MONOCLONAL ANTIBODY PRODUCTION AND THE APPLICATION OF MONOCLONAL ANTIBODIES TO THE STUDY OF TUMOUR CELL MEMBRANE ANTIGENS

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I hereby declare that the research described within this thesis is based entirely upon my own work

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Date 2/5/88

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Dedication

To Paula Ferrari,

for her love and friendship, kindness and understanding
No words can express the depth of my gratitude

ABBREVIATIONS

BSA	Bovine serum albumin
DMEM	Dulbecco's minimum essential medium
DMSO	Dimethylsulphoxide
ELISA	Enzyme-linked immunosorbant assay
FCS	Foetal calf serum
Fuc	D-Fucose
Gal	D-Galactase
GalNAc	N-Acetylgalactosamine
Glc	D-Glucose
GlcNAc	N-Acetylglucosamine
HAT	Hypoxanthine, aminopterin, thymidine
ip	Intra-peritoneal
iv	Intravenous
LAT	Landschütz ascites tumour
Man	D-Mannose
mw	Molecular weight
NC	Nitrocellulose
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
SA	Sialic acid
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

ABSTRACT

A murine monoclonal antibody, F10 1 E12, (IgM) was produced against Landschutz ascites tumour cells (LAT) This antibody exhibits preferential reactivity on tumour versus normal cells The antigen against which the antibody is directed was identified as a cell membrane glycoprotein of mw 205kD, designated p-205 Characterization of this antigen by Western immunoblot analysis, coupled with enzyme digestion studies, revealed that the epitope bound by F10 1 E12 is sensitive to the effects of trypsin, pronase and papain This suggests that an arginine and/or lysine amino acid residue may be located at this site Lipase and a number of glycosidases did not affect the antigen-antibody interaction Neuraminidase was found to destroy this interaction, however, it did lead to the immunodetection of a range of other glycoproteins of mw from 40kD to 60kD This may have been due to the effects of undefined proteases in the neuraminidase preparation used

The p-205 of LAT cells was not detectable on a range of normal mouse cells by Western immunoblot analysis, despite the fact that certain of these cells, notably normal mouse liver cells, exhibited strong reaction with F10 1 E12 in solid-phase ELISA studies This would suggest that the antigen detected on normal cells is different to that of LAT cells, or that the antigen as expressed by normal cells is more labile than that of tumour cells

The glycoprotein p-205 was also detectable by Western immunoblotting analysis using reagent polyclonal antibodies raised against high molecular weight fucopeptides derived from LAT cell surfaces These fucopeptides are well characterized in the literature and demonstrate a strong association with the neoplastic state This finding may provide some valuable clues as to the nature of the carbohydrate moieties of p-205

In the course of this project, developments were made in the areas of solid-phase ELISA, somatic cell fusion techniques using PEG and in the elimination of mycoplasma from infected hybridomas These methods and detailed accounts of the developments initiated during this research assignment will also be discussed

SECTION 1

INTRODUCTION

AN OUTLINE OF THE AIMS OF THIS CURRENT PROJECT

The objective of this project was to produce murine monoclonal antibodies against Landschutz ascites tumour cell surface antigens with a view to identifying a tumour specific or related antigen expressed by the LAT cells With this objective in mind the following problems had to be addressed

1 the determination of the optimal immunization protocol for the production of a strong immune response against LAT cells in Balb/c mice,

2 the development of a solid-phase ELISA suitable for the large scale screening of hybridomas secreting antibodies against LAT cells,

3 to study the existing methods for murine monoclonal antibody production with a view to increasing the frequency of hybridoma formation and thereby increasing the probability of generating a monoclonal antibody of the desired specificty,

4 to screen those monoclonal antibodies formed against panels of tumour and normal cells,

5 to characterize the antigen(s) bound by those monoclonal antibodies by Western immunoblot analysis, and

6 to isolate high molecular weight glycopeptides from LAT cells followed by their conjugation onto BSA for use in the production of reagent anti-glycopeptide polyclonal antibodies

Having done this, the potential value of the monoclonal antibodies produced here may then be examined in relation to the development of a model system for the study of *in vivo* tumour localization and antibody-mediated cancer therapy The LAT cell represents an ideal candidate for

such a model system because it can be grown both *in vitro* and *in vivo* *In vivo*, one can grow it either as a diffuse ascitic tumour in the peritoneal cavity or as a solid tumour when the cells are injected subcutaneously

As a part of the presentation of the results of the work carried out for this project, detailed discussions of the relevant techniques used will be given with reference to the current status of these methods and our own experiences in the development of these techniques for our own use In particular, detailed discussions will be given in the following areas

1 solid-phase ELISA for whole fixed cells,
2 the production of monoclonal antibodies,
3 the treatment of mycoplasma-infected hybridomas, and
4 Western immunoblotting

SECTION 1

MONOCLONAL ANTIBODY PRODUCTION AND THE APPLICATION OF MONOCLONAL ANTIBODIES TO THE STUDY OF CANCER

THE APPLICATION OF HYBRIDOMA TECHNOLOGY TO THE SEARCH FOR TUMOUR "SPECIFIC" ANTIGENS

1 1 GENERAL INTRODUCTION

Antibodies have long been noted for their tremendous specificity and resolving power However, the widespread application of antibodies was hampered by the major difficulties associated with production of even very small amounts of pure, high affinity antibodies of a predefined specificity In this regard, the advent of hybridoma technology, as first described by Kohler and Milstein (1975), represented a major advance in the field antibody-mediated analysis Monoclonal antibodies have since found applications in all areas of analytical science and medicine and the potential for further uses appear to be endless The highlights in somatic cell hybrid research leading up to this development are reviewed by Tom (1983)

Hybridoma technology is characterized by two major features

1 the ability to produce unlimited quantities of monoclonal antibody of a predefined specificity,

and 2 the ability to generate antibodies against previously unrecognized antigens in highly complex mixtures

The development of hybridoma technology has resulted in a major resurgence in the search for tumour specific antigens with a view to their exploitation in the diagnosis and treatment of cancer It is with this aspect of monoclonal antibody application which we will concern ourselves

1 2 TUMOUR SPECIFIC ANTIGENS REALITY OR MYTH?

The tumour cell surface represents the logical place to begin a search for specific differences between tumour and

normal cells with a view to the identification of specific tumour markers amenable to exploitation in antibody-mediated diagnosis and therapeutics One may well ask if tumour cells do possess truly specific antigens and if so, why does the body not mount an effective immune response against them? Potentially tumourigenic cells do occasionally arise in the body but are removed by the immune system before they can develop into real tumours Evidence for this comes from the fact that there is a higher incidence of lymphomas in immunosuppressed patients (Penn, 1970) Tumour patients have been shown to mount both cellular and humoral immune responses against their own tumours (Sikora and Wright, 1981, Glassy et al , 1983) Indeed, lymphocytes from tumour bearing patients have been used for the generation of human anti-tumour monoclonal antibodies (Sikora and Phillips, 1981)

The question to be asked now, is how do tumour cells escape the immune system? The problem facing the immune system is twofold Firstly, tumour cells are "self" As yet, no truly tumour specific antigens have been identified and close examination of many supposedly tumour specific antigens has revealed them to be differentiation antigens being expressed out of character A great many cell surface alterations in tumour versus normal cells are known to exist Many of these alterations involve changes in the carbohydrate moieties of the cell surface glycoproteins and glycolipids (see Reading and Hutchins, 1985, for a review of "Carbohydrate Structure in Tumour Immunity") However, no truly tumour specific alteration has yet been identified Given that this is the case, it appears unlikely that any major immune response could be mounted against such qualitative differences alone This does not rule out the possibility of immunological exploitation of major quantitative differences between tumour and normal cells for therapeutic and other purposes

Secondly, tumour cell populations develop a high level of heterogeneity (Heppner, 1984, Alexander, 1985) enabling

tumour cells to escape much of the effects of any immune responses raised against the tumour as a whole Tumour cell heterogeneity exists at many levels and the cells of a tumour may vary with respect to karyotype, antigenicity, immunogenicity, growth, biochemical properties and susceptibility to various chemotherapeutic agents The same extremes of heterogeneity are unlikely to exist amongst normal cells

Given that no truly tumour specific antigens have been positively identified, a further question must be asked concerning the validity of a continued search for their existence and, in the absence of truly specific tumour markers, whether there is a role for monoclonal antibodies in the detection and treatment of cancer Any given cell contains within its cell surface membrane, a multitude of different proteins and lipids A great deal of microheterogeneity also exists amongst these molecules (Stanley, 1981, Litin and Grimes, 1983) Only a small percentage of the total lipids and proteins at the cell surface have been subjected to any extensive study, so that the possibility, at least, must still exist for the presence of some truly specific marker

It is not an essential prerequisite that an antibody should exhibit absolute specificity for tumour versus normal cells in order for that antibody to be of use in the detection and treatment of a cancer An antibody needs only to be "operationally specific" (Feder et al , 1983) for the tumour cell Antibodies demonstrating only very low levels of reactivity with normal cells as opposed to tumour cells can be of use in the detection and in vivo localization of a tumour For therapeutic purposes, the

criteria for "specificity" must obviously be more stringent in order to avoid excessive damage to both normal and irreplaceable stem cells

1 3

CELL SURFACE ALTERATIONS IN CANCER

The transformation of a normal cell into a neoplastic cell involves many alterations to the cell in terms of morphology, biochemistry and behaviour Few of the many changes which do take place can be shown to have any real consistency with the tumourigenic state The most consistently observed alterations following the neoplastic transformation of a cell relate to changes in the patterns of cell surface glycosylation of glycoproteins and glycolipids The nature of these alterations has been the subject of many excellent reviews (Warren et al , 1978, Smets, 1980, Reading and Hutchins, 1985) The complex carbohydrate structures of the cell surface membrane molecules can act as important antigenic determinants in immune recognition processes in cancer (Feizi, 1985) Because of this they have become a major focus of attention in the search for tumour specific antigens which may be amenable to exploitation in the diagnosis, localization and treatment of cancer using monoclonal antibodies

1 3 1

SIALIC ACID AND METASTATIC POTENTIAL

The sialic acids represent a very important group of amino sugars with functional roles in the areas of cell-cell communication, contact and adhesion phenomena They may also play a major role in the mediation of immune responses against tumour cell surface molecules (Reuter et al , 1982) There is also increasing evidence for a correlation between sialic acid levels and the tumourigenic and metastatic potentials of tumour cells (Yogeeswaran and Salk, 1981, Collard et al , 1981) Increased sialic acid has also been implicated in the platelet activation activity of tumour cells (Pearlstein et al , 1980, Bastida et al , 1987) which is believed to be involved in the development of metastases (Gasic et al , 1973, Jamieson et al , 1982) The loss of metastatic

potential by tumour cells is also known to be accompanied by a decrease in the levels of cell surface sialic acids (Reuter et al , 1982)

Sialic acid, neuraminidase and sialyltransferases play an important role in the regulation anti-tumour cell immune responses (Reuter et al , 1982) Terminal sialic may act to directly or indirectly mask cell surface antigens from immunodetection Direct masking arises when an antigenic carbohydrate residue is blocked by a terminal sialic acid residue Indirect masking occurs when sialic acids on one molecule block access to an adjacent sialic acid-free molecule by means steric hindrance mediated, at least in part, by the charge of the sialic acid residues (Reuter et al , 1982)

The removal of sialic acid from tumour cell surfaces by treatment with neuraminidase has been shown to induce stronger in vivo immune responses against the tumour cells (Currie, 1967, Sanford, 1967, Currie and Bagshawe, 1971, Simmons and Rios, 1971, Rios and Simmons, 1973, Pincus et al , 1981, Brandt et al , 1981) It has also been shown that the removal of sialic acid can result in reduced transplantability of tumour cells in vivo (Sanford, 1967b, Smyth et al , 1977) Neuraminidase treated cells have also been used in experimental immunotherapy of cancer with some success (Rios and Simmons, 1973, Sedlack et al 1975, Pincus et al , 1981)

Increased sialylation of membrane proteins is not unique to tumour cells Embryonic neural cellular adhesion molecules also contain large amounts of sialic acid in direct contrast to the adult forms of these proteins (Rothbard et al , 1982) The release of mature granulocytes from the bone marrow into the peripheral blood compartment may also involve a transient increase in the sialylation of cell surface carbohydrates (Van Beek et al , 1984) These increases in sialylation are not a permanent feature of these cells, unlike the situation in tumour cells Alterations in the levels of sialic acid and/or the sialylation patterns of tumour cell surface

glycoproteins and glycolipids represent an important feature in the development of the tumourigenic state (Collard et al , 1981, Yogeeswaran and Salk, 1981)

1 3 2

HIGH MOLECULAR WEIGHT FUCOPEPTIDES

One the most consistent alterations to be observed following the transformation of normal cells into tumourigenic cells is a shift from low molecular weight trypsin sensitive glycopeptides (fucopeptides) to high molecular weight glycopeptides (reviewed by Warren et al , 1978, Smets, 1980) These glycopeptides essentially represent the carbohydrate moieties of the cell surface glycoproteins which are exposed on the outer surface of the cell membrane This shift towards higher molecular weight glycopeptides was first reported by Buck et al (1970) and has since been reported for a variety of rodent (Warren et al , 1978, Santer and Glick, 1979, Collard et al , 1985) and human tumour cell types (Van Beek et al , 1975) Their presence has been noted for cells transformed by chemical mutagens (Warren et al , 1978), oncogenic viruses (Santer and Glick, 1979) and by transfection with DNA from neoplastic cells (Collard et al , 1985) Their expression has also been related to the activation of the ras oncogene (Santer et al , 1984)

While the shift towards high molecular weight glycopeptides would appear to be a universal marker for tumour cells there is some evidence to suggest that this alteration is not universally present on tumour cells Rachesky et al , (1982) reported that neoplastic cells of mesenchymal origin lacked the higher molecular weight glycopeptides and exhibited glycopeptide patterns similar to those of normal renal mesenchymal cells They also suggested that cell lineage within the same organ may play a role in determining the presence or absence of this phenomenon Normal chondroblasts cultured in vitro as spread cells or in suspension were found to express high levels of the high molecular weight glycopeptides (Cossu et al , 1982) However, when these cells were cultured as

organ cultures or *in vivo*, they did not synthesize the high molecular weight glycopeptides, indicating some possible association with the mode of *in vitro* growth rather than tumourigenicity It had previously been shown that the size distribution of the surface glycopeptides was not markedly influenced by the cell density or the physiological state of the cells (Richards *et al* , 1981) It was also reported that the differences in the levels between normal and tumour cells in relation to the expression of high molecular weight glycopeptides was quantitative rather than qualitative (Glick, 1979, Blithe *et al* , 1980) Despite this, the increased expression of these glycopeptides has been shown to correlate with tumourigenesis (Glick, 1973, Richards *et al* , 1981), metastasis (Dennis *et al* , 1987), and the invasive capacity of neoplastic cells (Bolscher *et al* , 1986) Also, wide differences in the metastatic potentials of different cells need not necessarily be correlated with major qualitative changes in any single cell surface biochemical characteristic (Raz *et al* , 1980) and it is possible that appropriate subtle quantitative alterations of cell surface components may significantly contribute to the development of a tumour and its metastases

Overall, the presence of high molecular weight glycopeptides demonstrate a very strong correlation with the neoplastic state Whether or not specific qualitative differences in the structures can be uniquely associated with the neoplastic state remains to be seen It has been suggested that the type of glycoconjugates which are more highly expressed in tumour cells may be species specific (Santer *et al* , 1984) Also, the levels may of expression may be related to state of differentiation and the population density of the cells involved (Codogno *et al* , 1985) Nonetheless, the strong correlation of the high molecular weight with neoplasia must make this alteration a focus of attention for the presence of possible tumour specific or related antigens amenable to immunological exploitation

1 3 3

THE STRUCTURAL NATURE OF THE HIGH MOLECULAR WEIGHT GLYCOPEPTIDES

It was originally thought that the shift from low to high molecular weight glycopeptides was due solely to the increased sialylation of the glycopeptides on tumour cells (Warren et al , 1978) The removal of sialic acid from tumour cell surface glycopeptides resulted in their co-elution with the glycopeptides from normal cells, suggesting that sialic acid plays a key role in the nature of this alteration However, Ogata et al , (1976) showed that sialic acid alone could not account for the entire differences in molecular weight between the high and low molecular weight glycopeptides of tumour and normal cells respectively They suggested that the addition of up to 4 chains of sugars to give rise to a tetra-antennary structure, was required to account for the molecular weight differences These chains would consist of sialic acid bound to Gal-GLcNAc-Man-, as shown in figure 1 1 It has since been shown that tri- and tetra-antennary carbohydrate structures are a predominant feature on tumour cells as opposed to normal cells which possess predominantly bi-antennary carbohydrates (Figure 1 2) (Santer and Glick, 1983, Santer et al , 1984, Yamashita et al , 1985)

The high molecular weight glycopeptides are now known to possess complex asparagine (Asn) N-linked glycans The N-glycosyl units of glycoproteins contain a universal pentasaccharide core of Man3(GLcNAc)2 (see Figure 1 1) which is usually substituted with additional mannose residues or branches of Gal-β(1-4)-GLcNAc and sialic acid or polysialic acid units (Sharon, 1984) The high molecular weight glycopeptides were found to be highly branched at the tri-manrosyl cores with (1-6)-lactosamine antennae [i e Gal-β(1-4)-GLcNAc-β(1-6)-] (Santer et al , 1984) This is further supported by the findings of Yamashita et al , (1985) that the high molecular weight chains consist of GLcNAc- (1-6)-Man-α(1-x) while the low molecular weight glycopeptides contain GLcNAc-α(1-4)-4Man-α(1-x)

1 4

OTHER ALTERATIONS IN TUMOUR CELLS

1 4 1

GLYCOLIPIDS

The transformation of normal cells into neoplastic cells is also known to affect the cells surface glycolipids (reviewed by Reading and Hutchins, 1985, Hakomori, 1985) These alterations may involve changes in the expression levels of certain glycolipids as well as alterations in their glycosylation patterns Many of these alterations are now being defined by monoclonal antibodies In some cases, it has been found that the tumour cell surface glycolipids (and glycoproteins) are expressed in cryptic forms on normal cells These cryptic antigens on normal cells may not be readily detectable by immunological means (Hakomori, 1971, Wolf and Robbins, 1974) so that immunological studies alone may make it appear that a given antigen may be tumour specific while chemical analysis reveals a different story

As with the glycoproteins, most glycolipid alterations are often found to represent the aberrant expression of differentiation antigens or purely quantitative changes as opposed to qualitative differences between normal and tumour cells Qualitative differences have been reported on the sialylation positions and the expression of surface glycolipids in of high and low metastatic variants of murine lymphomas (Murayama et al , 1986) The low metastatic variants had increased levels of exposed ganglio-glycolipids Gg_3 and Gg_4 (see Figure 1 3 for structures), both of which were absent from the high metastatic variants However, in a similar study of metastatic variants of a rat hepatoma, the patterns of Gg_3 and Gg_4 expression were directly opposite to those of the murine system (Hirabayshi et al , 1978, Taki et al , 1979) The expression of the glycolipid GD_3 was found to be decreased in Rous Sarcoma virus transformed chick embryo cells (Hakomori et al , 1971) while it was increased in human melanomas where it was shown to have a

possible association with metastasis (Cheresh et al , 1984)

The above examples serve to illustrate the complexity and heterogeneity of antigenic expression patterns in tumour cells from different species and, possibly between tumour cells taken from different anatomical sites within a given individual Such heterogeneity adds greatly to the complex nature of cancer and also points to the need for the careful evaluation of each new antigen for variations in their expression profiles within different tumour systems Such an evaluation will be of critical importance where a given antigen is proposed as a potential target for antibody-mediated localization and drug-targetting in vivo

1 4 2

ENDOGENOUS LECTINS

The surface properties of the cell play a major role in the growth of the cells and their invasion into other tissues (Poste and Fidler, 1980) Recently, some attention has been focused on the role(s) of endogenous animal cell lectins capable of binding mono- and oligosaccharides expressed by glycoproteins, glycolipids and glycosaminoglycans in cell-cell recognition and adhesion processes

In the mid-1970's, a series of reports appeared on the existence of endogenous lectins in animal cells and their possible roles in cell-cell recognition and adhesion processes (Gartner and Podleski, 1975, Nowak et al , 1977, Simpson et al , 1978, Grabel et al , 1979, Powell, 1980) The endogenous lectins may also be shown to be developmentally regulated (Nowak et al , 1976, Powell and Whitney, 1980) The different levels of expression of the endogenous lectins on the surfaces of non-tumourigenic, tumourigenic and metastatic cells have also been reported (Raz et al , 1986) Furthermore, the endogenous lectins have been associated with tumour heterogeneity and the selective emergence of tumour cell sub-populations with

different lectin-binding profiles to the primary tumour (Kellokumpu, 1986) This suggests that these lectins may play a crucial role in the selective colonization of different anatomical sites in the body This is further supported by the findings of Meromsky et al , (1986), that a monoclonal antibody directed against a cell surface lectin of B16 melanoma and UV-2237 fibrosarcoma cells brought about a reduction of up to 90% of the levels of lung tumour colonization Roos (1984), using a monoclonal antibody against a different cell surface component found it to be an effective inhibitor of carcinoma-hepatocyte adhesion This suggests that the process of metastatic colonization of tissues is under the control of multiple factors and that the regulation of cell surface lectins and other factors by monoclonal antibodies may prove to be useful in the control of tumour progression in vivo

1 4 3

SHEDDING OF TUMOUR CELL SURFACE ANTIGENS

The shedding of tumour cell surface antigens in vivo represents a dynamic process involving the loss of cell surface components without affecting the cells viability (Black, 1980) Antigens may be shed in either molecular or particulate forms, often as vesicles The shedding of antigens may be a selective process with different antigens being shed at different rates (Emerson and Cone, 1979, Emerson and Cone, 1981) The exact mechanisms involved for shedding of surface antigens remain unclear Proteolysis (Kapeller et al , 1973, Doljanski and Kapeller, 1976) and membrane fluidity (Van Blitterswijk et al , 1975 and 1979, Nowotny, 1983) may be instrumental in determining which areas are to be shed However, shedding has been shown to occur in the absence of proteolysis (Plesser et al , 1980, Brennan et al , 1980) Some evidence suggests that the rate of sheading of antigen from tumour cells may be related to the degree of tumourigenicity of the cells (Black, 1980, Dennis et al , 1981) The rate of shedding is also increased in metastatic cells versus non-metastatic cells (Currie and Alexander, 1974) However, in such cells, it is not only

the tumour antigens which are shed at a higher frequency Davey et al , (1976) have shown that the levels of normal histocompatible antigen shedding are also increased

The functional relevance of antigen shedding is not yet clear It has been proposed that it may be associated with cell-cell communication (Emerson and Cone, 1979) and it has been shown to be the major mechanism for the clearance of surface antigens from lymphocytes (Hsu et al , 1980) In tumour cells, it may play a much more important role as a "self defence" mechanism to protect the cell from immunodestruction (Nicolson, 1976, Price and Baldwin, 1977) The shedding of tumour related antigens may help to induce a state of tolerance in which immunorecognition is blocked through the binding of shed antigen to circulating antibody (Doljanski and Kapeller,

1976, Nowotny, 1983) Vesicles shed from tumour cells are known to be enriched in tumour related high molecular weight glycoproteins (Van Blitterswijk et al , 1979, Walsh, 1984) and have been shown to be capable of preventing the interaction between tumour cells and macrophages in vitro (Raz et al , 1978)

The presence of tumour antigens in the serum and pleural effusions of cancer patients (Petitou et al , 1978) and the relationships between the degree of tumourigenicity (Black, 1980, Dennis et al , 1981) and the metastatic potential of malignant cells (Currie and Alexander, 1974) makes it reasonable to assume that the serum levels of such antigens could provide a means of detecting and monitoring the progression of a cancer However, some consideration must be given to the clearance of such antigens and antigen-antibody complexes from the circulation by phagocytosis in the reticuloendothelial system and by deposition in the renal glomeruli and other tissues (Benacerraf et al , 1959) The liver may also influence the profile of circulating antigens shed from tumour cells There is some evidence top suggest that the liver may preferentially clear certain types of antigens from the circulation, particularly galactose-terminating

basic glycopeptides (Baumann and Eldredge, 1982) The reuptake and autodegradation of shed antigens may also take place, as has been reported for human melanoma cells (Johnston and Bystryn, 1984) Serum-antigen levels may be further influenced by the pathological changes observed during the progress and development of different cancers (Rudman et al , 1969) Because of these factors, it is difficult to make definite correlations between between serum antigen and antigen-antibody complex levels and the development and clinical status of different types of cancer (Pierce and De Vald, 1975, Price and Baldwin, 1977) However, where a detailed knowledge is available concerning the source and fate of a given tumour related antigen which is shed from the tumour cell surface, it may be possible to devise appropriate immunoassays to quantify and relate the serum antigen levels to the clinical status of the patient In this regard, appropriate monoclonal antibodies would be of great value for the diagnosis and monitoring of cancer in vivo

1 5

APPLICATIONS FOR MONOCLONAL ANTIBODIES IN TUMOUR DETECTION AND MANAGEMENT

In 1913, Paul Ehrlich suggested that the treatment of disease may one day be tackled by means of a "magic bullet" consisting of a drug linked to a specific targetting agent in a single bi-functional molecule Such an approach to the treatment of cancer was never more than a dream largely due to the lack of specific targetting agents capable of distinguishing between tumour and normal cells The advent of hybridoma technology (Kohler and Milstein, 1975) has changed all of that by making it possible to generate unlimited amounts of target-specific antibodies of predefined specificities The only remaining problem lies in identifying a tumour specific antigen against which antibodies can be targeted It is becoming increasingly obvious that a truly specific tumour marker may not exist Nonetheless, many quantitative differences between tumour and normal cells have been identified and these differences may be exploited for the

detection and treatment of cancer The principles and applications of monoclonal antibodies to the management of cancer have recently been reviewed by Schlom (1986) For diagnostic purposes, total specificity for tumour cells is not a critical requirement for antibody-mediated diagnosis and *in vivo* localization of tumours The major requirement is that a significant quantitative difference exist, allowing the detection of tumour tissues against a relatively low level of background staining of normal tissues In the case of antibody-mediated targetting of drugs and toxins to tumour cells, the specificity of the target antigen for the tumour cell becomes more critical However, the target antigen need not be unique to the tumour cell in order to make possible the exploitation of that antigen in antibody-mediated therapy (Trowbridge and Domingo, 1981, Obrist, 1983)

1 5 1

IMMUNODIAGNOSIS OF CANCER

The widest application of monoclonal antibodies to date has been in the areas of *in vitro* analysis of leukemias and lymphomas using blood or bone marrow samples and tissue biopsy specimens (Levy and Miller, 1983) The major application of antibodies has been in determining whether or not a given tumour is benign or malignant and in the identification of the tissue of origin of the tumour (Mason *et al* , 1982, Borowitz and Stein, 1984) One of the major problems associated with the use of monoclonal antibodies in *in vitro* diagnostics relates to the fact that the epitopes against which the antibodies are directed may appear on other related but distinct molecules bearing a stronger correlation with the normal cell than with the tumour cell (Nigg *et al* , 1982) Also, allotypic variability and tumour cell heterogeneity (Heppner, 1984) may sometimes result in failure to detect tumour cell surface antigens on sub-populations, even when the antigen is present Most studies involving the use of monoclonal antibodies in the study of lymphoid tumours have been directed at the classification of the tumours (Greaves *et al* , 1982) Such classification of

cancers may provide valuable information in determining the course of treatment best suited to a given type of cancer

1 5 2

THE IN VIVO LOCALIZATION OF TUMOURS USING MONOCLONAL ANTIBODIES

Antibody-mediated localization of tumours *in vivo* represents a major advance in the management of cancer This involves the administration of an anti-tumour cell antibody labelled with a radionuclide (typically ^{131}I ^{111}In or ^{99m}Tc) This is allowed to accumulate in tumour tissues and the precise location of the tumour is then determined by scanning with a gamma-camera The antibodies used are selected so as to allow high tumour to normal tissue (T NT) ratios of bound antibody to be obtained High levels of background label are commonly encountered, the bulk of this arising from the presence of free labeled antibody in the serum and lymphatics or non-specifically bound to some normal tissues This problem is usually overcome by means of computer-aided subtraction methods A number of alternative methods have also been developed to allow improved imaging of tumours Firstly, a second antibody may be employed to remove any free labeled antibody which has not been bound by the tumour (Sharkey, 1984) Secondly, liposomally encapsulated radionuclides may be delivered to the tumours by means of antibody targetting of the liposomes (Caride, 1984) Finally, liposomes coated with antibodies directed against the labeled antibody can be used to clear free label from the body This latter methods seems to be the most promising because it offers the advantages of avoiding the removal of labeled antibody from the tumour surface The liposomes used are unable to penetrate the epithelial barriers of the blood vessels and cannot therefore gain access to the tumour tissues themselves They also represent large lattice structures which are more efficiently and rapidly cleared from the blood system (Proffitt *et al* , 1983, Barratt *et al* , 1984)

1 5 3

ANTIBODY-MEDIATED THERAPY OF CANCER

The in vivo antibody-mediated treatment of cancer makes use of radionuclides (Hammersmith Oncology Group Report, 1984, Schlom, 1986), drugs (Embleton, 1987) and toxins (Gilliland et al , 1980, Collier and Kaplan, 1984, Hara and Seon, 1987) conjugated onto anti-tumour antibodies The most serious problem encountered in antibody-mediated drug delivery systems is the non-specific uptake of antibody by normal tissues resulting the lack of total specificity of antibody for tumour cells This problem is further compounded by tumour cell heterogeneity and antigenic modulation (the disappearance of the antigen from the cell surface following interaction with antibody) (Obrist, 1983, Schlom, 1987) which may allow sub-populations of tumour cells to escape the effects of the therapy used It has been argued that the use of radionuclides may be superior to the use of drugs and toxins in the treatment of cancer on the grounds that locally high concentrations of radionuclide can be achieved by means of antibody targetting Such a concentration of radionuclide would destroy all of the cells in the immediate vicinity including those tumour cells which may have failed to bind the antibodies used (Schlom, 1987)

1 5 3 1

LIPOSOMES

The use of targeted liposomes containing encapsulated drugs may also prove useful in this respect Liposomes offer a means of allowing the slow release of drugs in the vicinity of the tumour and the potential exists to allow for the design of liposomes which can be activated to release their load at specific sites e g by localized heat treatment to rupture heat-sensitive liposomes Indeed, it has been shown by Heath et al (1984) that liposomes coated with surface antibodies may bind to tumour cells with greater affinities than the free antibody itself

1 5 3 2

COMPLEMENT DEPENDENT CYTOTOXICITY

Complement dependent cytotoxicity of tumour cells may also be mediated be monoclonal antibodies This has been taken to the treatment of metastases of a murine leukemia *in vivo* with no serious side-effects on normal lymphocytes (Bernstein *et al* , 1980, Ball *et al* , 1982) A similar report was made concerning the treatment of a melanoma tumour using a monoclonal antibody directed against GD_2 ganglioside (Irie and Morton, 1986) Different antibody classes have different efficiencies in the fixing of complement and this is reflected in the efficiency of different monoclonal antibodies in complement dependent cytotoxicity of tumour cells *in vivo* (Seto *et al* , 1983) IgG_{2a} was found to be the most effective followed by IgG_{2b} and IgG_1 Neither IgM nor IgA were found to be very effective in this role The efficiency of cell destruction by antibody-mediated complement cytotoxicity is also dependent on the nature of the antigen bound by the antibodies Some cells may lack the antigen while others may undergo antigenic modulation However, monovalent antibodies are known not to induce antigenic modulation and using this observation, Cobbold and Waldmann (1984) demonstrated increased complement-mediated cytotoxicity for monovalent monoclonal antibodies over the corresponding bivalent monoclonal antibodies The monovalent antibodies used in this study were generated by the fusion of immune rat spleen cells with the k-light chain secreting rat myeloma, Y3/Ag1 2 3 The resulting hybridomas secreted both monovalent and bivalent antibodies The monovalent antibodies were then isolated by affinity chromatography with an anti-Y3/Ag1 2 3 k-light chain specific monoclonal antibody (MRC OX 12) Anti-target cell bivalent antibodies did not bind to the MRC OX 12 antibody, since they lacked the Y3/Ag1 2 3 k-light chain

To overcome tumour cell heterogeneity, panels of monoclonal antibodies directed against a series of selected antigens may greatly increase the possibility of

killing all or, at least, a greater proportion of the tumour cells present in a given cancer

1 5 3 3

OTHER TRENDS IN ANTIBODY-MEDIATED CANCER THERAPY

The possibility of developing anti-tumour vaccines to protect against the development of tumours is currently under some investigation Whether or not this is feasible remains to be seen Given the generally weak immune responses elicited in tumour bearing individuals, the possibility of developing an anti-tumour vaccine must seem very remote The common explanation for the weakness of the immune responses elicited by tumours is tolerization to the tumour antigens (McBride and Howie, 1986) One possible means of overcoming this would be to present the critical antigens or epitopes in a new form capable of eliciting a greater immune response With the aid of monoclonal antibodies, it may be possible to identify and isolate such appropriate antigens from tumour cells The purified ganglioside, GM_2, has been used in this manner to elicit an immune response (Livingston et al , 1987) While the immune responses to GM_2 were short-lived, the results of this study suggested that melanoma recurrence in patients developing anti-GM_2 antibodies was delayed by comparison to those lacking anti-GM_2 antibodies

The use of anti-idiotypic antibodies offers a further possibility in this respect It has been shown that anti-idiotypic antibodies raised against a monoclonal antibody bear a mirror image of the human tumour antigen against which the monoclonal antibody was directed (Healyn et al , 1986) This opens the possibility of using anti-tumour monoclonal antibodies to elicit anti-idiotypic immune responses in cancer patients Based on the above evidence, such an immune response may also be expected to recognise the tumour antigens against which the monoclonal antibodies were first produced A major advantage of such a system is that large amounts of the appropriate monoclonal antibodies are readily prepared where only very limited amounts of tumour antigen may be available for

immunization purposes Using this approach, Kennedy et al , (1985) were able to inhibit the transplanting of a virus induced tumour in a model system Modulation of immune responses to tumours have also been reported following the administration of anti-idiotypic antibody (Forstrom et al , 1983) However, not all anti-tumour monoclonal antibodies may be suitable for the induction of a protective anti-idiotypic immune response (Forstrom et al , 1983, Raychaudhuri et al , 1987)

The generation of an immune response against murine monoclonal antibodies during antibody-mediated therapy of cancer represents a serious problem, with normal individuals generating anti-antibody immune responses in approximately 10 days (Obrist, 1983) It is unlikely that a single dose of antibody-drug conjugate will ever be sufficient for the treatment of a cancer In order to overcome this problem, patients receiving antibody treatments may need to undergo some form of immunosuppression to prevent this occurring Currently, a great deal of research is being directed towards the production of stable human monoclonal antibodies which can be expected to be much less immunogenic in humans Many problems remain to be overcome before appropriate antibodies are produced though significant advances have been made (Olsson and Brams, 1985, Glassy et al , 1985) A novel approach to the generation of a human monoclonal antibody was taken to this problem by Liu et al (1987) and Sun et al (1987) They genetically constructed mouse- human chimeric antibodies incorporating the binding sites of the mouse monoclonal antibodies with the constant regions of a human antibody The resulting chimeric DNA was transfected into Sp2/0 cells to give a monoclonal antibody secreting cell line The resulting antibodies retained the antigenic specificity of the parent mouse antibody A major advantage associated with this method may be the ability to predefine the class of antibody to be made which may be of great relevance to the intended applications of the chimeiic antibodies

1 6

CONCLUDING COMMENTS

To date, no truly specific marker for tumour cells has been identified Of the differences observed between tumour and normal cells, the majority are of a quantitative rather than a qualitative nature Alternatively, they represent the aberrant expression of differentiation antigens Few of these differences can be shown to have a strong correlation with the neoplastic state One of the exceptions to this are the observed alterations in the cell surface carbohydrates of both glycoproteins and glycolipids expressed by tumour cells (Warren et al , 1978, Smets, 1980, Busch, 1984, Reading and Hutchins, 1985) Even here, true specificity for the neoplastic state has yet to be demonstrated

The development of hybridoma technology (Kohler and Milstein, 1975) saw a major resurgence in the search for a tumour specific antigen However, while a great many new antigens have been identified, none can be said to be truly tumour specific In spite of this, monoclonal antibodies have continued to play a key role in the study of cancer For the exploitation of monoclonal antibodies as detection and therapeutic agents in the fight against cancer, absolute specificity of antibody for the tumour cells is not essential, though highly desirable (Lennox and Sikora, 1982) The conditions under which the antibody is to be used should be designed so as to render it "operationally specific" i e under conditions where the damage to normal cells is minimal compared to the damage caused to the tumour cells In order to achieve this status, the antigen to be exploited must be carefully assessed in relation to its expression by both tumour and normal cells This is further compounded by the heterogeneity of tumors cells within a given tumour mass (Heppner, 1984) The problem may be particularly serious in antibody-mediated treatment of cancer (Schlom, 1986) where sub-populations of tumour cells may not express the antigen being used for antibody targetting and, so might escape destruction In the future, we may see the use of

preselected panels of monoclonal antibodies for the treatment of cancer with a view to overcoming the problem of tumour cell heterogeneity

Finally, monoclonal antibodies have played a major role in advancing our understanding of the development of neoplasia and the nature of the cellular alterations which accompany the transformation of a normal cell into a neoplastic cell They have also contributed greatly to the earlier diagnosis and treatment of cancer and will, no doubt, play a key role in the furtherance of our ability to control cancer

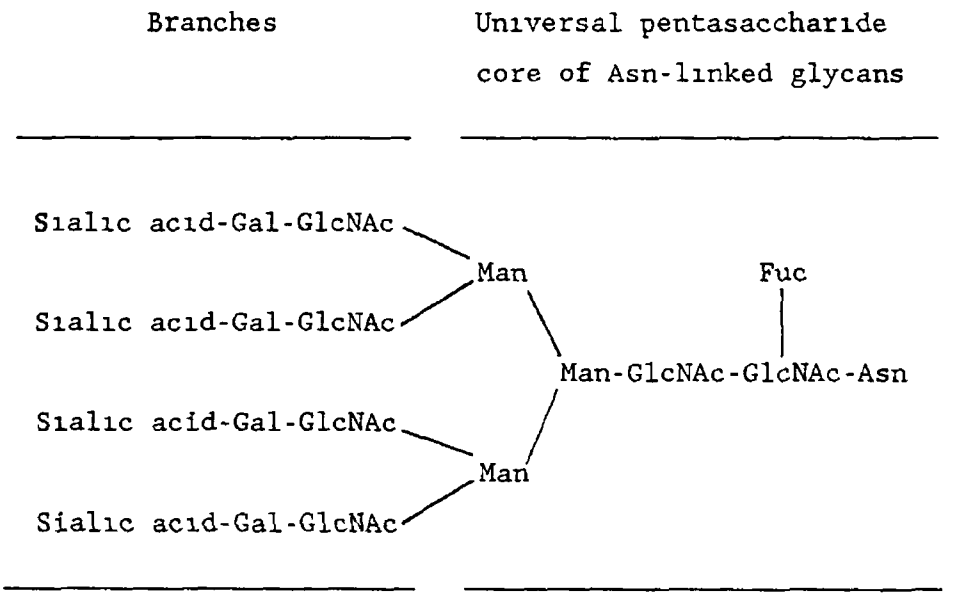

FIGURE 1 1

A structural model for the high molecular weight glycopeptides expressed by tumour cells, as proposed by Ogata et al , (1976) (Taken from Ogata et al , 1976)

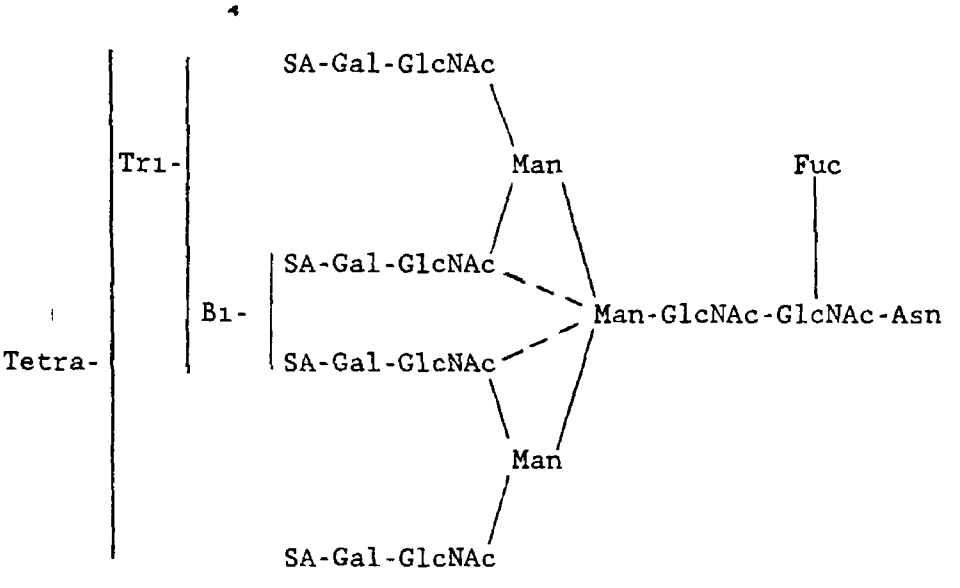

FIGURE 1 2

Cell surface complex carbohydrates showing Tetra-, Tri- and Bi-antennary structures

(A) αGalNAc-βGal(1-4)βGlc(1-1-OH)sphıngosıne of ceramıde

(B) αGalNAc-βGal(1-4)βGlc(1-1-OH)sphıngosıne of ceramıde
|
βGal

(C) βGal(1-4)βGlc(1-1-OH)sphıngosıne of ceramıde
/
αSA(2-8)αSA

FIGURE 1 3

Glycolıpıd structures (A) Gg_3, (B) Gg_4, and (C) GD_3 All three glycolıpıds belong to the ganglıo-serıes of glycolıpıds (Readıng and Hutchıns, 1985)

SECTION 2

MATERIALS AND METHODS

MATERIALS

A CELL CULTURE MATERIALS

All cell culture media, foetal calf sera, L-glutamine and penicillin-streptomycin solutions were obtained from Flow Laboratories

Cell culture vessels were obtained from Sterilin, Nunc and Costar

Cell lines

Cell line	Source of cells
Sp2/0 (mouse myeloma)	Flow Laboratories, Irvine, Scotland
A549 (human lung carcinoma)	Kindly donated by Drs M Clynes and M Dooley (NIHED)
NRK (normal rat kidney)	Kindly donated by Dr M Clynes and Miss S Neenan (NIHED)
Landschütz ascites tumour cells (murine ascites carcinoma)	Kindly donated by Dr H Smyth, University College, Dublin and Dr, M Conalty, MRCI Lab Trinity College, Dublin

B CHEMICALS

All of the chemicals used were of analar grade and supplied by Riedel-de Haen, Sigma and BDH Exceptions to this were absolute alcohol and methanol used for staining and destaining solutions in electrophoresis Both were of reagent grade and were supplied by Riedel-de Haen Acrylamide and Bis-acrylamide were of electran grade and were supplied by BDH Ready-solv EP scintillation fluid and scintillation vials were supplied by Beckman Liquid-N_2 was supplied by Cryogas CO_2 and N_2 gases were from Irish Industrial Gases ^3H-Fucose (1 µCi/ml) w s

purchased from Amersham, as were the β-galactosidase linked sheep anti-mouse antibody conjugates for ELISA Goat anti-mouse Ig conjugated to alkaline phosphatase was supplied by Sigma Antibody isotype analysis kits came from Zymed Neuraminidase was a gift from Dr H Smyth, UCD Other enzymes were supplied by Sigma Hoechst 33258 fluoresence mycoplasma stain was obtained from Calbiochem PBS (Dulbecco's A) was obtained from Oxoid

C EQUIPMENT

ITEMS	MANUFACTURER
Laminar flow cabinets	Nuaire and Microflow
CO_2-incubators	LEEC
Liquid-N_2 freezers	Union Carbide
Multichannel pipettes	Flow-titertek and Costar
Micropipettes	Gilson and BCL
Electrophoresis	LKB (2197 system)
Immunoblotting	Biorad (Transblot)
Centrifuges	Heraeus Christ (bench top) Sorvall RC-5B (high speed) Beckman (L8M, 70 Ti rotor, ultra)
Scintillation counter	Beckman (LS-800)
Chromatography	Waters HPLC (model no U6K) Waters Protein Pak Column 300SW LKB and Pharmacia gel filtration columns
Fraction collector	LKB (2070 Ultrorac)
Peristaltic pumps	LKB (2232 Microperpex-S)
Freeze-drier	Modulo-Hetosicc (CD 52 model)

2 2 TISSUE CULTURE METHODS

2 2 1 SP2/0 MYELOMA CELLS

SP2/0 myeloma cells (Flow Laboratories) were maintained in RPMI-1640 medium supplemented with 10% (v/v) FCS Cultures were initiated at cell densities of $2x10^5$ cells/ml, using 10mls per $75cm_2$ culture flask The cells were harvested when ~75% confluent by flushing them from culture surfaces using a sterile pasteur pipette All cell cultures (including those below), were incubated in a humid, 5% CO_2 atmosphere at 37°C

2 2 2 A549 AND MRC-5 LUNG CELLS

A549 cells were donated by S Neenan and Dr M Clynes, NIHED This is a human lung adenocarcinoma, developed by Girard et al , (1973), and has an epithelial-like morphology

MRC-5 cells were obtained from the American Type Culture Collection, Rockville, MD This is a normal human lung cell of embryonic origin with a fibroblast-like morphology The line was first described by Jacobs (1970)

Both A549 and MRC-5 cells were cultured under similar culture conditions Each was maintained in DMEM medium supplemented with 10% (v/v) FCS As before, cell cultures were established at $2 0x10^5$ cells per ml of medium in 75cm flasks

Because both A549 and MRC-5 are strongly adherent to culture surfaces, they require mild trypsinization to release them during harvesting and sub-culturing of cells All of the culture medium was decanted and 2 mls of trypsin EDTA solution (Flow Laboratories, 0 25% [w/v] trypsin 0 02% [w/v] EDTA in PBS) was added to the flasks

After 1 minute, a further 3-4 mls of trypsin/EDTA solution was added and incubated at 37°C for 10 minutes The cell suspension was decanted into 5-10 mls of complete culture medium to inactivate the trypsin Cells were washed in 10 mls of culture medium before use

2 2 3

NRK CELLS

The same procedures, just outlined (2 2 2), were used in the culture of NRK (normal rat kidney) cells with the exception that 5% (v/v) FCS was used instead of 10% (v/v) FCS This cell line was first described by Duc-Nguyen et al (1966)

2 2 4

CELL COUNTS AND VIABILITY STAINING

Cell counts were performed on an improved Neubauer haemocytometer slide Acridine orange and ethidium bromide were routinely used for the determination of cell viabilities (Mishell and Shiigi, 1985) Stain solution contained 0 1 mg acridine orange and 0 1 mg ethidium bromide in 100 mls PBS One volume of cells was added to an equal volume of stain and examined by U V flourescence microscopy Live cells stain green while dead cells stain orange

2 2 5

FREEZING AND RECOVERY OF CELLS

The 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% dimethyl-sulphoxide (DMSO, cryoprotectorant) One ml aliquots were placed in sterile cryotubes (Nunc) and frozen at a rate of approx 1°C per minute to -70°C on a freezing tray (Union Carbide) before being placed into liquid nitrogen

Recovery of cells from liquid nitrogen was achieved by thawing the cells rapidly to 37°C and then washing them twice in culture medium, or PBS, as appropriate (Osterling and Earley, 1985) Cells recovered from liquid nitrogen were approximately 90% viable

2 2 6

ISOLATION OF WHITE CELLS FROM WHOLE BLOOD

White cells (leucocytes) were isolated from human peripheral whole blood by density gradient centrifugation over Ficoll (Boyum, A , 1968)

Heparinized blood was diluted 1 1 with PBS containing 20mm sodium azide Two parts of this mixture were then layered over 1 part of lymphoprep (Ficoll-Metrizoate, ρ=1 077 g/ml) in a 10 ml centrifuge tube and centrifuged at 400xg for 35 mins at 20°C in a swinging bucket rotor (Heraeus Christ Labofuge 6000) The blood cells separated into 2 distinct fractions a) the red blood cells, which form a pellet, and b) the white cells which form a "buffy layer" between the lymphoprep and the plasma The white cells were carefully withdrawn using a pasteur pipette, avoiding the uptake of Ficoll-Metrizoate Cells were washed 3 times in PBS-azide (0 1% w/v) before use Where the cells are to be used for culture, azide should be omitted from all steps to ensure that viable cells are obtained

2 2 7

LANDSCHÜTZ ASCITES TUMOUR (LAT) CELLS

LAT cells were maintained by passage in Schofield mice as intraperitoneal (ip) ascites tumours The tumours were established by ip injection of $1x10^7$ cells into mice aged from 12 to 15 weeks old After 10 to 14 days, cells were harvested into 20mls of PBS and washed 3 times in PBS before use

2 2 8

MYCOPLASMA DETECTION - HOECHST 33258 FLUORESCENCE ASSAY

The use of Hoechst 33258 fluorescence stain for the detection of mycoplasma was first described by Chen, (1977) It employs the DNA-intercalator bisbenzimid (Hoechst 33258) to detect the presence of mycoplasma in cellular cytoplasm The following method is an adaptation of this procedure developed by M Dooley and M Clynes, NIHED (personal communication)

Coverslip cultures of mycoplasma-free NRK cells were established using $5x10^3$ cells in 1 ml DMEM containing 5% FCS Following overnight growth of cells, 1 to 2 mls of culture supernatant from the cells being tested (free of cells and debris) was added to the NRK cells, which were reincubated for a further 3 to 4 days, or until cells attained 75% confluency The coverslips were then washed 3 times in PBS (taking care to keep the cells on the uppermost surface all of the time) and fixed for 6 mins in a 1 1 methanol acetone solution at -20°C Coverslips were then rinsed 3 times in PBS and stained for 10 mins in Hoechst 33258 stain (0 05 µg/ml in PBS) The stain was then washed from the cells 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 oil-immersion and a B2 combination filter (Nikon)

2 3

LAT CELL ^{3}H-FUCOPEPTIDES THEIR PREPARATION, ISOLATION AND CONJUGATION TO BSA FOR THE PRODUCTION OF POLYCLONAL ANTIBODIES

2 3 1

THE IN VIVO LABELLING OF LAT CELLS WITH ^{3}H-FUCOSE

Seven day old LAT cell ascitic tumours from Schofield mice were metabolically labelled with 3H-fucose Each mouse received, by intraperitoneal injection, 40 µCi of ^{3}H-fucose (Amersham) ir 0 5 ml of PBS The cells were

harvested after 36 to 48 hours Only those samples free of contamination with red blood cells and greater than 90% viable were used The cells were washed 3 times in PBS before use

2 3 2

PREPARATION OF LAT CELL TRYPSINATES

Washed LAT cells were resuspended at $5x10^7$ cells/ml in PBS containing 0 1 mg/ml of trypsin (type III-S from bovine pancreas, E C 3 4 21 4, Sigma) and incubated at 37°C for 20 mins The cells were then removed by centrifugation at 400xg and the supernatant (trypsinate) incubated at 80°C to inactivate the trypsin (O'Kennedy, 1979, PhD thesis) The trypsinate was then dialyzed against distilled water and lyophillized

2 3 3

PRONASE DIGESTION OF LAT CELL TRYPSINATES

Lyophilized trypsinates from $1x10^{10}$ LAT cells were dissolved in 20 mls of PBS (10 mls for $1x10^9$ cells) containing 0 67 mg/ml of pronase (protease from Streptomyces griseus, type XIV, Sigma) and incubated at 37°C for 3 days on a rotary shaker table (LH Fermentation) Fresh additions of enzyme were made daily along with a few drops of toluene to help prevent microbial growth On completion of pronase digestion, the protein was removed by precipitation with 5% (w/v) trichloroacetic acid followed by centrifugation at 1000xg for 15 mins The final supernatants were dialyzed against distilled water and lyophilized The dried ^{3}H-fuco-peptide preparations were stored in a desiccator at -20°C

2 3 4

MOLECULAR SIZE FRACTIONATION OF ^{3}H-FUCOPEPTIDES

Lyophilized pronase digests of ^{3}H-fucopeptides from LAT cells were resuspended into a minimum volume of 0 1M Tris-acetate buffer, pH 9 0, containing 0 1% (w/v) sodium dodecyl sulphate, (SDS) 0 01% (w/v) EDTA and 0 1% (v/v) B-2-mercaptoethanol Samples were applied to a mixed column of Biogel P-10 Sephadex G-50 (2 1) preequilibrated in the above buffer (elution buffer) The column size was 94x1 5 cm (internal diameter) The void and total volumes (26 and 95 mls respectively) were determined using Blue dextran 2000 (2,000 kd) and phenol red (500 d) Samples were eluted at a flow rate of 0 2 mls/min and fractions of 1 0 ml were collected

2 3 5

RADIOACTIVITY COUNTS

Aliquots (100 µl) of column fractions were added to 10 mls of Ready-Solv EP scintillation fluid (Beckman) Samples were vigorously shaken before being counted in a Beckman LS-800 scintillation counter (10 mins per sample)

2 3 6

CONJUGATION OF ^{3}H-FUCOPEPTIDES TO BSA

^{3}H-fucopeptides, prepared and separated as described above were conjugated onto bovine serum albumin (BSA) by the method of Zopf et al (1978) and Smith et al (1978) Three distinct steps are involved 1 phenethylamine derivatization of ^{3}H-fucopeptides, 2 formation of isothiocyanate-phenthylamine-^{3}H-fucopeptide derivatives, and 3 the conjugation of the above derivatives to BSA

2 3 6 1

PHENETHYLAMINE DERIVATIZATION OF ^{3}H-FUCOPEPTIDES

Two mg of the ^{3}H-fucopeptides were added to 0 5 ml of -(p-aminophenyl)ethylamine (3 5 mM, Aldrich) and shaken in a sealed tube for 18 hours at 40C to achieve solution The viscosity was then reduced by the addition of 0 5 ml of absolute alcohol followed immediately by 1 0 ml of absolute alcohol containing 21 mg of sodium borohydride The opalescent suspension of sodium borohydride in absolute alcohol was prepared by sonication This mixture was stirred for at least 4 hours in a vented tube at room temperature and then diluted with 4 mls of distilled water and placed on ice The pH was adjusted to 5 6 by the dropwise addition of glacial acetic acid If there was any precipitation at this point, the mixture was left overnight to ensure that full reduction of the mixture by sodium borohydride took place

The ethanol was then removed by rotary evaporation and the mixture taken up into 5 mls of distilled water and applied to a column of Sephadex G-10 (50x1 6 cm, equilibrated with 1M acetic acid, adjusted to pH 5 0 with pyridine) The separation of the free amine from the phenethylamine derivatives of the ^{3}-H-fucopeptides was shown by a continuous UV-280nm monitor (LKB Uvicord) A flow rate of 0 3 mls/min was used and 3 0 ml fractions were collected The phenethylamine-^{3}H-fucopeptides were located by counting 100 µl aliquots of each fraction for radioactivity (Section 2 3 5) The fucopeptide fractions were then pooled, dialyzed against distilled water and lyophilized

2 3 6 2

P-ISOTHIOCYANATE-PHENETHYLAMINE DERIVATIVE FORMATION

Phenethylamine-^{3}H-fucopeptides were dissolved in 2 mls of 0 1 M $NaHCO_3$, pH 8 0 and layered over 2 5 mls of chloroform containing 5 µl of thiophosgene (65 µmol) in a tightly sealed vessel an shaken vigorously for 1 hour The aqueous layer was then extracted twice with 2 ml

volumes of chloroform to remove excess thiophosgene Nitrogen was bubbled through the final aqueous layer to remove any residual chloroform

2 3 6 3

CONJUGATION OF THE ^{3}H-FUCOPEPTIDES ONTO BSA

The aqueous layer from the previous step was added to 2 mls of 0 3 M NaCl in 0 1 M $NaHCO_3$, pH 9 5 containing 13 mg of BSA This was allowed to react for 18 hours at room temperature without stirring The unconjugated ^{3}H-fucopeptides were removed chromatographically on a Sephadex G-50 column (50x1 6 cm) equilibrated with PBS The eluate was monitored at 280nm to detect the BSA-^{3}H-fucopeptides A constant flow rate of 0 12 ml/min was used Fractions of 2 0 ml were collected and 100 ul removed from each to count for radioactivity The BSA-^{3}H-fucopeptides were pooled, dialyzed against distilled water and lyophilized before storage at -20°C

2 3 7

PRODUCTION OF REAGENT POLYCLONAL ANTIBODIES AGAINST BSA-^{3}H-FUCOPEPTIDES

Polyclonal antibodies were raised against ^{3}H-fucopeptide-BSA conjugates (as prepared above) by the method of Tung (1983), in which antibodies are induced in the ascitic fluids of mice

A stock solution of antigen (25 mg/ml in PBS) was diluted with 9 parts of complete Freunds' adjuvant Of this, 0 2 ml volumes were administered ip to Balb/c mice aged 8 to 10 weeks old (this age is critical) at the time of the first injection Further injections were given on days 14, 21, 28, and 35 On day 42, 0 5 ml of pristane was given ip to further enhance ascites production Ascites may begin to appear from day 2 onwards and mice should be checked regularly from this time

Ascites were collected by tapping the mice with a 19-gauge needle Pooled ascites fluids were filtered through glass wool and centrifuged at 400xg for 20 mins at 4°C followed by centrifugation at 15000xg for 20 mins at 4°C to remove cell debris and fibrin clots Supernatants were filtered as before and stored at -70°C until further use

2 4

IMMUNIZATION OF BALB/C MICE FOR THE PRODUCTION OF MONOCLONAL ANTIBODIES

For the production of monoclonal antibodies, Balb/c mice were immunized with LAT cells and A549 cells Lysed LAT cells or whole A549 cells were administered by ip injection to Balb/c mice aged from 8 to 12 weeks The A549 cells were freshly harvested from 75 cm culture flasks by mild trypsinization and washed in PBS LAT cells, freshly harvested from Schofield mice bearing ascitic tumours (Section 2 2 7), were washed in PBS and lysed, either by rapid freeze-thawing in liquid-nitrogen, or by resuspension in hypotonic buffer (5 mM Tris-HCl, pH 7 5) for 10 mins An aliquot of cells was stained with acridine orange ethidium bromide to ensure that no live cells remained This was to ensure that ascitic tumours did not develop in mice being immunized with LAT cells For both cell types, immunizations were performed using $1x10^7$ cells in 1 ml of PBS

The routine immunization regimen used involved ip injections of cells on days 0, 7, 28, and again, 3 days prior to the harvesting of splenocytes from the mice for use in somatic cell fusion experiments

2 5

ENZYME-LINKED IMMUNOSORBANT ASSAY

A protocol for the setting up and performance of a solid-phase enzyme-linked immunosorbant assay (ELISA) is described for use with fixed cells as target antigens The

procedure is described in two parts

1 cell fixation, and

2 ELISA

2 5 1

CELL FIXATION

Cell fixation was performed using the methods described by Stocker and Heusser (1979) and Cobbold and Waldmann (1981) with some modifications The method described here was designed for Landschutz ascites tumour (LAT) cells and may require some minor modification for use with other cell types (see Section 5 2 3)

Flat bottomed, flexible polyvinylchloride (PVC) plates (Cooke Dynatech) were treated with 0 1% (w/v, 100 µl/well) poly-L-lysine (ave mw 100 kd, Sigma) for 1 hour at 37°C This reagent may be re-used up to 10 times (Cobbold and Waldmann, 1981) Following the removal of the poly-L-lysine, the plates were washed in PBS and 50 µl of a $5x10^5$ cell/ml suspension of washed LAT cells was added to each well After 1 hour at 4°C, 50 µl of 0 05% glutaraldehydye (GA, precooled to 4°C) was added to each well to give a final concentration of 0 025% GA per well, taking care not to disturb the cell layer After 10 mins, the GA was removed and the plates washed 3 times in PBS Any residual GA was blocked by incubation of the plates in PBS containing 100mM glycine for 30 mins at 37°C (Epstein and Lunney, 1985) Following a final wash, 100 µl of storage/blocking buffer (200 µg gelatin per ml in PBS containing 0 1% sodium azide as a preservative) was added to each well These plates could be stored for up to 6 months at 4°C with no apparent loss of activity

2 5 2

ELISA

Antigen coated plates were washed in 0 1% (v/v) Tween-20 in PBS (wash solution) Non-specific binding sites were blocked with 1% BSA (w/v) in PBS (100 µl/well) for 1 hour at 37°C The plates were then washed and 50 µl of an

appropriate dilution of test serum or hybridoma supernatant was added The plates were then incubated for 1 hour at 37°C Free antibody was removed by washing the plates 3 times as before adding 50 µl of β-galactosidase-linked, sheep anti-mouse Ig, $F(ab')_2$ fragment (Amersham) and incubating at 37°C for 1 hour After a thorough washing to remove unbound conjugate, 100 µl of substrate (ONPG, O-nitrophenyl-β-galacto-pyranoside) was added to each well When sufficient colour had developed, the reaction was stopped by the addition of 50 µl of 1M sodium carbonate, which also enhances the colour in positive wells The absorbance was recorded at 405 nm on a Bio-tek EIA plate reader, model EL-307

2 6

SOMATIC CELL FUSIONS AND RELATED PROCEDURES

2 6 1

AZOGUANINE TEST FOR HAT SENSITIVITY OF MYELOMA CELLS

The method of Earley and Osterling (1985) was used Sp2/0 cells were placed in complete RPMI-1640 (10% FCS) supplemented with $1x10^{-4}$ M 8-azoguanine (2 0 µg/ml) for 3 days Only HAT sensitive cells will grow in this medium Should the cells fail to grow, they should be replaced immediately with fresh cells known to be HAT sensitive The Sp2/0 cells were subsequently grown in 8-azoguanine-free medium for 1 week prior to use in cell fusions

2 6 2

ISOLATION OF IMMUNE SPLEEN CELLS AND THE DEPLETION OF RED BLOOD CELLS

The spleen of an immunized mouse was aseptically removed and pierced several times with a 21-gauge needle Using a 26-gauge needle fitted to a 10 ml syringe, the spleen cells were flushed from the spleen into a sterile petri-dish using 10 mls of RPMI-1640 (free of serum) The resulting cell suspension was washed 3 times in RPMI-1640

Contaminating red blood cells (RBC) were removed by lysis in Gey's haemolytic solution as described by Mishell and Shiigi (1981) Briefly, spleen cells were resuspended into 1 ml of RPMI-1640 containing 5% FCS To this, 5 mls of Gey's haemolytic solution were added and the mixture held on ice for 5 mins The suspension was then carefully underlayered with 5 mls of FCS and centrifuged at 300xg for 10 mins The lymphocytes, which form a pellet, were washed 3 times in RPMI-1640 before use Viability was greater than 90% and each spleen yielded up to $1x10^8$ splenocytes

2 6 3

PREPARATION OF 50% (W/V) POLYETHYLENE GLYCOL (PEG)

Solutions of 50% PEG were prepared in each of the following ways

A Five grams of PEG-1540 (Riedel De Haen) were dissolved fully in 3 0 mls of serum-free RPMI-1640 and adjusted to a final volume of 10 mls with RPMI-1640 The solution was sterilized by filtration through a 0 22 μm filter (Gelman) and stored in 1 0 ml aliquots at -20°C

B Five grams of PEG-1540 were autoclaved to dissolve and sterilize the PEG Before the PEG resolidified (approx 40°C), it was diluted, aseptically, using an equal volume of serum-free RPMI-1640 and aliquotted into 1 0 ml lots for storage at -20°C

2 6 4

PREPARATION OF MOUSE MACROPHAGE FEEDER LAYERS

In a typical experiment, 4 Schofield mice (8 to 12 weeks old) were sacrificed by cervical dislocation and immersed in 70% ethanol The peritoneal wall was aseptically exposed and 10 mls of RPMI-1640 was injected using a 21-gauge needle The abdomen was then gently massaged, to bring the peritoneal cells into suspension, and the medium withdrawn The cells were pooled, washed and counted "Bloody" samples were discarded Each mouse yielded up to $1x10^7$ cells which were plated into 96-well culture

trays at a concentration of $1x10^4$ cells per well in complete RPMI-1640 Feeder layers were usually set up on the day prior to cell fusion experiments

2 6 5

CELL FUSION PROCEDURE

Sp2/0 cells, maintained in mid-log phase of growth for 7 days prior to fusion,and pre-tested for both HAT sensitivity (Section 2 6 1) and the presence of mycoplasma (Section 2 2 8), were pooled, counted (Section 2 2 4) and washed 3 times in serum-free medium Immunized spleen cells which had been depleted of RBC, were also counted and washed as above Spleen and Sp2/0 cells (in a ratio of 10 1) were mixed thoroughly and pelleted at 400xg for 10 mins The supernatant was fully decanted The cells were fused in 50% (w/v) PEG-1540 (Section 2 6 3) at 37°C One ml of the PEG solution was added to the pelleted cells over 1 minute with gentle mixing This was left for a further minute while the container was rotated with a continual, gentle swirling motion The PEG was then slowly diluted by the dropwise addition of 3 mls of RPMI-1640 (serum-free) over 3 mins , followed by 8 mls of RPMI-1640 over a further 3 mins The cells were pelleted at 300xg for 10 mins and resuspended at $2x10^5$ cells/ml in complete RPMI-1640 (20% v/v serum) containing 2X HAT (Section 2 6 6) The cells were plated at a concentration of $2x10^4$ cells/well of 96-well culture trays precoated with feeder cells (Section 2 6 4) and then incubated at 37°C in 5% CO_2 Clones become apparent after 7 to 10 days The HAT medium was replaced by HT medium (Section 2 6 6) after 7 days After the 14th day, the cells were cultured in RPMI-1640 (10% v/v FCS)

2 6 6

HAT SELECTIVE MEDIUM

HAT selective medium, as originally described by Littlefield (1964), consisted of complete culture medium (in this case, RPMI-1640) supplemented with 100μM hypoxanthine (H), 1 M aminopterin (A) and 16 μM thymidine

(T) The HT medium was identical to the HAT medium with the omission of aminopterin This was used to wean cells off HAT medium

2 6 7

CLONING OF HYBRIDOMAS BY LIMITING DILUTION

Cells producing antibodies of specific interest were expanded from 96-well trays into 1 ml cultures in 24-well cluster trays When confluent, the cells were flushed from their wells and counted An aliquot was frozen for storage in liquid-N2 (Section 2 2 5) The remaining cells were diluted in RPMI-1640 (10% FCS) and plated over four 96-well trays at concentrations of 10, 5 and 1 cell/well (Rener et al , 1985) Each well had been precoated with $2x10^4$ macrophage feeder cells (Section 2 6 4)

2 7

ISOTYPE ANALYSIS OF MONOCLONAL ANTIBODIES

This was performed by both ELISA and HPLC

2 7 1

ISOTYPE ANALYSIS BY ELISA

An antibody isotype ELISA analysis kit (Zymed) was used Ascitic fluid was diluted to a concentration of 100 µg of ascitic protein per ml in a coating buffer consisting of 18mM $NaHCO_3$ and 14mM Na_2CO_3, pH 9 5, and applied to a microtitre tray (100 µl/well) Antibody was allowed to bind overnight at room temperature (RT) Free binding sites were blocked by incubation with 1% BSA for 1 hour at 37°C The wells were then washed in 0 1% Tween-20 in PBS and rabbit anti-mouse subclass/chain specific antibody applied for 1 hour at 37°C Free antibody was removed and goat anti-rabbit Ig conjugated to horseradish peroxidase applied for 1 hour at 37°C Following washing to remove free conjugate, the enzyme substrate (10 mg orthophenylene-diamine and 5 1 H_2O_2 in 25 mls of 0 5M Na_2HPO_4 in 0 1M citric acid, pH 5 0) was added

(100 µl/well) and colour allowed to develop at 37°C for 15 to 20 mins The reaction was stopped by the addition of 50 µl of 20% (v/v) H_2SO_4 The O D was read at 490nm

2 7 2

HPLC DETERMINATION OF ANTIBODY CLASS

This method only allowed antibody class to be determined No information was gained on subclass or light chain type

A 10 µl volume of ascitic fluid in PBS, at a concentration of 1 µg/ml, was applied to a Waters Protein PAK 300SW column preequilibrated with elution buffer (0 1M phosphate, pH 7 1) A flow rate of 0 5 ml/min was used Samples of 1 0 ml volume were monitored for protein by continuous UV at 254nm A calibration curve was set up using horseradish peroxidase (45 kd), BSA (67 kd), lactate dehydrogenase (140 kd) and catalase (323 kd) as molecular weight markers

2 8

ELECTROPHORETIC ANALYSIS OF LAT CELL MEMBRANES

2 8 1

PREPARATION OF MEMBRANES

All of the following steps were carried out at 4°C or on
ice LAT cells, taken from Schofield mice (Section 2 2 7), were washed in PBS, pelleted and resuspended in hypotonic buffer (5mM Tris-HCl, pH 7 5) at $1x10^7$ cells/ml for 5 mins They were then pelleted at 400xg for 10 mins and resuspended to 25% w/v in homogenisation buffer (5 mM Tris-HCl, pH 7 5 containing 0 25M sucrose and 1mM PMSF (phenylmethylsulphonyl fluoride, protease inhibitor)) The cells were disrupted by 10 strokes of a Potter homogeniser, motor driven at 500 rpm The homogenate was centrifuged at 10,000xg for 15 mins (Sorvall RC-5B, GSA rotor) to remove cells and nuclei The supernatant was recentrifuged at 100,000xg for 90 mins

(Beckman L8-M ultracentrifuge, 70Ti rotor) The final membrane pellet was washed once in distilled water at 100,000xg for 90 mins The final pellet was resuspended into 10 mls of distilled water and 50 µl taken for protein concentration determination by the method of Lowry et al , (1951) The remainder of the sample was aliquotted into volumes containing 2 5 mg of protein and the samples were then lyophilized The dried samples were stored at -20°C until use

2 8 2

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

LAT cell membrane samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a modified Laemmli (1970) procedure Proteins were separated on a resolving gel containing 10% w/v acrylamide, 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, 0 08% w/v bis-acrylamide, 0 25% w/v Tris, 0 2% w/v SDS, 0 08% v/v TEMED and 0 08% w/v ammonium persulphate pH 6 8, was used The electrode buffer contained 0 025M Tris, 0 192M glycine, and 0 1% w/v SDS in distilled water, pH 8 3 Samples for electrophoresis were resuspended in a buffer containing 0 062M Tris, 2 0% w/v SDS, 10% v/v glycerol, 0 5% v/v 2-mercaptoethanol and 0 001% w/v bromophenol blue The samples, at a concentration of 2 5 mg/ml in this buffer, were boiled for 5 mins at 100°C before being applied to the gels (use 30 to 50 µl volumes) Electrophoresis was carried out at 100mA constant current for about 3 hours at 4°C 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 1 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 1 hour Destaining was carried out overnight in a solution of ethanol, acetic acid and distilled water (1 3 6)

2 8 3

WESTERN IMMUNOBLOTTING

The method of Towbin et al , (1979) was essentially followed Following SDS-PAGE, gels were placed onto sheets of nitrocellulose (NC) (0 45 µm pore size, Schleicher and Schuell) cut to the appropriate size and presoaked in transfer buffer (0 025M Tris, 0 192M glycine and 0 1% w/v SDS in 10% v/v methanol The gel-NC sandwich was then placed between two Scotch Brite pads soaked in transfer buffer and clamped between two tightly fitting plastic grids This complete unit was then placed into a Transblot transfer cell (Biorad) with the NC to the positive electrode Blotting was carried out overnight at 45 mA constant current at 10°C

2 8 4

VISUALISATION OF IMMUNOBLOTS

Two methods were used For the total staining of blots, amido black was used, while immunological staining was used for the specific detection of individual bands

2 8 4 1

AMIDO BLACK STAINING

Blots were reversibly stained with 0 1% (v/v) amido black in distilled water for 2 to 3 mins Complete destaining was achieved by frequent washing of the blot in distilled water

2 8 4 2

IMMUNOLOGICAL STAINING OF BLOTS

The blots were soaked in blocking solution (BS, 10mM Tris, 150mM NaCl and 0 5% v/v Tween-20 in aqueous solution, pH 7 5), or 1% w/v BSA in 10mM Tris and 150mM NaCl, to block all of the available protein binding sites on the NC After 1 hour at 37°C, ascitic monoclonal antibody, at an appropriate dilution in BS, was applied to the blot and incubated at 37°C for 1 hour The blot was washed

overnight in several changes of BS to remove all unbound antibody The second antibody, alkaline phosphatase-conjugated goat anti-mouse Ig (Sigma) was applied at a dilution of 1 1000 in BS for 1 hour at 37°C Again, the blot was washed overnight The pH of the blot was then brought to 8 9, by incubation in 10mM Tris-HCl, pH 8 9 for 15 mins The development solution was then added This consisted of

1 1 ml of 0 1% nitroblue-tetrazolium in 10mM Tris-HCl, pH 8 9,
2 0 4 ml of a 5 mg/ml solution of 5-bromo-4-chloro-3-indoylphosphate (toluidine salt) in dimethyl-formamide,
3 40 µl of aqueous $MgCl_2$, and
4 8 86 ml of 0 5M Tris-HCl, pH 8 9,

per 10mls of solution

Colour development was complete in 2 to 3 mins The reaction was stopped by washing the blot in a large excess of distilled water Stained blots were dried for storage by placing them between two layers of filter paper sandwiched between two heavy glass plates and incubating at 37°C overnight

SECTION 3

HIGH MOLECULAR WEIGHT FUCOPEPTIDES ON LAT CELLS THEIR ISOLATION AND CONJUGATION TO BSA FOR THE PRODUCTION OF REAGENT POLYCLONAL ANTIBODIES

HIGH MOLECULAR WEIGHT FUCOPEPTIDES ON LAT CELLS THEIR ISOLATION AND CONJUGATION TO BSA FOR THE PRODUCTION ON REAGENT POLYCLONAL ANTIBODIES

3 1 INTRODUCTION

The presence of high molecular weight fucopeptides on tumour cells shows a very strong correlation with the tumourigenic state (Section 1) They appear very soon after the transformation of a normal cell into a tumourigenic cell and have been observed on a wide variety of tumour cell types including LAT cells (O'Kennedy, 1979) The shift towards extra content of high molecular weight fucopeptides represents one of the more consistently observed cellular alterations following neoplastic transformation and have been detected in both rodent (Warren et al , 1978, Santer and Glick, 1979, Collard et al , 1985) and human tumour cells (Van Beek et al , 1975) Their appearence coincides with the transformation of normal cells by chemical mutagens (Warren et al , 1978), oncogenic viruses (Santer and Glick, 1979) and transfection with DNA from neoplastic cells (Collard et al , 1985) Because of their strong association with the tumourigenic state, it is reasonable to expect that this group of molecules might contain some antigenic species showing a greater than usual relationship with the tumourigenic state as compared to the normal cell state With this in mind, ^{3}H-fucopeptides were isolated from LAT cells, conjugated to bovine serum albumin (BSA) and used for the production of reagent polyclonal antibodies by the method of Tung (1983) (see Methods, Section 2 3 7) These antibodies were subsequently used in Western immunoblotting studies of LAT cells in order to determine which glycoproteins contained the high molecular weight fucopeptides The banding profiles obtained from these studies were then compared with banding patterns obtained with monoclonal anti-LAT

cell antibodies produced here (Section 7) If the bands stained by the monoclonal antibodies corresponded to those stained by the anti-^{3}H-fucopeptide antibodies, it would provide valuable information concerning the nature of the antigens involved, as well as providing some clues as to their structure

3 2

PREPARATION AND ISOLATION OF ^{3}H-FUCOPEPTIDES FROM LAT CELLS

Tritiated fucopeptides from metabolically labelled LAT cells were prepared as described in methods (Section 2 3) The final preparation of ^{3}H-fucopeptides was applied to a mixed column of Biogel P-10 Sephadex G-50 (2 1) to separate the ^{3}H-fucopeptides into high and low molecular weight species Cell surface fucopeptides, prepared as described in methods, and separated according to size on a mixed column of Biogel P-10 Sephadex G50, typically separate into two distinct operationally defined fractions, the high molecular weight (earlier eluting) components and the low molecular weight components The high molecular weight fucopeptides were defined as those fucopeptides eluting on the ascending arm of the first major peak to come off the column (see Figure 3 1) Both fractions are heterogenous mixtures of fucopeptides of similar molecular weight ranges Further purification or refinement of these fractions may be achieved by ion-exchange chromatography This, however, was not done here A typical elution profile for the separation of the fucopeptides according to size, and performed on a mixed column of Biogel P-10 Sephadex G-50 (Section 2 3), is given in figure 3 1 The ascending arm of the first major elution peak (fractions 12 to 24) were taken to represent the high molecular weight ^{3}H-fucopeptides (Walsh, 1984, PhD thesis) These fractions were pooled, dialyzed against distilled water and lyophilized A total of 10 mg (dry weight) of ^{3}H-fucopeptides were isolated for conjugation to bovine serum albumin (BSA)

3 3

CONJUGATION OF ^{3}H-FUCOPEPTIDES TO BSA

The methods of Zopf et al , (1978) were used for the conjugation of the isolated ^{3}H-fucopeptides to BSA (see Section 2 3, for full details) Three distinct steps were involved

1 the formation of phenethylamine derivatives of the ^{3}H-fucopeptides,
2 preparation of p-isothiocyanate-phenethylamine derivatives of the ^{3}H-fucopeptides, and
3 the conjugation of the above derivatives to BSA

The overall reaction sequence is summarized in figure 3 2

3 3 1

PHENETHYLAMINE DERIVATIZATION OF ^{3}H-FUCOPEPTIDES

The procedure outlined in methods (Section 2 3 6 1) was appropriately scaled up to cater for the 10 mg of ^{3}H-fucopeptides prepared (the method described is for 2 mg of starting material)

Reduction of the reaction mixture in sodium borohydride was carried out for 20 hours due to the presence of some precipitation (as recommended by Zopf et al , 1978) Following the complete reduction of the sample, it was applied to a column of Sephadex G-10 (50 x 1 6 cm) to separate the derivatized product (phenethylamine-^{3}H-fucopeptides) from the free amine The elution profile from this step is shown in figure 3 3 The product (fractions 10 to 19) was pooled, dialyzed against distilled water and lyophilized

3 3 2

P-ISOTHIOCYANATE DERIVATIZATION OF PHENETHYLAMINE-^{3}H-FUCOPEPTIDES AND THEIR CONJUGATION TO BSA

The phenethylamine derivitives of the ^{3}H-fucopeptides were further derivatized to form p-isothiocyanate-phenethylamine derivatives of the ^{3}H-fucopeptides The

final derivitive was reacted overnight at room temperature (approx 18°C) with 52 mg of BSA The ^{3}H-fucopeptides-BSA conjugates were exhaustively dialysed against distilled water and lyophilized The dried material was resuspended into 5 mls of PBS and applied to a column of Sephadex G-50 (40 x 1 6 cm) to separate any unconjugated ^{3}H-fucopeptides from the conjugated material (Figure 3 4)

In the final step of the reaction sequence, 21% of the total amount of derivatized ^{3}H-fucopeptide material used was found to conjugate onto the BSA (Table 3 1) This represents 19 01% of the total ^{3}H-fucopeptide starting material used for derivatization and conjugation onto BSA Similar values were obtained in three series of conjugation reactions carried out here using the same starting materials in each case These values are substantially lower than the 80% values reported by Zopf et al , (1978) The reason for this is unclear Thiophosgene is a possible source of the problem In initial trial experiments, only 3% of the total starting material was bound to BSA It was then discovered that the thiophosgene used had deteriorated in quality, possibly due to water absorbtion In subsequent reactions, fresh thiophosgene was used (kindly donated by Ita Bolger, School of Chemical Sciences, NIHED) with a resulting increase in the efficiency of binding of ^{3}H-fucopeptides onto BSA The material being used here was also different from that used by Zopf et al , (1978), which accounts for some of the differences in observed conjugation efficiencies

3 4 PRODUCTION OF REAGENT POLYCLONAL ANTIBODIES AGAINST ^{3}H-FUCOPEPTIDE-BSA CONJUGATES

Reagent polyclonal antibodies were produced against ^{3}H-fucopeptide-BSA conjugates by the method of Tung (1983) By this procedure, antibodies are produced in the ascitic fluids of Balb/c mice, which offers the advantage of allowing relatively large amounts of antibody to be

produced from a small amount of immunogen

In this study, 8 mice were used for immunization with ^{3}H-fucopeptide-BSA Three of the 8 mice produced good quantities of ascitic fluid (from 1 5 to 3 0 mls of fluid on each of 3 tappings) Four of the mice developed solid tumours with little or no fluid being obtained The eighth mouse did not appear to produce any tumours, as evidenced by the lack of abdominal swelling or fluid production The ascitic fluids from the first 3 mice were pooled and centrifuged at 25000 x g to remove cells and debris The supernatant was then passed through a glass wool filter to remove the fat layer The final solution was aliquotted into 1 0 ml lots and stored at -20^{o}C

One aliquot was retained for the determination of the antibody titre against BSA and LAT cells in a solid-phase assay A titre of 1 1500 was obtained against BSA alone In the assay against LAT cells, the plates were blocked with casein (against which no reaction was observed) instead of the more usual BSA Because of this, any reaction observed against the LAT cells should have represented the reactivity of the polyclonal antibodies against the ^{3}H-fucopeptides alone as they occur in their native form *in vivo* This is also the form in which the antibodies could be expected to encounter the fucopeptides in later immunoblotting experiments (Section 10) Also, this method of antibody titre determination served to eliminate reactivity of antibodies against fucopeptides which may have suffered the destruction of important structural epitopes during the process of conjugation to BSA The titre against the LAT cells alone was found to be 1 250, which represents approx 16% of the total antibody in the ascitic fluid These antibodies were not tested on any other cell types

More recently, a new method for the production of reagent polyclonal antibodies was described by Lacy and Voss (1986) in which ascitic tumours are induced by the inoculation of previously immunized mice with Sp2/0 cells This procedure offers the advantage of producing

tumours more rapidly than the method of Tung (1983) used here (10 to 14 days as opposed to 28 to 40 days) Comparable titres of antibody are achieved by both methods

TABLE 3 1

CONJUGATION OF ^{3}H-FUCOPEPTIDES TO BSA % RECOVERIES AT EACH STAGE OF THE REACTION

Reaction stage	Total counts*	% of total starting counts
Starting material	141790	100 00
Phenethylamine derivatives of ^{3}H-fucopeptides	125910	88 80
^{3}H-fucopeptide-BSA conjugates	26952	19 01

* counts per minute

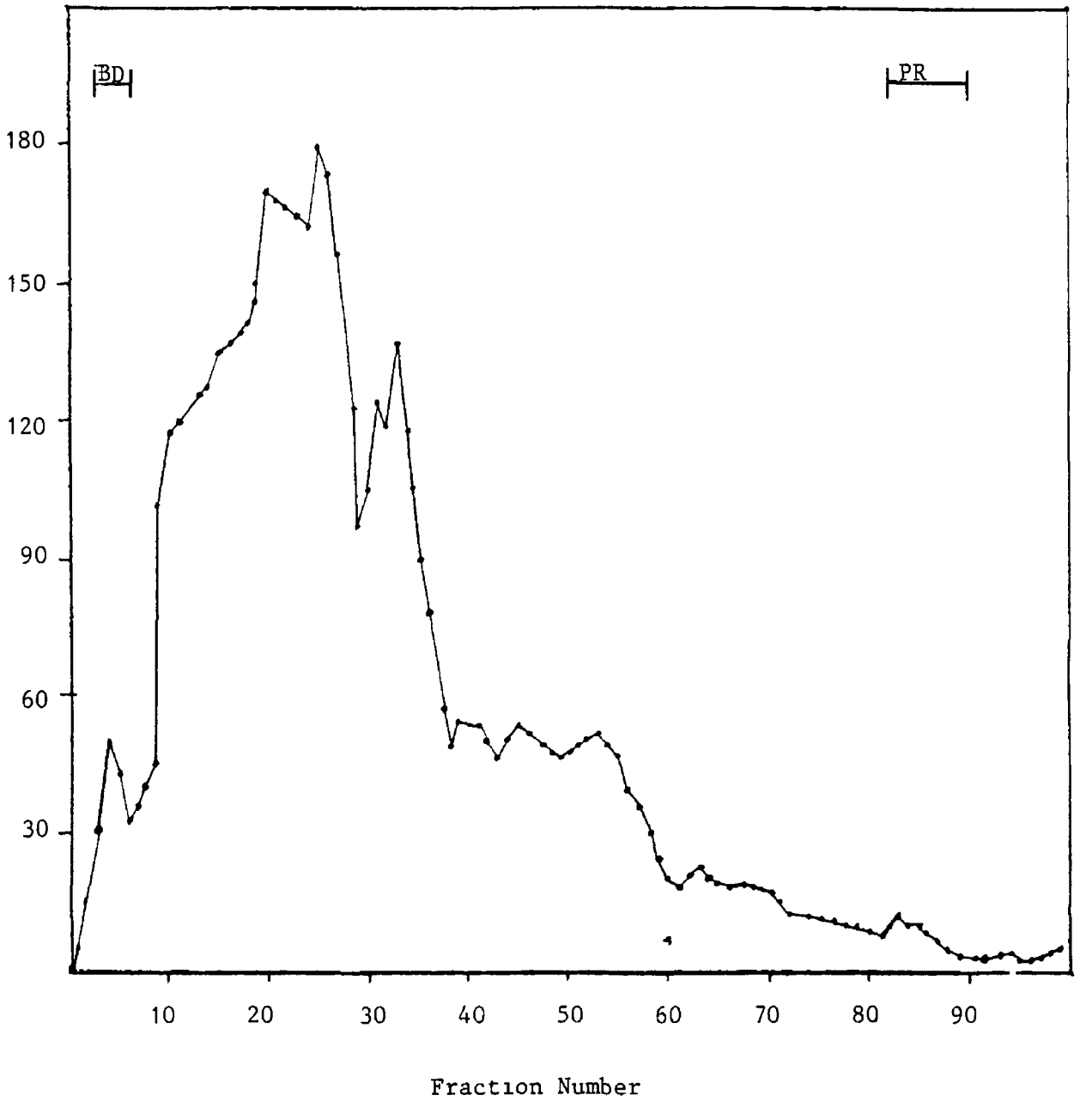

Figure 3 1

Separation by column chromatography of 3-H fucopeptides derived from LAT cell surface membranes on a mixed column of Biogel P-10 Sephadex G-50 (2 1)

Column size 94 x 1 5 cm (internal diameter)

Flow rate 0 2 mls/min

1 ml fractions were collected

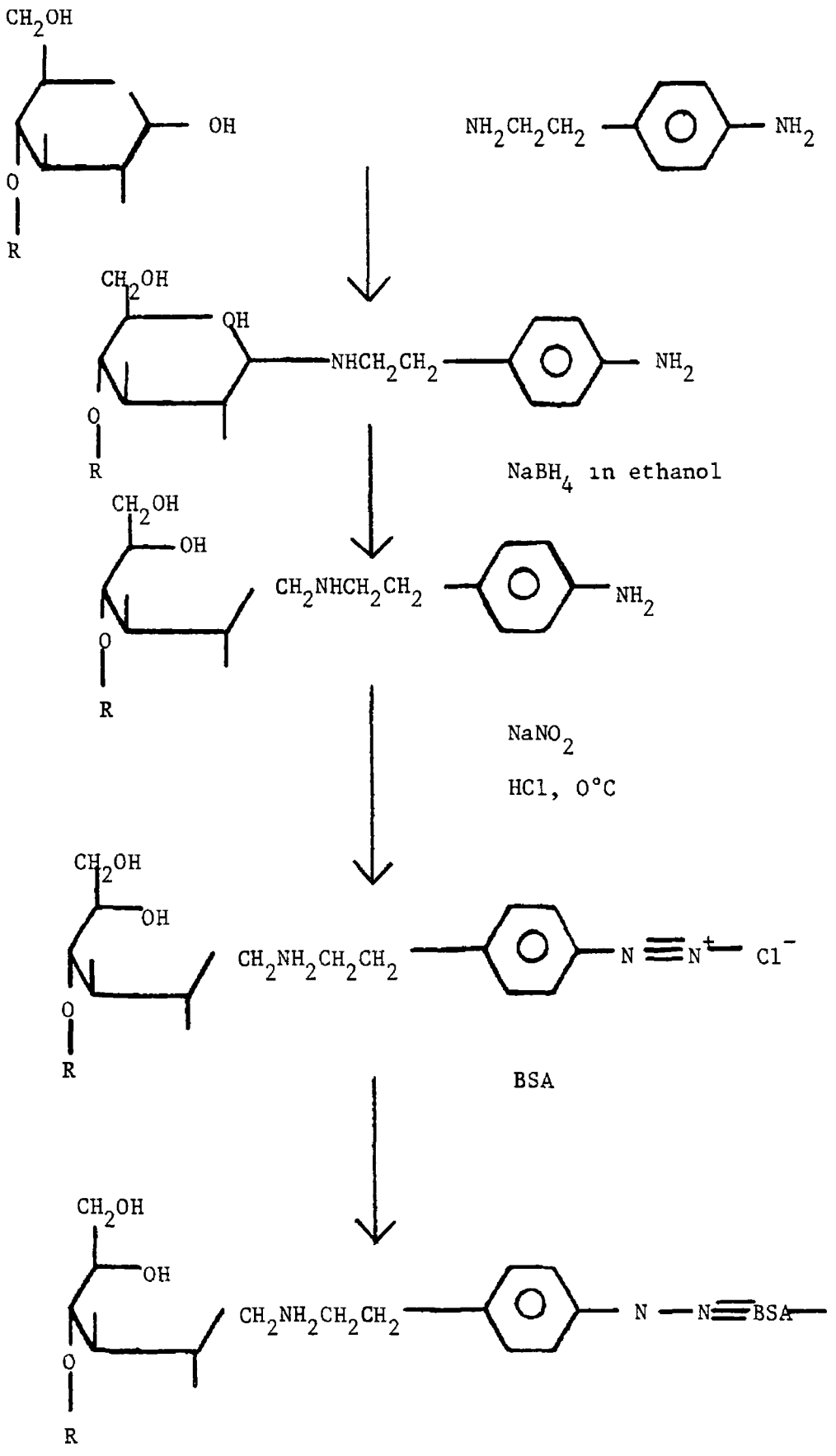

Figure 3.2

The reaction sequence involved in the conjugation of sialyloligosaccharides onto BSA (Taken from Smith et al , 1978)

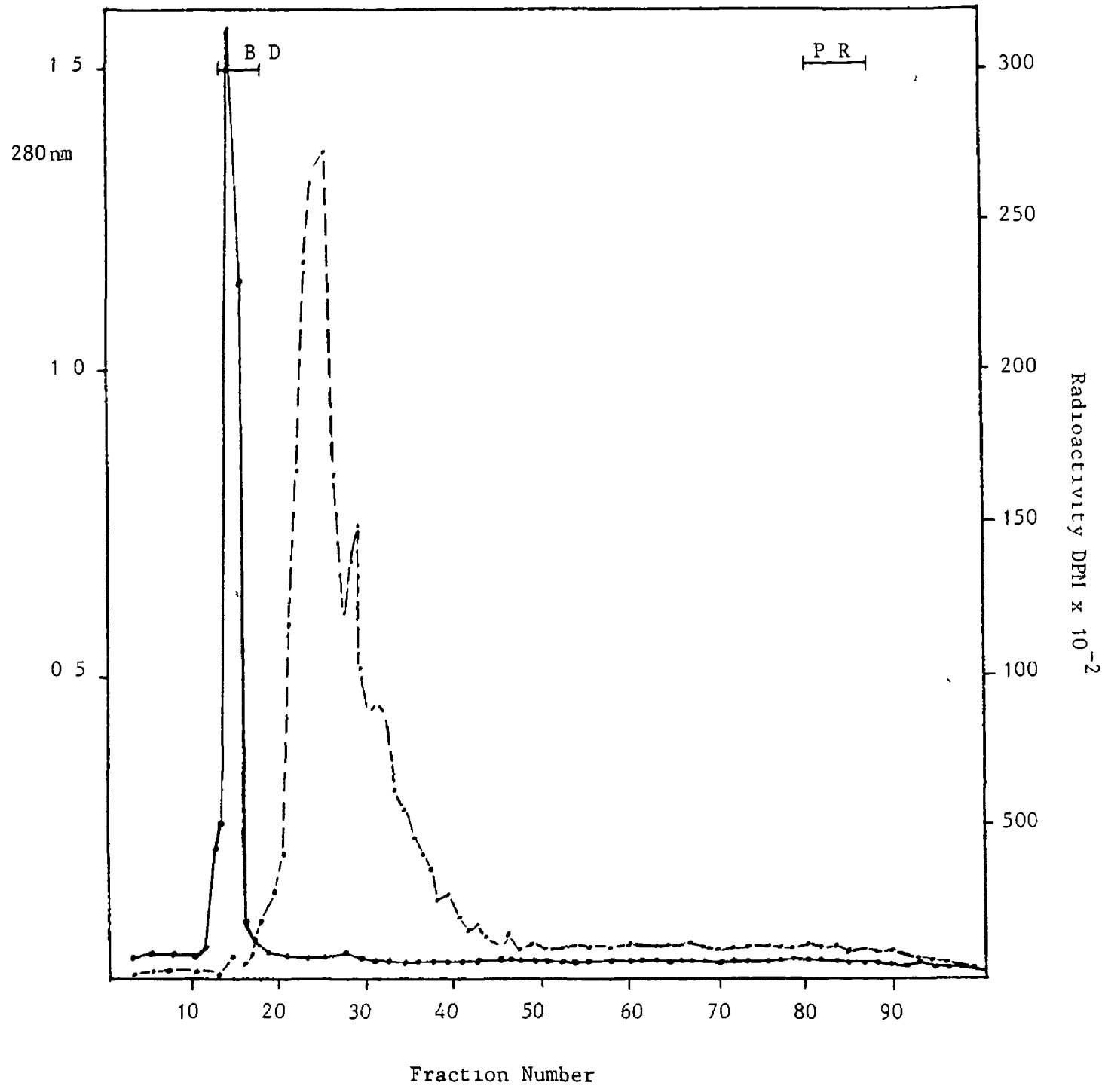

Figure 3 3

DPM

OD

Separation of free β-(p-aminophenyl)ethylamine from phenethylamine-derivitized 3H-fucopeptides by column chromatography using Sephadex G-10

Column size 50 x 1 6 cm (internal diameter)

Flow rate 0 3 mls/min

Fractions of 3 0 mls were collected

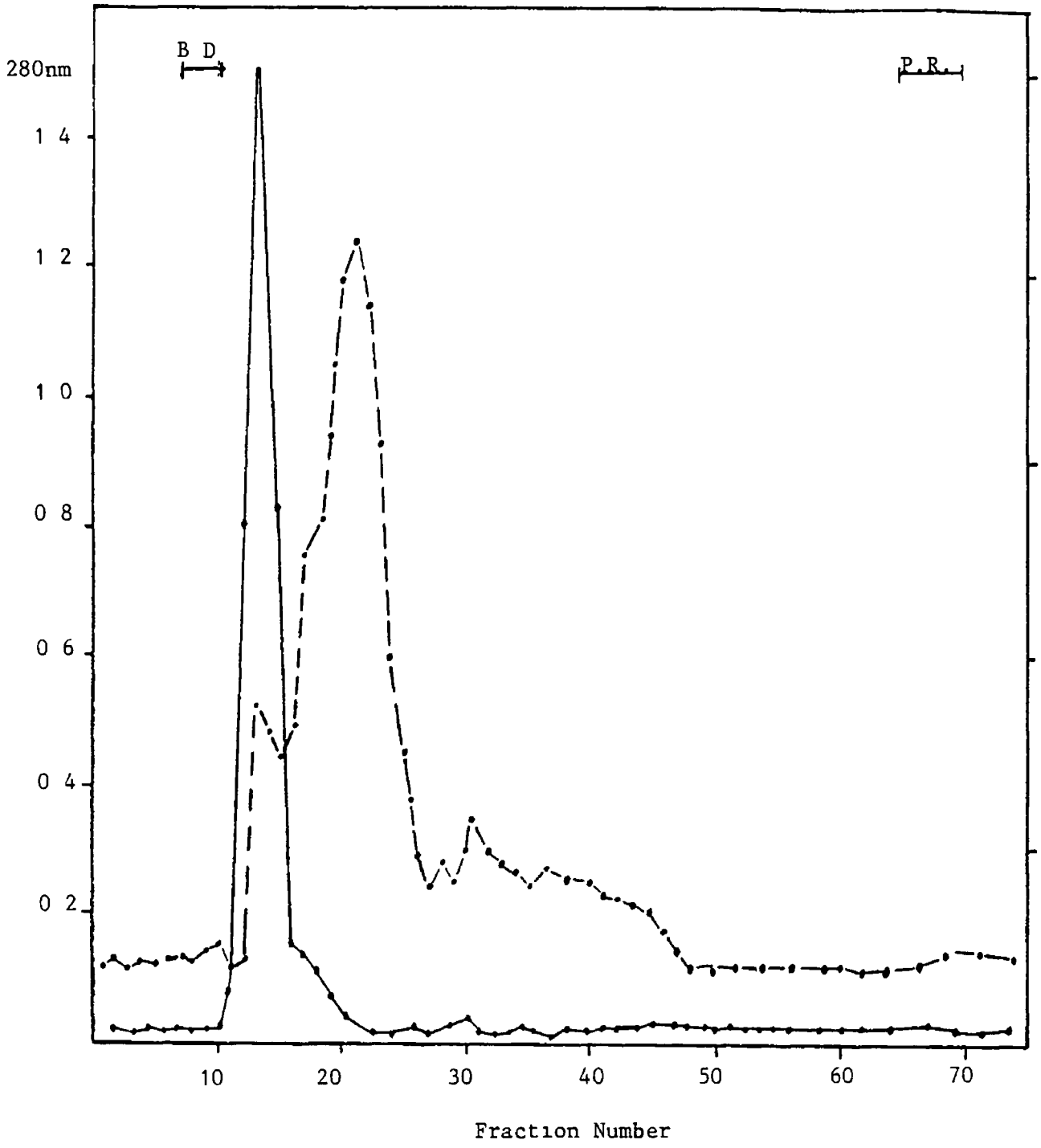

Figure 3 4

OD

DPM

Separation of unconjugated 3H-fucopeptides from BSA-3H-fucopeptides by column chromatography using Sephadex G-50

Column size 50 x 1 6 cm (internal diameter)

Flow rate . 0 12 mls/min

Fractions of 2 0 mls were collected

SECTION 4

DETERMINATION OF THE OPTIMUM IMMUNIZATION SCHEDULE FOR THE PRODUCTION OF MONOCLONAL ANTIBODIES TO LAT CELLS

DETERMINATION OF THE OPTIMUM IMMUNIZATION SCHEDULE FOR THE PRODUCTION OF MONOCLONAL ANTIBODIES TO LAT CELLS

4 1 INTRODUCTION

In the production of a monoclonal antibody, some consideration must be given to the selection of a suitable immunization regimen in order to ensure that an optimal immune response is generated against the antigen being used This will help to increase the probability of a successful fusion and the generation of an antibody of both suitable class and affinity

The choice of an immunization protocol will depend on the nature of the antigen being used For soluble antigens it is normal to use adjuvants to boost the immune response With cell surface antigens, however, it is not usually necessary to use adjuvants since such antigens tend to be highly immunogenic when presented on intact cells (Goding, 1980) Quantitative differences are known to exist with respect to the strength of the immune response to a given antigen As yet the mechanisms controlling the intensity of the response to a given molecule, or even to certain determinants within a given molecule, are not fully understood Amongst the factors which may contribute to a strong response are the following

1. the phylogenetic distance between the antigen and the recipient,
2. molecular size larger molecules tend to be more highly immunogenic than small ones,
3. repeating determinants favour a strong response, and
4. particulate antigens tend to be more effective than soluble ones

Because different animals vary in their responses to a given antigen, both the schedule of injections and the choice of animals become important factors in obtaining a suitable population of lymphoid cells for use in somatic

cell fusions to generate monoclonal antibodies It is therefore to be recommended that more than one regimen of immunization and several animals be used initially, in order to select those giving the highest serum titres of specific antibody for use in cell fusions (Galfre and Milstein, 1981) Once a suitable immunization protocol has been found it can be used routinely to raise antibodies against that antigen

4 2

CHOICE OF ANIMALS

In mouse x mouse cell fusions the Sp2/0 myeloma cell line is used routinely (Shulman et al , 1978) Because this cell line was derived from the Balb/c mouse, this strain of mouse is the animal of choice for immunizations leading to the production of mouse monoclonal antibodies This has the further advantage of allowing hybridomas to be grown as ascitic tumours in syngeneic mice where they grow particularly well Otherwise, there is no reason why other strains of mouse cannot be used (Bastin et al , 1982) However, hybridomas from the fusion of Sp2/0 cells with cells from non-Balb/c mice require the use of F1-generation mice (Balb/c x immunized mouse strain) for the production of ascites (Simmons et al , 1971)

4 3

PURITY OF ANTIGEN

The purity of the antigen used is not of the utmost importance, since hybridoma technology, coupled with careful screening and cloning of cells, makes possible the isolation of cells secreting antibodies against even minor antigenic species in complex mixtures Nonetneless, pure antigen is always desirable and becomes of greater importance when impure material allows only a poor immune response to some minor antigen of interest It is also of importance during the later stages of screening to allow for the elimination of antibodies with undesirable cross-reactivity on other antigens

4 4

EXPERIMENTAL

Both LAT and A549 cells were used in studies on immunization procedures However, most emphasis was placed on LAT cells as immunogens The immune responses against LAT cells were consistently poorer than those observed for A549 cells Cells were prepared for immunizations as described in section 2 4

All immunizations were administered ip to Balb/c mice aged 8 to 12 weeks at the time of their first injection The following regimens were used

A 4 once weekly injections,

B 9 once weekly injections - hyperimmunization, and

C 3 once weekly injections followed by a rest period of 3 weeks before the final boost

The efficiency of each method was determined by a comparison of the serum antibody titres of each group of mice against the injected antigen in a solid-phase ELISA (Sections 2 5 and 5)

4 5

RESULTS AND DISCUSSION

4 5 1

USE OF LAT CELLS IN DIFFERENT IMMUNIZATION REGIMENS

The administration of LAT cells to Balb/c mice in 4 once weekly injections elicited consistently poor immune responses Only with prolonged exposure to antigen were any appreciable serum antibody titres obtained (Table 4 1, Figure 4 1) An exception to this was when a resting period was introduced before the final boost was given

The highest serum antibody titres obtained in hyperimmunized mice were seen after 7 to 8 injections of cells It is unclear, however, whether or not this was due to an accumulation of antibody in the serum with time, or the result of the recent activation of new B-cells

This is of some importance, since available evidence suggests that only recently activated B-cells make good fusion partners in the production of monoclonal antibodies (Goding, 1980, Bastin et al , 1982, Spitz et al , 1984) Thus, high serum antibody titres do not necessarily indicate the prime time for harvesting of immune spleen cells for fusions, but should only be taken as an indication of the suitability of the immunization procedure for raising antibodies against an antigen

4 5 2

WERE ANTIGENS BEING MASKED BY SIALIC ACID?

It has been reported that sialic acid might mask some cell surface antigens either by direct chemical linkage or through simple charge effects (Jeanloz and Codington, 1976) In further support of this, the removal of sialic residues by Vibrio cholerae neuraminidase (VCN, E C 3 2 1 18) had been reported to reduce the transplantability of tumours (Currie, 1967, Sanford, 1967, Smyth et al , 1977, O'Kennedy et al , 1980) This was interpreted in terms of altered immunogenicity making the cells more immunogenic and therefore more susceptible to immune attack (Currie and Bagshawe, 1968) This finding has been contradicted by others (Grothaus et al , 1971, Hauschka et al ,1971, Kassulke et al , 1971, Simmons et al , 1971) Here, the use of neuraminidase treated cells (25 IU VCN per $1x10^7$ LAT cells in PBS, pH 7 4, at 37°C for 1 hour) for immunization was found not to cause any increase in the observed immune response elicited by these cells as compared to untreated cells, suggesting that some other factors were involved in maintaining a poor response to these cells

4 5 3

POSSIBLE REASONS FOR THE POOR IMMUNOGENICITY OF LAT CELLS

Why LAT cells should elicit poor immune responses in mice is unclear It may be due to the disruption of the cells prior to immunization with the result that cytoplasmic elements are released which would compete with membrane

antigens in eliciting an immune response The release of "blocking factors" by the LAT cells, possibly in the form of secreted sialo-complexes (O'Kennedy et al , 1980), may also play a role in suppressing the immune response Also, because the cells had been grown in vivo, it is possible that they may have become coated with antibody which would mask surface antigens during subsequent immunizations (Shulman et al , 1974) Furthermore, because LAT cells are murine in origin, they would be expected to share several antigenic determinants in common with the recipient Balb/c mice This may be seen as a disadvantage when one is only interested in high serum antibody titres, in which case, a different animal species could be used eg rabbits In the production of anti-tumour antibodies, this close phylogenetic relationship does have the theoretical advantage that any tumour related antigens present may be more readily recognised by the host mouse and not be masked the more predominant normal histocompatability antigens Such antigens would normally attract the lions share of the immune response

The use of electrophoretically purified membrane proteins for the production anti-tumour cell surface antibodies is only likely to be of value when specific band differences are known to exist between the tumour cell and its normal cell counterpart Furthermore, the isolation of pure membrane proteins usually requires that the molecule be denatured and there is no guarantee that such a molecule will elicit an immune response which will react with the molecule in its native form (Bastin et al , 1982) Crude membrane preparations can be expected to offer little advantage over intact cells as immunogens, since, on a mass basis, such preparations are unlikely to be any less heterogenous than the intact cell (Parham, 1983), though it may eliminate responses against subcellular antigens As a general rule, antigens should, where possible, be presented in a state as close as possible to that in which the desired antibodies are likely to encounter them

In general therefore, while a pure preparation of antigen is highly desirable for immunization and screening purposes, little can be done to overcome the complexity of the cell membrane when seeking antibodies against previously unrecognised antigens.

4.5.4

A549 CELLS AS IMMUNOGENS

The use of A549 cells as immunogens produced better overall immune responses. Serum antibody titres of up to 1:300 were readily obtained for A549 as compared to titres of 1:50 for LAT cells under the same conditions (4 once weekly injections). A comparison of results for different immunization regimens using LAT and A549 cells is provided in table 4.2 and figure 4.2. Part of the disparity between LAT and A549 cells may be due to the murine origin of the LAT cells as opposed to the human origin of A549 cells.

4.6

ALTERNATIVE METHODS OF IMMUNIZATION

Many different protocols have been described for the successful immunization of Balb/c mice against tumour cell surface antigens using whole cells as immunogens. In general, $1x10^7$ to $2x10^7$ cells in PBS are given intraperitoneally (ip) as a primary boost. The subsequent regimen of injections however, varies greatly from one laboratory to the next.

In some cases, only 1 or 2 injections are given, with the antigen being administered either intravenously (Trucco et al., 1978) or intrasplenically (Spitz et al., 1984). Intrasplenic immunization was attempted here, and while the procedure was relatively simple, it was found to be very severe on the mice. For this reason, its use was discontinued. The use of a primary boost given ip, followed by the iv, or intrasplenic injection of antigen may produce a better overall response with higher numbers of specific antibody-producing B-cells being generated

(Westerwoudt et al , 1983) The use of a single injection of antigen would favour the production of IgM class antibodies which are predominant in primary and, to a lesser degree, in secondary immune responses (Eshar, 1985) Such antibodies may not be desirable as an end-product and consideration should therefore be given to the desired class and function of the antibody to be produced when deciding on an immunization protocol

Hyperimmunization of mice has also been suggested as a means of boosting the frequency of specific hybrids from cell fusions Stahli et al , (1980) proposed the use of very high doses of antigen on each of the last 4 days prior to cell fusion However, this could be counterproductive by causing suppression of the immune response (Goding, 1980, Stahli et al , 1983) With complex antigens like cell membranes it may allow one to obtain antibodies against minor populations of antigens Also, it is generally recommended that hyperimmunized mice be given a rest period of up to 4 weeks before the final boost is given (Goding, 1980, Mishell and Shiigi, 1980)

Regardless of the methods used, a number of important points have emerged from the literature Firstly, the use of an iv injection of antigen for the final boost prior to cell fusion is favoured (Simmons et al , 1971, Trucco et al , 1978, Stahli et al , 1980) Secondly, spleens taken 3 to 4 days after the final boost are best for fusions At this point, serum antibody levels are low, suggesting that immature antibody secreting cells are best for hybridoma formation (Goding, 1980, Mishell and Shiigi, 1980) Splenocytes taken 6 to 8 days after the final boost tend to produce poor or negative fusions (Goding, 1980, Spitz et al , 1984) Finally, since so many factors are involved in the immune response, exhaustive studies on the best protocol to use for immunization can only be justified in special cases (Galfre and Milstein, 1981) It is best to keep systems simple and to use more than one protocol in several animals Those animals with the highest serum antibody titres can then be selected for use in cell fusions

4 7

CONCLUDING COMMENTS

In conclusion, LAT cells were found to be inefficient in eliciting immune responses in Balb/c mice Best results were obtained when a resting period of 3 weeks was introduced before the final immunization was administered This also serves to ensure optimal B-cell stimulation prior to the use of immune spleen cells in cell fusions

TABLE 4 1

A SUMMARY OF RESULTS OBTAINED FOR DIFFERENT IMMUNIZATION PROTOCOLS USING LAT CELLS AS IMMUNOGEN

	Number of Immunizations								
	1	2	3	4	5	6	7	8	9
A	-	-	25*	50	-	-	-	-	-
B	-	-	25	50	50	100	100	150	100
C	-	-	25	50	rest period			150	-

Values represent serum antibody titres as determined by solid phase ELISA Results are averages for 3 to 4 mice

TABLE 4 2

A COMPARISON OF SERUM ANTIBODY TITRES AGAINST LAT AND A549 DURING DIFFERENT IMMUNIZATION PROTOCOLS

	Number of Immunizations							
	1	2	3	4	5	6	7	8
LAT	-	25	50	-	-	-	-	-
A549	-	150	250	-	-	-	-	-
LAT	-	25	50	rest period				150
A549	-	100	250	rest period				300

Results represent an average titre for 3 to 4 mice as determined by a solid phase ELISA

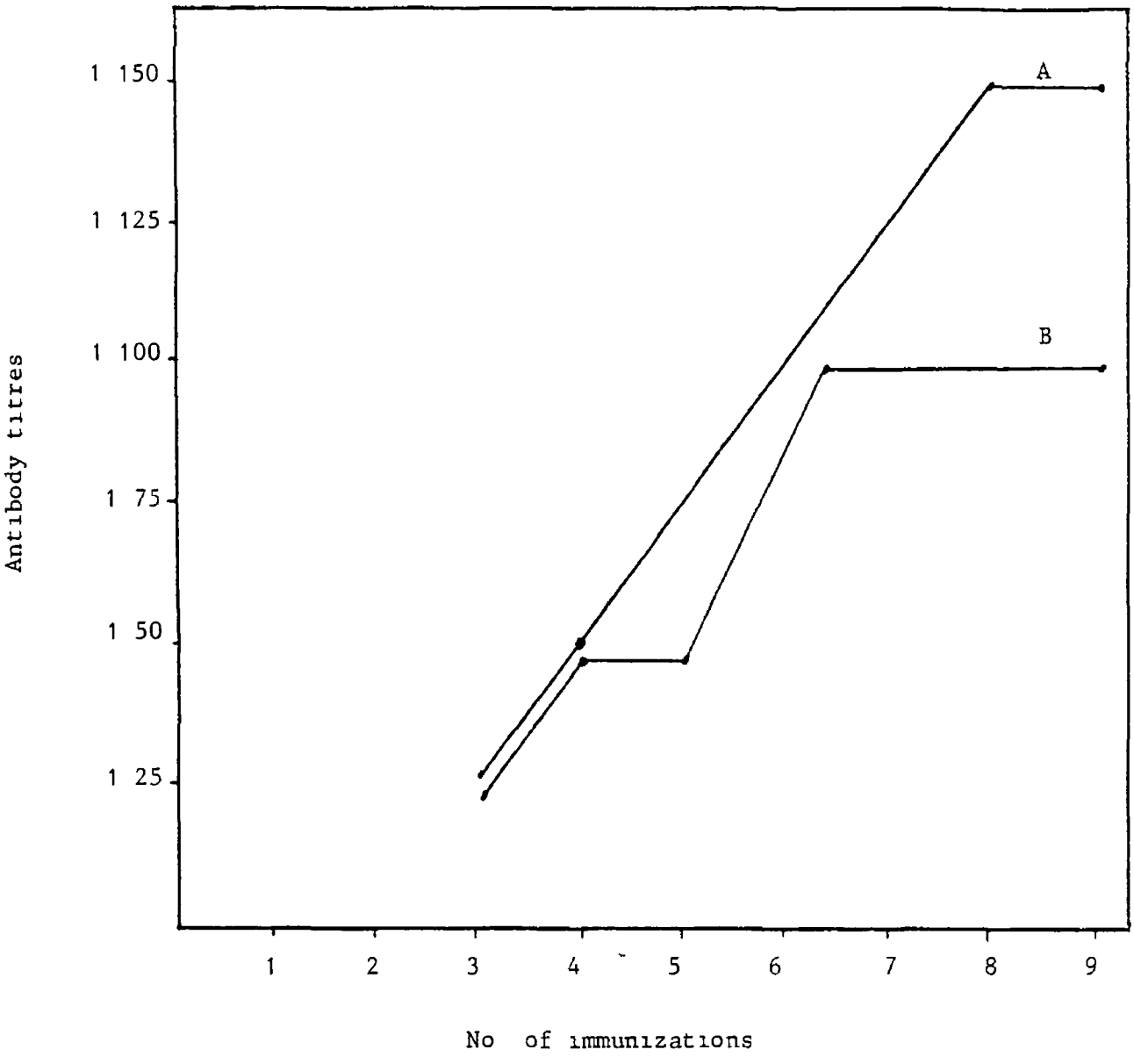

Figure 4 1

A rest

B no rest

Graph of antibody titre versus number of immunizations using LAT cells as immunogens

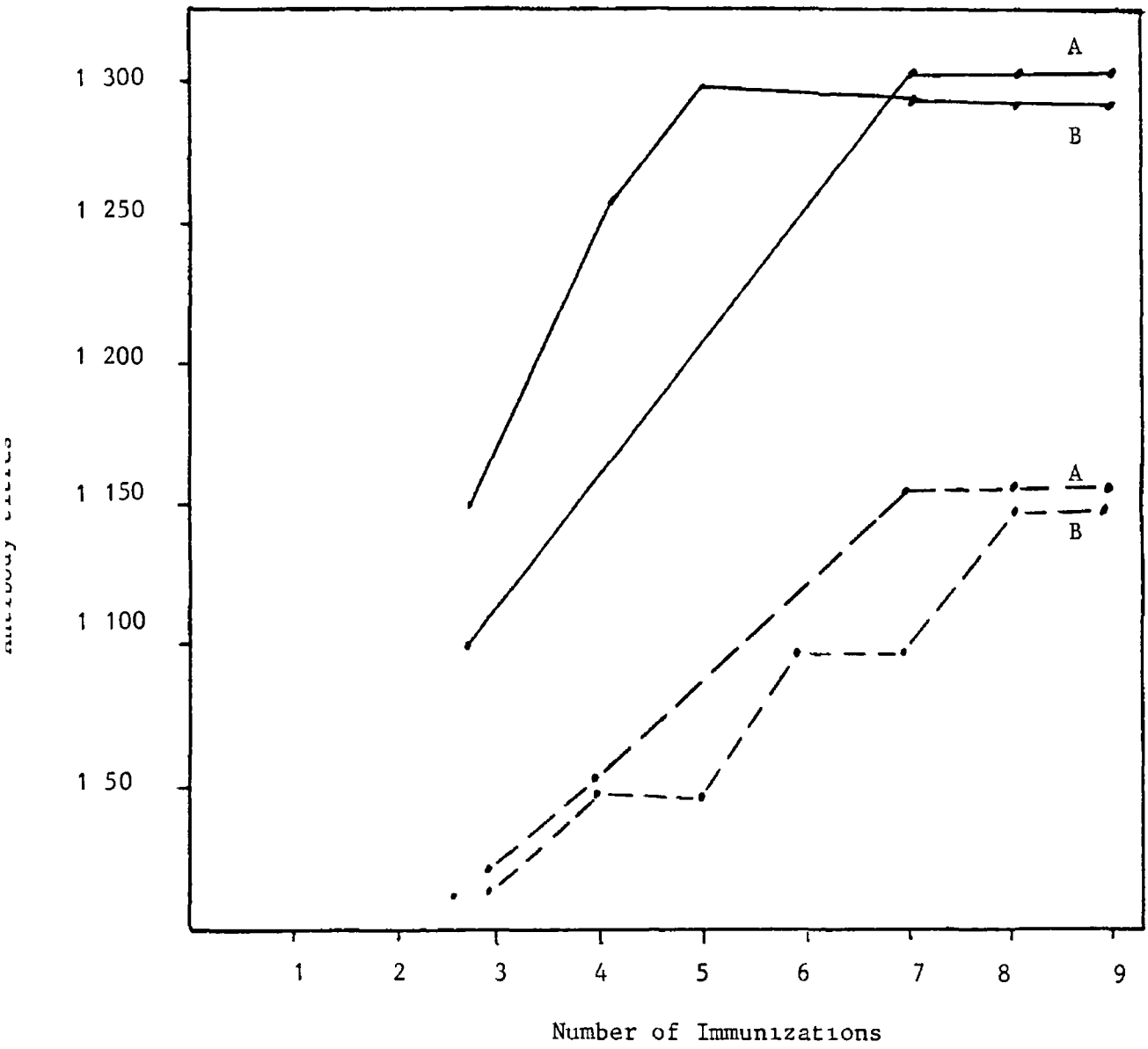

Figure 4 2

LAT

A549

A rest

B no rest

Comparison of serum antibody titres versus number of immunizations for LAT and A549 cells in different immunization procedures

SECTION 5

ENZYME-LINKED IMMUNOSORBANT ASSAY

ENZYME-LINKED IMMUNOSORBANT ASSAY

5 1 INTRODUCTION

The successful generation of a panel of monoclonal antibodies of a desired specificity depends largely on the efficiency of the screening assay used to identify the relevant hybridomas from amongst the large numbers of clones originally produced in a cell fusion Following a cell fusion, as many as 600 wells of microtitre cell culture trays may require screening for production of specific antibodies In the subsequent cloning of positive wells, as many as 1000 more wells may require screening All of this must be accomplished in a very short period of time in order to prevent loss of clones due to overgrowth by non-producing cells It also serves to eliminate large numbers of negative cultures with a consequent reduction in workload and expenditure of media and reagents

Because cells are routinely plated out so as to achieve approximately 1 clone per well, any antibody detected in culture wells is likely to be of a single type and specificity Consequently, the choice of screening assay must not be dependent on reactions involving multiple epitope binding by antibodies (eg immunoprecipitation) as they are poorly suited to hybridoma selection (Campbell, 1984)

Taking the above points into consideration, a suitable screening assay should possess the following features

1 it must be able to cope with large numbers of samples at once and under identical conditions,
2 it must be highly sensitive, being able to detect μg quantities of antibodies, and
3 it should be applicable to all classes of antibodies

Several assay systems lend themselves to these criteria, but the most commonly used are enzyme-linked immunosorbant assay (ELISA) and radioimmunoassay (RIA) Some of the disadvantages associated with RIA include

1 expensive equipment for detection,
2 the short half-life of reagents,
3 biohazards from radiation,
4 disposal of reagents, and
5 non-applicability the field

ELISA, on the other hand, offers the following advantages

1 very high levels of sensitivity, detectability and specificity,
2 relatively cheap equipment available for detection,
3 rapid, simple and highly reproducible,
4 applicable in the field,
5 no problems with radiation and reagent disposal, although some toxicity may be experienced from certain reagents used in ELISA (Gosling, 1980),
6 enzyme conjugates have long shelf-lives, and
7 takes full advantage of the potential of monoclonal antibodies in terms of their specificity and affinity for their target antigens

Recent developments in the amplification of ELISA will, no doubt, lead to even greater sensitivities and economy of reagents (Self, 1985, Johannsson et al , 1986, and Car et al , 1987)

It is imperative that the screening assay for use in the production of monoclonal antibodies be developed and any problems associated with it be sorted out before fusions are attempted The very heavy workload which follows a cell fusion experiment, and the need to rapidly screen cells for antibody production, will leave little or no time for solving problems in the screening assay

In this study a solid-phase ELISA was developed for the large scale screening of hybridomas There are two distinct stages to the setting up of this assay

1 cell fixation onto 96-well microtitre plates, and
2 the ELISA

The various problems encountered in the development of this assay will be discussed The methods finally used are described in section 2 5

5 2 RESULTS AND DISCUSSION

5 2 1 CELL FIXATION STUDIES

The stable fixation of cells onto microtitre plates represented a key part in the overall development of this assay The requirement for stable fixed cell monolayers arose from the need to be able to prepare antigen-coated plates in advance of screening hybridomas due to the unavailability of cells on a daily basis The advantages of using such a solid-phase include

1 stable monolayers of cells can be prepared which are are also stable on storage for several weeks at 4°C with no loss of activity,
2 ease of washing plates in ELISA with the elimination of time consuming centrifugation steps,
3 all classes of antibodies can be applied in ELISA,
4 relatively small amounts of cells and reagents are required,
5 many assays can be set up simultaneously, and
6 many steps are amenable to automation

The fixation of cells to microtitre plates is a relatively simple procedure but is subject to some variation depending on

1 the choice of the type of microtitre plate to be used,
2 the concentration of glutaraldehyde fixative to be used, and
3 the type of cells used

5 2 2

CHOICE OF PLATES FOR CELL FIXATION

The first stage in the development of any ELISA is the proper choice of microtitre plate This is especially true when whole cells are to be used as fixed target antigens, due to variations in the bindability of different cells on different plates From experience gained in this laboratory, it is clear that this must be done empirically for each cell type to be studied No one plate was found to be satisfactory for all of the cells being used in ELISA Consequently, it was found necessary to examine a number of plate types for each cell used while keeping the following points in mind

1 the ability of cells to bind to plates,
2 stability of bound cells during storage, and
3 levels of background colour development seen in ELISA due to nonspecific adsorption of antibodies by different plates

5 2 3

THE ABILITY OF CELLS TO BIND TO PLATES

In a limited study, LAT (Landschütz ascites tumour) and A549 (human lung adenocarcimoma) cells were plated onto each of 4 different plate types under identical conditions (see Methods, Section 2 5) to determine which plate best suited each cell type The plates used were

1 flexible polyvinyl chloride (PVC) plates (Cooke Dynatech),
2 hard PVC plates (Cooke Dynatech),
3 flexible activated-Titertek plates (Flow Labs), and
4 flexible hi-activated-Titertek plates (Flow Labs)

Good fixation of LAT cells was observed on flexible PVC and hi-activated-Titertek plates (Table 5 1) Background colour development on the hi-activated-Titertek plates was found to be unacceptably high during subsequent ELISA The other plates failed to provide adequate binding of cells Therefore, the flexible PVC plates were adopted for all future use with LAT cells For A549 cells the

same plates were found to give good binding of cells In this instance, however, the two plate types also compared favourably in ELISA Two points worth mentioning here are that

1 both of the plates found to be unsuitable for the fixation of LAT and A549 cells have been found to be suitable for the fixation of a number of other cell types, eg lymphocytes from some leukaemic patients,

& 2 within any given plate, some variation exists in relation to the abilities of different wells to bind cells, particularly amongst the outer rows of wells ("edge effects", Tijssen, 1985)

While this study was a limited one, it does serve to illustrate the need to assess different plate types for each cell being examined and is in keeping with the findings of others (Kenny and Dunsmoor, 1983) In our experience, the flexible PVC plates from Cooke Dynatech were the most satisfactory, although they did not bind all of the cell types used Interestingly, most fixation problems were encountered with anchorage independent cells (myelomas and leukaemic cells) To some extent, these problems could be overcome by centrifuging the cells onto the poly-L-lysine primed plates at 200xg for 10 min, although this was not successful in all cases

Flat bottomed plates were used routinely, though the successful use of U-bottomed plates has been reported (Stocker and Heusser, 1979, Cobbold and Waldmann, 1981) V-shaped wells were found to be unsatisfactory due to the formation of cell pellets as opposed to monolayers (Stocker and Heusser, 1979)

In relation to soluble antigens (proteins etc), no problems were encountered in binding them to any of the plates used here It would therefore appear that the choice of plate in such cases is less critical than is the case for whole cells as antigens

5 2 4

GLUTARALDEHYDE FIXATIVE CONCENTRATION

There has been much discussion in the literature on the use of glutaraldehyde (GA) in the fixation of cells for use in solid-phase ELISA There are those who feel that its use can lead to the destruction of some antigenic determinants and the generation of both false positive and false negative reactions (Douillard et al , 1980, Lansdorp et al , 1980, Posner et al , 1982, Drover and Marshall, 1986) This would indicate the need for caution in the interpretation of results obtained from solid-phase ELISA where GA was used as a fixative

Others claim that no adverse effects were observed due to the use of GA (Stocker and Heusser, 1979, Cobbold and Waldmann, 1981) A notable feature of this discussion is the broad variation in terms of the concentrations of GA used and the times and temperatures for which cells are exposed to the GA This may well account for some of the confusion surrounding the issue The range of concentrations of GA used vary from 0 5% to 0 025% (v/v, final concentration on the cells) The times and temperatures used range from 5 to 20 min and 4°C to room temperature (approx 18°C), respectively In the experiments described here, 0 025% GA was routinely used at 4°C for 10 min with no observed adverse effects on cell antigenicity, as seen from comparison of GA fixed cells and unfixed cells in ELISA

It is generally held that microtitre plates should be precoated with poly-L-lysine (0 1% w/v in PBS) prior to addition of cells to the plates This serves to bind the cells more firmly to the wells and also increases the range and diversity of cells which one can fix to plates (Cobbold and Waldmann, 1981) It was reported by (Epstein and Lunney, 1985) that poly-L-lysine alone rather than poly-L-lysine with GA could be used to fix cells to plates, but in our experience, this was unsuccessful (Table 5 2) and the few cells that did bind were stripped from the wells during the subsequent washing stages of ELISA

In establishing this assay, we examined the effects of a range of GA concentrations on the formation of stable cell monolayers and the effects on cell antigenicity, as reflected by the levels of nonspecific colour development in ELISA LAT cells were used throughout on flexible PVC plates (see Section 5 2) Exposure to GA was at 4°C for 10min throughout the experiment

Table 5 2 presents data on cell fixation at different concentrations of GA While markedly higher numbers of cells were bound with high concentrations of GA (greater than 0 1% v/v), these had to be ignored due to unacceptably high levels of nonspecific colour development in ELISA (Table 5 3) This would indicate that high concentrations of GA can in fact cause serious damage to cellular antigenicity, in keeping with the findings of Douillard et al , (1980), Lansdorp et al , (1980), and Drover and Marshall (1986, see below) Much more satisfactory results were obtained when using low GA concentrations (0 025% v/v) Cells fixed under these conditions compared very favourably with unfixed cells in ELISA The use of unfixed cells is undesirable due to the lengthy washing stages involved in the centrifugation of cells Also, in assays using cells fixed with 0 025% (v/v) GA, similar OD values (405 nm, generally less than 0 05) were observed when both PBS and non-immune sera were used as negative controls, indicating that little or no nonspecific binding of serum components was taking place Concentrations of GA less than 0 025% (v/v) were found to generate unstable cell monolayers with substantial stripping of cells during washing leading to large well-to -well variations in ELISA

Drover and Marshall (1986) recently reported that GA fixation caused artefacts in ELISA False negative results were found to be the result of destruction or modification to HLA-D region coded molecules False positive results were caused predominantly by the non-specific adhesion of IgM to the fixed cells These latter effects were less pronounced for IgG_{2a} and IgG_{2b} respectively They used cold 0 25% (v/v, final

concentration) GA for fixation at room temperature for 15 min This, we feel, is excessive and the use of milder conditions may eliminate such effects Also, we have routinely used IgM class antibodies in our ELISA without observing the above mentioned effects

Some authors have reported the successful use of GA concentrations of greater than 0 025% (v/v) with no adverse effects (Stocker and Heusser, 1979, Cobbold and Waldmann, 1981) We do consider it probable that the use of GA may destroy certain sensitive antigens eg HLA-D histocompatability antigens (Drover and Marshall, 1986) Thus, it might be recommended that the effects of GA on specific antigens be examined before deciding on a screening method for hybridoma selection In the case of monoclonal antibody production against tumour antigens, one is often unaware of the nature the antigen being sought In such an instance, the use of a solid-phase ELISA with cells fixed in 0 025% (v/v) GA may be considered for the initial screening of hybridomas For later screenings, some alternative method may be used to ensure that no false positive clones are selected

5 2 5

CELL NUMBERS USED FOR FIXATION

The final concentration of cells used for fixation is, to some extent, dependent on the size of the cells being used It is recommended that the cells should only form a monolayer on the wells because the use of excess cells may lead to unstably bound cells, with stripping of cells from the well surfaces during the vigorous washing of plates in ELISA This stripping of cells is highly variable from well to well and can lead to serious errors in ELISA Each cell to be used in ELISA should be titrated to determine the optimal cell concentration for stable monolayer formation. It was recommended by Cobbold and Waldmann (1981) that a cell concentration giving 25% of confluency be used, a recommendation which was adopted here Generally, adequate cell concentrations are $2\text{-}5 \times 10^5$

cells/ml for normal cells, and 0 2-2 0x10^5 cells/ml for tumour cells, though this may vary, depending on cell size (Cobbold and Waldmann, 1981)

5 3 ENZYME-LINKED IMMUNOSORBANT ASSAY

ELISA represents a highly sensitive assay system which combines the discriminatory power of antibodies with the catalytic power of enzymes Because of its very high level of sensitivity, it is also highly sensitive to nonspecific effects which can lead to both false positive and false negative results Amongst the sources of such effects are the following

1. plates (see Sections 5 2 2 and 5 2 3),
2. use of excess GA in the fixation of cells (see Section 5 2 4),
3. endogenous enzymes in the assay material,
4. unstable substrates,
5. electrostatic interactions between antibodies and plates or antigens, and
6. Fc-receptor binding of antibodies

Certain of these factors have already been dealt with in previous sections (as indicated) since they are largely related to the setting up of the assay The remaining factors are dealt with below

5 3 1 CHOICE OF ENZYME FOR ELISA

The problem of endogenous enzymes is most frequently encountered when using whole cells as antigens The most commonly used enzymes in ELISA are horseradish peroxidase, alkaline phosphatase and β-galactosidase A number of other enzymes have also been used (see Tijssen, 1985 for an account of them and their properties in relation to ELISA) β-galactosidase (EC 3 2 1 23) is often the enzyme of choice for use with whole cells as antigens because it is not commonly found in mammalian cells (Cameron and

Erlanger, 1976) It is further characterized by its high stability over a range of pH's and temperatures (Tijssen, 1985) The substrate used is o-nitrophenyl-β-D-galactopyranoside (ONPG) which is also quite stable but will autohydrolyse to give colour development by the release of free nitrophenol when stored for long periods at greater than 30°C (over 4 hours in our experience) However, it is generally freshly prepared immediately before use

The use of horseradish peroxidase has been reported with cells, though it requires the destruction of endogenous enzyme (Swirsky et al , 1983) This adds an extra step to the assay and offers no gains in terms of sensitivity

5 3 2

F(Ab')^2 VERSUS WHOLE ANTIBODY-ENZYME CONJUGATES

F(Ab')$_2$ antibody enzyme conjugates were used routinely Initially, however, whole antibody enzyme conjugates were used, but were later rejected in favour of F(Ab')^2 due to the unacceptable levels of nonspecific colour development observed during their use Fc-receptor binding of the whole antibodies was found to be partially responsible for these problems Comparisons between assays using whole antibodies and F(Ab')^2 fragments revealed lower levels of non-specific colour development for the whole antibodies The most likely source of Fc-receptors in this study comes from contaminating peritoneal macrophages in the LAT cell preparations Similar effects were not observed for A549 cells grown in vitro The presence of Fc-receptor containing cells in the LAT cell preparations presented a problem in that a certain amount of unavoidable non-specific binding of first antibody took place In general, however this was not found to cause excessive levels of background and could be blocked, to a large extent, by the use of non-murine serum as a blocking protein instead of the usual BSA

5 3 3

NON-SPECIFIC ELECTROSTATIC INTERACTIONS

The non-specific binding of antibodies to plates and antigens by electrostatic and hydrophobic interactions can represent a major source of errors in ELISA However, such reactions are largely reversible and preventable by the use of appropriate blocking agents and detergents which disrupt such interactions BSA at 1% (w/v) in PBS is the most commonly used blocking agent Antigen coated plates are simply incubated in BSA solution at 37°C for 1 hour prior to antibody incubations This provides effective blocking of most non-specific binding sites

The incorporation of a mild detergent (usually Tween-20, 0 1% v/v) in wash solutions and antibody diluents helps to prevent any further non-specific binding of antibodies during ELISA Tween-20 does not affect antigen-antibody interactions (Crumpton and Parkhouse, 1972,Dimitriadis 1979) The interactions mainly disrupted by Tween-20 are hydrophobic interactions

There are increasing numbers of reports in the literature on the removal of proteins from nitrocellulose (NC) caused by the use of Tween-20 in wash solutions during Western blotting (Hoffman and Jump, 1986) This, presumably, also applies to dot-immunobinding assays The use of Nonidet P-40 (Lin and Kasamatsu, 1983, Spinola and Cannon, 1985) and Triton X-100 (Batteiger et al , 1982, Gershoni and Palade, 1982) have already been widely discontinued for such purposes Tween-20 is, however, much less efficient in the removal of proteins from NC and does not affect all proteins equally (Hoffman and Jump, 1986) Whether or not the same effects apply to ELISA is unclear In the case of covalently bound antigens, this is unlikely to be the case However, for most soluble antigens used in ELISA, binding is by simple adsorption onto plates and may, therefore be affected as on NC

Wedege and Svenneby (1986) reported that the use of Tween-20 caused non-specific binding of antibodies on NC It was suggested by Gershoni and Palade (1982) that false

positive bands on NC may be due to hydrophobic interactions between antibodies and antigens mediated by detergents. To our knowledge, similar effects have not been reported for ELISA but the mounting evidence suggests the need for a revaluation of the use of detergents in ELISA. It was reported by Ashorn and Krohn (1986) that the use of "tap water" was equally as good as Tween-20 for washing in ELISA. Our experience, however, suggests that this is not the case.

5.3.4

EFFECTS OF TIME AND TEMPERATURE OF ANTIBODY INCUBATIONS ON BACKGROUND EFFECTS

During the establishment of this assay, polyclonal anti-LAT cell antisera from immunized Balb/c mice were used as positive controls in the absence of suitable monoclonal antibodies. Using these antibodies, it was found necessary to use incubation temperatures of 4°C for 1 to 2 hours in order to get optimal binding of antibodies with minimal nonspecific binding effects. Incubations at 37°C resulted in elevated levels of background colour development in negative controls using non-immune sera (Table 5.4). Although these levels of background were quite low, the use of 4°C incubations gave even lower backgrounds, allowing a better visual contrast between positive and negative tests, and, therefore, easier identification of positives.

Similar, though more pronounced effects were observed when long incubation periods were compared with short incubations for first and second antibodies (Table 5.5). Incubations of greater than 2 hours at 4°C resulted in elevated nonspecific colour developments which could only be partially accounted for by the autohydrolysis of the ONPG to free nitrophenol (coloured product).

In subsequent assays using monoclonal antibodies, these effects were not so noticable and 37°C incubations were used routinely. This may be due to higher affinities for the target cells and because of higher levels of specific

antibodies in the samples used, keeping in mind that anti-LAT cell antisera were commonly found to be of a low titre (see Section 4)

5 4 CONCLUDING COMMENTS ON CELL FIXATION AND ELISA DEVELOPMENT

In the course of developing this solid-phase ELISA, a number of important points came to light in relation to the setting up and performance of the assay The first is the choice of the correct type of plate to be used as a solid support for the particular target cells being used A great deal of variation exists between different plate types, and even among plates of the same batch, in their ability to bind different cell types Secondly, well-to-well variations exist in relation to their optical properties This can make accurate comparisions between individual wells somewhat difficult This effect is most pronounced amongst the outer rows of wells on plates (Tijssen, 1985) Where accurate quantitative comparisions are to be made, therefore, it is to be recommended that the outer rows of wells on plates be avoided This problem is not so serious when purely qualitative (+/-) results are needed, as in the case of the primary screening of monoclonal antibodies, since positive wells are often readily discernable on visual inspection of the plates Thirdly, antibody incubation periods should not be excessive when using whole cells as target antigens, due to the excessive non-specific adsorption of antibody onto the cells, leading to elevated levels of background colour and false positive results This was not observed to be the case when using soluble antigens in ELISA The non-specific uptake of antibodies would appear therefore, to be a function of cells when used as fixed target antigens

The final details of the assay developed here are described in section 2 5 Briefly, 50 µl of LAT cells ($5x10^5$ cells/ml in PBS) are applied to the wells of flexible PVC microtitre plates (Cooke Dynatech) pretreated with 0 1% (w/v) poly-L-lysine for 1 hour at

37°C The cells are allowed to bind for 1 hour at 4°C and are then covalently fixed to the plates by the addition of 50 l of 0 05% (v/v) GA at 4°C, to give a final concentration of 0 025% (v/v) GA in each well After 10 min the GA is removed and the plates washed in PBS Free binding sites on the wells are now blocked by the addition of 1% (w/v) BSA in PBS First antibody (immune serum or hybridoma supernatant) is now applied After 1 hour at 37°C, free antibody is removed by washing with 0 1% (v/v) Tween-20 in PBS Second antibody (sheep anti-mouse, $F(Ab')^2$-linked -galactosidase, Amersham) is then applied at an appropriate dilution and allowed to bind at 37°C for 1 hour The plates are then washed to remove any free antibody, followed by the addition of substrate (ONPG) Colour development is usually complete in 30 to 60 min OD at 405 mn is read after stopping the reaction by the addition of 1M sodium carbonate

This assay was found to give reproducible results when using both LAT and A549 cells as the target antigens In principle, this assay can be applied to any cell, but the correct choice of ELISA plates must be made empirically for each cell type to be examined Some variations may also exist within the ELISA itself, depending on the nature (class and specificity) of the antibodies being applied In this laboratory, however, no serious problems (other than the correct selection of plates for different cell types) have been encountered with this assay and it has been successfully applied to the study of antibody-antigen interactions on a wide variety of normal and tumour cell types

5 5

CURRENT AND FUTURE TRENDS IN IMMUNOASSAY

To date, plastic microtitre trays have been used predominantly as solid-phase surfaces in ELISA However, they do suffer from some limitations as described by Tijssen (1985)

1 they are immunoreagent consumptive, requiring up to

10x more materials for coating than some other surfaces (e.g. nitrocellulose, [NC]);

2. the avidity of some larger antigens for antibodies may be reduced by 1 to 2 orders of magnitude, possibly due to wide spacing of epitopes;
3. the rates of antibody-antigen reactions are slower for fixed antigens versus solutions (K_{ass} is largely dictated by diffusion rates);
4. plastics have lower absorbtion capacities per unit area (require µg quantities of materials versus pgs for NC);
5. up to 68% of antigen may desorb from plastic when non-covalently bound.

More and more frequently, NC is now being used as a solid-phase surface for immunoassays, particularly in dot-immunobinding assays for soluble (Hawkes et al., 1982; McDougal et al., 1983) and particulate (Fanci et al., 1986) antigens. This offers the advantages that:

1. very small sample volumes (<1µl; 100pg of pure antigen) can be used;
2. up to 100% of most antibodies and antigens are bound; and
3. ionic detergent solubilized samples can be applied.

However, NC is used only in the qualitative detection of antigens, although it can also be applied quantitatively. Also, in light of reports on the removal of some antigens from NC by the use of Tween-20 (Hoffman and Jump, 1986), some care should be taken to ensure that similar effects do not also apply to the antigen being used.

Recent developments in enzyme amplification for immunoassays allows even greater levels of sensitivity and detectibility to be achieved (down to 1 hundreth of an attomole; Johannsson et al., 1986). This limits the capacities of such assays to the affinities of the antibodies employed and not the weakness of the detection systems currently in use.

TABLE 5 1

BINDING OF CELLS TO MICROTITRE PLATES

TYPE OF MICROTITRE PLATE	CELL TYPE	
	LAT	A549
Flexible PVC	229*	206
Hard PVC	74	53
Activated-Titertek	128	72
Hi-activated-Titertek	195	221

* Values represent the numbers of cells bound per mm^2 as determined microscopically at 40x mag on a Nikon Diaphot inverted microscope fitted with a graticule eye piece These values are only representative of the cell numbers bound, since some well to well variations do exist The values were taken from a single count, but are typical of the values being observed

TABLE 5 2

EFFECTS OF DIFFERENT CONCENTRATIONS OF GLUTARALDEHYDE ON CELL FIXATION

CELL TYPE	GLUTARALDEHYDE CONCENTRATION (% V/V)				
	0 000	0 025	0 050	0 100	0 250
LAT	13*	245	302	295	413
A549	30	226	247	274	381

* Values represent the numbers of cells bound per mm^2 as determined microscopically using a Nikon Diaphot inverted microscope at 40x mag with a graticule All wells were pretreated with 0 1% v/v poly-L-lysine

TABLE 5 3

COMPARISON OF NONSPECIFIC COLOUR DEVELOPMENTS IN ELISA WITH LAT CELLS FIXED AT DIFFERENT GLUTARALDEHYDE CONCENTRATIONS

CONCENTRATION OF GLUTARALDEHYDE (% v/v)	ABSORBANCE AT 405 nm		
	POSITIVE SERUM *	NEGATIVE SERUM	PBS
0 025	0 211	0 065	0 062
0 250	0 344	0 138	0 093

* Anti-LAT, polyclonal antibodies raised in Balb/c mice Negative serum was taken from non-immunized Balb/c mice PBS was used as a further negative control Assays were performed in triplicate A typical result is shown
The ELISA was as described in methods (Section 2 5)

TABLE 5 4

EFFECT OF TEMPERATURE OF ANTIBODY INCUBATIONS ON BACKGROUND EFFECTS IN ELISA

Temperature (C)	Anti-LAT serum + *	Non-immune serum -	PBS - control
37	0 216 **	0 039	0 008
4	0 178	0 008	0 003

* These represent the first antibodies (positive and negative controls, as indicated) used in the ELISA The positive and negative antisera were taken from immunized and non-immunized Balb/c mice respectively

In all cases, the second antibody was sheep anti-mouse $F(Ab')^2$-linked β-galactosidase (Amersham)

** Values represent OD (405 nm) readings for a typical assay The assays were carried out in triplicate on 3 separate occasions It was not possible to obtain accurate mean values due to plate to plate variations arising from the nature of the plates (see Section 5 2 3)

First antibody was incubated for 1 hour and second antibody for 1 5 hours, both at 37°C

TABLE 5 5

EFFECT OF ANTIBODY INCUBATION TIMES ON BACKGROUND COLOUR DEVELOPMENT IN ELISA

Incubation times for each antibody	Anti-LAT serum + *	Non-immune serum - *	PBS - control
Ab-1 for 1 hour Ab-2 for 18 hours	0 688 **	0 415	0 150
Ab-1 for 18 hour Ab-2 for 1 5 hours	0 235	0 051	0 019
Ab-1 for 1 hour Ab-2 for 1 5 hours	0 205	0 066	0 011

* Anti-LAT and non-immune sera from immunized and non-immunized Balb/c mice respectively

** Values represent OD (405 nm) from a typical assay These assays were performed in triplicate on 3 separate occasions It was not possible to obtain accurate mean values due to plate-to-plate variations in readings arising from the nature of the plates (see Section 5 2 2)

Second antibody was sheep anti-mouse $F(Ab')^2$-linked β-galactosidase (Amersham)

This table illustrates how prolonged antibody incubations with target cells at 4°C can cause elevated levels of background, particularly when the 2nd antibody incubation period has been extended

SECTION 6

MONOCLONAL ANTIBODY PRODUCTION

A GENERAL REVIEW OF THE METHODS FOR SOMATIC CELL FUSION EMPLOYED IN THIS PROJECT

MONOCLONAL ANTIBODY PRODUCTION

6 1
INTRODUCTION

The generation of permanent cell lines capable of producing antibodies of predefined specificity was first described by Kohler and Milstein, (1975) This represented a major landmark in the entire field of antibody-mediated analysis The use of monoclonal antibodies has since become widespread in many areas of analytical biomedical sciences

The original work of Kohler and Milstein (1975) centered on the mouse, as is largely the case today There is, however, a growing interest in monoclonal antibodies from other species, especially human monoclonal antibodies (Roome and Reading, 1984, Carroll et al , 1986, James and Bell, 1987) In this discussion, we will concentrate on the mouse system, which was used here Excellent reviews on this area include those of Westerwoudt (1985) and Samoilovitch et al , (1987)

6 2
PRODUCTION OF MOUSE MONOCLONAL ANTIBODIES

The methods used for the production of mouse monoclonal antibodies are all basically alike in terms of the general approach taken in fusing cells However, the specific details tend to vary from one laboratory to the next, and no truly optimal method has as yet been described In the work carried out here, some of the factors were examined with a view to improving the frequencies of hybridoma formation being achieved in this laboratory This study was a very limited one, as a comprehensive study of all of the factors involved lay outside of the scope of this project

6 3

PREPARATION OF THE FUSION PARTNER

The Sp2/0 mouse myeloma cell line (Shulman et al , 1978) was used throughout This cell does not produce any antibody chains and is the most commonly used mouse myeloma cell line Sp2/0's are sensitive to to certain environmental factors and are particularly intolerant of severe dilution and alkaline pH (Goding, 1980) Their doubling time is from 19 to 22 hours (Fazekas de St Groth and Schreidegger, 1980) For all fusions, Sp2/0 cells were maintained in the mid-log phase of growth for at least 7 days prior to fusion because non-log phase cells, or overgrown cells, make poor fusion partners (Galfre and Milstein, 1981, Oi and Herzenberg, 1981)

Mycoplasma infection of cells will also lead to a reduction in, or a lack of hybridoma formation (see Section 8) In cases where mycoplasma infected cells were inadvertently used, the frequency of hybridoma formation was from 0 to 0 3 as compared to a frequency of 2 58 hybridomas per $1x10^5$ spleen cell input into the fusion mixture (Table 6 1) In cases where one fails to get any, or gets very few clones following a fusion, mycoplasma infection should be considered as a possible cause Indeed, prior to fusions, it is good practice to screen for mycoplasma (see Section 2 2 8) The Sp2/0 cells should also be checked for HAT sensitivity by growing them in 10^{-4} M 8-azoguanine for three days to approx 1 week prior to fusion (Earley and Osterling, 1985, see Section 2 6 1)

6 4

RATIO OF SP2/0 TO SPLEEN CELLS IN FUSIONS

The recommended ratio of Sp2/0 to spleen cells used in fusion mixtures varies from one laboratory to another Ratios of 1 1 (Goding, 1980) to 1 10 (Westerwoudt et al , 1984) have been used successfully A recent extensive review of fusion methods by Westerwoudt et al , (1984) suggests that the optimal ratio is 1 10, and that the

fusion frequency (the number of hybridomas formed) is more dependent on the spleen cell input This, under the conditions described, gave a fusion frequency of 2 clones per $1x10^5$ spleen cell input with approx 1 in 7 clones producing antibodies Here, under the same conditions (see Section 2 6 5), 2 58 clones were obtained per $1x10^5$ spleen cell input (Table 6 2) No data on the number of antibody producers is available It must be noted, however, that other factors may also have been involved (e g source of PEG, quality of feeder layers etc) Also, in our study, red blood cells (RBC) were removed by haemolysis from the preparations of spleen cells (Mishell and Shiigi, 1981, Earley and Osterling, 1985) This should serve to reduce the frequency of both Sp2/0 to RBC and immune B-cell to RBC fusions, and should, therefore, increase the fusion frequencies between Sp2/0 and B-cells This may account for the higher fusion frequency obtained here compared to that reported by Westerwoudt et al , (1984) No attempt was made to fuse cells at the ratio of 1 10 (Sp2/0 spleen cells) without the lysis of red blood cells

6 5

FUSOGENS

Polyethylene glycol (PEG), first introduced by Portecorvo (1976) for the somatic cell fusion of animal cells, has become the most commonly used fusogen PEG appears to act by promoting the close cell to cell contact necessary for cell fusion to occur, while additives within the PEG stimulate the fusion process (Wojcieszyn et al , 1983) This may explain why different lots and brands of PEG vary in their fusion efficiencies (Fazekas de St Groth and Schreidegger, 1980, Lane et al , 1984)

PEG's of different molecular weights (mw) and concentrations may also be used Cell monolayers favour the use of lower mw PEG's (1000 to 1500 mw), while cell suspensions favour higher mw (up to 4000 mw) (Fazekas de St Groth, 1980, Westerwoudt, 1985) PEG of mw less than 500 is ineffective, while PEG of mw greater than 4000 are

too viscous (Fazekas de St Groth and Schreidegger, 1980) The concentration of the PEG is a more critical factor and should lie in the range of 30 to 50% (v/v) Fifty % (v/v) is most commonly used (Goding, 1980, Oi and Herzenberg, 1981, Westerwoudt, 1985) Lower concentrations have also been used successfully (Hadas and Theilen, 1987)

The manner in which the PEG solution is prepared also varies Originally we prepared it by autoclaving solid PEG to dissolve and sterilize it When it had cooled to approx 40°C, it was aseptically diluted with an equal volume of sterile serum-free culture medium PEG prepared in this way has a pH of 7 0 to 7 4, but becomes very alkaline in storage However, it was reported by Westerwoudt (1985) that PEG prepared by dissolving the solid PEG in medium, followed by filter sterilization through a 0 22 μm filter (Millipore), allowed a higher frequency of hybridoma formation Also, the optimum pH was reported to be 8 0 Here, a comparison of fusion frequencies for PEG's (1540 and 4000 mw, both from Riedel-de Haen) prepared by the above two methods revealed that PEG-4000 prepared by filter sterilization gave higher fusion frequencies, 1 21 versus 0 74 for PEG-1540 and 2 58 versus 1 61 for PEG-4000 (Table 6 3) The different values observed between the PEG-1540 and the PEG-4000 may be accounted for by the fact that the cells used were in suspension, which is more suited to the use of PEG 4000 (Fazekas de St Groth and Schreidegger, 1980) These results suggest that some component(s) in the PEG, important to the fusion process, are damaged or destroyed during autoclaving

In the above cases, the cells were exposed to PEG for a total time of 2 to 3 min However, evidence presented by Lane (1985) suggests that this is suboptimal and that shorter fusion periods (30 to 60 secs) allows up to 5 times more hybrids to be obtained when using Sp2/0 and 30 times more when using FOX-NY cells as fusion partners The inefficiency of the longer fusion periods was attributed to the cytotoxic effects of the PEG on the newly formed hybridomas There are also reports that the

addition of 5 to 10 % (v/v) DMSO to the PEG solution can further enhance the fusion frequency (Norwood et al , 1976, Westerwoudt, 1985), although this has been disputed elsewhere (Fazekas de St Groth and Schreidegger, 1980) These factors were not examined here

6 6
FEEDER CELL LAYERS

The presence of a layer of feeder cells greatly increases the ability of cultured cells to grow at very low dilutions This is considered to be essential when plating newly formed hybridomas, and especially during the cloning of cells by limiting dilution More recently, increasing attention has been focussed on identifying the factors provided by feeder cells which stimulate the growth of cells at low dilutions The use of conditioned media (media in which cells have been actively grown) has also become more common (Tharakan et al , 1986, Nordan and Potter, 1986, Rathjen and Greczy, 1986) While the use of defined growth factors and conditioned media was not examined here, conditioned medium was occasionally used

Peritoneal macrophages (exudate cells) were used routinely as a source of feeder cells (see Section 2 6 4) Their use was first reported by Fazekas de St Groth and Schreidegger (1980) The optimal concentration of cells was found to be $1x10^4$ cells per well, in keeping with the findings of Fazekas de St Groth and Schreidegger(1980) The cells were untreated in any way The use of activated macrophages (obtained from mice who had received an ip injection of 1 ml of 10% v/v sodium thioglycollate 4 days prior to harvesting of the peritoneal cells) has been reported though they have been considered by others to be too active and destructive towards the emerging hybridomas (Campbell, 1984) The use of higher concentrations of cells did not confer any further advantage Indeed, when using greater than $1x\ 10^5$ cells per well, the number of clones was sometimes less than expected, possibly due to destruction of emerging clones by the macrophages This may be overcome by the use of other cell types such as

thymocytes (Westerwoudt et al , 1983) Macrophage conditioned medium was also used to successfully support hybridoma growth following fusion and during cloning This conditioned medium was prepared by allowing macrophages to grow for 48 hours in RPMI-1640 culture medium containing 10% FCS The medium was harvested and cells and debris removed by centrifugation It was then stored at 4°C until required (not more than 7 days) It was supplied to the hybridomas at a 1 1 concentration in complete RPMI-1640 containing 10% FCS

6 7

ALTERNATIVES TO FEEDER LAYERS OF CELLS

Concerns have been raised over the use of feeder cells in the production of monoclonal antibodies (Samoilovitch et al , 1987) These fears arise from the increased dangers of contamination of cells with microorganisms, due to the extra operations involved in their preparation Also, the feeder cells provide added competition for the growing hybridomas in terms of nutrient depletion and the accumulation of waste products A further concern lies with the possibility of the release of autolytic enzymes from dying cells (macrophages, being normal cells, have a finite lifespan in culture) which may damage both the hybridomas and their antibodies Such factors have stimulated the search for alternatives to feeder layers of cells for the promotion of hybridoma growth

Many factors other than mouse macrophages have been reported to potentiate and enhance the growth of emerging hybridomas eg human umbilical cord serum (HUCS, Westerwoudt et al , 1983), human low density lipoproteins

(Mao and France, 1984), mouse fibroblast conditioned medium (Walker et al , 1986) and irradiated human diploid fibroblast cells (notably MRC-5, reported to be approx 10 times more potent than mouse macrophages, Long et al , 1986) Regardless of the method used, it is clear that the optimum potentiator of hybridoma growth is more likely to be a cocktail of factors rather than any individual

factor alone This is an area worthy of further research, particularly due to the growing need for serum-free media for the large scale production of monoclonal antibodies

One further point to be mentioned here is that the yield of antibody secreting clones is dependent on the concentration at which the newly formed hybridomas are plated (Hadas and Theilen, 1987), the lower the plating density of cells, the higher the yield of secretors Also, the instability of antibody secretion by polyclonal cultures is due to genetic instability rather than overgrowth by non-secretors, as was previously thought (Westerwoudt et al , 1984, Hadas and Theilen, 1986)

6 8
CONCLUDING COMMENTS ON FUSION PROTOCOLS

The fusion protocol which we initially used was modified in the following respects,

1 PEG was filter sterilized instead of being autoclaved,
2 PEG-4000 was used instead of PEG-1500,
3 the ratio of Sp2/0 to spleen cells was increased from 1 1 to 1 10,
4 spleen cell suspensions were treated to remove red blood cells by haemolysis,
5 cells were plated out at $2x10^4$ as opposed to $2x10^5$ cells/ml in the original procedure

The final fusion protocol, as detailed in section 2 6 5, incorporates all of the above modifications and brought about a marked increase in the frequency of hybridoma formation (Table 6 4) In this study, the percentage of wells having viable clones was increased from 24% - 50% up to more than 80% (cells were plated out over six 96 well plates) The observed multiplicity of clones per well was generally less than 3, and frequently at 1 clone per well, indicating that the newly formed hybridomas were being plated at close to the optimum concentrations required to give a single clone per well This allows the maximum number of individual clones to be generated with a

subsequent increase in the probability of finding an antibody of the desired specificity

The observed increases in fusion frequencies due to these modifications increases the probability of generating a monoclonal antibody with the desired characteristics for its intended applications This is particularly important when trying to generate monoclonal antibodies against minor components of complex antigenic mixtures, as is the case when using whole cells as antigens (Hadas and Theilen, 1987) LAT cells, which were found to be weakly immunogenic in Balb/c mice (Section 4) were used as antigen for the production of monoclonal antibodies in this study (see following section) Little success was achieved using the original fusion protocol, possibly due to the low frequencies of hybridoma formation being observed Using the modified fusion method, greater success was achieved and a monoclonal antibody was generated which was found to be reactive on LAT cells and a number of other tumour cell types, but has limited reactivity on the range of normal cells examined (see Section 10) The increased frequency of hybridoma formation may well have contributed to this success

It is evident from the above discussion that, as yet, the procedures for somatic cell fusion have not been optimized This area remains as much an art as a science The optimization of the fusion protocol is made very difficult by virtue of the many parameters involved which lie outside of the control of the investigator Extensive variations may exist from laboratory to laboratory in terms of the precise methods being used e g variations in the time for which cells are exposed to fusogens, the quality of different preparations of PEG, the quality of the feeder layers (Fazekas de St Groth and Schreidegger, 1980) or of conditioned media prepared in different laboratories etc Clearly, accurate comparisons are difficult to make, even from one fusion to the next in a given laboratory The increasing availability of standardized commercial preparations of growth factors and conditioned media may help to reverse this situation

TABLE 6 1

EFFECT OF MYCOPLASMA INFECTION ON FUSION FREQUENCY

Sp2/0 cells +/- mycoplasma	Number of tests	Frequency of hybridoma formation per $1x10^5$ spleen cell input
-	3	1 61 to 2 58
+	3	0 to 0 30

TABLE 6 2

EFFECT OF DIFFERENT SP2/0 TO SPLEEN CELL RATIOS ON FUSION FREQUENCIES

Sp2/0 to spleen cell ratio		Frequency of hybridoma formation per $1x10^5$ spleen cell input
1 1	A	1 01 *
	B	0 74
1 10	A	2 58
	B	2 24

* Each value represents a different fusion experiment

TABLE 6 3

FUSION FREQUENCIES FOR PEG'S PREPARED IN DIFFERENT WAYS

PEG mw	Preparation of PEG (see text for details)		Fusion frequency clones per $1x10^5$ spleen cell input
1540	Autoclaved	A	0 74 *
		B	1 21
4000	Filtered	A	1 61
		B	2 58

* Each value represents a different fusion experiment

TABLE 6 4

PRODUCTION OF HYBRIDOMAS A COMPARISON OF THE ORIGINAL FUSION PROTOCOL WITH THE FINAL PROTOCOL ADOPTED IN THIS STUDY

FUSION PROTOCOL	SPLEEN CELL INPUT	NUMBER OF CLONES	% OF WELLS WITH CLONES
1	$1X10^7$	161 - 258	34 00 - 53 75
2	$1X10^7$	> 500	80 - 100

The differences between fusion protocols 1 and 2 were

1 autoclaved PEG was used in method 1 as opposed to filter sterilized PEG in method 2,

2 PEG-1500 was used in procedure 1 versus PEG-4000 in procedure 2,

3 the ratios of SP2/0 spleen cells used were 1 1 versus 1 10 in methods 1 and 2 respectively,

4 in protocol 2, the spleen cell preparation was depleted of red blood cells,

5 cells were plated at $2x10^4$ cells/ml in protocol 2 versus $2x10^5$ in protocol 1

SECTION 7

THE PRODUCTION OF ANTI-LAT CELL MONOCLONAL ANTIBODIES

THE PRODUCTION OF ANTI-LAT CELL MONOCLONAL ANTIBODIES

7 1 INTRODUCTION

The primary aim of this project was to produce a monoclonal antibody with specificity for some tumour-related antigen expressed by LAT cells This antibody would also be assessed for its reactivity on a number of other tumour and normal cell types in order to assess its value for the design of both *in vivo* and *in vitro* model systems of tumour localization and antibody-mediated drug delivery

In the course of this project, a number of somatic cell fusions were carried out with this end point in mind Primary screening of the hybridomas was performed on LAT cells in solid-phase ELISAs (Sections 2 5 and 4) Positive clones from this screening were then examined for reactivity with normal human leucocytes in solid-phase ELISA Only those clones showing reactivity with LAT cells alone were considered for further expansion and analysis Those clones showing strong reactivity on normal human leucocytes were discarded from further study This helped in the rapid elimination of clones producing antibodies to normal antigens and also in reducing the workload to more manageable levels

Only very brief details will be presented about those fusions which failed to produce an antibody of the desired specificity i e restricted reactivity for tumour cells over normal cells A more detailed account will be given for fusion 13, from which the antibodies used in subsequent studies were produced

7 2

FUSIONS TO PRODUCE ANTI-LAT CELL MONOCLONAL ANTIBODIES

In all, 5 fusions yielded hybridomas which were tested for reactivity against LAT cells Four of these fusions are considered here The fifth fusion, from which useful anti-LAT cell monoclonal antibodies were generated, will be considered separately in the following section

The results of the first 4 fusions are summarised in table 7 1 The majority of the hybridomas found to be reactive with LAT cells were also found to be cross-reactive on normal human white blood cells (70% of the LAT reactive clones from the first 4 fusions) and were therefore eliminated from any further expansion and more extensive screening This level of cross-reactivity is not surprising, since the use of whole cells as antigens inevitably leads to the bulk of the immune response being directed against normal antigens In all, 12 clones were found to be reactive on LAT cells, but not on normal human white blood cells (30% of the LAT- reactive clones, Table 7 2) Of those 12 clones, 7 ceased to produce antibodies following further expansion This was most probably due to the genetic instability of the newly formed hybridomas (heterokaryons) (Westerwoudt et al , 1984, Hadas and Theilen, 1987) The newly formed hybridomas are highly unstable heterokaryons which shed excess chromosomes in a random fashion in an effort to return to the diploid state During each cell division more chromosomes are lost These chromosomes may be involved in antibody production, processing (post-translational modifications), antibody secretion, or may be responsible for the ability of the cells to grow in vitro In any case, the end result is the loss of that clone

Of the 5 remaining clones, 4 were lost due to contamination, or the loss of the ability to continue growing in culture The remaining clone, 9AW, was expanded and cloned The subclones were, however, found to be contaminated with mycoplasma and efforts to rescue

this clone by passage in Balb/c mice were unsuccessful (see Section 8) This clone continues to produce only very low levels of antibody when grown as an ascitic tumour in Balb/c mice (titres of less than 1 50 versus titres of 1 500 achieved by other clones) Because of its persistent mycoplasma infection, this clone was not examined any further due to the serious risk of contamination posed to other uninfected cell lines

7 3

PRODUCTION OF ANTI-LAT CELL MONOCLONAL ANTIBODY F10 1 E12

Hereafter, clones generated in this fusion may be referred to without the use of the prefix F10 1 i e clone F10 1 E12 may be refered to as clone E12 etc

Following the failure of the previous fusions (above) to produce a suitable monoclonal antibody, a further fusion was carried out making use of the information gained in section 6 (i e improvements in the fusion protocol to achieve higher fusion frequencies) As a result of these modifications, a larger number of anti-LAT cell-reactive clones were generated from this fusion than was the case for any previous fusion Indeed, the number of clones obtained from this fusion equalled the total number of reactive clones obtained from the previous 4 fusions (40 clones) The results of this fusion are summarized in table 7 3 Of the 40 LAT-reactive clones generated, 22 showed strong reactivity on normal human white blood cells in solid-phase ELISA and were discarded from further studies A further 10 clones ceased to produce antibody, leaving a total of 8 clones for further consideration

7 3 1

CROSS-REACTIVITY STUDIES OF ANTIBODIES AGAINST A PANEL OF NORMAL MOUSE CELLS

The remaining 8 clones (i e those demonstrating strong reactivity on LAT cells, little or no reactivity with

normal human leucocytes and continuous production of monoclonal antibody in vitro) were tested against a panel of normal mouse cells in solid-phase ELISA These normal cells were taken from the heart, lung, liver, thymus, spleen and kidney of Balb/c mice Three separate lots of each cell type, each taken from a different mouse, were used in these studies, the results of which are summarized in table 7 4

Three of the 8 clones examined in this study (B11, C12 and H5) showed extensive and often strong reactivity with a number of the normal cells used, in particular those derived from the spleen, liver and thymus Because of this, these 3 clones were discarded from further study In general, the 5 remaining clones (E10, E12, B4, C3 and D1) demonstrated lower levels of reactivity on the normal cells used as opposed to LAT and other tumour cells cells (below) The most frequently observed cross-reactions exhibited by these clones were with cells derived from mouse spleen and liver tissues Clone D1 showed the least degree of cross-reaction with the normal cells followed by clone E12 (moderate cross-reaction on liver cells only) When the 5 clones were compared in relation to their levels of reactivity with LAT cells, 3 (E10,E12 and D1) were shown to be strongly reactive, while B4 was moderately reactive and C3 was only weakly reactive On the basis of these results, clones E10,E12 and D1 were selected for further expansion and study

7 3 2

CROSS-REACTIVITY STUDIES OF CLONES F10 1 E10, F10 1 E12 AND F10 1 D1 AGAINST A PANEL OF TUMOUR CELLS

Monoclonal antibodies produced by clones E10, E12 and D1 were examined in solid-phase ELISA for cross-reactivity with a panel of tumour cells of mouse, rat, dog and human origins The results of this work are summarized in table 7 5

From these results, it can be seen that all 3 antibodies

exhibit strong reactivity with the murine myeloma and ascites tumour cells used Strong reactions were also recorded against 2 of the human tumour cell lines used (RPMI- an adenocarcinoma an SCC-9, a squamous cell carcinoma) Reactions were also seen with NRK (normal rat kidney) cells

7 4

DISCUSSION

The results presented in this section are only preliminary results (particularly in the case of antibody F10 1 E12) and a more detailed discussion and characterization of both the antibodies and the antigens against which they are directed is provided in section 10 Section 10 provides a detailed account of the work in the characterization of the sub-clones of clone F10 1 E12 by means of ELISA and Western immunoblotting The results provided in tables 7 4 and 7 5 are also covered in the discussion of section 10 This approach has been adopted in order to avoid any lengthy repetition of the discussion of results

The results, as presented here, suggest that the antigenic determinants against which the antibodies (E10, E12 and D1) are directed are weakly expressed on normal cells as opposed to tumour cells As such, the antigens involved appear to represent a quantitative difference rather than a qualitative difference between tumour and normal cells Also, the relatively strong reactions observed for the antibodies against some normal cells would seem to indicate that the antigens involved may be normally expressed on cells in certain tissues, most notably liver and spleen cells, against which antibody cross-reactions were most frequently observed (see Table 7 4)

Results from such a qualitative assay as the solid-phase ELISA used in this preliminary study must be interpreted with caution The solid-phase ELISA used here can only provide a positive or negative result in relation to the

expression of a given antigen Immune reactivity alone should not be taken as a measure of the level of expression of the antigen in question It was reported by Hakomori et al , (1971) that the glycolipid, GM-3, was present in lower concentrations on Rous sarcoma virus transformed cells than on untransformed cells However, when the cells were examined using an anti-GM-3 antibody, the transformed cells showed the greater level of reactivity The implication of this study was that the determinant, against which the antibody was directed, was displayed to a greater extent on the transformed cells versus the untransformed cells, despite the lower overall content of antigen on the transformed cells This is further supported by the report of Wolf and Robbins (1974), who demonstrated increased reactivity of an antibody against the Forssman glycolipid in normal cells following treatment of the cells with trypsin Untreated cells exhibited lower levels of reaction with the same antibody This finding further illustrates how an antigenic determinant may be present on a cell, without being available for reaction with an antibody

Unfortunately, at this point, a serious problem arose with mycoplasma, resulting in the loss of 2 of the clones (F10 1 E10 and F10 2 D1) described here Copies of those clones which were moderately reactive on LAT cells (F10 1 B4) were also lost Following much effort, the last of the strongly reactive clones, F10 1 E12, was rescued by passage in Balb/c mice to remove the mycoplasma (see Section 8) These cells were recloned and used for the further characterization of the LAT cell surface antigen against which their antibodies are directed by means of ELISA and Western immunoblotting (Section 10)

TABLE 7 1

SUMMARY OF HYBRIDOMA PRODUCTION IN FUSIONS USING LAT CELLS AS ANTIGEN

Fusion number	2	5	8	9
Total number of clones	96	74	101	207
Number of clones positive for LAT and normal human leucocytes	8(8 3)	3(4 0)	-	17(8 2)*
LAT-positive only	10(10 4)	-	-	2(1 0)

* % of the total number of clones

TABLE 7 2

THE FATE OF LAT-POSITIVE CLONES FROM THE FIRST 4 FUSIONS

Total number of LAT-positive clones	40	
Clones found to be reactive on normal white blood cells	28	(70 0%)*
Clones which ceased to produce antibody	7	(17 5%)
Clones positive for LAT cells which failed to grow or became contaminated	4	(10 0%)
LAT-positive clones which were cloned	1	(2 5%)#

* % of total LAT-positive clones

\# clone 9AW - subsequently found to be infected with mycoplasma (see Section 8)

TABLE 7 3

SUMMARY OF THE RESULTS OF FUSION 13 PRODUCTION OF MONOCLONAL ANTIBODY F10 1 E12

Frequency of hybridoma formation per $1x10^5$ spleen cell input	2 58
Total number of clones	258
Clones reactive on LAT cells	40
Reactive clones which ceased to produce antibody	10
Clones cross-reactive on normal human white blood cells	22
LAT-positive clones expanded for further study	8

TABLE 7 4

CROSS-REACTIVITY STUDIES OF MONOCLONAL ANTIBODIES FROM FUSION 13 AGAINST NORMAL MOUSE CELLS

Tissue	Antibodies							
	B11	C12	E10	E12	H5	B4	C3	D1
Spleen	++++	+++	++	+	++++	+++	++	+
Thymus	++	+++	+	+	+++	+	+++	+
Lung	+	+	+	+	++	-	+	-
Liver	++++	++	+++	++	+++	++	-	+
Heart	++	+	-	-	-	+	+	-
Kidney	+	+	-	+	+	-	+	-

Values represent the relative degrees of reactivity shown by each antibody against each cell type in ELISA ++++ = O D 405nm of > 0 20, +++ = O D 405nm of > 0 15 etc

TABLE 7 5

CROSS-REACTIVITY OF MONOCLONAL ANTIBODIES FROM FUSION 13 AGAINST A PANEL OF TUMOUR CELLS

Cell type	Cell origin	Antibodies E10	E12	D1
LAT	Mouse ascites carcinoma	++++	++++	+++
Ehrlich	Mouse ascites carcinoma	+++	++++	++
Sp2/0	Mouse myeloma	++++	++++	+++
IR983F	Rat myeloma	-	-	-
NRK	Normal rat kidney	+++	++++	+++
MDCK	Marin-Darby canine kidney	+	-	-
A549	Human lung carcinoma	-	-	-
RPMI	Human adenocarcinoma	+++	++++	+++
SCC-9	Squamous cell carcinoma (H)	+++	++++	+++
EJ	Human bladder tumour	+	+++	++
MRC-5	Normal human lung (foetal)	+	+	-

Values represent the relative reactivities of each antibody against each cell type in ELISA ++++ = O D 405nm of > O 20, +++ = O D 405nm of > O 15 etc

SECTION 8

THE ELIMINATION OF MYCOPLASMA FROM INFECTED HYBRIDOMAS BY PASSAGING IN BALB/C MICE

THE ELIMINATION OF MYCOPLASMA FROM INFECTED HYBRIDOMAS BY PASSAGING IN BALB/C MICE

8 1 INTRODUCTION

Mycoplasma infections represent a major problem in the routine culture of all animal cells (see Smith, 1971 for a review of their biology) In the production of monoclonal antibodies mycoplasma can have devastating effects leading to unsuccessful fusions, failure to produce any clones, very poor performance in culture, and loss of antibody production ability Failure to produce clones in cell fusion experiments where mycoplasma-infected cells were used can be attributed to the high levels of thymidine kinase possessed by many mycoplasma This enzyme cleaves thymidine to its component sugar and base thus making HAT selection (Littlefield, 1964) of hybridomas ineffective (Campbell, 1984) This can lead to the complete loss of potentially valuable and irreplaceable cells It is therefore, of very great importance that mycoplasma be routinely screened for, so as to allow for their early detection and elimination before the problem can extend itself to other uncontaminated cells This, of course, applies to all animal cell cultures

The detection of mycoplasma can be achieved in several ways (see Hessling et al , 1980, for a comparision of methods) The direct fluorescent assay, using Hoechst 33258 fluorochrome (Chen, 1977), offers one of the most rapid and reliable methods for mycoplasma detection and was the method used here

The successful elimination of mycoplasma is not so simple, however, and it is generally felt that such attempts should only be undertaken for the treatment of irreplaceable cells because of the risk of spreading the infection to other cells The successful use of

antibiotics and other chemicals in the treatment of mycoplasma infections has been reported (Stewart et al , 1969, Adams, 1980, Schmidt and Erfle, 1984), but resistance can develop rapidly Also, different species of mycoplasma vary in their sensitivities to such agents, making the choice of mode of treatment more difficult On the other hand, all mycoplasma species would appear to be susceptible to treatment by passage in the nude mouse (van Diggelen et al ,1977) or in the histocompatible Balb/c mouse in the case of murine myeloma and hybridoma cells

The use of nude mice for the elimination of mycoplasma from infected cells offers a simple and reliable means of obtaining mycoplasma-free cells from contaminated cultures Here, in the absence of of a nude mouse colony, Balb/c mice were used for the elimination of mycoplasma from infected hybridomas Success was achieved in 4 of the 5 cases examined

8 2

HYBRIDOMAS TREATED FOR MYCOPLASMA INFECTIONS

Due to mycoplasma infections, all of the clones described in section 7 were lost with the exceptions of clones 9AW and F10 1 E12 Clone F10 1 E12 had been cloned just prior to this outbreak of mycoplasma infection and the subclones 7C1, 8B1 and 10E3 were successfully cleared of infection by passage in Balb/c mice as ascitic tumours Efforts to rid other cells of mycoplasma by means of antibiotic treatments using kanamycin, gentamycin, tylosin (tylocine) and tetracycline (all from Gibco) were uniformly unsuccessful

Mycoplasma were routinely screened for by means of the Hoechst 33258 fluorochrome assay as described by Chen (1977) and modified by M Dooley and M Clynes (N.I H E D , personal communication, Section 2 2 8) Hybridomas found to be infected with mycoplasma were, where possible, injected into pristane-primed Balb/c mice to grow as ascitic tumours (Section 2) In some cases

this was not possible due to the lack of sufficent cell numbers to allow tumour formation. The minimum cell number required was usually of the order of $1x10^4$ cells. In general, however, $1x10^6$ cells were used for tumour induction.

8.3

RESULTS

8.3.1

INDIRECT DETECTION OF MYCOPLASMA BY EXAMINATION UNDER ORDINARY LIGHT MICROSCOPY

The presence of mycoplasma in cell cultures is not always overtly evident upon routine examination of cells in ordinary light microscopy. Indeed, some cells may appear to be quite healthy despite the presence of chronic mycoplasma infections.

In our study, the presence of mycoplasma could be inferred from alterations in the appearance and growth characteristics of the hybridomas in culture. These alterations included:

1. reduced growth rates;
2. reduced production levels of antibodies;
3. a ragged appearance to the cell surface and the presence of unsual vesicle-like projections from the cell membrane;
4. a marked granular appearance to the cytoplasm which, at the later stages of infection resulted in some cells appearing almost totally black, followed by the disintegration of the cells; and
5. the almost complete failure of infected Sp2/0 cells to produce clones in somatic cell fusion experiments.

In the absence of any other more obvious cause, such as bacterial contamination or poor quality culture reagents, any combination of the above features may be indicative of mycoplasma infections. Subsequent verification should be carried out using the direct fluorescent stain. During this time, the suspect cells should be held in

isolation to prevent any spread of infection

8 3 2

DIRECT DETECTION OF MYCOPLASMA BY HOECHST 33258 STAINING OF CELLS

Five hybridomas were found to be infected with mycoplasma The NRK cells from these positive tests all contained small, bright fluorescent foci in their cytoplasms, distinctly separate from the much larger fluorescent nucleus (Figure 8 1) These bright foci, representing the presence of mycoplasma, were mainly located at the cell periphery In some cases, they may appear as thin filaments in the cytoplasm (Hessling et al , 1977) The NRK cells from negative control cells were consistently devoid of any cytoplasmic fluorescent foci or filaments (Figure 8 2) They contained only a large nucleus against a dark background

3 3

ELIMINATION OF MYCOPLASMA FROM INFECTED CELLS

Infected cells were harvested and the cell numbers adjusted to $1x10^4$ to $1x10^6$ cell/0 5 mls of PBS The cells were then injected i p into pristane-primed Balb/c mice to form ascitic tumours Depending on the cell numbers used, ascites were ready for harvesting after 14 to 32 days Subsequent passages of these cells did not require the use of pristane, as the cells were sufficiently well adapted to this mode of growth after the first passage Following 3 to 4 passages in mice, the cells were recovered back into culture and retested for mycoplasma as before Of the 5 cell types used in this study, all but one (9C1) of the cell types was found to be free of mycoplasma

It was shown by van Diggelen et al ,(1977), that the presence of mycoplasma in cells could lead to altered tumour initiation and growth rates of normally tumourigenic cells in the nude mouse Here, a similar effect was observed in the Balb/c mouse (Table 8 1) The initial passage of infected cells took from 18 to 20 days

for abdominal swelling to become obvious due to tumour growth when using $1x10^6$ cells (7C1 and 8B1) as the starting innoculum In subsequent passages, carried out in the absence of pristane, cells could be harvested after 14 to 17 days It is possible that this time could have been shorter had pristane been used The reduced growth rate of the infected cells in vivo correlates to their reduced growth rates in vitro as compared to the growth rate of uninfected cells on recovery from mice

8 3 4

EFFECTS OF MYCOPLASMA ON ANTIBODY PRODUCTION IN VIVO

The first samples of ascitic fluid taken from mice following the injection of mycoplasma-infected cells were found to have lower titres of specific antibody than samples taken at later passages (Table 8 2) The reduced levels of antibody after the first passage may be due to the presence of mycoplasma which can be expected to place a serious metabolic burden on these cells, leading to a reduced production of secretory products As the burden of mycoplasma fell in later passages, a concommittent increase in antibody production was observed in 3 of the cases studied The exceptions were for cells 9A and 10W In the case of 9A, the failure to see an increase in antibody titre can be attributed to the persistence of its mycoplasma infection which remained even after 4 passages of the cells in mice In the case of clone 10W, the low titres of antibody were found to be due to overgrowth of the antibody-producing cells by non-producers This became evident during recloning of the cells after recovery from mice and the elimination of its mycoplasma

8 4

DISCUSSION

Mycplasma infections of hybridomas can result in a reduction in, or a complete loss of antibody production capacity by those cells It may also lead to the death of the cells with the loss of valuable and irreplaceable

clones, particularly if the contamination arises in the early stages after cell fusion experiments and during HAT selection

The elimination of mycoplasma can be achieved by the passage of infected cells as ascitic tumours in Balb/c mice This method is simple and reliable with the added advantage that the cells are not being maintained in culture where they would pose a serious threat to other non-contaminated cells Here, 4 of the 5 cultures studied were cleared of infections Cells can be rendered free of mycoplasma in 4 to 5 weeks when using pristane, or possibly in a shorter period of time if Freund's adjuvant is used (Mueller et al , 1986, Gillette, 1987) As few as $1x10^4$ cells may be successfully used by these methods, enabling the eradication of mycoplasma from hybridomas (should the need arise) soon after their generation

The presence of mycoplasma in hybridomas was found to result in a reduction in the tumour initiation rate for these cells in vivo This is in agreement with the findings of van Diggelen et al , (1977) when using the nude mouse to clear cells of mycoplasma This situation is reversed however, on the elimination of mycoplasma from cells This would also appear to be the case in relation to other adverse effects of mycoplasma on cells Removal of mycoplasma from hybridomas resulted in increased production levels of specific antibodies In some cases, it is possible that infection of cells may cause a complete loss of antibody production Whether or not such cells will regain the ability to produce antibody on removal of mycoplasma is unclear In general, however, it should be possible to salvage valuable hybridomas from mycoplasma infections by the procedure described here

TABLE 8 1

COMPARISION OF THE IN VIVO GROWTH RATES OF HYBRIDOMAS DURING SEQUENTIAL PASSAGES IN BALB/C MICE

HYBRIDOMA	INOCULUM SIZE	PASSAGE NUMBER	NUMBER OF DAYS TO CELL HARVEST
7C1	$1x10^6$	1	19
	$1x10^6$	2	17
	$1x10^6$	3	16
8B1	$1x10^6$	1	20
	$1x10^6$	2	16
	$1x10^6$	3	14

TABLE 8 2

COMPARISION OF SPECIFIC ANTIBODY TITRES FOR ASCITIC FLUIDS DURING SEQUENTIAL PASSAGES OF MYCOPLASMA-INFECTED CELLS IN BALB/C MICE

PASSAGE NUMBER	HYBRIDOMA				
	7C1	8B1	9AW *	10E	10W **
1	1 100	1 75	1 50	1 75	1 50
2	1 250	1 250	1 100	1 150	1 100
3	1 500	1 500	1 100	1 300	1 250
4	1 500	1 500	1 100	1 300	1 100

* This cell was not cleared of mycoplasma, even after 4 passages in the mice The small increase in antibody titre may be due to a reduction in the levels of mycoplasma in the cells

** This cell was later shown to be polyclonal Overgrowth by non-producing cells would account for the reduced titres in the later passages

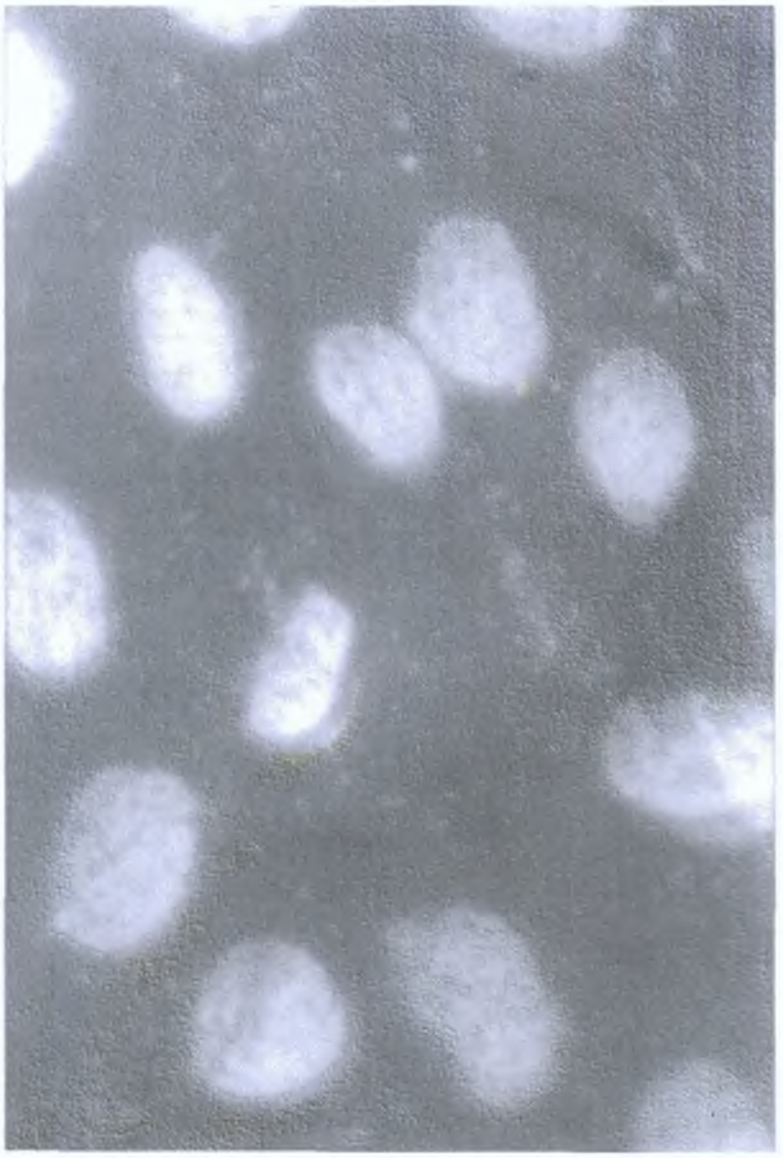

Figure 8.1

Fluorescent micrograph of NRK cells showing a positive result for the Hoechst 33258 fluorescence assay. Note the presence of small fluorescent foci outside the large nucleus, representative of mycoplasma infection. Compare this to Figure 8.2. Viewed under UV light at 100 x mag. with oil immersion.

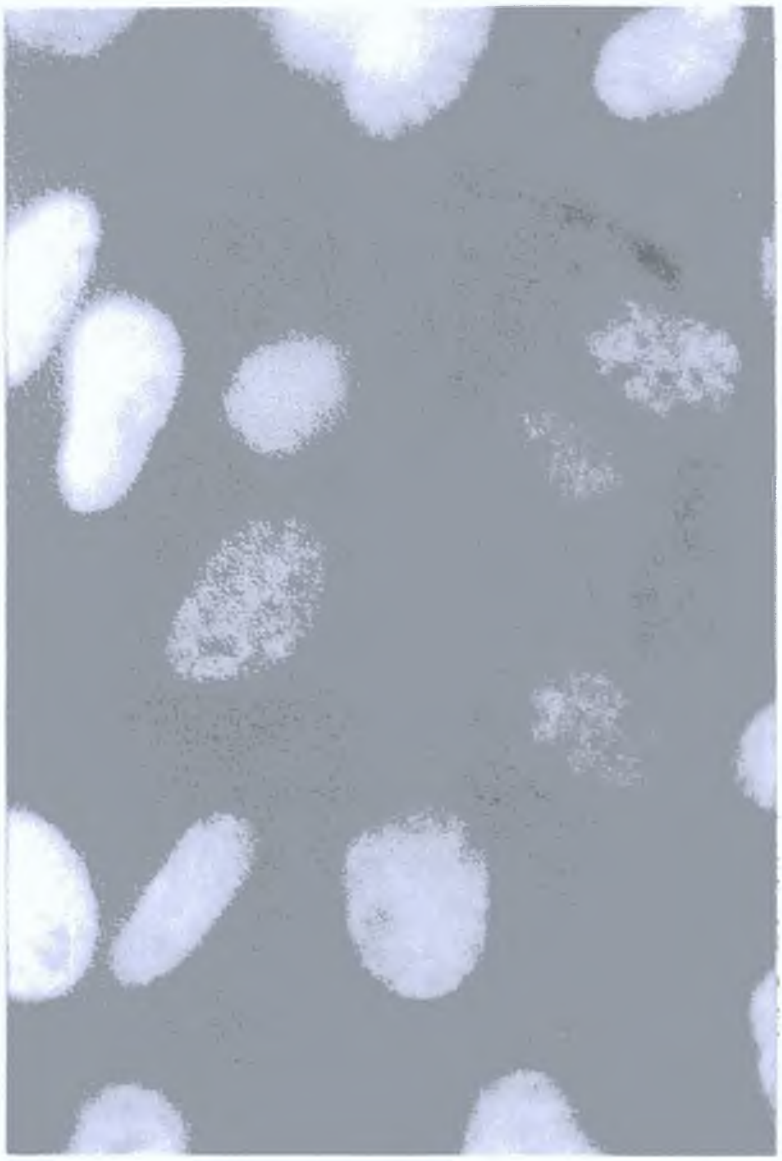

Figure 8.2

Fluorescent micrograph of NRK cells showing the absence of mycoplasma. Only the large cell nucleus is visible against a dark background.

Viewed under UV light at 100 x mag. with oil immersion.

SECTION 9

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN IMMUNOBLOTTING OF LAT CELL MEMBRANES A DISCUSSION OF THE METHODS USED

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN IMMUNOBLOTTING OF LAT CELL MEMBRANES A DISCUSSION OF THE METHODS USED

9 1 INTRODUCTION

Before going on to consider the characterization of the F10 1 E12 monoclonal antibodies and the antigen against which they are directed (Section 10), we feel it would be useful to briefly examine the major techniques of sodium dodecyl-sulphate polyacrylamide electrophoresis (SDS-PAGE) and Western-immunoblot analysis used in this project Particular emphasis will be placed on the subject of immunodetection of separated membrane proteins It is not the intention of this discussion to provide a detailed review of these procedures, but simply to examine them in the context of their use in this study The methods of SDS-PAGE and Western-blot analysis have been extensively reviewed (SDS-PAGE Hames, 1981, Blackshear, 1984, Western-blotting Towbin et al , 1979, Gershoni and Palade, 1983, Towbin and Gordon, 1984)

9 2 SDS-PAGE OF LAT CELL SURFACE MEMBRANE PROTEINS

9 2 1 SAMPLE PREPARATION

Crude preparations of LAT cell surface membranes were prepared as described in methods (Section 2 8 1) Solubilization of the membrane proteins was achieved by the addition of hot (100°C) SDS-electrophoresis buffer (0 62M Tris, 3 0% [w/v] SDS, 10% [v/v] glycerol, 0 5% [v/v] β-2-mercaptoethanol and 0 001% [w/v] bromophenol-blue) to the pelleted membrane material or to lyophilized preparations of membrane material which had been stored at -20°C prior to use The use of hot SDS sample buffer aids in the destruction of any proteases or other enzymes which may be present in the membrane samples (Blackshear, 1984)

The usefulness of PMSF (phenylmethylsulfonyl fluoride; 1mM) as a protease inhibitor in homogenization buffers has been called into question due to its inability to inhibit all proteases (McWilliams, 1986). Consequently, all steps in the isolation of LAT cell membranes were carried out on ice.

9.2.2

CONCENTRATION OF SDS IN SAMPLE BUFFERS

Membrane samples for SDS-PAGE were solubilized in SDS sample buffer (2mg protein/ml) and boiled for 5 min. They were then allowed to cool to room temperature before being centrifuged at 100,000xg for 90 min. In the original method of Laemlli (1971), the sample buffer contained 2% (w/v) SDS. Here , however, it was found to be necessary to use 3% (w/v) SDS in the sample buffer. This was due to high levels of particulate matter found in membrane samples solubilized in 2% (w/v) SDS sample buffer as evidenced by the the relatively large pellets obtained when such samples were centrifuged at 100,000xg for 90 min. When these samples were applied to SDS-PAGE gels, a dark band was seen at the sample application point, which was indicative of particulate or aggregated, insoluble material in the samples. The levels of insoluble material were reduced by using a 3% (w/v) SDS sample buffer, although tiny pellets of particulate matter were sometimes obtained following the centrifugation of samples prior to electrophoresis. The nature of the particulate matter was not investigated further. It may be possible that even greater solubility could be achieved by increasing the pH of the sample buffer above pH 6.8 (Hames, 1981).

9.2.3

ELECTROPHORESIS OF LAT MEMBRANE SAMPLES

Following the centrifugation of solubilized membrane samples to remove any particulate matter, 50µl sample volumes (containing 100µg of membrane protein) were applied to vertical slab SDS polyacrylamide gels (7.5% resolving gels with 4% stacking gels) for the

electrophoretic separation the component proteins Sample wells were 1 5mm x 10mm in size The use of a stacking gel offers the advantages of allowing large and/or dilute samples to be applied to the gel During the stacking process the proteins are concentrated into a very narrow starting band of locally high protein concentration This facilitates a high degree of resolution of the individual proteins in the resolving gel (Chrambach and Rodbard, 1971) Horizontal slab gel electrophoresis does not offer this facility (i e the use of a stacking gel) and results obtained using this method were much less satisfactory than those obtained for the vertical system

9 3

WESTERN-BLOTTING

For Western-immunoblotting experiments the protein concentrations of the samples applied to the electrophoresis gels were lower than those used when the gel itself was to be stained for total membrane protein profile In general, samples were diluted 1 10 in SDS-sample buffer for use in blotting experiments In other systems the appropriate dilution of sample to use will need to be determined empirically and will depend, to some degree, on the sensitivity of the antibodies used This also applies to other non-immunological detection systems e g the use of lectins etc Dilute samples are used in order to avoid excessive problems with non-specific uptake of antibodies leading to erroneous results In cases where undiluted LAT membrane protein samples (i e 2 0 mg protein/ml) were used, a number of bands were detectable on electroblots probed with rabbit anti-mouse Ig conjugated to alkaline phosphatase (Sigma) alone, indicative of the non-specific uptake of second antibody (i e the anitbody-enzyme conjugate) by the separated proteins This problem was significantly reduced when diluted (1 10) protein samples were used for electrophoresis

9.4

IMMUNOSTAINING OF WESTERN-BLOTS

9.4.1

BLOCKING SOLUTIONS FOR ELECTROBLOTS

BSA is the agent most commonly used for the blocking of non-specific binding sites on nitrocellulose (NC). However, there have been some reports questioning the suitability of any individual solution as a universal blocking agent in Western-blot analysis, particularly in relation to the use of antibody probes. It may well be that the choice of blocking agent will need to be determined empirically for each individual situation. Hauri and Bucher (1986) reported on the use of 4 different blocking agents (foetal calf serum, mammalian gelatine-Nonidet-P-40 [NP-40], fish-gelatine-NP40 and defatted powdered milk) in Western-blot studies. They found that different antibodies gave optimal results in the presence of different blocking agents. Given that a preparation of monoclonal antibody is a homogeneous solution, any factor affecting the reaction between the antibody and its antigen can be expected to have serious consequences in relation to the usefulness of the antibody under those conditions. With a polyclonal antibody these effects are likely to be less serious, since only a small proportion of the total antibodies are likely to be affected. Similar results to those reported by Hauri and Bucher (1986) have been reported by Wedege and Svenneby (1986) using both Tween-20 and BSA at different pH values (7.2 and 10.2).

Batteiger et al., (1982) reported that the use of Tween-20 alone provided adequate blocking of free protein-binding sites on NC. The findings of Hoffman and Jump (1986) indicate that Tween-20 may in fact remove some (though not all) proteins and antibodies from NC. Tween-20 was however, found to be much less efficient in the removal of proteins from NC than other detergents like NP-40 (Lin and Kasamatsu, 1983) and Triton X-100 (Batteiger et al., 1982; Gershoni and Palade, 1982). Based on these reports, the

use of both NP-40 and Triton X-100 has been widely discontinued in blocking solutions on NC It may be possible to overcome the stripping of protein from the blot matrix by covalently attaching the protein to the matrix Use of Diazophenylthio (DPT) and Diazobenzyloxymethyl (DBM) papers readily allow for this (Renart and Sandoval, 1984) However, the resolution obtainable on these support matrices tends to be less than that of NC due their coarseness

The use of Tween-20 as a blocking agent for ELISA and immunostaining of electroblots was prompted by the reports of Crumpton and Parkhouse (1972) and Dimitriadis (1979) that non-ionic detergents did not disrupt immune complexes More recent reports indicate that Tween-20 may cause some non-specific binding of antibodies and some false positive reactions observed on NC may be due to hydrophobic interactions between antigens and antibodies mediated by detergents (Gershoni and Palade, 1982, Wedege and Svenneby, 1986)

In the work carried out here, BSA, casein and Tween-20 were all used as blocking agents without any differences in immune reactivity being observed This may only be a reflection of the stability of the antibodies used (monoclonal antibodies 7C1, 8B1 and 10E3, Section 7) in the presence of these agents and under the conditions of use It is possible that other working conditions may not have been so favourable to the use of these antibodies

9 4 2

DETECTION SYSTEMS USED WITH WESTERN-BLOTTING

The most commonly used probes in Western-blot analysis are monoclonal antibodies Western-blot analysis has proven itself to be a powerful and highly sensitive tool for the study of antibody-antigen interaction Other detection (probing) systems used include lectins (WELLA Western enzyme-linked lectin analysis, Reading and Hickey, 1985) and the Biotin-Streptavidin complex for the quantitative estimation of glycoproteins on electroblots (O'Shannessy et al , 1987)

In the immune-detection of antigens on electroblots, most variation is centred on the choice of enzyme conjugate for the visualization of the antigen The most common enzymes are horseradish peroxidase and alkaline phosphatase though other enzymes have also been used e g glucose oxidase (Geoghegan et al , 1986) As an alternative to the use of enzyme based visualization, colloidal metals (gold or silver) may also be used (Moeremans et al , 1985) These are often used for the detection of total protein patterns as opposed to the detection of individual proteins The use of dual detection systems is also becoming increasingly more popular Electroblots are stained for total protein using a reversible stain like India ink or amido-black followed by the specific immunodetection of the antigen of interest (Glenney, 1986, Harper et al , 1986) This permits the accurate determination of the position of the specific band in relation to the total band pattern, which is of importance in molecular weight determinations Certain stains like Coomassie blue may interfere with immunoreactivty (Harper et al , 1986) Another alternative is to successively probe the blots with different antibodies, developing each one with an enzyme yielding a different coloured product Thus, a number of specific antigens may be detected on a single blot by probing it with a range of different antibodies (Daneels et al , 1986)

9 5

FAILURE TO DETECT SOME ANTIGENS BY WESTERN-BLOT ANALYSIS

There are a number of possibilities as to why an antibody may fail to detect a specific antigen following electrophoresis and electroblotting The most common reason is the denaturation of the antigen in SDS with the disruption and loss of many antigenic determinants, particularly structural epitopes Other possibilities may include the failure to resolve the particular antigen in the electrophoresis stage, too low a concentration of antigen being used, or the use of conditions incompatible with the antibodies being used for immunodetection These problems will be discussed in detail in the following sections

9 5 1

DENATURATION OF ANTIGEN

Where denaturation of the antigen results in the loss of the antigenic determinant bound by a given antibody, the use of non-denaturing conditions may be examined However, this option is not always available, particularly in the case of studies on membrane antigens which necessitate the use of denaturing conditions in order to be able to resolve the individual components by electrophoresis It may be possible to overcome this problem by first reacting the antibody with the antigen in its native state followed by the isolation of the immune complex This approach was successfully used by Hamada and Tsuruo (1987) who employed the use of a cleavable crosslinking agent (dithiobis[succinimidyl propionate]) to covalently link a monoclonal antibody to its antigen in an intact cell membrane The immune complex was then solubilized, immunoprecipitated and analysed by SDS-PAGE

Dunn (1986) reported on improved antibody recognitior of antigens when the electrotransfer step was performed in carbonate buffer at pH 9 9 as opposed to the more common Tris-glycine buffer, pH 8 3 The carbonate buffer system supposedly promotes the renaturation of the antigens, thereby allowing better antibody-antigen recognition to take place The incubation of the gel in a mild buffer (20% v/v glycerol and 50 mM Tris-HCl, pH 7 4) prior to transfer to NC and immunodetection was also reported to aid in the renaturation of antigens with subsequent improvements in antibody-antigen recognition Whether or not these methods can be further refined for more general use remains to be seen

9 5 2

FAILURE TO RESOLVE AN ANTIGEN BY ELECTROPHORESIS AS A REASON FOR FAILING TO DETECT THAT ANTIGEN

It may happen occasionally that the specific antigen bound by a given antibody is not being solubilized under the conditions used e g not all membrane proteins are

solubilized by NP-40, a non-ionic detergent commonly used in isoelectric focusing studies (Mescher et al , 1981) SDS is much more effective in the solubilization of proteins and is avidly bound by basic, neutral and acidic proteins alike (Blackshear, 1984) Even here, however, it is not uncommon to find some particulate matter in SDS-solubilized cell membrane preparations which is not resolved by SDS-PAGE

The incorrect choice of resolving gel may also result in one's failure to detect a given antigen If the molecular weight of the antigen being sought lies outside the limits of resolution of the gel used, then the antigen will not be detected on electroblots derived from this gel Indeed, it may be good practice to use gradient SDS-PAGE gels for the initial detection of an antigen Such an approach (providing the denaturation of the antigen did not present itself as a problem) could provide some indication as to the effective gel concentration required for the resolution of the antigen prior to more extensive studies being undertaken

9 5 3

LOW CONCENTRATION OF ANTIGEN AS A REASON FOR FAILURE TO DETECT THAT ANTIGEN

In some cases, the antigen being sought may represent a minor antigen in a complex mixture, as is often the case when dealing with cell surface antigens This fact may contribute to one's failure to detect that antigen The use of more concentrated samples for electrophoresis is the obvious solution to this problem but care must be taken to ensure that excessive non-specific uptake of antibodies does not occur as a result of this The use of appropriate controls (which should be employed in any case) is therefore of particular importance Amplification of the signal to noise ratio offers another possible solution to this problem The use of the alkaline phosphatase anti-alkaline phosphatase or the peroxidase anti-peroxidase (PAP) system, coupled with colour intensification of reaction products by the inclusion of

nickel and cobalt in the buffers (De Blas and Cherwinski, 1983), offers some possibilities Swerdlow et al , (1986) reported on increased immunosensitivity when electroblots were hydrated and heated prior to reaction with antibodies This was found to be particularly successful for the immunodetection of peptides, though some native proteins exhibited a decreased sensitivity under the same conditions Enzyme amplification systems developed for immunoassays (Johannsson et al , 1986, Carr et al , 1987) may also find applications in the area of Western immunoblotting should suitable enzyme substrates yielding insoluble precipitates as products become available

9 5 4

BLOCKING AGENTS AS A CAUSE OF FALSE-NEGATIVE RESULTS IN WESTERN IMUNOBLOTTING

As detailed above (Section 9 4 1), the choice of blocking agents may contribute to the failure to detect a given antigen Where possible, the conditions under which an antibody is used in immunoblot analysis should be kept as close as possible to those under which the antibody was used in previous assays (e g ELISA)

9 6

CONCLUDING COMMENTS

Western electroblotting has proven itself to be a powerful analytical technique combining the resolving power of electrophoresis with the specificity of antibodies and other probes (e g lectins) More recently, it has also been used to supplement isoelectric focusing studies (Otey et al , 1986, Matthaei et al , 1986) as well as studies on glycosphingolipids (Buehler and Macher, 1986) The method can also be extended to the study of proteolytic digests of specific antigens in order to ascertain the nature of the epitope(s) being bound by given antibodies The current trend towards sequential immunostaining (Daneels et al , 1986) also adds to the usefulness of this technique by allowing greater economy of antigen and reagents It also allows much greater amounts of information to be gleaned from individual experiments

SECTION 10

CHARACTERIZATION OF MONOCLONAL ANTIBODY F 10 1 E12

10 1 INTRODUCTION

Following the recovery of F10 1 E12 from mycoplasma infection (Section 8), this clone was returned to cell culture and recloned as described in Methods (Section 2 6 7) Three subclones from this cloning experiment, 7C1, 8B1 and 10E3, were selected for further study and the characterization of the antigen against which they were directed The characterization of these antibodies can be considered in four parts

1 isotype analysis of the antibodies,
2 further cross-reactivity studies against a panel of tumour and normal cells in solid-phase ELISA,
3 immunoblot analysis, and
4 enzyme digestion studies of the separated antigen on nitrocellulose (NC) coupled with immunostaining

10 2 ISOTYPE ANALYSIS OF THE ANTIBODIES

This was approached in two ways Firstly, by solid-phase ELISA using anti-mouse Ig subclass and light chain specific antibodies, and secondly, by HPLC

10 2 1 ISOTYPE ANALYSIS BY ELISA

A mouse antibody isotype analysis kit (Zymed) was used, making use of a solid-phase ELISA system (Section 2 7 1) Ascitic fluids, taken from Balb/c mice bearing tumours derived from cloned F10 1 E12 cells, were used as the source of mouse monoclonal antibodies for isotype analysis A range of dilutions of the ascitic fluid were used to coat each microtitre plate The reason for this approach was to permit the determination of the monoclonal antibody isotype without interference from

naturally occuring antibodies in the ascitic fluids of the tumour-bearing mice By reducing the amount of the natural antibodies to a level below the detection limit of the ELISA assay, the monoclonal antibody, due to its far higher concentrations in the ascitic fluid, would remain within the detection limits of the assay Hence, by this procedure, information could be gained about the isotype of the monoclonal antibody and the nature of its light chain Some information was also gained concerning the nature of the major classes of contaminating antibodies of mouse origin present in the ascitic fluids

From this study, it was found that the antibodies secreted by the clone F10 1 E 12 and its subclones, 7C1, 8B1 and 10E3 were of the IgM class with a k-light chain HPLC studies were used to confirm that the antibody was of the IgM class (see Figure 10 1)

10 2 2

ISOTYPE ANALYSIS BY HPLC

Ascitic fluid from a Balb/c mouse bearing a tumour derived from F10 1 E12 was analysed for antibody content by HPLC (Section 2 7 2) A sample from which the antibody had been removed by ammonium sulphate precipitation and the precipitated material were also examined by HPLC The neat ascites sample had a major peak corresponding to the elution position of IgM (Figure 10 1A), as well as other minor peaks, as expected from the results obtained from ELISA studies (above) Ascites fluid from which the antibody fraction had been precipitated, lacked this major peak (Figure 10 1B), as determined by molecular weight studies (horseradish peroxidase, 45kD, bovine serum albumin, 67kD, lactate dehydrogenase, 140kD, catalase, 323kD) The precipitated material, however, was found to elute in the IgM position, based on molecular weight studies (Figure 10 1C) Further analysis of this fraction by solid-phase ELISA against LAT cells, revealed anti-LAT cell reactivity in this fraction These findings support the observation that the antibodies being produced are of the

IgM class The HPLC method of isotype analysis could be further modified to allow light chain analysis, by examining antibodies under denaturing conditions Furthermore, by adding anti-isotype or anti-light chain specific antibodies to the test samples, one could look for shifts in sample peak positions, which would be indicative of the nature of the antibodies being examined This particular development may also be applied to the study of membrane antigens and other molecules by the coupling of antibody specificity to the resolving power of HPLC

10 2 3

CONCLUDING COMMENTS ON ISOTYPE ANALYSIS OF F10 1 E12 ANTIBODIES

The finding that the antibodies produced by F10 1 E12 and its subclones are of the IgM class was unexpected, since the mice used as a source of immune B-cells for the generation of these antibodies had received extensive immunization (4 injections over a 6 week period) IgM antibodies are normally associated with primary immune responses (Eshar, 1985) Given the extent of the immunization protocol used, one would have expected to see antibodies predominantly of the IgG class Why the antibodies generated here should be of the IgM class is unclear, but there are some possible explanations Firstly, the parent B-cell which gave rise to the F10 1 E 12 antibody may have been a newly sensitized cell with no previous exposure to the antigen Since newly sensitized B-cells are believed to make the best fusion partners (Goding, 1980, Spitz et al , 1984), it is conceivable that such cells would be predominant in the formation of hybridomas

Secondly, some antigens do not produce a secondary immune response (only producing IgM) Such antigens are often T-independent immunogens, often having repeating structures which directly stimulate B-cells In some non-responder animals, T-dependent antigens may also fail to produce secondary immune responses (Tijssen, 1985)

The fact that the antigen against which the antibody F10.1.E.12 is directed may have failed to stimulate a secondary immune response in Balb/c mice may be further supported by the apparently low titre of this antibody for the antigen. This is further reflected in the relatively low working titres for this antibody observed in ELISA (1:500 versus commonly reported titres of up to 1:10,000 for other antibodies). Primary response antibodies tend to be of a lower affinity than those produced in secondary responses (Tijssen, 1985), possibly due to the selection of higher affinity antibodies during the maturation of the immune response.

By using lower dilutions of ascitic fluids for the coating of microtitre plates prior to isotype analysis, interference was observed from naturally occurring antibodies in the ascites. From the levels of reaction observed for these antibodies, it was possible to arrive at some conclusions concerning the nature of the contaminating antibodies (Table 10.1). The most frequent contaminant was of the IgG_{2a} class followed by IgG_{2b} and IgG_1. In the case of 10E3, IgG_{2b} was the major contaminant, closely followed by IgG_{2a}. Antibody from clone 9AW (Section 7.2.1), was contaminated with IgA as well as IgG_{2a}. This information may be of value in the design of other experiments where unpurified monoclonal antibodies derived from ascitic fluids may be used.

10.3
STUDIES ON THE CROSS-REACTIVITY PROFILES OF THE ANTIBODIES 7C1, 8B1 AND 10E3 AGAINST A PANEL OF TUMOUR AND NORMAL CELLS IN SOLID-PHASE ELISA

Panels of both tumour and normal cells were established to study the cross-reactivity profiles of the three hybridoma cell lines 7C1, 8B1 and 10E3. This was to supplement the earlier studies outlined in section 7.2.1 (Tables 7.4 and 7.5), as well as to ensure that these clones exhibited the same general range of reactivities

as exhibited by the parental clone, F10.1.E12. The new panel of tumours cells included a selection of leukaemic cell samples (peripheral blood leucocytes) taken from a number of leukaemic patients. The reactivity of the antibodies was assessed by solid-phase ELISA (Section 2.4). The results of these cross-reactivity studies are summarized in tables 10.2, 10.3 and 10.4 (cross-reactivity on panels of normal cells tumour cell lines and human leukaemic cell samples respectively). Only antibodies 7C1, 8B1 and 10E3 were used in these studies. These results are also compared with those presented for F10.1.E12 in tables 7.4 and 7.5.

10.3.1

CROSS-REACTIONS OF ANTIBODIES ON NORMAL CELLS

It can be seen from the results in table 10.2 that all three antibodies exhibit a limited reactivity with certain of the normal cells used here. The notable exceptions were NRK cells, human tonsils and mouse liver cells which exhibited strong reactivities with all three antibodies. Antibody from 7C1 also exhibited reactivity with normal mouse spleen cells. For all of the other cells examined, the levels of reactivity, as determined by the solid-phase ELISA methods used here, were markedly lower than those observed for LAT cells.

10.3.2

CROSS-REACTIONS OF ANTIBODIES WITH TUMOUR CELLS

The spectrum of observed cross-reactions with tumour cells (Tables 10.3 and 10.4) was found to be much broader. Good reactivity, comparable to that seen for LAT cells, was observed for both the murine myeloma and ascites tumour cell lines examined (Sp2/0 and Ehrlich ascites tumour cells). Of the human tumour cell lines used (all grown in vitro), RPMI-2650 (adenocarcinoma) and SCC-9 (squamous cell carcinoma) and EJ (bladder tumour) gave strong reactions to each antibody, while Molt-4 (T-cell leukaemic cell line) reacted with 7C1 only. In no instance was the level of reaction on any tumour cell

as high as that recorded for LAT cells. In general, where reactivity was observed between the antibodies and the cells being examined, the levels of reaction varied from 50 to 65% of that for the LAT cells, although reaction levels with Ehrlich ascites tumour cells and Sp2/0 cells rose to as high as 85% of the level of reaction for LAT cells when using the 10E3 monoclonal antibody.

The human leukaemias (Table 10.4) showed the greatest degree of variability in terms of reactivity with all three antibodies used (7C1, 8B1 and 10E3). Of the leukaemic samples examined, 6 were CLL's and 2 ALL's (chronic lymphocytic leukaemias and acute lymphoblastic leukaemias respectively). Only 1 patient (P.H.) produced a consistently strong reaction wtih all 3 antibodies, which was comparable to that observed for LAT cells. A similar pattern was seen for N.O'N., although the degree of reactivity was lower (approx. 50%). Of the CLL samples, 5 out of 6 reacted with 7C1 (1 strongly and 4 moderately). For 8B1, 4 of the 6 samples reacted (1 strongly and 3 moderately) while 3 out 5 of samples reacted with 10E3 (1 strongly and 2 moderately). All 3 antibodies reacted with 1 of the ALL samples (K.M'C.). The reaction was greatest for 10E3. The second ALL sample failed to react with any of the antibodies.

10.3.3

DISCUSSION OF ELISA BASED CROSS-REACTIVITY STUDIES

The cross-reactivity studies carried out on the monoclonal antibodies 7C1, 8B1 and 10E3, against panels of normal and tumour cells (Tables 10.2, 10.3 and 10.4) revealed a restricted level of reaction for these antibodies with normal versus tumour cells. These findings are in keeping with the earlier findings reported in Tables 7.4 and 7.5 for antibody from the hybridoma F10.1.E12, the parent clone from which the subclones producing the monoclonal antibodies 7C1, 8B1 and 10E3 was derived. As expected, no major differences

were noted between the reactivity profiles of the parental clone, F10 1 E12, and its subclones 7C1, 8B1 and 10E3, though some differences did exist between the individual subclones In general, such differences were of a minor nature Since all 3 hybridomas were derived from a common ancestor, their antigenic specificities must be identical Therefore, any observed differences in reactivity must be a reflection of the different affinities of each antibody for its antigenic determinant This is further supported by the fact that the antibodies were used at concentrations designed to saturate all of the available binding sites on the cells used in these studies (i e antibodies were used at lower dilutions than the known titres of the antibody preparations, as determined by solid-phase ELISA using LAT cells as the fixed antigen)

The major finding from these antibody cross-reactivity studies is that the antigenic determinant against which the antibodies are directed is not specific for tumour cells alone but is also expressed by certain normal cells, notably mouse liver and spleen cells as well as NRK (normal rat kidney cells) Those normal cells on which the antigen is expressed, express it at a lower level than LAT cells and certain other tumour cell lines, notably Ehrlich ascites tumour cells and the Sp2/0 myeloma cell line) Other tumour cells which were found to react with the antibodies appear to express the antigen at the same level as normal cells which express this antigen However, the expression of this antigen was found to be more common among tumour cells, thus making this antigen potentially interesting as a target for antibody-mediated drug targeting and <u>in vivo</u> localization in a model system using LAT cell tumours in Schofield mice Furtner studies on the nature of the antigen(s) involved are needed before undertaking such studies (see following sections)

10 4

LIMITATIONS OF ELISA AS A SCREENING ASSAY

ELISA, as a means of screening hybridomas for the production of specific antibodies against a desired antigen, is of unquestionable value However, when complex antigenic mixtures like whole cells are being used and no other information is available on the nature of the specific determinant(s) being bound by the antibodies under study, ELISA can only provide very limited amounts of further information concerning the nature of the antigen Most of this information is of a qualitative nature, determining the positive or negative expression of the antigen involved and providing an indication as to the relative levels of expression of that antigen on the cells being examined Even where positive expression of the antigen is indicated by ELISA, some caution must be exercised in comparing the reactivities of an antibody with different cells

The antigens being detected by the monoclonal antibodies 7C1, 8B1 and 10E3 appear to represent a quantitative difference rather than a qualitative difference between tumour and normal cells Immune reactivity alone should not be taken as a measure of the level of expression of the antigen in question As pointed out earlier (Section 7 4), certain antigens may be present on the cell surface but in a form which is unavailable for reaction with antibodies (cryptic antigens) The report of Hakomori et al (1971) serves to illustrate this point very well They showed that the concentration of the glycolipid GM-3 was lower on Rous sarcoma virus transformed cells than on untransformed cells However, when the cells were examined using an anti-GM_3 antibody, the transformed cells demonstrated a higher level of reactivity This implied that the determinant against which the antibody was directed, was displayed to a greater extent on the transformed cells versus the untransformed cells, despite the lower overall content of antigen on the transformed cells This is further supported by the report of Wolf and Robbins (1974), who demonstrated increased reactivity

of an antibody against the Forssman glycolipid in normal cells following treatment of the cells with trypsin Untreated cells exhibited lower levels of reaction with the same antibody This finding further illustrates how an antigenic determinant may be present on a cell, without being available for reaction with an antibody

In order to gain further information on the nature of the antigen(s) being bound by the antibodies 7C1, 8B1, and 10E3, methods other than ELISA must also be used One of the most widely used techniques used for this purpose is Western immunoblotting of membrane proteins onto nitrocellulose (NC) (Towbin et al , 1979) followed by probing with monoclonal antibodies (see Section 9) in order to detect the specific antigens bound by the antibodies used

10 5

WESTERN IMMUNOBLOTTING STUDIES OF LAT CELL MEMBRANE PROTEINS USING MONOCLONAL ANTIBODIES 7C1, 8B1 AND 10E3 AS PROBES

LAT cell membranes were subjected to electro- phoresis in the presence of SDS and β-2-mercaptoethanol (see Section 2 8 and Section 9) The separated proteins were either visualized by Coomassie Brilliant blue R-250 staining or electrophoretically transfered onto NC membranes to be probed with monoclonal antibodies 7C1, 8B1 and 10E3 in order to detect the specific proteins bound by them The electroblots were either probed directly with antibody, or else treated with various enzymes prior to probing The enzyme treatments were designed to provide some insight into the chemical nature of the specific epitopes detected by the antibodies

10 5 1

TOTAL PROFILE OF LAT CELL MEMBRANE PROTEINS

LAT cell membrane preparations (Section 2 8) were resuspended in SDS electrophoresis sample buffer at 2 0mg protein/ml and separated on 7 5% SDS polyacrylamide gels

with a 4% stacking gel Both 5% and 10% resolving gels were also used, but best results were obtained with the 7 5% gels and these gels were used in all subsequent experiments Proteins detected on these gels ranged in molecular weights from 40,000 up to 250,000 daltons Figure 10 2 presents a typical separation profile of the LAT cell membrane proteins The molecular weight markers used on this and all other gels were myosin (205 kD), β-galactosidase (116 kD), phosphorylase-b (97 kD), bovine albumin (66kD), egg albumin (45 kD) (all supplied by Sigma, Kit no MW-SDS-200)

10 5 2

IMMUNOBLOTS OF LAT CELL MEMBRANE PROTEINS PROBED WITH MONOCLONAL ANTIBODIES 7C1, 8B1 AND 10E3

For the purposes of electroblotting, SDS-solubilized LAT membranes were diluted 1 10 in electrophoresis sample buffer prior to electrophoresis (see Section 9 3) This method was used to ensure that excessive levels of non-specific uptake of antibodies leading to false positive results were avoided Because immunological detection systems are generally much more sensitive than Coomassie blue staining (ng versus µg range), a 1 10 dilution of the initial sample does not significantly detract from the detection of an antigen by immunological methods

The monoclonal antibodies 7C1, 8B1 and 10E3 used in this and all subsequent experiments, were produced by the growth of the respective hybridomas as ascitic tumours in Balb/c mice The ascitic fluids were diluted 1 100 in blocking solution (BS 10mM Tris, 150mM NaCl, 0 5% v/v Tween-20, pH 7 5) for use in immunodetection of proteins on NC The working titres of the neat ascitic fluids, as determined by solid-phase ELISA were 1 500 The lower dilution of 1 100 used in immunoblotting was to ensure that all of the specific antibody binding sites on the NC would be occupied and also to allow for any loss of antibody activity arising from storage

When NC electroblots of LAT cell membrane proteins were probed using the monoclonal antibodies 7C1, 8B1 and 10E3, 3 specific protein bands were detected (Figure 10.3). The banding patterns were the same for all 3 antibodies, as was expected from the fact that they all arose from a common ancestor (F10.1.E12; Section 7). The molecular weights (mw) of these bands were determined from a plot of Log mw versus Rf values of the molecular weight standards used (Figure 10.4). The Rf value is determined by the equation:

$$Rf = \frac{\text{distance of migration of the protein}}{\text{distance of migration of the dye front}}$$

The specific bands detected by the monoclonal antibodi es were of apparent mw's: a). 205kD; b). 71kD; and c). 48kD. Henceforth, these bands will be referred to as p-205, p-71 and p-48 respectively.

Of the three proteins, p-205 was to prove itself to be the most interesting. Both the p-71 and p-48 bands were found to be artifacts arising from the non-specific uptake of the second antibody (anti-mouse Ig-linked alkaline phosphatase) by the separated membrane proteins. Electroblots of LAT cell membrane proteins which were probed with second antibody in the absence of any probing with anti-LAT monoclonal antibodies were found to have both p-71 and p-48 bands, but not p-205. The implication of this is that bands p-71 and p-48 both bound the second antibody. The p-48 band may represent a mouse Ig-heavy chain expressed at the cell surface by the LAT cells. In support of this, the molecular weight of the antibody heavy chain is known to be approximately 50kD and the same band was found to appear when mouse serum and ascitic fluids were probed under the same conditions (Figure 10.5). Under the electrophoretic conditions used here for the separation of proteins (i.e. SDS and 2-β-mercaptoethanol), the immunoglobulin heavy and light chains would have been dissociated from one another. Both serum and ascitic fluids contain naturally occuring antibodies which can be expected to give rise to such a band when probed with anti-mouse Ig conjugates.

The nature of the p-71 band is less clear, but some evidence suggests that it may represent an Fc-receptor protein. This band only appeared on those blots which were probed with whole antibody-enzyme conjugates and was not evident on blots probed with $F(ab')_2$ anti-mouse antibody enzyme conjugates. The source of these Fc-receptors is unclear but contaminating macrophages in the LAT cell preparations may be responsible. This problem was also encountered in the development of the ELISA assay as described in Section 5.3.2. These receptors should have no effect on the binding the monoclonal antibodies 7C1, 8B1 and 10E3 which were all found to be of the IgM class of antibodies (Section 10.2) and therefore lacking a free Fc portion for the binding of Fc-receptors.

10.5.3

COMPARISION BETWEEN LAT CELLS AND A PANEL OF NORMAL MOUSE CELLS BY WESTERN IMMUNOBLOT ANALYSIS

A panel of normal mouse cells (kidney, liver, spleen, thymus, heart and lung) were prepared for electrophoresis as described for LAT cells (sections 2.8 and 9). The object of this experiment was to determine whether or not the monoclonal antibodies 7C1, 8B1 and 10E3 would exhibit the same patterns of reactivity as seen in solid-phase ELISA cross-reactivity studies (Section 10.3.1). The human lung adenocarcinoma cell line, A549, was also included in this study.

Of the 3 proteins bands (p-205, p-71 and p-48) detected on LAT cells, only p-48 could be detected on the panel of normal cells, and then only for mouse spleen cells (Figure 10.3). In the light of the evidence to suggest that p-48 represents mouse antibody heavy chain protein (Section 10..5.2), the appearance of this band in spleen cells is not surprising, since spleen cells represent a rich source of immune B-cells, all expressing surface antibodies.

Normal liver cells, which showed the highest levels of reactivity with the monoclonal antibodies 7C1, 8B1 and 10E3 in solid-phase ELISA, failed to react with the antibodies following Western immunoblotting. This finding would seem to indicate that the antigen bound by the antibodies in normal cells is different from that expressed by the LAT cells. A second possibility is that it may be the same antigen, but expressed in a more labile form on normal cells as compared to LAT cells. A more labile antigen may possibly lose the structural epitope bound by the antibodies following the denaturation of the protein in SDS. Whether or not the use of different conditions for the separation and immunoblotting of the normal cell membrane proteins will allow the detection of some specific protein(s) remains to be seen. Unfortunately, sufficient cell numbers of other normal and tumour cell lines were not available to allow more extensive comparative studies with LAT cells to be performed. Such studies would be of interest to determine whether or not p-205 could be detected on other cell types.

10.5.4

WESTERN IMMUNOBLOT ANALYSIS OF MOUSE SERUM AND ASCITIC FLUID FROM LAT-TUMOUR BEARING MICE FOR THE PRESENCE OF P-205

Serum and ascitic fluids, taken from Schofield mice bearing both ascitic and solid tumours of LAT cells, were subjected to SDS-PAGE and electrotransfer onto NC to be probed with the monoclonal antibodies 7C1, 8B1 and 10E3, in order to see if the antigen p-205 (or any other antigen) was present. The shedding of antigens from the cell surface is a normal part of cell turnover (Price and Baldwin, 1977) and may play a crucial role in allowing tumour cells to escape the body's immunoregulatory systems. The shedding of cell surface tumour glycoproteins by LAT cells has been detailed elsewhere (Walsh, 1984, Ph.D Thesis). The work carried out by Walsh (1984) suggested that LAT cells (and possibly other tumour cells) may preferentially shed tumour related

glycoproteins into the ascitic fluids of tumour bearing mice The presence of tumour antigen in the body fluids would have serious implications in the event of the antibodies against them ever being used for *in vivo* studies (e g localization or drug targeting)

In each case, serum and ascitic fluids were collected from mice bearing 7 day-old tumours The tumours were induced by the injection of $1x10^7$ LAT cells in 0 5 ml volumes of PBS, intraperitoneally in the case of ascitic tumours and subcutaneously in the case of solid tumours The ascitic fluids were centrifuged at 400xg to remove cells before being lyophilized The dried ascitic fluid proteins were resuspended at 2 0mg protein/ml (as determined by the method of Lowry *et al* , 1951) in SDS electrophoresis sample buffer The neat sera were diluted 1 2 in the SDS sample buffer Both samples were electrophoresed as described for LAT cell membranes (Section 2 8)

The results of Western immunoblot analysis of the serum and ascitic fluids revealed the presence of two bands in both the serum and ascites (Figure 10 5) One of these two bands corresponded to the p-48 seen on LAT cell membranes and most likely represents naturally occuring antibody found in both serum and ascitic fluids The second band corresponded to the p-71 protein of LAT cells, having the same approximate molecular weight Once again, this band was found to be artifactual, being detectable on blots probed with antibody-enzyme conjugate alone The nature of this band was not examined any further In no instance could the p-205 protein be detected in either serum or ascitic fluids of tumour bearing mice Its possible occurence in normal sera was not investigated

10 5 5

PROBING OF WESTERN IMMUNOBLOTS WITH POLYCLONAL ANTI-3H-GLYCOCONJUGATES FROM LAT CELL MEMBRANES

Reagent polyclonal antibodies directed against the tumour related high molecular weight glycopeptides of LAT cells (Section 3) were used to probe western immunoblots of total LAT cell membrane proteins The object of this experiment was to determine whether or not the p-205 protein detected by monoclonal antibodies 7C1, 8B1 and 10E3 might belong to this group of molecules If p-205 were found to belong to this group, then it may provide some valuable information relating to the chemical structure of p-205

The polyclonal antiserum was raised against ^{3}H-glycopeptides conjugated onto BSA as a carrier protein Because of this, the electroblots in this experiment were blocked with casein which was not found to be reactive with the polyclonal antibodies in solid-phase ELISA When the separated LAT membrane proteins were probed with this polyclonal antiserum a number of bands were found to be stained over the full separation range of the gel (i e in a molecular weight range from 40 kD to 250 kD) (Figure 10 6) This included a protein band corresponding to the p-205 protein bound by the monoclonal antibodies 7C1, 8B1 and 10E3 However, this cannot be taken to mean that p-205 definitely belongs to those membrane glycoproteins expressing the trypsin-sensitive high molecular weight glycopeptides SDS-PAGE only separates proteins on the basis of size and it is possible that several proteins of any given molecular weight may exist within a given membrane system

10 6

ENZYME DIGESTION STUDIES OF SEPARATED LAT CELL MEMBRANE PROTEINS FOLLOWED BY IMMUNODETECTION WITH MONOCLONAL ANTIBODIES 7C1, 8B1 AND 10E3

Having identified the antigen against which the monoclonal antibodies 7C1, 8B1 and 10E3 are directed,

some enzyme digestion studies of the antigen were undertaken to try to gain some further insight into the structural nature of the p-205 antigen. The approach adopted here was to electrophoretically transfer the separated LAT cell membrane proteins onto NC which was then treated with various enzymes prior to immunostaining to determine what effects, if any, the enzymes had on the ability of the monoclonal antibodies to bind the antigen. Using this information, coupled with a knowledge of the specificities of the enzymes used, an indication could be gained as to the nature of the epitopes being bound by the antibodies. The enzymes used were trypsin (EC 3.4.21.4), pronase, papain (EC 3.4.22.2), β-galactosidase (EC 3.2.1.23), neuraminidase (EC 3.2.1.18), collagenase (EC 3.4.24.3), amylo-1-6-glucosidase (EC 3.2.1.3), β-glucuronidase (EC 3.2.1.31), and lipase (EC 3.1.1.3). The proteases trypsin, pronase and papain were all used at concentrations of 0.1 mg/ml while the glycosidases were all used at concentrations giving 0.1 IU/ml (according to the suppliers instructions). Of these enzymes, only the three proteases and Vibrio cholerae neuraminidase affected the binding of the monoclonal antibodies to the separated membrane proteins.

10.6.1

THE EFFECTS OF PROTEASES ON THE BINDING OF MONOCLONAL ANTIBODIES 7C1, 8B1 AND 10E3 TO THE ANTIGEN P-205

Western blots of LAT cell membrane proteins were treated with the proteases trypsin, papain and pronase prior to probing with the monoclonal antibodies 7C1, 8B1 and 10E3. This resulted in the complete loss of the p-205 epitope(s) bound by the antibodies (Figure 10.7). This would suggest that the antibodies are binding to the protein backbone of p-205. No effects were noted on the p-48 or p-71 bands.

Both trypsin and papain cleave proteins at the basic amino acids, arginine and lysine. Papain also cleaves proteins at the peptide bond next but one to the -COOH group of phenylalanine residues.

Pronase represents a mixture of proteases including a trypsin-like activity Given that all three proteases bring about the destruction of the p-205 epitope(s) bound by the monoclonal antibodies and that they share a common site of protein cleavage in arginine and lysine residues, it is reasonable to believe that either one of these amino acids lies at, or near to the affected epitope

10 6 2

THE EFFECTS OF NEURAMINIDASE ON THE BINDING OF THE MONOCLONAL ANTIBODIES 7C1, 8B1 AND 10E3 TO LAT CELL PROTEINS

Neuraminidase treatment of the separated LAT cell proteins produced the most interesting results Firstly, it resulted in the loss of the p-205 band, indicating that p-205 is a glycoprotein and that sialic acid is structurally important in the epitope(s) bound by the monoclonal antibodies Secondly, and more surprisingly, neuraminidase treatment resulted in the immunodetection of a range of previously unseen glycoproteins of molecular weights ranging from 40kD to 60kD (Figure 10 8) These bands stained more strongly than the p-205 band observed in the absence of neuraminidase treatment (compare Figures 10 3 and 10 8) At first sight, the implication from this result is that the epitope bound by the monoclonal antibodies 7C1, 8B1 and 10E3 is commonly expressed by a range of different glycoproteins but is normally protected from immune detection by the masking effects of sialic acids Masking of antigens by sialic acids has been reported in other circumstances in relation to both LAT (Currie, 1967, Currie and Bagshawe, 1968, Smyth et al , 1977) and other cells (Pincus et al , 1981) This masking is usually accounted for by steric hindrance arising from the ionic charge of sialic acids

In the case of p-205, the opposite effect would appear to be the case, with the antigen being destroyed by the neuraminidase treatment It may be possible to explain this anomaly by taking into account the fact that the neuraminidase preparation contained some protease

activity (0 1 mU of protease activity/ml, according to the manufacturers instructions, Erlanger et al , 1961) These proteases may have destroyed the protein backbone of the p-205 glycoprotein at some site distant, though structurally important to the integrity of the epitope bound by the antibodies This structural site may not exist in the novel glycoproteins (40kD to 60kD molecular weights) and in their case, the proteases may serve only to expose previously hidden epitopes to allow interactions with the antibodies to take place In support of this, other investigators have reported similar findings in relation to other antigens For example, Wolf and Robbins (1974) reported that the Forssman glycolipid is more readily detected in normal cells after trypsinization If this were also the case in this instance, then sialic acid itself may not necessarily be directly involved in the masking of these new epitopes from interaction with antibody

It is strange that all of the novel glycoproteins detected following neuraminidase treatment were of relatively low molecular weights This may be seen to be suggestive of proteolysis of the membrane proteins during the isolation and solubilization of the LAT membrane proteins Thus, it may be argued that proteolytic digestion of the membrane glycoproteins may have been responsible for the appearance of the novel glycoproteins on the neuraminidase treated Western blots However, several points would indicate that this was not the case Firstly, a number of precautions were taken to minimize and prevent the effects of proteases of LAT cell origin (i e the inclusion of the protease inhibitor phenyl methylsulfonyl fluoride (1mM) in the homogenization buffers, the maintenance of membrane samples on ice at all stages of memorane isolation and the use of hot SDS-electrophoresis sample buffers for the resuspension and solubilization of the membrane samples) Secondly, and probably more importantly, if extensive proteolytic digestion of membrane proteins had taken place, then one would expect that multiple protein banding patterns arising from p-205 fragments would have been found on

those blots which were probed with antibody in the absence of neuraminidase

10 7 FINAL DISCUSSION ON THE CHARACTERIZATION OF THE MONOCLONAL ANTIBODIES 7C1, 8B1 AND 10E3

Murine monoclonal antibodies were generated against LAT cells and the antibodies were screened against panels of both normal and tumour cells in solid-phase ELISA and by Western immunoblot analysis using SDS-solubilized membrane proteins The monoclonal antibodies used were 7C1, 8B1 and 10E3 They were all derived from a common ancestral clone, F10 1 E 12 Not surprisingly, they were found to share similar patterns of reactivty against a range of tumour and normal cells both in ELISA and Western immunoblot analysis

The antibodies secreted by these hybridomas were found to be of the IgM class by solid-phase ELISA and HPLC (Section 10 2) This may have serious implications if these antibodies are to prove themselves to be of value for tumour localization and drug-targetting in vivo IgM antibodies are particularly sensitive to inactivation when conjugated to other molecules such as drugs, although the degree of inactivation will depend on the type of drug used (Embleton, 1986) F(ab)-antibody fragments can be prepared from IgM antibodies (Bidlack and Marbie, 1986) and these may prove to be more useful, particularly since smaller conjugates are prefered for the localization and targetting of tumours (Embleton, 1987) This reduces the likelihood of capillary blockage by large molecular complexes The isolation and purification of IgM antibodies does not present any major problems Crude preparations of antibody are readily obtained from ascitic fluids by ammonium sulphate precipitation Higher yields and better purity are reportedly obtainable when precipitation in polyethylene glycol-6000 is used (Neoh et al , 1986)

The cross-reactivity profiles of the antibodies against panels of normal and tumour cells in solid-phase ELISA indicate that the antigenic determinant bound by the antibodies is expressed on both types of cells, but appears to be more highly expressed on tumour cells Thus, the antigen being identified is not a truly tumour specific antigen but represents a quantitative difference between the tumour and normal cells The interpretation of such immunological analysis must be done with some degree of caution since higher levels of immunoreactivity may not neccessarily be reflected in higher levels of antigen as determined by chemical methods An antigen may be present on a cell but in a form that is unavailable for interaction with an antibody (Hakomori et al , 1983)

Of the normal mouse cells tested for reaction with the monoclonal antibodies 7C1, 8B1 and 10E3 in solid-phase ELISA, normal mouse liver and spleen cells showed the highest levels of reaction (Table 10 2) However, following the SDS-solubilization and electrophoretic separation of the membrane proteins of these cells, the monoclonal antibodies failed to detect any proteins in Western immunoblots, despite the fact that the antibodies were able to detect a glycoprotein of molecular weight 205kD on LAT cells This indicates that the antigen being recognized on the tumour cells may be different from that expressed by the normal cells Alternatively, the same antigens may be involved, but those on the normal cells are more sensitive to the denaturing effects of the SDS-buffers used for the solubilization of the membrane proteins

The p-205 glycoprotein antigen detectable on LAT cells probed with the monoclonal antibodies 7C1, 8B1 and 10E3 is not detectable in the serum or ascites of Schofield mice bearing either solid or ascitic LAT cell tumours It would therefore appear that this antigen is not shed from the LAT cell surface

Preliminary work on the structural nature of the p-205

antigen revealed that it is sensitive to the effects of the proteases trypsin, papain and pronase, all of which destroy the ability of the monoclonal antibodies to interact with the antigen Lipase and a number of glycosidases had no effect on the antigen-antibody interaction Neuraminidase was an exception to this The treatment of Western immunoblots of LAT cells with this enzyme prior to probing with monoclonal antibody resulted in the loss of p-205 detection while a range of previously unseen proteins of molecular weights from 40kD to 60kD was revealed It is difficult to explain the reason for this result but it is felt that proteases contained in the preparation of neuraminidase may be involved These proteases may possibly bring about the loss of the p-205 antigen, while unmasking antibody binding sites on other proteins Proteolysis of the p-205 antigen during the preparation of the LAT cell membranes was discounted as a possible source of these new bands, largely because they could not be detected in the absence of neuraminidase-treatment of the Western blots prior to probing with antibody

Polyclonal antisera, raised against high molecular weight glycopeptides (see Section 3 4) derived from LAT cell surfaces were also used to probe Western blots of SDS-PAGE separated membrane proteins The high molecular weight glycopeptides represent a cell surface alteration commonly seen in tumour cells (Smets, 1980 for a review) In this study, these antisera were used to see if any of the bands they might detect amongst separated LAT membrane proteins would correspond with the p-205 band detected by the monoclonal antibodies 7C1, 8B1 and 10E3 This was found to be the case and would indicate that the carbohydrate portion of p-205 is synonymous with the high molecular weight glycopetides characteristic of tumour cells

If p-205 does belong to the group of cell surface membrane glycoproteins which possess the high molecular weight glycopeptides, then we can postulate as to the actual chemical structure of the carbohydrate portion of

the p-205 glycoprotein On the basis of available data, we know that the high molecular weight glycopeptides are trypsin sensitive (*) (Buck et al , 1970) and are commonly enriched in sialic acid residues (Warren et al , 1978) They contain a universal carbohydrate core of Man3(GlcNAc)2-Asn (Figure 1 1, Sharon, 1984) and are predominantly tri- and tetra-antennary complex carbohydrates (Santer and Glick, 1983, Yamashita et al , 1985) with branches containing Gal- β(1-4)-GlcNAc and sialic acid (Figure 1 2, Sharon, 1984) This information may prove to be the basis for further deeper analysis of the nature of the p-205 glycoprotein using such techniques as WELLA (Western enzyme-lectin linked analysis, Reading and Hickey, 1985)

The exact location of the p-205 glycoprotein detected by the monoclonal antibodies 7C1, 8B1 and 10E3 is not clear from the studies carried out here Clearly p-205 is a membrane glycoprotein, being present in microsomal membrane preparations from LAT cells, as demonstrated in the Western immunoblotting studies performed here (Sections 10 5 and 10 6) What is not clear is whether or not p-205 is expressed at the cell surface or on the intercellular membranes which also form a part of the

* Addendum

Since completing this work it has been brought to my attention that the moieties shed from the cell surface following treatment with trypsin are not simply trypsin-sensitive glycopeptides Instead they include intact glycoproteins in large vesicles (McGuinness and Smyth, 1987) They may also be protected from proteolytic degradation due to an "umbrella type" conformation assumed by their highly sialylated components (Montreuil, 1984) Thus, these fucopeptides may be better described as "trypsin-releasable" components

membrane preparations used here In support of a cell surface location for p-205 is the fact that a p205 band was detectable when Western immunoblots of LAT cell membrane proteins were probed with the polyclonal antibodies raised against high molecular weight fucopeptides derived from LAT cell surfaces (Sections 3 4 and 10 5 5) However, it must be mentioned that the high molecular weight fucopeptides can also be found on intercellular membranes, although at much lower levels (Warren et al , 1978) Thus the exact cellular location of p-205 remains to be determined

In conclusion, monoclonal antibodies were raised against LAT cells which show a greater preference for tumour cells over normal cells Preliminary research into the nature of the antigen has provided some insight into possible chemical structure of this antigen It now remains to be seen if this antigen can be usefully exploited as a target for the in vivo localization of tumours and the subsequent targetting of anti-cancer drugs to tumour cells

TABLE 10 1

THE NATURE OF CONTAMINATING ANTIBODIES IN MONOCLONAL ANTIBODIES PRODUCED IN ASCITIC FLUIDS OF BALB/C MICE

Antibody subclass and light chain type	Monoclonal antibodies 9AW	7C1	8B1	10E3
IgG_1	-	-	-	+
IgG_{2a}	++ *	-	++	+
IgG_{2b}	+	-	-	++
IgG_3	-	-	-	-
IgA	+	-	-	-
IgM	+++++	+++++	+++++	+++++
Kappa-light chainT	+++	+++	+++	+++
Lamda-light chain	-	-	-	-

* Values represent the relative degrees of reactivity observed in an ELISA to determine the isotypes of ascitic antibodies

+ = O D 405nm of >0 05, ++ = O D 405nm of >0 10, +++ = O D 405nm of >0 15, ++++ = O D 405nm of >0 20, +++++ = O D 405nm of >0 25

Clones 7C1, 8B1 and 10E3 are all subclones of F10 1 E12 Clone 9AW was produced in a separate fusion experiment (see Section 7 2 1)

TABLE 10 2

CROSS-REACTIVITY OF ANTIBODIES 7C1, 8B1 AND 10E3 ON A PANEL OF NORMAL CELLS

Origin of cells	tissue/ organ	No of samples	Antibodies 7C1	8B1	10E3
Mouse	LAT ascites#	3	+++++	+++++	++++++
Mouse	Spleen	3	* ++	+	+
	Thymus	3	+	+	+
	Lung	3	+	+	-
	Heart	3	-	-	-
	Kidney	3	-	-	-
	Liver	3	++	++	++
Human	Leucocytes	3	-	-	-
	Tonsil	1	+++	++	++
	Liver	1	+	+	+
	MRC-5	3	+	+	+
*Rat	NRK (normal rat kidney)	3	+++	+++	+++

Reactivity against LAT cells is given for comparative purposes only

* Values represent the relative degrees of reactivity of each antibody against each cell type

++++ = O D 405nm of >0 20, +++ = O D 405mn of >0 15 etc

TABLE 10 3

CROSS-REACTIVITY OF ANTIBODIES 7C1, 8B1 AND 10E3 ON A PANEL OF TUMOUR CELL LINES

Cell origin	Cell name	Cell type	Antibodies 7C1	8B1	10E3
Mouse	LAT	Ascites tumour	+++++	+++++	++++++
	Ehrlich	Ascites tumour	+++	++++	+++++
	Sp2/0	Myeloma	+++	++++	+++++
Rat	IR983F	Myeloma	-	-	-
Dog	MDCK	Canine kidney	-	+	-
Human	A549	Adenocarcinoma	-	-	-
	RPMI-2650	Adenocarcimoma	+++	++++	+++
	SCC-9	Squamous cell carcinoma	+++	+++	+++
	EJ	Bladder	+++	+++	+++
	Molt-4	T-ALL leukaemia	++	+	-

Values represent the relative degrees of reactivity of each antibody with the given cell type

++++ = O D 405mn of >0 20, +++ = O D 405nm of >0 15 etc

TABLE 10 4

CROSS-REACTIVITY OF ANTIBODIES 7C1, 8B1 AND 10E3 ON A PANEL OF HUMAN LEUKAEMIC CELL SAMPLES

Leukemic cell type	Patient name	Antibodies 7C1	8B1	10E3
CLL*	NO'N	++	++	++
CLL	MM	++	++	+
CLL	FO'C	+	++	+
CLL	JC	++	+	++
CLL	PH	++++	+++	++++
CLL	WC	++	+	-
ALL@	KMcC	++	++	++++
ALL	PI	+	+	+
LAT#		+++++	+++++	++++++

Values presented represent the relative reactivities of each antibody against the given cell type

++++ = O D 405nm of >0 20, +++ = O D 405nm of >0 15 etc

* Chronic lymphocytic leukaemia, @ Acute lymphoblastic leukaemia, # Values for LAT cells are provided for comparative purposes only

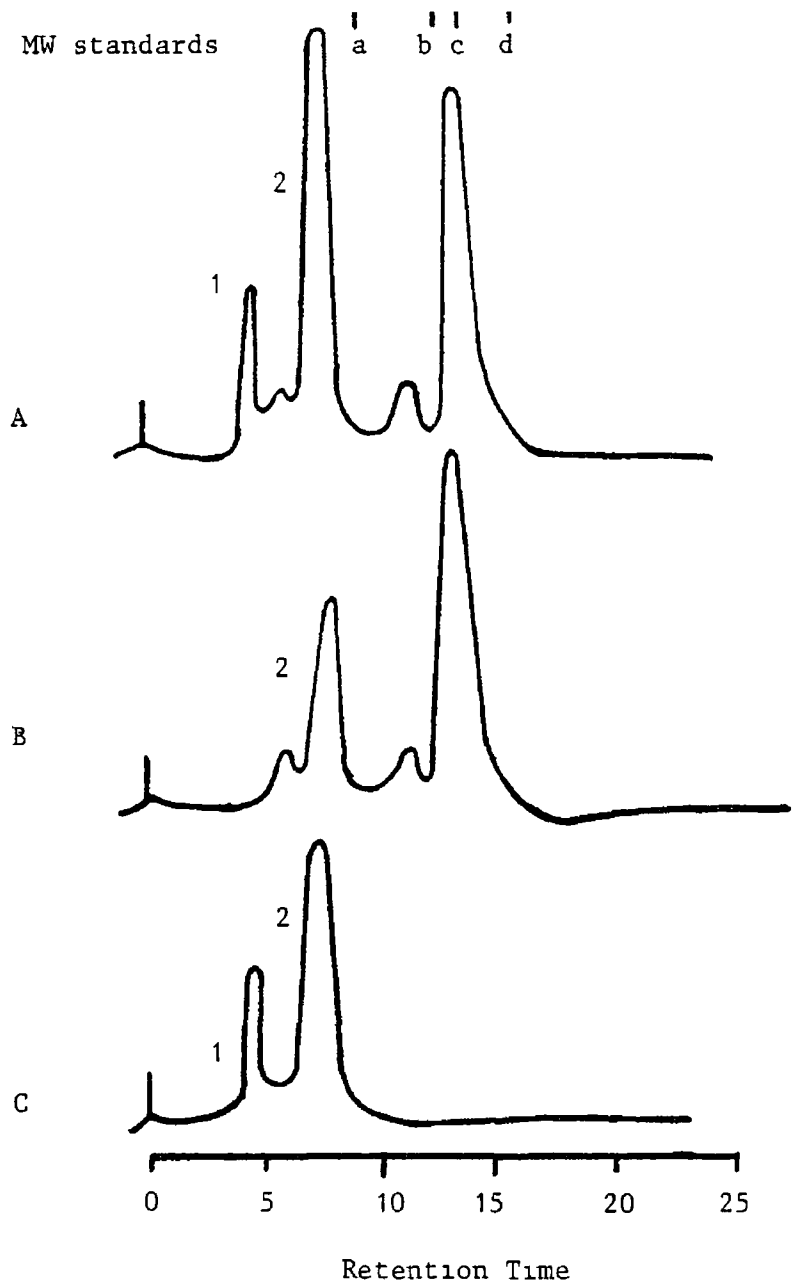

Figure 10 1

HPLC analysis of ascites fluid from mice bearing tumours derived from the hybridoma F10 1 F12, (IgM antibody secretor)

A Whole ascites
Peak 1 corresponds to IgM, peak 2 corresponds to IgG abs and albumin

B Ascitic fluid following ammonium sulphate precipitation to remove antibody Note the loss of peak 1 (IgM) and the reduction in the size of peak 2 (IgG and albumin)

C The ammonium sulphate precipitated material

Molecular weight standards

a) Catalase

b) lactate dehydrogenase

c) bovine serum albumin and

d) horseradish peroxidase

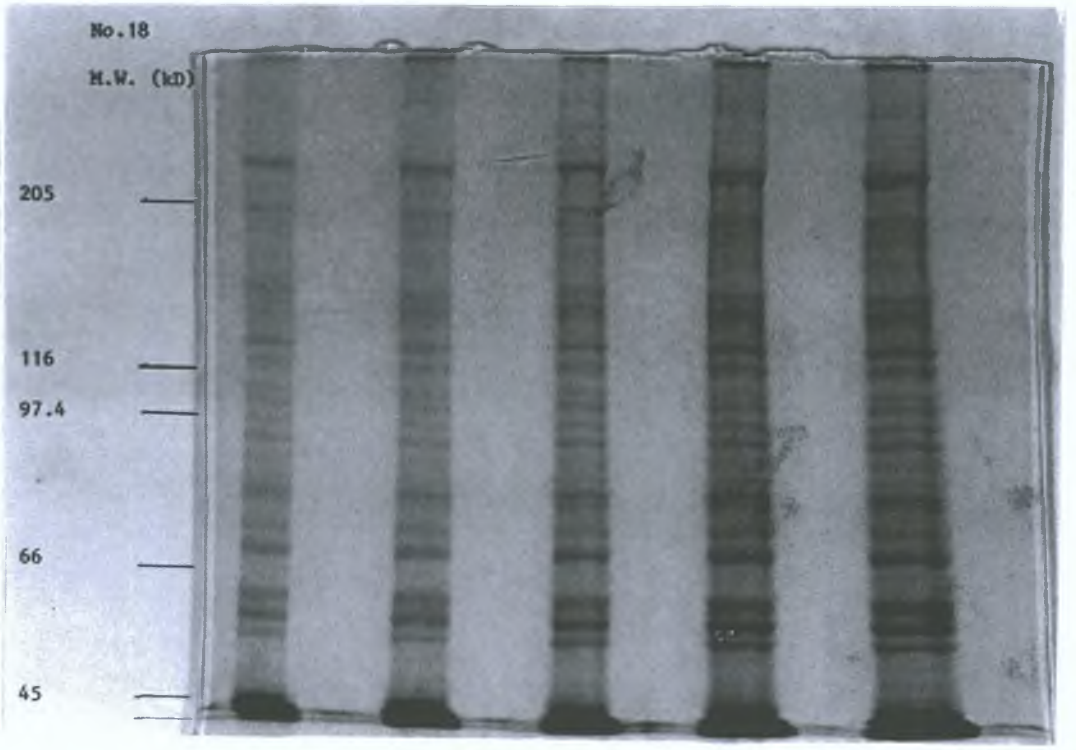

Figure 10.2

A total profile of LAT cell membrane proteins separated on a 7.5% polyacrylamide gel in the presence of SDS and mercaptoethanol.

Molecular weight markers (Sigma kit no. MW-SDS-200) for this and all subsequent figures were : myosin (205kD); β-galactosidase (116kD); phosphorylase (97kD); BSA (66kD) and egg albumin (45kD).

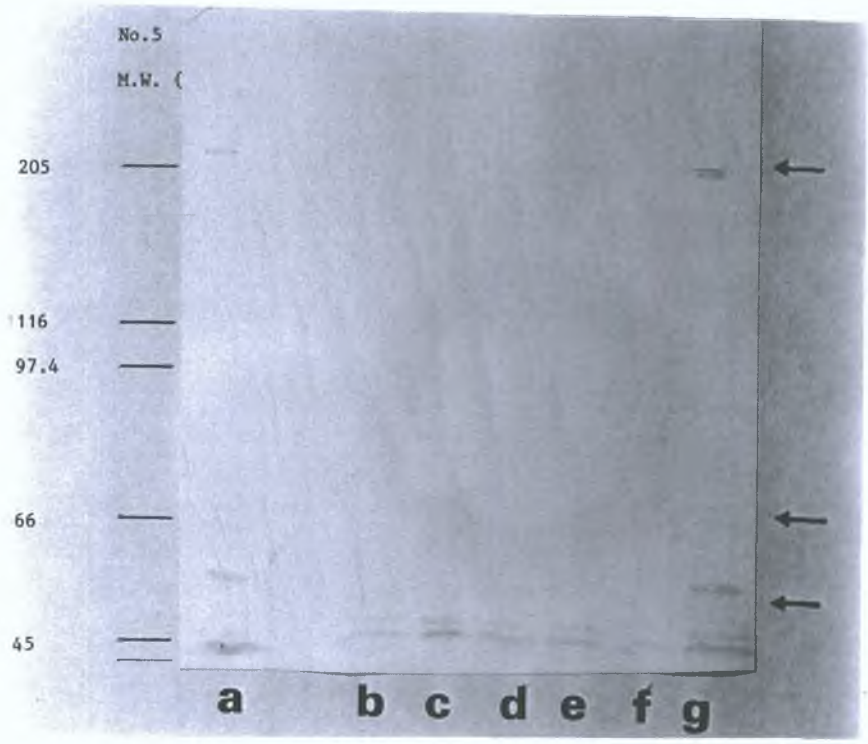

Figure 10.3

Western immunoblot of normal and LAT cell membranes.
Western immunoblot of LAT cell membrane proteins derived from normal mouse (a) spleen, (b) heart, (c) liver, (d) lung, (e) and kidney cells; (f) A549 human lung adenocarcinoma and (g) LAT cells. Note the presence of the protein bands p-205, p-71 and p-48 in LAT cells only. p-48 also appeared in normal mouse spleen cells.

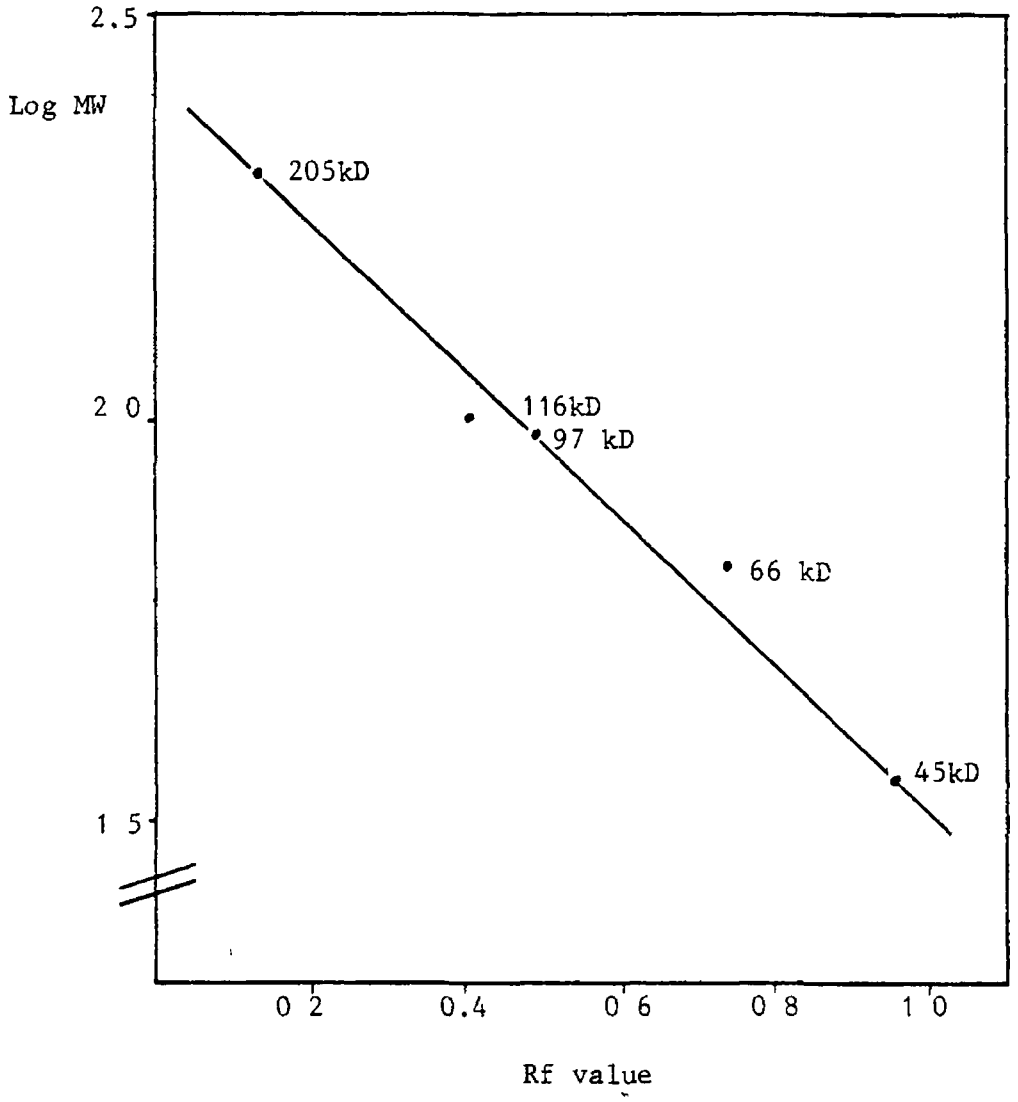

Figure 10 4

Molecular weight calibration curve from 7 5% SDS-PAGE using the Sigma MW-SDS-200 calibration kit
Data points 205kD myosin, 116kD β-galactosidase, 97kD phosphorylase b, 60kD BSA and 45kD egg albumin

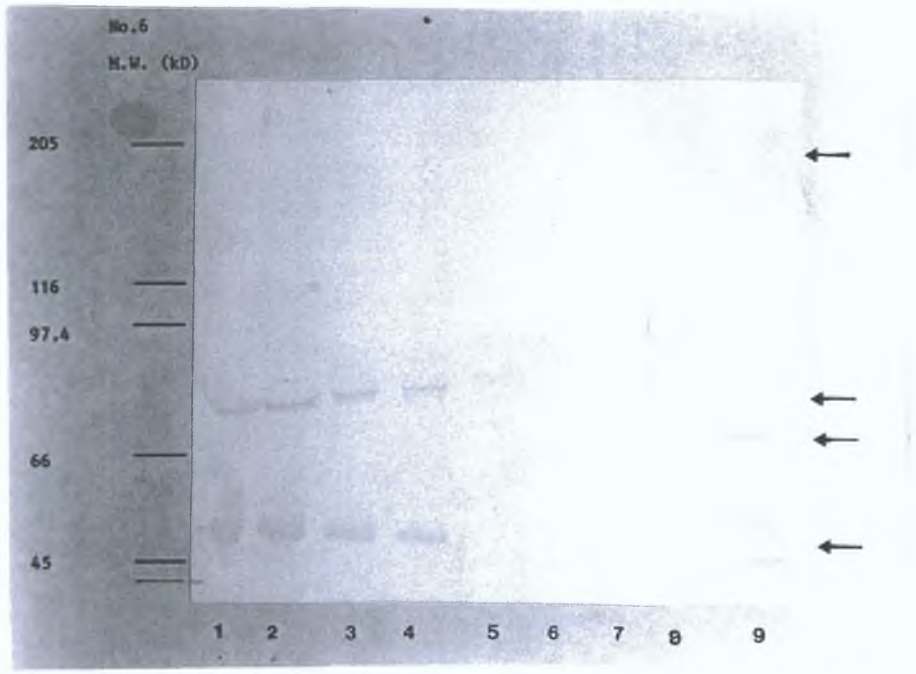

Figure 10.5

Western immunoblot of serum and ascitic fluids taken from Schofield mice bearing LAT cell tumours. Lanes 1-3: serum diluted 1:2; lane 4: serum diluted 1:5; and lane 5: serum diluted 1:10. Lanes 6, 7, 8: ascitic fluid diluted 1:10, 1:5 and 1:2 respectively; lane 9: whole LAT cell surface membranes. Note the presence of both p-48 and p-71 in serum and ascites. p-205 did not appear in these fluids.

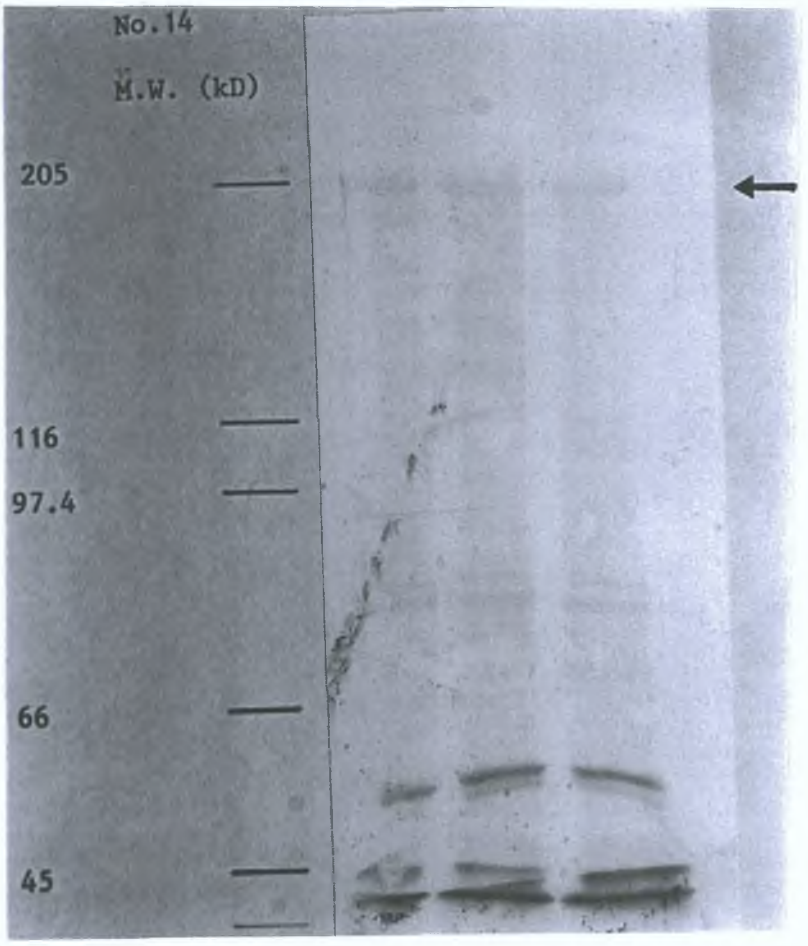

Figure 10.6

Western immunoblots of LAT cell membrane proteins probed with polyclonal anti-high molecular weight 3-H-fucopeptides derived from LAT cells (see Section 3).

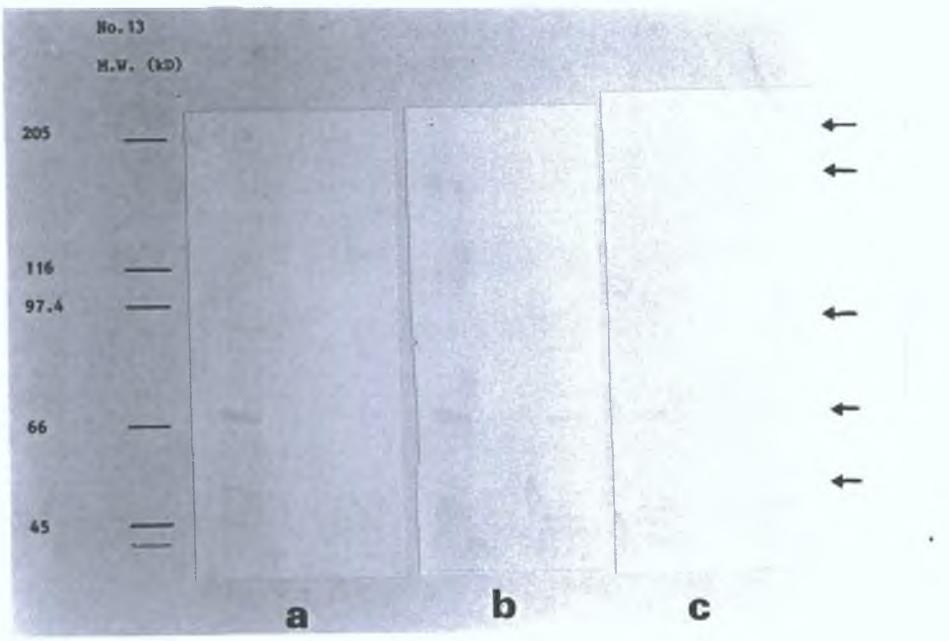

Figure 10.7

The effects of (a) trypsin, (b) papain and (c) pronase on the interaction between p-205 and monoclonal antibody F.10.1.E12. Note that p-48 and p-71 were not affected, while the detection of p-205 was impaired.

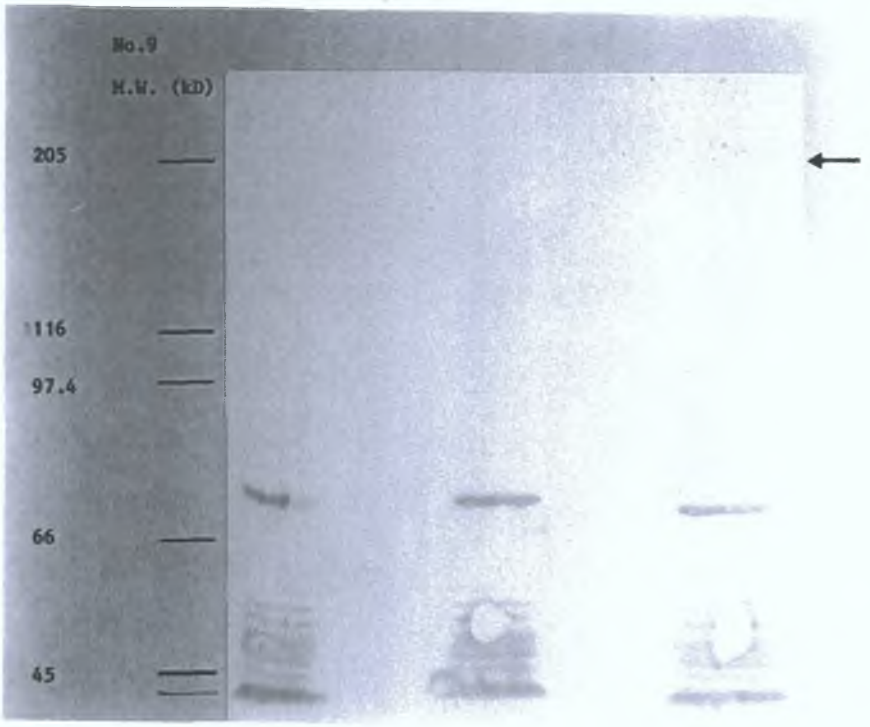

Figure 10.8

The effect of neuraminidase on the interaction of monoclonal antibody F.10.1.E12 with LAT cell membrane proteins. Note the disappearance of p-205 and the detection of the novel glycoproteins of mw from 40kD to 60kD.

SECTION 11

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