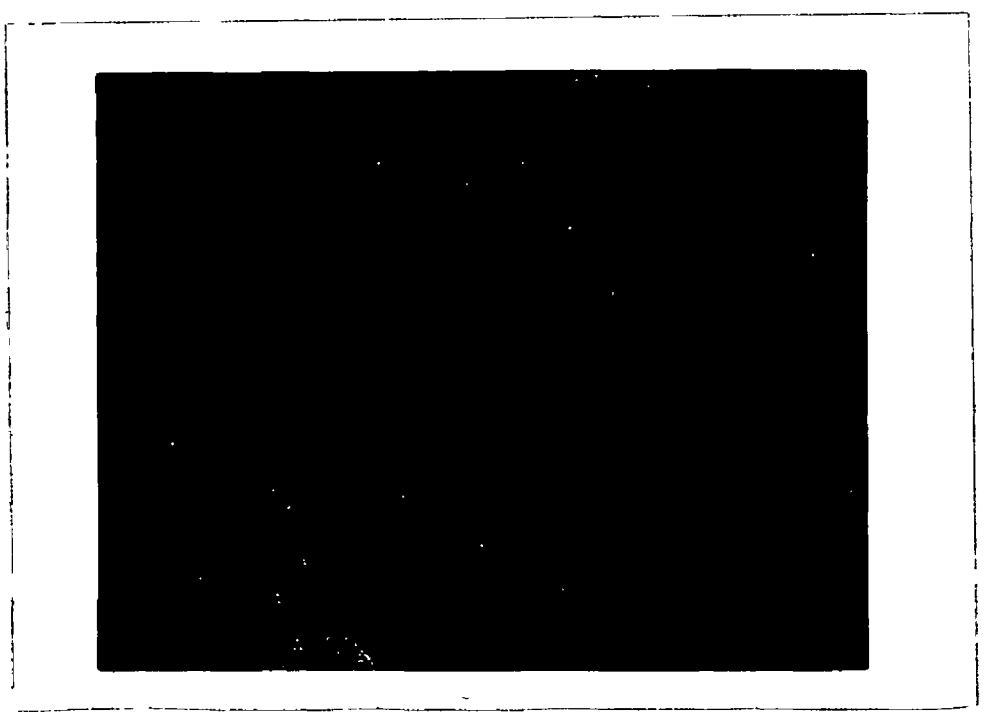
efficient cDNA synthesis (Pharmacia, 1991) This method is rapid, but has the disadvantage that it requires 1/3 of the mRNA sample A total of 4 71µg mRNA was obtained This was sufficient, as 0 5µg was required per cDNA reaction

5 2 7 Results of cDNA synthesis and PCR

The mRNA was immediately used as a template for the synthesis of the first strand of cDNA using reverse transcriptase (Section 2 3 30 9) The primers were used in the combinations outlined in Table 5 2 1 for the three PCR reactions (Section 2 3 30 6) As a negative control, the PCR reactions were preformed without template DNA The results of these PCR reactions are shown in Figure 5 2 7

Table 5 2 1 Primer combinations used in the three cDNA and PCR reactions (RXN)

RXN 1	RXN 2	RXN 3
VKFOR VKCBAC2	1HEAVY HCH1BAC2	1HEAVY HCH2



1 2 3 4 5

Figure 5 2 6 Determination of the presence of the 5 different DNA primers following their elution from primer columns The primers are as follows, Lane (1) HCH2, (2) VKCBAC2, (3) 1HEAVY, (4) VKFOR and (5) HCH1BAC2

1353 bp
1078
872
603
310
271, 281

1 2 3 4 5 6 7 8 9 10

Figure 5 2 7 Results of the initial PCR experiments to amplify regions of the 3BH2A3 genomic DNA The gel was loaded as follows, Lane (1) RXN3, (2) RXN3, negative control, (3) RXN2, (4) RXN2, negative control, (5) RXN1, (6) RXN1, negative control, (7) ϕX174/*Hae* III MWM, in base pairs (bp), (8) and (9) Positive controls and (10) Negative control

5 2 8 Optimisation of PCR reactions

From Figure 5 2 7 , a band can be seen for reactions 2 and 3 and no band is seen in their negative controls, these bands correspond to 660 and 735 base pairs (bp), respectively, which agree with the base pair lengths of the corresponding amplified DNA regions shown in Figure 5 2 2 The light chain reaction was initially unsucessful, which could be due to an inability of the light chain primers to bind the template DNA The three PCR reactions were optimised and the results are shown for the heavy chain reactions in Figure 5 2 7 and for the light chain reactions in Figure 5 2 8

The heavy chain reactions both gave one defined band on a gel (Figure 5 2 7) and were purified from contaminating amplification primers and nucleotides using the Magic PCR preps™ purification system from Promega (Section 2 3 30 16) Due to the lack of success in the PCR amplification of the light chain reaction in Figure 5 2 8 , the DNA from the band corresponding to the molecular weight of this fragment (660bp) was excised carefully from the gel and was also purified using the "Magic" PCR preps™ purification system (Section 2 3 30 16) This was used as a template for PCR *but again* no band was seen

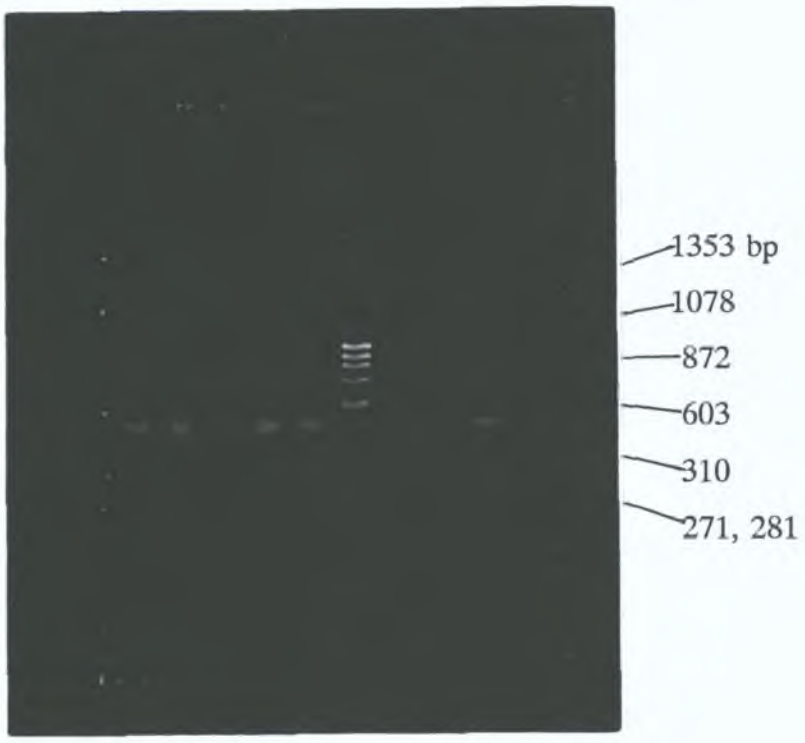


Figure 5.2.8. Results of the optimised PCR experiments to amplify regions of the 3BH2A3 heavy chain genomic DNA. The gel was loaded as follows; Lane (1) RXN3, (2) RXN2, (3) and (4) RXN3, (5) and (7) RXN2, (6) ϕX174/*Hae* III MWM (bp), (8) and (9) negative controls for RXN3 and RXN2 respectively. Both PCR reactions in lanes (1) and (2) produced one clear band at a molecular weight of approximately 735bp, in lane 1, and 660bp, in lane 2. The PCR reactions in lanes (3), (4), (5) and (7) were under different conditions to those in lanes (1) and (2) and were unsuccessful.

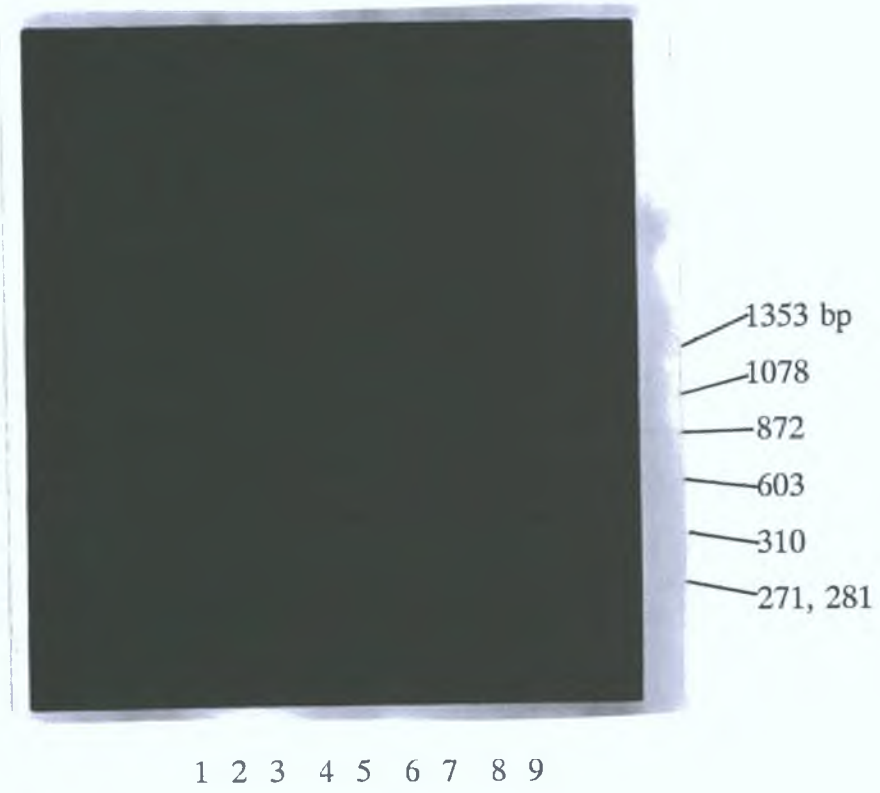


Figure 5.2.9. Results of the optimised PCR experiments to amplify regions of the 3BH2A3 light chain genomic DNA. The gel was loaded as follows; Lane (1), (2) and (3) RXN1, (4) and (5) RXN1, which showed a series of bands (6) RXN1, negative control, (7) and (8) negative controls, (9) ϕX174/*Hae* III MWM (bp).

5 2 9 Results of the cloning of the amplified DNA

The purified PCR fragments were digested with their appropriate restriction enzymes (Section 2 3 30 6) before being ligated to their respective plasmids (Section 2 3 30 19) The heavy chain reactions were ligated to the pTACO1H plasmid, which carries the carbenicillin resistance gene and the light chain was ligated to pTCO1 which carries the chloramphenicol resistance gene These plasmids were specially prepared for ligation by treatment with calf intestinal phosphatase (Section 2 3 30 18) which is shown in Figure 5 2 10

The plasmids were prepared according to Section 2 3 30 18 (Birnboim and Doly, 1979) 20ng were used at 10ng/µl in the electroporation procedure (Section 2 3 30 21) Positive and negative controls were performed at all stages of the experiment The ligated plasmids were transformed by electroporation (Section 2 3 30 21) into electrocompetent *E coli* TG1 cells (Section 2 3 30 20) Antibiotics were prepared according to Section 2 3 30 23 and the transformed *E coli* cells were spread onto agar plates containing the appropriate antibiotic

Colonies were obtained on the test antibiotic plates and these were screened for inserts according to Section 2 3 30 22 by purifying the plasmid DNA from the *E coli* cells, restricting the plasmid with the appropriate restriction enzymes and observing the digestion pattern on a 4% agarose gel The orientation of the insert in the plasmid is also important The antibody fragments will only be produced if the insert is in the correct orientation and position with regard to the promoter (Figure 5 2 4 (b)) The orientation of inserts can be found by PCR cloning (Innis *et al* , 1988) and analysis of their restriction patterns (Sambrook *et al* , 1989) However, of the clones obtained, no clone contained the heavy chain insert in the correct orientation Due to time constraints, this transformation was not repeated These results are further discussed in Section 5 2 10

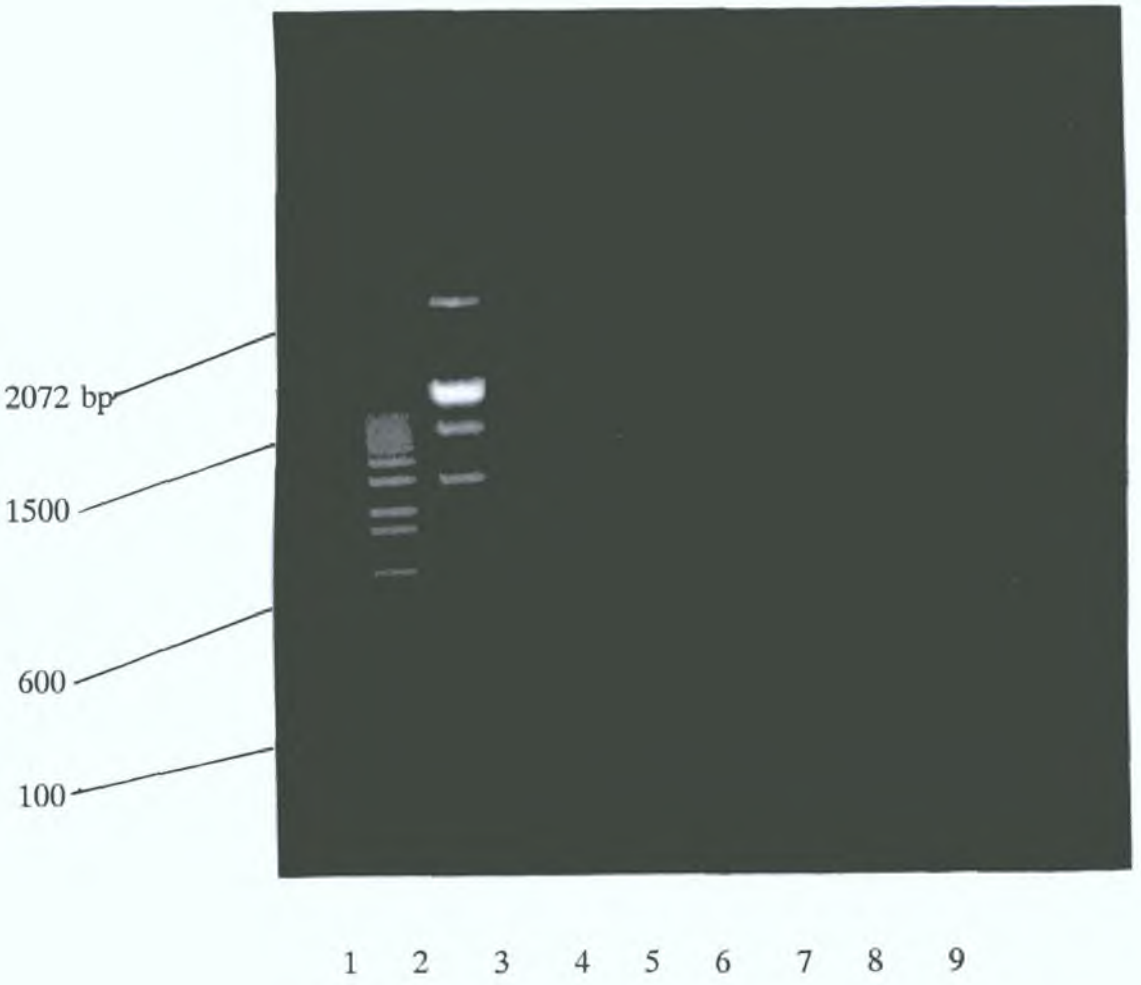


Figure 5.2.10. Gel showing preparation of plasmids pTACO1H and pTCO1 for ligation with purified PCR fragments. Lane (1) MWM (Gibco 1Kb ladder), (2) pTCO1, (3) pTCO1 RNase treated, (4) pTCO1 RNase treated cut with *Xba*I and *Sac*I, (5) pTCO1 RNase treated cut with *Xba* I and *Sac* I and treated with calf intestine phosphatase, (6) pTACO1H, (7) pTACO1H RNase treated, (8) pTACO1H RNase treated cut with *Xho* I and *Spe* I, (9) pTACO1H RNase treated cut with *Xho* I and *Spe* I and treated with calf intestine phosphatase.

5 2 17 Discussion

Hand *et al* (1994) reviewed the development of novel immunoglobulin forms and proposed that such genetic modifications may result in more useful diagnostic reagents and in the production of more stable immunoconjugates with the characteristics of more efficient tumour cell killing While the use of phage to express antibodies is still a new technology, many groups are already using bacterial expression vectors for the production of antibody fragments Using the phage system (Cunningham and Harris, 1992) (Figure 5 2 2), each phage mimics a B cell in that each phage displays an antibody fragment on its surface Only 1–10mg of antibody fragments per litre of culture broth could be produced using routine laboratory culture methods Antibody–fragment production in the gram per litre range appears to be achieveable when fermentor systems are used The combination of using technology for generating novel antibodies and bacterial–host expression systems using fermentation techniques to produce large quantities of Fab or Fab'_2 fragments could mean that, before long, traditional murine immunisation protocols may become redundant Pharmacia, in collaboration with Cambridge Antibody Technology Ltd , U K , have developed a Recombinant Phage Antibody System designed for the cloning and expression of recombinant antibody fragments in bacteria This approach is based on that developed by McCafferty *et al* (1990) and Winter and Milstein, (1991) This approach relies on a phage–display system in which fragments of antibodies are expressed as fusion protein on the phage surface The outline of this system is shown in Figure 5 2 2 (Pharmacia, 1992)

The choice of host for the antibody genes is paramount In eukaryotes, the heavy and light chains are secreted into the lumen of the endoplasmic reticulum where folding and disulphide bond formation occurs (Pluckthun *et al* , 1988) The host cell should, if possible, mimic this process if functional antibody products are to be obtained

For a bacterial host to express functional antibodies or antibody fragments it must, (i) synthesise approximately equal numbers of heavy and light chains, (ii) transport the chain precursors to the periplasm (functionally equivalent to the endoplasmic reticulum), (iii) align the chains correctly, (iv) fold correctly, (v) form disulphide bonds and (vi) form heterodimers These criteria were realised in *E coli* (Pluckthun, 1990) It is also important that the gene sequences transfected contain many unique restriction sites and do not contain any codons considered foreign to *E coli*

Yeast could also be used as a host since it does not have the problems associated with

folding, bond formation, etc as in *E. coli*, and its expression systems are well understood The secreted antibody from yeast has, however, been found not to bind to the antigen (Horowitz *et al* , 1988) Further studies have been undertaken using insects (Hasemann and Capra, 1990) and transgenic plants with varying degrees of success Hiatt *et al* (1989) showed that it was possible to express antibodies in plants with 1 3% of total leaf protein found to be functional antibody

Transfection is another difficulty, especially with yeast cells The whole process is very inefficient with less than 10^{-4} cells being stably transformed Calcium phosphate precipitation of the DNA (Chu and Sharp, 1981) is a common method but the success rate is very low (Oi and Morrison, 1986) Protoplast fusion provides a better method (Oi and Morrison, 1986) with frequencies of up to 10^{-3} and better were obtained with the myeloma line J558L (Gillies *et al* , 1983) Electroporation is another technique that can be used It is quick and simple but the DNA has to be specially prepared (Toneguzzo *et al* , 1986)

In this project, mRNA from the 3BH2A3 hybridoma (Section 3) was successfully isolated using the QuickPrep mRNA purification kit from Pharmacia (Section 2 3 30 9) This was quantified spectrophotometrically and a total of 4 71µg mRNA was obtained This was sufficient, as only 0 5µg was required per cDNA reaction The cDNA was prepared immediately, as RNA is less stable than DNA The cDNA was amplified using specifically developed primers to amplify the regions of interest (Table 5 2 1) by PCR Following PCR, it was seen that single bands were obtained for reactions 2 and 3, both heavy chain reactions No band was seen for the light chain reactions (RXN1) This could be due to the degradation of the mRNA for this reaction, or more probably, the light chain primers were not specific for this region (Section 2 3 30 5) Since it has been reported by Orlandi *et al* (1989) and Ward *et al* (1989) that much of the specific antigen-binding capability resides in the variable region of the heavy chain, and they have produced single domain antibodies, consisting only of the variable heavy chain region (Figure 5 2 1), it was decided to attempt to clone and express the heavy chain The plasmid DNA was prepared (Figure 5 2 9) and the purified DNA was ligated and transformed into *E coli* The plasmid DNA from the clones produced (Section 2 3 30 22) was screened for the presence of inserts in the correct orientation by restriction enzyme analysis (Sambrook *et al* , 1989) No inserts in the correct orientation were obtained

This transformation was not repeated due to insufficient time Future work would involve repeating the transformation to obtain a clone containing the DNA encoding the variable regions of the 3BH2A3 monoclonal antibody heavy chain, with and without the hinge region (Figure 5 2 1) Had an insert in the correct orientation been found, it would have been digested with the appropriate restriction enzymes and would be cloned into the pComb expression vector The clones which contained the V_H and V_k gene inserts would have been confirmed by sequencing as most of the recombinants would be expected to carry the correct inserts Any functional antibody obtained, would have been detected by ELISA (Mixter *et al* , 1986) and the results compared to the parent anti-G-CCM antibody (Section 3) As an alternative to sequencing independent clones, the amplified cDNA could be sequenced directly (Innis *et al* , 1988) Work was halted on this project due to insufficient time and may be resumed in the future

Antibody engineering has created new exciting fields of study and is helping to optimise the older ones Bifunctional antibodies have potential for many uses including imaging, therapy and diagnostic kits (Section 1 18) There are two main methods for their production, chemical and biological The chemical method involves the chemical linkage of two F(ab') fragments (Nolan and O'Kennedy, 1990, Section 5 1) and the biological is based on somatic cell hybridization Such bifuctional antibodies are active in their native state and need no chemical alteration to bind either the target or the functional agent In the future, it may also be possible to create tri- and multi-functional antibodies using antibody engineering

In summary, an unsuccessful attempt was made to express the anti-G-CCM antibody, 3BH2A3, (Section 3) in *E coli* (Section 5 2) mRNA was isolated from the hybridoma cells, cDNA was produced Primers were constructed to amplify the regions encoding the antibody heavy and light chains from the cDNA by PCR This was successful for the heavy chain PCR reactions but not for the light chain reactions The DNA from the heavy chain gene reactions was ligated to the pTA01H plasmid and transformed into *E coli* No inserts in the correct orientation were obtained

SECTION 6 PRODUCTION AND CHARACTERISATION OF CATALYTIC ANTIBODIES

6 Outline

This section is concerned with the application of polyclonal and monoclonal catalytic antibodies which would catalyse the hydrolysis of the mixed aryl 4-nitrophenyl carbonate (I), but would not catalysis the hydrolysis of the isomeric 2-nitrophenyl carbonate (II) The polyclonal antibody raised in sheep which catalysed this reaction was obtained from Dr Gerry Gallacher (Table 2 3) and was purified and analysed for catalysis The results obtained were in close agreement with those obtained by Gallacher *et al* (1991) A number of attempts were made to produce monoclonal antibodies of the IgG isotype, but only IgM antibodies were obtained The most promising of these were grown as an ascites (Section 2 3 17), the antibody was purified and characterised by ELISA, HPLC, SDS-PAGE and catalytic activity

6 1 Catalytic antibodies, transition states and their analogues

Recent developments and applications of catalytic antibodies have been discussed (Section 1 20) In the short period of time which has elapsed since the first reports of antibody catalysis in 1986 (Pollack *et al* , 1986, Tramontano *et al* , 1986) a considerable number of reactions have been catalysed using antibodies The substrate specificities of antibody catalysed reactions are high and rate accelerations of up to 10^6 have been achieved (Tramontano *et al* , 1988) A number of general stratagies have also evolved for generating catalytic antibodies including transition state stabilisation, catalysis by approximation, introduction of catalytic groups via hapten-antibody complementarity and chemical modification, generation of cofactor binding sites and site-directed mutagenesis (Shokat and Schultz, 1990) Characterisation of catalytic antibodies is providing insights into fundamental notions of enzymatic catalysis

The concept of catalytic antibodies is by no means a new one In 1946, Linus Pauling hinted at the possibility (Pauling, 1946) In 1952, Woolley suggested that if 'antibodies were challenged continuously and for long periods of time, they might evolve to become enzymes' (Woolley, 1952) In 1969, Jenks stated that it should be possible to find antibodies with catalytic properties The first attempts to elicit such antibodies were made by Raso and Stollar in 1975 Although unsuccessful, their research prompted other groups to begin working in the area but it was not until Kohen *et al* (1980) demonstrated that monoclonal antibodies against 2,4,-di-mtro-phenyl (DNP) were able to enhance the hydrolysis of DNP-ε-amino-caproyl-umbelliferone conjugate implying that

these antibodies have quasi-enzymatic properties akin to those observed in anti-steroidal antibodies Apart from their fundamental importance, such antibodies are interesting as they may prove useful as 'made-to-measure' reagents to induce specific hydrolytic reactions The first catalytic antibodies were produced by Tramontano *et al* (1986) and Pollack *et al* (1986) Since then, antibodies have been generated against a variety of haptens to catalyse various reactions including carbonate, ester and amide hydrolysis (reviewed in Nakayama and Schultz, 1991) Antibodies which catalyse Claisen rearrangements (Hilvert *et al* , 1988), redox reactions (Shokat *et al* , 1988), disfavoured reactions (Danishefsky, 1993, Janda *et al* , 1993) and cationic cyclisation (Li *et al* , 1994) have now been produced

Pauling stated that the fundamental difference between antibodies and enzymes was that enzymes bind transition states while antibodies bind ground states (Pauling, 1946) He also predicted that a stable substance might mimic the transition state in shape and charge He suggested that one way in which enzymes lower the activation energy of a reaction is to bind most strongly to the transition state and, thereby, stabilising it Less energy is then needed for its formation and thus, the reaction is accelerated (Lerner and Tramontano, 1988) Enzymes use a variety of methods to lower the activation energy of a reaction including transition state stabilization, general acid-base catalysis, strain and proximity effects Although enzymes use several of these mechanisms simultaneously, catalytic antibodies usually employ just one However, the specificity of antibody-catalysed reactions may be very high and oftens rivals or even exceeds that of enzyme-catalysed reactions (Lerner *et al* , 1991) The reactions are not catalysed to the same extent as enzymatic reactions as yet, but typically they proceed 10^3-10^6 times faster than the uncatalysed reactions

The whole idea of catalysis is to lower the activation energy of a given reaction Without a catalyst, most biochemical reactions would be impossibly slow Many enzymes have evolved to provide an active site environment that is sterically and electronically complementary to the rate determining transition state (Pauling, 1946) Enzymes catalyse chemical reactions without being used up themselves and antibodies recognise a diversity of substances, yet both exert their effect by binding to another molecule Some antibodies may bind more readily and stabilise the transition state of a reaction to be catalysed, in preference to the resting or ground state, thereby decreasing the energy needed for the reaction and, thus, increasing its rate Whereas

intermediate and ground state species of a chemical reaction lie in the free energy wells and correspond to structures in which bonds are fully formed or fully broken, the transition state configuration corresponds to a species in which bonds are partially formed and partially broken (Schatz, 1993, Section 1 20)

The transition state can be defined as being located at a free-energy minimum along a reaction pathway between reactants and products (Schatz, 1993) It is the highest energy species in the reaction, has a lifetime on the order of a bond vibration (10^{-13}sec) and is, therefore, unable to act as an immunogen However, antibodies with such specificities for this transition state can be produced by immunising with a transition state analogue During the transition state period, the length of the bonds from the central carbon increase to 120% of their normal size To provide an analogue for this, phosphorous is substituted for the central carbon Its bonds are roughly 20% longer than those of carbon and the resulting compound, known as the phosphate ester, is stable

6.2 Initial investigations with catalytic antibodies

In the initial stages of this project, in collaboration with Dr Mark Searcy, St Lukes Cancer Research Institute, an attempt was made to produce a monoclonal antibody to a transition state analogue Our proposed phosphate ester, was a prodrug that could be cleaved on interacting with an appropriate monoclonal to 7-OH-coumarin (Figure 6 1) It was envisaged that the proposed catalytic antibody would cleave the prodrug (Figure 6 1 (a)) resulting in free 7-OH-coumarin The applications of 7-OH-coumarin as an anti-cancer agent have been reviewed by Egan *et al* (1990)

Had this antibody been produced, it could have been a novel method to target drugs to the tumour site, if the catalytic antibody and the anti-G-CCM antibody were used to make a heteroconjugate, the anti-tumour part of this heteroconjugate could localise it to the tumour *in vivo* and the catalytic side could catalyse the prodrug to drug conversion at the tumour site The prodrug, being non-toxic, would not damage non-tumour tissue However, Dr Searcy's group failed to produce such a transition state analogue and this work was discontinued The assistance and encouragment of Dr Searcy is gratefully acknowledged and appreciated

Dr Gerald Gallacher's group in London have produced a transition state analogue which was prepared by substitution of a phosphorous atom for a central carbon atom in the transition state's tetrahedral group of atoms to form a stable phosphate ester (Gallacher

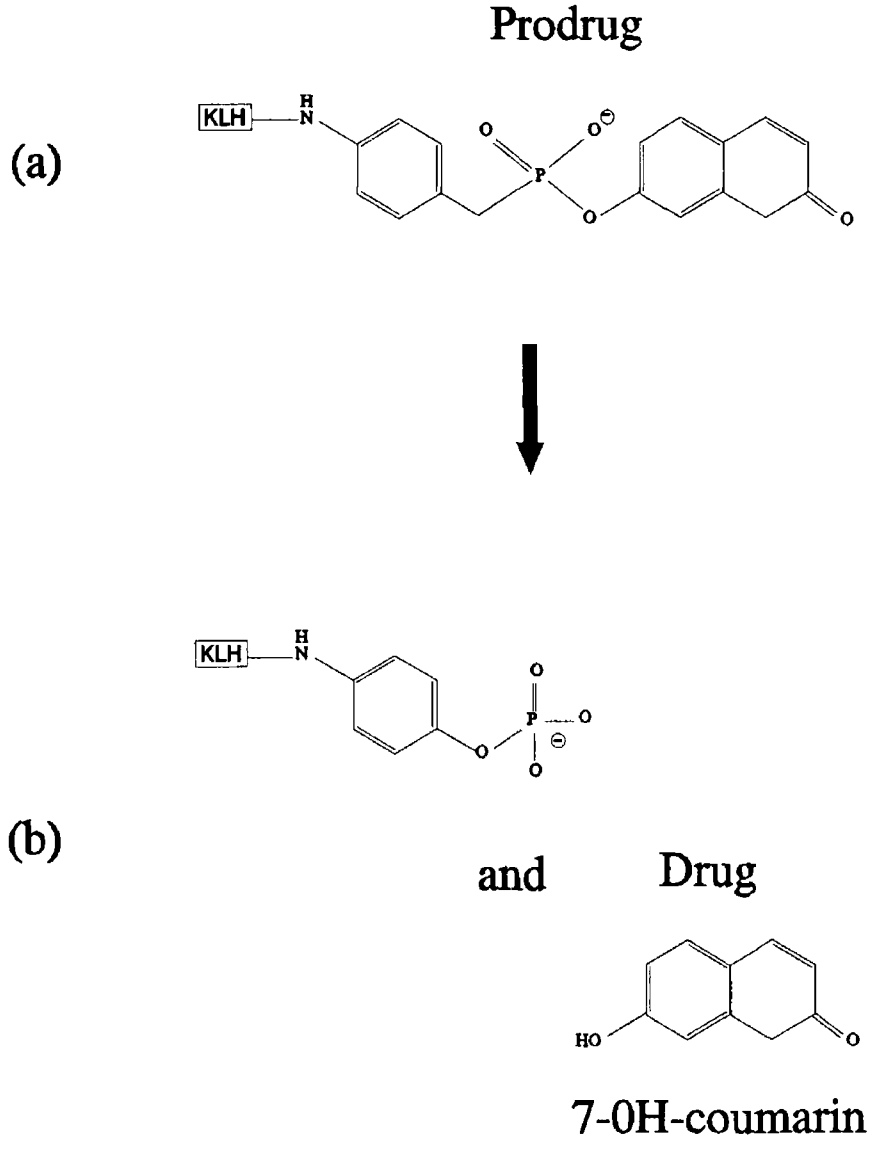


Figure 6.1. Postulated scheme of prodrug to drug conversion using catalytic monoclonal antibodies (a) Phosphate ester attached to KLH, the carrier protein This is the putative prodrug (b) Postulated scheme of prodrug to drug conversion using catalytic antibodies resulting in 7-OH-coumarin

et al , 1991) This was conjugated to KLH and was known as the phosphate immunogen, compound (XI) The distribution of charge on the oxygen resembles that of the transition state, in addition, the phosphorus–oxygen bonds are about 20% longer than ordinary carbon–oxygen bonds, which enables the analogue to mimic the elongated bonds of the transition state Further work in this area was continued using this transition state analogue

6 3 Polyclonal catalytic antibodies

The first incidence of the production and characterisation of a catalytically competent polyclonal antibody preparation from a sheep was by Gallacher *et al* (1991) In this instance, a sheep was immunised with the hapten–KLH conjugate (Phos–KLH) (sheep 271) and this preparation of IgG catalysed hydrolysis of the mixed aryl 4–nitrophenyl carbonate (I) but not of the isomeric 2–nitrophenyl carbonate (II)(Figure 6 2) The catalysed reaction obeys Michaelis–Menten kinetics

This result demonstrates that, whatever structural heterogenity exists in the IgG, the catalytic characteristics of the active IgG species are all sufficiently similar that differences between them are not readily detected as deviations from a single–site saturation model

Gallacher argued that the polyclonal method was preferable as it is easier to produce polyclonal antibodies and that the investigation of catalytic antibody responses in animals could also be studied The incidence of contamination with enzymes was ruled out in this study after extensive analysis

The structures of the phosphate immunogen (XI), the carbonate substrate (I), the amide substrate (III), the inhibitor and the alternative substrate are shown in Figure 6 2 and are described below

The phosphate immunogen (Figure 6 2 (a)), known as compound (XI) (all compounds are as described in Gallacher *et al* , 1991, unless otherwise referenced) and compound (II) (Gallacher *et al* , 1992), involved the carboxy group of 4–nitrophenyl 4'–carboxy–methylphenyl phosphate and an amino group of KLH and was designated 4–nitrophenyl 4'–(3–oxa–2–oxobutyl)phenyl hydrogen phosphate The carbonate substrate (Figure 6 2 (b)), known as compound (I), was labelled 2–nitrophenyl 4'–3(–aza–2–oxoheptyl) phenyl carbonate This is a chromogenic substrate for the investigation of catalytic activity The amide substrate (Figure 6 2 (c)), known as compound (III), was called N–

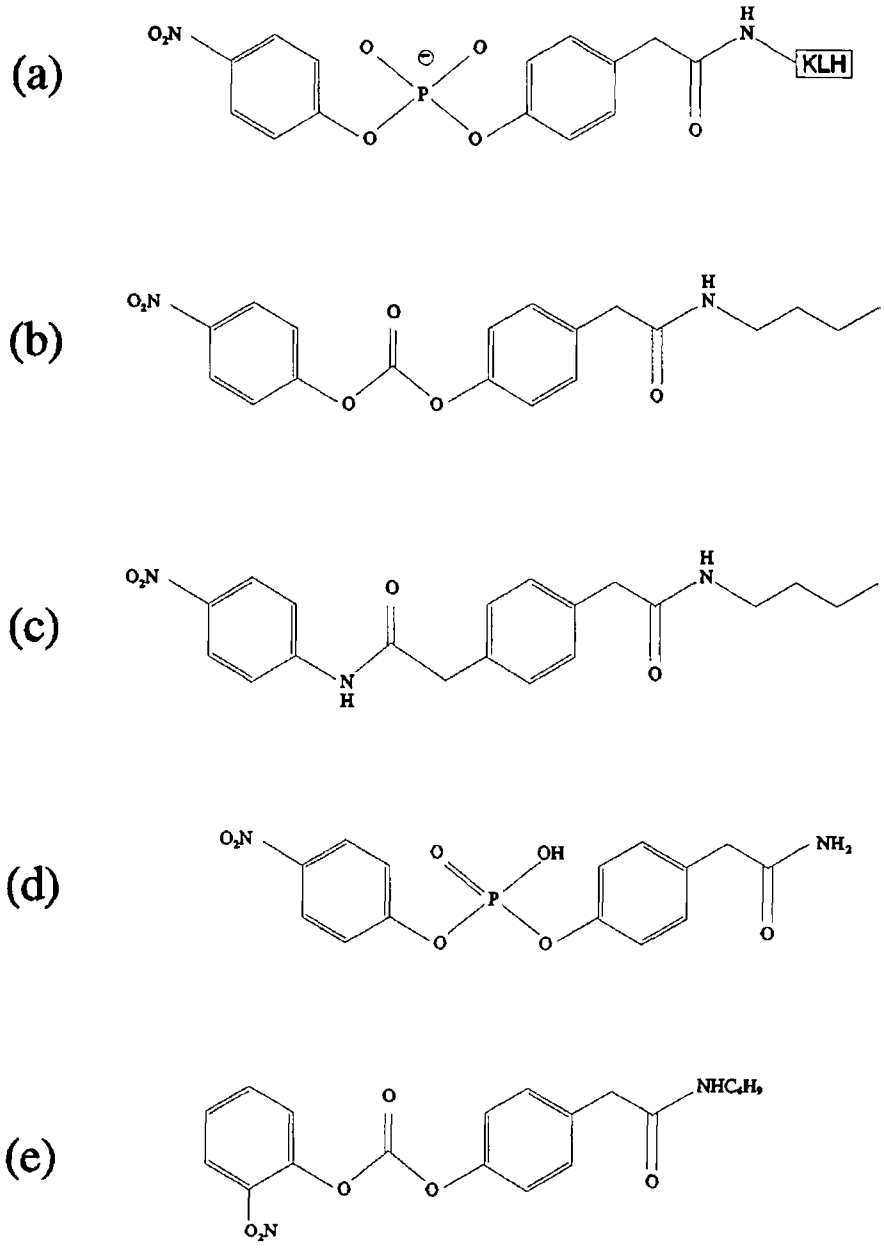


Figure 6.2. The structures of (a) the phosphate immunogen (XI), (b) the carbonate substrate (I), (c) the amide substrate (III), (d) the inhibitor and (e) the alternative substrate are shown Detailed descriptions are given in Section 6 3

(4-nitrophenyl)N'-butyl-1,4-phenylenediacetamide The inhibitor (Figure 6 2 (d)) was termed compound (XXI) This acts as a competitive inhibitor of the catalysed hydrolysis of compound (I), the carbonate substrate The alternative substrate is shown in Figure 6 2 (e) Hydrolysis of this substrate could be catalysed by contaminating enzymes, like esterases, but will not be catalysed by antibodies (G Gallacher, *personal communication*)

6 4 Techniques used in the characterisation of polyclonal and monoclonal catalytic antibodies

In order to detect catalysis of the monoclonal antibodies produced during this project, it was first necessary to repeat the procedure outlined in Gallacher *et al* (1991) to determine the kinetics of hydrolysis of the carbonate ester (Figure 6 2 (b)) In order to do this, the crude normal sheep serum and the antibody fraction from the serum of the immunised sheep 271 was isolated by sodium sulphate precipation and chromatography on Protein G-Sepharose (Sections 2 6 1 and 2 6 3, respectively) (Gallacher *et al*, 1991) Kinetics of the hydrolysis of the carbonate ester were monitored by the release of 4-nitrophenolate from the carbonate ester in sodium phosphate buffer, pH 8 0, containing 0 67% (v/v) acetonitrile at 25°C by recording the increase in the absorbance at 400nm (A_{∞}) with the use of a Shimadzu UV-160A UV-Visible recording spectrophotometer and quantified by using $\varepsilon_{400} = 1\ 65 \times 10^{4} M^{-1}cm^{-1}$ Stock solutions of the substrate were prepared in acetonitrile and their concentrations were adjusted so that, by addition of stock substrate solution and acetonitrile solvent to 20µl in a final reaction volume of 3ml, final concentrations in the approximate range of 0 2-16µM were achieved (Section 2 6 4) In some cases, reaction mixtures also contained 0 2µM IgG prepared from sheep 271 or 0 2µM IgG prepared from the non-immunised sheep or from hybridoma serum or from mouse ascitic fluid, control or sample

6 5 Initial evaluation of sheep polyclonal catalytic antibody

The kinetic data presented below were obtained for an IgG preparation of sheep antiserum (number 271) supplied by Dr Gallacher, which had been immunised with the Phos-KLH immunogen Sheep were chosen as the source of IgG because of the large volumes of antiserum they can provide

The concentration of IgG in the elutate from the Sephadex G-25 column was

determined by using ε_{280}=2 0x10^{5}M 'cm ^{1} and was found to be approximately 3μM from Gallacher *et al* (1991), using the following equation,

$$A=\varepsilon cl$$

where ε is the extinction coefficient of proteins (M 'cm^{-1}),

c is the concentration (M)

l is the path length = 1cm

A is absorbance

The concentration of IgG obtained using the procedure outlined in Section 2 6 4 was determined to be 3 86μM, as an absorbance of 0 077 was obtained at 280nm To calculate the total background hydrolysis, this was repeated for 20μl of the 1mg/ml stock of the substrate in acetonitrile in 3ml of carbonate buffer, pH 12 0, at 400nm (Section 2 6 4) This was repeated for the same concentration of substrate in the same buffer at pH 8 0 at 400nm, which was used to determine background hydrolysis The concentration of substrate was calculated by subtracting the value at pH 8 0 from the value at pH 12 0 using ε_{400} = 1 65x10^{-4}M'cm ^{1}

The total background hydrolysis was obtained by monitoring the absorbance at 400nm of carbonate buffer pH 12 0 with 20μM substrate and repeating this for carbonate buffer pH 8 0 with 20μM substrate The results obtained were as follows,

A_{400}(pH 12 0) = 0 0210,

c = 1 273x10^{-6}M,

A_{400}(pH 8 0) = 0 0149,

c = 9 03x10 ^{7}M

The total background hydrolysis was 1 273x10 ^{6}M − 9 03x10^{-7}M = 3 07x10^{-7}M

Initial rates (ϑ) were determined from slopes of linear progress curves From Gallacher's paper, $\vartheta=k[S]$, since the same [S] is used throughout the experiment, it is assumed that $\vartheta \propto k$ Thus, for the reactions carried out (i) in the absence of IgG, in the presence of carboxylesterase, DMEMS$_0$, control ascitic fluid (Section 2 3 17) or control whole sera or (ii) in the presence of IgG prepared from non-immunised sheep, the initial change in absorbance increased linearly with increased substrate concentration [S] and were closely similar at any given [S] The initial rates obtained in the presence of IgG from non-immunised sheep were subtracted from those obtained at the same values of [S] in

the presence of the IgG prepared from sheep number 271 to provide the rates of the polyclonal catalytic antibody-catalysed reaction Other parameters such as ϑ_{max} and K_m can be calculated (Gallacher *et al* , 1991)

In this system, the apparent background first order rate constant for hydrolysis in pH 8 0 buffer at 400nm was determined as follows, 20µl of substrate was added to 3ml carbonate buffer, pH 8 0, and the change in absorbance at 400nm (dA) was monitored over 40 seconds (s,dt) dA/dt=(0 0071-0 0001)/40s = 1 75x$10^{-4}s^{-1}$ This compares well with the 1 87x$10^{-4}s^{-1}$ obtained by Gallacher The next step was to do the same experiment with 0 2µM IgG from the purified normal sheep serum which was found to be [(0 0081-0 0002)/40s] 1,975x$10^{-4}s^{-1}$ again comparing with 1 99x$10^{-4}s^{-1}$ in Gallacher's paper Then the same experiment was carried out using purified sheep 271 serum and the results obtained are shown in Table 6 3 It was hoped a monoclonal catalytic antibody would improve on these results

6 6 Advantages of catalytic monoclonal antibodies

Characterisation of the kinetic parameters, specificity, mechanism and structural properties of catalytic antibodies is complicated and difficult to mtrepret unless a monoclonal antibody is used In addition, reproducibility becomes an important concern when a polyclonal antibody preparation is generated It should be noted that nearly 20 years ago, Raso and Stollar (1975) attempted to use pyridoxamine binding antibodies to catalyse Schiff base formation and transamination reactions Their lack of success may have been due to the use of polyclonal rather than monoclonal antibodies The importance of monoclonal antibodies is further discussed in Section 6 19

6 7 Immunisation of mice with the phosphate immunogen, Phos-KLH (XI)

The protocols used in this project to generate catalytic monoclonal antibodies are generally no different than those used for the production of monoclonal antibodies for other uses (Kohler and Milstein, 1975, Goding, 1986), and are described in Section 2 6 5 An amide conjugate involving the carboxy group of the 4-nitrophenyl 4'-carboxy-methylphenyl phosphate and an amino group of KLH was synthesised as outlined (Gallacher *et al* , 1991) This phosphate immunogen (Phos-KLH) (XI) used to immunise the 6 balb/c mice was produced and supplied by Dr Gerard Gallacher (Table 2 3) The mice were tail bled (Section 2 3 7) and their sera tested by ELISA on Phos-

BSA and BSA coated plates The results obtained are shown in Table 6 1

Table 6 1 Testing of sera (1 100 dilution) from 6 mice immunised with Phos-KLH, used in the 6 fusions performed during this project, by ELISA against Phos-BSA and BSA coated plates read at 620nm (A_{620}) Standard deviations are in brackets, (n=3)

Fusion number	Phos-BSA coated plates (10μg/ml) (A_{620})	BSA coated plates (10μg/ml) (A_{620})
1	0 474 (0 009)	0 002 (0 001)
2	0 114 (0 003)	0 002 (0 000)
3	0 108 (0 004)	0 004 (0 000)
4	0 101 (0 003)	0 008 (0 001)
5	0 087 (0 012)	0 037 (0 007)
6	0 072 (0 001)	0 003 (0 000)

The controls (n=3) used for this ELISA, with standard deviations in brackets, included,

PBS 0 0036 (0 0035),

normal mouse serum, diluted 1 100 with 0 01M PBS, pH 7 3, 0 0125 (0 008),

DMEM$_0$ 0 0175 (0 003) and

SP2/0 supernatant 0 014 (0 006)

6 8 Results of the initial fusions and screening of anti-Phos monoclonal antibodies

Of the six mice immunised, initially three were used An *in vitro* immunisation was also performed as outlined in Section 2 3 11 5 The spleen cells from this *in vitro* immunisation were fused with SP2/0 cells using the PEG method (Section 2 6 5) (Fusion 1) The success of this *in vitro* immunisation was assessed using the ELISA-spot assay This assay showed that 131 hybridoma cells producing the desired antibody were obtained by this method For the other two fusions performed, PEG was also used These results are outlined in the following scheme

IN VITRO IMMUNISATION

↓

PEG FUSIONS

↓

FUSION 1 5 plates, 164 clones

FUSION 2 5 plates, 74 clones

FUSION 3 5 plates, 79 clones

↓

These 317 clones were screened by ELISA using anti-mouse IgG as antigen (Section 2 3 16 4), the clones which gave positive ELISA values (> 0 150) on the sandwich ELISA were expanded further and screened against BSA, KLH, Phos-BSA and Phos-KLH The most interesting clones are outlined below,

FUSION 1 PL2G7 and PL4B1

FUSION 2 PL1E7, PL3A10, PL3C8, PL3D1, PL4C2, PL4D2, PL4F6, PL5C4 and PL5H2

FUSION 3 PL1E4, PL1E7, PL1F5, PL2A10, PL2C5, PL2D5, PL3D1, PL3D7, PL3D8, PL4A2, PL4C3, PL4D2, PL5B2 and PL5E11

↓

These antibodies were screened by ELISA for cross-reactivity with BSA-hapten, for inhibition of binding to the BSA-hapten conjugate by free hapten and for lack of cross-reactivity with KLH Those with maximum binding affinity for the hapten carrier conjugate were further characterised

↓

9 clones were found to be positive against the phosphate antigen In order to find which of these antibodies may be catalytic, an attempt was made to detect hydrolysis of the carbonate ester by the monoclonal as described for sheep polyclonals (Gallacher *et al* , 1991) (Section 6 4)

↓

The most interesting antibodies were F1PL4B1, F2PL1E7, F3PL1E4, F3PL1F5, F3PL2C5, F3PL2D5, F3PL4C3, F3PL5B2, F3PL5E11 These hybridomas were cloned according to Section 2 3 15 and the results of their screening by ELISA against Phos-BSA, BSA, Phos-KLH and KLH are shown in Table 6 2

These results indicate that these antibodies may be against the phosphate immunogen and an attempt was made to determine whether they catalysed the hydrolysis of the carbonate ester (Figure 6 2 (b)) as described in Section 6 9

Table 6 2 Supernatants taken from cloned hybridomas, diluted 1 100 with 0 01M PBS, pH 7 3, were screened by ELISA against plates coated with Phos-BSA, BSA, Phos-KLH and KLH The absorbance was read at 620nm (A_{620}) and n=1

Clone	Phos-BSA	BSA	Phos-KLH	KLH
F1PL4B1	0 107	0 101	0 029	0 054
F2PL1E7	0 103	0 095	0 036	0 119
F3PL1E4	0 117	0 057	0 026	0 011
F3PL1F5	0 313	0 217	0 194	0 322
F3PL2C5	0 268	0 062	0 130	0 071
F3PL2D5	0 096	0 118	0 074	0 092
F3PL4C3	0 032	0 072	0 010	0 043
F3PL5B2	0 087	0 025	0 050	0 073
F3PL5E11	0 006	0 000	0 104	0 021

The controls used for this ELISA were as described for Table 6 1

6 9 Detection of catalysis in hybridoma supernatants

Following the choice of hapten, the hybridoma selection procedure is the most crucial phase in the successful production of catalytic monoclonal antibodies The generation of catalytic antibodies makes use of the same basic procedures that have been used for the generation of binding monoclonal antibodies, yet the process involves an additional crucial step screening for catalytic activity Tawfik *et al* , (1992) addressed the unique problems involved in the detection of inefficient catalytic activity that is accompanied by uncatalysed background reactions, as discussed in Section 6 19

In order to ascertain if it is possible to detect the hydrolysis of the carbonate substrate (I) (Figure 6 2 (b)) by monoclonal antibodies in the supernatant of the hybridomas produced from these fusions, initial rates (ϑ) were determined from slopes of linear progression curves These are slopes of curves obtained when 20μM of substrate was added to 3ml of control or sample and blanked against carbonate buffer or $DMEMS_{10}$, as appropriate The absorbance was read at 400nm and the change in absorbance taken for a time period of 40 seconds (s, dA/dt) 40s was chosen as the absorbance remained constant after this time The supernatants from the hybridomas which appeared interesting by ELISA were monitored to ascertain if it was possible to detect a potential catalytic antibody using this method If this procedure was successful, it would save time, money, animals and expertise, in that only the interesting hybridomas would be grown in ascitic fluid (Section 2 3 17), purified and characterised The results are shown in Table 6 3

Table 6 3 Determination of monoclonal antibody-catalysed hydrolysis of the carbonate substrate (20μl) by detecting a change in absorbance at 400nm (dA) over 40s (dt) of controls and sample supernatants, at pH 8 0, unless otherwise stated.

Sample (pH 8 0) + 20μl substrate	Change in Absorbance at 400nm (dA)	ϑ = dA/40s =dA/dt (s^{-1})
Normal sheep serum	0 0079	$1\ 975x10^{-4}$
271 Sheep serum	0 0796	$1\ 99x10^{-3}$
Carbonate buffer	0 0070	$1\ 75x10^{-4}$
Carbonate buffer (pH 12 0)	0 2147	$5\ 368x10^{-3}$
$DMEMS_{10}$	0 1307	$3\ 268x10^{-3}$
F1PL4B1	0 1457	$3\ 643x10^{-3}$
F1PL4B1 (pH 7 2)	0 0158	$1\ 295x10^{-3}$
F2PL3C8	0 2444	$6\ 11x10^{-3}$
F3PL1E4	0 1228	$3\ 07x10^{-3}$
F3PL1E4 (pH 7 2)	0 0726	$1\ 815x10^{-3}$
F3PL1F5	0 1845	$4\ 62x10^{-3}$
F3PL2A10	0 2428	$6\ 07x10^{-3}$
F3PL2C5	0 1666	$4\ 165x10^{-3}$
F3PL2C5 (pH 7 2)	0 0473	$1\ 18x10^{-3}$
F3PL1E4 (pH 7 2)	0 0726	$1\ 815x10^{-3}$
F3PL2D5	0 0426	$1\ 065x10^{-3}$
F3PL2D5 (10μl)	0 0228	$5\ 7x10^{-4}$
F3PL4C3	0 0524	$1\ 31x10^{-3}$
F3PL5B2	0 2476	$6\ 19x10^{-3}$
F3PL5E11	0 2300	$5\ 75x10^{-3}$

6 10 Discussion of the results obtained in Table 6 3

The results obtained for (i) the apparent background first order rate constant for hydrolysis in pH 8 0 buffer was 1 75x10^{-4}s^{-1} concurring with that obtained by Gallacher *et al* (1991) of 1 87x10^{-4}s^{-1} and (ii) normal sheep serum of 1 975x10^{-4}s^{-1} agrees well with that obtained by Gallacher *et al* (1991) of 1 99x10^{-4}s^{-1} The result obtained using purified 271 sheep serum was 1 99x10^{-3}s^{-1} These results are further discussed in detail in Section 6 19

6 11 Isotyping of the initial anti-Phos monoclonal antibodies

These antibodies were isotyped according to Section 2 3 18 and all were shown to be IgM, probably due to MHV contamination (Section 3 19) For this reasons discussed in Section 6 19 , it was decided an IgG antibody was preferable and, therefore, a number of strategies were tried to produce an anti-Phos monoclonal antibody which was IgG, including using the Cel Prime *in vitro* immunisation 'High IgG Yield' kit from Immune Systems (Section 2 3 11 6)

6 12 Attempts to produce IgG anti-Phos monoclonal antibodies

One of the certified disease-free mice purchased from Harlan and Olac was used in an *in vitro* immunisation (Section 2 3 11 5, Fusion 4) The fifth fusion was carried out using the Cel-Prime High IgG yield' kit from Immune Systems (Section 2 3 11 6 , Fusion 5) This kit advocated the discarding of any excess support cells but instead these cells were frozen and used in the sixth fusion with 95% viability

Fusion 6 used the same procedure as Fusion 5 except, since the immunisation medium supplied with the kit had been exhausted, the immunisation medium used in Fusion 4 was used instead The support cells were incubated in this medium with antigen as outlined in Section 2 3 11 6 and the spleen cells were incubated as for Fusion 5 for a further 3 days

All fusions were performed using PEG 1500 (Section 2 3 13 1) and the clones were selected using HA (Section 2 3 14) This sixth fusion proved to be the most successful The spleen used in Fusion 4 had only been immunised once with 50μg of Phos-KLH before the *in vitro* immunisation whereas Fusions 5 and 6 had been immunised twice with 50μg of Phos-KLH The results of the ELISA on the pre-immunised sera for these mice are given in Table 6 1

For each fusion, the spleen cells were counted (Section 2 3 3), pooled and were fused using the PEG method

8 56x10' spleen cells were fused with 1 22x10' SP2/0 cells at a 7 1 ratio of spleen cells to myeloma cells (Fusion 4)

Fusion 5 was a fusion of 3 7x10' spleen cells and 3 72x10' SP2/0 cells, at a 10 1 spleen myeloma cell ratio

Fusion 6 was a fusion of 6 7x10' spleen cells and 1 0x10' SP2/0 cells, at a 6 7 1 spleen myeloma cell ratio

The cells from each of these three fusions were plated onto 5 plates which were monitored for growth of clones after 10 days The hybridomas were screened by ELISA against Phos-BSA, BSA, Phos-KLH and KLH (Section 2 3 16 3 and 6 13)

6 13 Results of *in vitro* immunisations and fusions

The results of the second set of anti-Phos fusions are outlined below

IN VITRO IMMUNISATIONS

↓

PEG FUSIONS

↓

FUSION 4 5 plates, 52 wells contained clones

FUSION 5 5 plates, 64 wells contained clones

FUSION 6 5 plates, 77 wells contained clones

↓

These 193 clones were expanded into 24 well plates, and were screened by ELISA using anti-mouse IgG as antigen

↓

40 clones gave positive ELISA values (> 0 150), these were expanded and screened against Phos-BSA, Phos-KLH, BSA and KLH

↓

FUSION 4 A10E5

FUSION 5 E7C11, F5C11

FUSION 6 B2C7, B2D6, D5A6, D5A10

These 7 clones were found to be the most promising as they gave positive results against the phosphate antigen and not against the carrier proteins Their initial screening of

hybridoma supernatants against Phos-BSA, Phos-KLH, BSA and KLH are shown in Table 6.4. for a dilution of 1:100.

↓

A10E5, B2C7, B2D6, D5A6, D5A10, E7C11 and F5E11 were found to be stable, specific and good producers of antibody. These hybridomas were subcloned twice, screened for anti-Phos activity, grown in ascitic fluid, frozen as stocks and initially characterised by HPLC, with B2C7 being analysed by SDS-PAGE and for catalytic activity.

Table 6.4. Supernatants taken from cloned hybridomas from the second set of fusions, diluted 1:100 with 0.01M PBS, pH7.3, were screened by ELISA against plates coated with Phos-BSA, BSA, Phos-KLH and KLH. The absorbance of one reading, at 620nm (A_{620}), is presented.

Clone	Phos-BSA	BSA	Phos-KLH	KLH
A10E5	0.247	0.127	0.198	0.130
B2C7	0.271	0.074	0.222	0.091
B2D6	0.237	0.070	0.231	0.118
D5A6	0.192	0.155	0.214	0.184
D5A10	0.165	0.064	0.078	0.097
E7C11	0.253	0.140	0.212	0.163
F5E11	0.065	0.062	0.101	0.064

The controls for this ELISA were as described for Table 6.1.

6.14. Results of isotyping the second set of anti-Phos monoclonal antibodies.

A10E5, B2C7, B2D6, E7C11 and F5E11 were found to be the best producers and the most stable during freeze-thawing. These antibodies were further characterised and will be referred to as the `anti-Phos' antibodies. These antibodies were isotyped using the SEROTEC isotyping kit (Section 2.3.18.4.) along with the isotyping strip method

(a) B2C7
Ig
(b) B2D6

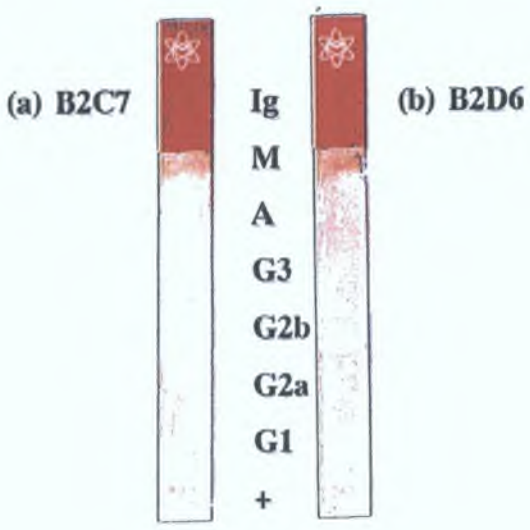


Figure 6.3.

Results obtained using isotyping strips from Sigma of neat supernatant taken from (a) B2C7 and (b) B2D6. Neat hybridoma supernatant was added to a tube provided and the procedure was as outlined in Section 2.3.18.4. Both hybridomas isotyped as IgM. (Section 2.3.18.3.) and all of the clones tested isotyped as IgM.

B2C7 and B2D6 were isotyped twice as they showed some positivity for IgG2b when the SEROTEC kit was used, but on repeating the test using the isotyping strips, both these hybridomas gave a definite IgM result This result was disappointing, especially as it was obtained despite importing certified mice and using the Cel-Prime `High IgG yield' kit from Immune Systems (Section 2 3 11 6) (Fusion 5) which claimed a 55% IgG yield, without prior immunisation These isotyping results are shown in Figure 6 3

6 15 Results of ELISAs on purified polyclonal and monoclonal anti-Phos antibodies

The anti-Phos monoclonal antibodies were grown as ascitic fluid (Section 2 3 17) and purified according to Gallacher *et al* (1991) The results of screening crude and Protein G-purified (i) normal sheep serum, (ii) catalytic serum, and crude and Protein G-purified ascitic fluid from (iii) A10E5, (iv) B2C7, (v) B2D6 and (vi)E7C11 are shown in Table 6 5 All samples were adjusted to a stock concentration of 2mg/ml before dilution and the results using a 1 1000 dilution is given Each antibody shows cross-reactivity with KLH, as would be expected, and all show some reactivity against BSA

Table 6 5 1 1000 dilution of Protein G-purified polyclonal and monoclonal anti-Phos antibodies screened against Phos-BSA, BSA, Phos-KLH and KLH All samples were brought to a stock concentration of 2mg/ml The absorbance was read at 620nm (A_{620})

Sample	Phos-BSA	BSA	Phos-KLH	KLH
Normal sheep serum	0 044	0 007	0 065	0 011
271 Sheep serum	0 253	0 017	0 414	0 136
A10E5	0 389	0 099	0 412	0 108
B2C7	0 372	0 077	0 344	0 183
B2D6	0 282	0 063	0 308	0 086
E7C11	0 347	0 133	0 408	0 104

The controls used in this ELISA were as described in Table 6 1

6 16 HPLC analysis with anti-phosphogen antibodies

Seven monoclonal antibodies, A10E5, B2C7, B2D6, D5A6, D5A10, E7C11 and F5E11, were grown as ascitic fluid in mice (Section 2 3 17) and were analysed by HPLC, along with mouse IgM and IgG as standards for comparison

When commercial IgM was loaded on the HPLC, two peaks were obtained at 10 08mins and 17 25mins (Figure 3 2 1) IgM has a molecular weight of 900kD which is higher than the fractionation range of the column used and, therefore, it comes off the HPLC column in the void volume (10 08min) The first peak represents intact IgM (900kD) and the second peak may represent the breakdown of IgM into its component of five IgG1 immunogloublins which have a molecular weight of 180kD each A peak at 16 21min was observed when commercially available IgG was loaded (150kD)

SAS-precipitated B2C7 antibody-containing ascitic fluid was loaded on to the HPLC column and the result is shown in Figure 6 4 1 The major peak detected had a retention time of 17 01mins which may represent the IgG1 constituents of the IgM antibody The peak at 9 92min represents proteins eluting in the void volume and may contain the intact IgM antibody A small peak was seen at 24 70min and may be denatured or contaminating protein If this analysis of the HPLC chromatogram presented in Figure 6 4 1 is correct, it would imply that this ascitic fluid may contain a mixture of IgM and IgG molecules which would correspond to these two peaks

SAS-precipitated ascitic fluid from the following hybridomas, A10E5 (16 49min), B2D6 (16 51mm), D5A6 (16 52min), D5A10 (16 64min), E7C11 (16 48min) and F5E11 (16 82min) were also examined by HPLC The respective major peak retention times are given in brackets

A second set of experiments were performed to analyse the interactions of these antibodies with the Phos-protein conjugates The binding of the antibody to the unconjugated phosphogen or the carbonate substrate were not used, as the change in molecular weight would not be detected by HPLC

All samples were adjusted to 2mg/ml and were incubated with the Phos-protein conjugate (20µg/ml) for 30min at 37°C

SAS-treated B2C7 antibody-containing ascitic fluid was incubated with Phos-BSA for 30min at 37°C and the major peak detected had a retention time of 16 94mins (Figure 6 4 2) which may represent the IgG1 constituents of the IgM antibody, as previously discussed The peak at 9 85min represents proteins eluting in the void volume and may

contain the intact IgM antibody A small peak was seen at 25 21min and may be denatured or contaminating protein An extra peak was observed at 14 03min which distinguishes the Phos-BSA-B2C7 complex which has a molecular weight of approximately 250kD (180kD for IgG1 + 70kD for Phos-BSA)

A similar chromatogram was obtained when B2C7 was incubated with the Phos-KLH conjugate, while no peak at a retention time of 14 0min was observed when B2C7 was incubated with KLH or BSA

This experiment was also carried out using A10E5, which was incubated with KLH and BSA The chromatograms obtained were scrutinised for a new peak at the retention time of 13 6min - 14 1min which would indicate the formation of an antibody-antigen complex BSA gave no new peak but KLH gave a peak at 13 70min A distinct peak was seen when incubated with Phos-KLH at 13 66min and a broader, less distinct peak was observed on incubation with Phos-BSA (13 75min)

B2D6 gave no peak when incubated with KLH or BSA, a distinct peak was seen when incubated with Phos-KLH (14 05min) and a larger one was seen on incubation with Phos-BSA (14 11min) This result suggests that this antibody reacts with the phosphogen portion of the Phos-protein conjugate

D5A6 ascitic fluid showed a distinct new peak at 14 34min and 14 46 when incubated with Phos-KLH and Phos-BSA, respectively D5A10 ascitic fluid showed a distinct new peak at 14 29min and 13 75 when incubated with Phos-BSA and Phos-KLH, respectively E7C11 ascitic fluid was treated as above but no Phos-protein-antibody conjugate was observed F5E11 gave a new peak at 13 88min and 13 99mm when incubated with Phos-KLH and Phos-BSA, respectively

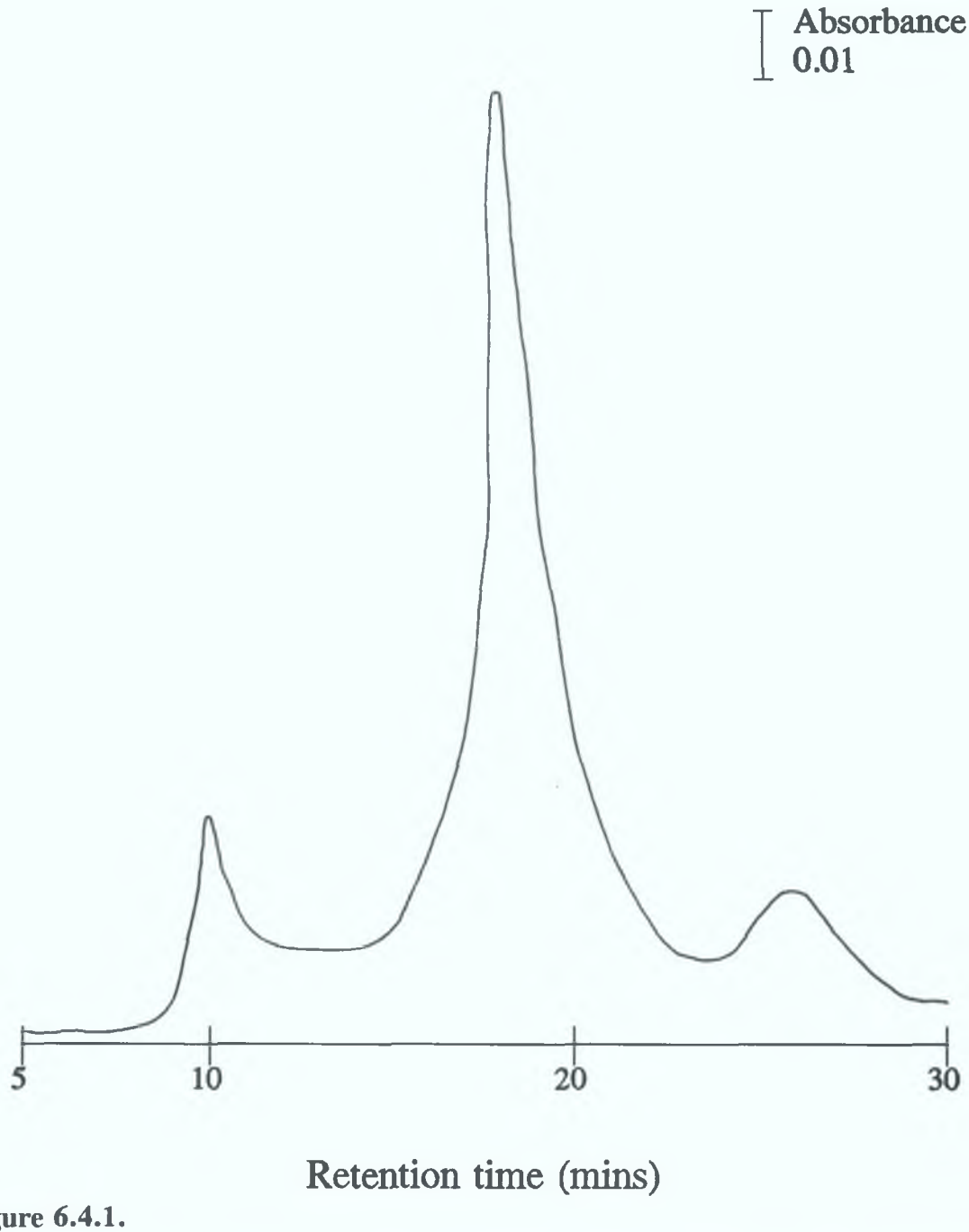


Figure 6.4.1.

HPLC chromatogram of SAS-precipitated B2C7 antibody-containing ascitic fluid. The column used was a 10μm Protein Pak SW 300 column with a mobile phase of 0.1M phosphate buffer, pH 7.0 and a flow rate of 0.5ml/min. Absorbance was detected at 280nm. The major peak detected had a retention time of 17.01mins which may represent the IgG1 constituents of the IgM antibody. The peak at 9.92min represents proteins eluting in the void volume and may contain the intact IgM antibody. A small peak was seen at 24.70min and may be denatured or contaminating protein.

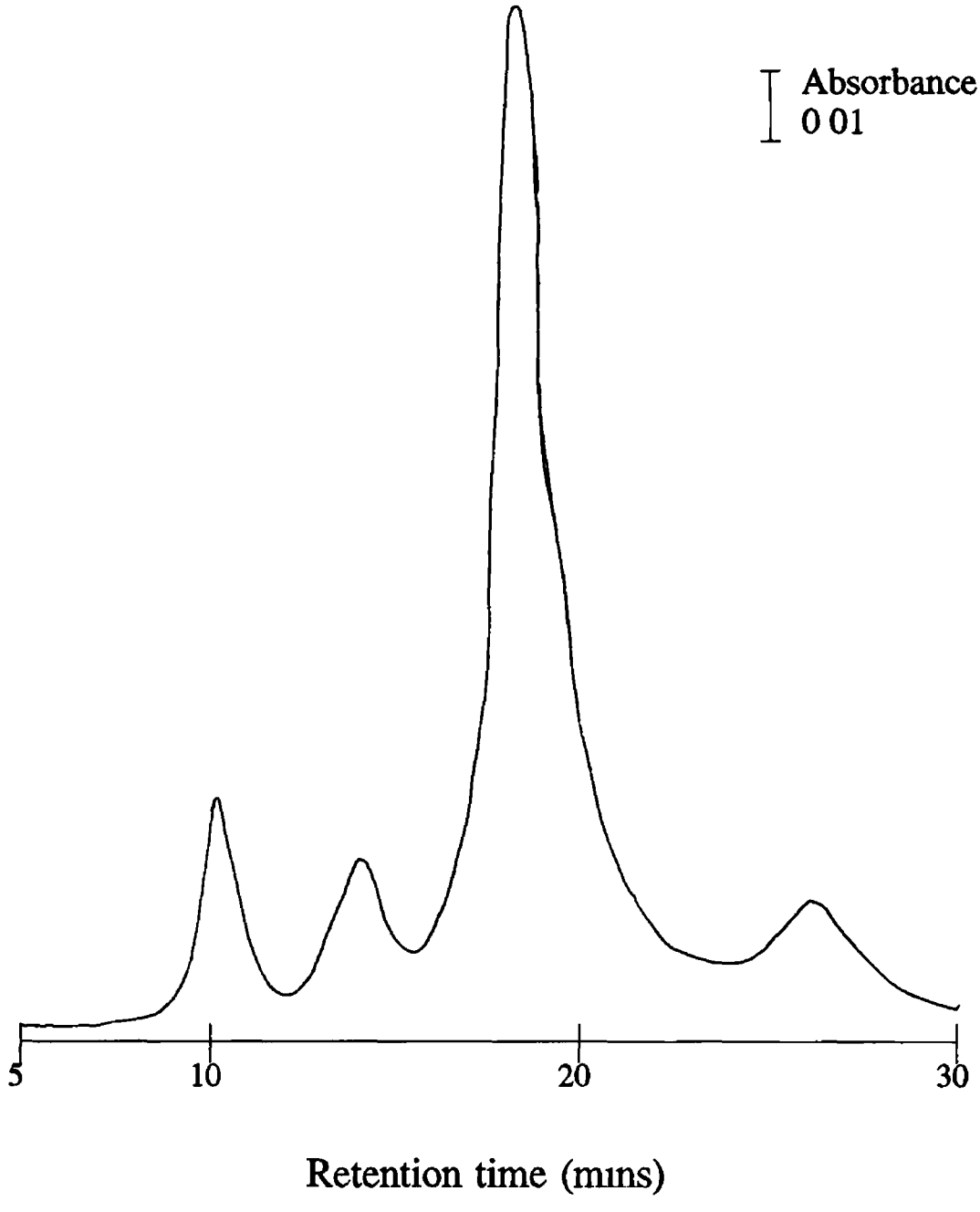


Figure 6.4 2. HPLC chromatogram of SAS-purified B2C7 antibody-containing ascitic fluid (2mg/ml) incubated with Phos-BSA (20μg/ml) for 30min at 37°C The column used was as described in Figure 6 4 1 Absorbance was detected at 280nm The major peak detected had a retention time of 16 94mins The peak at 9 85min represents proteins eluting in the void volume A small peak was seen at 25 21min and may be denatured or contaminating protein An extra peak was observed at 14 03min which distinguishes the Phos-BSA-B2C7 complex which has a molecular weight of approximately 250kD (180kD for IgG1 + 70kD for Phos-BSA)

6.17 Electrophoresis of B2C7 ascitic fluid purified by SDS-PAGE

B2C7 was purified using anhydrous sodium sulphate (Section 2 6 1) and protein G affinity chromatography (Section 2 6 3) and was analysed by SDS-PAGE (Section 2 4 17) The results are shown in Figure 6 5 and are discussed in Section 6 19

6 18 Detection of catalytic activity using the purified anti-Phos ascitic fluid

The antibody fraction from the anti-Phos ascitic fluid was precipitated using anhydrous sodium sulphate and Protein G 20µl of carbonate substrate was added to 20µl of A10E5 and read for 40sec (and 10mins) on a Shimadzu UV-160A UV-Visible spectrophotometer and the results obtained are presented in Table 6 6 These results are discussed in Section 6 19

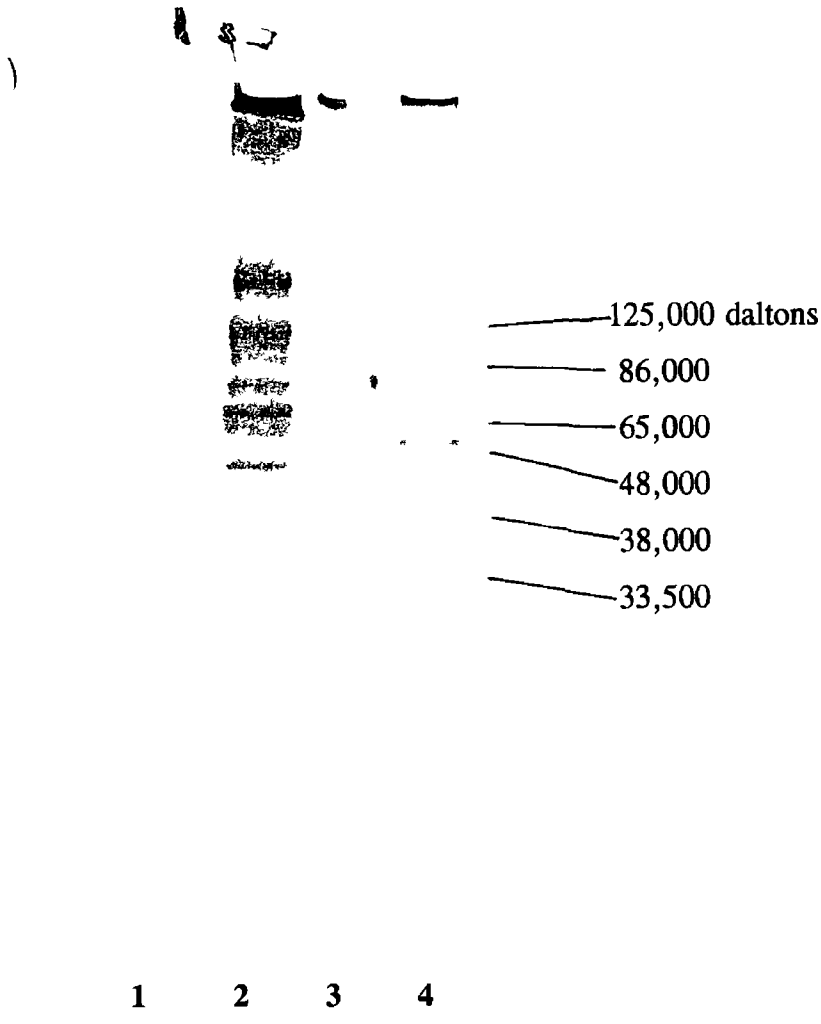


Figure 6.5. A 5-20% (w/v) gradient PAGEL^{R} precast polyacrylamide gel from ATTO was loaded as follows, Lane (1) protein G-purified B2C7 monoclonal antibody-containing ascitic fluid, (2) anhydrous sodium sulphate-precipitated B2C7 antibody fraction, (3) IgG standard, this sample was too dilute to be seen on the gel, the bands seen may be protein which leaked from lane 2, and (4) molecular weight markers (in daltons) The gel was run at 20mAmps per gel and the results are discussed in Section 6 17

Table 6 6 Determination of monoclonal antibody-catalysed hydrolysis of the carbonate substrate (20μl) by detecting a change in absorbance at 400nm (dA) over 40s (dt) of protein G-purified ascitic fluid, at pH 8 0

Sample (pH8 0) + 20μl substrate	Change in Absorbance at 400nm (dA)	ϑ = dA/40s =dA/dt (s^{-1})
A10E5	0 0015	3 66x10 $^{5}s^{-1}$
B2C7	0 0012	3 01x10 $^{5}s^{-1}$
B2D6	0 0014	3 42x10 $^{5}s^{-1}$

The controls for this experiment are as described in Table 6 3

6 19 Discussion

Two unique points are crucial for the successful production of catalytic monoclonal antibodies (i) the design of the hapten and (ii) the process of selecting the few monoclonal antibodies that are catalytic from among the many antibodies which bind the hapten but do not catalyse the reaction The attempts to produce a transition state analogue for the proposed phosphate ester by Dr Mark Searcy (Figure 6 1), which would have been catalysed to produce 7-OH-coumarin as the post-catalysed product, were unsuccessful It was decided to produce monoclonal antibodies which catalysed the hydrolysis of the mixed aryl 4-nitrophenyl carbonate (I) (Gallacher *et al* , 1991) instead, as this phosphate ester was readily available

Once the methods of catalytic screening were established in this project (Section 6 5), it was possible to screen hybridoma supernatants and ascitic fluid to determine the rate of hydrolysis of the amide substrate by an antibody Catalytic antibodies are currently raised by immunisation of mice with a transition-state analogue-protein carrier conjugate and subsequent fusion, the resulting hybridoma cells are then screened in order to select those antibodies exhibiting binding affinity for the hapten An alternative method for selecting potential catalytic monoclonal antibody-secreting clones is on the basis of their affinity to a short "transition state analog" (Tawfik *et al* , 1990) These potentially positive clones are then propagated in large quantities, generally as ascitic fluid preparations, and the antibodies purified to allow their screening for catalytic

activity

In this project, fusions were performed using PEG (Section 6 7 and 6 8) and the nine most interesting hybridomas were cloned and their supernatants were screened by ELISA (Table 6 2) and for catalytic activity The techniques used in the characterisation of polyclonal and monoclonal catalytic antibodies were as described by Gallacher *et al* (1991) The concentration of the protein G–purified sheep serum (271) obtained during this project was in agreement with the value of 'approximately 3μM' found by Gallacher *et al* (1991) Then the total background hydrolysis was determined to be 3 07x10 ^{7}M The apparent background first order rate constant for hydrolysis obtained was 1 75x10^{-4}s^{-1} (Section 6 5) This is in excellent agreement with the value of (1 87x10^{-4}s^{-1}) obtained by Gallacher Repeating this experiment using purified normal sheep serum the value obtained (1 975x10^{-4}s^{-1}) compares well with the 1 99x10^{-4}s^{-1} in Gallacher's paper This experiment was repeated using purified sheep 271 serum and 1 99x10^{-3}s^{-1} was obtained (Section 6 5)

The selection of hybridoma supernatant, by screening for catalytic activity, is crucial in the successful production of catalytic antibodies Since the hybridomas were grown in DMEMS$_{10}$, the next measurements were to detect the apparent background first order rate constant for hydrolysis in DMEMS$_{10}$ (pH 8 0) The pH of this medium decreased when exposed to the air for any length of time, which made the accurate attainment of these results difficult It was found that when 20μl of substrate were added to the DMEMS$_{10}$, and this was blanked against DMEMS$_{10}$, an initial rate of 3 268x10^{-3}s^{-1} was obtained To eliminate this, each hybridoma sample was blanked against DMEMS$_{10}$ which had 20μl of substrate added at the same time as the substrate was added to the sample and the readings were then taken (Table 6 3) This represents the rate of hydrolysis of the amide substrate When supernatant from the anti–G–CCM hybridoma, 3BH2A3 (Section 3), was tested in a similar manner, the change in absorbance was 0 025 and the initial rate was 6 25x10^{-4}s^{-1}

The hybridomas which appeared interesting from ELISA gave initial rates of hydrolysis of the substrate [Figure 6 2 (c)] by the antibody in the supernatant in the order of 4x10^{-3}s^{-1}which was higher than the result obtained for the polyclonal sheep catalytic antibody (1 99x10^{-3}s^{-1}) This initial result showed that perhaps the antibody in this hybridoma supernatant was catalysing this reaction The initial rate was, however, in the same order as the polyclonal antibody

The results obtained in Table 6 3 may, or may not be, due to catalysis For a single substrate concentration, the expected observation would be a rapid reaction that slows as the substrate is used up A reaction burst, as was seen here, might indicate that substantial amounts of substrate are used up quickly due to very rapid reaction Because this was the case the initial rate (ϑ) was taken from a linear part of each graph near the origin, (i e the first 40 seconds) If the reaction slows down while there is still a substantial (90%) amount of substrate, this may be a catalysed reaction that is not turning over (i e it is stoichiometric)

The hybridoma supernatants were isotyped (Section 2 3 18) and all were IgM The reasons for this are discussed in Section 3 19 At a discussion at the Ciba Foundation catalytic antibody symposium (1991), Janda and Lerner stated that both their groups had obtained some catalytic activity with IgM antibodies but they were orders of magnitude lower in affinity At this meeting, Schultz stated that of all the IgM antibodies obtained in his group, none were catalytic For this reason it was decided an IgG antibody was preferable and therefore, a number of strategies were tried to produce an anti-Phos monoclonal antibody which was IgG Despite the attempts to produce anti-Phos catalytic antibodies of the IgG isotype, the isotyping results obtained using hybridoma supernatant from B2C7 and B2D6 were also IgM (Figure 6 3) This result was unexpected since the Cel-Prime 'High IgG yield' kit from Immune Systems was used which claimed a 55% IgG yield, without prior immunisation

The ascitic fluid from the best four hybridomas was purified according to Gallacher *et al* (1991) and was screened by ELISA (Table 6 5), which showed continued reactivity against the phosphogen hapten The reaction of the phosphate immunogen with the seven monoclonal antibodies was analysed by HPLC (Section 6 16) and demonstrated in the cases of B2C7 B2D6, D5A6 and D5A10 that these antibodies were indeed reacting with the phosphogen immunogen

The results of the SDS-PAGE analysis of the protein G-purified B2C7 ascitic fluid, under non-reducing conditions, showned one band at 150kD (Figure 6 5) corresponding to the molecular weight of IgG Since this antibody isotyped as IgM, this band may be due to the breakdown of the IgM antibody into its constituent IgG1 antibodies B2C7, B2D6 and A10E5 were then tested for catalytic activity and the results are shown in Table 6 6 These results were in the order of the results obtained using the polyclonal antibody, which indicate that these antibodies bind the phosphate immunogen but are not

catalytic as a sharp increase in the initial rate would be expected if these antibodies catalysed the of the mixed aryl 4-nitrophenyl carbonate (I) substrate (Dr G Gallacher, *personal communication*)

These antibodies isotyped as IgM but on HPLC and SDS-PAGE analysis the appeared to be IgG It is speculated that perhaps these antibodies were a mixture of IgM and IgG and that the isotype was not the reason that these antibodies did not catalyse the desired reaction Much more than seven monoclonal antibodies would need to be screened in order to find a catalytic monoclonal antibody (Shokat and Schultz, 1990) Since an IgG antibody was required, perhaps it would have been more successful if the immunisation scheme proposed by Tawfik *et al* (1990) had been tried using certified mice immunised in isolators Tawfik *et al* (1990) used a short immunisation protocol using two foot pad injections of balb/c mice over an 18 day period (25μg/injection), followed three days later by draining lymph nodes and fusing these cells to NSO myeloma cells (Eshhar, 1985) Draining lymph node cells was found to yield a greater repertoire of clones than spleen cells when a short immunisation protocol was used Perhaps this method would give better results if another attempt is made to produce this monoclonal antibody It would have a high probability of success if it was used with certified mice in an isolator

Relative to enzymes, antibody-mediated catalysis in still very modest, the largest turnover number, K_{cat}, reported for a catalytic monoclonal antibody is 0 3 min^{-1} (Tramontano *et al* , 1986), while K_{cat} values as high as $10^6 min^{-1}$ are not uncommon for enzymes (Fersht, 1985) In addition, the nature of most of the reported antibody catalysed reactions is such that they proceed with measurable rates even in the absence of any catalyst The catalytic power is generally described in terms of K_{cat}/K_{uncat}, i e the rate enhancement of the reaction, rather than the absolute rate, K_{cat}, commonly used to describe enzymatic activity Rate enhancements by catalytic monoclonal antibodies are generally in the range of $10^2 - 10^5$, which is still lower than those of enzymes by orders of magnitude The detection of catalysis by antibodies therefore presents unique difficulties which were addressed by Tawfik *et al* (1992)

The most important factors involved in being able to detect antibody-mediated catalytic activity are (i) the concentration and purity of the monoclonal antibody in the preparation used for the assay and (ii) the choice (structure) and concentration of the substrate Tawfik *et al* (1992) showed that the detection of antibody-mediated catalysis

requires not only relatively high concentrations of purified monoclonal antibodies but also the use of an appropriately designed substrate Unlike enzymes, catalytic antibodies are tailormade catalysts The structure of the substrate must obviously be related to the structure of the hapten used and the reaction that is to be catalysed by the resulting antibodies, yet various possibilities for the design of the substrate always exist Other important points involved in the detection of catalytic antibodies include the sensitivity of the assay to be used, the solubility and reactivity of the substrate, the pH and the buffer

If it is known that an enzyme catalyses the reaction of interest, it is important to purify the antibody to ensure any catalysis of the fraction observed is due only to the antibody and not due to a contaminating enzyme For example, if the turnover number, k_{cat}, for a catalytic (IgG) antibody is $1min^{-1}$, and a natural enzyme has a k_{cat} of $5x10^{4}min^{-1}$, contamination of the antibody with $1x10^{-3}$% (on a mol/mol basis) of the natural enzyme would lead to the deduction that the antibody is catalytic when in fact the contaminating enzyme is responsible for the rate enhancement observed Kinetic analysis, specificity or inhibition data cannot always distinguish between catalysis by an antibody and catalysis by an enzyme impurity Rigorous checks of specific activity must be performed after each purification step, along with a comparison between different methods of purification and comparison of whole antibody versus isolated F_{ab} For example, Shokat and Schultz (1990) found it very difficult, even after considerable purification, including affinity chromatography, to remove glycosidase, adenosine deaminase and ribonuclease impurities from what appeared, by gel electrophoresis, to be homogeneous antibody

The long-range goal is to be able to directly screen the antibodies present in each of the hybridoma supernatant solutions for catalysis However an analysis that takes into account the amount of antibody present in such supernatants, the detection sensitivity, and the degree of non-specific reaction (Tawfik *et al* , 1990) indicates that (a) a reaction which proceeds at any appreciable rate without antibody catalysis (as a result of non-catalysed reaction, enzyme impurities in the medium, etc) cannot be considered for direct screening of catalytic monoclonal antibodies at the low antibody concentration in the media and (b) if no background reaction exists, direct screening for catalysis is only possible for efficient catalytic monoclonal antibodies and when a particularly sensitive as well as selective assay (able to detect small amounts of product in the presence of a 10^{4}–10^{6} excess of unreacted substiate) is used Other stratagems to screen for potential

catalytic clones must therefore be sought

Novel methods, such as combinatorial variable-region cloning in phage (Winter and Milstein, 1991), have been used to generate catalytic antibodies (Section 5.2.) Yet future application of these methodologies for obtaining catalytic antibodies depend upon appropriate screening. Analysis of the unique problems involved in direct screening of hybridoma supernatants for antibody-mediated catalysis led Tawfik's group to develop an ELISA to detect catalytic antibodies in supernatant, called catELISA (Tawfik *et al*, 1992), an assay involving a substrate-protein conjugate immobilised on microtitre plates. Antibody-catalysed conversion of any "solid phase" substrate to a product is then detected by ordinary ELISA, using binding anti-product antibodies. Employing this catELISA, Tawfik's group were able to rapidly screen thousands of hybridoma clones elicited against both a phosphate transition state analog and an amide substrate to detect catalytic cleavage of the corresponding *p*-nitrobenzyl ester.

A prerequisite to the design and engineering of catalytic antibodies is the knowledge of their structure and in particular which residues are involved in binding and catalysis. Individual residues important for ligand binding and catalytic activity have been identified by site-directed mutagenesis and computer modelling, using a three-dimensional model of the antibody variable region using an antibody structural database (Stewart *et al*, 1994, Roberts *et al*, 1994). The results of these studies allow the reaction mechanism of antibody catalysed reactions to be elucidated.

The three-dimensional structure of a catalytic antibody with chorismate mutase activity has been determined to 3.0Å resolution as a complex with a transition state analogue (Haynes *et al*, 1994). The structural data suggest that the antibody stabilizes the same conformationally restricted pericycle transition state that occurs in the uncatalysed reaction. The structure of this antibody-hapten complex provides confirmation that the properties of an antibody catalyst faithfully reflect the design of the transition state analogue which could be an aid in determining their structure.

Genetic selections or screens have yet to be exploited for the generation of catalytic antibodies which may allow the generation of catalytic antibodies from substrate specific antibodies. However this approach will require more efficient yeast and bacterial expression systems. Clearly the most promising method for the generation of new catalytic antibodies with rate enhancements comparable to enzymes is the combination of two or more highly successful strategies. New approaches will become increasingly

important as different strategies are combined to make efficient, tailorable catalysts for application in biotechnology, chemistry and medicine and for environmental and industrial applications

The paradigm of Jencks (1969), " it should be possible to synthesise an enzyme by [preparing] an antibody to a haptenic group which resembles the transition state of a given reaction " is proving to be very fruitful for an increasing variety of model reactions The use of improved technologies combined with direct screening methodologies such as those presented in Tawfik *et al*, (1993) promises to afford useful antibody-based catalysts for more demanding chemical reactions and challenging biomedical applications

In summary, monoclonal antibodies were produced which reacted with the phosphate immunogen (XI) (Gallacher *et al*, 1991) The sheep polyclonal antibody obtained from Dr Gallacher was purified and the results obtained for the catalysis of the hydrolysis of the mixed carbonate substrate (I) were in close agreement with those obtained by Gallacher *et al*, 1991 Monoclonal antibodies were produced which reacted by ELISA with the phosphate immunogen (I) and did not react with the carrier proteins, KLH or BSA These monoclonal antibodies isotyped as IgM An attempt was made to detect catalysis of these antibodies in cell culture supernatants, which was unsuccessful Ascitic fluid was produced, purified and characterised by ELISA, SDS-PAGE and HPLC This fluid was analysed for catalytic activity

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