UNIVERSITY OF SOUTHAMPTON

THE RECRUITMENT OF A RIBOSOMAL INACTIVATING PROTEIN OR T CELLS BY ANTIBODY DERIVATIVES IN THE TREATMENT OF B CELL LYMPHOMA

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UNIVERSITY OF SOUTHAMPTON
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ABSTRACT

Doctor of Philosophy

THE RECRUITMENT OF A RIBOSOMAL INACTIVATING PROTEIN OR T CELLS BY ANTIBODY DERIVATIVES IN THE TREATMENT OF B CELL LYMPHOMA

by Harry Michael McBride

An idiotope associated with the surface IgM of a little studied murine B cell lymphoma (A31) was used as a highly specific target for immunotherapeutic studies.

The A31 IgM was recovered by somatic fusion of the A31 tumour cells and a murine plasmacytoma, NS-1, producing a hybridoma that secreted pentameric A31 IgM in culture (rescued idiotypic IgM). A xenogenic fusion using a murine plasmacytoma line and splenocytes from a rat immunised with idiotypic IgM led to the successful isolation of anti-idiotypic monoclonal antibodies. One of the latter, a rat IgG2a designated Mc39-16, was chosen for detailed immunotherapeutic studies.

Scatchard plot analysis of radioiodinated Mc39-16 binding to A31 cells produced a functional association constant of 3.8×10^8 M^{-1} and enumerated the surface IgM molecules of A31 cells at approximately 3.8×10^5.

Use of the antibody in immunohistology revealed that growth of the tumour was restricted initially to the B cell areas of the spleen. General invasion of splenic tissue occurred between 10-15 days post inoculation, at which time significant liver involvement was manifest. Coincident with the latter was a rapid rise in serum idiotype levels from <10 µg/ml to a maximum level of ~70 µg/ml immediately prior to death.

Therapy with Mc39-16 given 24 hr after tumour inoculation yielded long-term survivors in animals receiving $<5 \times 10^3$ cells but limited survival of animals given $>2.5 \times 10^4$.

The ribosome-inactivating protein saporin was delivered to the tumour cells *in vitro* using either an immunotoxin (IT) or bispecific $F(ab')_2$ (anti-tumour x anti-saporin) antibodies constructed using Mc39-16. The IT and one of the $F(ab')_2$ constructs enhanced the toxicity of saporin by 1800-2900 fold. Despite this the IT was capable of curing mice injected with the tumour whilst the bispecific antibodies gave only marginal therapeutic benefit.

A bispecific $F(ab')_2$ (anti-tumour x anti-CD3), designed to induce T-cell killing of tumour, was also constructed using Mc39-16. This antibody gave significantly greater protection than Mc39-16 IgG at tumour cell inoculations of $2.5\text{-}5 \times 10^4$ cells/mouse including long term survival in a proportion of the mice.

These studies demonstrate that A31 is likely to be a highly suitable model for the study of new immunotherapeutic treatments for human lymphoma.

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Finally I would like to thank my parents and brother for their encouragement and support throughout this work.

ABBREVIATIONS

A	absorption
ADCC	antibody-dependent cell-mediated cytotoxicity
BiP	heavy chain binding protein
BrdUrd	5-bromo-2-deoxyuridine
BSA	bovine Serum Albumin
C	constant region
CD	cluster of differentiation
CEA	carcinoembryonic antigen
CFA	complete Freund's adjuvant
cpm	counts per minute
^{51}Cr	radioactive chromate isotope
CR	complement receptor
CTL	cytotoxic T lymphocyte
D	diversity region
DAF	decay accelerating factor
DEAE	diethylaminoethyl (cellulose)
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol

EDTA	ethylenediaminetetra-acetic acid, disodium salt
ELISA	enzyme-linked immunosorbent assay
$F(ab')_2$	or $F(ab'\gamma)_2$ denotes a bivalent antigen-binding fragment derived from IgG (by digestion with pepsin)
Fab	fragment antigen binding
Fab'	or Fab'γ denotes a univalent antigen-binding fragment derived from IgG (by reduction of $F(ab')_2$)
$Fab'\gamma_{mal}$	maleimidated Fab'γ
FACS	fluorescence activated cell sorter
Fc	fragment crystallizable
FcR	Fc receptor
FCS	fetal calf serum
FdUrd	5-fluoro-2-deoxyuridine
FITC	fluorescein isothiocyanate
H	heavy (chain)
^{125}I	radioactive iodine isotope
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
Ia	MHC class II molecule
IAA	iodoacetamide

Id	idiotype
IFA	incomplete Freund's adjuvant
Ig	immunoglobulin
J	joining region
κ	kappa
kDa	kiloDaltons
λ	lamda
l	light (chain)
LAK	lymphokine activated killer
LGL	large granular lymphocyte
MAC	membrane attack complex
MCP	membrane cofactor protein
2-ME	2-mercaptoethanol
MHC	Major Histocompatibility Complex
mRNA	messenger Ribonucleic acid
NHL	non-Hodgkin's lymphoma
NK	natural Killer cell
OD	optical density
OPD	o-phenylenediamine
oPDM	N,N'-o-phenyldimaleimide

PBS	phosphate buffered saline
PDS	pyridyl disulphide (dithiodipyridine)
RIP	ribosome inactivating protein
S-S	disulphide bond
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH	sulphydryl group
T_3-Ti	CD3 and TCR complex
TAA	tumour associated antigen
T_C	T cytotoxic cell
TCR	T cell receptor
T_H	T helper cell
TNFα	tumour necrosis factor alpha
TNFβ	tumour necrosis factor beta
TRIS	tris[hydroxymethyl]aminomethane
TSA	tumour specific antigen
V	variable gene region

DEDICATION

This thesis is dedicated to the memory of Ian Alexander McBride (1950-1980).

CHAPTER 1

1.1. INTRODUCTION

If you have designed a therapy that is capable of destroying 99.999% of all the tumour cells then in a 60lb child with leukaemia having a tumour load of 2×10^{12} you will have 2×10^{7} cells still remaining. This is the equivalent of reducing the tumour cell volume from one litre down to < one ml of cells. This may sound quite impressive but unfortunately it is this remaining population of cells, often being refractory to further attack, that will grow out and kill the patient (Frei and Freireich, 1964).

There are a number of malignant tumours for which total eradication of the neoplastic cell population using modern therapeutic regimes has lead to much higher survival rates. Included in this group are Hodgkin's disease, cancer of the testis and the acute leukaemias in children (Review of the National Cancer Registration System, 1990). There remain, however, malignancies for which the total elimination of those final few resistant cells has proven difficult to achieve, leading to failure of the therapies and death of the patents.

In some cases of cancer, the cell population can become resistant to conventional radio- and chemotherapeutic treatment and novel methods of therapy are being examined to eradicate the disease (reviewed by Jain, 1989).

This thesis is concerned with the characterisation recently described murine B cell lymphoma that has certain analogies with the human disease. This murine lymphoma has been used as an experimental model to develop therapies utilising monoclonal antibodies and the ribosomal-inactivating protein, saporin.

1.2. PREVALENCE OF LYMPHOMAS AND LEUKAEMIAS

Of the deaths from neoplasms within England and Wales for the year 1989 a total of 5134 males and 4779 females succumbed to tumours of the lymphatic and haematopoietic tissues. Using data from the office of population censuses and surveys mortality reports for 1989, the latest complete

year for which data has been made available, this can be placed in the context of the total notifiable neoplasms (Table 1.1.), and of the death rates per million population from various diseases (Table 1.2.).

TABLE 1.1.: NEOPLASMS NOTIFIED BY YEAR IN ENGLAND AND WALES

year	86	87	88	89
neoplasms male	73 717	74 325	75 312	75 172
neoplasms female	67 084	68 126	68 126	69 948
lymphatic neoplasms male + female	9004	9489	9718	9913

TABLE 1.2.: DEATH RATE PER MILLION POPULATION FOR VARIOUS DISEASES IN 1989

DISEASE	ENGLAND AND WALES		WORLD
Malignant neoplasms	male female	2840 2570	1804 1285
Malignant neoplasms of lymphatic and haematopoietic tissues	male female	197 177	136 90
Leukaemia	male female	74 65	53 35
Neoplasm of breast	female	525	296
Neoplasm of trachea bronchus and lung	male female	922 415	572 207
Ischaemic heart disease	male female	3122 2387	1972 891

Table 1.2 indicates that cancers of the lymphatic and haematopoietic tissues are less common than some other neoplasms and disorders afflicting mankind but that they are of such a level as to contribute a major drain on the resources of a community, not ignoring the personnel trauma involved.

1.3. CLASSIFICATION OF NON-HODGKIN'S LYMPHOMA

The term non-Hodgkin's lymphoma is a generalised category for a group of neoplasms that have proved very difficult to classify. A large number of classification schemes have arisen in attempts to describe the histological and clinical pictures and to correlate these with staging of the disease. Some of these classifications relied purely on morphological criteria whilst others, although still relying on the morphology of the tumour, include elements of immunohistology and molecular biology to give a more scientific basis to the classification (Stansfeld et al., 1988; Falzon and Isaacson, 1990). Unfortunately this has led to a wide divergence in the terminology used to describe these tumours. The list in Table 1.3. describes the most predominant classifications and reflects the number of such schemes used.

TABLE 1.3.: CLASSIFICATION SCHEMES FOR MALIGNANT LYMPHOMA

YEAR	SOURCE
1942	Gall and Mallory[1]
1966	Rappaport classification[2]
1974	Dorfman[3]
1974	Kiel classification[4]
1974, 1978	British national lymphoma investigation classification[5]
1974	Lukes and Collins[6]
1976	WHO classification[7]
1980	Stanford working classification [8]

REFERENCES TO TABLE 1.3.

(1) Gall and Mallory, 1942
(2) Rappaport, 1966.
(3) Dorfman, 1974, 1977.
(4) Gerard-Marchant et al., 1974.
(5) Bennett et al., 1974; Henry et al., 1978.
(6) Lukes and Collins, 1974.
(7) Mathe et al., 1976.
(8) The non-Hodgkin's lymphoma pathological classification project. National Cancer Institute sponsored study of lymphomas. Summary and describtion of a working formulation of clinical usage, (1982). Cancer 49, 2112-2135.

The Stanford working formulation arose following an international study undertaken in 1976 to retrospectively examine well-documented pathological specimens. The aim was to produce a clinical working formulation for non-Hodgkin's lymphomas that could be correlated with the biological and morphological data (Standford, 1980). However some doubt has been expressed over its true worth as a guide in scientific investigations (Lennert and Feller, 1992). This doubt has resulted in many groups, particularly in Europe, retaining the Kiel classification, the original version of which has been slightly updated by the separation of T and B cell lymphomas into separate groups (Stansfeld et al., 1988; see table 1.4.).

In general terms the follicular lymphomas are very rare in children, uncommon below the age of twenty and occur with increasing frequency in older people. They are virtually unknown in the negro race and are rare in the Chinese. A detailed description of the clinical and histopathological features of the lymphomas will not be given here, the interested reader is referred to "a colour atlas of thymus and lymph node histopathology" (Henry and Farrer-Brown, 1981) and the many excellent textbooks available on the subject.

1.4. B AND T CELL ONTOGENY

Pathogens and tumour cells must elude certain defensive mechanisms in order for them to successfully colonise their intended host. The leucocytes that form the protective sentinels that these invaders must avoid begin their lives as pleuripotent stem cells within the foetal liver and adult bone marrow, and under the influence of either the thymic or bone marrow environments differentiate into T or B lymphocytes respectively (Meager, 1990; Nikolic-Zugic, 1991; Boyd and Hugo, 1991; Melchers et al., 1993). The stem cells can initially take one of two routes of differentiation, the myeloid lineage or the lymphoid lineage (Figure 1.1), the latter being described in greater detail in the following sections.

TABLE 1.4.: UPDATED KIEL CLASSIFICATION OF NON-HODGKIN'S LYMPHOMA

B	T
Low grade Lymphocytic-chronic lymphocytic and prolymphocytic leukaemia; hairy cell leukaemia Lymphoblastic/cytoid (LP immunocytoma) Plasmacytic Centroblastic/centrocytic -follicular diffuse -diffuse	*low grade* Lymphocytic-chronic lymphocytic and prolymphocytic leukaemia Small,cerebriform cell-mycosis fungoides, Sezary's syndrome Lymphoepithelioid (Lennert's lymphoma) Angioimmunoblastic (AILD, LgX) T zone Pleomorphic, small cell (HTLV-1)
High grade Centroblastic Immunoblastic Large cell anaplastic (Ki-1+) Burkitt lymphoma Lymphoblastic *Rare types*	*High grade* Pleomorphic, medium and large cell (HTLV-1) Immunoblastic (HTLV-1) Large cell anablastic (Ki-1) Lymphoblastic *Rare types*

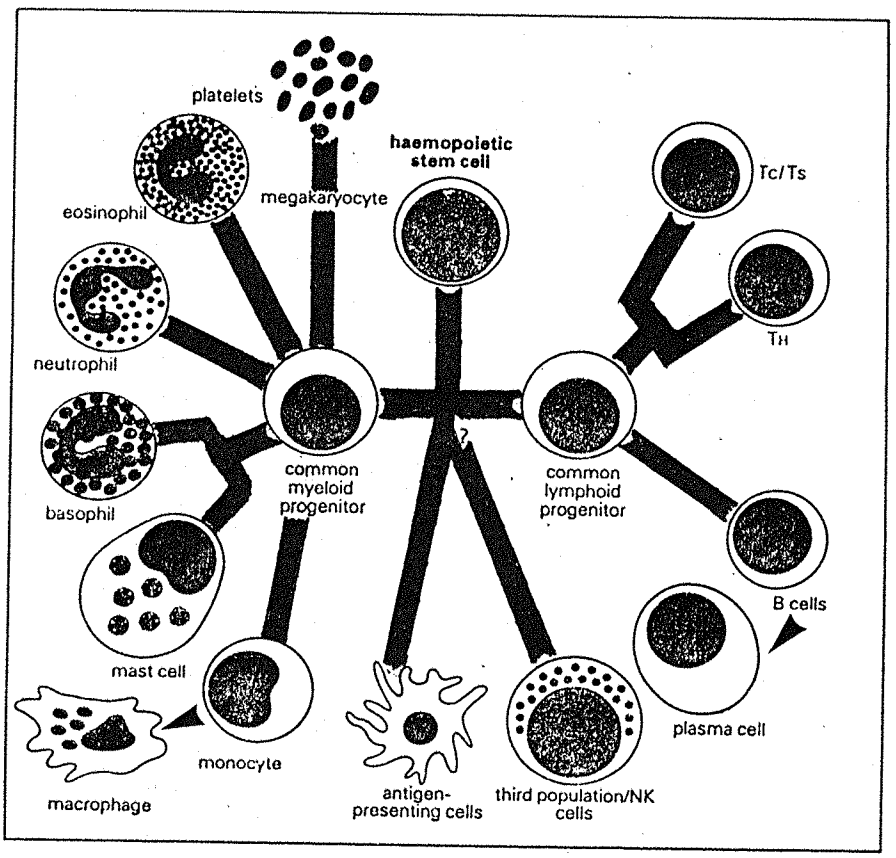


Figure 1.1. Differentiation pathways for haemopoietic stem cells. The third population of cells (also called null cells) represents large granular lymphocytes (LGLs). Not shown but also derived from the myeloid lineage are the cells that form erythrocytes.

As published by; Immunology by Roitt I., Brostof J., and Male D., (1989), Gower Medical Publishing (Pub), London, U.K., pp 2.1.

1.4.1. B CELL ONTOGENY

The maturation of B lymphocytes is determined by genetic events. These not only commit the pluripotential stem cell of the foetal liver or adult bone marrow to the B cell lineage but direct the B lymphocyte as it passes down the differentiation pathway. The earliest cell of the B cell lineage is the progenitor cell (pro-B cell) which is characterised in the mouse by the presence of the B220 surface antigen and germ line configuration of the immunoglobulin gene segments. Rearragements of the immunoglobulin gene segments produces mature functional genes leading first to the cytoplasmic expression of μ heavy chain as found in the precursor B cell (pre-B cell) followed by the surface expression of IgM with or without IgD as found in the immature B cell. After a short lag period, when the lymphocytes only express surface immunoglobulin of the IgM class, the surface expression of FcR and CR antigens can be detected (Chan and Osmond, 1979), whilst the expression of Ia parallels the expression of surface IgD (Rahal and Osmond, 1981). Further genetic rearrangements later in the ontogenetic pathway invoke class and subclass immunoglobulin switching with antigen driven selection leading to an overall increase in antibody affinity and the differentiation of the mature activated B cell into either a long lived memory cell or a terminally differentiated plasma cell. Class switching is influenced by interaction with T cells and the concomitant production of cytokines (Mayer et al., 1985; Sideras et al., 1985; Meager, 1990).

The heamopoietic stem cell that is destined to become a B lymphocyte rearranges its immunoglobulin genes in a set sequence. The first DNA manipulation that occurs is the recombination of one of about 20 D segments to one of 6 J_H segments. Next one of a few hundred V_H sequences is joined to the DJ_H combination to form the complete VDJ gene. Numbers quoted are for the human genome and are likely to be revised as more information becomes available. The mouse has over 1000 known V_H genes the organisation of which resembles the human genome (Krawinkel et al., 1989). The VDJ gene is finally joined to the constant region of the heavy chain during mRNA splicing. Thus in the earliest precursor B cell

(pre-B cells) only cytoplasmic μ chains are detected (Cooper, 1981). With the successful production of functioning heavy chains and their surface expression in the context of surrogate light chains (Melchers et al., 1993), light chain rearrangements can occur. Unlike the heavy chain gene rearrangements the first recombination found in light chain genes is between V_L and J_L segments, there being no D_L sequences. In the mouse, recombination initially involves the kappa light chain segments and only if non-functional rearrangements occur in both kappa loci will the cell go on to attempt rearrangment of its lambda light chain segments. As soon as a legitimate recombination occurs further rearrangements of the light chain genes are inhibited. The V_LJ_L sequence is joined to the constant region of the light chain by splicing of the mRNA transcript. The μ heavy chain upon translation and transport into the endoplasmic reticulum is bound by the heavy chain binding protein (BiP) via the μ chains first constant domain (Bole et al., 1986). It remains bound until the BiP is displaced by light chain. It should be noted that although heavy chains appear within the cytoplasm first (Raff et al., 1976) during B cell development, the heavy chains are inherently unstable and will usually be degraded if they do not combine with a light chain. In the absence of successful genetic rearrangements cell death occurs and it has been estimated that up to 75% of pre-B cells generated within the bone marrow are destroyed (Gallagher and Osmond, 1991).

Besides the diversity generated by the various V, D, and J segments, further variation is generated by a number of mechanisms. First the V, D and J regions do not join together at precisely the same points each time, generating slightly different codons at these locations leading to the insertion of different amino acids around these junctions. In addition, heavy chain genes may have additional, non-template encoded, bases (N-sequences) inserted on either side of the D segment (Desiderio et al., 1984; Lewis et al., 1985).

Later in the life of the B cell and after antigen driven proliferation further variations occur by the process of somatic mutation. Under the influence of selection pressures caused by the presence of the antigen these somatic mutations

allow fine tuning of the immune response and lead to the production of high affinity antibodies (Berek et al., 1991).

1.4.2. POPULATION DYNAMICS OF B LYMPHOCYTES

In a 2-4 week old mouse there are between 6.25-6.5 x 10^5 small lymphocytes per mm^3 of bone marrow, while in a mouse of 8-10 weeks of age this has fallen to approximately 5 x 10^5 per mm^3. Assuming an average body weight of 10 gram for a mouse of this age and a total bone marrow volume of 120 mm^3 this gives a total bone marrow population of 1.2 x 10^8 small lymphocytes (Miller and Osmond 1974; Osmond 1986). Within the bone marrow the cytoplasmic μ-chain positive cells exhibiting no surface IgM account for 30% of the small lymphocytes, while the surface IgM positive small lymphocytes account for 50%. In addition there are 5-8% T cells leaving approximately 10% of the cells with unknown lineage (Osmond, 1986). Although mitotic arrest and immunofluoresence labelling techniques indicate a rapid production rate of cells within the $c\mu^+s\mu^-$ population 75% of these cells are post-mitotic and mature into $sIgM^+$ lymphocytes without further division. The rapid turnover of cells is actually due to the remaining 25% of cells that have a division rate of approximately 3%/h (Landreth et al., 1981; Opstelten and Osmond, 1983). Approximately 2 x 10^7 IgM^+ B cells survive the post mitotic selection process within the bone marrow and are exported to the peripheral lymphoid tissue each day (Osmond 1993). These newly formed small lymphocytes leave the bone marrow within the first 1-2 days but it should be emphasised that the emerging cells contain both $sIgM^+$ cells and immature cells.

Transit time in the blood of the emerging B cells is short, about 30 minutes in the mouse (Yoshida and Osmond, 1978). The vast majority of these cells localise to the spleen while there is a 1-2 day lag period before newly formed B cells colonise the lymph nodes. Much smaller numbers can occasionally be found in the cortico-medullary junction of the thymus (Brahim and Osmond, 1970). Once established in the secondary lymphoid organs and given the appropriate circumstances the B cells are able to respond to antigens. The majority of antigen stimulated B lymphocytes differentiate into short-lived antibody producing cells

however a minority, after clonal proliferation in germinal centres, go on to form relatively long-lived memory cells. It is these cells that produce the high titre secondary antibody responses. Although the bone marrow contains memory B cells these are generally unresponsive to antigen stimulation *in situ*, and it is memory cells within the spleen and lymph nodes that account for the secondary response. Once stimulated in the spleen however these cells, after a 2-4 day lag, migrate to the bone marrow as plasma cells to make it the major site of antibody production during a secondary response (Benner et al., 1981; Koch et al., 1981).

1.4.3. T CELL ONTOGENY

Just as the bone marrow micro-environment induces the maturation of B lymphocytes the local environment within the thymus determines the development of T lymphocytes. Pro-thymocytes entering the thymus from the bone marrow locate to the subcapsular region however they soon migrate to the cortex and it is within this region that the majority of the cell proliferation occurs. As with B lymphocytes in the bone marrow the transit of T Lymphocytes through the thymus is accompanied by changes in the expression of surface markers (Strominger, 1989; Haynes et al., 1989; Nikolic-Zugic, 1991), as well as substantial cell death. The mature T cells are to be found in the medulla but, as with B lymphocytes within the bone marrow, cells within the cortex and medulla can be sufficiently mature to leave the thymus and enter the peripheral circulation on route to the secondary lymphoid tissues.

Within the thymus the T lymphocytes are both positively and negatively selected although the exact timing of these events has not been fully elucidated, the following description therefore is based on a possible sequence of events that has recently been proposed (Finkel et al., 1991). Positive selection occurs within the cortex with negative selection being a predominantly medullary process requiring the presence of bone-marrow derived macrophage\dendritic cells. T cells enter the thymus as $CD4^-$ $CD8^-$ (double negative) precursor cells and while within the thymic cortex acquire the surface molecules CD4 and CD8 (double positive: a

brief description of these T cell surface markers is given at the end of this section). Still within the cortex rearrangement of the T cell receptor (TCR) occurs, those cells unable to accomplish this successfully being eliminated. After successful TCR rearrangement and surface expression, positive selection occurs of those cells that can interact with major histocompatability complex (MHC) class I or II on the cortical epithelium. Cells unable to bind the MHC complex are eliminated. The cells continue to mature and now enter a negative selection stage; that is those cells that bind avidly to self-MHC or self-MHC plus self antigen, and would therefore be auto-reactive are eliminated or suppressed. Successful avoidance of elimination at this stage allows maturation into immunocompetent antigen receptor high, $CD4^+$ or $CD8^+$ (single positive) T cells. T cell maturation is a complex process and in addition to the reference given above two excellent reviews of this aspect of T lymphocytes have recently been published (Nikolic-Zugic 1991; Boyd and Hugo, 1991).

The TCR for the MHC-antigen complex is now well characterised. Two separate structures have been identified namely the alpha/beta and the gamma/delta receptors. In a similar fashion to the immunoglobulin genes the alpha and gamma chains are formed from V, J and C segments, whilst the beta and delta chains are formed from V, D, J and C segments. Once again additional diversity can be generated by non-stringent joining of the exons and by insertion of additional bases however unlike immunoglobulin V genes somatic mutation does not play any role in the final receptor repertoire.

The T cell receptor genes rearrange themselves in a set sequence as seen with the immunoglobulin genes. First to be rearranged are the gamma and delta segments to generate a γ/δ complex at the cell surface. In the mouse VJ_γ rearrangements are first seen on day 14 of gestation and are complete by day 16. This is paralleled by rearrangement of V_δ, D_δ and J_δ exons. Full length gamma and delta transcripts are seen on day 14 but reach a maximum on day 15 and then decline until birth on day 20. The surface expression of γ/δ receptors appears on day 15, reaches a maximum on day 17 and declines thereafter until day 20. Similarly a short DJ_β transcript can

be detected by day 14 but the full length VDJ_β transcript does not appear until day 16 reaching a maximum at day 20. A short J_α product can be detected by day 16 although the full length VJ_α transcript does not appear until day 17 reaching a maximum by day 20. Surface expression of the alpha/beta receptor starts on day 17 and is maximal by birth (Strominger, 1989). There are relatively high numbers of cells bearing the γ/δ type of receptor early in foetal life but these numbers decline rapidly so that by birth they will only represent 1-10% of peripheral blood T cell receptors in the mouse. The selection of a particular T cell receptor may define the role that a T lymphocyte will play within the body. Thus there is accumulating evidence that γ/δ bearing cells have a major function in surveillance at mucosal surfaces (Janeway, 1988; Janeway et al., 1988) and that specific epithelial surfaces may be patrolled by T cells having restricted gamma/delta repertoires (Asarnow et al., 1989; Itohara et al., 1990).

The T cell receptor is always associated with the CD3 molecule which is a complex of three single polypeptide chains and a disulphide linked homodimer (ζ-chain) and/or a disulphide linked heterodimer ($\zeta\eta$-chains). This macro-molecular complex appears to play a key role in signal transduction following TCR cross-linking. In addition to CD3 there are a number of other well-characterised surface molecules associated with the function of the T cell receptor including the CD4 and CD8 molecules that delineate helper/inducer and cytotoxic/suppressor T cells respectively. As mentioned earlier in terms of ontogeny of the T cell response these molecules show differential expression during maturation of the T cell population. The first cells appearing in the thymus, at about day 11 in the mouse, are double negatives for CD4 and CD8 ($CD4^-CD8^-$) and most of the gamma/delta receptor positive cells remain $CD4^-CD8^-$ even after leaving the thymus, with a small number becoming $CD8^+$ whilst an even smaller proportion will be $CD4^+$. Early alpha/beta cells are $CD4^+CD8^+$ these cells either proceeding to form α/β^+ $CD4^+CD8^-$, $CD4^-CD8^+$ or $CD4^-CD8^-$ cells the latter appearing late in the development of the T cell population. T cell ontogeny is a complex phenomenon and many questions

still remain to be answered.

1.5. STRUCTURE AND FUNCTION OF IMMUNOGLOBULINS

The final physical structure and functional characteristics of individual immunoglobulin molecules depends upon a complex series of molecular and cellular events that are influenced by the immune system as a whole. The next three sections will describe the formation of functional immunoglobulin molecules from their genetic components and how these mechanisms can be utilised experimentally to produce therapeutic monoclonal antibodies.

1.5.1. GENERATION OF IMMUNOGLOBULIN MOLECULES

In a previous section the rearrangements of immunoglobulin gene segments during B cell ontogeny were described. Processing of this rearranged DNA produces a primary RNA transcript with a combined VDJ sequence and heavy chain constant region domains separated by introns. Processing by small ribonucleoproteins removes these introns to give the full length mRNA. Differential splicing at this stage will produce either heavy chains destined for export as secreted immunoglobulin or heavy chains containing membrane insertion sequences that will form cell surface immunoglobulin.

Upon leaving the nucleus the leader sequence is translated first on cytoplasmic ribosomes, further translation only occurring upon binding to the docking protein on the endoplasmic reticulum. As mentioned previously upon entering the endoplasmic reticulum immunoglobulin heavy chain is bound by BiP until the latter is displaced by either surrogate light chain molecules or lamda/kappa light chain. The assembled molecule is finally glycosylated in the Golgi apparatus and transported to the surface of the cell.

1.5.2. FUNCTION AND MOLECULAR STRUCTURE OF IMMUNOGLOBULINS

The basic immunoglobulin molecule is constructed from a four chain configuration containing two identical light chains (22-23 kDa) and two identical heavy chains (50-77 kDa).

Immunoglobulin molecules can be divided into two

functional ends, at the amino-terminus there are two identical antigen binding regions each formed by the juxtapositioning of light and heavy chain variable regions whilst towards the carboxyl-terminal end are the constant region domains that interact with various effector mechanisms of the immune system (Figure 1.2.). Antibodies of the IgG, IgA and IgD classes have three constant domains and a flexible hinge region between the antigen-binding Fab arms and the effector-recruiting Fc region (Fig 1.2.). Immunoglobulins of the IgM and IgE classes have an addition region formed from a pair of domains inserted at the level of the hinge region (Pumphrey, 1986; reviewed by Burton, 1987). Whilst IgG, IgE, and IgD exist as the basic immunoglobulin monomer shown in Figure 1.2., IgM and IgA can also exist as polymers usually being pentameric and dimeric respectively.

Upon treatment with the enzyme papain (Fig 1.2.) the IgG molecule can be divided into the two separate antigen binding regions, Fab arms, and a fragment containing most of the constant region domains, the Fc fragment. Cleavage with the enzyme pepsin on the other hand leads to the formation of a $F(ab')_2$ fragment.

The general structure of an immunoglobulin domain is shown in Figure 1.3. As can be seen each domain consists of a 7-9 stranded beta-barrel in the middle of which is an invarant intra-chain disulphide bond which has the tendency to flatten the barrel (Richardson, 1981). In the variable domains of the heavy and light chains three hyper-variable loops form the complementarity determining regions (CDR), these are indicated by residues 27, 68 and 94 in Figure 1.3. It is these loops in the heavy and light chains that are mainly responsible for forming the antigen-binding surface of the antibody. Separating the CDR's are less variable sequences called framework regions (FR). The variable domains of the immunoglobulin molecule can act as antigens in their own right and antibodies that react with this structure, termed anti-idiotypic, may react with the variable or framework regions. Those antibodies capable of reacting with the antigen-binding surface of the immunoglobulin are termed anti-paratopes.

The various immunoglobulin classes and subclasses vary at

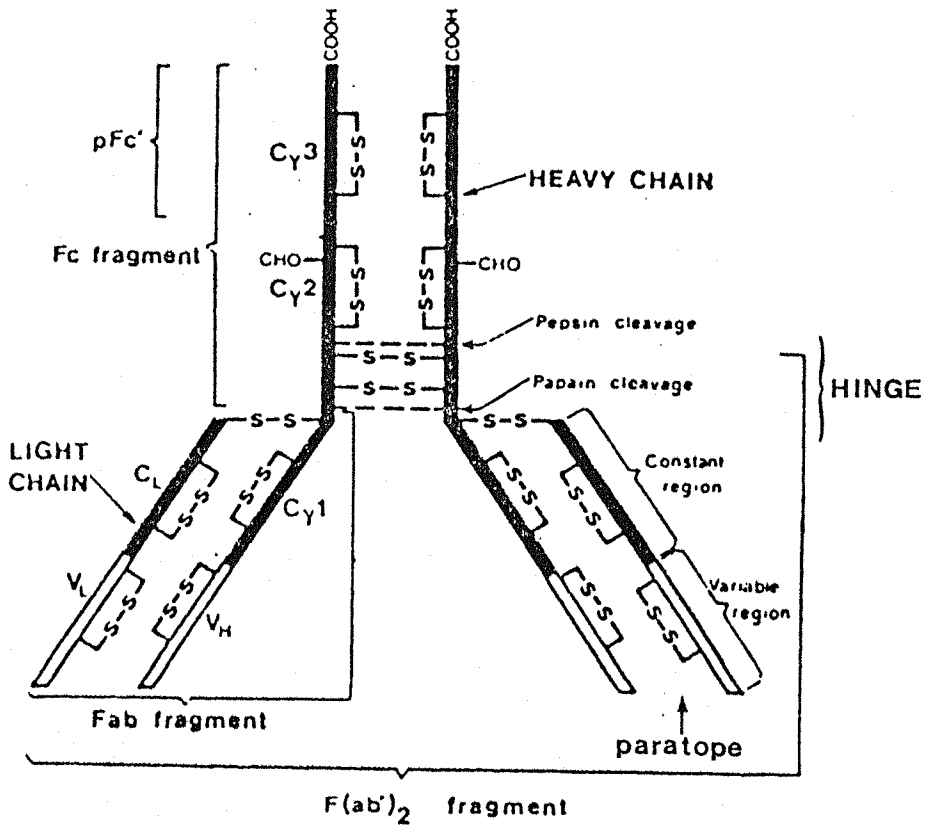


Figure 1.2. A typical Immunoglobulin Molecule.

The diagram does not represent a particular class or subclass of immunoglobulin and a full description of immunoglobulin structure is given within the text. Major enzymic cleavage points are depicted but further sites of digestion also exist.

Adapted from; Burton D.R., (1987), Structure and function of antibodies. In: Molecular genetics of immunoglobulins. Calabi F., and Neuberger M.S., (Eds), pp1-50. Elsevier, Amsterdam.

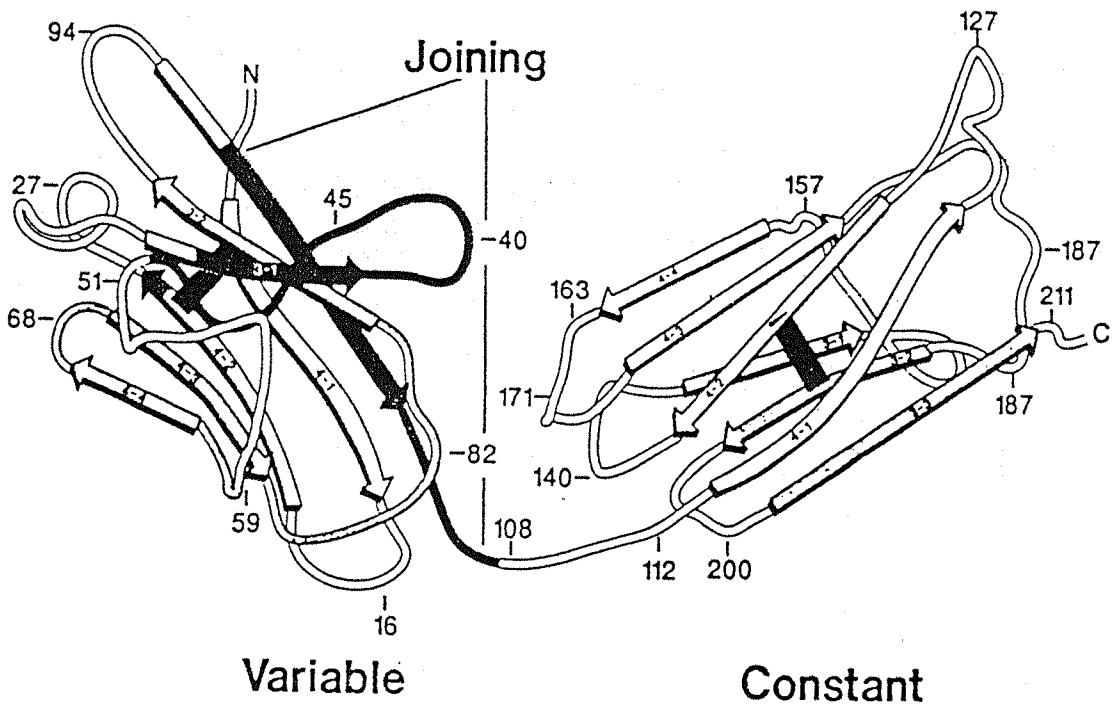


Figure 1.3. Peptide chain folding of variable and constant immunoglobulin domains. The broad arrows represent β-pleated sheet whilst the joining seqments represent α-helices, bends and other structures. The solid black non-arrowed band depicts the intra-chain disulphide bond. The hyper-variable loops are situated around residues 27, 68, and 94 of the variable domain.

As published by; Marchalonis J.J., and Schluter S.F., (1989), Evolution of variable and constant domains and joining seqments of rearranging immunoglobulins. FASEB 3, 2469-2479.

the molecular level by specific differences within the amino acid sequences of the heavy chain constant domains. For instance an arginine at position 12 of the C-terminal octadecapeptide is specific for IgG3 whilst leucine in position 2 is specific for IgG4 (Natvig and Forre, 1986). Immunoglobulin light chains may be separated into two types dependent on the amino acid sequence of the constant regions, these types being referred to as lambda or kappa light chains. In addition, the constant domains of kappa light chains as well as IgG1, IgG2, IgG3, IgA2 and IgE heavy chains have been shown to carry allotypic markers (Loghem, 1986).

The hinge region of immunoglobulins may give a certain degree of flexibility to the molecule allowing the Fab arms to move independently of each other. Within the hinge region, and linking the two heavy chains together, are situated inter-chain disulphide bonds the number of which varies not only between species but also within the subclasses of the same species. Thus within the human IgG subclasses, IgG_1 and IgG_4 have two, IgG_2 has four and IgG_3 has 11 (Burton et al., 1986). Each light chain is joined to the heavy chain by a single disulphide bridge except for the case of IgA2 where the light chains are joined by a disulphide bond (Burton, 1987).

The constant domains contain the sequences which permit the immunoglobulin molecule to interact with the rest of the immune system. It is within these regions that are found complement binding activity and sites recognised by the FcR carried by various cells of the immune system.

1.6. MONOCLONAL ANTIBODIES

The previous sections described how antibodies are derived from genetic elements within the mammalian B lymphocyte and how fine adjustments to the molecular structure can lead to high affinity antibodies. This section gives a brief description of how the production of antibodies specific for particular epitopes of an antigen can be achieved by *in vitro* manipulations.

The very fact that it is possible to grow and select cells producing specific antibodies is due to the work of Köhler and Milstein (1975). Since their description of the

methodology for the generation of continuous cultures of fused cells was disseminated a vast array of new immunological reagents and procedures have appeared. These have been of major benefit to the biological sciences in general and the medical field in particular, as well as generating a new industry within the commercial world. It is perhaps of no surprise that Köhler and Milstein were awarded the Nobel prize for medicine in 1984.

1.6.1. PRINCIPLE OF MONOCLONAL ANTIBODY PRODUCTION

Mice injected with a protein immunogen will produce a range of antibodies each reactive with a particular antigenic determinant (epitope) expressed on the protein. Subsequent injection of the same antigen, separated by a few weeks, will generate a secondary response and antibodies of various immunoglobulin classes and subclasses will be detected, in addition the affinity of the antibodies will tend to increase (affinity maturation). Once the mouse has been primed in this way an intravenous injection of the immunogen will cause a blastogenic response that will peak three to four days later. Unfortunately B cells taken from the spleen of such an animal will not survive *in vitro* using current tissue culture procedures. If the B lymphocytes from this spleen however are fused to a murine plasmacytoma cell line, which has been adapted to cell culture, it is possible to obtain a hybrid cell (a hybridoma) that has the antibody-producing potential of one parent and the immortal growth characteristics of the other. To ensure the immortalised parent does not overgrow the hybridoma a selective media is used which specifically kills the parent neoplastic line. Careful cloning of the products of these fusions makes it possible to isolate populations of cells which constitute the immortal progeny of a single antibody secreting B lymphocytes. The antibody secreted from such a population is therefore referred to as a monoclonal antibody and will recognise a single epitope on the antigen (Littlefield, 1964; Köhler and Milstein, 1975, Galfre et al., 1977; Shulman et al., 1978; Kearney et al., 1979).

Using this technique it has been possible to fuse various combinations of cells to form mouse-mouse, mouse-rat, rat-

rat, mouse-human and human-human hybridomas. There are various advantages and disadvantages with each of these hybridomas. The mouse-mouse hybridoma is the most common combination, it is easy to grow *in vivo* as an ascitic tumour and can produce high affinity monoclonal antibodies. However it can be difficult to obtain syngenic antibodies, i.e. a mouse monoclonal antibody that recognises a mouse protein. This can be overcome by mouse-rat and rat-rat fusions. The former is quite successful but has the disadvantages that the resulting hybridoma is difficult to grow *in vivo* due to rejection of the tumour and the antibody produced is of rat origin which can produce problems when working with mouse models. The rat-rat fusions (Bazin et al., 1972; 1973) have a number of advantages over mouse-mouse fusions. The reversion of parent lines to non-secretors is lower compared to the mouse cell lines and there is a higher frequency of production of spleen immunoglobulins compared to the mouse-mouse fusions (Clark et al., 1983). The larger size of the rat makes it possible to obtain much larger volumes of ascitic fluid (Galfre et al., 1979). However it should be stated that rat-rat hybridomas can prove difficult to establish although this does improve with experience (Galfre and Milstein, 1981).

Human-human fusions have lagged behind those using rodent partners principally due to the limited primed B cells available and the low fusion frequency, (immunisation of humans with antigens other than those approved for vaccination requires careful consideration. see Koyama et al., 1990), and also due to the limited number of appropriate cell lines to act as fusion partners (see Campbell, 1985). The search for the latter continues and together with the advances in the development of *in vitro* immunisation the production of human monoclonals in the future may not be as problematical as it has been in the past (Olsson and Kaplan, 1980; Reviewed by Markham and Sikora, 1987). Advances in molecular biology such as the humanisation of murine monoclonal antibodies and the future potential for further manipulations of the immunoglobulin gene repertoire hold great potential (reviewed by Winter and Milstein, 1991).

Monoclonal antibodies have allowed researchers to dissect

and manipulate many biological processes. Included in this work are monoclonal antibodies reactive with surface molecules found on effector cells such as receptors for immunoglobulin Fc together with antibodies reactive with the T3-Ti complex of cytotoxic T cells (Knapp et al., 1989). It is also possible to produce monoclonal antibodies that specifically recognise tumour markers. These antibodies can therefore be used to mark the tumour for the "kiss of death". A powerful technique is to construct molecules that combine both attributes, i.e. molecules that not only specifically attach to the tumour but also have an intrinsic ability to organise the destruction of that tumour cell. These antibodies may achieve killing by either the recruitment of effector cells or other therapeutic reagents. These concepts will be discussed in the following sections, the first of which will consider the recognition of tumour cells.

1.7. TUMOUR ASSOCIATED AND SPECIFIC ANTIGENS

1.7.1. NON-LYMPHOID TUMOURS

Only in a limited number of cases are antigen(s) expressed by a tumour cell unique to that tumour population. The non-virus-producing Schmidt-Ruppin strain of Rous sarcoma virus, (RSV), produces both non-viron and viron related tumour specific antigens (Kuzumaki et al., 1984), the expression of these TSAs being associated with the RSV *src* gene. A similar oncogene is involved with the expression of a further TSA. The *ras* gene encodes a group of membrane associated proteins with a molecular weight of 21 kDa that bind guanine nucleotide and have low GTPase activity (McGrath et al., 1984; Sweet et al., 1984; Gibbs et al., 1984). The proteins expressed by this gene are found in most mammalian cells and are thought to be involved in a signal transduction pathway (Barbacid, 1987) however a single amino acid mutations within these proteins can lead to transformation of both murine and human cells. The production of monoclonal antibodies that are specific for these point mutations has allowed them to be recognised as TSAs in colorectal cancers, pancreatic and lung carcinomas as well as acute myelogenous leukemia and preleukemic syndromes (Reviewed by Carney,1988).

The use of chemical carcinogens can also lead to the expression of tumour-specific transplantation antigens that have a specificity, often to a fine degree, that is unique to the tumour created (Reviewed by Daar and Lennox, 1987). However as it has been shown that these antigens are due to re-combinations of retro-viral proteins this system does not constitute a model that represents a common occurrence in neoplasms of man (Lennox et al., 1981).

Upon transformation certain cells re-express antigens, termed oncofoetal antigens, that are normally present only on foetal cells. The most studied example of which is probably the carcinoembryonic antigen (CEA) a 180 kDa glycoprotein found on normal foetal colonic mucosa and certain gastrointestinal carcinomas (Gold and Freedman, 1965). Despite a wide range of monoclonal antibodies having been raised to this protein it has been found that there is variation in their cross-reactivities to normal foetal and adult tissues (reviewed by Schlom, 1986).

More commonly antigens expressed by carcinomas are also expressed by a limited number of other tissues these antigens being referred to as tumour-associated antigens (TAAs). Such an antigen is the Tag-72 protein that is found on colonic adenocarcinomas, invasive ductal cancer of the breast, non-small cell carcinoma of the lung, ovarian, pancreatic, gastric and oesophageal cancers (Schlom, 1986) but has also be reported to be expressed in as high as 84% of normal colonic mucosa (Listrom et al., 1989). Exacting studies of the reaction patterns of monoclonal antibodies initially thought to represent markers for tumour-specific antigens can find responses, usually of a weak nature, with normal tissue thereby down grading these markers to the level of tumour-associated antigens (Stein et al., 1991).

Differentiation antigens distinguish between different cell types and tissues and also between the same cells at different stages in ontogeny. As such they represent normal cellular markers however the loss, over-expression or inappropriate appearance of these antigens can allow them to be used as tumour markers (reviewed by Daar and Lennox, 1987; Daar, 1987) or indeed as targets for therapy. This latter concept can be illustrated by the melanoma associated p97

antigen which can be expressed at a level of fifty times that seen on normal tissue (Brown et al., 1981) or the epidermal growth factor (EGF) receptor which exists in much higher numbers on squamous cell carcinomas, gliomas and some breast cancers (Masui et al., 1989). It is also possible that when a cellular differentiation antigen has expression on a limited population of normal as well as malignant cells the destruction of the normal population may be an acceptable price to pay for the removal of the tumour. This is best illustrated by malignancies of the lymphoid system and will be discussed in the next section.

1.7.2. LYMPHOID TUMOURS

One of the most commonly recognised human virally-induced lymphoid neoplasm is that associated with the Epstein-Barr virus (Burkitt and Wright, 1970). The infection of human B cells with this virus can, through multi-factorial events, lead to the development of Burkitt's lymphoma. The EBV genome produces at least six proteins that can be detected within the nucleus of infected cells, (EBNA antigens), however although these are of diagnostic value as they are not expressed at the surface they do not provide a therapeutic route for antibody. Peptides from EBNA antigens can be expressed at the cell surface in association with MHC class I and may play an important role in control of the infection by CTL (Rickinson et al., 1981) providing a possible mechanism for development of peptide based vaccines (Murry et al., 1990; Schmidt et al., 1991; Burrows et al., 1992).

Differentiation antigens can provide target molecules for the treatment of lymphoid tumours. Most B-lineage chronic lymphocytic leukaemias (CLLs) express the CD5 marker that was originally associated with T cells (Jones et al., 1986). The CALLA (CD10: common acute lymphoblastic antigen) is found on B cell precursors, including pre-B leukaemias, some mature lymphomas and leukaemias and the occasional T-ALL. However it can also be found on renal and other epithelia (Metzgar et al., 1981). The CD19 antigen has broad expression on virtually all normal and neoplastic B cells with the exception of plasma cells (Nadler, 1986) while the CD22 marker is B lymphocyte restricted but has a complex

expression when specific lineages of B cells are examined (Reviewed by Zola, 1987). Pan T cell depletion of donor marrow grafts has made major differences in graft versus host disease (Filipovich et al., 1987) and this technique was refined by the use of monoclonal antibodies against certain subsets of T cell (Uckun et al., 1989). The above only indicates some possible differentiation antigens that can be used as target molecules and a look at compilations of surface markers for B, T, and myeloid-cells would give the reader a good indication of other surface molecules that could be exploited for therapy. As mentioned above because the differentiation markers are not restricted to just the neoplastic population a careful selection has to be made for a potentially useful target molecule taking into account the possible destruction of normal cells and tissues carrying the markers. This may not be so important if, for instance, the normal population can be re-generated from stem cells.

It is fortunate that neoplasms associated with immature and mature B and T cells provide the researcher with target molecules that are unique to the neoplastic population, i.e. the idiotypes associated with their antigen receptors. The idiotopes that define the idiotype are antigenic in their own right and may act as tumour-associated antigens (TAAs). Antibodies raised against these structures (anti-idiotypes) therefore can act as tumour markers. Not only can anti-idiotypic monoclonal antibodies be used in detection of neoplastic populations and in the monitoring of treatment regimes but also as therapeutic weapons. The anti-idiotypic antibodies may be of use in their native state or can be modified by conjugation to radioisotopes, chemotherapeutic agents, toxins or as part of bispecific antibodies. The next section will describe the effector mechanisms that can be exploited by the use of antibodies in the treatment of neoplastic disease.

1.8. EFFECTOR MECHANISMS IN THERAPY

Before 1947 every attempt at influencing the course of acute leukemia in patients failed (Watkins, 1947). In 1947 and 1948 investigations on the action of folic acid conjugates and later folic acid antagonists resulted in the

first planned induction of remissions of children with acute leukemia (Farber et al., 1948; Farber, 1949). It is remarkable that almost thirty years later the same drug, aminopterin, through its selection of HAT sensitive myeloma lines, played its part in the establishment of monoclonal antibodies as therapeutic agents. In the interim many cytotoxic drugs and radiotherapy had been used for the treatment of neoplasms and in many cases these types of treatment proved successful. Unfortunately cytotoxic drugs and radiotherapy lack specificity and these treatments are often associated with severe side effects. The advent of monoclonal antibodies brought hope of more effective and specific methods of treatment with reduced side effects, and while this end has not been achieved it remains the ultimate goal.

The natural killer cell (NK cell) has an intrinsic ability to recognise certain virally infected or transformed cells and may play a role in the first line of surveillance/attack against these potentially catastrophic occurrences within the host organism (Versteeg, 1992; Moretta et al., 1992). Similarly the cytotoxic T cell (CTL) can distinguish virally infected cells in the context of peptides presented on MHC molecules but, whilst they can be found infiltrating tumour sites, the nature of the recognition of autologous tumours by CTL and the importance of the infiltration in controlling tumour progression remains unclear (Knuth et al., 1984; Topalian et al., 1989). In addition naturally occurring and *in vitro* produced antibodies can also elicit effector mechanism that may be exploited for the treatment of cancer (Herlyn et al., 1985; Fanger et al., 1989). Both polyclonal and monoclonal antibodies can exert their effect in a number of ways including complement activation, antibody-dependent cellular cytotoxicity (ADCC) and macrophage-mediated cytostasis, these effects being mediated through the Fc portion of the immunoglobulin molecule. Antibodies can be used to deliver toxic molecules such as ricin directly to unwanted cells through the use of immunotoxins (Wawrzynczak and Derbyshire, 1992). The antigen binding potential of antibodies can be utilised via manipulations of the immunoglobulin molecules to form

bispecific antibodies able to recruit effector cells (Fanger et al., 1990;1991) or toxins to the tumour target (Raso and Griffin, 1981).

1.8.1. COMPLEMENT ACTIVATION

The general formation of the membrane attack complex subsequent to complement activation by the classical or alternative pathways is well known (reviewed by Kinoshita, 1991) and will not be repeated here. In terms of monoclonal antibodies a few considerations are required for selection of antibodies suitable for human therapy. Not all classes or subclasses of immunoglobulin will bind the C1q component of complement and thereby activate the complement cascade, this point being illustrated in table 1.5..

TABLE 1.5.: SPECIES SPECIFIC ANTIBODIES AND COMPLEMENT ACTIVATION VIA CLASSICAL PATHWAY

ANTIBODY	COMPLEMENT ACTIVATION
HUMAN IgG1	+
HUMAN IgG2	-
HUMAN IgG3	+
HUMAN IgG4	-
HUMAN IgM	+
MOUSE IgG1	-
MOUSE IgG2a	+
MOUSE IgG2b	+
MOUSE IgG3	-
MOUSE IgM	+
RAT IgG1	+
RAT IgG2a	+
RAT IgG2b	+
RAT IgG2c	+
RAT IgGM	+

NOTE: All the above complement reactions are with the homologous complement source and the absolute levels of lysis have not been indicated.

In addition to having the correct class or subclass of antibody it is necessary to achieve aggregates of immunoglobulin at the target cell surface in order to activate the classical complement pathway. The necessity for aggregates can be explained when it is realised that the binding affinity of a single immunoglobulin molecule for C1q is only in the order of 1-5 x 10^4 M^{-1} and dissociation of the C1q will occur before activation of the lytic pathway is achieved. The binding of two antibody molecules raises the binding constant to 5 x 10^7 M^{-1} whilst three molecules will achieve 1 x 10^{10} M^{-1} the latter two binding constants being high enough to activate the classical pathway (Hughes-Jones, 1977).

In order for C1 activation to occur at least two binding-sites on C1q must be occupied this being acheived for example by a single molecule of pentameric IgM bound to its antigen therby creating a high enough affinity for activation of the complement cascade. Once the C1q has been held evidence suggests that distortion of at least two C1q arms is also required to bring C1r and C1s catalytic subunits together in order to initiate the complement cascade (Hoekzema et al., 1988; Burton and Woof, 1992).

Whether complement deposition is due to antibody-antigen complexes and the classical pathway or via interaction with the surface of a target through the alternative pathway the central feature is the formation of C3 convertase. The Fragment of C3 termed C3b is central in the formation of C3 and C5 convertases leading to formation of the terminal membrane attack complex and may also give rise to further degradation products (iC3b, C3c, C3d and C3dg) located on the target surface. Fragments such as these are recognised and bound by various cell types via receptors (complement receptors 1-5; CR1-CR5) within their plasma membranes (see Table 1.6.).

The interaction of complement coated antigen complexes with these receptors has important consequences in the clearance of immune complexes and in the interaction between immunocompetent cells. The presence of CR1 and CR2 on antigen presenting cells may help to concentrate antigen to these cells while the same type of receptors on T cells may reduce

the level of antigen necessary to induce stimulation (Arvieux et al., 1988). Complement receptors could also be important in helping to maintain B cell memory as it has been proposed that marginal zone B cells may trap antibody-antigen-complement complexes on their surface and transport these complexes to the follicular dendritic cells situated within the folliculi. The latter are known to be important in the long term maintenance of unprocessed antigen (Klaus and Humphrey, 1986; Gray and Skarvall, 1988).

TABLE 1.6.: RECEPTORS FOR COMPLEMENT COMPONENTS

RECEPTOR (CD number)	LIGAND	RECEPTOR-POSITIVE CELLS
C3aR	C3a (C4a)	Mast cells, monocytes, Macrophages, T cells, neutrophils, basophils
CR1 (CD35)	C3b (iC3b, C3c, C4b)	Erythrocytes, B and T cells, monocytes, macrophages, follicular dendritic cells, neutrophils, eosinophils
CR2 (CD21)	C3d, C3dg (iC3b)	B cells, thymocytes, B and T cell lines, follicular dendritic cells
CR3 (CD11b/CD18)	iC3b (C3d)	Monocytes, Macrophages, neutrophils, eosinophils, follicular dendritic cells, K and NK cells
CR4 (CD11c/CD18)	iC3b	Monocytes, macrophages, neutrophils, K and NK cells
CR5	C3dg, C3d	Neutrophils, platelets

Note: Taken from Erdei, Fust and Gergely (1991).

Immobilised C3b and C3d, through their interaction with CR1 and CR2, can also induce pre-activated B cells to enter the S phase of the growth cycle which may have important

consequences during interaction with macrophages within germinal centres and during encounters with immune complexes (Melchers et al., 1985). Interaction between C3 and complement receptors of macrophages, K and NK cells as well as CTL whilst thought not to be absolutely essential in the induction of cytolysis, may play a role as accessory molecules in the formation of effector to target cell contacts. In this role the fragments of C3 and the respective receptors will act as adhesion molecules (Perlmann, 1982; Ramos et al., 1985; Erdei et al., 1985).

In contrast to the above description of insoluble or cell surface bound complexes of C3 fragments it should be noted that soluble forms of these fragments far from enhancing the immune response may be more likely to suppress it. This suppression may involve inhibition of an early event in B cell activation via soluble C3d (Weiler et al., 1982; Melchers et al., 1985; Kuraya et al., 1990) and/or inhibition of IL-2 dependent T cell proliferation (Walker et al., 1986).

1.8.2. ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY (ADCC)

Once a target cell has become coated with antibody the Fc fragment can recruit various effector cells that will destroy the target. The binding of the effector cell to the target requires the former to have appropriate Fc receptors (FcR) in its membrane that are capable of interacting with coating antibody (Perlmann et al., 1972). Cellular cytotoxicity may be broken down into a number of stages; (1) Recognition of target (2) Conjugation formation (3) Triggering of cytotoxic mechanisms (4) Target cell lysis (5) Recycling of the effector cell. A number of different types of effector cell have been shown to mediate ADCC including lymphocytes (Perlmann et al., 1972); K/NK cells (Karpovsky et al., 1984; Anasetti et al., 1987); monocytes and macrophages (Holm and Hammarstrom, 1973; Mantovani et al., 1977); neutrophils (Levy et al., 1979), and eosinophils (Kazura and Grove, 1978). The types of FcR found and some of their characteristics are shown in Table 1.7.

1.8.3. NATURAL KILLER CELLS

These cells belong to a group termed large granular lymphocytes (LGLs) and included in this population, in contrast to the NK cells ability to recognise certain virally infected or transformed cells, are those cells responsible for most ADCC activity against tumour targets. It has been suggested in the past that NK cells and cytotoxic T cells may share a common progenitor cell pathway (Ritz et al., 1985).

TABLE 1.7.: Fc RECEPTORS OF MAN AND MOUSE

CD number	present and (prior designations)	characteristics
HUMAN		
CD64	huFcγRI (FcγRhi, FcγRp72)	High affinity on monocyte, macrophage, activated neutrophils (mol.wt 72 000)
CD32	huFcγRII (FcγRp40)	Low affinity on monocyte, neutrophil, eosinophil, platelets, B cells (mol.wt 42 000)
CD16	huFcγRIII (FcγRlo) (FcγRp50-70)	Low affinity on NK cells, activated neutrophils, eosinophils and low levels on monocytes and macrophages (mol.wt 75 000)
MURINE		
NA	moFcγRI (Fcγ2aR)	High affinity on macrophage
NA	moFcγRII (Fcγ2b/γ1R)	Low affinity, widely distributed, binds IgG2b, IgG1, IgG2a (mol.wt 50 000-70 000)
NA	moFcγRIII (moFcγ3R)	Not well characterised, but present on macrophages

*Adapted from Unkeless, Scigliano and Freedman, 1988

Recent evidence however suggests that they may be separate lineages (Garni-Wagner et al., 1990) or that T cells evolved from NK-like cells (Versteeg, 1992). Freshly isolated NK cells have germ-line T cell receptor genes (Tutt et al., 1986) however they do express the FcRIII (CD16) receptor (Lanier et al., 1983) and the CD2 cell surface marker (Meuer et al., 1984), both of which are associated with the ζ-chain as found in CD3-Ti complexes (Anderson et al., 1989; Lanier et al., 1989) and are involved in cellular activation giving the NK cell the additional ability to perform ADCC (Spruyt et al., 1991). It has been shown that at least 60% of peripheral blood $CD16^+$ NK cells can form conjugates with NK-sensitive cells (Perussia, 1983).

The cellular target(s) for NK cells are unknown although the leukocyte integrin LFA-1 is probably involved in stabilising the conjugation between NK and target cells (reviewed by Springer et al., 1987). Conflicting evidence exists on the role of the CD56 marker (an isoform of the neural adhesion molecule, N-CAM) in NK-mediated cytotoxicity. A monoclonal antibody recognising CD56 has been shown to block NK cytotoxicity (Nitta et al., 1989b) whilst N-CAM loss mutants and N-CAM transfectant studies have indicated no major role for CD56 in NK-mediated cytotoxicity (Lanier et al., 1991). The original concept that NK cells are able to kill allogenic cells as well as certain tumours and virally infected targets in the absence of antibody coating and without involvement of MHC may need revision in the light of more recent experimental data.

There follows a brief description of these new ideas, more detailed explanation of the concepts can be obtained from two recent reviews of the subject (Versteeg, 1992; Moretta et al., 1992). Evidence is accumulating that self-MHC products may protect cells from NK mediated killing and mis-matched MHC or inappropriately expressed MHC may mark a cell for NK cell recognition and elimination. A number of conceptual models have been proposed to explain NK cell recognition of target cells. The available evidence could be explained by NK cell recognition of a protective peptide within the self-MHC grove. In this model recognition of the self-peptide protects the cell from lysis whilst non-

recognition, by expression of a viral peptide or down regulation of MHC in a tumour cell, would lead to NK cell mediated lysis. According to this model the putative NK receptor would recognise the appropriate MHC molecule. Alternatively the NK recognition molecule that induces lysis could be a self epitope that is normally masked by self-MHC. An inappropriate MHC allele, as in allogenic cells, or lack of expression of the protective allele, as in virally infected cells or some tumour cells, would once again mark the cell for destruction. The basis for the molecular recognition of alloantigen is still unresolved although monoclonal antibodies recently obtained suggest that a 58 kDa family of proteins may be responsibly.

Cell to cell contact during NK cell recognition is a Mg^{++}-dependent process, which is followed by a Ca^{++}-dependent programming stage and finally a $Mg^{++}Ca^{++}$-independent cell disintegration phase (Hiserodt, 1977). Triggering of the NK cell is required for lysis to proceed and this is probably stimulated by the cell surface receptors of the effector cell, possibly the 58 kDa family of proteins. Cross-linking of CD2 and CD16 on the surface however can provide the necessary signal, the latter being dependent to a certain extent on the NK cell lines examined (Schmidt, 1985). After binding of the target cell occurs, degranulation of the NK cell releases a pore-forming protein monomer called perforin (cytolysin) into the intercellular space. In the presence of calcium this monomer polymerises into the target cell membrane to form pores, 5-20 nm in diameter, leading to possible disruption of intracellular osmotic homoeostasis or allowing access of additional cyto-disruptive molecules to the cytoplasm. It should be noted that perforin has been shown to have a degree of homology with the C9 component of complement (Young et al., 1986; Tschopp et al., 1986). In addition to the perforin released various other enzymes have been detected, including serine esterases, although whether these enzymes are involved in pore formation or are active in target cell killing, or both, remains to be elucidated.

Target cells can also be killed by a calcium-independent pathway (Tirosh and Berke, 1985) and it has been shown that freshly isolated peritoneal exudate lymphocytes (PEL) do not

contain perforin or serine esterases (Berke and Rosen, 1987), yet retain their ability to kill tumour cells. Cells attacked by the calcium-independent route show distinctive fragmentation of their DNA into 200 kB units. The cell surface undergoes zeiosis and eventually disintegrates into separate membrane-bound pieces, this complete process being known as apotosis. A soluble factor from NK cells and CTLs has been put forward as a mediator of this mode of cell death. Lymphotoxin (LTNα or TNFβ) has been shown to allow CTLs to kill target cells in a calcium-free and perforin-independent manner (Young et al., 1987) and has been identified in human NK cells (Degliantoni et al., 1985; Peters et al., 1986). The action of this molecule, or molecules with a similar function, on susceptible tumour targets is relatively slow taking several hours to achieve DNA fragmentation. Cell death achieved when intact effector cells are used is much more rapid. Such results suggest that TNFβ alone cannot account for programmed cell death. A membrane bound form of TNF has recently been identified in murine CTLs and has been proposed as a method of killing mediated through direct cell-cell contact (Liu et al., 1989). A complete understanding of the mechanisms involved in the killing of susceptible targets by NK cells has not yet been achieved (Clark, 1988).

As previously mentioned NK-cells posses CD16. K-cell activity, Fc mediated ADCC exhibited by NK-cells, can be inhibited by blocking CD16 without affecting the recognition and lysis of NK-sensitive cells. The experimental investigation of ADCC *in vitro* is complicated by a number of factors including the following. Original descriptions of ADCC used mouse effector cells (Moller, 1965) but data from a number of systems indicates that mouse effectors are generally poor mediators of ADCC when compared to human effectors (Berger and Amos, 1977). The results of Herlyn and colleges, however, using an extensive range of eighty-seven murine monoclonal antibodies to a range of human tumour cell targets, suggested that the ADCC activity of various effector populations from different species contradicted the original conclusions of Berger and Amos. The effector cells included mouse splenocytes, thioglycolate-elicited mouse peritoneal

macrophages, nonadherent human peripheral blood lymphocytes and human monocytes. Of the effector cell populations mouse macrophages showed the highest cytotoxicity whilst the various immunoglobulin isotypes exhibited individual, characteristic patterns of activity with the various effector cells that was independent of the target cell type (Herlyn et al., 1985).

Herlyn and colleges also demonstrated that individual monoclonals of all IgG sub-classes could be found that would mediate ADCC with IgG2a and IgG3 inducing lysis with all effector cell populations. Efficient ADCC by murine IgG3 has also been shown using switch variants of an anti-Thy-1.1 monoclonal antibody and human LGLs against murine SL2 thymoma target cells (Anasetti et al., 1987). The rank order of the variants was IgG3, IgG2a, IgG2b with IgG1 giving minimal cytotoxicity. The importance of sub-class has also been shown with rat monoclonal antibodies using hapten modified Chang cells as targets. The sub-class IgG2a was most effective with IgG1 and IgG2b also being proficient, although this was only demonstrable with rat effector cells as human effectors gave effective ADCC only with IgG2b monoclonals (Chassoux et al., 1988). Similarly Kaminski and colleagues used a murine B cell lymphoma model and isotype switch variants to show the importance of the Fc region in therapy demonstrating a parallel effectiveness with *in vitro* ADCC (Kaminski et al., 1986).

In experimental systems the short term *in vitro* ADCC (4-5h) is mainly mediated by K/NK cells whilst longer incubations, 24-48 hours may be required for human macrophages (Mantovani et al., 1977) and approximately three days for murine macrophages (Herlyn et al., 1985). The detection system used to determine cytotoxicity can also influence experimental results. The most common methods to determine cell killing in ADCC is release of ^{51}Cr or [^{3}H]TdR from pre-labelled cells and although these test have been used by many workers a note of caution is required. It has been shown that activated macrophages may phagocytose their targets intact thus preventing the release of the radiolabel (Gardner et al., 1987; Munn and Cheung, 1989). The latter authors described an Enzyme-Linked Immuno-sorbant assay

(E.L.I.S.A.) for determination of cell killing but this procedure has certain technical aspects which limits its general use. Another variable that has been shown to influence cytotoxicity assays is the composition of the medium used for the culture of the cells. In particular Dulbecco's modified Eagles medium with high glucose was found to be inhibitory to lysis and a pronounced inhibition was found if amphotericin B was included in the medium (Hasday and Crawford, 1988).

In summary when considering ADCC it is important to take into account (1) the isotype of the antibodies being used (2) the type and species source of effector cells (3) the target cells, this is especially important when using targets and effectors from different species and (4) experimental conditions being employed during analysis.

1.8.4. MACROPHAGE-MEDIATED CYTOTOXICITY AND CYTOSTASIS

The ability of macrophages to lyse tumour cells has been mentioned in the previous section and will now be discussed further. Although both monocytes and macrophages can cause lysis by ADCC freshly isolated monocytes will mediate low-level cytotoxicity. However the efficiency of this lysis is greatly improved by prior activation (Fidler and Schroit, 1984; Nathan et al., 1983).

The activation of monocytes and macrophages has been shown to be influenced by a number of factors including interferon-γ, IL-1, IL-2, AND IL-4 (reviewed by Fidler, 1985), GM-CSF (Grabstein et al., 1986) and M-CSF (Sampson-Johannes and Carlino, 1988). Of these interferon-γ is the main macrophage activating factor (Celeda et al., 1984; Schreiber et al., 1985) however it is likely that the other factors synergise to enhance monocyte/macrophage tumour killing (Sampson-Johannes and Carlino, 1988). The experimental activation of monocytes/macrophages in animals is usually achieved by the administration of glycogen, thioglycollate, muramyl dipeptide, or mineral oils given intraperitoneal (i.p.) (Reviewed by Ogmundsdottir and Weir, 1980; Johnston, 1988). In addition activation of these cells can be accomplished by the use of polyinosinic:polycytidylic acid both *in vivo* and *in vitro* (Alexander and Evans, 1971).

Activated monocytes/macrophages contain over one hundred chemicals that have been identified as being secreted by these cells (Nathan, 1987) and includes an arsenal with which to attack tumour cells including; lysosomal enzymes, oxygen metabolites, nitric oxide, cytolytic proteases, and TNFα. In addition these cells may directly phagocytose the target cells (reviewed by Johnston, 1988).

Perhaps one of the most important characteristics of these effector cells is their ability to migrate rapidly to the site of inflammation and tumour growth following a chemotactic pathway of complement factors, lymphokines released by neutrophils and lymphocytes and chemicals released by the tumour cells (Levy and Wheelock, 1974; Shin et al., 1975).

In addition to the killing of tumour cells, macrophages have an ability to stop proliferation of the tumour population in the absence of cell lysis (Keller, 1976; Evans and Alexander, 1976), a property referred to as cytostasis. This cytostatic mechanism has been divided into an essential single hit macrophage to target cell contact event followed by a cell to cell contact independent process which leads to suppression of growth (Pasternack et al., 1978a;1978b). Confirmation of the essential nature of the cell-cell contact has been demonstrated by showing that macrophage interaction with syngeneic fibroblasts via lectin binding to surface antigens leads to a decrease in ^{3}H-thymidine incorporation in the absence of cell lysis (Cabilly and Gallily, 1981). However it should be mentioned that [^{3}H]TdR release assays described in this latter report may have been compromised by the error described at the end of section 1.8.3.

1.8.5. CYTOTOXIC T CELLS

The recognition of target cells by cytotoxic T lymphocytes (CTL) differs from that of NK cells in that the CTL has a requirement for recognition of peptides within the binding grove of MHC molecules. The initial adhesion to the target cell may be promoted by interactions between pairs of receptors associated with the target and effector cell surface including CD2 and CD58; CD43 and CD54; CD45 and CD22; CD5 and CD72; and CD28 and B7 (reviewed by Altmann et al.,

1990). Increasing stabilisation of the contacts between the two cells allows the possible engagement of the T cell receptor (TCR) with the MHC presented peptide (Martz, 1987).

The lytic mechanisms available to the T lymphocyte may include the perforin/lymphotoxin molecules as described for the NK cells however the same arguments against these being the sole mode of killing exist for the T cell as they did for the NK cell (Berke, 1989). Lymphocyte induced internal damage of the target cell (Russell, 1983), is a programmed cell death initiated by the CTL and involves signalling in the target cell, possibly through elevated intra-cellular calcium levels (Tirosh and Berke, 1985). A recent review proposed that a prelytic halt mechanism to stop viral propagation may account for some of the experimental data generated with cytotoxic T cells. In this hypothesis cell lysis takes on a secondary role to mechanisms that stop viral replication (Martz and Howell, 1989).

One interesting feature of NK/CTL killing is the resistance of the effector cells to self-destruction by released perforin (Liu et al., 1989). A number of mechanisms have been proposed to explain this resistance to self-injury including; the release of slow diffusing chondroitin-sulphate A from NK-cell granules at the same time as release of perforin with the former neutralising any perforin permeating back towards the effector cell (Tschopp and Conzelmann, 1986); CD3-TCR complex, CD8, LFA-1 and CD2 molecules within the membranes of cytotoxic granules conferring exclusive binding to the MHC-antigen of the target cell (Peters et al., 1990); the presence of an effector cell surface protein that interfers with the binding of perforin to the effector cell membrane (Jiang et al., 1990). The exact mechanism(s) involved in self-protection of effector cells remains elusive as indeed does the importance of perforin as opposed to programmed cell death for target cell destruction (see Berke, 1991 and Krahenbuhl and Tschopp, 1991).

Although activation of cytotoxic T cells normally involves interaction between the CD3-Ti complex and a peptide-MHC molecule on the target this route may be artificially circumvented. Certain plant lectins such as Con A or PHA (Beven and Cohn, 1975) or mild oxidation of the cell

surface (Novogrodsky and Katchalski, 1972; Novogrodsky, 1975; Schmitt-Verhulst and Shearer, 1976) can cause lysis of virtually any target cell. It is also possible to induce killing via reverse ADCC, sideways killing, by the use of monoclonal antibodies attached to the target cell through an FcR. If this same antibody has specificity for the CD3-Ti complex or some other surface molecules on the CTL which are capable of activating the latter then the target cell will be destroyed (Leeuwenberg et al., 1985; Ito et al., 1989).

1.8.6. ANTI-IDIOTYPE THERAPY OF HUMAN B CELL LYMPHOMA

The use of anti-idiotypic antibodies for the treatment of patients with B cell lymphoma was pioneered at the Tenovus research laboratory in Southampton under the guidance of Professor G.T. Stevenson (Hamblin et al., 1980). These early attempts at therapy gave transient falls in circulating tumour which was unfortunately followed by a rapid resurgence in leukemic cells.

One of the most comprehensive studies of anti-idiotype therapy for patients with malignant B cell lymphoma has been carried out by the Stanford group of Ronald Levy. From 1981 to 1989 over thirty patients have been treated (Brown et al., 1989). The first patient received 400 mg of a IgG2b monoclonal anti-idiotype and after resolution of his disease remained in complete remission for over six years.

This successful therapy stimulated many workers to examine the use of anti-idiotype therapy in treatment of lymphomas however since this first therapy the results have been disappointing. Within the Stanford group of patients fifteen received anti-idiotype therapy alone and of these only one additional remission lasting greater than six years has been reported. In a second group of eleven patients anti-idiotype therapy was combined with interferon treatment and of these two remained in complete remission for over 23 months. The treatment of patients directly with monoclonal antibodies has proved disappointing and researchers have turned to ways of augmenting the action of the antibodies by various manipulations.

1.8.7. BISPECIFIC ANTIBODIES AND THERAPY

The term 'bispecific antibody' refers to antibodies that have binding sites for two distinct antigenic determinants. Attempts to form such molecules using nonspecific binding procedures such as protein A or glutaraldehyde produces ill-defined complexes of two intact immunoglobulins (Ghetie and Mota, 1980; Guesdon et al., 1983). Prior to these reports the chemical cross-linker N-succinimidyl-3-(1-pyridyldithio)propionate (SPDP) had been used to produce functionally bispecific antibodies however these were heterogeneous in terms of chemical linkage (Staerz et al., 1985).

Production of the first true bispecific antibodies had been accomplished in the 1960's by Nisonoff and colleges who used Fab fragments of rabbit IgG and linked separate Fabs through their hinge-region disulphides (Nisonoff and Rivers, 1961; Nisonoff and Palmer, 1964). This procedure produces hetero- and homo-dimers of the parent Fab' fragments, which can be separated by the appropriate immunoadsorbants, but was limited in its applications for monoclonal antibodies because of the multiple disulphides in murine IgG1. Both of these drawbacks were overcome by the prevention of intrachain disulphides with the formation of dithiol arsenite complexes from which Fab Thionitrobenzoate (TNB) derivatives can be formed. Combination of this derivative with a second reduced Fab produces a bispecific antibody of defined structure (Brennan et al., 1985).

Using a different approach to the problem of producing bispecific antibodies several workers have used hybridoma-hybridoma fusion techniques (Milstein and Cuello, 1983). A major drawback to this approach however is the difficulty in separating the resulting mixture of immunoglobulins with various combinations of heavy and light chains.

In 1987 a new method for the production of bispecific antibodies was established at the Tenovus laboratory (Glennie et al., 1987) that not only simplified the production of the derivatives but also allowed the formation of trispecific molecules (Tutt et al., 1991).

It had been shown early on that TNP-derivatised erythrocytes were effectively lysed by human neutrophiles in

the presence of anti-DNP x anti-FcRIII heteroantibodies (Karpovsky et al., 1984). Similarly FcRIII bearing lymphocytes can be redirected to kill melanoma cells *in vitro* and *in vivo* in nude mice if heteroantibodies with the correct binding specificity are used (Titus et al., 1987).

In extensive studies Fanger and collaborators have used chicken RBC as targets for various effector cells recruited using heteroantibodies with specificity for different FcγRs (Summarised by Fanger et al., 1989). Both FcγRI and FcγRII were shown to be cytotoxic triggering molecules on monocytes and polymorphonuclear cells whilst FcγRIII although only weakly stimulatory for PMNs was strongly stimulatory for LGLs. This latter finding has been linked to the presence of a transmembrane form of FcγRIII on LGLs and a phosphoinositol (PI) glycan-linked form in the membranes of PMNs (Lanier et al., 1988; Ueda et al., 1989). Monocyte ADCC remains at a low level unless prior stimulation with IFN-γ occurs when binding through the three receptors shows enhanced killing, in particular increased expression of FcγRI initiates substantially higher cytotoxicity. This latter finding has also been shown for neutrophil-mediated lysis of chick RBC were IFN-γ induces high expression of FcgRI. By chosing antibodies that bind to the FcR at sites away from its ligand binding domain it is possible to achieve killing in the presence of serum IgG (Segal and Wunderlich, 1988; Palazzo et al., 1990), an important consideration when contemplating *in vivo* therapies. Experimental results on the use of FcRs as target ligands for directed cytotoxicity have been summarised in recent reviews (Fanger et al., 1989; Fanger et al., 1990).

Bispecific antibodies have been made using the SPDP linkage with either antibodies against an allotype of the TCR or CD3 and a second antibody against various targets (Staerz et al., 1985; Perez et al., 1985). A comparative study of bispecific antibodies with one arm directed against CD3 and the other arm recognising gp85 on K562 and Daudi tumour cell lines in order to recruit T cell effectors to the tumour indicated that bispecifics made using SPDP linkage were less effective than those using the TNB derivatives (Nitta, 1989a). Further *in vitro* studies have indicated the potential of bispecifc antibodies in the recruitment of T cells bearing

the γ/δ receptor to ovarian carcinoma cells (Ferrini et al., 1989), of the re-targeting of CD3 positive cells to multidrug-resistant cells (Van Duk et al., 1989) and the potential of propagating tumour infiltrating lymphocytes (Wong et al., 1989).

The TNB procedure has been used to construct anti-CD3 x anti-glioma-associated antigen bispecific antibody which was shown to enhance the *in vitro* LAK cell activity of PBMC from glioma patients (Nitta et al., 1990a). These *in vitro* studies have recently been extended to the clinical situation by the treatment of glioma patients with IL-2 stimulated LAK cells and bispecific antibody. Four out of ten patients showed tumour regression and in another four eradication of remaining tumour after surgical resection was indicated. When patients received LAK therapy alone ninety per cent had reoccurrence of tumour within twelve months whilst patients receiving LAK plus bispecific therapy remained tumour free for at least 8-18 months (Nitta et al., 1990b).

With the identification of many cell surface markers in recent years a little consideration of the possible combinations that may be used to construct bispecifics indicates the potential for this form of therapy in the localisation of tumours and the recruitment, and activation, of effector cells to those tumours. In addition the technology can also be applied to immunoassays, staining reagents for light and electron microscopy, enzyme electrodes and biosensors (Paulus, 1985).

1.9. TOXINS IN THERAPY

Discussion on the use of immunotoxins and bispecific antibody mediated delivery of toxins in therapy will be preceded by a brief description on the structure and function of the commonly used toxins. The following account is based on two recent reviews (Olsnes and Sandvig, 1988; Stirpe and Battelli, 1990).

The ribosomal-inactivating proteins (RIP) are classified as type 1 or type 2 depending on their molecular structure. The type 1 RIPs are represented by such molecules as gelonin, saporin, momordin, pokeweed antiviral protein (PAP) and bryodin whilst the type 2 toxins include ricin, diphtheria

toxin, Shigella toxin, Pseudomonas exotoxin A, abrin, and modeccin. The former RIPs are single chain proteins whilst the latter proteins contain two peptide chains, (see Figure 1.4.). It should be noted that diphtheria toxin although formed as a single chain protein is only active when the molecule is cleaved and is converted into a two peptide chain structure (Fig 1.4.). In the type 2 RIPs the B chain has lectin like properties that allow it to bind to galactosyl-terminated residues on the target cell and also facilitates the entry of the toxin to the cytosol. The actual toxic moiety resides on the A chain which has the ability to inactivate ribosomes but does not have cell targeting properties. The type 2 toxins, with their intrinsic ability to bind to cell surfaces, are therefore much more toxic in the native form than the type 1 RIPs. The only cells that appear to be sensitive to unmodified type 1 RIPS are macrophages that have high rates of pinocytosis thereby allowing receptor independent access to the cytosol.

The normal route of entry into cells for type 2 RIPs is by endocytosis via coated pits and coated vesicles however ricin also seems to have an additional route of entry not involving clathrin-coated pits (Moya et al., 1985), the exact nature of this alternative route however remains unclear. The entry of diphtheria toxin into the cytosol requires that it encounters a pH below 5.3 within the endosome in order for hydrophobic domains on the B chain to be exposed which then insert themselves into the endosomal membrane and facilitates the entry of the A chain to the cytosol. This requirement for low pH within the intracellular compartment is also shared by Pseudomonas exotoxin A and modeccin. In contrast to the low pH required by these latter toxins ricin and abrin have increased toxicity in cells that have been treated to increase the pH of their endosomes (Sandvig et al., 1979; Mecada et al., 1981).

The exact site of translocation of toxins to the cytosol has been the subject of a number of studies. Hybridoma cells secreting anti-ricin antibodies demonstrated that ricin could interact with this antibody before the toxin had chance to enter the cytosol thereby conferring resistance to intoxication on the hybridoma cells. As the first site of

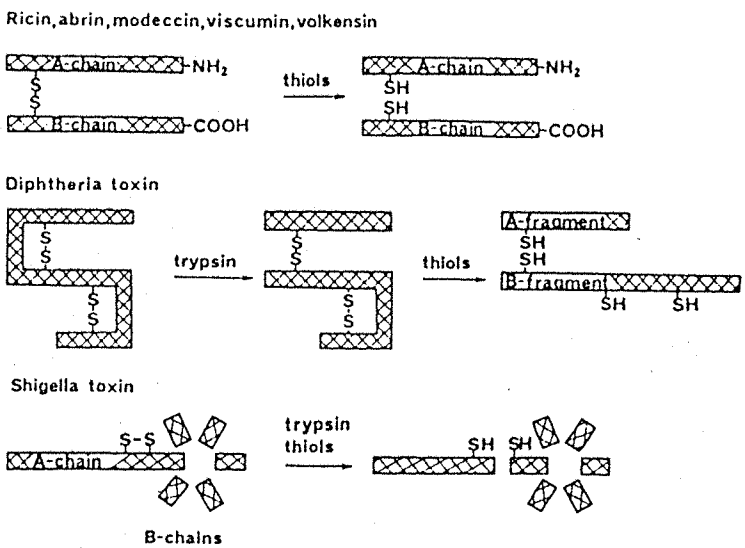


Figure 1.4. Peptide chain structure of type 2 ribosomal-inactivating proteins. Cell-binding activity is conferred by the B chain whilst the toxic moiety is the A chain.

As published by; Olsnes S., Sandvig K., (1988), How protein toxins enter and kill cells. In: Immunotoxins, Frankel A.E., (Ed), Kluwer Academic Publishers (Pub), pp 39-73.

inter-connection between the endosomal pathway and secretory pathways is the golgi apparatus this was considered to be the point at which ricin enters the cell (Youle and Colombatti, 1987). Further evidence of the site of ricin entry to the cytosol was forthcoming the same year using electron microscopical techniques. Using vero cells the investigators visualised gold and horseradish peroxidase (HRP) conjugates of ricin entering the cells via coated pits and coated vesicles. After internalisation sorting of the conjugates was seen to occur within the endosomal system with native ricin and a monovalent HRP conjugate reaching the golgi apparatus whilst the gold and polyvalent HRP conjugates did not (van Deurs et al., 1986). The approach of using anti-ricin hybridomas to assess intracellular trafficking was used by Kornfeld and colleagues (Kornfeld et al., 1991) were they came to the conclusion that a compartment proximal to the pre-golgi was important in translocation of a ricin immunotoxin to the cytosol. The fungal metabolite brefeldin-A, which blocks golgi function (Misumi et al., 1986; Lippincott-Schwartz et al., 1989,1990; Ulmer and Palade 1989), was used to study the role of the golgi in translocation of holotoxins and immunotoxins (Hudson and Grillo, 1991). At concentrations of brefeldin A below those needed to block holotoxin intoxication (see Yoshida et al., 1991) and inhibition of IL-2 secretion there was an enhancement of immunotoxin intoxication. It was found that immunotoxin processing was not effected but that it was at the point of translocation of the RIP to the cytosol that brefeldin A had an effect. These reports therefore indicate that translocation of ricin to the cytosol occurs proximal to or within the golgi apparatus. The endosomal reticulum is a very complex network of tubular cisternae containing multivesicular bodies (Hopkins et al., 1990). The trans golgi network receives membrane material from this endosomal pathway (Farquhar and Palade, 1981; Kornfeld and Mellman, 1989) and in the presence of brefeldin A has a high degree of continuity with the plasma membrane (Lippincott-Schwartz et al., 1991). Therefore in the presence of the latter drug disruption of the endosomal-golgi junction may make it difficult to precisely locate the actual site of

translocation. Dispite this caveat the evidence accumulated for ricin suggests the toxin is transported to the golgi complex via endosomes where it accumulates within the trans-golgi reticulum with significant amounts being transported back to the cell surface (Sandvig and Olsnes, 1979). Toxin that is not exocytosed can enter the cytoplasm from the trans-golgi compartment and inhibit protein synthesis (van Deurs et al., 1986; Sandvig et al., 1986; van Deurs et al., 1987; Youle and Colombatti, 1987; Marsh, 1989).

The main cellular location of disulphide cleavage reactions, a process that is essential for cleavage of the A chain from the B chain within the ricin molecule and which appears to be a rate limiting step in terms of ricin cytotoxicity (Lewis and Youle, 1986) and possibly leads to exposure of hydrophobic sites (Ischida et al., 1983; Utsumi et al., 1984), has also been studied. A synthetic probe, [^{125}I]iodotyramine-SS-poly($_{L}$-lysine), was used to study this aspect of immunotoxins. It was found that the most likely site of disulphide bond cleavage was the golgi apparatus with a small amount of reductive cleavage occurring at the cell surface (Feener et al., 1990).

1.9.1. MECHANISMS OF PROTEIN INHIBITION

Diphtheria toxin and Pseudomonas exotoxin A share the same mechanism of protein inhibition by carrying out an enzymic cleavage of NAD with linkage of the larger part, ADP-ribose, to elongation factor 2 with concomitant release of nicotinamide (Pappenheimer, 1977). The exact site of the ADP-ribosylation is an unusual amino-acid only found in elongation factor 2 called diphthamide that has a post-translationary modified side chain (Moehring et al., 1984). In contrast to this mechanism plant toxins such as ricin, gelonin and possibly saporin attack the ribosome directly to exert their effect. In a highly specific manner these RIPs cleave the bond between adenine and ribose in the nucleotide A4324 at the 3' end of the 28s ribosome subunit (Endo et al., 1987; Endo and Tsurugi, 1987). Removal of this adenine residue leaves the RNA backbone intact but results in reduced binding of elongation factor 2 and a consequent reduction in protein synthesis (Fernandez et al., 1976).

1.9.2. IMMUNOTOXINS

As early as the 1970s antibody-linked cytotoxic agents had been used to selectively kill target cells using either diphtheria toxin or drugs such as daunomycin and adriamycin (early immunotoxins referenced by Krolick et al., 1982; Hurwitz et al., 1975; 1979). Although useful results were obtained the production of antibody-drug conjugates was limited by the difficulties of purifying tumour specific polyclonal antibodies and of maintaining structural integrity of the antibody and drug. By the 1980's the ability to make monoclonal antibodies together with increasing knowledge of bacterial and plant toxins resulted in considerable interest in the production of toxin-antibody conjugates and which are now called immunotoxins. Since 1985 a number of chemical cross-linking agents have been described which have advantages in ease of production of immunotoxins and the resulting stability of the thioether bonds (Worrel et al., 1986; Thorpe et al., 1987).

One of the first immunotoxins produced utilised ricin A chain chemically linked to anti-IgD, anti-IgM or to an anti-idiotype of BCL_1. All these immunotoxins were able to kill target cells *in vitro* (Krolick et al., 1980) with later studies extending this work to include treatment of bone-marrrow removed from mice bearing BCL_1 lymphoma and the subsequently transfer of this laundered marrow into lethally irradiated naive mice. After twelve weeks 3 out of 20 mice were leukemic indicating that 99.9% of the tumour cells had been eliminated (Krolick et al., 1982). Treatment of advanced disease in the same model by cytoreduction (irradiation and splenectomy) followed by an anti-idiotype ricin A chain immunotoxin produced prolonged survival and possibly cured some mice (Krolick et al., 1982).

With these promising results it was not surprising that clinical trials in humans followed. Recently a phase I trial was carried out on 20 patients with advanced metastatic melanoma, a tumour that is especially difficult to treat by conventional means. Ricin A chain immunotoxin therapy only induced one complete remission (lasting >12 months) and one brief mixed response (Gonzalez et al., 1991).

Although treatment of neoplastic disease with immunotoxins of various types has generally been disappointing with few complete remissions achieved, an area that holds considerable potential is that of graft versus host disease (GVHD). In a recent study 12 of 15 bone marrow transplant patients had significant reduction in their GVHD with three patients obtaining complete resolution of symptoms (Byers et al., 1987). One unforeseen complication identified however is that if all the T cells within the allogenic bone-marrow are eliminated by immunotoxin treatment rather than enhancing the success rate of engraftment it was found that the failure rate had actually increased (Patterson et al., 1986; Martin, 1992). This is thought to be due to removal of T cells that produce factors that enhance the homing and engraftment of the bone marrow cells or removal of donor T lymphocytes that have a "veto" effect on host T cells (Fink et al., 1988). Removal of T-cells also eliminates the potential for a graft versus leukaemia effect were the donor T cells within the graft attack and destroy the neoplastic population within the host (Poynton, 1988). These phenomena emphasis the fact that careful selection of the specificity of such powerful agents as immunotoxins is required in order to avoid the removal of important non-target-cell populations. In the case of GVHD recent work has included the selection of specific sub-populations of T cells for removal by immunotoxin in order to avoid the impairment of the engraftment (Uckun et al., 1989).

The use of immunotoxins in patients with GVHD or cancer is not without its problems. In a phase I trail of an anti-breast cancer immunotoxin given by continuous infusion toxic effects noted included fluid overload and sensorimotor neuropathies induced by binding of the antibody to Schwann cells. Unfortunately there was also no therapeutic response in these patients (Gould et al., 1989). In a similar therapy of metastatic colon cancer side-effects observed included decreased serum albumin, flu-like symptoms, mild fever, reversible proteinuria and mental status changes (Byers et al., 1989). Once again only minor therapeutic responses were achieved in these patients. Many of these side effects were probably due to inappropriate binding of the antibody, the

use of glycosylated ricin A chain and instability of the immunoconjugates, factors that can hopefully be solved by appropriate modifications to the immunotoxins.

A number of studies on immunotoxins have examined the use of the single chain type 1 RIP, saporin. Lymphoma cells expressing the surface marker Thy 1.1 experienced a 50% reduction in protein synthesis when exposed to *in vitro* concentrations as low as 3×10^{-12} M of an anti-Thy 1.1-saporin immunotoxin. The reduction in tumour within mice carrying the AKR-A lymphoma after treatment with the immunotoxin indicated that 99.999% of the tumour cells had been eliminated (Thorpe et al., 1985). The cells emerging from this therapy did not display a difference in surface Thy 1.1 and remained sensitive to the action of the same immunotoxin. This finding is in contrast to the results seen during treatment of the guinea pig leukaemia L_2C with an immunotoxin composed of an anti-idiotype monoclonal and saporin (Glennie et al., 1987). This experimental therapy resulted in the emergence of both surface immunoglobulin negative variants and idiotope loss variants making the escaping cells more refractory to the action of the immunotoxin.

Selective elimination of $CD4^+$ and $CD8^+$ T cells has been achieved using saporin containing immunotoxins, the authors stating that the immunotoxin used appeared to be more effective than a similar immunotoxin utilising Ricin A chain to remove CD4 and $CD8^+$ cells reported by another group. The suggestion was that the greater effectiveness was due to enhanced internalisation of the saporin immunotoxin (Barbieri et al., 1989a). Significant protein synthesis inhibition by saporin immunotoxins have also been reported for B cell lines and B-CLL cells targeted through CD5, CD19 or CD22 (Barbieri et al., 1989b; Siena et al., 1989). The potential use of saporin immunotoxins for bone marrow purging in multiple myeloma has also been examined. Lack of toxicity towards myeloid precursors was a positive finding however the use of a single target antigen did not eliminate all clones of myeloma cell lines indicating that a mixture of immunotoxins reacting with different antigens may be required (Barbieri et al., 1989b).

1.9.3. BISPECIFIC ANTIBODIES AND TOXINS

A logical application for bispecifics is the targeting of toxins to tumour cells, autoimmune effectors or autoreactive T cells involved in GVHD, an extension of immunotoxin therapy. The Nisonoff procedure has been used for the production of bispecific antibodies with specificity for human IgG and ricin A chain, the antibody being found to enhance the toxicity of the ricin to human lymphoblastoid cells (Raso and Griffin, 1981). Ricin A chain has also been targeted to a prostate carcinoma cell line, (PC3), using bifunctional antibody recognising the ricin and a prostate-restricted antigen (Webb et al., 1985).

In a series of papers Corvalan and colleagues described the production from hybridoma-hybridoma fusion of bispecific antibodies with reactivity to CEA and vinca alkaloids, a cytostatic drug. The product of the fusion was shown to specifically localise the drug to the human colorectal carcinoma cell line MAWI, grown as a xenograft in nude mice (Corvalan and Smith, 1987; Corvalan et al., 1987a,1987b; Corvalan et al., 1988).

Saporin has been directed to kill L_2C cells via their surface idiotype with the use of a bispecific antibody. The therapeutic antibody was extremely efficient at inhibiting protein synthesis *in vitro* and had a profound effect on the growth of the cells *in vivo* subjecting the tumour to a high enough selection pressure that the only surviving tumour cells had lost expression of the idiotype (Glennie et al., 1987; Glennie et al., 1988).

1.10. HUMAN THERAPIES WITH TOXINS

The ultimate aim of the experimental investigations described in this dissertation is the development of treatments for human subjects with cancer. The following is a summary of the majority of trials on patients in which immunotoxins or bispecific antibodies played a major role and includes there use in bone marrow laundering. This summary is intended to give an understanding to the current effectiveness of these procedures.

1985: Gorin et al.,; three patients with non-Hodgkin's lymphoma and one with lymphoblastic leukemia: Ricin A chain immunotoxin clearance of autologous bone marrow: Side effects; slow recovery of lymphocytes after transplant and severe infection in two patients: Clinical outcome; hematopoietic recovery in all cases.

1986: Laurent et al.,; T-ALL and B-CLL: Ricin A chain immunotoxin given i.v.: Side effects; none: Clinical outcome; T-ALL transient decrease in tumour cells; B-CLL 40% reduction in lymphocyte count stable for two weeks.

1987: Hertler et al.,; Spitler et al: 22 melanoma patients: Ricin A chain immunotoxin: Side effects; anti-RTA and HAMA in 17/22. Immune mediated allergic responses: Clinical outcome; 1 CR; 4 mixed responses; 5 stable disease; 11 progressive diseases.

1987: Filipovich et al.,; Acute leukemia or CML: Allogenic bone marrow purging with anti T cell-ricin immunotoxin: Side effects; 4/17 grade II skin GVHD: Clinical outcome; 13/17 complete and sustained engraftment however 8 of these eventually relapsed; 4/17 autologous bone marrow recovery or graft rejection.

1988: Hertler et al.,: 4 patients with CLL: Ricin A chain immunotoxin: Side effects; mild fever and 1/4 anti-ricin A chain response: Clinical outcome; 4/4 rapid fall in wbc <24 hours duration, no sustained benefit.

1989: Durrant et al.,; Byers et al.,: 17 patients with colorectal cancer: Ricin A chain immunotoxin given i.v.: Side effects; anti A chain and HAMA responses; Hypoalbuminemia, mild fever, flu-like symptoms, proteinuria and mental status changes: Clinical response; 5/17 showed tumour regression at some sites only.

1989: Gould et al.,: 5 patients with breast cancer: Ricin A chain immunotoxin given i.v.: Side effects; fluid overload, debilitating sensorimotor neuropathies: Clinical response;

none.

1990: Nitta et al.,: 10 patients with malignant glioma: Bispecific anti-CD3 targeted LAK cells: Side effects; 1/10 transient fever; 2/10 increase in intracranial pressure. These symptoms were also present in a control group given LAK cells only: Clinical response; 4/10 regression of tumour; 4/10 eradication of tumour cells left after surgery.

1990: Mischak et al.,; Oratz et al: Melanoma: Ricin A chain immunotoxin or ricin A chain immunotoxin + cyclophosphamide: Side effects; IT alone- 20/21 anti ricin A chain response and 15/21 HAMA response; RTA + cyclophosphamide- no reduction in anti A chain or HAMA responses: Clinical outcome; 20% overall reduction in pulmonary and soft tissue nodules.

1990: Filipovich et al.,: 29 patients with leukaemias: Allogenic bone marrow purging of T cells with ricin A chain immunotoxin: Side effects; N/A: Clinical outcome; 28/29 engrafted with significant reduction in GVHD.

1990: Byers et al.,: 34 patients with pre-existing AGVHD: Ricin A chain immunotoxin: Side effects; Fatigue, myalgias, hypoalbuminemia, weight gain and 6/23 with low titre anti-IT response: Clinical response; 16/34 complete or partial responses.

1991: Vitetta et al.,: 15 patients with B cell lymphoma: Fab-ricin A chain immunotoxin: Side effects; 3/15 anti ricin A chain response; 1/15 with an additional HAMA response; Vascular leak syndrome, fever, anorexia, myalgia, pulmonary edema/effusion, expressive aphasia, rhabdomyolysis: Clinical response; 38% PR transient not lasting longer than 4 months.

1991: Gobbi et al.,: 14 patients with multiple myeloma: Autologous bone marrow purging with momordin immunotoxins. Side effects; N/A. Clinical response; 8/13 platelet recovery but 9/14 died with 1 complete remission and 3 partial remissions.

1991: Antin et al.,: 74 patients with hematologic malignancies: allogenic bone marrow T cell purging with ricin A chain immunotoxin: Side effects; N/A: Clinical response; 95% depletion of T cells, primary and late failure of graft in 4% of patients with effecctive control of GVHD in histocompatible patients.

1991: Gonzalez et al.,: 20 patients with melanoma treated with ricin A chain immunotoxin given i.v.: Side effects; Fatigue, myalgias, arthralgias, hypoalbuminemia, weight gain, peripheral edema, mild hypotension, flu-like syndrome, and 2/20 allergic response: Clinical outcome; 1 CR of >12 months, 1 mixed response lasting 3 months.

1992: Grossbard et al.,: 25 patients with B cell malignancies treated with 'blocked' ricin immunotoxin: Side effects; Hypoalbuminemia, thrombocytopenia, fevers and 9/25 produced an anti-ricin and HAMA response: Clinical response; 1 CR, 2 PR and 8 mixed or transient responses.

1992: Falini et al.,: 4 patients with Hodgkin's disease treated with anti-CD30-saporin immunotoxin: Side effects; Fever, malaise, anorexia, fatigue, myalgia (1 patient) and increase in body weight with oedema of lower limbs (1 patient) + other transient abnormalities, anti-saporin and HAMA responses: Clinical response; Transient reduction in tumour mass lasting no longer than 10 weeks.

A number of points can be made from the above survey; Most of the immunotoxins utilised ricin as the toxin moiety with saporin and momordin being the only other RIPs reported. The treatment of malignancies with these immunotoxins has been generally disappointing with perhaps the exception of the glioma study. The immunotoxins and bispecific antibodies do appear to have a use in GVHD and bone marrow purging.

1.11. TUMOUR ESCAPE MECHANISMS

Sections 1.8. and 1.9. described the effector mechanisms that can be utilised to kill tumour cells *in vivo* and *in vitro*. Unfortunately transformed cell populations seldom accept attack upon themselves without displaying some form of escape mechanisms. The major ways in which tumour cells may escape attack are:

Inaccessibility of cells
Extracellular antigen
Sparseness of surface antigen
Modulation of surface antigen
Mutants lacking surface antigen
Mutants with increased resistance to effector mechanisms
Appropriate effectors not recruited
Exhaustion of effectors
Anti-antibody

1.11.1. ESCAPE FROM COMPLEMENT LYSIS

Evidence accumulated so far suggests that complement mediated lysis does not play a major part in the rejection of autologous tumours (Lanier et al., 1980; Miller et al., 1982). The reasons for this can be found within the escape mechanisms utilised by cells to avoid attack by autologous complement.

Pore formation by the terminal membrane attack complex (MAC) within the membrane of unnucleated erythrocytes leads to lysis of the cell and requires that only one pore be formed to condemn the cell (Mayer, 1953) whilst in contrast nucleated cells require multiple hits with MAC to achieve lysis (Koski et al., 1983). The relative resistance of nucleated cells depends on the position within the cell cycle those cells in the stationary phase being more susceptible to attack than those cells in the exponential phase indicating that metabolically active cells are more resistant (Cikes, 1970a,b). Various studies have suggested that nucleated cells possess membrane-related mechanisms of defence against complement killing related to membrane repair (Yoo et al., 1980; Ohanian and Schlager, 1981). The use of patch clamp

techniques has shown that MAC channels open and close rapidly in the membranes of nucleated cells suggesting that only by overwhelming repair mechanisms can such cells be lysed (Stephens et al., 1980; Jackson et al., 1981). What are the mechanisms used to ensure cell survival?

Physical removal of the MACs by vesiculation has been shown to be efficient at expelling sufficient complexes to ensure survival of human neutrophils as was the mechanism of endocytosis and intracellular degradation of MACs (Morgan et al., 1987). It has been long known that cells are more resistant to attack by homologous than heterlogous serum (Muir, 1911). It has recently been shown that proteins exist within the membranes of cells that bind to complement components and thus disrupt the formation of the final complex. These factors include the following; decay-accelerating factor (DAF), which prevents association of C3b with factor B and of C4b with C2; membrane cofactor protein (MCP), which has cofactor activity with factor I and accelerates the cleavage of C3b to iC3b; CR1, which has cofactor activity for breakdown of iC3b to C3dg; C8 binding protein (C8bp = homologous restriction factor HRF); CD59 which binds to C8 and C9 in the assembling MAC. Some of these factors have been shown to exhibit a certain degree of species restricted (Nicholson-Weller et al., 1982; Schonermark et al., 1986; Zalman, et al., 1986; Lachmann, 1991). In some nucleated cells non-lethal complement effects have been noted. In these cases the escape and repair mechanisms allow the cell to survive but the increase in intracellular Ca^{++} can cause the cell to produce inflammatory mediators including reactive oxygen metabolites, metabolites of arachidonic acid and cytokines all of which may be implicated in disease processes (reviewed by Morgan, 1989).

1.11.2. RESISTANCE TO CELLULAR CYTOTOXICITY

Whereas the host must protect itself from attack by its own evolutionary primitive complement defence mechanism the cytotoxic cells have evolved to recognise altered self therefore there is no intrinsic protective mechanisms able to oppose attack by the hosts effector cells. For instance restriction factors similar to DAF and HRF have not been

identified that protect cells from cell-mediated lysis by ADCC, NK cells or perforin (Hollander et al., 1989). For tumour cells to survive attack by host cells they must avoid or block specific recognition by antibody or effector cell antigen receptors.

1.11.3. ANTIGENIC BARRIER

The release of large amounts of antigen into the circulation can prevent the antibody reaching the target cell, an antigen barrier. This has been shown in a number of tumours including colorectal carcinomas (Gold and Freedman, 1965; Herlyn et al., 1982); Carcinoma of the prostate (Wang et al., 1979); Ovarian cancer (Bast et al., 1981); Gastric and pancreatic carcinomas (Herlyn et al., 1982); Breast carcinoma (Kufe et al., 1984) and B cell lymphomas (Nadler et al., 1980). This has relevance in both the imaging of tumours (Zalutsky et al., 1989) and in their therapy (Alexander, 1974; Glennie and Stevenson, 1986). In the case of the B cell lymphomas not all tumour cells produce large amounts of circulating antigen (Gordon et al., 1984) and it has been suggested that patient selection could be based on the *in vitro* secretion of immunoglobulin by their tumour cells (Miller et al., 1987; Brown et al., 1989). Another alternative involves plasmaphoresis of the patient although this has variable results (Hamblin et al., 1980). In the absence of these procedures the antigenic sites may be blocked by excess antibody or the tumour load within the patient may be reduced by chemotherapy, radiotherapy or surgery. Obviously rather than trying to compete with the antigen barrier a better approach is to select surface markers that are not secreted into the circulation in high amounts yet remain reasonably specific for the tumour. In addition to the antigen barrier the formation of immune complexes can lead to exhaustion of effector mechanisms such as complement and phagocytosis capacity (Gordon et al., 1984) but does not seem to lead to critical renal damage (Nadler et al., 1980).

1.11.4. ALTERATIONS IN SURFACE ANTIGEN

Without sufficient surface antigen to bind the level of antibody deposition may be too low to initiate effector mechanisms. This may be because the physical distances between Fc regions are too great to activate complement or to stabilise cell-cell contact between target and effector cells. Non-heritable, non-cell-cycle related heterogeneity (intraclonal fluctuations) in idiotype expression over time can influence the susceptibility of individual B cells to attack (Taupier et al., 1983).

A specific mechanism that has to be considered when targeting antibodies to cell-surface structures is antigenic modulation (Boyse et al., 1963). Since the demonstration by these authors that the rapid resistance to attack by antibodies directed at the TL antigen from the surface of murine leukemia cells was due to removal of the antigenic site a large number of surface antigens have been shown to modulate in the presence of specific antibody (Chatenoud and Bach, 1984). A major exception to this is the major histocompatibility antigens although minor internalisation of MHC class II molecules has been reported (Neefjes et al., 1990). The exact mechanisms involved in this modulation however vary depending on the surface component involved. The TL antigen, for instance, is not internalised but forms microaggregates within the plasma membrane and becomes refractory to antigen specific antibodies (Esmon and Little, 1976). In contrast CALLA is rapidly removed from the surface of cells exposed to specific antibody and internalised (Ritz et al., 1980; Pesando et al., 1981).

The route of internalisation also varies depending on the antigen under consideration. In a recent study the modulation of CD4, 5, 7, and 150 kDa membrane antigens were investigated using monoclonal antibodies (Carriere, 1989). Over 55% of the CD7 antigen was internalised rapidly via clathrin-coated pits while in contrast CD4 was internalised four-times as slowly and was routed via non-coated microinvaginations. The third route observed involved the modulation of the 150 kDa membrane antigen which redistributed within the membrane to form clusters over a four hour period followed by internalisation via wide, smooth invaginations.

Experimental modulation of surface immunoglobulin from B lymphocytes is usually initiated by the binding of specific antigen or anti-immunoglobulin antibodies. Upon cross-linking of surface immunoglobulin there is rapid patching of receptor-ligand complexes within the membrane followed by capping of the complexes to one pole of the B cell leading to endocytosis (Taylor et al., 1971; Loor et al., 1972). The initial rapid phase of endocytosis lasts approximately 15-30 minutes and accounts for the internalisation of between 50 and 60 per cent of the surface immunoglobulin with the remaining antigen/anti-immunoglobulin being internalised more slowly (Myers and Vitetta, 1989; Drake et al., 1989). The rate of internalisation does not seem to be dependent on the size of the bound complexes at least in the molecular weight range of 40-240 kDa (Drake et al., 1989). The experiments performed by Drake and colleagues also indicated that if extensive cross-linking is achieved by using a second antibody, although the extent of the capping observed is increased, the rate of endocytosis is reduced significantly demonstrating that endocytosis can be unlinked from capping. It should be noted that internalisation of monovalent Fab fragments directed at membrane immunoglobulins has been demonstrated in the absence of cross-linking and capping (Goud and Antoine, 1984; Metezeau et al., 1984).
Another important consideration is the fate of the internalised proteins. Some cell surface receptors are recycled back to the plasma membrane with their attached ligand after internalisation the classical example being the transferrin receptor (Klausner et al., 1983). The same is true for antibodies directed at many cell surface proteins (Schneider et al., 1979) although antigen bound to surface immunoglobulin dissociates internally and the empty receptor returns to the surface. It should be born in mind that re-expression of surface immunoglobulin may also involve use of preformed cytoplasmic immunoglobulin pools (Drake et al., 1989). The path taken by antigen bound to its immunoglobulin receptor and that taken by anti-immunoglobulin-immunoglobulin complexes may be different as degraded antigen may reappear at the cell surface within 15 minutes whilst anti-Ig fragments take up to one or two hours to appear (Myers and

Vitetta, 1989). Surface receptors such as that for epidermal growth factor and human gonadotropin are degraded intracellularly and therefore do not recycle. This differential trafficking of receptors requires sorting of internalised components as some must be returned to the surface intact while others must be directed to fuse with lysosomes. Recent evidence indicates that the endosomal compartment is an extensive tubular reticulum with expanded swellings along its length (Hopkins et al., 1990). The work of Hopkins and colleagues suggests that receptors that are to be returned to the cell surface remain within the reticulum while those receptors that are to be degraded are sequestered within multivesicular bodies which are transported at a relatively slow rate to the pericentriolar area. The internalised proteins remain imprisoned within vesicles that are routed through the cell with access to the cytosol being very restricted.

1.11.5. ESCAPE FROM THERAPY BY MUTATION

Most tumour populations will have within them individual cells carrying variations of cell surface or intracellular proteins that may make them less susceptible to immunological control or attack. Under the selective pressure exerted by a therapeutic regime these variants may proliferate to become the dominant cell type. A number of mathematical models have been developed to try and describe the emergence of tumour variants during therapy and although great caution has to be observed when describing biological processes by these means it is apparent that even for small genetic drift constants the probability of a tumour remaining homogeneous rapidly approaches zero as the tumour size increases (Goldie and Coldman, 1979; Michelson et al., 1989).

These variations may involve antigenic determinants, metastatic potential, drug resistance, or growth rate and morphology (Dexter et al., 1978; Fidler, 1978; Olsson and Ebbesen, 1979; Fidler et al., 1981). There is no absolute requirement for a genetic sequence change to achieve these mutations as demonstrated by the fact that demethylation can be passed on as a hereditary trait and may lead to increased metastatic potential (reviewed by Gjedde, 1988).

Superinfection of highly metastatic murine large cell lymphoma cells with an endogenous virus from the low metastatic subline of the same tumour can produce a gradual decrease in the metastatic capabilities of the tumour. This has been linked to genes introduced by the virus that increase the expression of a surface glycoprotein thereby enhancing the tumour cells sensitivity to macrophage-mediated cytostatic factors. In addition analysis of RNA blots indicated that the highly metastatic cell line expressed ten times the normal subunit levels of a mitochondrial NADH molecule which was a target for a macrophage-released cytostatic factor (LaBiche, 1988).

A stepwise development of tumour resistance to effector mechanisms has been demonstrated in which the initial resistance to macrophage-mediated attack was followed by loss of a cell surface antigen recognised by one set of cytotoxic T cells followed by the loss of a second cell surface marker also recognised by a population of T cells (Urban et al., 1986). A further resistance mechanism has been identified by using limited dilution techniques by which non-tumourigenic variants where isolated from a population of a murine lymphoma and shown to express higher levels of TAA and Ia antigen (Fuji and Iribe, 1986).

It has recently been demonstrated that a proportion of lymphomas, including the majority of fresh Burkitt's lymphomas, express no or reduced levels of LFA-1 on the cell surface. These cells are poor stimulators of autologous and allogenic T cell responses indicting that the loss of this cellular adhesin may circumvent tumour rejection (Clayberger et al., 1987).

Common variations seen on initiating immunotherapy of malignant lymphoma with anti-idiotype antibodies is loss of idiotopes and/or total loss of surface immunoglobulin. When mouse myeloma cell lines are examined for spontaneous light or heavy chain loss variants a relatively high mutation rate of between 1×10^{-3} and 2×10^{-4} per cell generation has been found with additional deletion, nonsense, frameshift, point, subclass switch and antigen binding mutants being identified (reviewed by Morrison and Scharff, 1981). The mutation rate for drug resistance was less than 10^{-6} per cell generation

which is a figure similar to that found in non-myeloma cells. This indicates that the high mutation rate is a property associated with the genetic control and structure of the immunoglobulin genes. Mice immunised with idiotypic immunoglobulin from MOPC-315 and MOPC-460 and subsequently challenged with the relevant plasmacytoma generated Ig-negative variants. Immunisation of mice with idiotypic IgM before challenge with tumour has been examined in the BCL_1 tumour model. A stable variant was isolated that was surface IgM negative yet expressed cytoplasmic idiotype possibly being due to changes in the stability of the IgM or transport pathways within the cell (George et al., 1988). Immunoselection of variants of the murine lymphoma WEHI 279.1 suggests that the most common mutation seen is a complete loss of both membrane expression and synthesis of μ heavy chain within the cells (Sibley and Andrews-Wagner, 1983; Irick et al., 1986). The same outcome has been seen with immunotoxin therapy in the guinea-pig L_2C system were the most common variant identified was membrane IgM negative. In this latter case variants with loss of specific idiotopes were also seen (Glennie et al., 1987a). This latter finding had been identified earlier during anti-idiotype therapy of patients with malignant B cell lymphoma (Meeker et al., 1985). Of eleven patients treated with anti-idiotypic antibodies two were identified that had lost the idiotope to which the therapeutic antibody was directed and in addition one of these patients had also class-switched some of their tumour cells to IgG expression. The loss of the idiotope was due to somatic mutations within V_L and V_H regions of the active immunoglobulin genes (Levy et al., 1988).

1.11.6. ANTI-ANTIBODY

The infusion of repeated doses of large amounts of murine monoclonal antibodies into patients may produce an immune response, human anti-mouse antibody (HAMA), against those antibodies severely limiting the effectiveness of the therapy (Schroff et al., 1985; Shawler et al., 1985; Brown et al., 1989). Data from a number of studies suggests that the circulatory half life of a murine monoclonal antibody in patients is between fifteen and thirty hours and that an

immune response to the murine monoclonal antibody will peak between 2-4 weeks after administration (reviewed by LoBuglio et al., 1989). Both anti-constant region and anti-idiotype responses have been reported (Levy and Miller, 1983; Rowe et al., 1985). A number of approaches have been suggested in order to limit or prevent immunological responses to administered monoclonal antibodies. Immunosuppression of patients with B cell malignancies has been proposed as a reason for the often low level responses to xenogenic antibodies seen in these patients (Brown et al., 1989) and a similar state can be induced with the use of cyclosporin A which has been shown to inhibit host responses to murine antibodies (Ledermann et al., 1988).

The production of a chimeric univalent derivative from a murine anti-idiotype antibody utilising a human IgG antibody (FabIgG) failed to produce an immune response during treatment of a patient with follicular lymphoma (Hamblin et al., 1987). Development of more refined chimeric antibodies has been made possible by the use of genetic engineering. Construction of antibodies using these new techniques involved the attachment of the mouse variable regions to human constant regions (Morrison et al., 1984; Boulianne et al., 1984; Neuberger et al., 1985). Although this procedure reduces the non-human content of the immunoglobulins there are still significant murine sequences that may elicit an immune response. A more sophisticated technique is the insertion of only the rodent immunoglobulin hypervariable sequences into the genes for human immunoglobulin thus constructing an antibody that is essentially human but retaining the antigen specificity of the rodent monoclonal (Jones et al., 1986; Verhoeyen et al., 1988; Richmann et al., 1988).

1.11.7. INACCESSIBILITY OF TUMOUR CELLS

Even the most sophisticated genetically engineered antibodies are useless if they can not reach their targets. The problems of antigenic barriers, mutation and modulation have been described but the actual physical structure of solid tumours may also provide another barrier to successful therapy. The following account is taken from two reviews on

the physiology of solid tumours (Jain, 1989; Cobb, 1989).

Even when the therapeutic antibody has reached the lumen of the intra-tumour blood vessel it must still penetrate the tumour mass. The nature of the endothelium is important as that of the liver, spleen and bone marrow are relatively permeable to antibody whilst that of the skin and lung are impermeable (Cobb, 1989). It should be noted that in large tumours the drainage of intra-tumour fluid may be restricted thus generating high pressures within the tumour which may inhibit extra-vascularation of the antibodies.

Upon leaving the capillary the antibody must diffuse through the intercellular space the ease of which will depend on the matrix surrounding the cells for instance high levels of proteoglycans and glycosaminoglycans will restrict diffusion and convection of antibodies. In general terms connective tissues, muscle, haemopoetic and lymphoid tissues are composed of cells that are not bound close together by tight junctions and desmosomes and antibody has free access to the cells. Tissues such as epithial and parenchymatous glandular cells are rich in these cell to cell contacts and often show complex interdigitations severely restricting the diffusion of the antibodies. Antibody that successfully penetrates these obstacles will, on encountering the tumour, bind to its antigen thus a form of antigenic barrier may be present within the tumour itself if there are sufficiently large amounts of antigen at the periphery of the tumour to stop the antibody reaching the centre of the carcinoma. In addition a large tumour may be poorly vascularised and close to a necrotic centre may be viable cells that are protected from contact by therapeutic immunoglobulin (Cobb et al., 1987). This problem may be overcome by the use of mixtures of high and low affinity antibodies and IgG, $F(ab)_2$ and F(ab) fragments (Fujimori et al., 1989).

1.11.8. ESCAPE FROM IMMUNOTOXIN THERAPY

Many of the mechanisms described above will obviously play a role in limiting the effectiveness of immunotoxin therapy and a few examples are given below. In a study of 22 patients receiving a ricin A chain immunotoxin for treatment of metastatic melanoma, 17 of the subjects had detectable

levels of anti-toxin and anti-mouse antibodies in their circulation. In a limited number of patients who received infusion of immunotoxin in the face of circulating antibody an allergic response has also been demonstrated (Hertler et al., 1987).

Isolation of variants with resistance to the action of RIPs are rare events and those mutants that have been obtained have generally been the result of forced mutagenic experiments. The variants identified appear to have acquired resistance by one of the following mechanisms: alterations in ribosomal proteins restricting access of the toxin to adenine 4324; mutations in proteins or rRNA involved in ribosome-toxin interactions; and reduction in the passage of the toxins across the plasma membrane (Wellner et al., 1984; Goldmacher et al., 1987; Sallustio and Stanley, 1990).

The route of administration of immunotoxins can also be important and may be dependent on the localisation of the tumour within the host. An IgG immunotoxin, anti-CD5-Ricin A chain, therapy of an ascitic human tumour in nude mice has been shown to be much more effective when the therapeutic antibody was administered i.p rather than i.v. reflecting easier access to the tumour and longer survival of the IT within the host when administered by the former route (Rostaing-Capaillon and Cadellas, 1990). Improved delivery of the IT via the i.v. route was achieved by using a $F(ab')_2$ fragment of the antibody and partial deglycosylation of the ricin A chain. Higher tumour localisation with this derivative was explained by both better extravasation and decreased plasma clearance. However, when given i.v. this IT did not reach the binding levels on the tumour cells within the ascities seen with the unconjugated monoclonal antibody. A possible reason for this was the instability of the disulphide bond between the toxin and the monoclonal antibody such that once dissociated the smaller $F(ab')_2$ was cleared from the circulation at a faster rate. In addition to these problems, a non-specific inhibition of binding of the antibodies to the antigen that did not correlate with release of CD5 antigen was also noted (Rostaing-Capillon and Casellas, 1990).

1.12. AIMS

The A31 tumour is a B-cell tumour that arose in 1971 in a female CBA/H mouse that had been injected with ^{90}Sr i.p. 19 months previously (Cobb et al., 1986). Initial appearance of the tumour was as a mediastinal mass together with splenic involvement and the tumour can be routinely passaged via transfer of splenic tissue to naive animals. Little was known about this murine B cell lymphoma, the aim of this study was therefore to obtain data on the characteristics of the tumour and to examine its potential as a model for experimental therapies using antibody derivatives. The idiotype carried by the surface IgM of the neoplasm was to be used as a tumour specific marker and therefore the initial aim was to raise anti-idiotypic monoclonal antibodies that could then be used in therapy.

These antibodies were to be used to construct immunotoxins (ITs) and bispecific antibodies that were able to deliver the ribosomal-inactivating protein saporin to the tumour to allow the assessment of the therapeutic potential of these reagents. Bispecific antibodies differ significantly in their mode of action compared to ITs. The binding of the bispecific antibody to its target is via a single Fab arm compared to the divalent binding of the IT therefore differences in binding affinity to, and modulation of, surface target molecules may be expected. In addition IgG-ITs have an Fc region which may not only influence modulation parameters but also *in vivo* survival compared to bispecific antibodies constructed from separate Fab's. As the toxin moiety is held by covalent forces within the IT but by non-covalent bonds in the bispecific antibody further differences could be expected in terms of the efficiency of delivery to and release within the target cells. Covalent linkage of a toxin molecule to an IgG molecule may damage both the antigen binding site of the antibody and the RIP however this is not a consideration with bispecific antibodies. Although the smaller $F(ab)_2$ bispecific antibody may be cleared from the circulation faster than the IT the former will have a more rapid extravasation and therefore perfuse solid tumour more efficiently. A murine model of lymphoma may prove invaluable in determination of the importance of these parameters in

successful therapy of cancer by antibody derivatives.

Finally, as described previously, the bispecific antibody technology can be used to construct molecules capable of recruiting effector cells to the tumour. The availability of a monoclonal antibody (KT3) that binds the CD3 molecule of murine T lymphocytes made it possible to examine the therapeutic potential of recruiting T cells to the A31 tumour.

CHAPTER 2
MATERIALS AND METHODS

2.1. BUFFERS AND SOLUTIONS

Buffers and solutions were prepared from 'Analar' grade chemicals using glass distiled water. The pH of each buffer was checked before use and any adjustment required was achieved using the appropriate acid or base. Unless stated otherwise all chemicals were purchased from BDH or Sigma (BDH, Merck Ltd, Hunter Boulevard, Magna Park, Lutterworth, Leics, U.K.; Sigma Chemical Co Ltd, Fancy Rd, Poole, Dorset, U.K.). Many of the chemicals and procedures used are hazardous therefore they fall within The Control of Substances Hazardous to Health regulations (COSHH) and will require COSHH risk assessment forms to be completed.

(1) Phosphate Buffered Saline (PBS) pH 7.3

	g/l
NaCl	7.02
Na_2HPO_4	3.44
KH_2PO_4	0.79

(2) 0.044 M Phosphate - 0.004 M EDTA pH 7.3 (Papain activating buffer)

	g/l
Na_2HPO_4	6.25
KH_2PO_4	1.5
Na_2EDTA	1.49

(2a) Activation of papain

Papain requires the reduction of an intra-chain disulphide bond to obtain full enzymic activity. To achieve this dithiothreitol (DTT) was dissolved to 15.4 mg/ml in activating buffer and an equal volume of papain was added (10 mg/ml in activating buffer) and the solution incubated in a water bath at 37°C for 30 min.

(3) PBS - 1% (w/v) BSA - 20 mM NaN_3 (FACS wash buffer)

	g/l PBS
BSA (Fraction V)	10.0
NaN_3	1.3

(4) 0.06 M Phosphate - 0.002 M EDTA pH 7.3 (0.06M PE7.3)

	g/l
Na_2HPO_4	6.2
KH_2PO_4	2.04
Na_2EDTA	0.74

(5) 2 M Tris-HCl - 0.1 M Na_2EDTA (2 M TE8)

	g/l
Tris-HCl	242.0
Na_2EDTA	37.2
HCl (5 M)	200 ml

(6) 0.1 M NaCl - 0.02 M Tris-HCl - 0.001M EDTA (Tris-NaCl) pH 8.0

	g/l
NaCl	5.84
Tris-HCl	2.42
$Na_2EDTA.2H_2O$(EDTA)	0.372
1 M HCl	10 ml

(7) 0.5 M NaCl - 0.1 M Tris - HCl - 0.005M EDTA (High molarity Tris-HCl) pH 8.0

As for Tris-NaCl x 5

(8) 0.2 M Tris-HCl - 0.01 M EDTA pH 8.0 (0.2M TE8)

	g/l
Tris-HCl	24.2
Na_2EDTA	3.72
5 M HCl	20 ml

(9) Ammonium Sulphate (saturated)

770 grams $(NH_4)_2SO_4$ + 1 litre TE8 stirred for at least 30 minutes before use

(10) 0.2 M Sodium citrate pH 6.5

	g/l
Citric acid	42.0
NaOH	23.0

(11) 2 M Sodium acetate pH 3.7

	g/l
CH_3COONa	17.2
CH_3COOH	103 ml

(12) 0.05 M Sodium acetate - 0.0005 M EDTA pH 5.3 (0.05 M AE 5.3)

	g/l
CH_3COONa	4.102
Na_2EDTA	0.186

(13) 1 M Ethanolamine - HCl pH 9.5

60 ml ethanolamine/litre of distiled water; pH adjusted with HCl

(14) ELISA REAGENTS

(a) ELISA COATING BUFFER pH 9.6

	g/l
Na_2CO_3	1.59
$NaHCO_3$	2.93

(b) BLOCKING BUFFER: BSA/PBS 1%(w/v)

	g/l
Bovine Serum Albumin (Fraction V)	10.0
PBS	1 litre

(c) WASHING BUFFER: PBS/Polyoxyethylene sorbitan (Tween-20, Sigma)

	ml
Tween-20	2.5
PBS	5 litres

(d) OPD SUBSTRATE

STOCK SOLUTIONS: CITRIC ACID AND PHOSPHATE

	g/l
(a) Citric acid	19.2
(b) Na_2HPO_4	28.4

WORKING SOLUTION pH 5.0

	ml
Citric acid solution	24
Na_2HPO_4 solution	26
H_2O	50
	mg
OPD	20

Just before use 20 µl of H_2O_2 (60%w/v) was added. Reaction stopped with 0.25 volume of 5M H_2SO_4 after 30 min.

2.2. CELL CULTURE MEDIA

All cells including hybridomas were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco), supplemented with 100 U/ml penicillin (Glaxo), 100 µg/ml streptomycin (Evans), 2 µg/ml amphotericin B (Fungizone, Squibb & Sons), 2 mM fresh L-glutamine (Gibco), 1 mM pyruvate (Gibco) and either 10 or 15% (v/v) Myoclone plus Foetal Calf Serum (FCS, Gibco). In some instances cells were also cultured for sort periods in RPMI-1640 medium containing 25 mM HEPES buffer (Gibco) supplemented as above including 10% (v/v) Myoclone FCS.

2.3. ANIMALS

Balb/c, CBA, Balb/c x CBA (F_1) mice and Louvain rats were bred on these premises. Nude mice (MF1-Nu/Nu/OLA/HSD) were obtained at 8 weeks of age (Harlan Olac Ltd, Bicester, U.K.) and housed within a class 1 laminar flow hood. All water, feed and bedding was irradiated, the environmental temperature was controlled at 28°C and strict aseptic technique was maintained during routine handling and experimental procedures. Where mice were used for the production of ascitic fluid the animals received an injection of 0.2 ml of pristane i.p. and were left a minimum of three days before use.

2.4. A31 TUMOUR

The A31 tumour is a B-cell tumour that arose in 1971 in a female CBA/H mouse that had been injected with ^{90}Sr i.p. 19 months previously (Cobb et al., 1986). Initial appearance of the tumour was as a mediastinal mass together with splenic involvement. Tumour was passaged by transfer of splenic tissue to recipient mice and the tumour used in the present study represents stocks transferred to the Tenovus laboratory at passages 100-110 and immediately frozen in liquid nitrogen. Experiments described within this thesis were initiated with cells isolated within the first six passages after recovery of the cells from cryopreservation unless detailed otherwise within the text. Routine passages involved the injection of 5×10^5 tumour cells, isolated from the spleens of terminally ill animals, i.p. into each mouse.

2.5. MURINE TUMOUR AND HYBRIDOMA CELL LINES

The murine tumours and hybridoma cell lines used for production of immunoglobulins during this study are listed in table 2.1. Ascitic tumours were grown within the peritoneal cavity of pristane primed mice and the ascitic fluid collected from the peritoneal cavity after sacrifice of the animal. The murine lymphoma BCL_1 was grown within Balb/c mice and routinely passaged as described for the A31 tumour.

2.6. HOLLOW FIBRE BIOREACTOR CULTURE

Culture of a mouse-rat hybridoma within a hollow-fibre bioreactor (Endotronics AcuSyst-R, Northumbria Biologicals Ltd, Cramlington, U.K.) followed the manufacturer's recommended procedures.

2.7. CELL COUNTS AND VIABILITY

Cells were counted using an Industrial D model Coulter Counter fitted with a 140 µm orifice tube (Coulter Electronics Ltd, Luton, Beds, U.K.). Contaminating red blood cells were lysed using "zaponin" detergent (Coulter Electronics). Cell viability was determined by exclusion of 0.2% (w/v) Trypan Blue in PBS and enumeration of viable cells by visual examination using a haemocytometer. All cells still excluding the dye after two minutes were considered viable.

2.8. PREPARATION OF IDIOTYPIC IgM BY SURFACE DIGESTION OF A31 CELLS

Five CBA mice carrying the A31 tumour and in the terminal stage of the disease were sacrificed by cervical dislocation. Each spleen was transferred to a petri dish containing 15 ml of DMEM and cut-up into small pieces with forceps and scissors.

TABLE 2.1.

MONOCLONAL ANTIBODIES USED IN STUDY

Designation	Antigen or cell	Species	IgG Subclass	Source
HB58	Mouse κ-chain	Rat	IgG1	ATCC
GK1.5	L3T4	Rat	IgG2b	Dr.E.Shevach
53-6.7	Ly-2	Rat	IgG2a	Dr.E.Shevach
53-7.313	Ly-1	Rat	IgG2a	Dr.E.Shevach
M1/42 3.9.8	H-2	Rat	IgG2a	ATCC
M5/114.15.2	I-A&I-E	Rat	IgG2b	ATCC
BU-1	5-bromo-2-deoxyuridine	mouse	IgG2a	Dr.Gonchoroff
M6-3D10	L_2C Idiotype	mouse	IgG2aκ	Dr.M.Glennie
RJD-2A10	L_2C Idiotype	mouse	IgG1	Tenovus
MOPC-104E	dextran	mouse	IgMλ	Dr.Richardson
M38-7	guinea pig IgM	mouse	IgMλ	Dr.M.Glennie
BCL_1	murine leukemia	mouse	IgMλ	Dr.S.Slavin
TIB200	Hybridoma line	mouse	IgMκ	ATCC
Mc10 6A5	BCL_1 Idiotype	rat	IgG2a	H.M.McBride
KT3	murine CD3	rat	IgG2aλ	Dr.Tomonari
SI-1	saporin	mouse	IgG1	Dr.M.Glennie
DB7-18	saporin	mouse	IgG1	Dr.D.Brennand
M35-9F10	rat κ-chain	mouse	IgG1	Dr.M.Glennie

Samples of antibodies donated by Dr.E.M.Shevach and Dr.Gonchoroff were kindly supplied by Dr. F. Stevenson of the Wessex Regional Immunology Service, Tenovus Research Laboratory, Southampton General Hospital.

REFERENCES TO TABLE 2.1.

(1) HB58; Hybridoma 1: 5-11, 1981
(2) GK1.5; Wilde et al. J.Immunol. 131: 2178-2183, (1983)
(3) 53-6.7 and 53-7.313; Ledbetter and Herzenberg. Immunol. Rev. 47: 63-90, (1979)
(4) M1/42 3.9.8; Monoclonal antibodies, R.Kennett et al. (Eds), pp. 185-217, Plenum Press, 1980.
(5) M5/114.15.2; Bhattacharya et al. J.Immunol. 127, 2488-2495, 1981.
(6) BU-1; Gonchoroff et al. Cytometry. 6: 506-12, 1985.
(7) M6-3D10, RJD-2A10 and M38-7; Elliott et al. J.Immunol. 138: 981-988, 1987.
(8) MOPC 104E; McIntire et al., Science 150: 360-163.
(9) BCL_1; Slavin and Strober. Nature. 272: 624-626, 1978.
(10) TIB200; Abo and Balch. J.Immunol. 127: 1024-1029, 1981.
(11) Mc10-6A5; George et al. Hybridoma 10: 219-227, 1991.
(12) KT3; Tomonari. Immunogenetics 28: 455-458, 1988.
(13) DB7-18; Glennie et al. J.Immunol 141: 3662-3670, 1988.
(14) SI-3; French et al. Cancer Research 51: 2353-2361, 1991.

Using the plunger from a 2 ml disposable syringe the splenic tissue was teased through a metal gauze (3 cm x 3 cm; Expanded Metal Company., Hartlepool, U.K., Type 940, 200 mm wide.), to produce a single cell suspension. The latter was transferred to a universal and allowed to stand for a few minutes to allow the larger aggregates to settle before the supernatant was withdrawn using a glass pasteur pipette and transferred to the top of a 50 ml centrifuge tube containing an equal volume of Ficoll-Isopaque (Lymphoprep, Nycomed, Oslo). After centrifugation (450 g, 30 min, 20°C) the lymphocyte layer was recovered from the interface and washed twice by centrifugation at 200 g for 10 minutes at 20°C in 50 ml of PBS. The final cell pellet was then resuspended into 50 ml DMEM + 10% FCS and the tumour cells incubated at 37°C in a CO_2 incubator for 30 minutes to allow removal of surface absorbed immunoglobulin. Cells were recovered by

centrifugation as above and resuspended to 5 x 10^8 cells/ml in DMEM and transferred to a conical flask in preparation for surface digestion. Viability counts of cells prepared using the above method indicated that at least 95% of the tumour cells remained viable.

2.8.1. DIGESTION

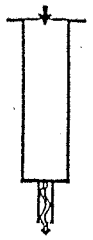
To each 9 ml of cell suspension was added 0.1 ml of micrococcal nuclease solution (Sigma; 0.5 mg/ml in PBS), the mixture briefly swirled and then incubated at 37°C for 5 minutes without further agitation. Activated papain was added (0.9 ml) and the mixture placed into a shaking water bath at 37°C (Grant Instruments, Cambridge, U.K.; 75 strokes/min). Agitation was continued for 30 minutes after which time the cells were removed by centrifugation (200 g, 10 min). To the supernatant was added 1.0 ml of iodoacetamide (37 mg/ml in PBS) and the solution incubated for 15 minutes at room temperature. Before storage of the surface digest at -70°C a carrier solution of sheep Fabγ (20 mg/ml) plus micrococcal nuclease (0.5 mg/ml) in 0.2M TE8 was added in order to reduce non-specific losses of the A31 IgM on subsequent manipulations.

2.8.2. SHEEP ANTI-A31 IgM COMPLEXES

Improved immunogenicity of the A31 IgM was obtained by isolation of A31 Fabμ as complexes with sheep anti-Fabμ using a automated chromatography system designed at the Tenovus laboratory (Stevenson, Smith and Hamblin, 1983). The schematic of this system is shown in figure 2.1. The cell surface digest, after centrifugation, was passed through; (1) a DEAE-cellulose column to remove acidic material and membrane fragments followed by (2) elution onto a G50 column were removal of solutes and the papain from the digestion stage occurred. In stage (3) material that would bind non-specifically during subsequent stages was retained on a blank immunosorbent column before the remaining solution was loaded onto the specific immunosorbent (4). The latter bound the A31 Fabμ and with this immunoglobulin still on the immunosorbent

FIGURE 2.1. ISOLATION OF A31 IgM COMPLEXES

30 ml OF SURFACE DIGEST
(CENTRIFUGED FOR 20 MINUTES AT 400 g)

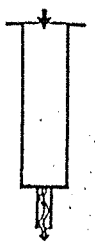


1

DEAE-CELLULOSE
(0.9 x 16 cm)
BUFFER = 0.2 M TE8

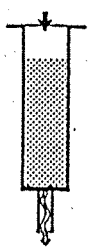
2

SEPHADEX G50
(1.5 x 90 cm)
BUFFER = 0.2M TE8



3

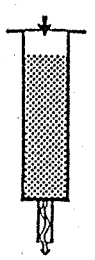
BLANK IMMUNOSORBENT
(SEPHAROSE 6B-SHEEP IgG)
(0.9 x 4 cm)



4

SEPHAROSE 6B-SHEEP ANTI-MOUSE Fdμ
(0.9 x 1 cm)
BUFFER = 0.2 M TE8

A31 Fab RETAINED



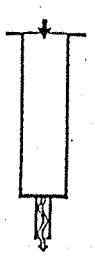
5

SAME COLUMN AS IN (4)
SHEEP ANTI-MOUSE Fabμ (2 ml at 1 mg/ml)
LOADED ONTO COLUMN
BUFFER = 0.2 M TE8

A31 Fab-SHEEP ANTI-MOUSE Fabμ
RETAINED

COLUMN WASHED EXTENSIVELY WITH 0.2 M TE8

ELUTED WITH 0.5 M NH_4OH/1 M KSCN (0.3 ml)



6

SEPHADEX G25
BUFFER = 0.2 M TE8

COMPLEXES (APPROX 2 ml)

a solution of sheep anti-Fabμ was run through the column (stage 5) thereby allowing the sheep anti-mouse Fabμ IgG to bind the retained A31 immunoglobulin fragments to form complexes. After extensive washing release of the A31 Fabμ-Sheep anti-Fabμ from the column was by reverse elution using 0.5M NH_4OH/1M KSCN and produced a single peak which was immediately passed through a G25 column equilibrated with 0.2M TE8 (stage 6). The complexes were collected and stored at 4°C. Elution of proteins from the columns was monitored by LKB Uvicord (LKB-Produkter AB, Bromma, Sweden) at 280 nM. Estimation of the antigen content of the complexes was calculated using the recorded elution peak to determine total protein content and assumed a weight ratio of sheep IgG to A31 Fabμ of 8:1.

2.8.3. IMMUNISATION OF SHEEP

The inoculum using the A31 IgM complexes was prepared by emulsifying equal volumes of complexes (11 μg of A31 Fabμ) and Complete Freund Adjuvant (CFA; Difco laboratories, Detroit, Michigan, USA). The sheep (Barren Ewe, Allington Farm, Porton, Wilts) received a primary injection of complexes at subcutaneous sites located distally in all four limbs followed 28 days later by a secondary immunisation also of 11 μg of Fabμ containing complexes in CFA and using similar injection sites. The animal was bled one week after this final injection.

A sheep had been immunised with purified rat IgG and subsequently bled prior to the start of this study. Briefly this animal had received a primary injection of 1 mg in CFA followed five weeks later by an aqueous booster injection of 1 mg and a final booster 3 months later once again with 1 mg of rat IgG given in aqueous solution. The animal was bleed seven days later.

2.8.4. ISOLATION OF SHEEP IgG FROM BLOOD

The isolation of IgG from sheep blood was required for the production of sheep anti-A31 immunoglobulin and the construction of a sheep anti-rat affinity column necessary

for the isolation of rat monoclonal antibody from culture supernatants. The IgG for the latter was already available and was prepared using the same method that will now be described for the isolation of the IgG fraction from the blood of the sheep immunised with A31 Fabμ complexes. Blood from the sheep was allowed to clot around glass beads (6 mm, Gallenkamp) for two hours at 20°C and the serum recovered by filtration through a buchner funnel with any remaining RBCs being removed by centrifugation (400 g, 10 min). To each 100 ml of serum was added, slowly with mixing, 60 ml of saturated ammonium sulphate. The mixture was stirred for 30 minutes at room temperature and then the precipitate recovered by centrifugation (1500 g, 30 min, 20°C). The pellet was resuspended to the original serum volume with 0.2M TE8 and the precipitation procedure repeated with fresh saturated ammonium sulphate. Finally the pellet was resuspended to the original serum volume with 0.06M PE pH 7.3 and extensively dialysed against the same buffer at 4°C over-night. The γ-globulin fraction was centrifuged (2000 g, 20 minutes) at 20°C and then applied (150 ml aliquots) to a DEAE-cellulose column (1 litre) equilibriated in 0.06M PE pH 7.3 and 8 ml fractions collected. The fractions from the first major peak of the anion exchange column were pooled and checked by serum agarose gel electrophoresis (Beckmann) to ensure purity of the material. The concentration of IgG within the pooled fractions was determined by its absorption at 280 nm using an extinction coefficient of 1.35.
In addition to these sheep polyclonal immunoglobulin fractions a number of other polyclonal antibody preparations were used during this study and these are listed in table 2.2.

2.9. MONOCLONAL ANTIBODY FROM ASCITIC FLUID

Murine IgM monoclonal antibodies were prepared from ascitic fluid by slow addition of an equal volume of saturated ammonium sulphate with constant stirring at room temperature. After 30 minutes the precipitate was harvested by centrifugation (2000 g, 30 min) and the resultant pellet was resuspended into high molar Tris-NaCl pH 8.0 and dialysed

Table 2.2

POLYCLONAL ANTIBODIES USED IN THIS STUDY

ANTIGEN	SPECIES	CONJUGATE	SOURCE
A31 idiotype	sheep	-	H.M^{C}Bride
A31 IgM	rat	-	H.M^{C}Bride
mouse Fdμ	sheep	-	Tenovus
mouse Fabμ	sheep	-	Tenovus
mouse μ-chain	goat	HRP	Serotec
mouse IgM	rabbit	HRP	Tenovus
mouse F(ab)$_2$	rabbit	-	Tenovus
mouse Fcμ	donkey	FITC	Stratech
rat Fcγ	sheep	HRP	Serotec
rat Ig class and subclass	rabbit	-	Sera-lab
rat IgG	rabbit	-	Tenovus
rat IgG	rabbit	HRP	Tenovus
rat Fcγ	mouse	HRP	Stratech
rat Fcγ	mouse	FITC	Stratech

against TE8 buffer. Insoluble material was removed by filtration through 0.2 µm filter before further purification by means of size exclusion on an Ultragel ACA 22 (LKB Pharmacia) column.
Murine and rat IgG monoclonal antibodies were precipitated from the ascitic fluid as described above and subjected to dialysis against 0.02 M TE8. Insoluble material was removed as described above and the solution applied to a DEAE-Sepharose FF ion-exchange column (LKB Pharmacia) equilibrated in the same buffer. The monoclonal IgG was recovered by elution from the column using a linear gradient of NaCl (0-350 mM) in 0.02 M TE8, monitored by a Uvicord at 280 nm (LKB).

2.10. POLYCLONAL MOUSE IMMUNOGLOBULIN

Mice F_1 (Balb/c x CBA) were anaesthetised with metofane (C-Vet Ltd, Bury St. Edmunds, U.K.) and exsanguinated by cardiac puncture. The blood was allowed to clot at 4°C before centrifugation (10 000 g, 2 min) and the serum removed. The immunoglobulin fraction was isolated by ammonium sulphate precipitation as for sheep serum and the recovered pellet dialysed into 0.1 M $NaHCO_3$/ 0.5M NaCl pH 8.3 buffer prior to coupling to CNBr-activated Sepharose 4B.

2.11. COUPLING OF IMMUNOGLOBULINS TO SEPHAROSE-4B

Proteins were coupled to cyanogen bromide activated Sepharose-4B (Pharmacia LKB, Sweden) following the manufactures procedure and using a protein concentration of 10 mg/ml of gel.

2.12. SECRETED A31 IgM

A single cell suspension of A31 tumour cells was prepared as described in 2.8. and finally resuspended in supplemented DMEM containing 10% FCS to a finally density of 1.4×10^7 cells/ml and cultured at 37°C for seven hours in a humidified CO_2 incubator (7.5% CO_2). After this culture period the cells were removed by centrifugation (200 g, 20 min) and the

supernatant containing secreted A31 IgM stored at -20°C until required.

2.13. IMMUNODIFFUSION

Double immunodiffusion was performed by the agar plate method of Ouchterlony (Ouchterlony, 1958) and precipitin lines were interpreted as discussed by Ouchterlony and Nilsson (1986). A 1% (w/v) purified agar solution (Oxoid) in 0.15 M NaCl-0.02M TE8-1.0% thiomersal, pH 8.0 was poured into plastic Petri dishes (Sterilin) to a depth of 3 mm. After congelation of the agar layer a template of the appropriate well pattern was placed under the Petri dish and a cork borer used to cut the required wells. The central well contained 120 µl of antisera and the outside wells approximately 50 µl of protein solution, antigen. Precipitin lines were allowed to develop at room temperature in a moist atmosphere for 24-48 hours and the resultant precipitation lines photographed using indirect lighting and a Polaroid MP-3 Land Camera with Polaroid 107 Land Film.

2.14. PURIFICATION OF RAT ANTI-A31 IDIOTYPIC MONOCLONAL ANTIBODY BY AFFINITY CHROMATOGRAPHY

Due to the large volume of culture supernatant derived from the hollow-fibre bioreactor isolation of a rat anti-A31 idiotypic monoclonal antibody from this source was achieved by use of affinity chromatography. The immunoglobulin fraction from the serum of a sheep immunised with rat immunoglobulins was collected and prepared as described in section 2.8. This was then linked to Sepharose-4B as described in section 2.11. and the immunosorbent packed into a chromatography column (3.5 x 10 cm). The column was equlibirated into high molarity Tris/HCl pH 8.0 and then the culture supernatant run onto the column which was then washed with the equilibirating buffer. The anti-idiotype was eluted of the column with 0.5 M NH_3/1 M KSCN pH 11.5 and immediately collected into a conical flask kept on ice and which contained 2 ml of high molarity Tris/HCl pH 8.0. The elutant was immediately dialysed into 0.2 M TE8.

The purity of all eluted immunoglobulin was routinely assessed by running a serum protein electrophoretic strip (Beckman, U.K.).

2.15. ELISA PROTOCOLS

A number of different ELISAs were used in the course of this study and these are described below in the form of protocols.

2.15.1. DETERMINATION OF THE SPECIFICITY OF THE SHEEP ANTI-A31 IDIOTYPIC IgG FRACTION

(1) Sheep anti-A31 idiotype IgG fraction coated onto plates (Nunc-immunoplate maxisorp F96, Gibco Ltd,) 100 µl at 50 µg/ml o/n at 4°C.
(2) Plates flicked out.
(3) Plates blocked with 150 µl of PBS/BSA.
(4) Incubated at 37°C for 1 hr.
(5) Plates washed x 2. All washing was carried out automatically using an inter-med NK-350 immunowasher (Damon IEC U.K. Ltd, Bedfordshire, England).
(6) Appropriate antigens loaded into wells of first row on ELISA plate and double diluted down the plate using PBS/BSA as diluent. The antigens used were BCL_1 (IgMλ) and MOPC 104E (IgMλ) at 500 ng/ml; Normal mouse serum diluted at 1:1000; A31 terminal serum diluted 1:1000; and A31 tumour cell culture supernatant used neat. All dilutions were made in PBS/BSA.
(7) Incubated 37°C for 90 min.
(8) Plates washed x 5.
(9) Peroxidase added (Goat anti-mouse µ, diluted 1:1000 in PBS/BSA; Serotec, Oxford, U.K.) 100 µl/well.
(10) Plates incubated 37°C for 90 min.
(11) Plates washed x 5.
(12) OPD substrate added (100 µl/well).
(13) Reaction stopped with 5M H_2SO_4 (50 µl/well) after 30 min.
(14) ELISA plates read at 490 nm (Dynatech 580 ELISA reader, Dynatech, Sussex, U.K.).

2.15.2. IDENTIFICATION OF RAT ANTI-IDIOTYPIC MONOCLONAL ANTIBODIES

(1) Antigens used were; (1) Idiotypic IgM fraction from AcA 22 chromatographic run of culture supernatant from rescue hybridoma (Mc22) diluted 1 + 3 in coating buffer; (2) IgM from TIB 200 hybridoma diluted to 200 ng/ml in coating buffer. Each well of the ELISA plates (Nunc-immunoplate maxisorp F96, Gibco Ltd,) received 100 µl and the plates were left o/n at 4°C.
(2) Plates flicked out.
(3) Plates blocked with 150 µl of PBS/BSA.
(4) Incubated at 37°C for 1 hr.
(5) Plates washed x 2.
(6) For each well on the fusions plates a 50 µl sample of culture supernatant was loaded into the appropriate well on an ELISA plate coated with (1) A31 IgM and (2) TIB 200 IgM.
(7) Incubated 37°C for 90 min.
(8) Plates washed x 5.
(9) Peroxidase added, Sheep anti-rat Fcγ (Serotec, Oxford, U.K.), diluted 1:1000 in PBS/BSA and 100 µl/well.
(10) Plates incubated 37°C for 90 min.
(11) Plates washed x 5.
(12) OPD substrate added (100 µl/well).
(13) Reaction stopped with 5M H_2SO_4 (50 µl/well) after 30 min.
(14) ELISA plates read at 490 nm (Dynatech 580 ELISA reader, Dynatech, Sussex, U.K.).

2.15.3. RAT AND MOUSE MONOCLONAL ANTIBODY ISOTYPE DETERMINATION

(1) Plates coated with rabbit anti-class or subclass specific IgG (100 µl per well at 50 µg/ml; Serotec), incubated 4°C o/n.
(2) Plates flicked out and unreacted sites blocked with 150 µl/well BSA/PBS for 1 hr at 37°C.
(3) Plates washed five times
(4) Culture supernatants diluted 1:100 or 1:500 with

BSA/PBS and added to individual wells, plates incubated 90 minutes at 37°C.

(6) Plates washed five times

(7) Rabbit anti-rat or anti-mouse IgG Peroxidase (Tenovus in house; 1:1000 in BSA/PBS) added to each well and incubated for 90 minutes at 37°C.

(8) Plates washed five times

(9) OPD substrate added and plates incubated for 30 minutes in the dark at 20°C.

(10) Reaction stopped with 5N H_2SO_4 and plates read on ELISA reader (Dynatech 580).

2.15.4. DETERMINATION OF IMMUNOGLOBULIN LEVELS WITHIN RAT MONOCLONAL ANTIBODY CULTURE SUPERNATANTS

This ELISA was essentially the same as in 2.15.3. with the exceptions that the plates were coated with a polyclonal rabbit anti-rat IgG fraction (50 µg/ml; Tenovus in-house) and the supernatants were double diluted across the plates. A purified rat IgG2a monoclonal antibody, Mc10 6A5 anti-BCL_1 idiotype (George et al., 1991), was used to establish a standard curve.

2.15.5. ELISA FOR DETERMINATION OF SERUM A31 IDIOTYPIC IgM LEVELS

(1) Plates coated with anti-idiotypic monoclonal antibody Mc39-16 (5 µg/ml in coating buffer), incubated 4°C o/n. See results section for isolation of Mc39-16.

(2) Plates flicked out and unreacted sites blocked with BSA/PBS for 1 hr at 37°C.

(3) Plates washed twice.

(4) Serum samples (80 µl) from A31 mice at the early stage of the disease were diluted with 120 µl of BSA/PBS and double diluted across the ELISA plate whilst samples from mice in the terminal stage (cardiac bleeds) were diluted 1:50 with BSA/PBS before making the dilution series across the plate, all plates were then incubated for 90 minutes at 37°C. Purified A31 IgM was used to establish a standard curve and normal mouse serum and

IgM were used as negative controls.

(6) Plates washed five times

(7) Rabbit anti-mouse IgM Peroxidase (Tenovus in house; 1:100 in BSA/PBS + 1% normal rabbit serum) added to each well (100 µl) and incubated for 90 minutes at 37°C.

(8) Plates washed five times

(9) OPD substrate added and plates incubated for 30 minutes in the dark at 20°C.

(10) Reaction stopped with 5N H_2SO_4 and plates read on ELISA reader (Dynatech 580, Dynatech, Sussex, U.K.).

(11) A standard curve was plotted using the readings given from the purified A31 IgM and the unknown values for the serum samples obtained taking into account all dilutions made from the original blood sample.

2.16. PREPARATION OF THYMOCYTE FEEDER LAYERS

A mouse (4-8 weeks old) was sacrificed in a lethal chamber and the thymus removed using good aseptic technique. A single cell suspension was prepared as described for splenocytes (section 2.8) The suspension was transferred to supplemented DMEM containing 15%(v/v) FCS + Hypoxanthine (10^{-4}M) + Aminopterin·($4x10^{-7}$M) + Thymidine ($1.6x10^{-5}$M) (DMEM-HAT) to give a final volume of 100 ml and 200 µl aliquots were dispensed into 5 x 96-well trays in preparation for addition of hybridoma cells from the fusions.

2.17. PRODUCTION OF MONOCLONAL ANTIBODIES

Hybridomas were produced by PEG mediated fusion between the murine myeloma line P3X63-Ag8.653 (653) or P3/NS1/1.Ag4.1 (NS-1) and either A31 tumour cells in the case of 'rescue' fusions or immunised rat splenocytes in the case of anti-idiotype monoclonal antibody production. Fusion between the myeloma line and the splenocytes immortalises the latter which would normally die in culture. After the fusion of the two cell populations any remaining myeloma cells , which would overgrow the cultures, are eliminated by the addition of aminopterin. The latter blocks the enzyme dihydrofolate

reductase which is essential for the function of the main biochemical pathway for DNA synthesis. A salvage pathway allowing DNA synthesis can function but only if the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) is present within the cell. The myeloma cells used in the fusions are $HGPRT^-$ therefore in the presence of aminopterin will die. The hybrid cells inherit HGPRT from the spleen cells and therefore can grow in the presence of aminopterin. To ensure efficient functioning of the salvage pathway hypoxanthine and thymidine are included in the selection medium. Essentially each fusion was by the method of Kohler and Milstein, (1975) as described by Stevenson et al., (1984).

Immunised animals or, in the case of 'rescue fusions', animals carrying the A31 tumour were sacrificed by cervical dislocation and the spleen removed. A single cell suspension was produced as described for cell surface digestion (Section 2.8.) and after isolation on Ficoll-isopaque the cells were washed twice in supplemented DMEM without FCS. The 653 or NS-1 myeloma cell lines (depending on the fusion) was harvested from tissue culture flasks (260ml flasks, GIBCO) and similarly washed three times in DMEM without FCS. Counts of each cell suspension were made and adjusted to give a tumour cell to myeloma cell ratio of 2:1 with a total cell number of 1.5×10^8 and the mixed cell suspension transferred to a 50 ml centrifuge tube. Centrifugation (200 g, 5 min) was used to recover the cells after which all the media from above the pellet was removed using a pasteur pipette. The centrifuge tube was placed into a beaker of sterile water warmed to 37°C and 1 ml of sterile PEG (0.48 gram in 0.52 ml DMEM) was slowly added over the period of 1 minute. After the addition of 0.5ml the pellet was disturbed in order to break it away from the bottom of the centrifuge tube. On completion of the addition of the PEG the pellet was gently stirred for a further 90 seconds before the slow addition of 10 ml of warm DMEM over a period of 5 minutes. The cells were immediately centrifuged (100 g, 5 min) and the PEG containing supernatant removed by pasteur pipette followed by the slow addition of 16 ml of warm DMEM-HAT. After disturbing the pellet the centrifuge tube was placed into a CO_2 incubator (7.5% CO_2,

37°C) for 30 minutes. This incubation step made it relatively easy to break up the cell pellet to form a single cell suspension, one drop of which was placed into each well of five 96-well tissue culture trays containing a thymocyte feeder layer. The cultures were feed by the addition of fresh DMEM-HAT media every second or third day until day 10, for the next two feeds the cells received supplemented DMEM + 15% FCS + Hypoxanthine (10^{-4}M) and Thymidine ($1.6x10^{-5}$M) (DMEM-HT media), and all subsequent feeds were with supplemented DMEM + 15% FCS.

Once the colonies were of a suitable size (typically 10-14 days) the production of monoclonal antibody within the individual wells was tested for using the appropriate ELISA as described in section 2.15. Wells giving a strong reaction on the screen were cloned out onto feeder layers and re-assayed by repeated ELISAs until two full clonings were achieved in which single colonies gave positive ELISA results. These individual hybridoma lines were expanded and frozen down in liquid nitrogen.

2.18. FLOW-CYTOMETRY

Two separate flow cytometers were used during this study; A Fluorescence Activated Cell Sorter (FACS III), and a FACScan (Becton Dickinson, Oxford, U.K.). As data from either machine was plotted in a similar manner the individual machine used for a particular set of data will not be given.

(1) A31 (including variants) and BCL_1 cells were isolated as described in section 2.8. and finally suspended to 5 x 10^7 cells/ml in cold PBS.

(2) 100 µl of each culture supernatant or purified antibody (50 µg/ml) under test was placed into plastic test tubes (75 x 13 mm, Midland Laboratory Equipment, Staffs, U.K.). For blocking experiments each antibody solution was pre-incubated with 50 µl of one of the following (a) 50 µl of PBS (b) 50 µl of serum from a terminal A31 mouse or (c) 50 µl of serum from a normal CBA mouse. After mixing the samples were incubated on ice for 30 min.

(3) To each tube was added 100 µl of the A31 cell suspension

and after mixing the tubes were returned to the ice bath for a further 30 min.

(4) FACS wash buffer was added to each tube (4 ml) and the cells pelleted by centrifugation (400 g, 4°C, 5 min). The tubes were inverted to remove the supernatant and while still inverted the tops of the tubes were blotted onto paper tissue. The cells were resuspended by drawing the tubes across the top of a plastic rack three times.

(5) Step (4) was repeated.

(6) After disrupting the cell pellet as above one drop (1:40 dilution in PBS) of a mouse anti-rat Fcγ specific FITC (Stratech Scientific Ltd, Luton, Beds, U.K.) was added from a pasteur pipette. The cells were returned to the ce bath for 30 min.

(7) Step (4) was repeated and the cells resuspended into 0.5 ml of FACS wash buffer.

(8) Analysis was carried out on the FACS III or FACScan. Dead cells were gated out by foreword scatter, the fluorochrome was excited at a wavelength of 488 nm and fluorescent intensity measured at 515-545 nm using the appropriate band-pass filter. The results were plotted as a histogram with cell numbers on the y-axis and fluorescent intensity on the x-axis (see figure 2.2.)

2.18.1. DETERMINATION OF MODULATION BY FLOW-CYTOMETRY

A31 tumour cells were prepared as described in 2.8. and finally resuspended in RPMI-1640 medium containing 25 mM HEPES and 15% myoclone foetal calf serum (Gibco) at a cell density of 5 x 10^7 cells/ml. From this cell suspension 100 µl was transferred to an appropriate tube and these were placed within an ice-bath at 4°C. The antibodies (5 µg/ml) to be tested were pre-incubated at 4°C for 15 minutes and then 100 µl of each was added to the pre-selected tube and when all the antibodies had been aliquoted into the correct tubes they were placed into a water bath maintained at 37°C. At various time intervals tubes were removed from the water bath and immediately washed as described in 2.18. All tubes were finally left with 0.5 ml of FACS wash buffer in them and stored at 4°C until all the time points had been collected.

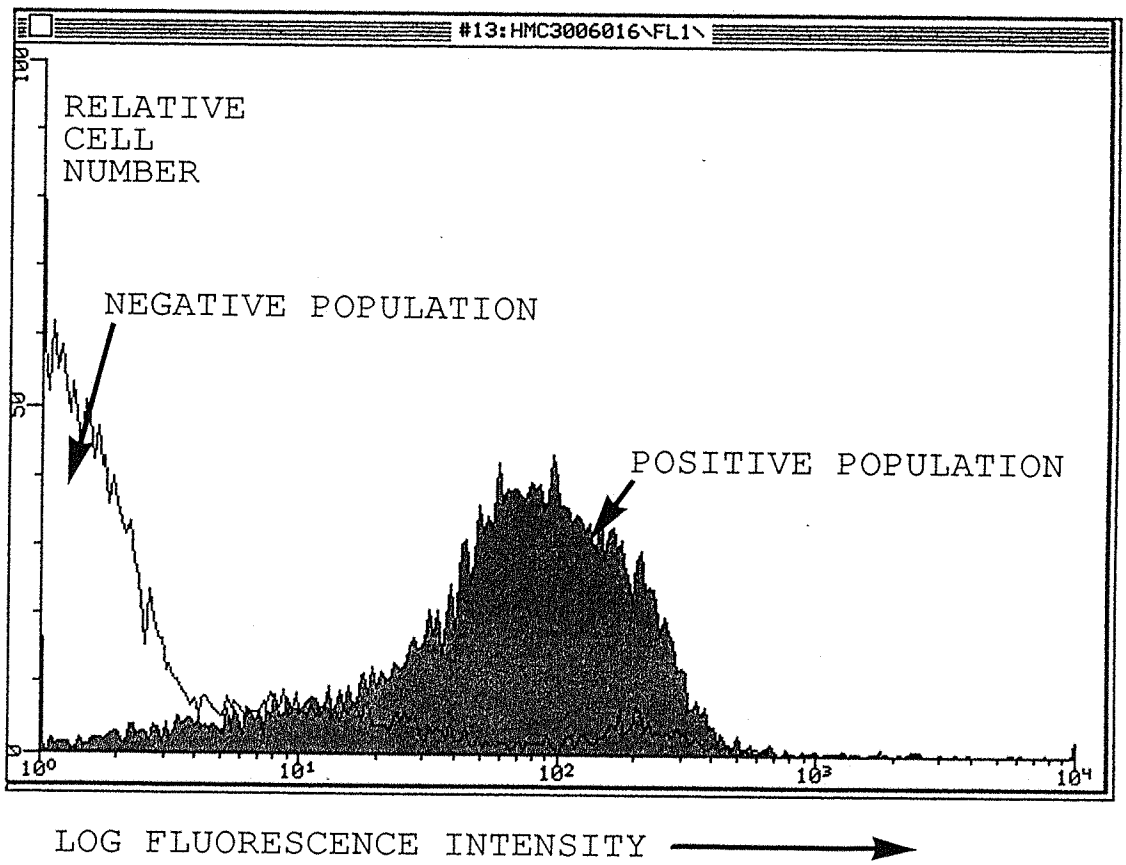


Figure 2.2. Sample flow cytometric display. All flow cytometric data presented in this thesis will follow this format. Note Y-axis represents relative cell numbers and X-axis represents log fluoresence intensity. Any movement in the fluorescent peak towards the right indicates an increase in fluorescent signal and therefore an increase in the antigenic determinant being screened for.

The cells were centrifuged as before and resuspended by drawing the bottom of the tubes across a plastic rack three times. To each tube was added one drop from a pasteur pipette of a solution of Donkey anti-mouse Fcμ specific FITC conjugate (1:80 dilution in PBS; Stratech Scientific Ltd, Luton, Beds, U.K.) and the tubes incubated on ice for 30 minutes. The cells were washed once as previously described and finally resuspended into 0.5 ml of FACS wash buffer and the cells examined on a flow cytometer.

2.19. COLLECTION OF SERUM FROM A31 MICE

Individual mice were identified by coding using an ear punch, blood samples (20 μl) being collected by tail bleeds into capillary tubes at set times during the course of the disease. The collected blood was aspirated into 80 μl of PBS and immediately centrifuged (7,000g, 5 min, 20°C) and 80 μl of serum collected from above the pellet and stored at -20°C. Animals in the terminal stage of the disease were anaesthetised with metofane (C-Vet Ltd, Bury St. Edmunds, U.K.) and exsanguinated by cardiac puncture. The blood was allowed to clot before centrifugation as above and the serum collected and stored at -20°C.

2.20. COMPLEMENT MEDIATED KILLING (^{51}CHROMIUM RELEASE ASSAY)

2.20.1. PREPARATION OF ^{51}CR-LABELLED TARGETS

A31 cells were harvested and prepared as described in section 2.8 and finally resuspended in DMEM to a cell density of 3 x 10^7 cells/ml. ^{51}Chromium (500 μCi of sodium ^{51}Chromate; Amersham International Plc, Bucks, UK) was added to 3 ml of cells which were then incubated at 37°C for 30 minutes. Radiolabelled cells were recovered by centrifugation (100 g, 10 minutes) and washed four times with cold DMEM containing 10% FCS before being resuspended to 5 x 10^4 cpm/ml.

2.20.2. COMPLEMENT-MEDIATED CYTOTOXICITY ASSAY

100 µl of ^{51}Cr-labelled A31 cells were aliquoted into plastic test tubes (9 x 44 mm polytubes; Luckhams, Sussex, UK) which were kept on ice before test and control monoclonal antibodies were added to the appropriate tubes to give duplicates at each dilution. The tubes were incubated for 30 minutes at 0°C and then 300 µl of a fresh rat complement source (rat serum diluted 1 + 2 with DMEM + 10% FCS + 20 mM sodium azide) was added. After mixing the tubes were transferred to a 37°C water bath for 30 minutes and then centrifuged at 1500 x g for 5 minutes before 200 µl was removed for counting. The maximal ^{51}Cr release was determined by incubating 100 µl of cells with 400 µl 1% Nonidet P40 (NP40; BDH) for 30 minutes at 37°C. Specific chromium release was expressed as a percentage using the following equation

$$\frac{\text{Test cpm - negative control cpm}}{\text{Maximum cpm - negative control cpm}} \times 100$$

where
(1) test cpm = cpm in presence of test antibodies
(20 negative control cpm = cpm in presence of anti-BCL_1 monoclonal antibody
(3) maximum cpm = cpm in presence of NP40

2.21. IMMUNOHISTOLOGY

In all cases Balb/c x CBA F1 crosses were used and sacrificed at the appropriate time after tumour inoculation by exposure to CO_2 in a lethal chamber. The various organs of interest were removed and blocks of tissue cut from them using a single edged blade. Tissue blocks were immediately wrapped into aluminium foil and dropped directly into a reservoir of liquid nitrogen before transfer to the vapour phase of a liquid nitrogen refrigerator for storage.
Serial sections (10 µm) were cut from the blocks on a Reichert-Jung Mod.2700 frigocut cryostat using a chamber temperature of -25°C and a stage temperature of -30°C. The

tissue sections were collected onto glass slides pre-treated with Vectabond (Vector Laboratories, Peterborough, U.K.) to eliminate the loss of sections from the slides during processing. The sections were processed as follows:

(1) Sections were air dried overnight at 20°C.
(2) An area around each section was marked using a Dako Pen (Dako Ltd, Bucks, U.K.) and allowed to dry for 15 minutes.
(3) The slides were immersed into dry acetone for 10 minutes.
(4) Sections were washed in three changes of PBS over 15 minutes.
(5) To the appropriate slides were added the antibodies either as neat culture supernatants or as purified solutions (50 μg/ml) in PBS and the slides incubated in a humidified chamber for 1 hr at 20°C. A rat monoclonal antibody of the same isotype as the test antibodies and having specificity for the idiotype on BCL_1 cells was used as a negative control.
(6) The sections were washed as in step (4).
(7) A 1:80 dilution of mouse anti-rat Fcγ specific peroxidase conjugate (Stratech Scientific Ltd, Beds, U.K.) diluted in PBS was added to the slides which were incubated in a humidified chamber for 1 hr at 20°C.
(8) A solution of 3,3' diaminobenzidine (DAB) was prepared as follows: 10 mg DAB tablet [3,3' diaminobenzidine tetrahydrochloride, Sigma] dissolved in 0.1 M Tris-HCl pH 7.6 (20 ml). Once the tablet had dissolved the solution was allowed to settle for 30 minutes and then the top 10 ml was removed to a clean plastic universal. To this latter solution and just before use was added 100 μl of a 0.03% (v/v) solution of H_2O_2.
(9) Slides were washed as in step (4).
(10) Each slide was flooded with the DAB solution and incubated for a further 10 minutes in the dark.
(11) After a quick rinse in PBS the slides were rinsed under running tap water for 10 minutes.
(12) Sections were next immersed into a solution of Harris Haematoxylin ('Gurr' BDH, Hants, U.K.) for one minute

and then immediately returned to tap water for a further 10 minutes.

(13) The sections were dehydrated and processed for mounting using the following steps

(a) 70% ethanol for 1 minute
(b) 70% ethanol for 1 minute
(c) 100% ethanol for 1 minute
(d) 100% ethanol for 1 minute
(e) 100% Xylene for 1 minute
(f) 100% Xylene for 1 minute
(g) 100% Xylene for 1 minute

(14) Finally the sections were mounted in DePeX ('Gurr' BDH, Hants, U.K.) using glass cover slips. The sections were examined on a Leitz orthoplan microscope and photographed using Kodak 64T film at a lamp current of 8 amps and CC10b, N4 and N16 colour correction filters.

2.22. RADIOIODINATION

Radioiodination of immunoglobulins and saporin was performed using carrier free ^{125}I (IMS/30 Radiochemical centre, Amersham, U.K.) using the following protocol:

(1) Purified protein (0.5 ml) at 1 mg/ml in PBS pH 7.4 was placed into 1.5 ml screw-capped tubes (Scotlab, Bellshill, Scotland).
(2) To each tube was added one iodo-bead (Pierce Chemical Company, Chester, U.K.).
(3) Each sample received 2.5 µl of ^{125}I (250 µCi total per tube) and the reaction was allowed to proceed at 20°C for five minutes with occasional mixing.
(4) The reaction was stopped by transfer of the antibody solutions to dialysis tubing followed by extensive dialysis against PBS pH 7.4.

Under these conditions maximum incorporation of ^{125}I did not exceed 70% of the total iodine added and in the case of saporin and an immunotoxin the incorporation was 20% of maximum. Comparison of iodinated anti-idiotypic antibody with unlabelled antibody in binding ELISA using A31 IgM as the

coating antigen suggested that radiation damage, if occurring at all, was negligible (Data not shown).

2.23. EQUILIBRIUM BINDING ANALYSIS

The association constant for anti-idiotypic monoclonal antibody binding to A31 cells was determined by a method modified from the work of Dower et al., (1981) as described by Elliot et al., (1987):
A31 cells were prepared as previously described (section 2.8.) and were finally resuspended to 1 x 10^7 cells/ml in DMEM containing 10% (v/v) heat inactivated foetal calf serum, 2-deoxyglucose (50mM; BDH) and sodium azide (15mM). The latter two components were included to abolish endocytosis of cell-bound antibody (Metezeau et al., 1984). The cells were incubated at 37°C for 30 minutes and in the meantime serial dilutions (1:1.6 in DMEM containing 10% foetal calf serum; final volume 0.5ml) of the antibodies were made into screw-capped tubes with a starting concentration of 5 µg/ml down to a final concentration of 0.0516 µg/ml. After the 30 minute incubation 0.5 ml of the cell suspension was added to each tube and then incubated at 37°C for two hours with occasional mixing by inversion. Duplicate 400 µl aliquotes were layered onto a 4:1 mixture of 2-dibutyl phthalate: dinonyl phthalate oil (BDH) and centrifuged (13 400g; Micro Centaur centrifuge, MSE, Crawley, U.K.) for 40 seconds. The tubes were removed from the centrifuge and lowered into a shallow bath of liquid nitrogen until frozen and the portion containing the cell pellet removed by cutting through the plastic tube with dog nail trimmers (Resco No 727, Tecla Company Inc, Walled Lake, MI 48088, U.S.A.). The cut lower portion of the tubes (containing the cell pellet) were collected into counting vials and the total counts within the cell pellet and the counts in the equilibrium mixture remaining were obtained by placing the tubes in a rackgamma counter (LKB).

2.24. IMMUNOTHERAPY PROCEDURES

Early therapies were carried out before purified monoclonal anti-idiotypic antibody was available therefore culture supernatants that had been concentrated by ultrafiltration under positive pressure (Amicon) and in which the level of rat IgG had been determined (section 2.15.4.) were used. The use of control concentrated culture supernatants in early therapies and purified rat anti-idiotypic monoclonal antibody at a later date confirmed that the results using culture supernatants were indistinguishable from those treatments utilising purified antibody.

Unless stated otherwise in the text groups of age-matched CBA or F1 (CBA x Balb/c) mice were inoculated inter-peritoneally with 5 x 10^5 A31 tumour cells prepared as described in 2.8. Although preliminary experiments indicated that the disease ran the same course in CBA or F1 mice the strain of mouse was kept the same for each therapeutic trial. The therapies were initiated twenty-four hours later by an inter-peritoneal injection of 100 µg of the anti-idiotypic antibody whilst control groups received a sham injection of PBS or control culture supernatant containing 100 µg of a rat monoclonal antibody (Mc10-6A5) of the same isotype as the anti-idiotypic antibody but with specificity for the idiotype expressed by BCL_1 cells. Animals were monitored daily for signs of illness and were sacrificed by exposure to CO_2 in a lethal chamber when clinical signs indicated that they were in the terminal stage of the disease and would therefore succumb within twelve hours.

Therapies using immunotoxin and bispecific antibodies were conducted in a similar fashion however the exact details of these therapies will be given in the appropriate sections.

2.25. S-PHASE ANALYSIS OF TUMOUR CELLS

If cells are incubated in the presence of 5-fluoro-2-deoxyuridine (FdUrd) they metabolise the compound to form fluorodeoxyuridylate which in turn binds to the enzyme thymidylate synthetase and blocks the endogenous thymidine production. If BrdUrd is included in the culture medium it is incorporated into the cellular DNA in place of the absent thymidine. The use of a monoclonal antibody reactive with BrdUrd allows the detection of the incorporated BrdUrd and therefore the enumeration of those cells in the S-phase of the cell cycle (Gratzner et al., 1975; Gratzner, 1982).

(1) A31 and A31-LTS cells were isolated and washed as described in section 2.8. and resuspended to a cell density of 5 x 10^5 cells/ml in 5 ml of RPMI-1640 + 10% (v/v) FCS.
(2) To this cell suspension was added BrdUrd to a final concentration of 10 µM and FdUrd to a final concentration of 1 µM all dilutions being made in tissue culture medium
(3) The cells were incubated at 37°C in a CO_2 incubator for 1 hour.
(4) On completion of the incubation the cells were washed twice in warm RPMI-1640 + 10% (v/v) FCS, cells being recovered by centrifugation each time (400 g, 10 min) and finally resuspended to a cell density of 1 x 10^6 cells/ml.
(5) This suspension was used to prepare cytopreps by placing 6-7 drops into the rotor of a cytocentrifuge (300 rpm, 10 minutes, cytospin 2, Shandon, Runcorn, U.K.).
(6) The slides were left to air dry overnight.
(7) After carefully marking around the cells with a diamond marker they were fixed in 95% (v/v) ethanol for 5 minutes.
(8) Slides washed 3 x 5 minutes in PBS.
(9) Monoclonal antibody BU-1 (kind gift of F.Stevenson, Regional Immunology Service, Tenovus laboratory, Southampton) was added to each slide, 13 µl of a 1:1.5

dilution of culture supernatant in PBS.

(10) Slides incubated in a humidified atmosphere for 60 minutes.

(11) Slides washed x3 in PBS.

(12) A rhodamine conjugated goat anti-mouse antibody was added to each slide (kind gift F.Stevenson).

(13) Slides incubated in a humidified atmosphere for 60 minutes.

(14) Slides washed x3 in PBS.

(15) The area up to the circle marking the position of the cells was dried and then the cells were mounted into 95% glycerol and the edges of the cover slip sealed with nail varnish.

(16) Examination of the cells was with a fluorescent microscope using a mercury vapour lamp (Dialux 20 EB, Leitz, Luton, U.K.). The percentage S-Phase cells was determined by two observers, the author and a researcher experienced in the technique (Dr. J. North, to whom I am most grateful). For each cell population five separate fields were counted on each of two slides with a total count of approximately 400 cells.

2.26. POLYACRYLAMIDE GEL ELECTROPHORESIS

Protein preparations were examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), according to the method of Laemmli (1970) using the buffers and reagents listed below.

Proteins to be analysed by SDS-PAGE were prepared as follows: 50 µl of protein at approximately 1 mg/ml was added to 50 µl of sample buffer and heated to 100°C for 2 minutes over a boiling water bath. Where reduction of disulphide bonds was required prior to separation by SDS-PAGE the samples were heated in sample buffer containing 10% (v/v) 2-mercaptoethanol. SDS-PAGE analysis was carried out using a vertical electrophoresis system (LKB, Sweden).

A separating gel (140 x 140 x 1.5 mm) using a linear polyacrylamide gradient of 7.5-15% (w/v) was formed using standard LKB gel moulding apparatus and gradient mixing

chamber (LKB). To ensure polymerisation occurred at the top of the cast gel a thin layer of butan-1-ol was overlayered to ensure anaerobic conditions. Once the separating gel had set the iso-butanol was poured of and the top of the separating gel rinsed with distilled water and dried using filter paper. A stacking gel (5% (w/v) acrylamide) was then layered over the separating gel and a well forming comb inserted before polymerisation occurred. This stacking gel allowed the loading of relatively large volumes of sample yet retaining good resolution of separated proteins due to the concentration of the proteins into narrow bands between the leading chloride ions and trailing glycine ions during migration through the stacking gel (Hames, 1981).

Immediately prior to the electrophoretic run the well forming comb was removed and the individual wells rinsed with running buffer. Samples (100 µl) were carefully loaded into the wells and then overlayered with running buffer taking care not to disturb the samples within the wells. The gel was then placed into the electrophoresis tank and the top and bottom reservoirs filled with Tris-glycine running buffer.

Electrophoresis was carried out through the stacking gel at 100 v before increasing the voltage to 300 v which was maintained until the dye front reached to within 0.5 cm of the bottom of the gel (approximately 4 h). The gel was kept cool during this time by the circulation of cold water through a cooling jacket within the apparatus.

Gels were stained with 0.3 % (w/v) Coomassie brilliant Blue R (Sigma) in destain solution o/n at room temperature before being destained with several changes of destain solution over a period of 18 hours.

Electrode buffer pH 8.3

	g/l
Glycine	144.13
Tris-HCl	30.28

Buffer diluted 1/10 with deionised water and 10 ml/l of 10% (w/v) SDS added before use.

SDS stock

	g/l
SDS	100

Stock ammonium persulphate

Ammonium persulphate 0.15 g/10 ml prepared immediately before use.

Sample buffer

0.25 M Tris-HCl pH 6.8 (30.28 g/l)
4% (w/v) SDS
20% (v/v) glycerol
0.02% (w/v) Bromophenol Blue
(+ 10% (v/v) 2-Mercaptoethanol if required)

Stock acrylamide

A 50% (w/v) solution of acrylamide was prepared using LKB PrePAG mix, containing acrylamide and bis-acrylamide at a ratio of 37:1.

Stain solution

100 ml Glacial acetic acid
200 ml Iso-propanol
700 ml Distilled water
0.5 g Brilliant Blue

Destain solution

100 ml Glacial acetic acid
100 ml Iso-propanol
800 ml Distilled water

Composition of separating gels

	7.5%	15%
Stock acrylamide	6	12
1 M Tris (pH 8.8)	15	15
Water	17.7	9.7
Stock SDS	0.4	0.4
Glycerol (50% v/v)	-	2
Stock APS	0.9	0.9
TEMED	25 µl	25µl

Composition of stacking gel

	5%
Stock acrylamide	1
1 M Tris (pH 6.8)	1.25
Water	7.3
Stock SDS	0.1
Stock APS	0.35
TEMED	10 µλ

(All volumes given are ml unless stated otherwise)

2.27. PREPARATION OF IMMUNOTOXIN

The immunotoxin was prepared by Professor F.Stirpe (Istituto di Patologia Generale, de'll Universitandi Bologna, 1-40126 Bologna, Italia), to whom I am most grateful. Briefly, Saporin was prepared from the seeds of *Saponaria officinalis* (soapwort) as described previously (Stirpe et al., 1983). The toxin was coupled to the anti-A31 idiotypic monoclonal antibody Mc39-16 by disulphide linkage using N-succinimidyl-3-(2 pyridyldithio) propionate (SPDP) essentially as described by Thorpe and colleges (Thorpe et al., 1981; 1985), and depicted in figure 2.3. Step 1 in the reaction is the introduction of 2-pyridyl-disulphide groups into the antibody by reaction of the N-hydroxy-succinimide group of SPDP with primary amines of the antibody molecule. Reduction of the 2-pyridyl-disulphide by DTT introduces free sulfhydryl groups into the antibody (figure 2.3 step 2). The reaction of this thiolated antibody with 2-pyridyl-disulphide activated saporin (prepared as in step 1) results in exchange

of the -SH group of the antibody with a 2-pyridyl-disulphide group, leading to formation of a disulphide bond between the antibody and the toxin (figure 2.3 step 3).

The final product was determined to have the following average composition:

RIP : Ab = 1.8 mol : 1 mol
Antibody = 233 µg/ml in PBS
RIP = 84 µg/ml in PBS

2.28. PREPARATION OF BISPECIFIC ANTIBODIES

2.28.1. PREPARATION OF F(ab')$_2$ FRAGMENTS.

The immunoglobulin to be digested (at approximately 14 mg/ml in Tris-NaCl) was dialysed into 0.1 M Na acetate pH 7.5 at 4°C o/n and then the pH lowered to 4.2 using 2 M Na Acetate pH 3.7. Pepsin was dissolved into 0.1 M Na acetate pH 4.2 to give a concentration of 10 mg/ml and both the antibody and pepsin solutions were then warmed to 37°C. With both reactants at 37°C, the pepsin was added to the immunoglobulin to equal 3% of the amount of antibody present, a sample was removed immediately to obtain an HPLC profile (LKB 2150 HPLC system using a Du Pont Zorbax Bio Series GF-250 column: Pharmacia LKB Biotechnology division, Milton Keynes, U.K. and Hichrom Ltd, Reading, U.K.) and the mixture returned to the 37°C incubator. A further sample was taken after four hours had elapsed and each subsequent hour until the digestion was complete. To stop the reaction the pH was raised to 7.5 by the addition of 2 M TE8 and the temperature reduced to 4°C. The F(ab')$_2$ was isolated from the mixture by size exclusion chromatography using two S200-HR columns (2 x 2.5 x 92 cm) joined in series and equilibrated in 0.2 M TE8 containing 10 mM EDTA. Cycling between the two columns was continued until sufficient separation had occurred (normally 3-4 cycles) for the 'pure' F(ab')$_2$ to be collected.

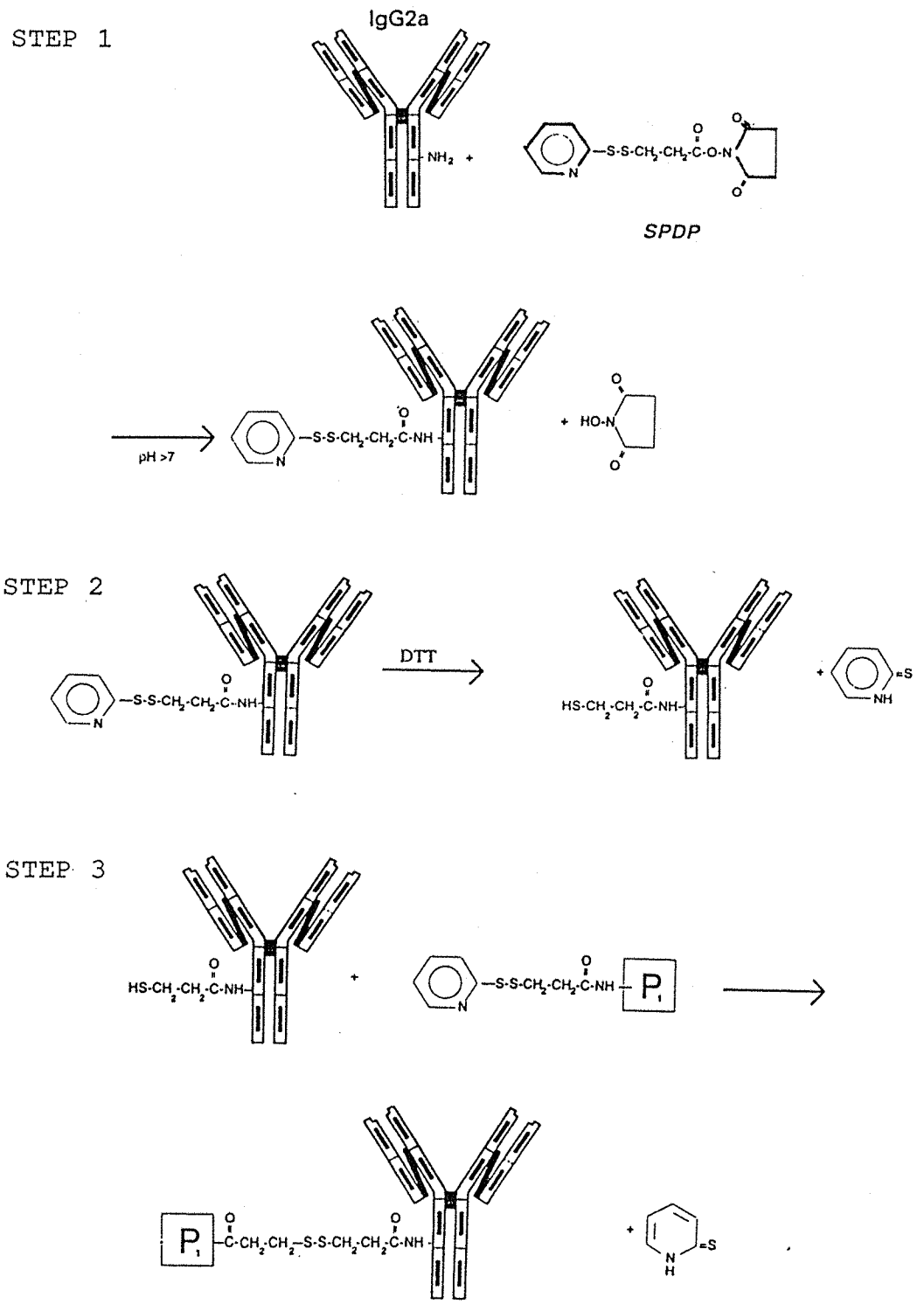


Figure 2.3. Schematic diagram of the covalent linkage of the ribosomal-inactivating protein, saporin, to a rat anti-A31 idiotypic monoclonal antibody using the cross-linking reagent SPDP. P_1 = saporin.

2.28.2. DETERMINATION OF AVAILABLE THIOL GROUPS IN Mc39-16.

100 µl of Mc39-16 F(ab')$_2$ at 7 mg/ml was reduced with 10 µl of DTT (66 mM) at 37°C for 30 minutes followed by isolation of the Fab' by passage through 0.6 ml Sephadex G25$_{course}$ in a 1 ml syringe. The elutant was collected as 200 µl fractions, the OD_{280} of which was determined by dilution of a 50 µl sample with 450 µl of 0.2 M TE8 before measurement in a Pye unicam SP1700 ultraviolet spectrophotometer. Using the fraction giving the maximum OD_{280} reading 150 µl were taken (0.048 mM Fab$_{SH}$) and to this was added a 5 M excess of 2,2' dithiodipyridine (PDS; Sigma) i.e. 15 µl of 0.24 mM PDS. The reaction mixture was incubated at 37°C for 15 minutes before dilution to a final volume of 500 µl with 0.2 M TE8 and measurement of the production of 2-thiopyridone at OD_{343} using an extinction coefficient of 8.08.

2.28.3. CONSTRUCTION OF BISPECIFIC ANTIBODIES.

The F(ab')$_2$ preparations of the two antibodies to be linked as Fab' fragments via thioether bonds were concentrated to 10 mg/ml (ultrafiltration under nitrogen pressure using Diaflo PM10 membranes in Amicon chambers, Amicon Ltd, Stroud, Bucks, UK). Both F(ab')$_2$ preparations were reduced to Fab$_{SH}$ by the addition of 0.1 volumes of a mixture of 0.22 M 2-mercaptoethanol (2-ME, BDH) and 0.085 mM EDTA dissolved in H_2O. The reduction was carried out at 37°C for 30 minutes after which both solutions were chilled to 4°C, a temperature that was maintained throughout the following manipulations including the chromatography purification steps. The Fab'γ_{SH} were isolated from the reduction mixture by passage through a Sephadex G-25 (Pharmacia) column equilibirated in 50 mM sodium acetate, pH 5.3, a sample (45 µl) of the Fab'γ_{SH} was removed at the top of the peak (monitored at 280 nm by a Uvicord) and after the addition of 5 µl of iodoacetamide (IAA: BDH) a HPLC profile was obtained (Du Pont Zorbax Bio Series GF-250 column). The first Fab'γ_{SH} was collected onto ice in a conical flask and a half volume of 12 mM o-phenylenedimaleimide (16.1 mg o-PDM dissolved in 5 ml dimethylformamide [BDH] kept in methanol/ice bath until

required) added rapidly with mixing. This reaction mixture was placed on ice for 30 minutes and in the meantime the second Fab'γ_{SH} was collected from the Sephadex G-25 column, once again a sample being checked as before by HPLC, and the bulk stored on ice until needed. The maleimidated Fab'γ_{mal} was isolated from the reaction mixture by passage through a Sephadex G-25 column equilibirated in 0.05 M AE 5.3 and was collected directly into an Amicon chamber to which was also added the Fab'γ_{SH} component and this mixture immediately concentrated down to 5 mg/ml under nitrogen at 4°C. After o/n incubation the pH was adjusted to 8 using 1 M Tris-HCl pH 8.0 and the mixture reduced by the addition of 2-Mercaptoethanol (2-ME) to a final concentration of 20 mM and incubation at 37°C for 30 minutes. After this time IAA was added to a final concentration of 25 mM and the bispecific F(ab'γ)$_2$ isolated from the other reaction products by size exclusion chromatography by passage through Ultrogel AcA44 equilibirated in 0.2 M TE8. An overview of the reaction steps is shown in figure 2.4.

2.29. ^{3}H-LEUCINE INCORPORATION ASSAYS: TITRATION OF ANTIBODIES, THEIR DERIVATIVES AND SAPORIN.

A31 tumour cells were isolated as described in 2.8. except that they were finally re-suspended into RPMI-1640 leucine-free medium (Gibco) at a cell density of 5 x 10^6/ml. Antibodies and their derivatives were titered by dilution across 96-well flat bottomed tissue culture trays (Gibco) from an initial concentration of 1000 ng/ml to a final concentration of 0.48 ng/ml using leucine-free medium containing 50 ng/ml of saporin as the diluent. Saporin (kind gift from F.Stirpe, Istituto di Patologia Generale, de'll Universitandi Bologna, 1-40126 Bologna, Italia), was titered out at a constant antibody concentration (final concentration 1 µg/ml) in a similar way. Triplicate wells were used at each dilution. In addition, a series of wells were included that contained either cells with no saporin (total incorporation) or no cells (non-specific background) the final volume in each well being 100 µl. The antibodies and saporin were

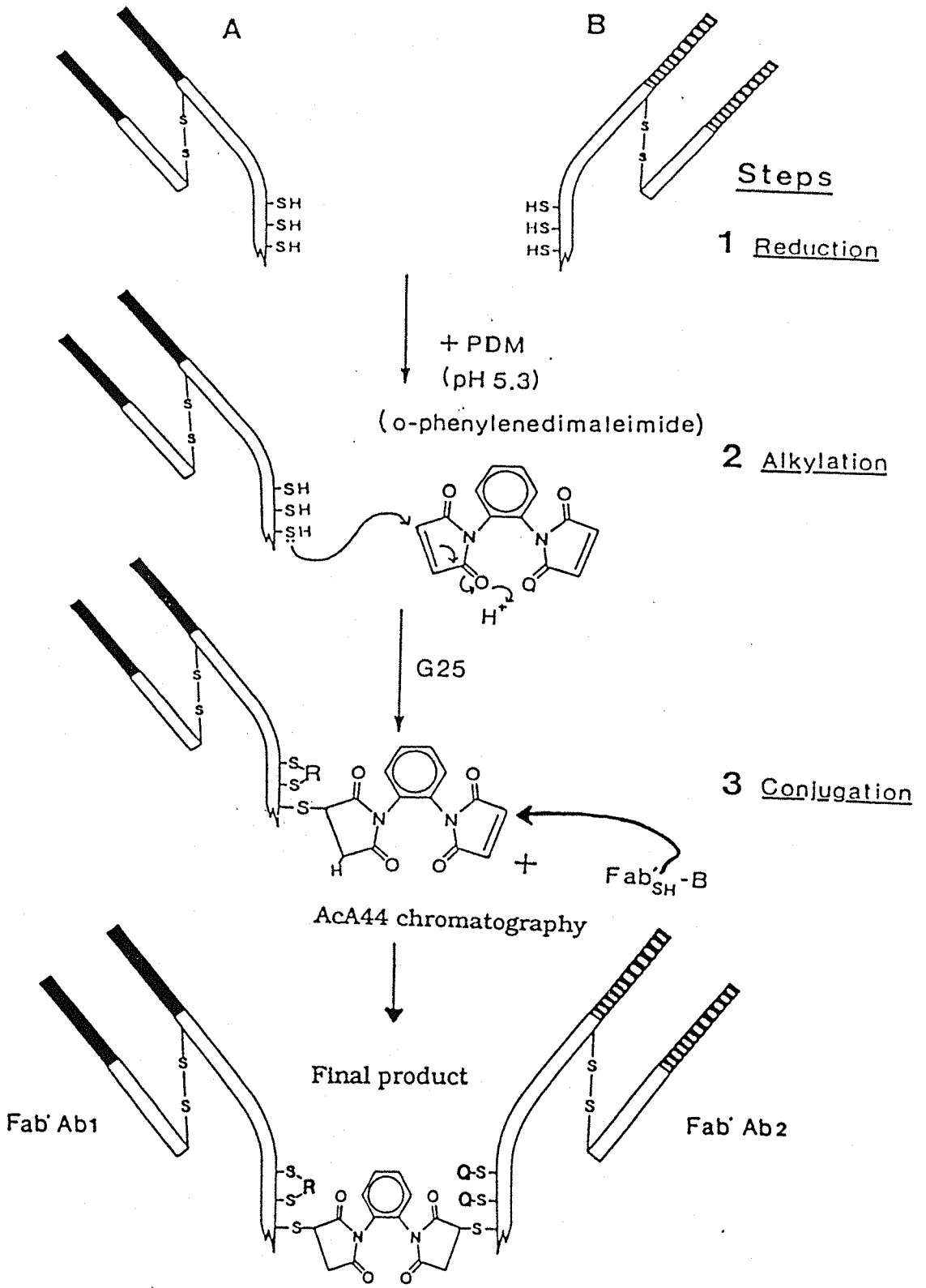


Figure 2.4. Schematic diagram of the formation of a bispecific antibody from two separate Fab' molecules using the cross-linking agent *o*PDM,
R = o-phenylenedisuccimimidyl.
Q = carboxyamidomethyl

incubated for one hour at 37°C before the addition of the cells. To each well was added 100 µl of the A31 cell suspension (i.e. 5 x 10^5 cells/well) and the plates placed in a humidified box which in turn was placed inside a 37°C incubator containing 7.5% CO_2. After 10-12 hours 50 µl of ^{3}H-leucine (0.5 µCi/well: Amersham International, Aylesbury, U.K.) was added to each well and the plates returned to the incubator for a further 10-12 hours. Just before harvesting the uptake of ^{3}H-leucine was blocked by the addition of 50 µl isoleucine (10 mg/ml in medium was added) and the plates transferred to an ice-tray. The cells with protein-associated ^{3}H-leucine were harvested using an Automash 2000 (Dynatech, Billingshurst, U.K.) onto glass filter discs (Whatman, Maidstone, U.K.) and the discs allowed to dry at 37°C. Once dry the discs were placed into scintillation vials (Pharmacia Wallac, Milton Keynes, U.K.) and 1 ml of Scintillation fluid (OptiScint 'Hi Safe', LKB, Loughborough, U.K.) added and the radioactivity present determined in a scintillation counter (1216 Rackbeta, LKB, Loughborough, U.K.).

CHAPTER 3: ISOLATION OF ANTI-IDIOTYPIC MONOCLONAL ANTIBODIES

3.1 INTRODUCTION

In order to raise monoclonal antibodies to the IgM of the A31 tumour a number of possible approaches could have been taken including; (a) Immunisation with whole cells (b) Isolation of immunoglobulin by enzymic surface digests of whole cells followed by affinity purification (Eady et al., 1974) or (c) "rescue" somatic cell fusion between the tumour cells and a murine plasmacytoma line such as NS-1 (Levy and Dilley, 1978; Brown et al., 1980). The first procedure induces a broad immune reaction to a large number of antigens and requires the development of a superior screening system to isolate those rare clones from the subsequent hybridoma fusion producing monoclonal antibody reactive with the A31 IgM-idiotype. The second approach requires large amounts of tumour to yield sufficient Idiotype for immunisation, whilst the third procedure requires development of an idiotype specific ELISA to screen the fusion products. For the current work it was decided to combine the latter two approaches. Using IgM from a papain surface digest of intact tumour cells, a sheep polyclonal antiserum was raised and made idiotype-specific by absorption on a column of normal mouse IgM and murine monoclonal immunoglobulin. With this reagent an idiotype specific ELISA was developed to screen A31 x NS-1 rescue fusions. If this approach was successful it would provide both a polyclonal anti-idiotypic reagent that would be invaluable in future work and a permanent cell line that would be a consistent source of A31 IgM.

3.2. SHEEP POLYCLONAL ANTI-IDIOTYPE IMMUNOGLOBULIN

3.2.1. MATERIALS AND METHODS

A sheep was immunised and boosted with A31 IgM according to the procedure given in chapter 2 and after analysis of the serum by Öuchterlony the IgG fraction of the serum isolated as also described in chapter 2.

To remove anti-constant region activity from the sheep polyclonal antibody the 7s IgG fraction was passed

exhaustively through the following immunosorbant columns: (a) M6-3D10-Sepharose 4B (Mouse IgGκ); (b), MOPC-104E-Sepharose 4B (mouse IgMλ); (c), M38-7-Sepharose 4B (mouse IgMλ); and (d), Pooled normal mouse Ig-Sepharose 4B. Where repeated runs through columns was required the latter were regenerated by elution with ammonium isothiocyanate (0.5M NH_3/1M KSCN pH 11.5). At various times during the absorption the sheep IgG antibody was tested for A31 specificity and lack of reaction to other mouse immunoglobulins using the ELISA as described in section 2.16.1.

3.2.2. RESULTS

Analysis of the serum by Öuchterlony prior to isolation of the IgG showed that the immune serum was highly reactive with A31 IgM, other mouse IgG and IgM fractions. There was a lack of reactivity towards isolated mouse lamda chains with a positive reaction towards isolated kappa light chains as would be expected after immunising with IgM isolated from tumour cells expressing a monoclonal immunoglobulin. Strong precipitation lines were obtained against mouse IgMλ and IgMκ indicating the presence of anti-μ antibodies. Determination of cross-reactivity of the absorbed IgG fraction by ELISA gave the result shown in figure 3.1.

Extensive absorption of the sheep IgG fraction on various mouse immunoglobulin columns was required before a preparation was obtained that reacted specifically with A31 IgM by ELISA (fig 3.1.). Absorption was continued until minimal reaction ($OD_{490} \leq 0.1$) to the non-specific antigens in the above ELISA was obtained and therefore represented an anti-idiotypic immunoglobulin fraction. It was noticeable from the ELISA results that the medium removed from cultured A31 tumour cells gave a higher titre than serum isolated from the blood of a terminally ill A31 mouse. This points towards the low secretion of idiotypic IgM *in vivo* demonstrated by the A31 tumour. With this anti-idiotypic immunoglobulin available for screening purposes it became feasable to rescue the A31 IgM by fusion of tumour cells with a mouse plasmacytoma line.

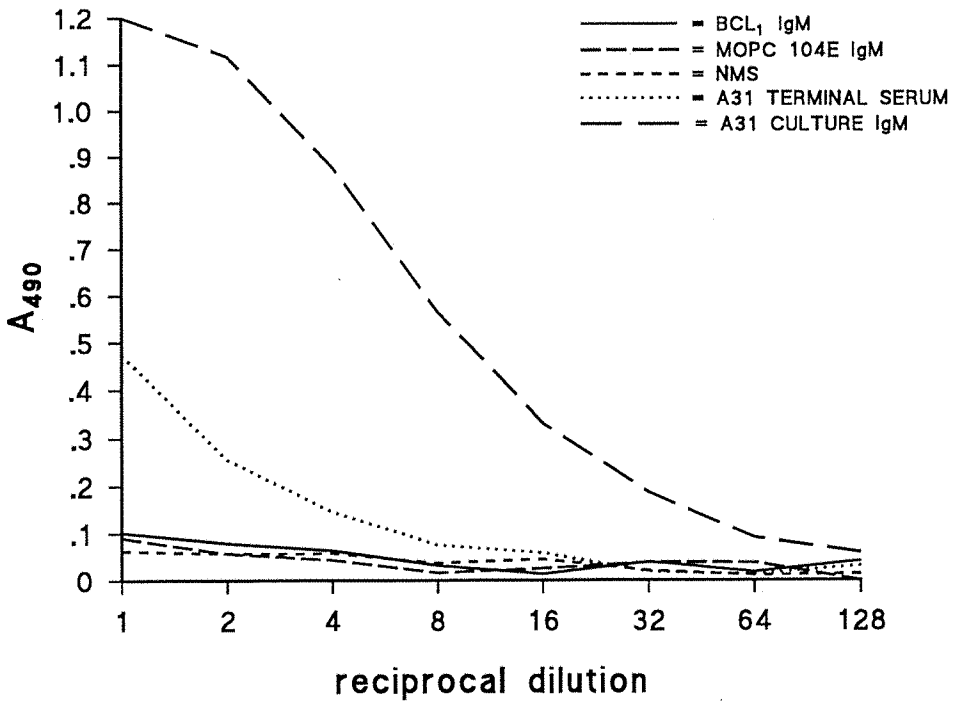


Figure 3.1. ELISA to determine specificity of sheep anti-A31 Idiotypic immunoglobulin fraction after immunoabsorption against monoclonal and normal mouse immunoglobulins. The ELISA was performed as described in section 2.15.1.

3.3. A31 RESCUE FUSIONS

Two attempts were made to produce a hybridoma cell line that would secrete A31 idiotypic IgM. Both attempts gave very similar results.

3.3.1. Mc1 FUSION

Hybridomas were produced by PEG mediated fusion between the murine myeloma line P3X63-Ag8.653 (653) and A31 tumour cells as described in chapter 2. Once the colonies were of a suitable size A31 IgM production was tested within the individual wells using the ELISA described in section 2.15.1. Wells giving a strong reaction on the screen were cloned onto thymocyte feeder layers and re-assayed by repeated ELISAs until two full clonings had been achieved. These individual hybridoma lines were expanded and frozen down in liquid

nitrogen. Isotype analysis (Section 2.15.3.) of culture supernatant from these hybridomas indicated that they contained murine IgM and would be suitable for growth in pristane primed mice in order to obtain ascitic fluid containing the A31 IgM at an enriched level. The ascitic fluid obtained from these mice contained only a weak monoclonal band by agarose gel electrophoresis (Paragon SPE system, Beckman: data not shown) compared with ascities from mice carrying conventional hybridoma cells, however attempts to purify A31 IgM by size exclusion chromatography failed to produce any material representative of 19s IgM (Data not shown).

3.3.2. Mc22 FUSION

The weak nature of the monoclonal band within the Mc1 ascites may have reflected the low secreting nature of the tumour, therefore an attempt was made to enhance production of IgM from the tumour cells by pre-culture in the presence of LPS prior to hybridoma isolation. It is known that LPS can cause the murine lymphoma line BCL_1 to increase its secretion of IgM (Knapp et al., 1979b; Strober et al., 1979). Similarly the B cell lymphoma WEHI 231, which resembles a resting B cell with low secretion of IgM, can be induced to secrete 4-8 fold more immunoglobulin upon exposure to 3 µg/ml of LPS with the maximal effect obtained after 3 days (Boyd et al., 1981). The fusion of LPS stimulated splenocytes with the plasmacytoma Ag8.653 produces a high frequency of immunoglobulin secreting hybridomas the majority of which secreted large quantities of IgM (Lawton et al., 1990).

3.3.2.1. RESULTS

Fresh tumour cells were prepared as described in section 2.8. A total of 1 x 10^8 cells were re-suspended into 40 ml of DMEM + 10% FCS containing Escherichia coli 0111:B4 LPS (a gift from Dr. J. Heckels, Department of Microbiology, University of Southampton) at a concentration of 100 µg/ml and the cells incubated for three days in a humidified atmosphere of 7.5% CO_2 at 37°C. Cell viability (trypan blue exclusion) at this time had dropped to 4% of that of the original inoculumn. A fusion using the same conditions as

previously described was performed with the exceptions that P3/NS1/1.Ag4.1 (NS-1) was used as the plasmacytoma partner. The cell numbers were adjusted to account for the lower viability and only three 96-well trays were inoculated with the cells from the fusion. Subsequent ELISA on the culture supernatants indicated three hybridomas that gave ODs of >1.0. These cells were cloned and frozen as usual. Once again the hybridomas were transferred into pristane primed mice for the production of ascities.

Agarose gel electrophoresis indicated a strong monoclonal band within the ascitic fluid. Fluid collected from mice inoculated with one of these hybridomas (Mc22-D5) was precipitated with ammonium sulphate and the immunoglobulin enriched pellet then dialysed into 0.1M Tris-NaCl pH 8.0 buffer. Agarose gel electrophoresis of this material indicated removal of the albumin band and the continued presence of the monoclonal IgM band (Figure 3.3. Tracks 3 and 4). Further purification was performed by means of size fractionation on an ultragel AcA 22 (Pharmacia LKB). Chromatographic profiles (figure 3.2.) contained a number of peaks each of which was collected and analysed by agarose gel electrophoresis and ELISA (section 2.15.1.). The major peak eluting from the AcA 22 column that gave a band on agarose gel electrophorisis closest to, but not identical with, the monoclonal band visible within the ascitic fluid (figure 3.3. track 7) was found as part of the final major peak represented by fractions 63-65 (figures 3.2.) and this together with an SDS-PAGE profile obtained at a later date when purified A31 IgM had become available (figure 3.4.) indicated that the protein responsible for the monoclonal band was of small molecular weight (44 000 kDa). In addition an ELISA which detected idiotype positive material indicated that the idiotype was not within this monoclonal band but represented a very minor peak on the AcA 22, of higher molecular weight (figure 3.2. approximately fraction 40).

In contrast AcA 22 chromatography and peak analysis by ELISA of culture supernatant from Mc22-D5 showed the presence of idiotype positive material eluting in the position expected of 19s IgM (figure 3.5.). Taking the results together it would appear that the Mc22 fusion did produce

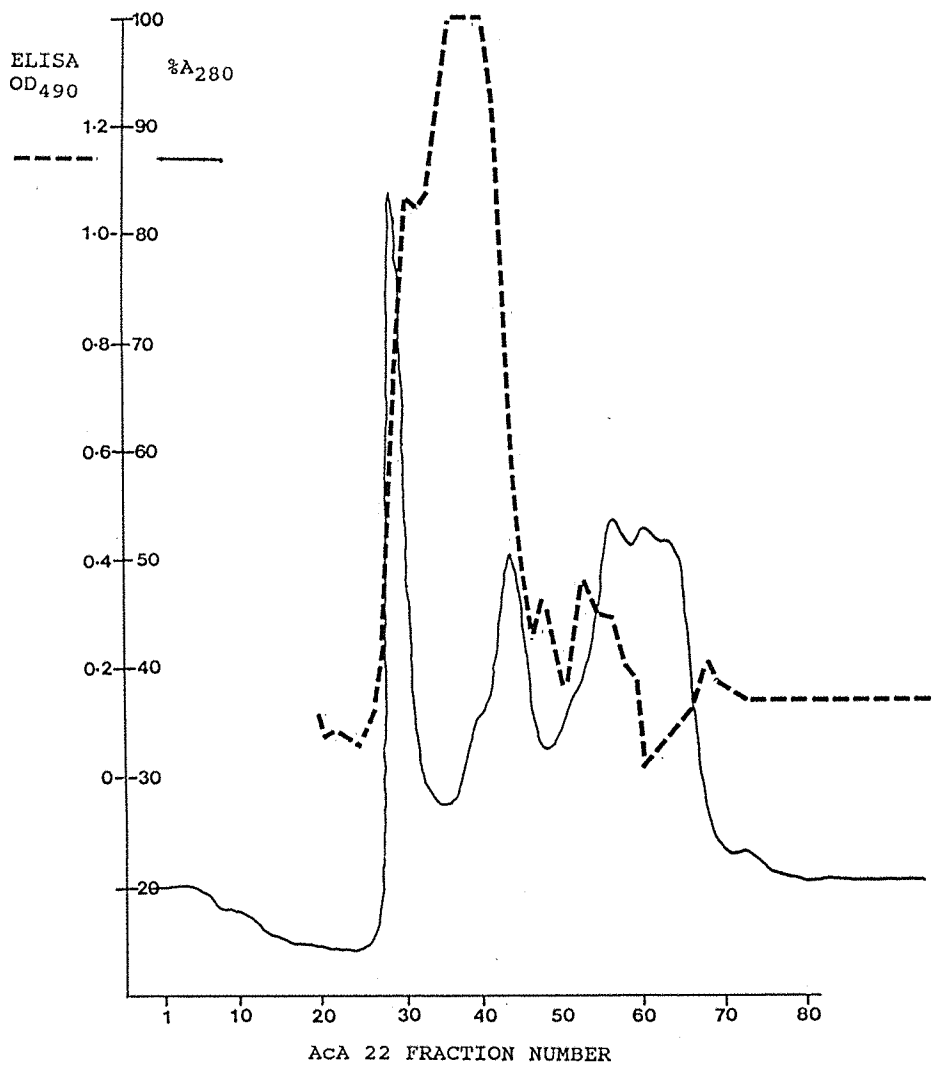


Figure 3.2. AcA 22 chromatographic profile of ammonium sulphate precipitated Mc22 D5 ascities. The location of A31 idiotypic material was determined by ELISA of individual fractions and has been superimposed onto the chromatographic profile (dotted line).

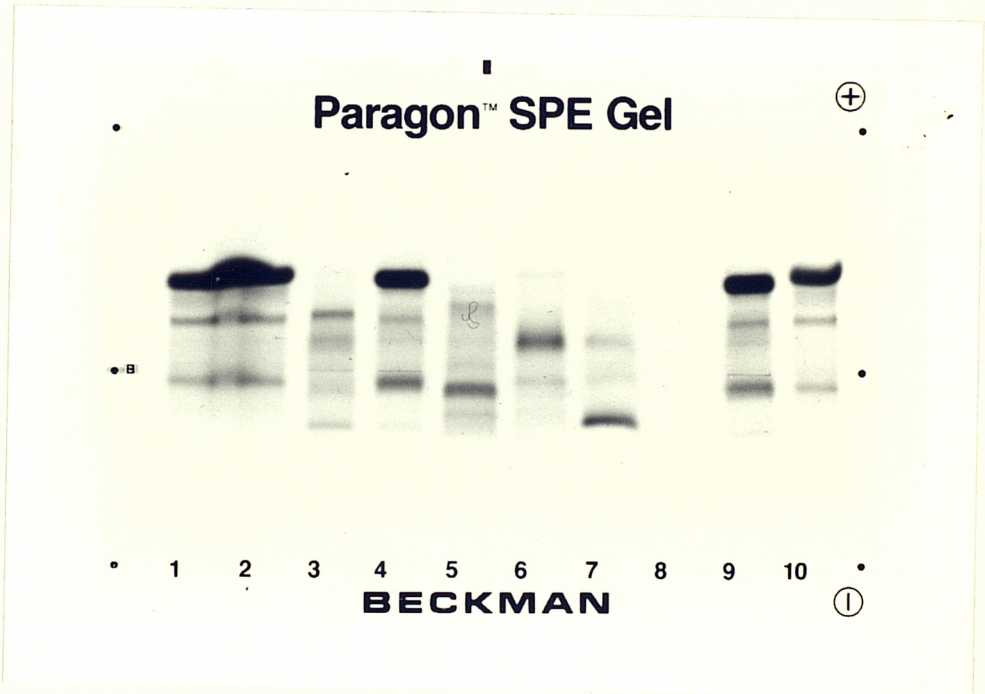


Figure 3.3. Agarose gel electrophorisis of concentrated, pooled, fractions from AcA 22 chromatographic run of ammonium sulphate precipitated Mc22 D5 ascites (fig. 3.2). Tracks (1) and (2) = Normal mouse serum; Track (3) = Mc22 D5 ammonium sulphate precipitated ascities; Track (4) = Mc22 D5 ascities; Track (5) = Fractions 55-58; Track (6) = Fractions 60-61; Track (7) = Fractions = 63-65; Track (8) = Blank; Track (9) = Mc22 D5 ascities; Track (10) = Normal mouse serum.
Note: First major peak in Figure 3.2. when examined by agarose gel electrophorisis gave a band in the $alpha_1$-globulin zone (fast migration) and is not shown above.

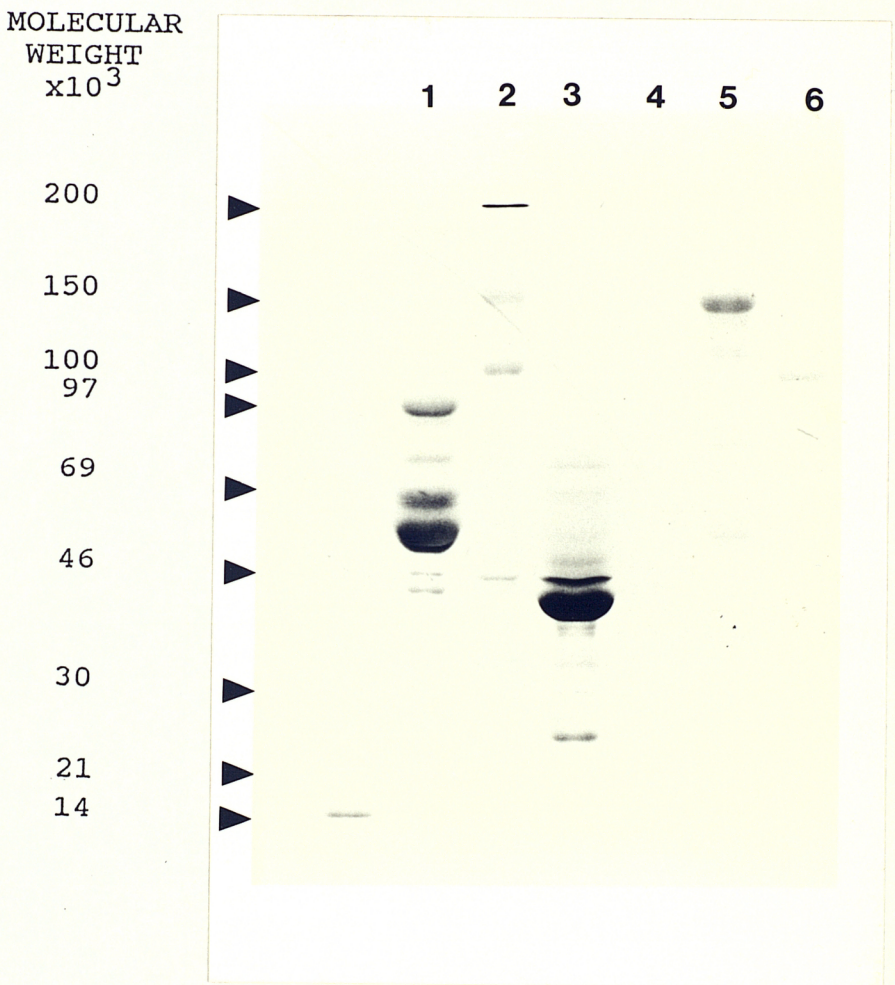


Figure 3.4. SDS-PAGE (non-reduced) of concentrated AcA 22 fractions 63-65. Track (1) = Mc22 D5 ascities; Track (2) = A31 IgM; Track (3) = AcA 22 fractions 63-65; Track (4) = Blank; Track (5) = KT3 IgG; Track (6) = KT3 $F(ab')_2$.

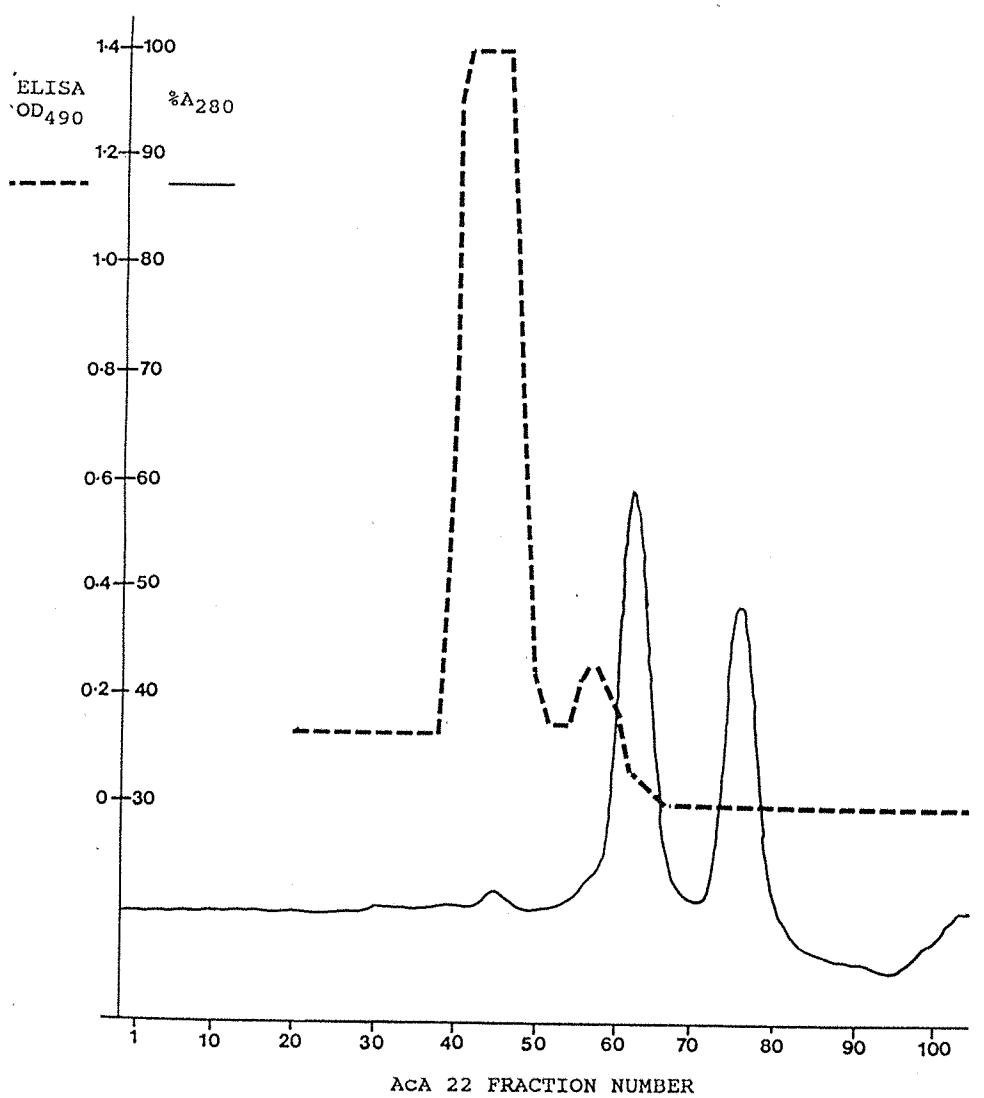


Figure 3.5. AcA 22 chromatographic profile of Mc22 D5 culture supernatant. The location of A31 idiotypic material was determined by ELISA of individual fractions and has been superimposed onto the chromatographic profile (dotted line).

hybridomas secreting 19s A31 IgM but that this production, as with the Mc1 fusion, was unstable when the cells were transferred to pristane primed mice. Attempts at production of 19s IgM in irradiated and hydrocortizone treated pristane-primed mice were also unsuccessful. Additionally cells isolated from ascitic fluid and re-cloned *in vitro* failed to secrete idiotypic immunoglobulin. In the absence at this time of alternatives such as nude mice or hollow-fibre bioreactors it was decided to continue with the project using culture supernatant as a source of IgM idiotype.

3.4. PRODUCTION OF MONOCLONAL ANTI-IDIOTYPIC ANTIBODIES.

INTRODUCTION

To overcome the lack of purified idiotypic IgM, a means of immunisation using the relatively low levels of A31 IgM in the Mc22 hybridoma culture supernatant was required. This was achieved using complexes formed in the same way as the polyclonal sheep anti-A31 idiotypic immunoglobulin (section 2.8.2.) with the exception that the Mc22 culture supernatant replaced the surface digest material. The major difficulty with this approach was the production of unwanted antibodies against the sheep component of the complexes thereby excluding the A31 specific ELISA described in 2.15.1. as a means of screening for monoclonal anti-idiotype antibodies. To overcome this difficulty an ELISA was designed in which IgM idiotype was coated directly onto the plate without a 'capture' antibody using material pooled from AcA 22 chromatography runs. Preliminary experiments using HB58 (Rat anti-mouse kappa chain) monoclonal antibody culture supernatant as a positive control indicated that the pooled AcA 22 material gave good results when coated at a dilution of 1+3 in coating buffer and detected using a sheep anti-rat Fcγ specific peroxidase.

Two attempts were made at this time to produce syngenic monoclonal anti-idiotypes by immunisation of mice with the sheep-A31 complexes. Despite the detection of anti-idiotypic antibodies within the sera of these mice, attempts at isolating monoclonal antibodies failed to produce hybridomas

secreting antibody to the idiotypic IgM. Further attempts at a later date to raise monoclonal antibodies using purified A31 IgM conjugated to KLH also failed to produce syngenic anti-idiotypic monoclonal antibodies. Due to the lack of success in raising antibodies to the A31 IgM in the murine system attention was switched to raising a response in rats.

3.4.1. Mc39 FUSION: RAT ANTI-A31 IDIOTYPIC MONOCLONAL ANTIBODIES

ANTIGEN:		SHEEP ANTI MURINE IgM-A31 IgM COMPLEXES
IMMUNISATION SCHEDULE:	1°	8/10/87; 10.7 µg in CFA given s/c
	2°	6/11/87; 13.0 µg in IFA given s/c
	3°	4/12/87; 6.0 µg in IFA given s/c
	4°	29/1/88; 12.0 µg in PBS given i.v.
SPLEEN DONOR:		LOU RAT
DATE OF FUSION:		1/2/88

The fusion was carried out as described in 2.17. with the exceptions that immunised rat splenocytes replaced the tumour cells in the procedure and P3/NS1/1.Ag4.1 cells were used as the plasmacytoma partner. Screening of the fusion was by ELISA as described in 2.15.2. From the five primary fusion plates 37 positive wells were selected and each semi-cloned onto one half of a 96-well plate. Once sufficient growth had occurred in the first two rows of the semi-clone plates the supernatants from these wells were harvested and reactivity with A31 cells determined on a FACS (FACS III, Becton Dickinson, Oxford, U.K.) using the method described in 2.18.

From the FACS results and analysis by ELISA against AcA 22 material twenty wells were chosen and the cells from each cloned out across a 96-well tray.

Further screening consisted of FACS analysis against A31 and BCL_1 cells including blocking experiments using normal mouse serum and terminal A31 serum giving the results shown in Figures 3.6. and 3.7. The reduction in fluorescent signal, signified by the peaks moving to left in figures 3.6. and 3.7. in the presence of normal mouse serum indicates that the monoclonal antibody being tested has been blocked in its binding to the surface of the tumour cell. This suggested that the particular monoclonal antibody being tested is reactive to a normal component of normal mouse serum and therefore is likely to be anti-kappa light chain or anti-μ chain. Comparison of this monoclonal antibodie's reactivity to BCL_1 cells, surface positive IgMλ, then allows discrimination between anti-kappa and anti-μ reactivity. The blocking, in the presence of serum isolated from a terminal A31 mouse of a monoclonal antibody that was not blocked by normal mouse serum and was not reactive with BCL_1 cells is indicative of an anti-idiotypic monoclonal antibody.

Consider monoclonal antibody Mc39-12 in figures 3.6. and 3.7., it is clearly reactive to A31 cells but was blocked by both normal mouse serum and terminal A31 serum. This suggests that it is reactive to either kappa light chain or μ-chain. Examination of figure 3.7. shows that this same antibody binds to the surface of BCL_1 cells (IgMλ) and must therefore be an anti-μ chain specific monoclonal antibody. Monoclonal antibody Mc39-16 on the other hand was reactive to A31 cells but was not blocked by normal mouse serum and did not bind to the surface of BCL_1 cells. Blocking of this antibodies reactivity to A31 cells by terminal A31 mouse serum confirmed its reactivity as anti-idiotypic. Similar FACS analysis of the other monoclonal antibodies isolated from the Mc39 fusion together with ELISA analysis using A31 IgM and control murine IgM's (data not shown) allowed the designation of the isolated monoclonal antibodies into the specificities given in table 3.1. Note that the slight blocking of Mc39-28 by normal mouse serum suggests that it is reactive to a restricted idiotype (i.e. an idiotope that is present in

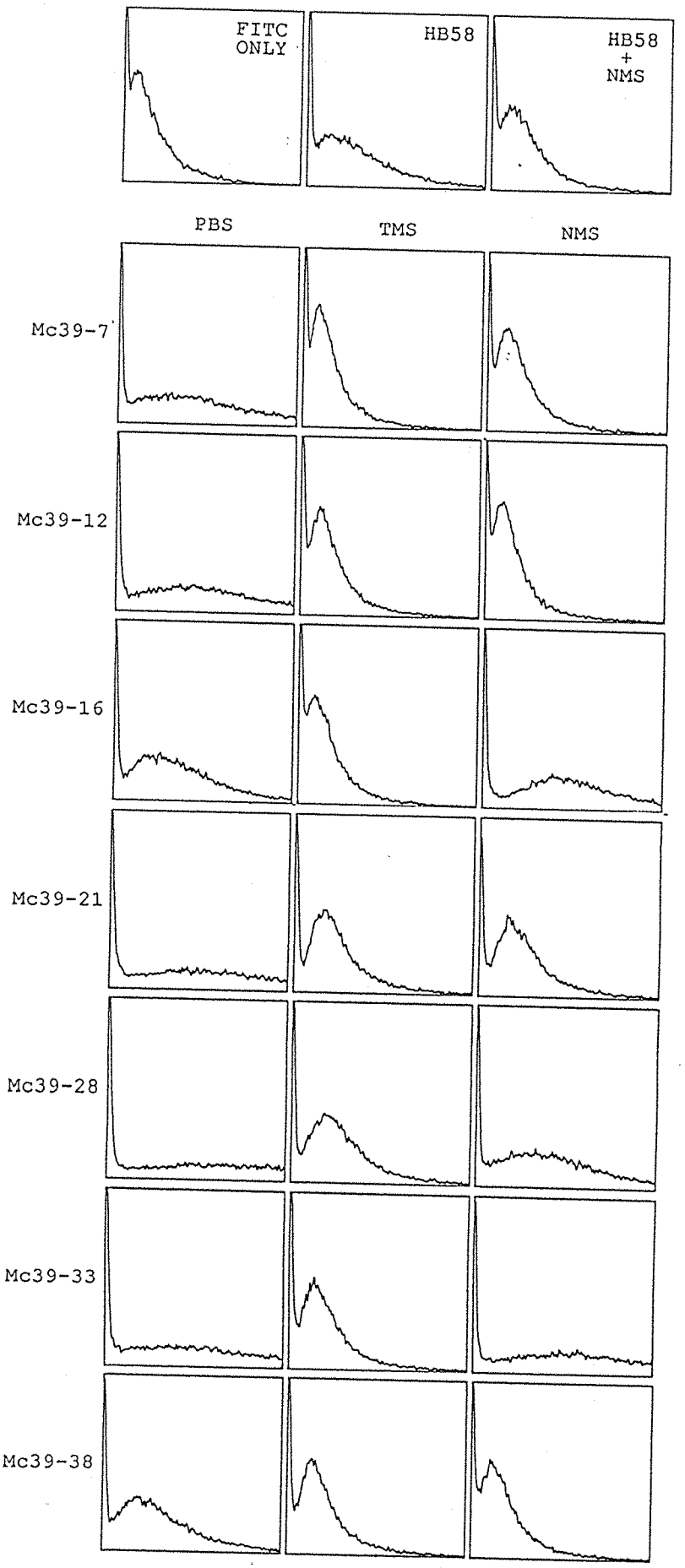


Figure 3.6.FACS analysis of monoclonal antibodies isolated from Mc39 fusion showing serum blocking studies. A31 cells used throughout. PBS = addition of PBS; TMS = addition of serum from terminal A31 mouse; NMS = addition of serum from normal CBA mouse; HB58 = anti-mouse kappa light chain.

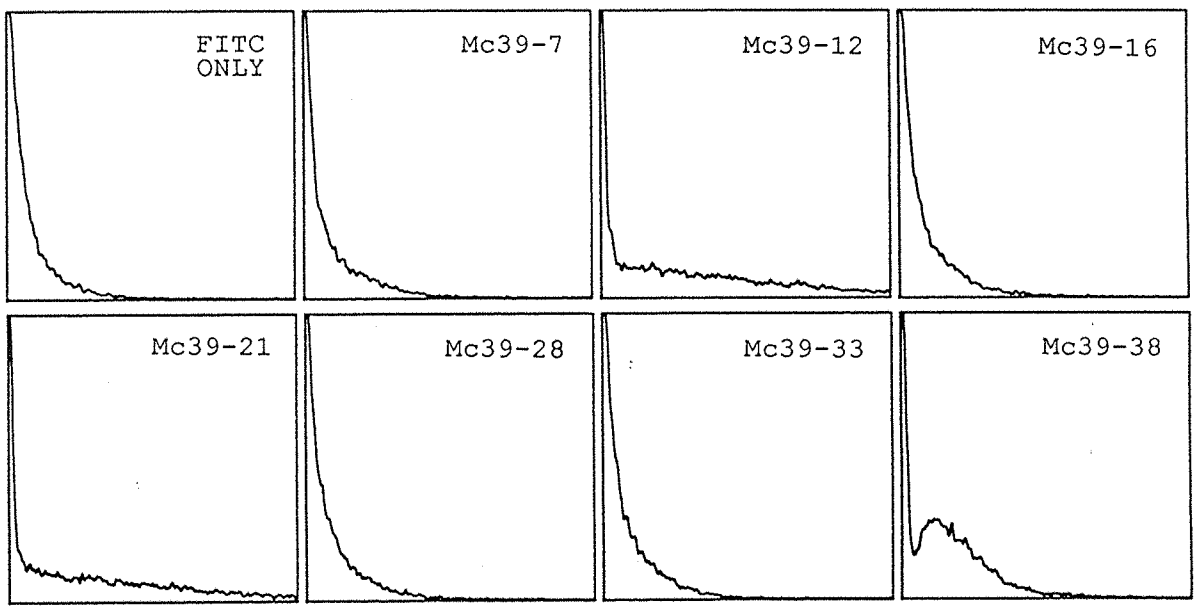


Figure 3.7. Mc39 monoclonal antibody staining of BCL_1 cells.

small amounts in normal serum).

TABLE 3.1. SPECIFICITY OF Mc39 MONOCLONAL ANTIBODIES

CLONE	SPECIFICITY
Mc39-7	anti-kappa light chain
Mc39-12	anti-μ heavy chain
Mc39-16	anti-idiotypic
Mc39-21	anti-μ heavy chain
Mc39-28	anti-cross reactive idiotype
Mc39-33	anti-idiotypic
Mc39-38	anti-μ heavy chain

3.5. ISOTYPE DETERMINATION OF Mc39 MONOCLONAL ANTIBODIES

The isotype of the individual monoclonal antibodies isolated from the Mc39 fusion were determined using the ELISA described in 2.15.3.

3.5.1. RESULT

All hybridomas were found to be secreting rat IgG2a immunoglobulin.

3.6. COMPLEMENT-MEDIATED LYSIS OF A31 CELLS BY ANTI-IDIOTYPIC MONOCLONAL ANTIBODIES

To further characterise each antibody their ability to lyse A31 tumour cells in the presence of rat complement was determined. The lack of purified immunoglobulin from each of the hybridomas required concentration of each culture supernatant by ultrafiltration under nitrogen pressure using Diaflo PM10 membranes in Amicon chambers (Amicon Ltd, Stroud, Bucks, UK). Antibody levels within the concentrated

supernatants were determined by an ELISA as described in section 2.15.4.

3.6.1. ^{51}CHROMIUM RELEASE ASSAY

A standard ^{51}Cr-release assay was used to determine the levels of complement activity (section 2.20.) with each antibody.

3.6.2. RESULTS

Figure 3.8. shows that of the antibodies tested only the serum from the rat used for the Mc39 fusion and monoclonal antibodies Mc39-12 and Mc39-16 gave significant complement-mediated lysis of tumour cells. At equivalent concentrations of antibody (10 µg/ml) the anti-idiotypic monoclonal antibody Mc39-16 gave higher lysis of tumour cells (48%) than the anti-µ chain specific antibody Mc39-12 (9%). The final end points (were the curves cross the x-axis) for Mc39-12 and Mc39-16 were 0.625 µg/ml and 0.31 µg/ml respectively.

3.7. ISOLATION OF MC39-16

INTRODUCTION

It was apparent that purified anti-idiotypic monoclonal antibody was required for further studies and as the hybridomas were produced by fusing a mouse cell line to rat immune spleen cells it would be of little use to inject the cells directly into normal rats or mice as each would reject the hybridoma cells. Because of the difficulties existing for the purification of monoclonal antibodies from rat-mouse heterohybrids it was decided at this stage to concentrate on Mc39-16 for further studies. Therefore in order to produce an ascitic fluid containing the monoclonal antibody some form of immunosuppression was required.

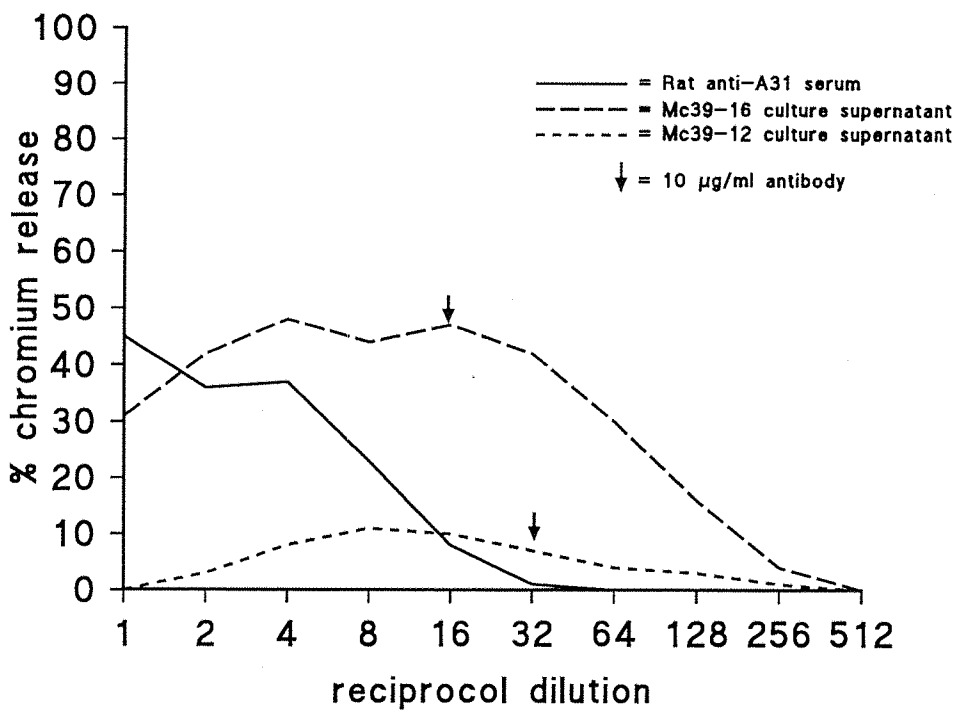


Figure 3.8. Complement lysis of A31 tumour cells mediated by polyclonal and monoclonal antibodies as determined by chromium release assay.

NOTES: Mc39-21, Mc39-28 and Mc39-33 gave minimal lysis of tumour cells.

3.7.1. Mc39-16 FROM IMMUNOSURPRESSED MICE

Initial experiments indicated that hydrocortosone injection and irradiation was insufficient to produce consistently positive ascites. To increase the degree of immunosuppression induced, the L_3T_4 and Ly-2 sub-populations of murine T cells were reduced by the injection of the rat cytotoxic monoclonal antibodies GK1.5 (anti-L_3T_4; Wilde et al., 1983) and 53-6.7 (anti-Lyt-2; Ledbetter and Herenberg, 1979). The following procedure was established:

(1) Day 0: Balb/c mice were given 0.2 ml of pristane (2,6,10,14- tetramethylpentadecane, Aldrich chemical company, Dorset, England) i.p.
(2) Day 7: each mouse received 3 mg hydrocortosone (Hydrocortosone sodium succinate, Organon laboratories Ltd, Cambridge, England) intramuscular (i.m.).
(3) Day 8: mice given whole body irradiated (300 rads)
(4) Day 9: animals treated with GK1.5 (anti-L_3T_4) and 53-6.7 anti-Ly-2 (100 µg of each i.p.) monoclonal antibodies (kind gift from F.Stevenson, Regional Immunology Service, Tenovus, Southampton)
(5) Day 10: monoclonal antibody treatment repeated and 2h later Mc39-16 cells (5×10^7/mouse) given i.p.

The animals were monitored daily and the ascitic fluid from each mouse analysed by agarose gel electrophoresis to determine the presence of a monoclonal band as not all mice produced significant levels of immunoglobulin. Pooled ascites was also examined by ELISA and FACS analysis to confirm reactivity with isolated A31 IgM and cells respectively.

Isolation of Mc39-16 by the above method did not give large amounts of antibody due to only a proportion of the collected ascitic fluid containing a monoclonal band so other methods were examined in order to increase the yeilds of Mc39-16.

3.7.2. GROWTH OF Mc39-16 IN NUDE MICE

Animals were housed as described in section 2.3. and hybridoma inoculation and ascities removal was carried out as described in 2.5. As with immunosuppressed mice not all mice produced ascitic fluid containing the antibody of interest, although the proportion of positive mice was higher than with mice immunosurpressed using the regime described in 3.7.1.

3.7.3. HOLLOW FIBRE BIOREACTOR CULTURE

The Mc39-16 hybridoma was later cultured within a hollow-fibre bioreactor as given in section 2.6.

3.8. PURIFICATION OF Mc39-16

Ion exchange chromatography was performed as described in section 2.9. with examination of the fractions coming off the column indicating that Mc39-16 eluted as the first major peak (figure 3.9.) and was essentially free from contaminating proteins when examined by agarose gel electrophoresis (figure 3.10).

3.9. SERUM LEVELS OF A31 IDIOTYPIC IgM

With the isolation of purified anti-idiotypic monoclonal antibodies further characterisation of the tumour was possible. A polyclonal antiserum had been used to determine the levels of IgM produced by tumour cells during short term *in vitro* culture (Cobb et al., 1986) and now the anti-idiotypic monoclonal antibody Mc39-16 was used to determine the serum secretion profile of the tumour *in situ* by collection of serum samples (section 2.19.) and using the ELISA described in 2.15.5.

The development of circulating idiotype IgM in A31 mice during the course of the disease is shown in figure 3.11. Serum levels of idiotypic IgM remained very low for the first 2 weeks after inoculation of the tumour cells followed by a rapid rise in levels which peaked at about 70 µg/ml during the terminal phase of the disease.

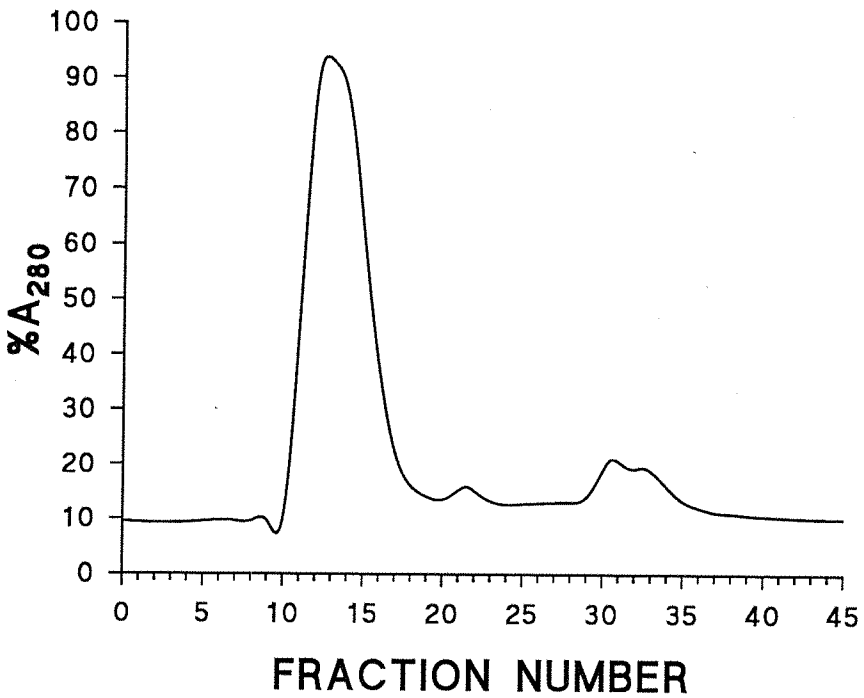


Figure 3.9. DEAE ion-exchange chromatographic purification of Mc39-16. The immunoglobulin component of ascitic fluid containing Mc39-16 was obtained by ammonium sulphate precipitation and further purified by DEAE chromatography. The first major peak (fractions 10-17) contained the monoclonal antibody free of contaminating proteins.

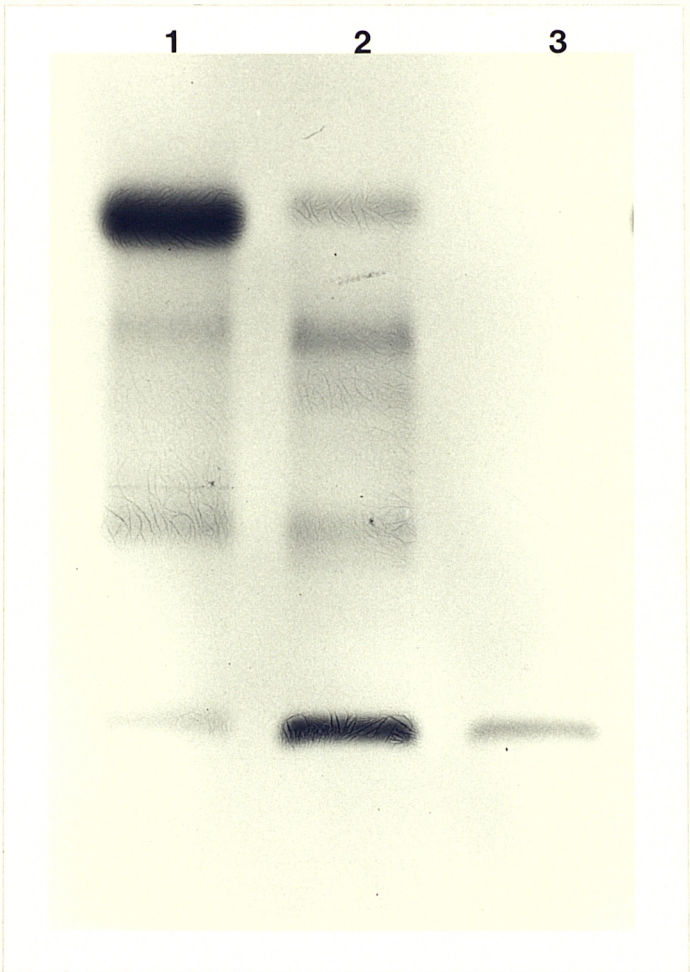
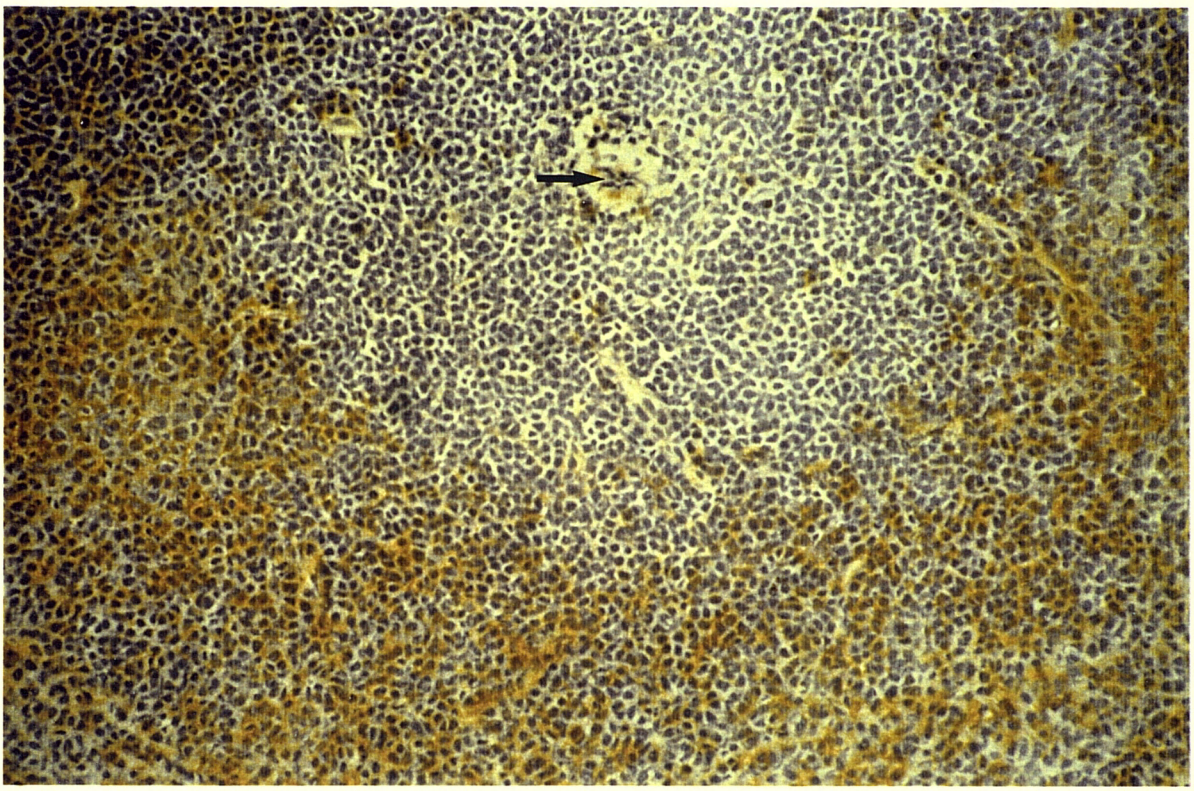
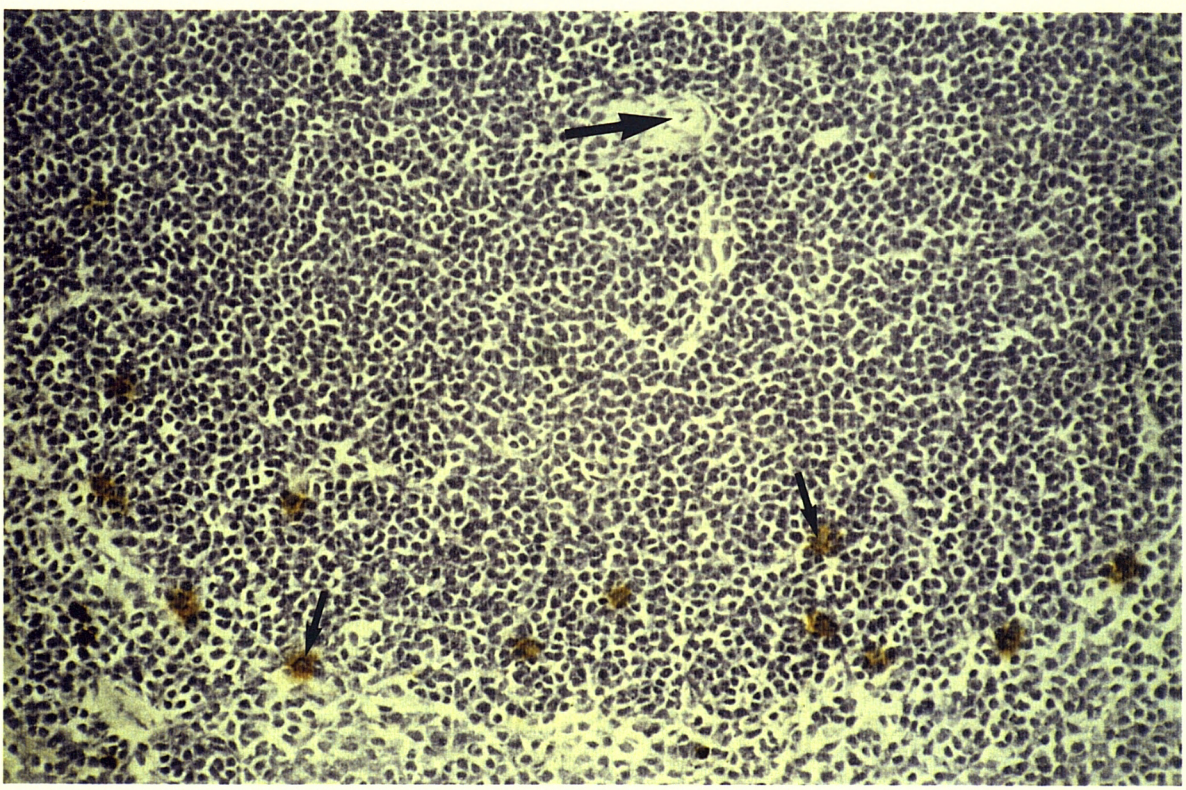


Figure 3.10. Agarose gel electrophoresis of DEAE-purified Mc39-16 IgG.
Track 1 = Mc39-16 mouse ascities
Track 2 = Mc39-16 ascities post ammonium sulphate precipitation
Track 3 = Pooled fractions (10-17) from DEAE chromatography.





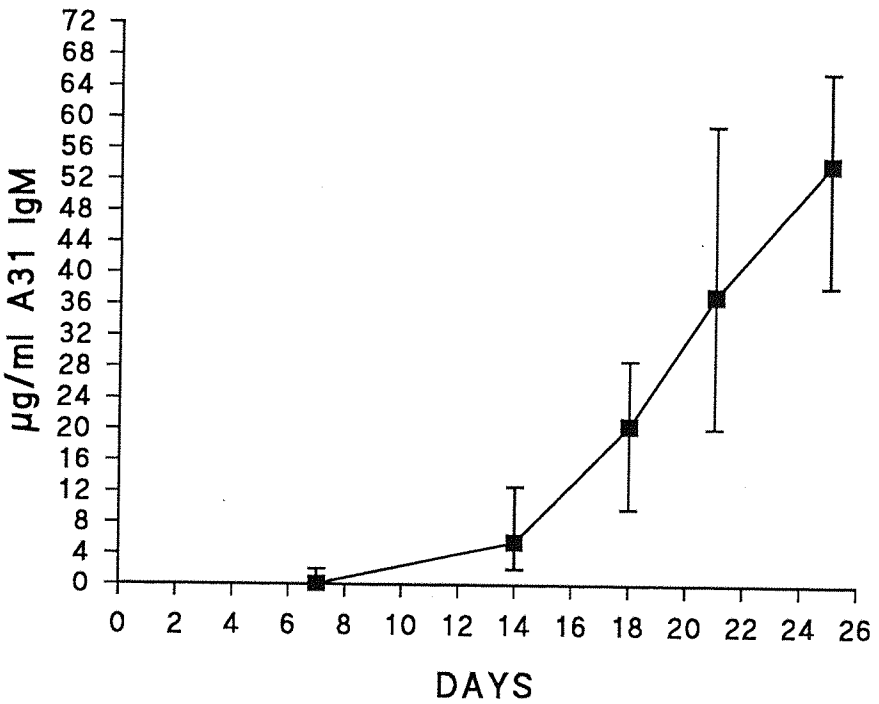


Figure 3.11. Idiotypic IgM levels within the serum of tumour bearing mice during progression of the disease.

NOTES:

(1) Day 7, 14, and 18 represent an average of ten mice; day 22 is an average of nine mice and day 26 is an average of three mice.

(2) Range bars are given at each point.

3.10. DETERMINATION OF THE Ka FOR MC39-16 BINDING TO A31 CELLS

To further characterise Mc39-16, its binding affinity for the surface IgM on A31 cells was determined using radiolabelled antibody following the method of Dower et al., (1981). Binding assays using Mc39-16 IgG, although giving reasonable binding curves, tended to give higher than expected binding at high antibody concentrations therefore the association constant determination described below was obtained using the $F(ab)_2$ fragment of Mc39-16. The $F(ab')_2$ fragments were prepared by standard pepsin digestion using our usual method (Glennie et al., 1987).

The radioiodination of antibodies and the method employed for the determination of the assocation constant for Mc39-16 binding to A31 cells have been described in sections 2.22. and 2.23. respectively. Briefly A31 cells that had been made incapable of endocytosis were exposed to radiolabelled antibody at 37°C for two hours. Separation of antibody bound to the cells from antibody in free solution was acheived by centrifugation of the cells through oil. Cell pellets were then recovered and the bound antibody measured in a gamma counter.

The data collected was first plotted as a saturation binding curve (insert Figure 3.12.) and then analysed by the method of Scatchard (Scatchard, 1949; Tinoco et al., 1978) to obtain the binding curve shown in main part of figure 3.12. from which an equilibrium binding constant (Ka) of 3.8×10^8 M^{-1} was obtained. The intercept on the x-axis suggests that the average number of IgM molecules on the A31 cells is in the order of 3.8×10^5. The Ka obtained was in good agreement with that obtained for the IgG molecule if the non-linearity for the latter at high concentrations was ignored. An irrelevant rat IgG2a monoclonal antibody with specificity for the idiotype on BCL_1 cells (Mc10-6A5) was used to determine the degree of non-specific binding. A full derivation of the Scatchard plot is given in appendix 1.

To confirm that the saturation observed above was likely to be genuine a further plot was constructed as recommended by Klotz (1985) and this plot is shown in figure 3.13.

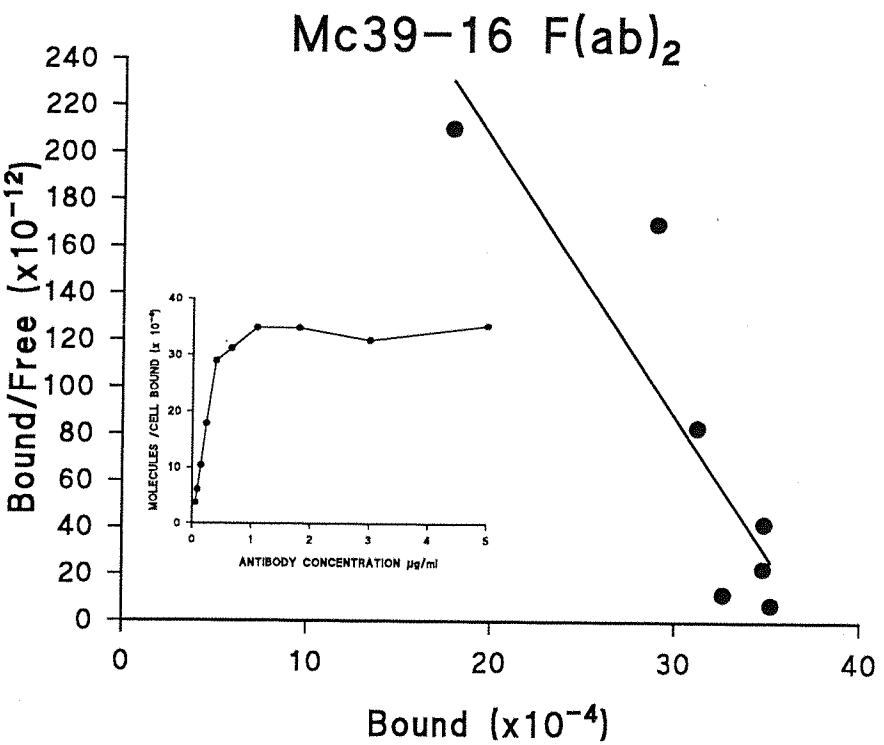


Figure 3.12. Saturation binding curve (inset) and Scatchard plot for Mc39-16 $F(ab)_2$ binding to A31 cells at 37°C.

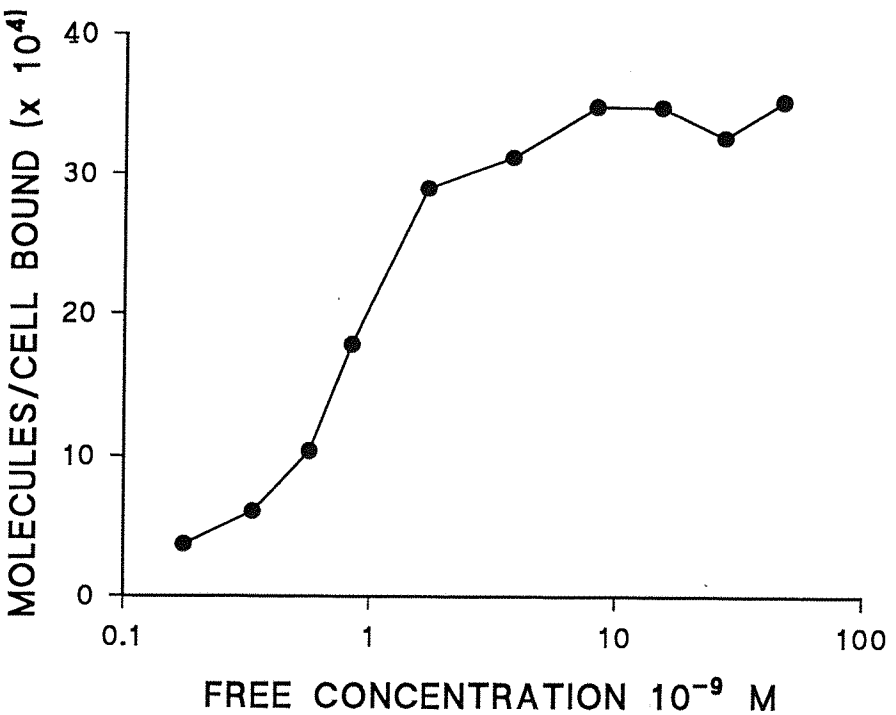


Figure 3.13. Binding of Mc39-16 to A31 cells at 37°C plotted on a semi-logarithmic graph. Asymptotic plateau suggests the saturation point has been approached.

By plotting the number of molecules bound as a function of the free ligand concentration as suggested by Klotz it is apparent that the curve plotted does approach an asymptotic plateau (figure 3.13.) suggesting that the saturation observed was genuine.

3.11. IMMUNOHISTOLOGY OF A31 TUMOUR

Two of the antibodies obtained from the Mc39 fusion were used, together with the anti-CD3 monoclonal antibody KT3 (Kind gift from K.Tomonari, MRC Clinical Research Centre, Middlesex, U.K.) to study the progression of the A31 tumour *in situ*. The availability of the anti-idiotypic monoclonal antibody made it possible to detect individual A31 tumour cells even when surrounded by a 'sea' of normal cells.

Preliminary investigation suggested that the usual tumour cell inoculation that was used for therapy studies (5×10^5 cells/mouse) was too low for detection of tumour cells within

the first two days. Therefore for tissue sections at 1 hour and 24 hours mice were used that had received 1 x 10^8 tumour cells by i.p. injection. Tissue sections taken beyond 24 hours were from mice that had received 5 x 10^5 tumour cells injected by the same route. Immunohistological slides were prepared from the various tissues as described in section 2.21.

3.11.1. RESULTS

The progressive growth of the A31 tumour within the host animal is depicted by figures 3.14.-3.20. Staining with the control antibody Mc10-6A5 was negative in all cases and is not shown.

Normal hepatic and splenic architecture was observed one hour post tumour inoculation with no evidence of A31 cells at these sites. Additionally tumour cells could not be observed within lung sections at this time (Figures 14-15). A31 tumour was first detected within the spleen of animals twenty-four hours post tumour inoculation (figure 3.16(b).). These tumour cells appeared to reside mainly within an area at the outer edge of the peripheral white pulp possibly representitive of marginal zone sinuses. A few tumour cells could also be observed located further in towards B-cell areas of the lymphatic nodules. By day four the peripheral distribution of the tumour cells was no longer evident as they appeared to occupy B-cell areas within the lymphatic sheaths (figure 3.17(c).). Large swaths of these predominatly B-cell areas became invaded by day ten post tumour inoculation although the T-cell areas within the spleen remained relatively spared by all but a few tumour cells (figures 3.18(a) & (b).). Some evidence of tumour cells within the red pulp was also evident at this time. Despite the large numbers of A31 cells residing in the spleen, there was no detectable tumour within the liver (not shown). This was in contrast to the picture five days later were the splenic archtecture was severly disrupted with invasion of white and red pulp areas (figure 3.19(b).). Diffuse areas staining with KT3 could still be discerned around central arterioles indicating that limited T cell zones still existed (figure 3.19(a).). Immunohistology at day fifteen also clearly showed that there was now significant

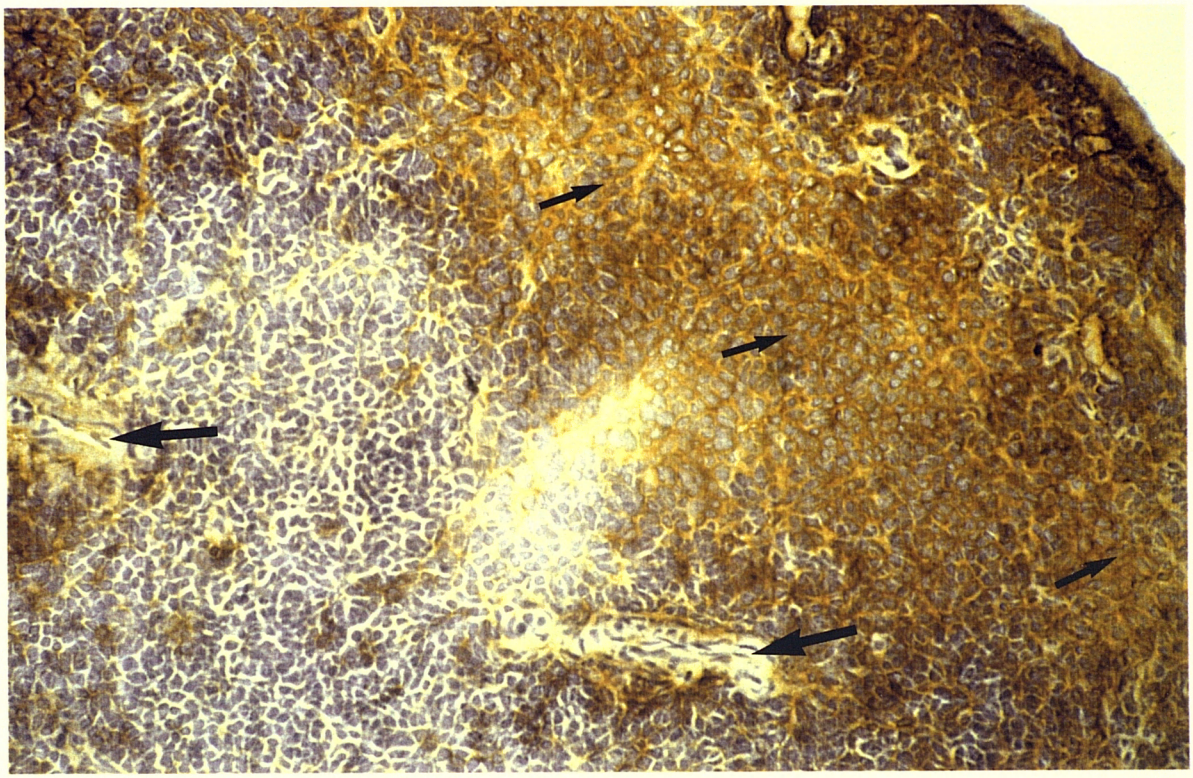
tumour load within the liver of these animals (figure 20.). From day fifteen onwards until the animals succumb the spleen continued to enlarge and the liver became progressively more involved with tumour.

3.12. DISCUSSION

The inability to obtain usable levels of A31 IgM from "rescue" hybridomas when grown in vivo, even when immunosurpressive regimes were used, was disappointing and greatly complicated this project. It should be stated that since these fusion were carried out a further rescue fusion has resulted in a hybridoma line that does produce stable A31 IgM when grown *in vivo* within pristane-primed mice, however the yields are very low and difficulty has been encountered during purification of the IgM (Dyke et al., 1991; T.Long personal communication). It is not clear why this last fusion should have been more successful. Although the three rescue fusions utilised different plasmacytoma partners it seems unlikely that this should influence the outcome since all three, NS-1, 653, and NS0, are known to give stable, productive hybridomas. There were also difficulties encountered in attempts to isolate syngeneic anti-idiotypic monoclonal antibodies after immunisation with sheep anti-murine IgM-A31 IgM complexes or A31 IgM-KLH complexes. The analysis of the serum from the immunised mice showed that there had been an immune response to the immunoglobulin however this did not translate into hybridomas secreting anti-idiotypic monoclonal antibodies. These early attempts at production of anti-idiotypes may have suffered from lack of immunising IgM as this had to be captured from culture supernatant, however the later fusion utilised purified A31 IgM linked to KLH and the mouse received an initial injection of 62 µg followed by a boost of 100 µg before the final i.v. injection and fusion. Despite this no anti-idiotypic monoclonal antibodies were isolated. The feasibility of raising syngeneic anti-idiotypes to murine immunoglobulins has been documented: Maloney and colleagues isolated such antibodies against the idiotype carried by the 38C13 murine lymphoma by immunisation with the idiotypic IgM linked to KLH (Maloney et al., 1985; Kaminski et al., 1986) as did Damanet

Figure 3.14(a). Spleen 1 hr post tumour inoculation stained with Mc39-12 (anti-μ). Brown staining indicates B cell domains (small arrows). The splenic capsule is visible in the top right corner and the central arterioles are idicated by large arrows. Magnification approximately x288.

Figure 3.14(b). Spleen 1 hr post tumour inoculation stained with KT3 (anti-CD3). Same lymphatic nodule shown in figure 3.14(a). Brown staining indicates T cell domains (small arrows). The splenic capsule is visible in the top right corner and the central arterioles are idicated by large arrows. Magnification approximately x288.



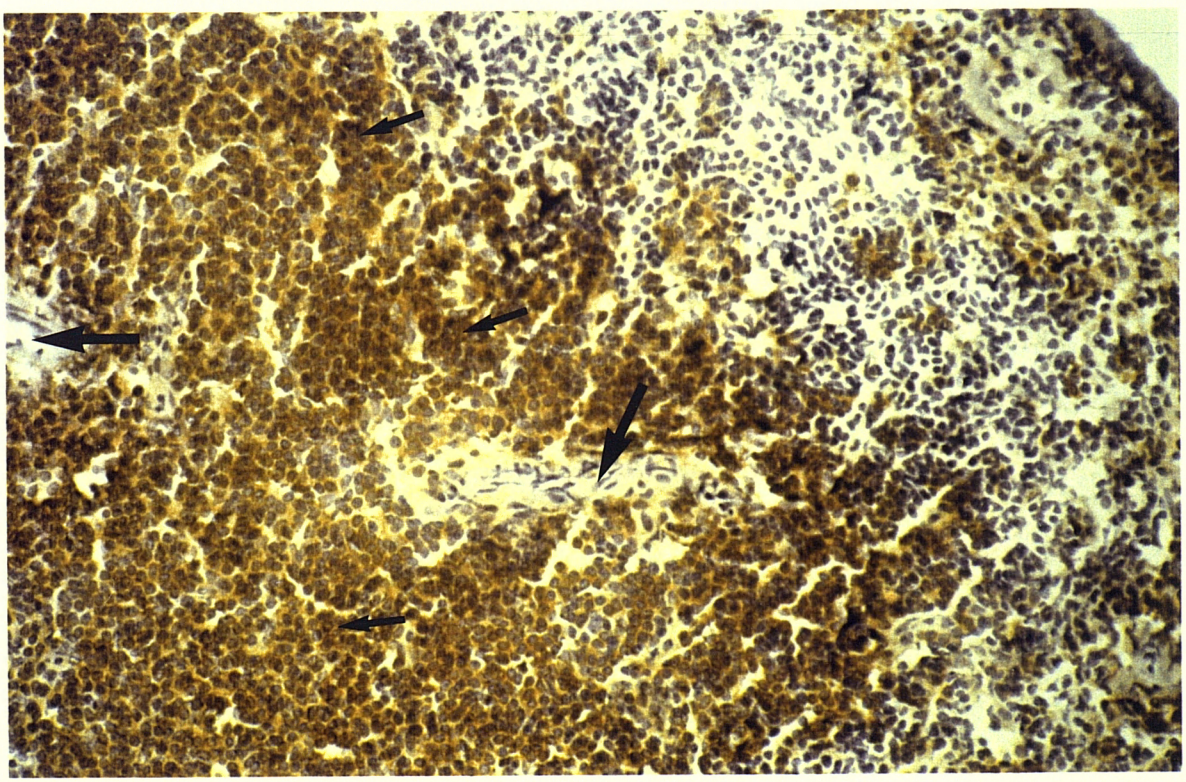


Figure 3.14(c). Spleen 1 hr post tumour inoculation stained with Mc39-16 (anti-Id). Same lymphatic nodule shown in figures 3.14(a) and 3.14(b). No visible staining to indicate the presence of A31 tumour. The splenic capsule is visible in the top right corner and the central arterioles are idicated by large arrows. Magnification approximately x288.

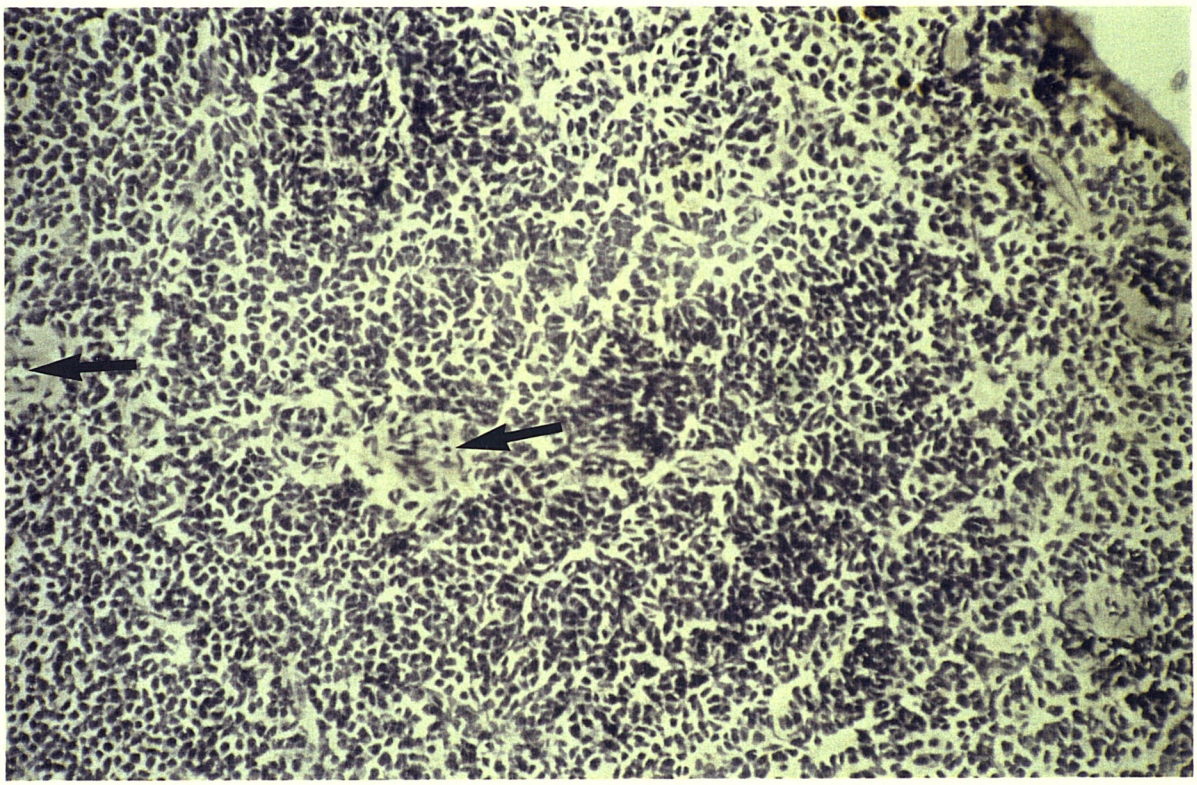
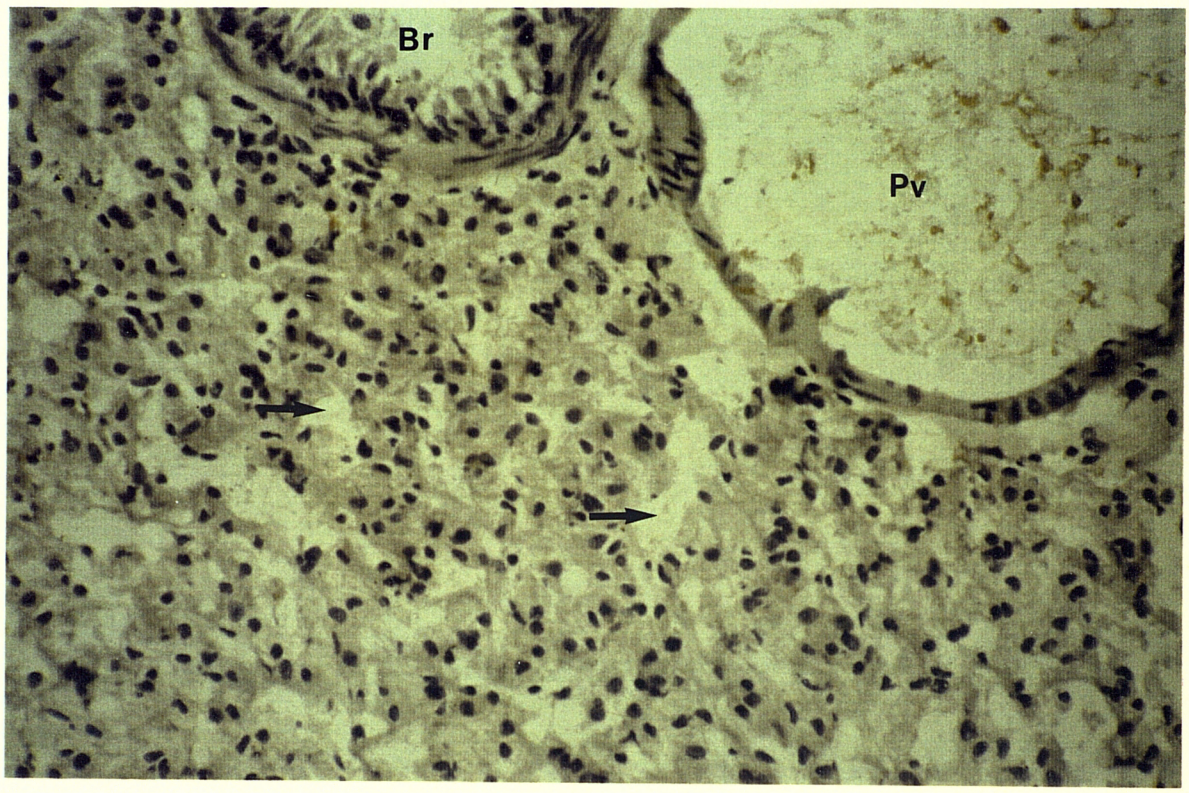


Figure 3.15(a). Lung 1 hr post tumour inoculation stained with Mc39-16 (anti-Id). No visible brown staining to indicate presence of A31 tumour. A pulmonary venule (Pv) and bronchiolus (Br) are visible within the section. Alveolar sacs can also be seen (small arrows). Magnification approximately x360.

Figure 3.15(b). Liver 1 hr post tumour inoculation stained with Mc39-16 (anti-Id). No visible staining to indicate presence of A31 tumour. Portal area is marked (large arrows) as are paraenchymal cells (small arrows). Magnification approximately x360.


Br
Pv

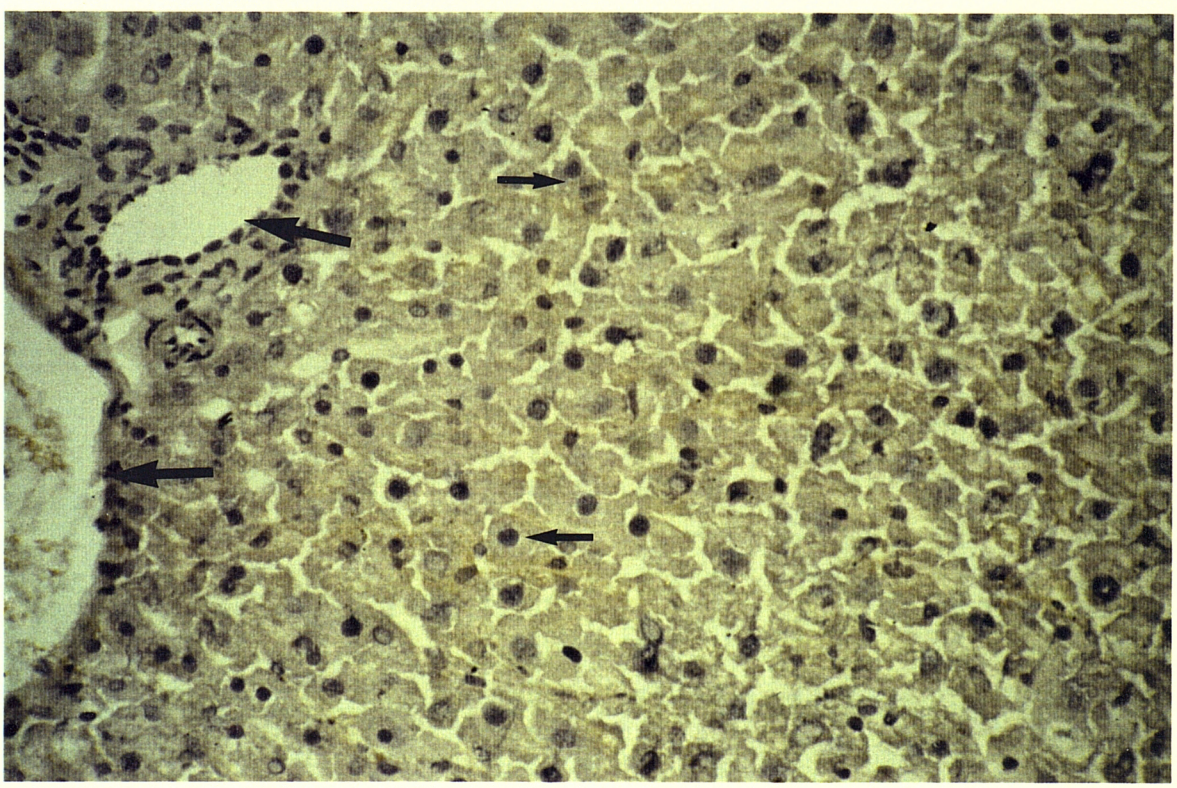
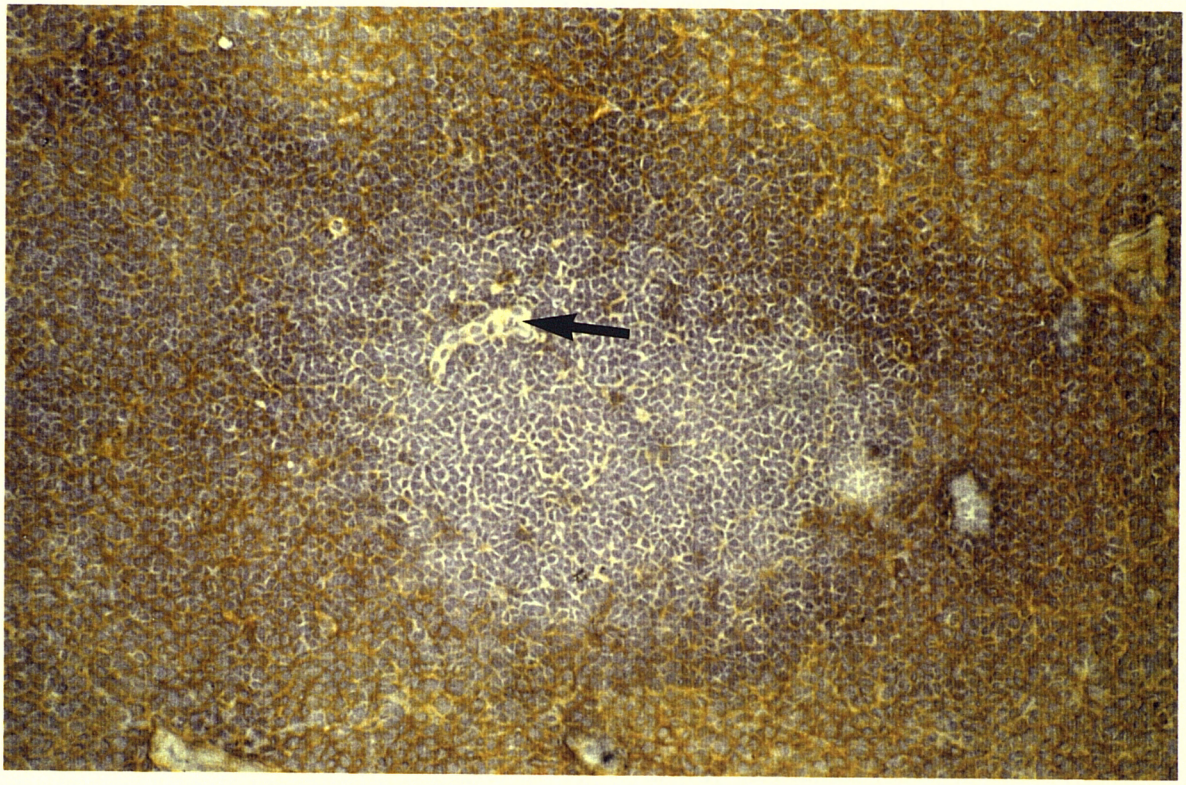


Figure 3.16(a). Spleen 24 hr post tumour inoculation stained with Mc39-12 (anti-μ). Brown staining indicates B cell domains. Central arteriole is marked by small arrow. Magnification approximately x288.

Figure 3.16(b). Spleen 24 hr post tumour inoculation stained with Mc39-16 (anti-Id). Same lymphatic nodule as shown in figure 3.16(a). A31 tumour cells are marked (small arrows). The distribution of tumour is mainly to the periphery of the lymphatic nodule close to the marginal zone sinuses. Central arteriole is marked by large arrow. Magnification approximately x288.

Figure 3.17(a). Spleen 4 days post tumour inoculation stained with Mc39-12 (anti-μ). Brown staining indicates B cell domains. Central arteriole is marked by large arrow. Magnification approximately x226.

Figure 3.17(b). Spleen 4 days post tumour inoculation stained with KT3 (anti-CD3). Same lymphatic nodule as shown in figure 3.17(a). Brown staining indicates T cell domains. The central arteriole is marked by large arrow. Magnification approximately x226.



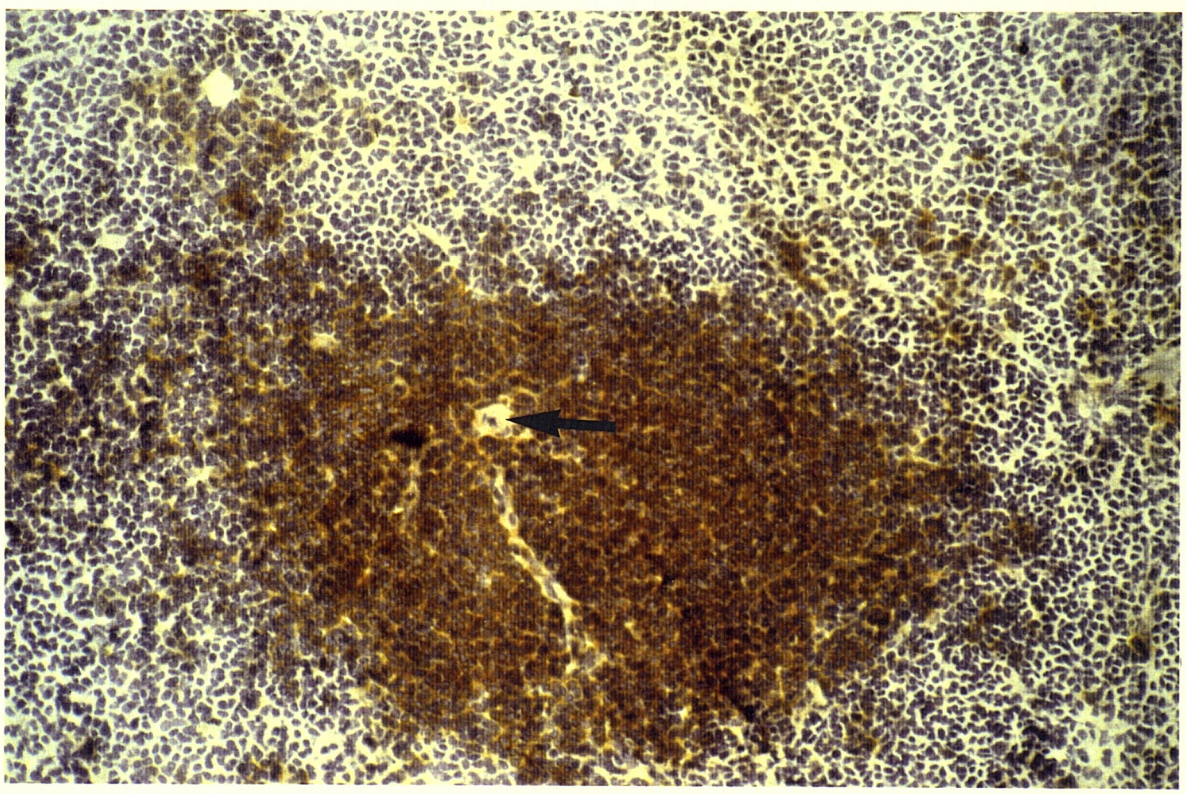


Figure 3.17(c). Spleen 4 days post tumour inoculation stained with Mc39-16 (anti-Id). Same lymphatic nodule as shown in figure 3.17(a) and 3.17(b). A31 tumour is marked by small arrows. Central arteriole is marked by large arrow. Magnification approximately x226.

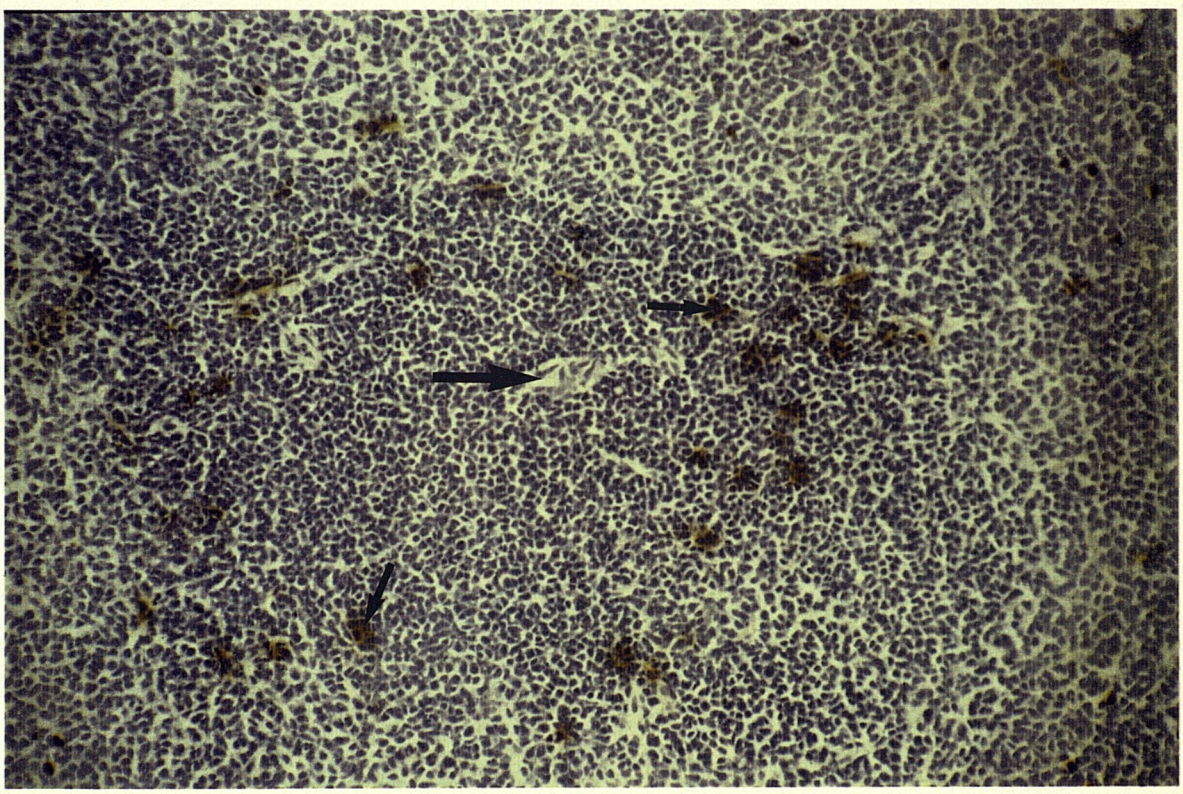
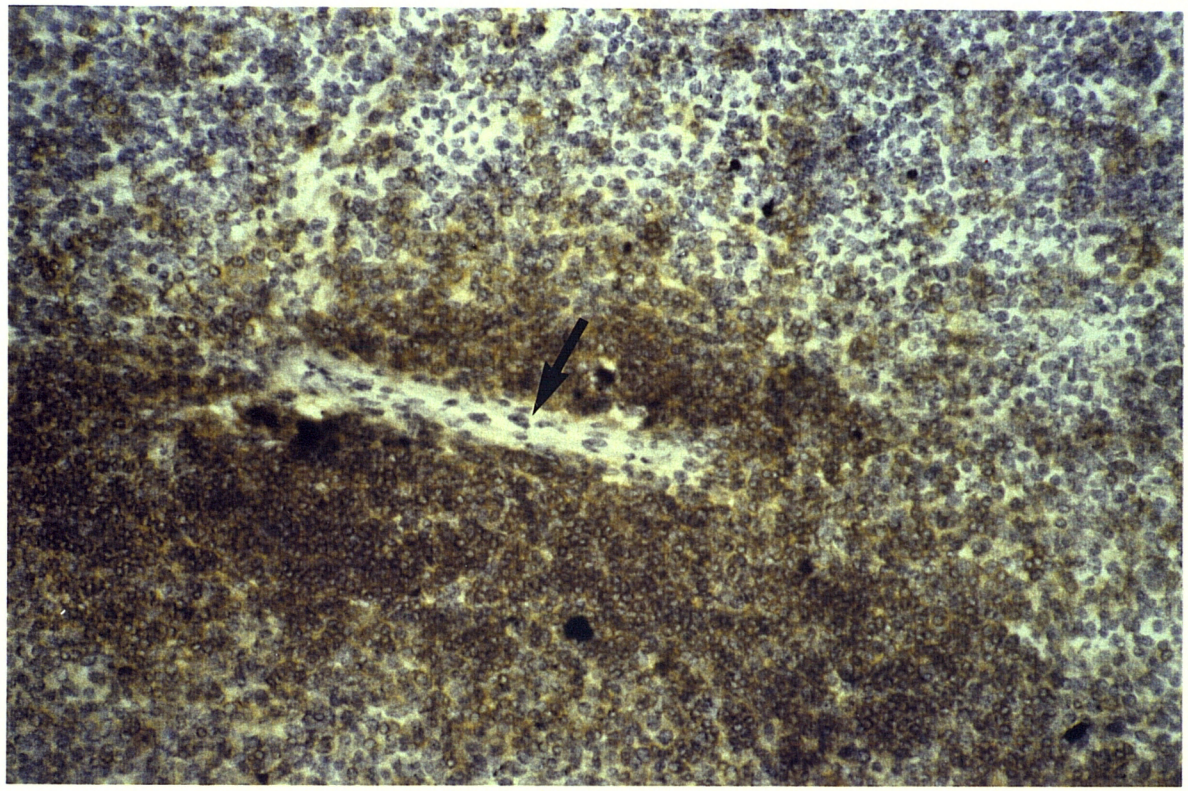


Figure 3.18(a). Spleen 10 days post tumour inoculation stained with KT3 (anti-CD3). Brown staining indicates T cell domains. Splenic capsule is marked by small arrows and central arteriole by large arrow. Magnification approximately x115.

Figure 3.18(b). Spleen 10 days post tumour inoculation stained with Mc39-16 (anti-Id). Same lymphatic nodule as shown in figure 3.18(a). Brown staining gives location of A31 tumour cells. although still mainly confined to B cell domains there is significant tumour within T cell areas and the red pulp. Splenic capsule is marked by small arrows and the central arteriole by large arrow. Magnification approximately x115.

Figure 3.19(a). Spleen 15 days post tumour inoculation stained with KT3 (anti-CD3). Brown staining indicates T cell domains. The central arteriole has been cut transversly (large arrow). Magnification approximately x288.

Figure 3.19(b). Spleen 15 days post tumour inoculation stained with Mc39-16 (anti-Id). Same lymphatic nodule as shown in figure 3.19(a). Brown staining indicates location of A31 tumour cells. The tumour has infiltrated most of the spleen although limited T cell areas remain. Central arteriole is marked by large arrow. Magnification approximately x288.



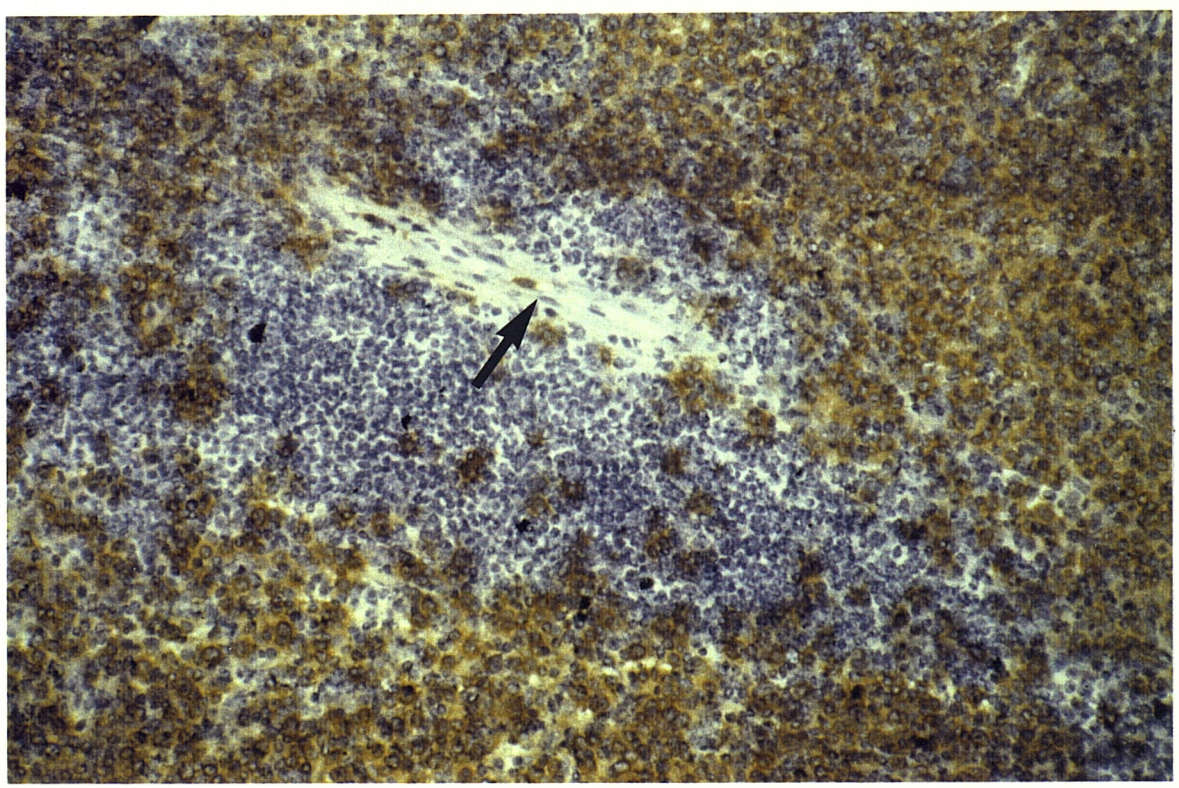
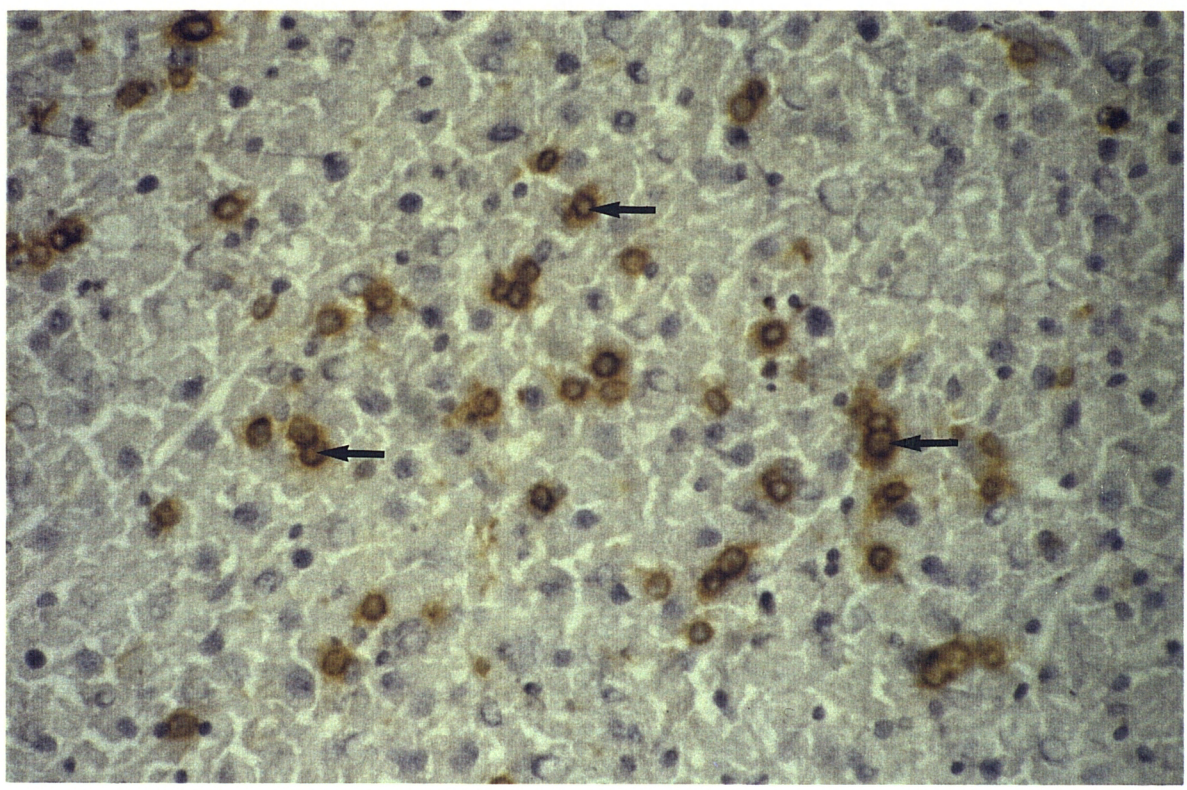


Figure 3.20. Liver stained 15 days post tumour inoculation stained with Mc39-16 (anti-Id). A31 tumour cells are marked by small arrows. Magnification approximately x360.



and colleagues (Damanet et al., 1991) and similarly antibodies have been described with reactivity against the idiotype carried by MOPC-460 cells (Sanchez et al., 1982). Syngeneic anti-idiotypic monoclonals have also been isolated after immunising with antibodies against CEA (Monestier et al., 1989) and various hapten and protein antigens (Brown and Sealy., 1986; Zanetti et al., 1986; Tanaka et al., 1986). It is not clear why anti-idiotypic antibodies could not be raised against A31 IgM. We have recently had good success with intra-splenic immunisation and this technique may increase our chance of success in the future.

In contrast to the attempts at production of syngeneic anti-idiotypic antibodies the immunisation and subsequent fusion using a Lou rat produced a number of anti-idiotypic monoclonal antibodies. Determination of the isotype of these antibodies showed them all to be IgG2a, a not unexpected result as immunisation of rats with antigen in CFA preferentially induces this isotype (Mota, 1986). Further analysis of these antibodies indicated that Mc39-28 was slightly blocked in its binding to A31 cells by normal mouse serum suggesting that it recognised a restricted idiotype, while the chromium release assay clearly showed that Mc39-16 gave the greatest cell lysis in the presence of rat complement. As there would be great difficulty in production of large quantities of these monoclonal antibodies for further studies it was decided at this stage to concentrate on Mc39-16.

The association constant ("functional affinity") for Mc39-16 binding to A31 cells was determined to be 3.8×10^8 M^{-1} a value within the mid-range of affinities described for a panel of murine monoclonal antibodies with specificity for the idiotopes carried by the L_2C guinea pig leukemia (Elliott et al., 1987). The intercept on the x-axis of the Scatchard plot gives an average value of 3.8×10^5 IgM molecules on the surface of each A31 cell, a value similar to that described for the L_2C leukemia (Elliott et al., 1987) and BCL_1 lymphoma (Stevenson et al., 1984) and the first formal demonstration of this characteristic of A31 cells. With regard to the secretion of A31 IgM within the host animal determination of idiotypic IgM levels using Mc39-16 confirmed earlier work

(Cobb et al., 1986) on the low level of IgM production by this tumour with serum levels below 10 µg/ml for the first fourteen days post tumour inoculation followed by a rapid rise in levels to a peak not exceeding 68 µg/ml. This terminal idiotypic IgM serum level is in contrast to that of the BCL_1 tumour were levels as high as 1-2 mg/ml have been reported (Tutt et al., 1985).

Purely visual observations of A31 tumour growth within the host gave a classic picture of murine B-cell lymphoma with a propensity for early colonisation of splenic tissue followed only later by growth at other sites, predominantly the liver with enlarged lymph nodes being discernible in very late stage disease only. Immunohistological examination of lung sections 1 hour and 24 hours post i.p. injection of tumour cells failed to detect A31 cells indicating that the vast majority of the injected cells traverse the pulmonary capillary beds very quickly and do not become trapped. This result is in contrast to that found with the BCL_1 tumour where large numbers of tumour cells were located within the pulmonary interstitium five minutes post i.v. injection and moderate numbers were seen at 18 hours post injection (Warnke et al., 1979). This dissimilarity may reflect differences in experimental procedures as the route of administration of the two tumours was different and in the case of the BCL_1 tumour the cells were radiolabelled and detected by autoradiography. The latter procedure may have increased the sensitivity of detection or the *in vitro* manipulations during the labelling may have altered the characteristics of the tumour cells.

Although A31 tumour cells were not detected within the liver 24 hours post tumour inoculation this was in contrast to the spleen were they were clearly visible using the anti-idiotypic antibody Mc39-16. Tumour cells were located close to the marginal zone sinuses or within the outer edge of the B cell domains with very few cells located within periarteriolar lymphatic sheaths, T-cell areas. By four days post tumour inoculation the marginal zone pattern was no longer discernible and the tumour was spread more randomly within the B-cell domains of the follicles. Over the next six days there was progressive growth of the tumour but this growth still resided mainly within the B-cell domains with a

few cells now appearing within the periarteriolar lymphatic sheaths and red pulp. Beyond ten days post tumour inoculation splenic architecture became progressively disrupted although T-cell areas could still be identified. It was during this period that significant tumour first appeared within the liver most probably due to spill over from the spleen and, coincidentally there was a rapid rise in serum idiotype-bearing IgM levels. Beyond fifteen days splenic tissue was virtually replaced by tumour and there was progressive growth within the liver until the animals succumbed. The pattern described here closely resembles that described for the BCL_1 tumour (Strober et al., 1979; Krolick et al., 1979) and although not conclusive suggests the A31 tumour cells do not tend to recirculate but home to the spleen and only when the tumour load becomes extensive within this organ does further invasion of other tissues occur.

CHAPTER 4

Mc39-16 IgG AS A THERAPEUTIC AGENT

4.1. INTRODUCTION

One of the main aims of this study into the A31 tumour as an animal model of lymphoma was to assess the therapeutic potential of an anti-idiotypic monoclonal antibody in the form of an immunotoxin or as the target-specific arm of chemically constructed bispecific antibodies. It was therefore important to establish the effectiveness of the native anti-idiotypic monoclonal antibody when used directly to treat the A31 tumour thereby setting the therapeutic standard to be reached, and hopefully, surpassed. A series of experiments to characterise the ability of Mc39-16 IgG to kill A31 tumour cells *in vivo* were performed.

4.2. THERAPY 1

4.2.1. IMMUNOTHERAPY PROCEDURE.

The procedures for initiation of passive therapy of animals inoculated with the tumour have been described in 2.24.

4.2.2. RESULTS

The results from the first therapy using Mc39-16 are shown in figure 4.1. It is clear that the anti-idiotype monoclonal antibody produced a significant therapeutic effect. A median survival of these animals of 87 days compares favourably with the 72 days that had previously been reported using cyclophosphamide to destroy A31 tumour (Cobb et al., 1986). Administering multiple doses of Mc39-16 to mice injected with A31 cells (figure 4.2.) gave a median survival of 88 days suggesting that the therapeutic limit, under these particular experimental conditions, had been reached by the single administration of antibody.

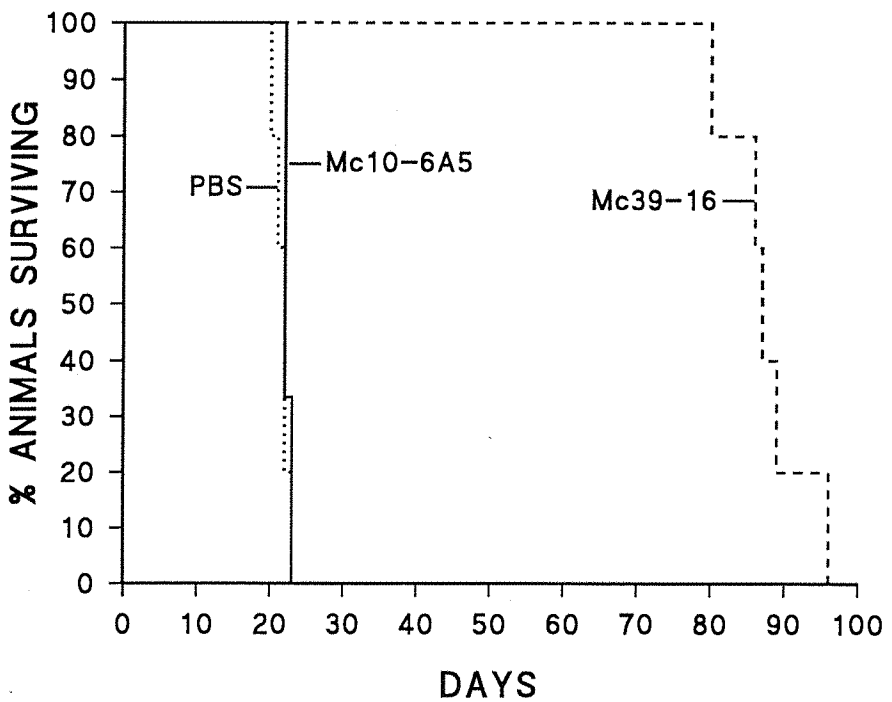


Figure 4.1. Therapy of A31 tumour using a rat monoclonal antibody (Mc39-16) reacting with the A31 idiotype.
Each mouse received 5 x 10^5 A31 cells i.p. on day 0 followed by therapy 24 hours later.
Control (PBS) group contained three mice.
Control (Mc10-6A5; anti-Id of BCL_1) group contained five mice.
Therapy (Mc39-16) group contained five mice.

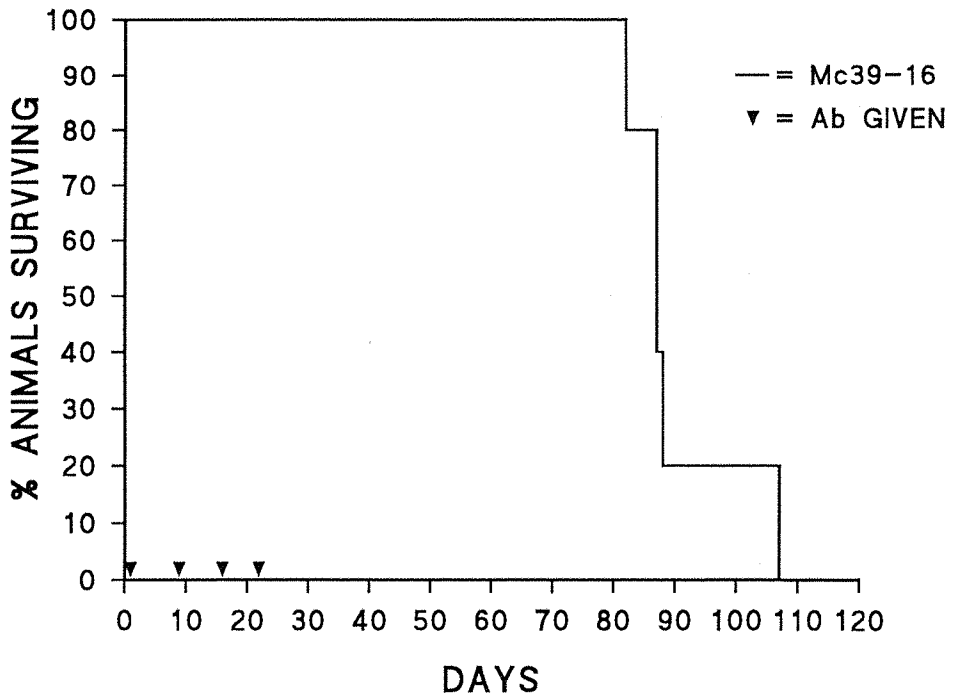


Figure 4.2. Effect of multiple doses of Mc39-16 on survival of mice with A31 tumour.
Each mouse received 5 x 10^5 A31 cells i.p. on day 0 followed by 100 µg of Mc39-16 i.p. at times indicated by arrows.
Therapy group (Mc39-16) contained five mice.

It was possible that the anti-idiotypic monoclonal antibody had exerted a selection pressure on the tumour population to cause the emergence of a sub-population with an altered surface phenotype (Badger and Berstein, 1983; Bridges et al., 1984). To test this hypothesis, tumour cells from the long term survivor (LTS) animals treated with a single dose of Mc39-16 were isolated on Ficoll-Isopaque and prepared for analysis by FACS (section 2.18). The surface phenotype of these cells shown in figures 4.3a. and 4.3b. show no detectable changes from the wild type tumour. The second mouse to die within the single-dose Mc39-16 treated group had, in addition to an enlarged spleen, evidence of ascities the cells from which were also isolated and examined (Fig 4.3a, row C). In all cases there was no change in the surface phenotype detectable with the

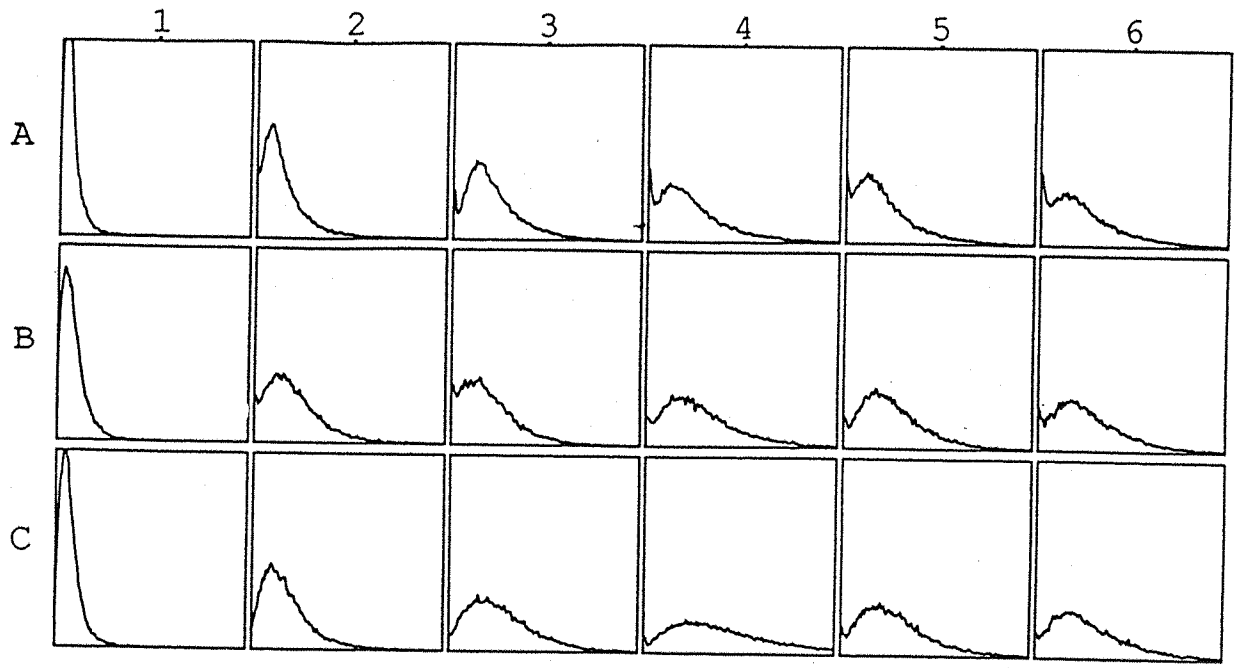


FIG 4.3a FACS analysis of second mouse to die in Mc39-16 treated group.
(A) A31 tumour cells from an untreated animal (B) Spleen cells from treated animal (C) Ascitic cells from treated animal (1) Stained with FITC conjugate only (2) Stained with HB58 (anti-kappa) (3) Stained with Mc39-12 (anti-μ) (4) Stained with Mc39-16 (5) Stained with Mc39-28 (6) Stained with Mc39-33.

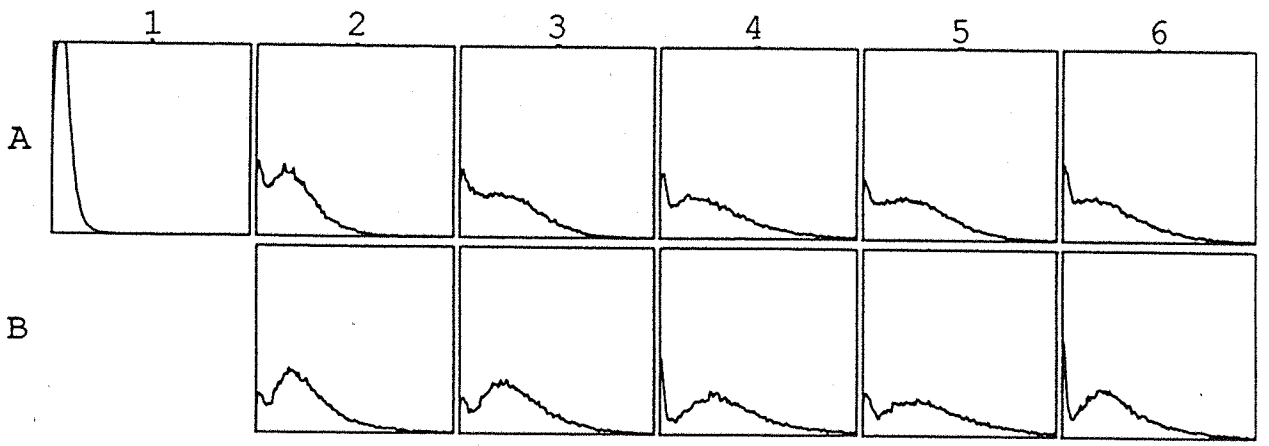


FIG 4.3b FACS analysis of last mouse to die in Mc39-16 treated group.
(A) A31 tumour cells from an untreated animal (B) Spleen cells from treated animal (1) Stained with FITC conjugate only (2) Stained with HB58 (Anti-kappa) (3) Stained with Mc39-12 (anti-μ) (4) Stained with Mc39-16 (5) Stained with Mc39-28 (6) Stained with Mc39-33.

available surface markers, in particular there was no reduction or loss of the idiotope recognised by Mc39-16. Examination of the ascitic fluid by agarose gel electrophoresis failed to reveal the presence of a monoclonal band.

4.3. IMMUNOTHERAPY OF LTS CELLS FROM THERAPY 1

4.3.1. INTRODUCTION

The failure to eradicate all tumour cells with Mc39-16 could be due to a number of possibilities including; (1) insufficent antibody and/or effector mechanisms to destroy all the available A31 cells *in vivo* (2) sequestering of tumour cells to privileged sites within the animal (3) escape from therapy by variants not detectable by the available antibodies or (4) short-term modulation of surface idiotype while anti-idiotype persists. The lack of therapeutic antibody was unlikely as multiple doses of Mc39-16 did not result in an increase in survival. Possible disparity in the degree or speed of surface modulation in the presence of Mc39-16 were examined and found not to be significantly different between A31 or A31-LTS cells (data not shown). On the other hand if exhaustion of effector mechanisms or sequestering of tumour cells alone were responsible for the final failure in therapy it would be expected that repeating therapy 1 using the emerging tumour cells should produce a similar survival curve. Therefore in order to gain further insight a second therapy was initiated using the tumour cells isolated from the spleen of the final animal in the single-dose Mc39-16 group of therapy 1. Two groups of four mice were each injected directly into the peritoneal cavity with 5×10^5 cells. Twenty-four hours later each animal in one group received an i.p. injection of 100 µg of monoclonal antibody reactive to the idiotype of BCL_1. The animals within the remaining group each received an i.p. injection of 100 µg of Mc39-16.

4.3.2. RESULTS

The results of this second therapy are shown in figure 4.4. It should be noted that one animal receiving an injection of Mc39-16 immediately leaked some antibody solution from

the injection site which probably accounts for the early death (28 days post tumour inoculation) of mouse 1 in this group.

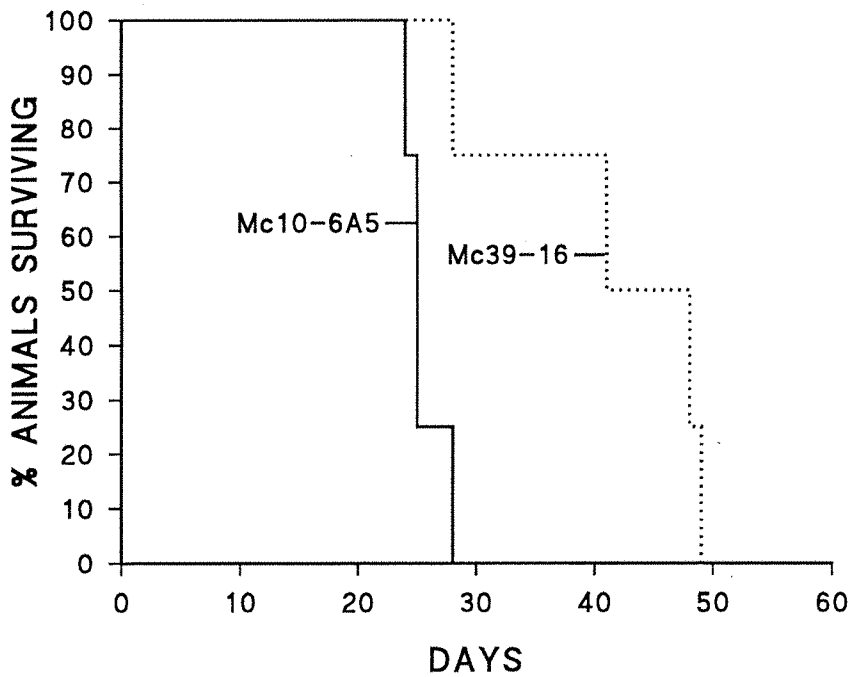


Figure 4.4. Effect of a single 100 µg dose of Mc39-16 on survival of mice injected with A31-LTS cells.
Each mouse received 5 x 10^5 A31-LTS cells i.p. on day 0 followed by therapy 24 hours later.
Control (Mc10-6A5) group contained four mice.
Therapy (Mc39-16) group contained four mice.

Animals within the control group treated with Mc10-6A5, anti-BCL_1 idiotype, succumbed to tumour between 25-28 days post tumour inoculation whereas those animals treated with the anti-A31 idiotypic monoclonal antibody Mc39-16 succumbed between 41-49 days post tumour inoculation, ignoring the early death mentioned above. As in therapy 1 the emerging cells from animals 3 and 4 were examined by FACS the results of which are shown in figure 4.5. The emerging cells from this second therapy did not show any decrease in surface expression of IgM or idiotype. It was clear that this common mechanism of escape from anti-idiotypic therapy had not played a role in the emergence of

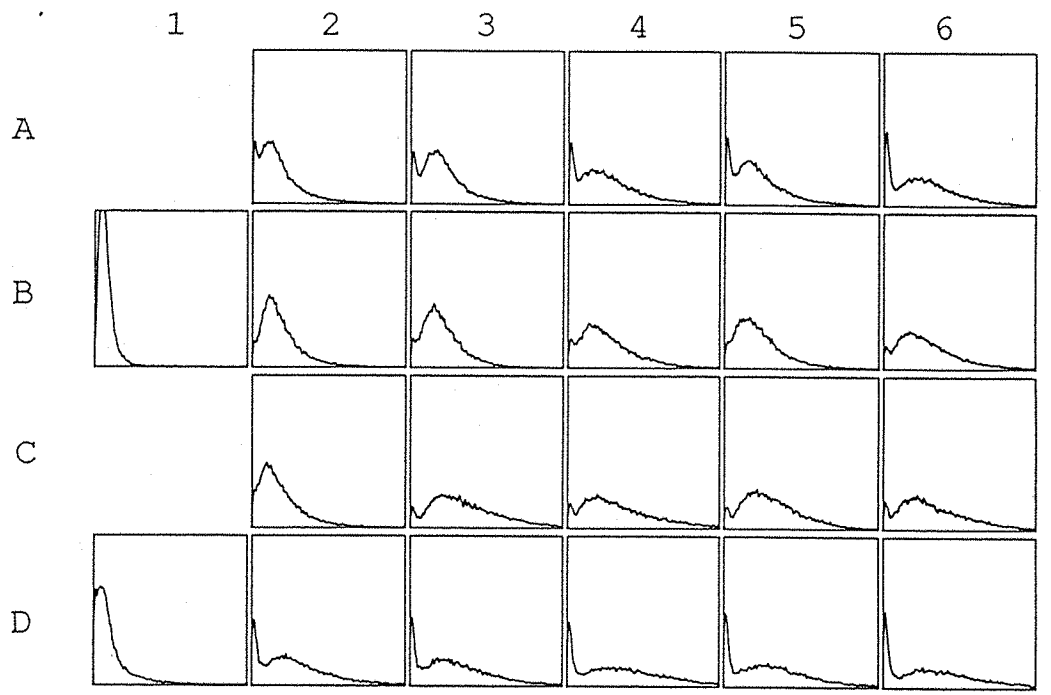


Figure 4.5. FACS analysis of tumour cells from mice 3 and 4 from Mc39-16 therapy 2.
(A) A31 tumour cells from an untreated animal; (B) Spleen cells from mouse 3; (C) Spleen cells from mouse 4; (D) Peripheral blood lymphocyte's (PBLs) from mouse 4.
(1) stained with FITC conjugate only; (2) Stained with HB58 (anti-kappa); (3) Stained with Mc39-12 (anti-μ); (4) Stained with Mc39-16; (5) Stained with Mc39-28; (6) Stained with Mc39-33.
Note: Cells from mice 3 and 4 were processed and examined on separate days.

A31-LTS cells.

It was also apparent that some change had occurred within the A31-LTS tumour cells that, while not reflected by a reduction in the presence or level of idiotope recognised by antibody Mc39-16 (fig 4.5.), had resulted in partial resistance of emerging tumour cells compared to the wild type tumour.

4.4. ^{51}Cr-RELEASE ASSAY WITH A31 AND A31-LTS CELLS.

4.4.1. INTRODUCTION

In an attempt to identify a difference between the wild type A31 cells and the A31-LTS cells, they were compared in a complement mediated lysis assay.

In this assay the ability of the anti-idiotypic monoclonal antibody Mc39-16, the anti-μ monoclonal antibody Mc39-12, and a polyclonal rabbit anti-mouse $F(ab')_2$ IgG to kill either A31 or A31-LTS cells in the presence of rat complement was examined.

4.4.2. RESULTS

The assay was carried out as described in section 2.20. using concentrated culture supernatants of Mc39-12 and Mc39-16 and 100 μg/ml of the rabbit anti-mouse $F(ab)_2$ IgG. The negative control antibody was Mc10-6A5 and the complement source was a 1 : 2 dilution of rat serum in DMEM.

The results of this assay are shown in figure 4.6a. and 4.6b. Although there was some variation in the maximum percentage release in chromium between the A31 and A31-LTS cells observed with Mc39-16, 45% compared with 39% respectively, this small difference is unlikely to account for the observed partial resistance of A31-LTS to *in vivo* therapy. The protection to lysis exhibited at high concentrations of Mc39-12 and Mc39-16 by both cell populations is almost certainly due to protection against isotonic shock endowed by the concentrated, hypertonic culture supernatants that had to be used due to the lack of purified antibodies.

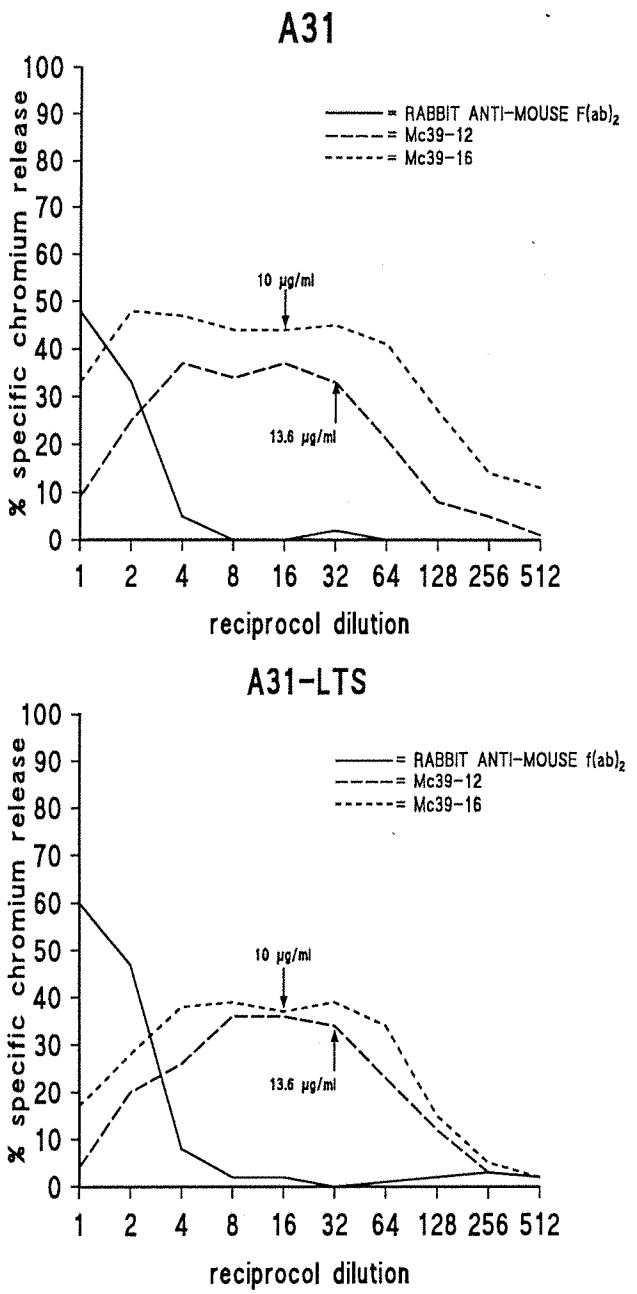


Figure 4.6. Chromium release assay for A31 and A31-LTS cells. ^{51}Cr-labelled cells were coated with appropriate antibodies at 4°C before exposure to rat serum at 37°C for 30 minutes. The degree of cell lysis was determined by the measurement of specific release of ^{51}Cr into the culture supernatant.

4.5. S-PHASE CELLS WITHIN A31 AND A31-LTS POPULATIONS

4.5.1. INTRODUCTION

Extensive attempts to produce a reliable ADCC assay using a range of target cell labels, including ^{51}Cr-release, MTT-reduction, ^{3}H-thymidine incorporation or PHK-26 membrane-label proved unproductive due to the poor uptake of reactants and/or low viability of tumour cells when maintained in culture. One curious, but consistant, observation during these attempts was the higher uptake of ^{51}Cr and ^{3}H-thymidine by the LTS cells compared with the wild type A31 cells. In view of this it was decided to examine the two populations in terms of their S-phase cells as determined by 5-Bromo-2-deoxyuridine (BrdUrd) incorporation.

4.5.2. RESULTS

The preparation and treatment of cells has been described in 2.25. Briefly cells were isolated from the spleens of animals in the terminal stage of the A31 or LTS tumours and incubated *in vitro* in the presence of 5-fluoro-2-deoxyuridine (FdUrd: to block endogenous thymidine incorportion) and 5-Bromo-2-deoxyuridine (BrdUrd: incorporates into DNA in place of thymidine). Cytopreps, (single cell suspension spun onto glass microscope slides), were prepared from the cells and BrdUrd detected with a mouse anti-BrdUrd monoclonal antibody (BU-1). Bound BU-1 was then reacted with a rhodamine-conjugated goat anti-mouse second antibody before examination under a fluorescent microscope. The results of this analysis are shown in table 4.1. with the typical staining pattern of BrdUrd-labelled cells being shown in figure 4.7.

TABLE 4.1. PERCENTAGE S-PHASE A31 AND LTS TUMOUR CELLS

SAMPLE	% CELLS IN S-PHASE	
	OBSERVER 1	OBSERVER 2
A31 SLIDE 1	20	32
A31 SLIDE 2	22	33
LTS SLIDE 1	39	53
LTS SLIDE 2	41	54

The above results do appear to show a higher S-phase within the A31-LTS population compared to the A31 wild type cells.

4.6. IMMUNOTHERAPY OF LTS CELLS FROM THERAPY 2.

4.6.1. INTRODUCTION

The reduction in the effectiveness of therapy 2 compared with therapy 1, although significant, was not complete in that there was still an approximately 20 day extension in survival of treated animals compared to control animals. A question that came to mind was whether this represented the gradual adaptive change of the tumour to the therapy or the selection of a variant from the original population. A number of further therapies were carried out in order to obtain further data to answer this question.

4.6.2. THERAPIES 3 and 4.

Tumour cells from animal 3 of therapy 2 were passaged on into four animals which were treated with Mc39-16 as before, with animals succumbing to the tumour between days 38-40. Comparative flow-cytometric analysis between these A31-LTS cells and wild-type A31 was carried out using antibodies reactive to IgM, the A31 idiotype, CD4, CD5, CD8, MHC class I and MHC class II surface antigens (figure 4.8.). If the cells with a fluorescent signal of $>10^1$ are

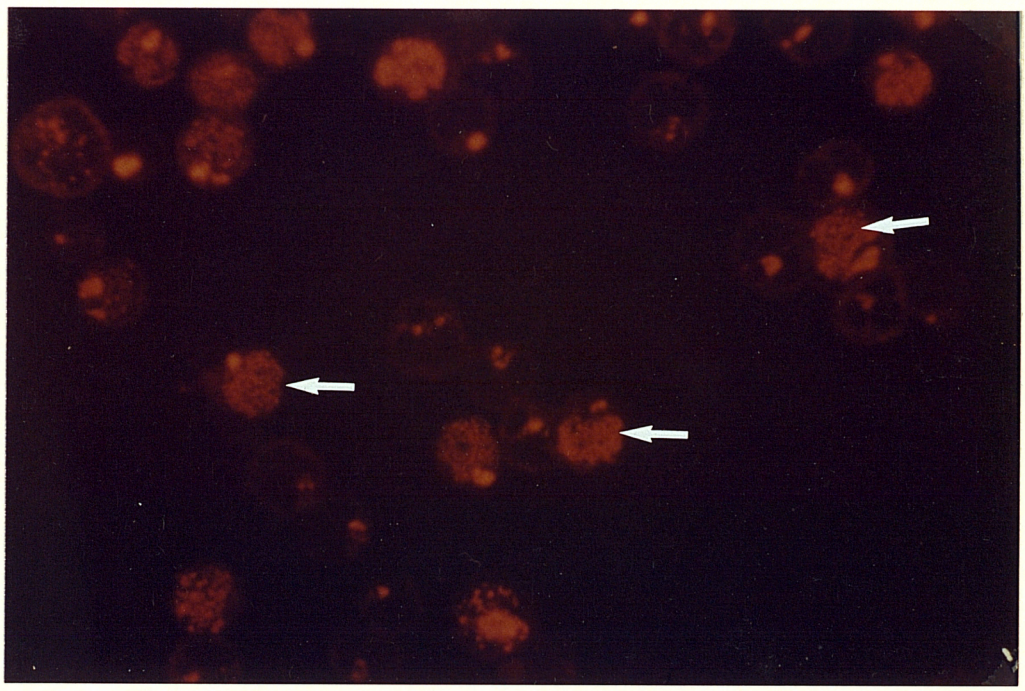
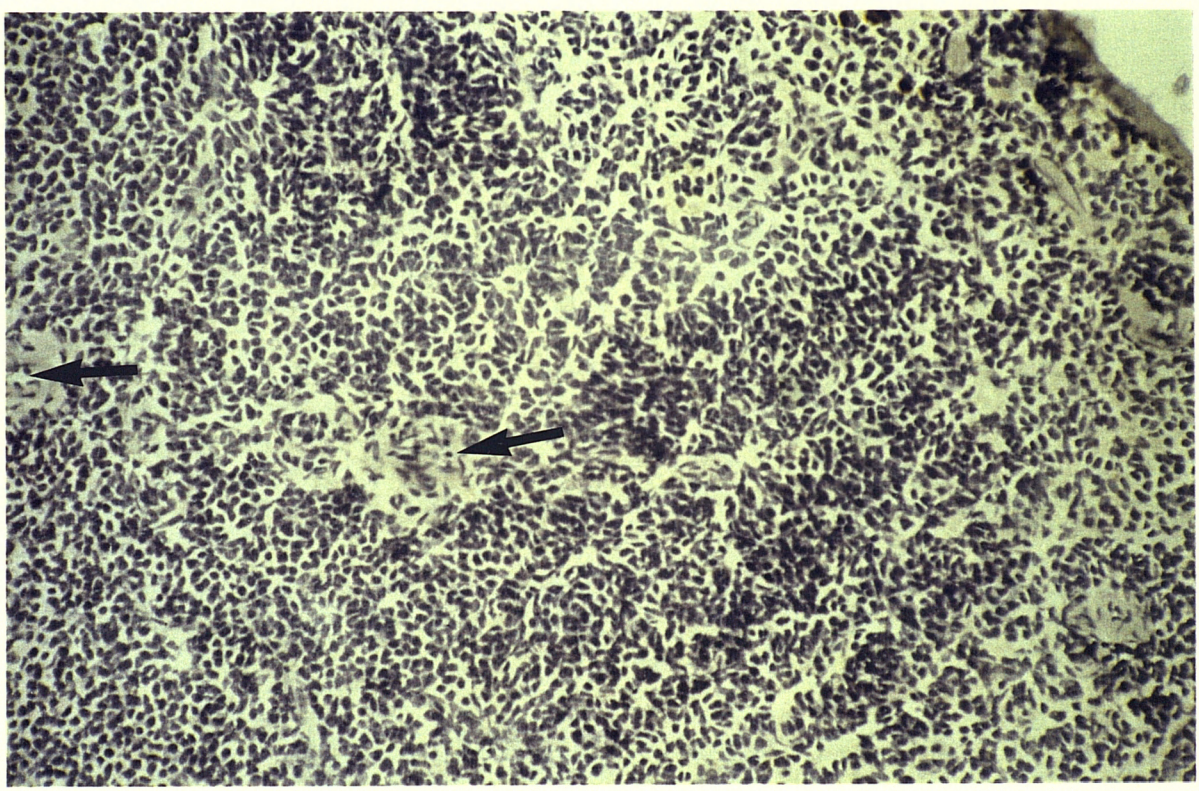


Figure 4.7. S-phase analysis of A31 cells.
Figure shows a single field photographed using a suitable filter for rhodamine excitation, showing the BrdUrd-positive nuclei (arrows) of cells in S-phase.
Magnification 500x.
Photograph kindly supplied by Dr. R Dyke.

Figure 3.14(c). Spleen 1 hr post tumour inoculation stained with Mc39-16 (anti-Id). Same lymphatic nodule shown in figures 3.14(a) and 3.14(b). No visible staining to indicate the presence of A31 tumour. The splenic capsule is visible in the top right corner and the central arterioles are idicated by large arrows. Magnification approximately x288.

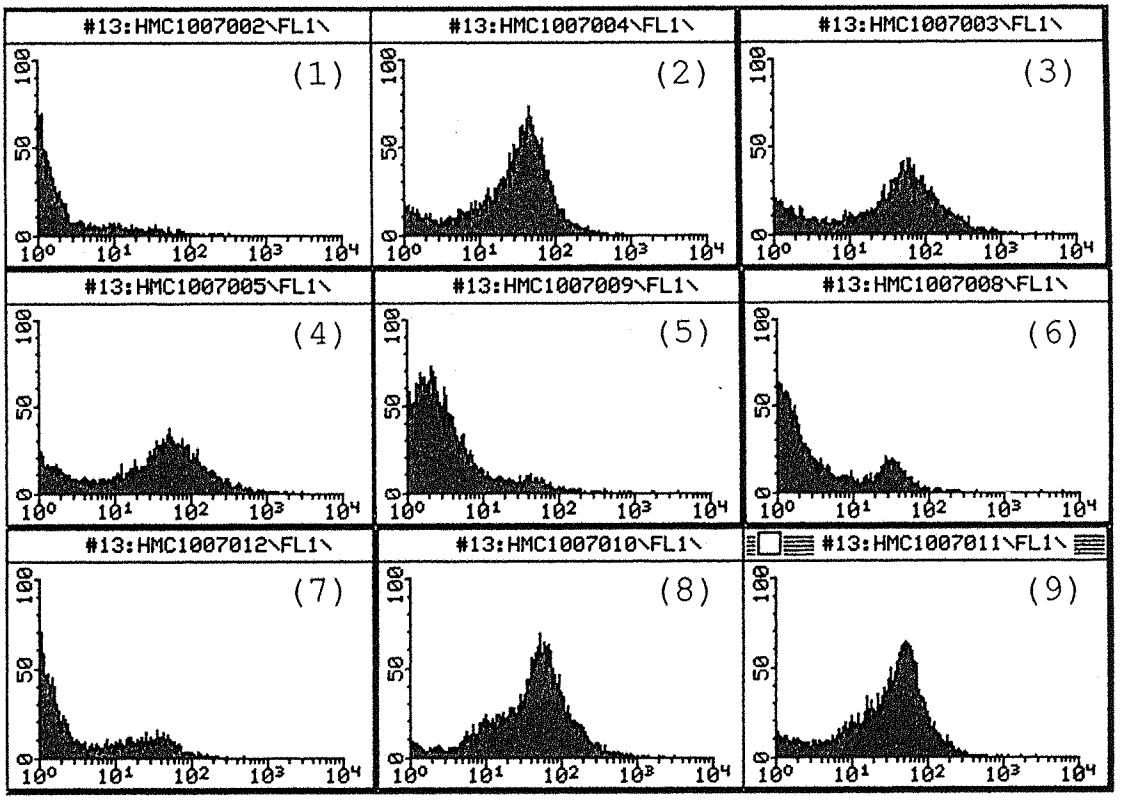


considered positive then the spleen from the A31-LTS animal contained more Mc39-16 positive cells than the A31 animal (48.6% compared to 39.3%) however using Mc39-33 as the idiotope marker the difference was not so great (35.5% compared to 37.5%). Although $CD8^+$ ($Lyt\text{-}2^+$) cells in the A31 and A31-LTS populations were similar (8.6% compared to 7.6%) there did appear to be more $CD4^+$ ($L_3T_4^+$) cells in the A31 population (11% versus 5.8%). A31 cells were clearly $CD5^-$ (Ly-1 marker) the slightly higher number of $CD5^+$ cells in the A31 compared to the A31-LTS populations probably being due to the greater number of $CD4^+$ cells present. Major histocompatability antigen (MHC) type 1 positive cells were comparable between the two populations (57.6% for A31 and 58.9% for A31-LTS) however greater numbers of MHC class II positive cells were present in the A31-LTS splenocyte population (64.1% versus 52.3%).

The tumour cells from the last animal to die in therapy 3 were passaged on with or without antibody treatment (therapy 4) and the results are depicted in figure 4.9. The FACS analysis of the cells was as seen in previous therapies (i.e. no change; data not shown). In addition to these cell passages the A31-LTS cells were passaged without therapy for four generations and then re-exposed to therapy with Mc39-16 (100 μg/mouse) upon the fifth passage. Under these conditions the A31-LTS tumour cells caused the animals to succumb to tumour with the same survival profile of tumour cells continually passaged in the presence of Mc39-16 (data not shown).

It was evident from therapies 3 and 4 that there was not a progressive increase in resistance to the anti-idiotypic treatment with each exposure. Lack of reversion to a wild type phenotype during passage of A31-LTS cells in the absence of anti-idiotype suggests that this partial resistance to therapy was a stable characteristic.

A



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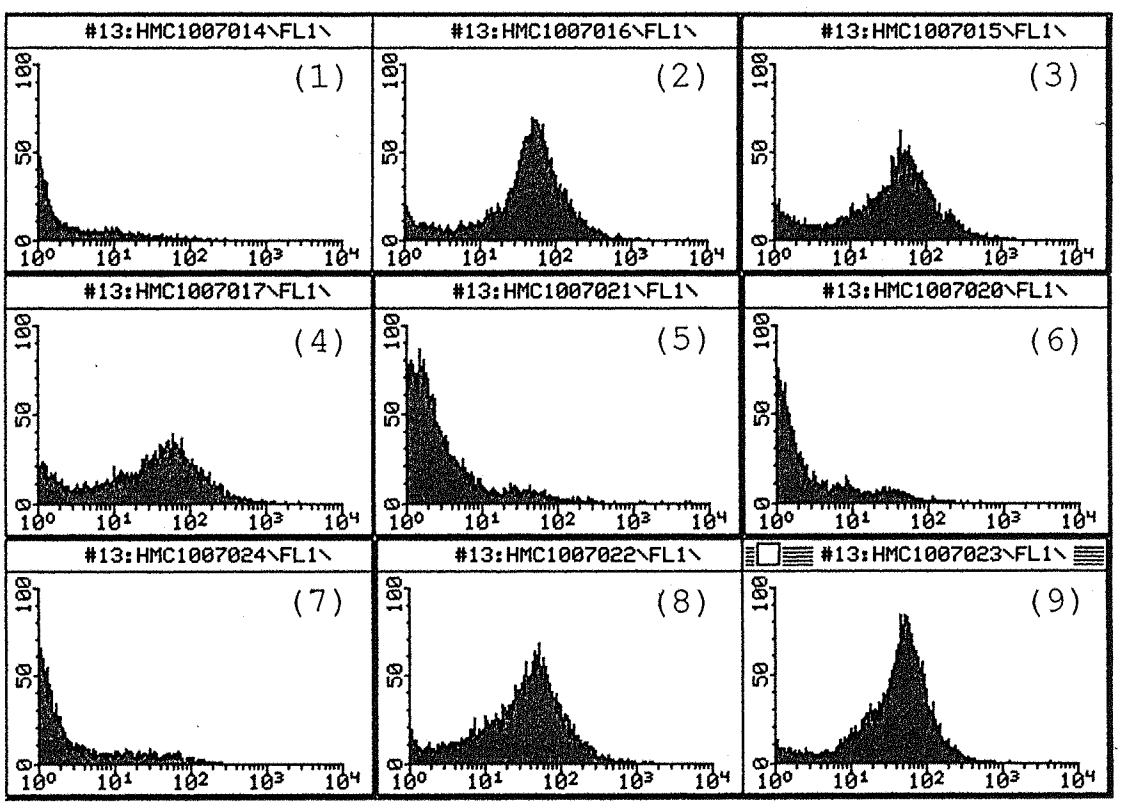


Figure 4.8. Comparative FACS profiles of A31 and A31-LTS cells.

A = A31; B= A31-LTS.

Antibodies used were: (1) Mc10-6A5 (anti-BCL_1 Id); (2) Mc39-12 (anti-μ); (3) Mc39-16 (anti-A31 Id); (4) Mc39-33 (anti-A31-Id); (5) Lyt-2 (anti-CD8); (6) L_3T_4 (anti-CD4); (7) Ly-1 (anti-CD5); (8) anti-MHC class I; (9) anti-MHC class II.

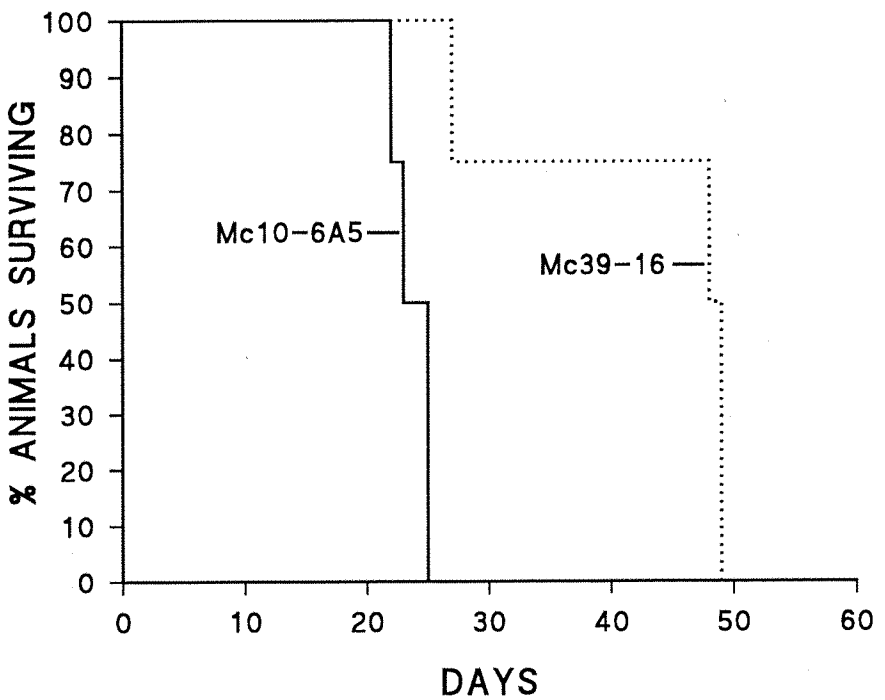


Figure 4.9. Effect of a single 100 µg dose of Mc39-16 on survival of mice injected with tumour isolated from therapy 3.

Each mouse received 5 x 10^5 A31 cells i.p. on day 0 followed by therapy 24 hours later.

Control (PBS) group contained four mice.

Therapy (Mc39-16) group contained four mice.

Note: The early death of animal one in the Mc39-16 treated group does not reflect previous or subsequent therapies and is probably due to experimental error.

4.7. TISSUE TRANSFER EXPERIMENT

4.7.1. INTRODUCTION

It was of interest to know whether the LTS tumour cells resided in particular sites within the host after antibody therapy. One approach to this was to radiolabel the cells either intrinsically or by surface iodonation. Problems do exist with these procedures, not only may the labelling cause cellular damage but also low numbers of tumour cells may not be detected. It was decided therefore to use a bioassay approach. Tissues removed from the animals at a certain time after administration of the antibody were transferred to naive mice and the reappearance of the tumour noted.

4.7.2. EXPERIMENTAL PROCEDURE

LTS tumour cells from the final mouse dying in therapy 3 were prepared as described in section 2.8. and four CBA mice were injected i.p. with 5 x 10^6 cells. Twenty-four hours later two of these mice were treated with 100 µg of Mc39-16 whilst the other two mice received a sham injection of PBS. A further six days later these mice were anaesthetised (metofane, C-Vet, Bury St. Edmunds) and the animals exsanguinated by cardiac puncture. The following tissues were removed:

(1) Whole blood, from cardiac puncture
(2) Lymph nodes, Inguinal
(3) Spleen
(4) Liver
(5) Adrenals
(6) Bone marrow, removed by cutting both ends of femers and flushing out with a 30 gauge needle and syringe filled with DMEM.

The lymph nodes, spleen, liver and adrenal glands were made into single cell suspensions by cutting into small pieces and teasing through a metal gauze as described in section 2.8. Each tissue, including the whole blood, was then injected i.p. into two CBA mice. Thus for each tissue from each mouse in the original treatment groups two naive mice

were injected, therefore a total of four mice received the same type of tissue from each treatment group. All the animals were then monitored for signs of illness.

4.7.3. RESULTS

Survival curves for the animals are shown in Figure 4.10. and in each case when an animal succumbed confirmation of the presence of A31 tumour was determined by FACS (data not shown).

The appearence of tumour in mice receiving tissues isolated from spleen, liver, lymph node, and adrenal glands clearly indicates that significant tumour populations exist in these areas six days after therapy. As all of the animals succumbed 30-40 days after transfer of tissue, it could be argued that the tumour loads at these sites may have been similar.

All but one of the remaining animals that received whole blood eventually developed tumour, but with an extended survival curve indicating that at least within the blood group fewer cells may have been transferred to the recipients. In contrast one animal receiving blood and all the animals receiving bone marrow from antibody treated groups remained in good health for 328 days (figure 4.10.). At the end of this time these surviving animals were sacrificed and the splenic tissue, which appeared normal, was made into single cell suspensions. The cells from each spleen were injected into naive mice. Three of these animals became ill at between 52-58 days post tumour inoculation. The last of these animals was sacrificed and a single cell suspension of the splenocytes examined by flow cytometry. This confirmed the presence of A31 tumour (data not shown). Thus, at least one animal receiving bone marrow, and probably some mice receiving whole blood from anti-idiotype treated animals, had established dormant A31 tumour within their spleens.

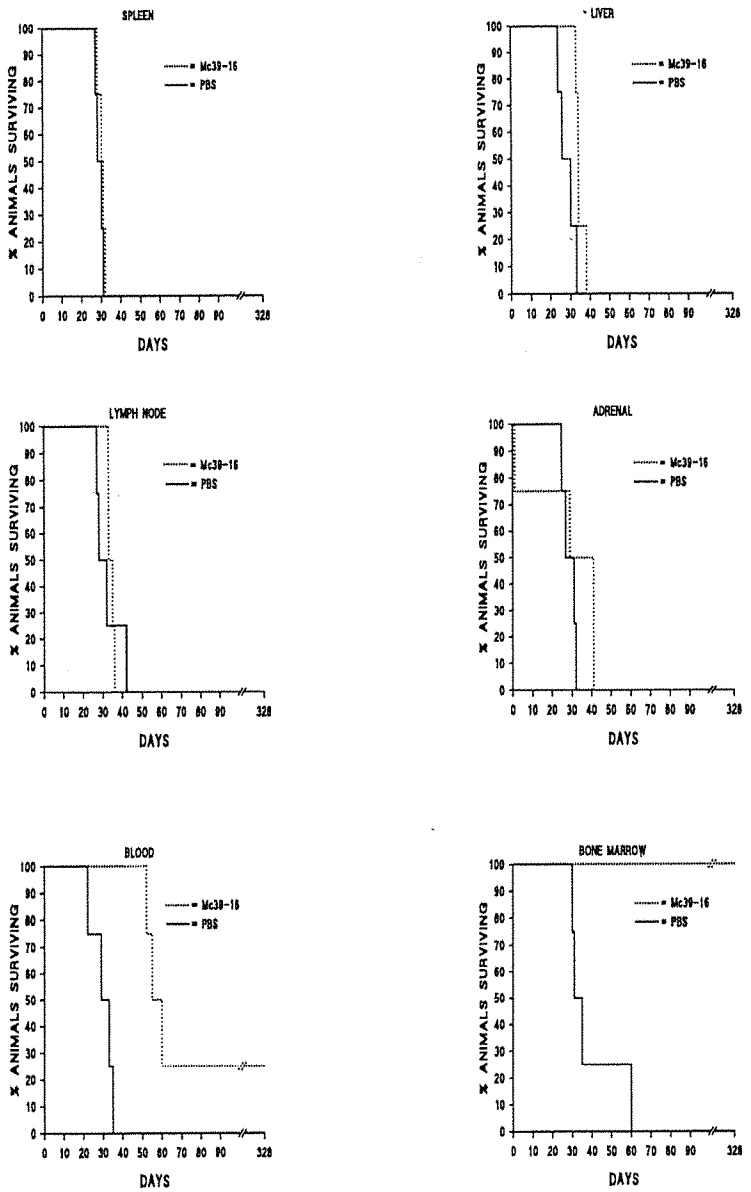


Figure 4.10. Transfer of tissue into naive mice from Mc39-16 treated and untreated A31-LTS animals.
Mice received an injection of a single cell suspension of the indicated tissue removed 6 days after animals carrying the A31-LTS tumour (5 x 10^6 cells given on day 0) had been injected with Mc39-16 (100 µg) or PBS 24 hours after tumour inoculation.

4.8. DISCUSSION

The therapeutic efficacy of the A31 anti-idiotypic monoclonal antibody Mc39-16 in passive treatment of the tumour has been examined. The usual course of A31 lymphoma leads to the death of animals, injected with 5 x 10^5 tumour cells i.p., in 26 ± 6 days, whereas survival is increased by approximately 60 days following a single injection of monoclonal anti-idiotype (100 µg) on day 1. Multiple injections of Mc39-16 covering the first three weeks post tumour inoculation did not extend this survival. This suggested that the maximal therapeutic benefit of Mc39-16 IgG therapy under these conditions had been reached. In terms of other animal models of leukemia and lymphoma the level of protection elicited by Mc39-16 was appreciable.

In the guinea pig B cell lymphoblastic leukaemia, L_2C, early investigations utilising polyclonal xenogenic anti-idiotype immunoglobulins gave modest therapeutic effects, a single 10 mg i.p. injection given six hours after 2 x 10^5 tumour cells prolonged the lives of the animals by about 3 days (Stevenson et al., 1977). Similarly injection of 1 mg of a murine monoclonal anti-idiotypic antibody given to animals 24 hours after receiving 10^5 tumour cells, extended the survival of the animals by 5 days (Glennie et al., 1987).

Studies with murine lymphomas however give a more pertinent comparison of passive immunotherapy with antibodies directed against the idiotype of the A31 lymphoma. Passive immunotherapy of the CH1 murine lymphoma using rabbit polyclonal anti-idiotype serum leads to a more pronounced effect then that seen in the guinea pig model. Mice inoculated with 10^3 tumour cells were given an i.p. injection of 0.2 ml anti-idiotype serum two days later, followed by a further injection of 0.7 ml 21 days post tumour inoculation. This regime produced mice surviving in excess of 150 days compared with 30 days for untreated animals. Higher doses of serum or tumour cells however resulted in failure of therapy (Haughton et al., 1978). The extensively studied murine lymphoma, BCL_1, has been the subject of polyclonal xenogenic therapy with variable results depending on the conditions used in the

experiments. With 10^4 BCL_1 cells given i.v. and doses of antibody from 1-10 μg/mouse, injected 24 hours prior to and days 3 and 21 post tumour inoculation, total eradication of the tumour growth has been achieved. However animals that had received 10^6 tumour cells two weeks preceding splenectomy followed by injection of 10 μg/mouse anti-idiotype immunoglobulin post splenectomy did not show any retardation of tumour growth (Krolick et al., 1979).

Anti-idiotype therapy of BCL_1 using a rat IgG2a monoclonal antibody has been investigated by injecting 2 x 10^5 tumour cells i.m. followed immediately by injection of various doses of antibody i.p. Antibody doses of between 50-200 μg/mouse produced a significant therapeutic effect with extension in survival of approximately 30 days over controls (George et al., 1991). Despite difficulties in comparing therapeutic results in these various animal models where such disparity in experimental protocols exist there can be little doubt that Mc39-16 alone produced a significant therapeutic effect.

In a similar fashion to the studies of passive anti-idiotype therapy of L_2C and BCL_1 the effect of Mc39-16 alone was to only delay the growth of the tumour *in vivo*. The tumour cells that emerged after this delay retained the same idiotype profile as the parent tumour cells. It has long been known that tumour cells emerging from passive anti-idiotype therapy, in both animal models and humans, may display changes in their surface expression of idiotype (Sibley and Andrews-Wagner, 1983; Meeker et al., 1985; Irick et al., 1986; Glennie et al., 1987a; George et al., 1988).

During therapeutic trials in humans the administration of xenogenic polyclonal anti-idiotypic immunoglobulin has been shown to induce modulation of the surface idiotype contributing to failure in therapy (Gordon et al., 1984). In a separate trial the administration of murine monoclonal antibodies directed against the idiotype of B-cell lymphomas lead to the emergence of tumour variants in which the tumour continued to express surface immunoglobulin but had lost the idiotope recognized by the therapeutic antibody (Meeker et al., 1985). Similarly a number of animal models of lymphoma have shown the tumour cells to be

capable of escaping therapy, passive or active, by either losing their surface immunoglobulin entirely or by loss of individual idiotopes (Lynch et al., 1972., Ghosh and Bankert, 1984., Bridges et al., 1984., Glennie et al., 1987., George et al., 1988). It is clear from the data, however, that some other mechanism must be operating to account for the tumour emerging from passive anti-idiotype therapy in the A31 model. The evidence suggests the tumour has not altered or lost the idiotope recognised by the therapeutic antibody.

On subsequent therapies the emerging tumour was not as sensitive to the anti-idiotypic monoclonal as the original stock of A31 tumour. It had not, however, become totally resistant to the antibody as the animals succumb to tumour some 20 days later than sham injected controls. This partial resistance did not increase with subsequent passages in the presence of the antibody. This change also appears to be stable as A31-LTS cells grown in the absence of Mc39-16 for four generations followed by administration of the antibody produced a survival curve consistent with that of A31-LTS cells grown in the continual presence of Mc39-16.

The data from S-phase analysis, taken in isolation, may only represents experimental variation in the two tumour populations. However, when examined in conjunction with the additional, and consistent, evidence of differences in ^{3}H-thymidine and ^{51}Chromium uptake by A31-LTS cells compared to A31 cells suggests this difference may be genuine. Combined evidence of the S-phase analysis and ^{3}H-thymidine incorporation implies that A31-LTS cells were dividing at a higher rate than the wild type cells. The former cells, however, did not cause animals to succumb any quicker than the wild type cells when grown in the absence of Mc39-16 arguing against the simple explanation of a faster division rate altering the profile of the survival curve.

The stability of this partial resistance in the absence of selection pressure from the anti-idiotype monoclonal antibody may argue for an inheritable trait. The latter may endow the LTS cells with the ability to produce a higher rate of division when induced directly by the presence of the antibody, or indirectly by interactions between the

tumour, antibody and effector cell populations. The increased tumour cell growth-rate combined with the inability of the host to up-regulate cell killing to the same degree should produce a reduced therapeutic effect.

In addition to the increased S-phase, the higher uptake of ^{51}Cr suggest that the LTS cells have a higher metabolic rate than the wild type cells. An increase in the number of molecules that are the usual target for effector cell killing mechanisms may give a cell partial resistance to attack. Some evidence for this phenomena has been obtained for tumour cell escape from host effector mechanisms in murine large cell lymphoma. In this animal model examination of differentially expressed genes within a highly metastatic subline indicated that a mitochondrial gene for a subunit of the NADH dehydrogenase enzyme was expressed at ten times the level seen in the parental cell line. This enzyme is known to be a target for an activated macrophage-released cytostatic factor and the authors suggested that this may partially account for the high metastatic phenotype of this line (Labiche et al., 1988). Upregulation of similar molecular targets within A31-LTS cells could also account for partial resistance to effector mechanisms.

An alternate explanation could be that the A31-LTS cells have a preference for a particular tissue site within the host. In this model therapeutic pressure causes the destruction of the majority of tumour within the host only leaving tumour cells within a privileged site. These cells can then expand and re-inoculate other sites within the host. The privileged site may represent an area were the growth characteristics of the tumour within that site give partial protection from the therapy. Once the tumour escapes from this location the growth characteristics may be entirely different and invasion of the rest of the host would be rapid. The identical survival curves for A31 and A31-LTS cells in the absence of therapeutic antibody would be a reflection of the extensive tumour load throughout the host.

The transfer into naive mice of various tissues from mice that had been inoculated with A31-LTS cells and then either injected with Mc39-16 or PBS did not clarify the

situation however. Under the experimental conditions chosen it was clear that significant tumour cell populations existed in spleen, liver, lymph node and adrenal glands in both treated and untreated mice. In antibody-treated animals, however, there were either significantly fewer cells in the blood and bone marrow or the tumour cells from these sites had a reduced ability to colonise the naive recipients. If the ability to colonise is truly reduced then the presence of therapeutic antibody must play a role, as blood and bone marrow taken from control animals caused the naive animals to succumb in the same time interval as animals in the other groups. In the belief that after treatment tumour levels would be low within the tissues, the tumour inoculum in this experiment was one log higher than that normally used for therapy. Additionally the tissue was harvested six days after antibody treatment. The combination of these factors may have concealed the true nature of the initial tissue distribution in treated animals. It is worth noting however that the presence of tumour at sites other than the spleen, even after antibody therapy, may indicate that the immunohistology described in chapter 3 may have missed low tumour cell numbers within the liver.

An interesting finding was the identification of dormant tumour within the spleen of an animal that had received bone marrow tissue from an anti-idiotype treated mouse and had remained well for approximately one year. The flow cytometry data indicated that these dormant tumour cells still retained the idiotope recognised by Mc39-16.

It is probably unwise to draw too many conclusions from the flow cytometry data of A31 and A31-LTS cell populations. Certainly the A31-LTS population had not reduced or lost the Mc39-16 idiotope during therapy, Figure 4.8. suggesting, if anything, an actual increase over that observed in the A31 wild-type tumour. Similarly there did appear to be more $CD4^+$ T cells within the latter tumour population. Although the spleens were taken from terminally ill animals and were comparable in size it can not be assumed that they contained the same density of tumour. If the higher number of Mc39-16^+ A31-LTS cells was genuine, the lower number of $CD4^+$ cells within the spleen taken from

that animal may simple reflect slightly greater disruption of the normal architecture of the spleen by tumour cells. To confirm any differences in the cellular content of A31 and A31-LTS spleen populations after therapy would require analysis of a large number of animals, at a number of time points post-inocultion, to identify any significant disparity between the two populations.

The treatment of the A31 tumour with an anti-idiotype monoclonal antibody produced significant therapeutic effect limited by the emergence of tumour variant(s). Although some limited data has been obtained on these variants considerable effort would be required to determine the mechanisms involved in tumour escape under these conditions and as the remit of this study was the development and study of immunotoxin and bispecific antibodies in the treatment of the A31 tumour this line of enquiry was taken no further. The following chapter describes the use of Mc39-16 in the construction and use of immunotoxin and bispecific antibodies for the delivery of the ribosomal inactivating protein saporin to the tumour.

CHAPTER 5

ANTI-IDIOTYPE DERIVATIVES RECRUITING SAPORIN TO THE A31 TUMOUR

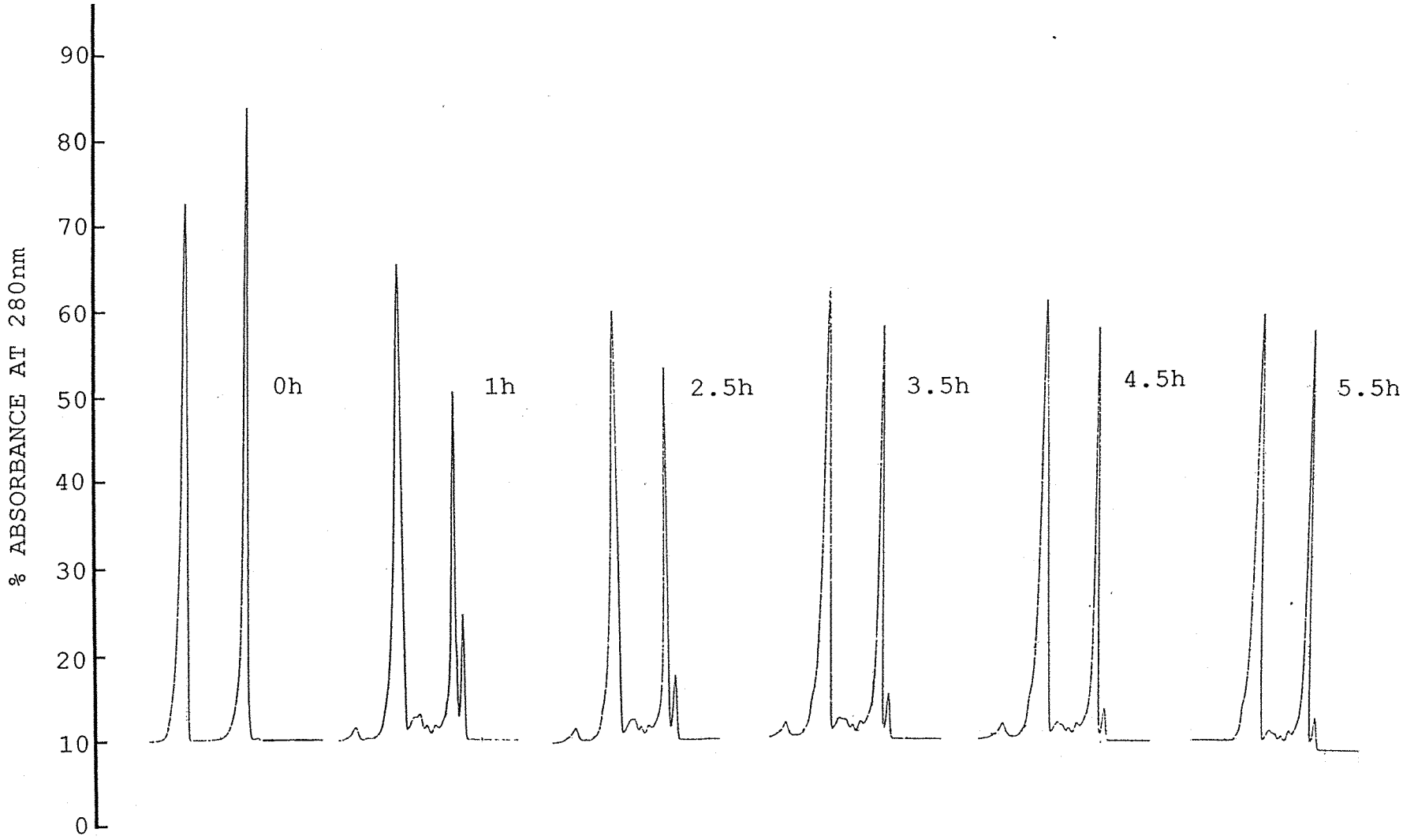
5.1. INTRODUCTION

Two types of derivative were produced from Mc39-16 in order to target the ribosomal-inactivating protein, saporin, to the A31 tumour. The first approach involved the formation of bispecific antibodies using a Fab' arm isolated from Mc39-16 joined by a stable thioether linkage to a Fab' fragment from antibodies with specificity for saporin whilst the second approach used a direct disulphide linkage between the antibody and saporin to form an immunotoxin.

5.2. PEPSIN DIGESTION OF Mc39-16

The rate and degree of digestion of rat immunoglobulins by pepsin is dependant on a number of reaction factors principally the pH, temperature, time and concentration of reactants. It has been suggested that optimum pepsin digestion of rat IgG2a molecules depends on a high enzyme to protein ratio and pH values below 4.5 (Rousseaux et al., 1983). As no previous information existed on the pepsin digestion pattern of Mc39-16 it was important to establish its sensitivity to this enzyme prior to preparation of the bispecific antibodies. A preliminary digestion indicated that using pepsin at an amount equal to 3% of the quantity of immunoglobulin present, a pH of 4.2 and with the immunoglobulin at 10 mg/ml produced almost complete digestion to $F(ab')_2$ within 4-6 hours (Figure 5.1.). The complete pepsin digestion procedure is given in section 2.28.1. These conditions were also found suitable for the generation of $F(ab')_2$ fragments from DB7/18 (anti-saporin), SI-1 (anti-saporin) and KT3 (anti-mouse CD3).

Figure 5.1. Six HPLC profiles obtained with samples taken at times 0, 1, 2.5, 3.5, 4.5, and 5.5 h during pepsin digestion of Mc39-16, a rat IgG2a immunoglobulin. Antibody at 12 mg/ml was digested with 3% pepsin at pH 4.2. At time 0 h the digestion mixture shows a single IgG peak (right-hand side) followed by the 'salt' peak. By 1 h the IgG has been substantially converted to $F(ab')_2$ which forms the major peak as a doublet with the IgG peak. By 5.5 h the bulk (>95%) of the IgG (very small leading peak) has been converted to $F(ab')_2$ (single major peak) together with smaller fragments and peptides. This latter material elutes with the 'salt' peak (left hand peak).


% ABSORBANCE AT 280nm
90
80
70
60
50
40
30
20
10
0
0h
1h
2.5h
3.5h
4.5h
5.5h

5.3. DETERMINATION OF AVAILABLE THIOL GROUPS IN Mc39-16.

As there was only limited data on the number of hinge region disulphide bonds present in rat IgG2a (Bruggemann, 1988) an estimate was made using reduced Fab (Fab'_{SH}) of Mc39-16 by the method of Grassetti and Murry (1967); and Stuchbury et al., (1975). The determination of available free thiol-groups by this method relies on the reduction of a disulphide bond within a 2,2' dithiodipyridine molecule by free thiol within the Fab molecule. This thiol-disulphide interchange results in the release of 2-thiopyridone which has an extinction coefficient of 8.08 at 343 nm. Each thiol group present at the start of the reaction will release one 2-thiopyridone molecule therefore determination of the number of molecules of the latter released gives a direct measurement of the number of thiol groups present in the reduced Fab.

5.3.1. RESULT

A total of 4.6 SH groups per molecule of Mc39-16 Fab_{SH} was found. Assuming two SH groups were contributed from the heavy-light chain disulphide bond the result would suggest that the hinge of Mc39-16 IgG may contain up to three disulphide bonds. We know from experence in preparing bispecific antibodies using mouse Fab'_{SH} that three hinge-region SH groups are ideal for this preparation (Glennie et al., 1987).

5.4. ANTIBODIES USED TO CONSTRUCT BISPECIFICS AND IMMUNOTOXINS

The antibodies used in the construction of the bispecifics and immunotoxin are listed in Table 5.1. Antibody designations will be used in subsequent graphical plots and text. Immunotoxins are designated by the antibody designation followed by -SO6.

5.5. BISPECIFIC ANTIBODIES

The construction of the saporin-recruiting bispecific antibodies used during this study has been described in section 2.28. HPLC profiles of the final products after reduction and alkylation but before separation on AcA 44 are shown in figures 5.2a and 5.2b. Bispecific antibodies were isolated from both preparations although the anti-A31 x anti-SAP1 preparation contained almost an equal amount of $F(ab)_3$ product as $F(ab)_2$ material in contrast to the anti-A31 x anti-SAP3 preparation where the $F(ab)_2$ was the principal moiety.

TABLE 5.1. MONOCLONAL ANTIBODIES USED IN THE CONSTRUCTION OF BISPECIFIIC ANTIBODIES

ANTIBODY NAME	ANTIBODY DESIGNATION	ANTIBODY SPECIFICITY
Mc39-16	ANTI-A31	A31 anti-Idiotype
DB7-18	ANTI-SAP1	anti-saporin
SI-1	ANTI-SAP3	anti-saporin
RJD-2A10	ANTI-L_2C1	L_2C anti-idiotype
M6-3D10	ANTI-L_2C2	L_2C anti-idiotype

Note: SI-1 and DB7-18 bind to separate and non-blocking epitopes on the saporin molecule (French et al., 1991). The designation of the anti-saporin monoclonal antibodies and that of the anti-L_2C anti-idiotypic monoclonal antibodies was first used by French and colleagues (1991) and to maintain consistency has been retained within this dissertation.

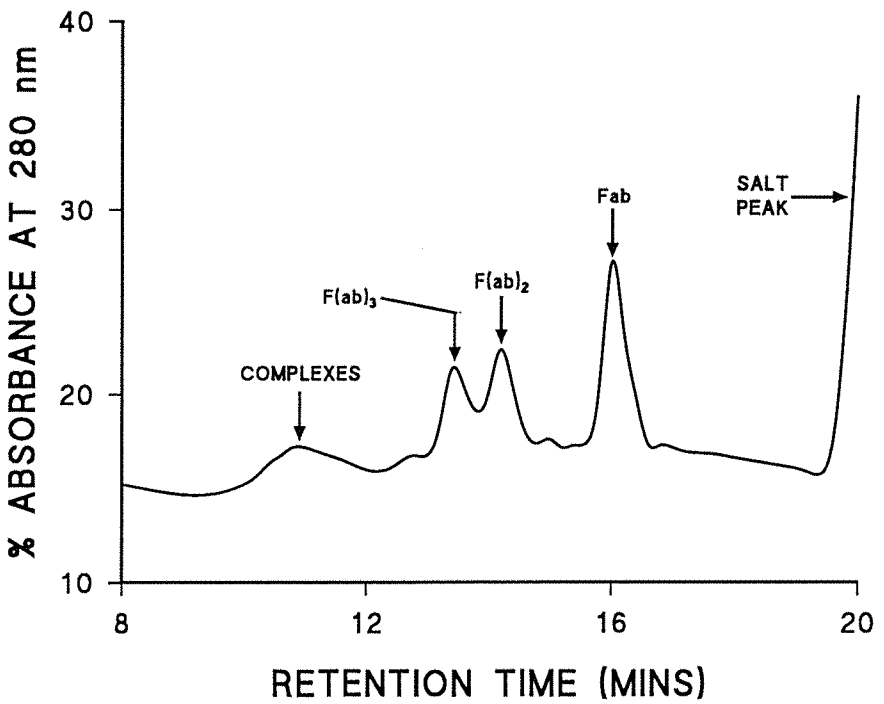


Figure 5.2a. HPLC profile of bispecific antibody preparation anti-A31 x anti-SAP1 after final reduction but before separation by AcA44 chromatography.

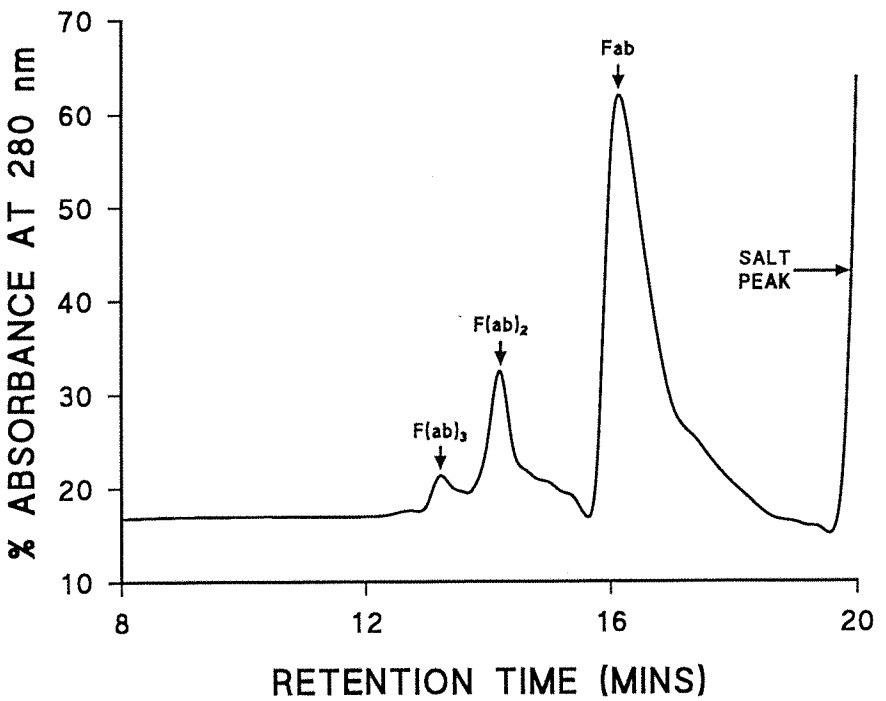


Figure 5.2b. HPLC profile of bispecific antibody preparation anti-A31 x anti-SAP3 after final reduction but before separation by AcA44 chromatography.

One possible explanation for the higher $F(ab)_3$ content within the anti-A31 x anti-SAP1 preparation is the slightly higher ratio of anti-SAP1 $F(ab')_2$ to anti-A31 $F(ab')_2$ (1.7 : 1) used compared to the ratio of anti-SAP3 $F(ab')_2$ to anti-A31 $F(ab')_2$ used (1.5 :1). If the final yields of bispecific antibody recovered are expressed as a percentage of the total $F(ab')_2$ used in their contruction then anti-A31 x anti-SAP1 represented 9% and anti-A31 x anti-SAP3 represented 16% of the total $F(ab')_2$ used in their preparation respectively. The lower percentage of the former reflecting the greater amount of $F(ab)_3$ produced. In both cases however the final yeilds were lower than is routinely produced in this laboratory using mouse $F(ab')_2$ as the starting material (50% of $F(ab')_2$ recovered as bispecific antibody; R.French personal communication).

5.6. TITRATION OF ANTIBODIES AND THEIR DERIVATIVES AGAINST A31 CELLS AT A CONSTANT SAPORIN CONCENTRATION.

5.6.1. INTRODUCTION

As saporin can alone can cause inhibition of protein synthesis it was of importance to titre bispecific antibodies out using a saporin concentration that caused minimal disturbance to the titration curve.

5.6.2. RESULTS

The ^{3}H-Leucine incorporation assay used to identify protein synthesis inhibition has been described in section 2.29. An initial experiment was performed to determiine the effect of saporin, at concentrations ranging from 10 µg/ml to 5 ng/ml, on the ^{3}H-leucine incorporation of A31 cells. The results demonstrate that inhibition of protein synthesis began to occur above a saporin concentration of 100 ng/ml. In the light of this evidence it was decided to titre the antibody derivatives against a constant saporin concentration of 25 ng/ml (Figure 5.3.).

The bispecific derivatives appear to reach their maximium inhibition of ^{3}H-leucine incorporation at an antibody concentration of between 100-500 ng/ml. Throughout the dilution series the bispecific antibody anti-A31 x anti-SAP1

or a mixture of anti-A31 x anti-SAP1 + anti-A31 x anti-SAP3 acheived significantly higher inhibitions of leucine incorporation than anti-A31 x anti-SAP3. It was noted that the cocktail of the two bispecific antibodies did not give a higher IC_{50}, concentration of saporin in presence of bispecific antibody that achieves $\frac{1}{2}$-maximal inhibition of protein synthesis, than anti-A31 x anti-SAP1.

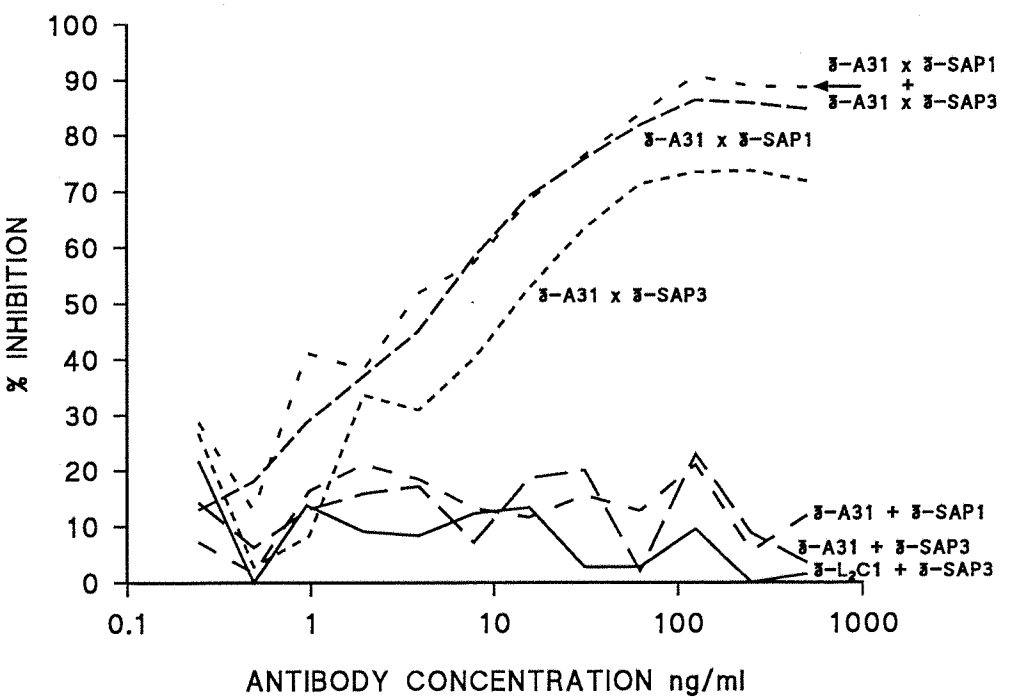


Figure 5.3. Titration of antibody derivatives against A31 cells at a constant saporin concentration (25 ng/ml). The results are plotted as a percentage inhibition of the ^{3}H-leucine incorporation in the absence of saporin.

5.7. TITRATION OF SAPORIN TOXICITY AGAINST A31 CELLS IN PRESENCE OF BISPECIFIC ANTIBODIES OR AS PART OF AN IMMUNOTOXIN.

5.7.1. INTRODUCTION

Having established the titration curve for the antibodies and their derivatives at a constant saporin concentration the next step was to titre the saporin out, either as part of the immunotoxin anti-A31-SO6, or in the presence of the antibodies and their bispecific derivatives.

5.7.2. RESULTS

The experimental procedure was as described in 2.29. Titration of the immunotoxin is shown in figure 5.4. and that of the bispecific antibodies in figure 5.5. From these plots the IC_{50} for each derivative was obtained and the results are shown in Table 5.2.

Conjugation of saporin to form the immunotoxin Ab1-SO6 or recruitment of saporin via bispecific antibody anti-A31 x anti-SAP1 increased the saporin's ability to inhibit protein synthesis by approximately a factor of 1800-2900. Although the IC_{50}s produced by the immunotoxin Ab1-SO6 and the bispecific antibody anti-A31 x anti-SAP1 showed a dramatic decrease compared to saporin alone, this contrasted with the derivative anti-A31 x anti-SAP3 which gave significantly lower toxicity (230-fold increase relative to saporin alone). compared to the immunotoxin and anti-A31 x anti-SAP1. When anti-A31 x anti-SAP1 and anti-A31 x anti-SAP3 were used in the form of a cocktail there was an additional decrease in the IC_{50} relative to the best bispecific antibody used alone (anti-A31 x anti-SAP1) to give an increase in effectiveness of 3800-fold relative to saporin.

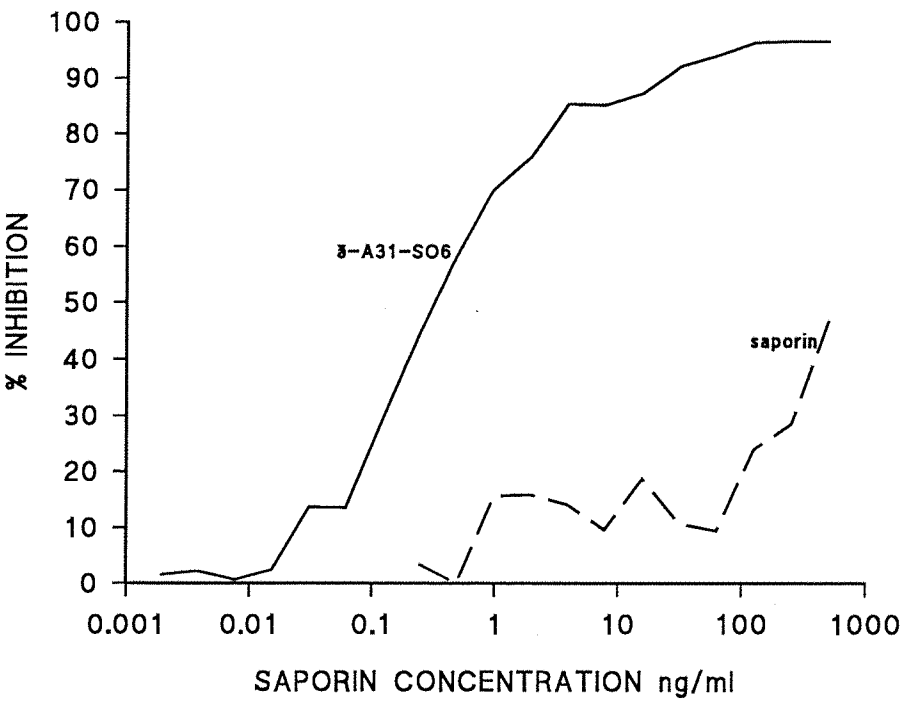


Figure 5.4. Inhibition of ^{3}H-leucine incorporation into A31 cells by immunotoxin anti-A31-SO6. The results are plotted as a percentage inhibition of the ^{3}H-leucine incorporation in the absence of saporin.

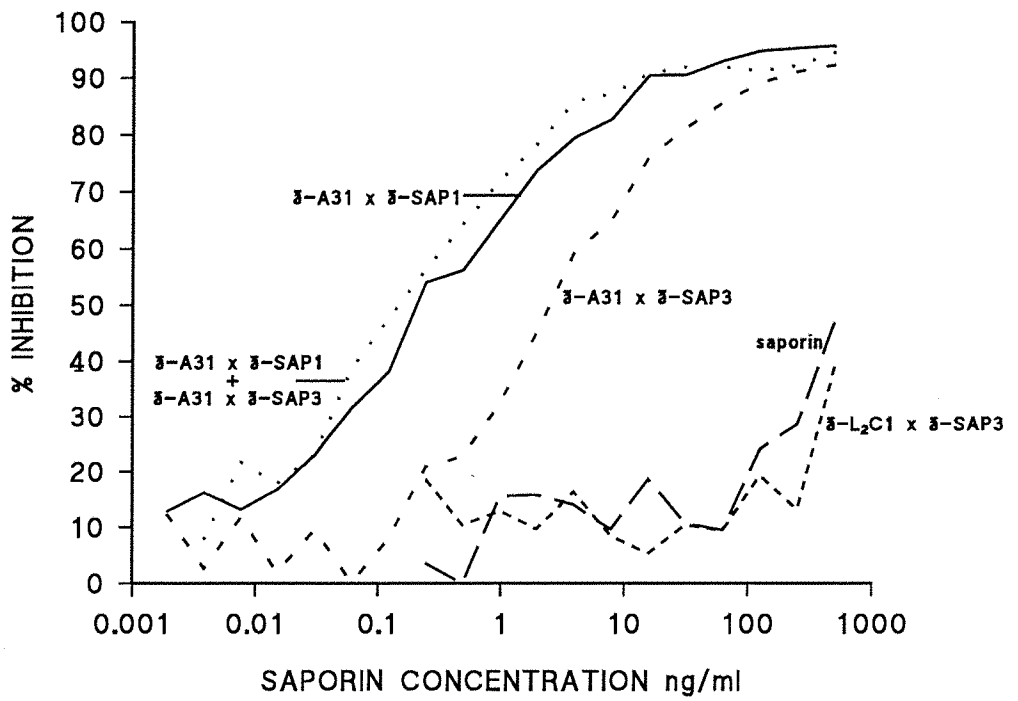


Figure 5.5. Inhibition of ^{3}H-leucine incorporation into A31 cells by antibody derivatives. The results are plotted as a percentage inhibition of the ^{3}H-leucine incorporation in the absence of saporin. In addition to the control antibody mixture shown (anti-L_2C1 x anti-SAP3) two other combinations were used, (anti-A31 + anti-SAP1) and (anti-A31 + anti-SAP3), in both cases the profiles were essentially the same as anti-L_2C1 x anti-SAP3 and therefore for clarity have not been shown.

TABLE 5.2. IC_{50} FOR ANTIBODY DERIVATIVES

ANTIBODY DERIVATIVE	IC_{50}	'FOLD' INCREASE OVER SAPORIN ALONE
SAPORIN	575 ng/ml	0
anti-L_2C1 x anti-SAP3	600 ng/ml	0.96
anti-A31 x anti-SAP1	0.2 ng/ml	2875
anti-A31 x anti-SAP3	2.5 ng/ml	230
anti-A31 x anti-SAP1 + anti-A31 x anti-SAP3	0.15 ng/ml	3833
anti-A31-SO6	0.32 ng/ml	1797

5.8. TITRATION OF TUMOUR CELL NUMBER AT CONSTANT SAPORIN AND ANTIBODY CONCENTRATIONS.

5.8.1. INTRODUCTION

It was possible that the IC_{50} values shown in Table 5.2. did not reflect the true point at which half maximal inhibition of ^{3}H-leucine incorporation occurred, but were due to depletion of all available saporin at low concentrations of the latter by the cells. If the IC_{50} values were correct, then reduction of the cell numbers should not cause a significant increase in inhibition. However, if the saporin was limiting for the cell numbers used, then an increase in the percentage inhibition should be observed as the number of cells was reduced.

5.8.2. RESULTS

The experimental procedure was as described in 2.29. except that saporin was maintained at either 0.1 or 0.5 ng/ml and the antibodies and derivatives were maintained at 1 µg/ml. Triplicate samples were used for each antibody. The A31 tumour cells were diluted from 5×10^5 to 1×10^5 cells/ml. The inhibition induced by each antibody/saporin mixture was calculated using saporin-free cultures to assess maximun uptake of ^{3}H-leucine. The results shown in Figure 5.6 demonstrate that no increase in inhibition occurred as the cell numbers were reduced indicating that the IC_{50} values obtained using 5×10^6 cells/ml were correct.

Having obtained data on the function of the immunotoxin and bispecific antibodies *in vitro* it was decided to compare their action in the treatment of animals carrying the A31 tumour.

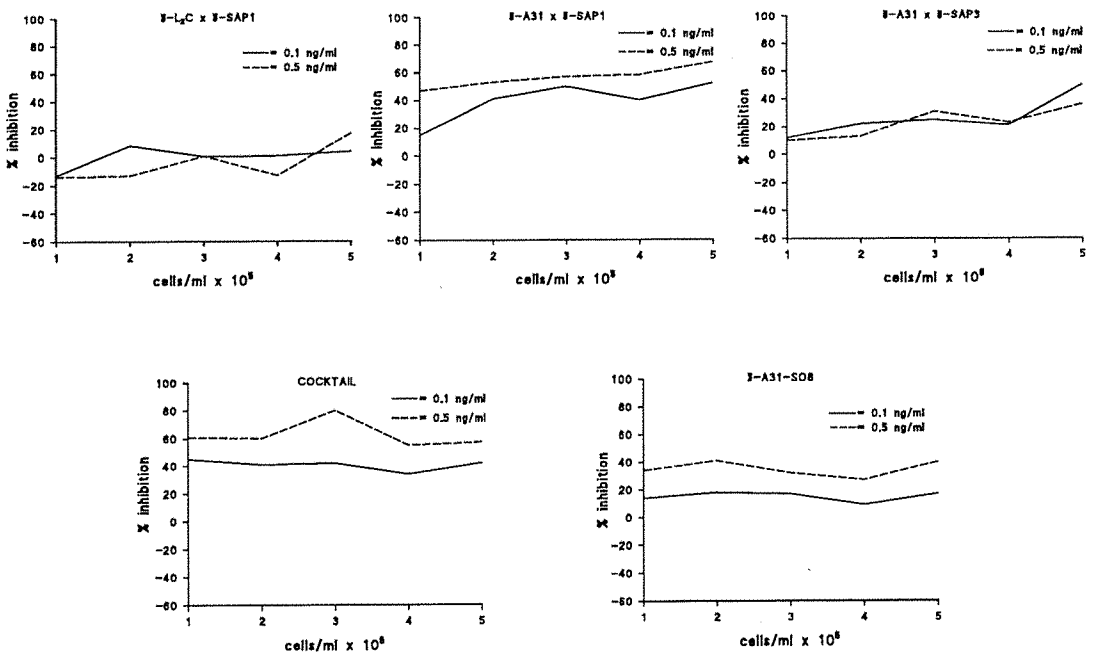


Figure 5.6. Titration of cell numbers at constant saporin and antibody levels. The results are plotted as a percentage inhibition of the ^{3}H-leucine incorporation in the absence of saporin at each cell concentration. the cocktail was an equal mixture of anti-A31 x anti-SAP1 and anti-A31 x anti-SAP3.

5.9. IMMUNOTHERAPY OF MICE CARRYING THE A31 TUMOUR WITH ANTI-IDIOTYPE SPECIFIC IMMUNOTOXIN

5.9.1. INTRODUCTION

The immunotoxin Mc39-16-SO6 (anti-A31-SO6) was available before the bispecific antibodies therefore this derivative was examined *in vivo* first.

5.9.2. RESULTS

Immunotherapy procedures have been described in 2.24. Briefly all mice received 5 x 10^5 A31 tumour cells i.p. on day 0 followed twenty-four hours later by the therapy also given as an i.p. injection. A total of seven groups of mice were used as shown in Table 5.3. Unless stated otherwise the doses of saporin administered (either as free saporin or as part of an immunotoxin) was 1 µg per mouse. The amount of

anti-A31 given as free antibody was equivalent to that present in anti-A31-SO6 containing 1 µg of saporin, approximately 2.77 µg of antibody. Animals were monitored for signs of illness, their deaths recorded and the results plotted as a survival curve (Figure 5.7.)

TABLE 5.3. IMMUNOTOXIN THERAPY 1 TREATMENT GROUPS

GROUP	THERAPY ADMINISTERED	NUMBER OF MICE IN GROUP
1	PBS	9
2	saporin	9
3	anti-A31	10
4	anti-L_2C2-SO6	9
5	anti-A31-SO6	11
6	anti-A31-SO6 0.1 µg	10
7	saporin + anti-A31	8

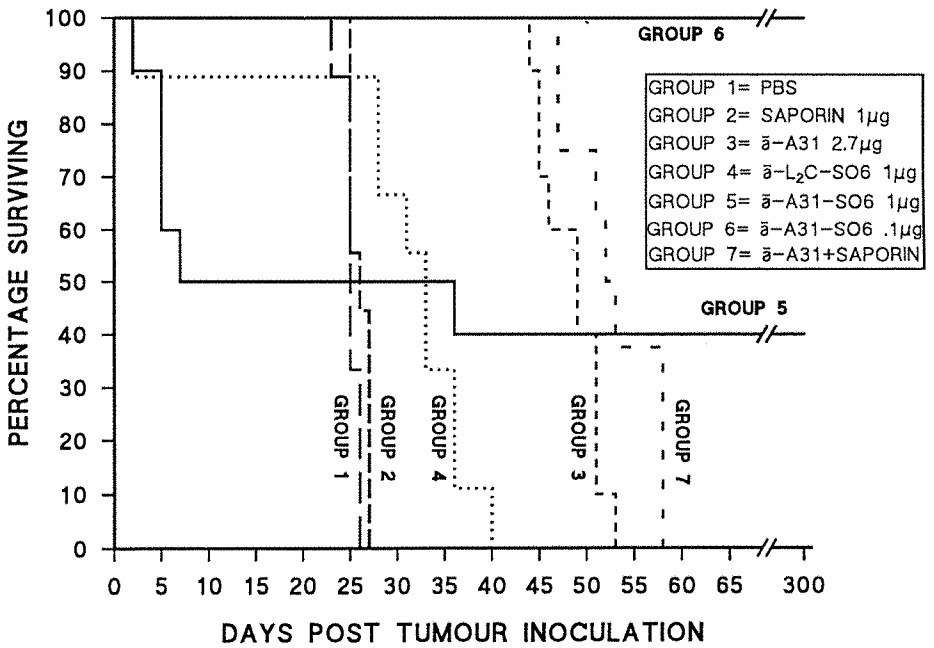


Figure 5.7. Immunotoxin therapy 1: Immunotoxin therapy of mice injected i.p. with 5 x 10^5 A31 tumour cells on day 0 followed twenty-four hours later with the therapy indicated.

The results of this immunotherapy show that while the anti-A31-SO6 immunotoxin was highly effective as an anti-lymphoma agent, this was not achieved without some degree of toxicity becoming apparent.

Groups 1 (PBS) and 2 (saporin) were control groups showing the normal progression of disease leading to deaths of the animals at approximately 26 days post tumour inoculation. Group 3 (2.7 µg of anti-A31 idiotypic immunoglobulin per mouse) clearly shows the expected extension in survival of the treated mice to about 45-54 days post tumour inoculation. A non-specific immunotoxin (anti-L_2C2-SO6) was also able to induce limited destruction of tumour cells thereby extending the survival of the animals by approximately 14 days (group 4). Administration of saporin together with the anti-idiotypic monoclonal antibody Mc39-16 (group 7) indicated that there was a slight syngeneic effect, (amounting to 2-4 days prolonged survival) compared to the effect of anti-A31 immunoglobulin given alone. The last

animal to die in group 7 did have an enlarged spleen and flow cytometry revealed the presence of A31 tumour cells with a normal surface phenotype (data not shown).

The first five animals to succumb in group five did not have enlarged spleens but there was in all cases a very pale liver. This apparent hepatic toxicity observed within group 5 animals occured at a level of conjugated saporin lower than had been reported from other investigators (Thorpe et al., 1985). To examine this toxicity further an i.p. injection of 1 μg of immunotoxin was given to mice not carrying the A31 tumour and their survival noted. These injections did not result in any deaths, the animals being monitored for in excess of 200 days post immunotoxin administration.

When a lower dose of anti-A31-SO6 was given (0.1 μg/mouse i.p.; group 6) all animals survived in excess of 300 days. Flow cytometry of splenocytes isolated from mice within group 6 failed to identify any A31 tumour and passage into naive animals of splenic tissue from group 6 did not induce A31 tumour in the recipient mice.

To examine the possibility of whether the destruction of the tumour within the animals of group 5 had induced any long term protection directed towards the tumour, the five surviving animals in group 5 were injected i.p. with 5×10^5 A31 tumour cells at 308 days post tumour inoculation. The survival of these animals together with that of a control group of naive mice injected with tumour at the same time is shown in Figure 5.8. Although the ex-immunotoxin group did survival slightly longer than the control group of animals (approximately 4-7 days) this level of protection is minimal and may not be significant. It seems likely therefore that, not unexpectedly, clearance of tumour by immunotoxin did not induce any long term immune-protection against the tumour.

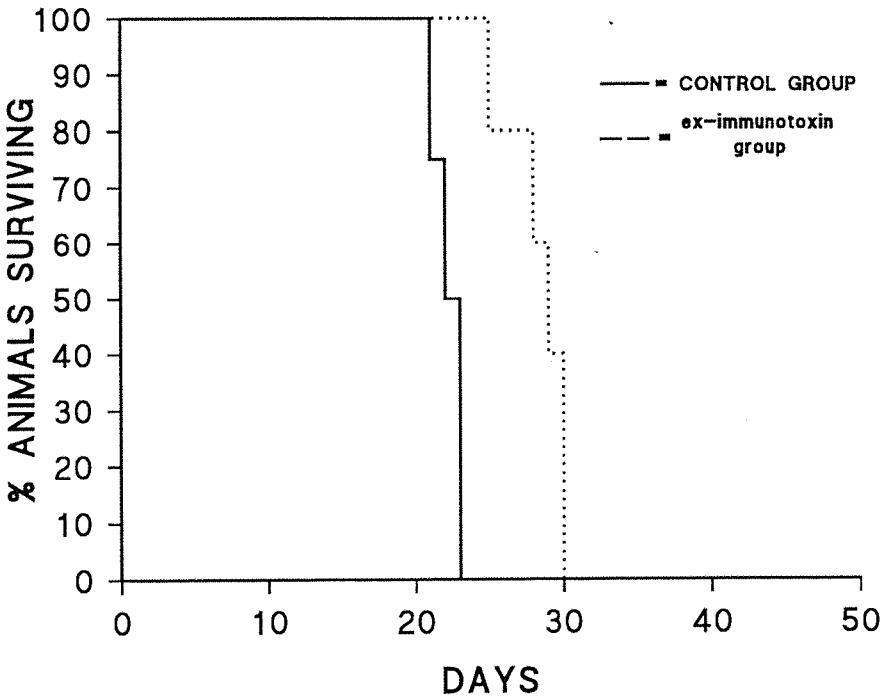


Figure 5.8. Re-inoculation of tumour into animals from which tumour had been eradicated by the initial immunotoxin therapy. Mice surviving from group 5 of antibody derivative therapy 1 were given 5 x 10^5 fresh A31 tumour cells i.p. 307 days after the first inoculation of tumour had been treated with immunotoxin.

5.10. IMMUNOTOXIN THERAPY 2.

5.10.1. INTRODUCTION

To determine how the immunotoxin would perform with tumour that had been established for a longer period a second therapy was initiated in which the therapy was delayed for set periods after the tumour was given.

5.10.2. RESULTS

Three groups of ten mice were each given 5 x 10^5 tumour cells i.p. on day 0. Treatment consisted of 0.1 µg of saporin in the form of immunotoxin and was given on specific days post tumour inoculation as described in Table 5.4.

TABLE 5.4. DELAYED THERAPY TREATMENT GROUPS

GROUP	DAYS THERAPY GIVEN (post tumour inoculation)
1	4
2	4,9,14,19,24
3	9,14

Failure to cure the mice of tumour occurred even with multiple doses of immunotoxin given to mice that had received tumour four days previously (see Figure 5.9.). There are two possible explanations for failure of this therapy. The tumour cells may have reached protected sites within the animals before initiation of the therapy or the increased numbers of tumour cells that were present after four days growth within the animal was sufficient to overwhelm the levels of immunotoxin given. To test this hypothesis two groups of ten mice were inoculated with 100 A31 cells then, after a delay of four days, a single i.p. injection of 0.1 μg immunotoxin was given to one group. The untreated animals succumbed to tumour between 32 and 35 days post tumour inoculation while the treated group remained well for over one year (Data not shown). Therefore a small tumour load can still be treated and cured on day 4 but delay in treatment at higher tumour loads renders the immunotoxin therapy ineffective. The first successful therapy of A31 tumour using the immunotoxin Ab1-SO6, (figure 5.7.), appears to have been administered close to the optimal 'window' of tumour load. These results suggest that any delay in therapy, longer than 24-hours post inoculation of 5×10^5 tumour cells, is likely to fail to cure the mice. The effective therapy, by IT four days after administration of 100 A31 cells, suggests that the tumour load present at the time of therapy may be the important factor. However, the 'seeding' of tumour cells to protected sites at high tumour cell inoculation can not be excluded.

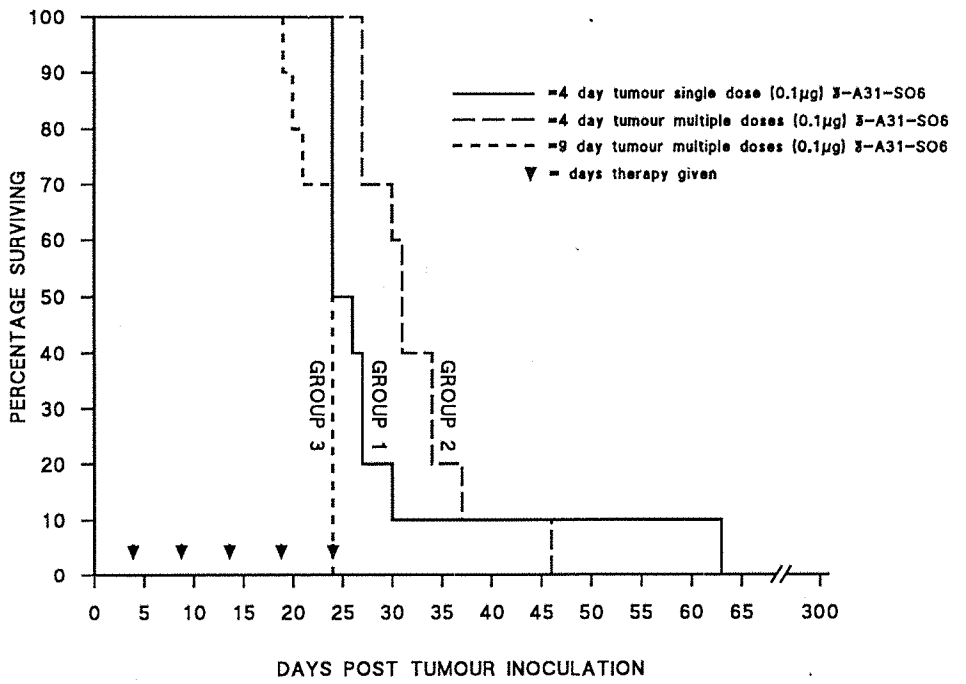


Figure 5.9. Delayed immunotoxin therapy for mice given 5 x 10^5 A31 tumour cells i.p. on day 0. The arrows indicate the days on which therapy was given to the groups as described in Table 5.4.

5.11. BISPECIFIC ANTIBODY THERAPY 1.

5.11.1. INTRODUCTION

Having established the protective capability of the immunotoxin preparation, the next therapy compared this protection with that given by therapy with the bispecific derivatives. These therapies were performed as described in Section 2.24. with a total of six groups as described in Table 5.5. To avoid the rapid loss of saporin, likely to occur if injected separately to the bispecific antibodies, the latter were pre-mixed with saporin at 37°C for 60 minutes prior to injection into the animals.

TABLE 5.5.BISPECIFIC ANTIBODY 1 TREATMENT GROUPS

GROUP	THERAPY ADMINISTERED	NUMBER OF MICE IN GROUP
1	anti-A31 x anti-SAP1 (1 μg) + saporin (0.1 μg)	10
2	anti-A31 x anti-SAP1 (10 μg) + saporin (1 μg)	9
3	anti-A31 x anti-SAP3 (1 μg) + saporin (0.1 μg)	10
4	anti-A31 x anti-SAP1 (0.5 μg) + anti-A31 x anti-SAP3 (0.5 μg) + saporin (0.1 μg)	9
5	anti-L_2C1 x anti-SAP1 (1 μg) + saporin (0.1 μg)	5
6	anti-A31-SO6 (0.1 μg)	5

5.11.2. RESULTS

The result of this therapy are shown in Figure 5.10. A control bispecific antibody with specificity for the idiotype of L_2C (group 5) gives no protection and all animals succumbed to tumour by day 21 which is the same as for unteated animals. Group 3 (A31 specific derivative using SI-3 to recruit saporin) gave a slight increase in survival to 31 days compared to group 5. Group 1 also utilised an A31 specific derivative but in this case saporin was bound via DB7-18 and for the majority of animals induced an approximate 4 days improvement over group 3. When the latter bispecific

derivative and saporin were injected at ten times the dose given to group 1 an increase in survival of animals within this group (group 2) of between 2 and 12 days compared to group 1 was observed. Despite this high dose, however, the last animal within group 2 had still succumbed by day 42, with flow cytometric analysis of splenocytes isolated from this animal revealing normal A31 tumour cells. In spite of the early losses of animals in group 4 (a cocktail of the SI-1 and DB7-18 containing A31 specific derivatives) the survival curve of the remaining animals suggests no enhancement of therapeutic effect had occured. Immunotoxin administered to tumour inoculated animals led to eradication of the tumour (group 6).

Thus the most striking feature of the results from the first bispecific antibody therapy was the limited effect the bispecific derivatives had in reducing the tumour load. Even 10 μg of anti-A31 x anti-SAP1 + 1 μg of saporin could only extend the survival of the last mouse within that group by approximately 20 days. This was only slightly better than that achieved with 2 μg of Mc39-16 IgG injected into tumour-bearing mice at the same time as the above groups and of which all but one had succumbed by day 34 (not shown in figure 5.10. to improve clarity). As in the *in vitro* assays anti-A31 x anti-SAP3 did not perform as well as anti-A31 x anti-SAP1.

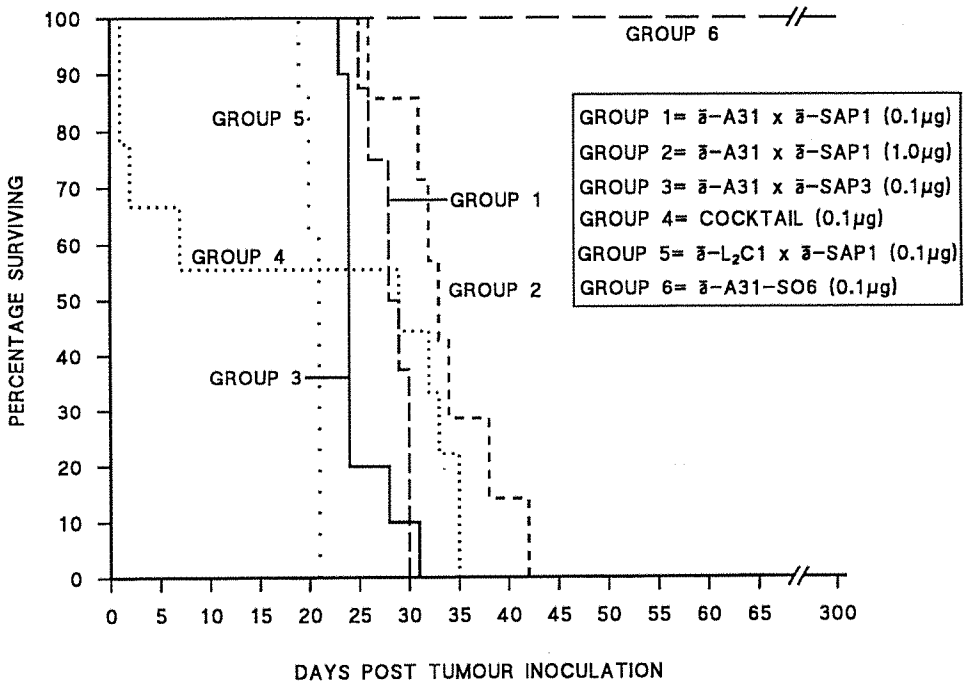


Figure 5.10. Therapy with bispecific antibody derivatives recruiting saporin to the tumour. Each animal received 5 x 10^5 A31 tumour cells i.p. on day 0 followed by antibody therapy i.p. twenty-four hours later as indicated by the groups in Table 5.5. The early deaths of animals in group 4 were probably due to experimental error with no signs of liver toxicity apparent. The amount of saporin administered to each animal within a particular group is indicated within the box.

5.12. WINN ASSAY *AND* CO-ADMINISTRATION OF TUMOUR AND BISPECIFIC ANTIBODY *IN VIVO.*

5.12.1. INTRODUCTION

There was obviously a discrepancy between the *in vitro* ^{3}H-leucine incorporation results which suggested that the bispecific antibody anti-A31 x anti-SAP1 and the immunotoxin Ab1-SO6 had similar IC_{50} values and the *in vivo* therapy results were the bispecific antibodies performed poorly. It was possible that the therapeutic failure may have been due to a short functional half-life of the bispecific antibodies within the animals therefore a further series of therapies were set up, that is; (a) tumour cells were exposed to the immunotoxin or saporin loaded bispecific antibodies *in vitro* before transfer to the animals i.e. a Winn assay (Winn, 1961) and (b) the saporin pre-loaded bispecific antibodies and the tumour cells were given i.p. into the animals at the same time.

5.12.2. RESULTS

Conditions were chosen to give maximal killing while avoiding the effect of non-specific toxicity (see Figures 5.3., 5.4. and 5.5.).

WINN ASSAY PROTOCOL:

A31 tumour cells (4 ml) at 2.5 x 10^{6}/ml were placed into a well in a 24-well tissue culture tray and the relevant antibody, either anti-L_2C1 x anti-SAP1 or anti-A31 x anti-SAP1, at a final concentration of 400 ng/ml, and saporin, at a final concentration of 50 ng/ml,were added. The immunotoxin Ab1-SO6 was incubated with tumour cells under the same conditions also at an antibody concentration of 400 ng/ml (144 ng/ml saporin) The cells were incubated within a CO_2 incubator for 12 hours before being harvested by centrifugation (400 x g, 10 min) and washed twice with DMEM containing 10% v/v FCS. The cells were then resuspended to 4 ml and 200 µl injected i.p. into the mice which were then monitored for signs of illness.

CO-ADMINISTRATION OF TUMOUR AND ANTIBODIES:

Tumour cells (5 x 10^5) were injected i.p. into the mice immediately followed by an i.p. injection at a different site of a pre-mixed (1 hour, 37°C) solution of saporin and the bispecific antibody anti-A31 x anti-SAP1 or control bispecific antibody anti-L_2C1 x anti-SAP1. For both derivatives the amount of antibody and saporin injected was 1 µg and 0.1 µg respectively. The immunotoxin anti-A31-SO6 was injected i.p. to give a equivalent amount of saporin (2.77 µg of antibody) and the animals were monitored for signs of disease.

The results of this experiment are shown in Figure 5.11. from which it appears quite clear that the failure in the bispecific antibody therapies is a failure to kill the tumour cells even when exposed to the antibodies *in vitro* for twelve hours before transfer to the animals. Therefore despite the ^{3}H-leucine incorporation data giving similar IC_{50} values for the immunotoxin (Figure 5.4.) and the bispecific antibody (anti-A31 x anti-SAP1; Figure 5.5.) the latter was not killing all the tumour cells present. A possible explanation for this discrepancy can be obtained by re-examining the data used to construct Figure 5.6. and simply plotting numbers of tumour cells against ^{3}H-leucine incorporation in the absence of saporin (Figure 5.12.).

As can be seen the detection level of ^{3}H-leucine incorporation occurs above 1 x 10^4 cells therefore significant numbers of tumour cells can remain viable and undetected with this assay.

To determine further possible differences between the bispecific antibodies and the immunotoxin an attempt was made to determine the number of saporin molecules being delivered to the cell surface by each derivative *in vitro*.

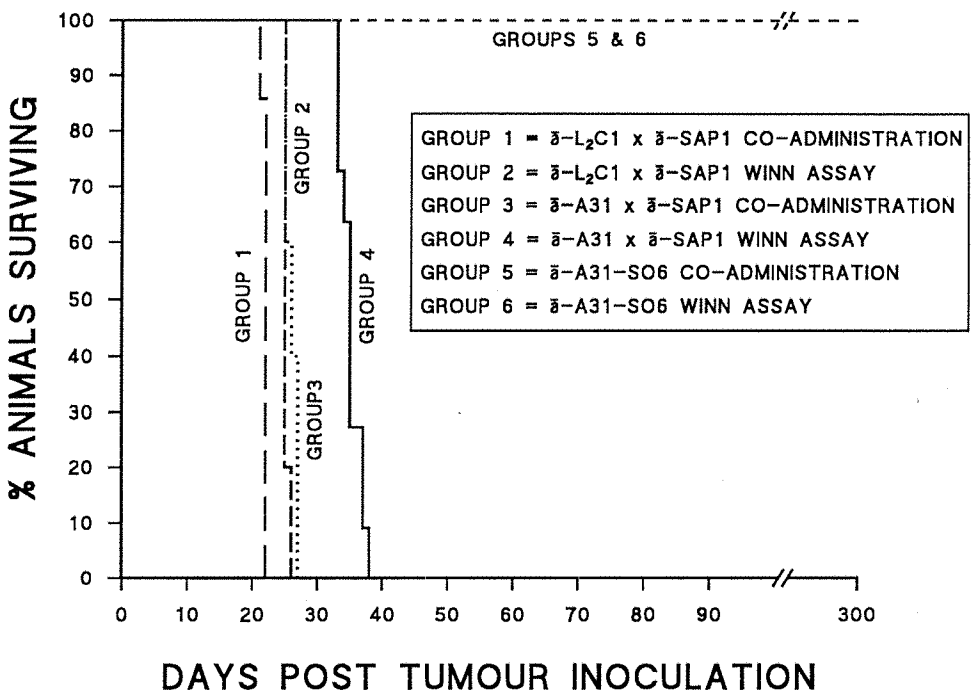


Figure 5.11. Winn assays and *in vivo* co-administration of tumour and antibody derivatives. A31 tumour was (1) treated with the relevant antibodies in vitro for twelve hours before passage into animals or (2) injected i.p. simultaneously at different sites with the saporin loaded bispecific antibody or the immunotoxin.

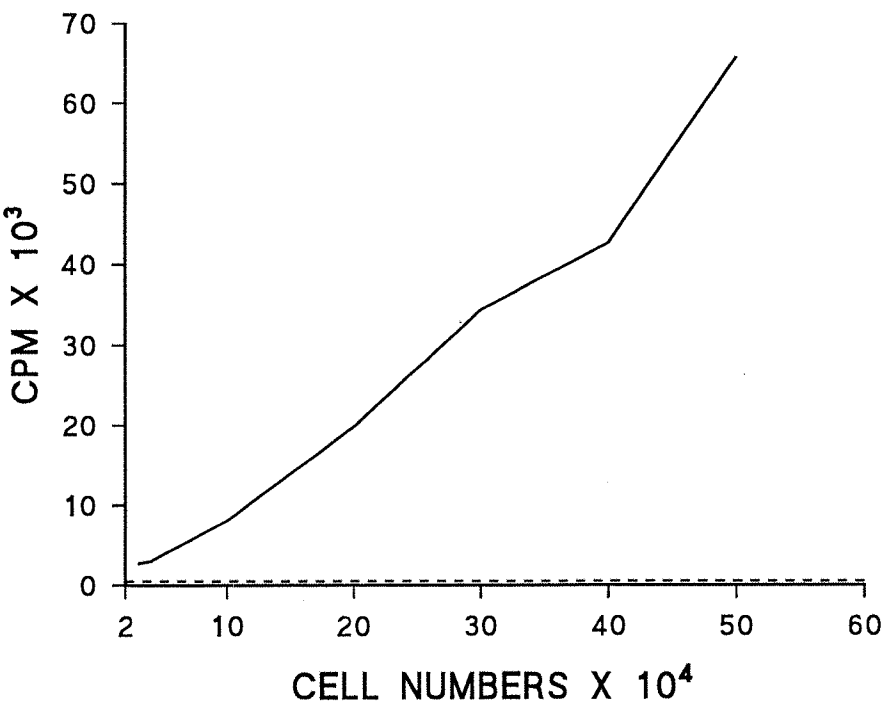


Figure 5.12. Number of A31 cells as a function of ^{3}H-leucine incorporation. The conditions were those used for determination of IC_{50} values of the bispecific antibodies and immunotoxin (Figures 5.4. & 5.5.). The dotted line represents the background cpm in the absence of cells.

5.13. BINDING OF ^{125}I-SAPORIN TO A31 CELLS IN THE PRESENCE OF BISPECIFIC ANTIBODY.

5.13.1. METHOD

Saporin at 1 mg/ml in PBS and Ab1-SO6 at 0.85 mg/ml in PBS were radiolabelled using iodobeads as described in 2.22. Approximately 20% of the total ^{125}I available for each reaction was incorporation into the saporin and the immunotoxin indicating that the iodonation was relatively mild. Binding to the cell surface was determined essentially as described in 2.23. with the exceptions that the final cell concentration in the reaction mixture was 6.25 x 10^6/ml, and the bispecific antibodies were kept constant at 1 µg/ml throughout the saporin dilutions, the bispecific antibodies and saporin being incubated at 37°C for 60 minutes before addition of the cells. The radiolabelled saporin was serially diluted from 1 µg/ml to 1.95 ng/ml whilst the radiolabelled immunotoxin (anti-A31-SO6) was serially diluted from 1 µg/ml to 1.95 ng/ml of antibody, equivalent to diluting the saporin from 360.5 ng/ml to 0.7 ng/ml. A bispecific antibody capable of recruiting saporin and having specificity for the idiotype on guinea pig L_2C cells (anti-L_2C1 x anti-SAP1) was used to determine non-specific binding to the A31 cells.

5.13.2. RESULTS

The cpm recorded for each cell pellet at a given starting saporin concentration, either as free saporin for the bispecific antibodies or conjugated saporin for the immunotoxin, were converted to give molecules of saporin binding per cell. These values were then plotted against the initial saporin concentration. The results shown in Figure 5.13. are as would be predicted from the knowledge of each derivative. The bivalent immunotoxin gives a higher functional affinity than the bispecifics and this together with the covalent linkage of the saporin leads to high surface binding at low concentrations. The stabilisation of the saporin on the cell surface by the combination of the two bispecifics each binding saporin through different epitopes accounts for the increased delivery of the RIP over the separate bispecifics. The more potent activity of anti-A31 x

anti-SAP1 over anti-A31 x anti-SAP3 is in line with the ^{3}H-leucine inhibition assay, and previously published data on anti-SAP1 and anti-SAP3 as components of bispecific antibodies, but does not reflect the identical functional binding affinities published for these two antibodies (French et al., 1991).

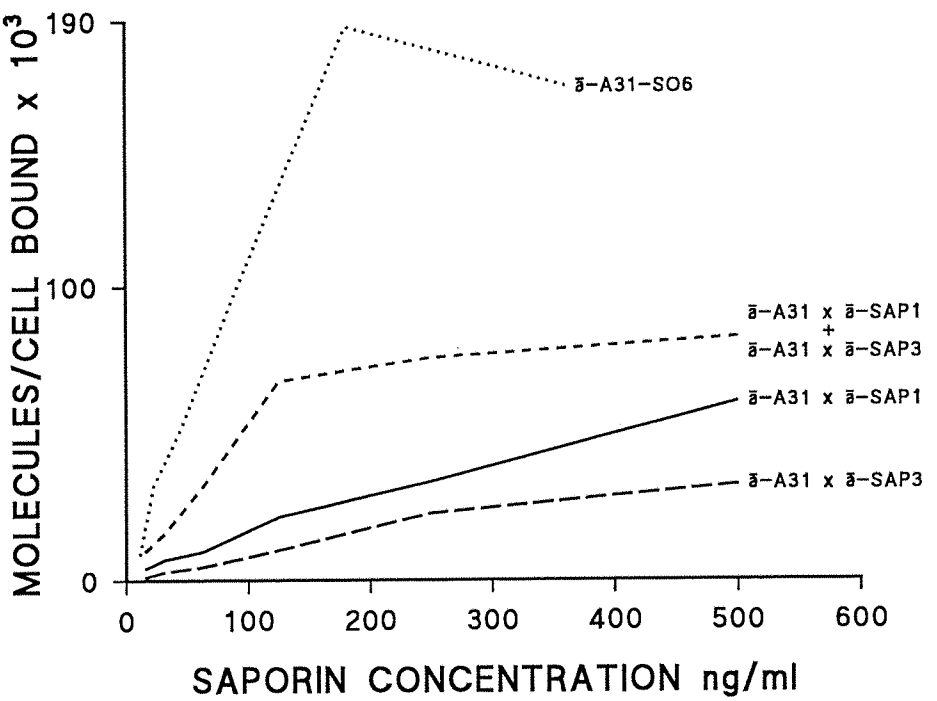


Figure 5.13. Binding of ^{125}I-saporin in the presence of bispecific antibody or ^{125}I-Ab1-SO6 to A31 cells. Bound radiolabelled saporin or immunotoxin was separated from that in free solution by centrifugation of cells through phthalate oils and the radioactivity in the pellets counted.

5.14. DISCUSSION

Developing bispecific antibodies using a rat IgG2a monoclonal antibody to give specificity for the tumour was new territory in that little is known about the molecular structure of the hinge region of this isotype of antibody (Bruggemann, 1988; H.Bazin, personal communication). Available evidence on the sensitivity of this isotype of immunoglobulin to pepsin digestion indicated that it was relatively resistant to pepsin attack (Rousseaux et al., 1983). Rousseaux and colleagues, (1983), suggested pre-treatment of the immunoglobulin at pH 2.8 before digestion with 1% (w/w) pepsin at pH 4.5. for 4 hours. In a preliminary digestion of Mc39-16 it was found that by carrying out the digestion at a slightly lower pH of 4.2 and using 3% (w/w) pepsin for 4 hours the antibody could be efficiency cleaved, and the low pH pre-treatment avoided.

The hinge region disulphide bonds of the rat IgG2a molecule are very sensitive to reducing conditions (Rousseaux et al., 1979) producing almost exclusively the HL product. This is in contrast to the mouse IgG2a molecule in which H_2L and H_2 fragments are formed (Williamson and Askonas, 1968), but is similar to the reduction of human IgG4 (Virella and Parkhouse, 1973). It was not surprising, therefore, that a good Fab reduction product was recovered prior to construction of the bispecific antibodies.

Production of the bispecific antibodies was straight-foreword although the final yields were smaller than previous experience with mouse immunoglobulins (Glennie et al., 1987). Examination of the final products by HPLC indicated the presence of a minor peak in the $F(ab)_3$ position for the Mc39-16 x SI-1 (anti-A31 x anti-SAP3) derivative and a similarly positioned peak almost equal in amount to the $F(ab)_2$ peak in the Mc39-16 x DB7-18 (anti-A31 x anti-SAP1) derivative. The presence of these $F(ab)_3$ peaks suggesting that a minimum of three hinge region disulphide bonds were available. This confirms the determination of the presence of 5 thiol groups in reduced Fab of Mc39-16 by measurement of the production of 2-thiopyridone from 2,2'dithiodipyridine (PDS) and the gene sequencing work of Bruggemann (1988) .

Initial titration of free saporin with A31 tumour cells in culture gave an IC_{50} of 575 ng/ml with concentrations below 50 ng/ml being essentially non-toxic. This IC_{50} figure is lower than that determined for the tumour cells from the guinea pig leukemia, L_2C, which gives an IC_{50} of between 1-18 µg/ml (Glennie et al., 1987; 1988; French et al., 1991). The IC_{50} of saporin toxicity in A31 cells was also lower than values obtained for AKR-A and EL4 lymphoma cell lines, and AKR T-cells (range 1.8-6 µg/ml) but higher than the IC_{50} of 0.3 µg/ml as seen with AKR-B cells (Thorpe et al., 1985).

Overall comparisons of the IC_{50} values reported in the literature for saporin immunotoxins and bispecific antibodies suggest that similar molecules constructed from Mc39-16 were particularly potent reagents. The former immunotoxin and one of the bispecific antibodies (anti-A31 x anti-SAP1) were capable of reducing the IC_{50} for saporin by approximately 1800 and 2900-fold respectively compared to saporin alone. The other bispecific antibody (anti-A31 x anti-SAP3) only reduced the IC_{50} by 230-fold. The higher IC_{50} value of anti-A31 x anti-SAP3 cannot be explained in terms of affinity differences in the anti-saporin arms as both antibodies have similar Ka values; 4.1-5.2 x 10^7 M^{-1} for anti-SAP1 and 2.8-5.2 x 10^7 M^{-1} for anti-SAP3 (French et al., 1991).

In the same study by French and colleagues it was found that the lowest IC_{50} values were obtained by a bispecific antibody constructed from rabbit polyclonal anti-saporin immunoglobulin or a mixture of bispecific antibodies constructed using monoclonal antibodies, both cases giving values in the region of 1.5 x 10^{-11} M (French et al., 1991). These combinations always out performed individual bispecific constructs.

The IC_{50} value quoted is somewhat higher than those values obtained for anti-A31 x anti-SAP1, the cocktail and the immunotoxin in the present study which were in the region of 2-5 x 10^{-12} M. It is in closer agreement, however, with that given in an earlier paper in which the saporin IC_{50} using rabbit anti-saporin Fab's as a component for delivery of toxin to L_2C cells was 7 x 10^{-12} M (Glennie et al., 1988). Both of these values are lower than the IC_{50} reported for a bispecific derivative delivering saporin to a human acute T-

cell leukaemia cell line, HSB-2, utilising anti-SAP1 as the saporin specific arm (Flavell et al., 1991).

Cazzola et al., (1991) found an anti-transferrin-saporin immunotoxin was effective at completely suppressing the colony formation of the human cell lines K-562 and HL-60 at concentrations ranging between 10^{-7}-10^{-10} M. IC_{50} values for anti-Thy 1.1.-saporin immunotoxins ranged between 1.5 x 10^{-11} and 3 x 10^{-12} M depending on the target cells used (Thorpe et al., 1985). Finally the IC_{50} for an OKT-1-SO6 immunotoxin has been reported to be 3.2 x 10^{-10} M in the absence, and 8 x 10^{-11} M in the presence of amantadine (Siena et al., 1988).

It was noted that the bispecific antibody anti-A31 x anti-SAP1 performed equally as well as the immunotoxin Ab1-SO6 and the cocktail of anti-A31 x anti-SAP1 + anti-A31 x anti-SAP3 in suppressing the incorporation of ^{3}H-leucine by A31 cells. A single bispecific antibody would have a lower functional affinity than an immunotoxin using the same anti-idiotype arms. The stabilisation of saporin to the cell surface by a cocktail of two bispecific antibodies reacting to separate, non-blocking epitopes would be expected to out-perform the single bispecific antibodies as at least two arms would need to become free in order to dissociate from the cell surface (figure 5.14.). Indeed the data displayed in Figure 5.13. agrees exactly with these predictions, however, anti-A31 x anti-SAP1 was just as efficient as the immunotoxin and bispecific antibody cocktail at suppressing ^{3}H-leucine incorporation.

The low IC_{50} values for saporin, the immunotoxin, anti-A31 x anti-SAP1, and the cocktail of anti-A31 x anti-SAP1 + anti-A31 x anti-SAP3 suggests that A31 is particularly sensitive to the effects of this particular RIP, when compared to the L_2C tumour for instance. This attribute in itself may explain why anti-A31 x anti-SAP1 performed as well as the immunotoxin and the cocktail of the two bispecific antibodies. If relatively few saporin molecules need to be delivered to the A31 cell surface to achieve the IC_{50}, the binding advantage of the immunotoxin and the cocktail of two bispecific antibodies may not be that important. Later steps in the intoxication of the cell, such as translocation to the cytosol, may play a more important role. This may explain the

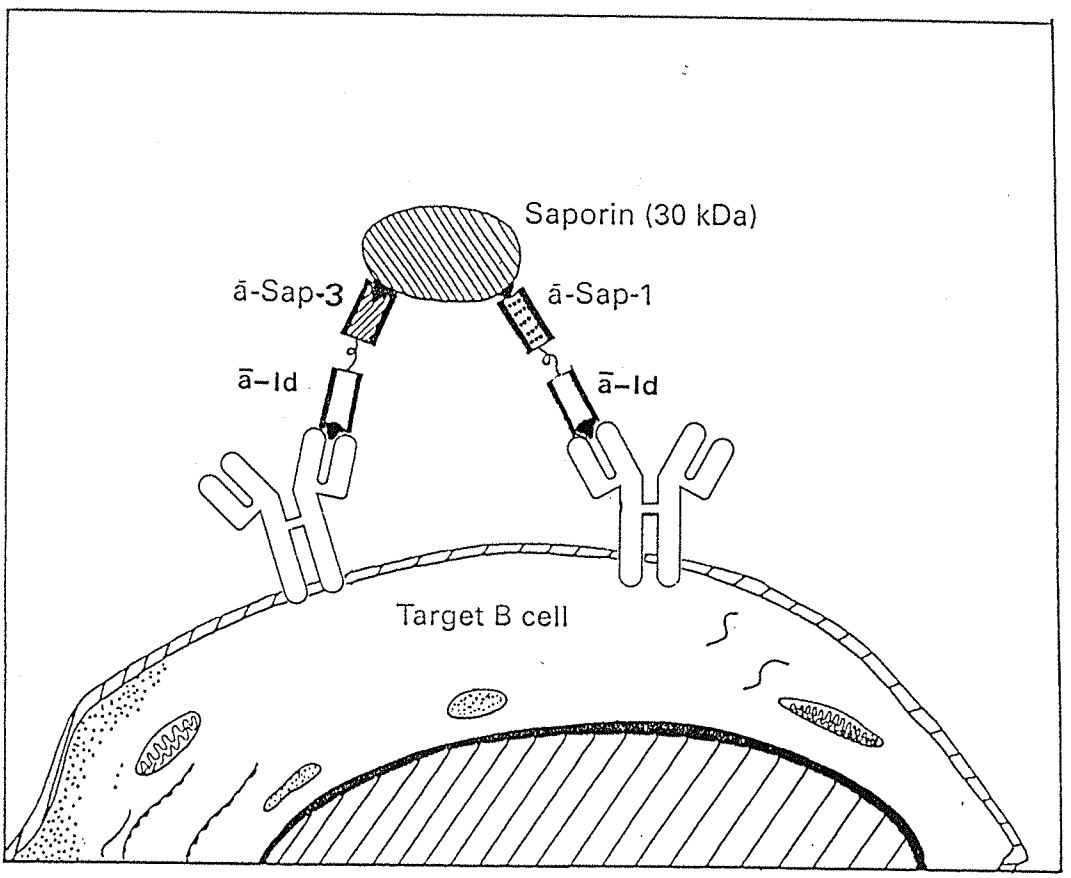


Figure 5.14. Cocktail of two bispecific antibodies recognising different epitopes on the saporin molecule but the same idiotope on the surface IgM of the target B cell.

slightly higher IC_{50} for the immunotoxin compared to the cocktail.

Despite the *in vitro* ^{3}H-leucine inhibition data, when tested *in vivo* in tumour bearing mice the bispecific antibodies failed to protect whilst the immmunotoxin was capable of eradicating tumour. The results of the immunotoxin therapy suggests that this derivative was able to kill all tumour cells in mice inoculated twenty-four hours previously with 5×10^5 A31 cells. If the tumour was left for four days, however, elimination of the tumour was not possible even if multiple injections of immunotoxin were given. Examination of the mice given the latter regime suggested that the injections were well tolerated with no signs of tissue damage at the site of the injections.

Inoculation of fewer cells with a delay of four days before commencement of therapy resulted once more in complete cure suggesting, but not proving, that the failure in therapy may have been simply a factor of too many cells to control with the systemic level of immunotoxin available. Attempts at increasing the therapeutic response by increasing the dose of IT given from 0.1 µg to 1 µg/mouse resulted in the deaths of a number of animals from acute liver damage. The lack of such damage in mice given the same dose of IT, but in the absence of tumour, may indicate that complex formation between tumour idiotype and IT, possibly as a direct result of tumour destruction, may be responsible. Alternatively tumour-free animals may be more resistant to the toxic effects of saporin.

Acute splenic and hepatic damage to mice has been previously reported for saporin conjugates but not at levels as low as 1 µg/mouse. In studies of anti-Thy 1.1.-saporin derivatives, the conjugation to antibody was found to increase the median lethal dose of saporin from 6.8 mg/kg to 0.425 mg/kg (Thorpe et al., 1985). The authors reported that this increase also applied to derivatives formed from $F(ab)_2$ fragments and did not involve recognition of carbohydrates, saporin is not glycosylated, in the immunotoxin or cross-reactive antigens on target tissue (Thorpe et al., 1985; Stirpe et al., 1987).

Evidence exists that of all the RIPs examined saporin may

be particularly toxic for liver tissue in mice. It has been shown that ricin A-chain immunotoxins, even when given to mice at five times the dose of equivalent saporin IT, do not cause the liver damage seen with the latter. Saporin and saporin ITs were also shown to be 30 and 6-fold more toxic to primary cultures of mouse liver parenchymal cells than were ricin A-chain IT, despite the fact that the cells bound 4-5 fold less saporin IT (Blakey et al., 1988).

Investigation into the poor *in vivo* performance of the bispecific antibodies revealed that this was not due to inadequate delivery of saporin to the tumour but due to a failure to kill all the tumour cells even when the latter were treated *in vitro* with bispecific antibody for twelve hours before transfer to the experimental mice. The discrepancy between the observed ^{3}H-leucine incorporation inhibition mediated by the bispecific antibodies and the poor therapeutic effect is difficult to reconcile. The most likely explanations for this lack of effect are either the existence of a population of resistant cells or the ability of saporin intoxicated cells to recover. Further discussion of these possibilities will be postponed until the final discussion at the end of this dissertation.

CHAPTER 6

ANTI-IDIOTYPE X ANTI-CD3 BISPECIFIC ANTIBODY RECRUITING T-CELLS TO THE A31 TUMOUR

6.1. INTRODUCTION

The use of hybrid antibodies to destroy target cells via CTLs is a relatively recent development (Staerz et al., 1985; Perez et al, 1985). These first reports described the use of T-cell specific antibodies (anti-allotype or anti-T3) coupled by SPDP to form heteroaggregates with anti-target cell antibodies, such derivatives were shown to both arm and direct the T-cells to kill the relevant targets.

After these initial reports there emerged a plethora of studies directing T lymphocytes (either mouse or human) against a variety of tumours including colon, ovarian and renal cell carcinomas as well as lymphoma by the use of hybrid antibodies (Jung et al., 1986; Clark and Waldmann., 1987; Lanzavecchia and Scheidegger., 1987; Rammensee et al., 1987; Titus et al., 1987a; Barr et al., 1988; Canevari et al., 1988; Mezzanzanica et al., 1988; Pupa et al., 1988; Scott et al., 1988; Barr et al.,1989; Ferrini et al., 1989; Gravelle and Ochi., 1989; Roosnek and Lanzavecchia., 1989; Van Dijk et al., 1989). Because many of these derivatives were formed by aggregation of intact antibodies or by hybrid hybridoma techniques they contained intact Fc regions raising the possibility for inappropriate killing of Fc receptor bearing cells within the host or destruction of the effector cells, therefore derivatives were sought that were formed from Fab fragments of the individual antibodies (Glennie et al., 1987b; Nitta et al., 1989). The former paper reported the use of bispecific antibodies formed from individual Fab arms linked by oPDM and showed the targeting of human PBL to the guinea pig leukemia L_2C via anti-Fc receptors on the PBL cells.

The $F(ab')_2$ fragment of a hybrid antibody with specificity for both ovarian carcinoma cells and CD3 of human CTL clones has been shown to perform equally as well as the intact antibody in mediating target cell lysis and allowed the CD3 directed lysis to be distinguished from Fc-receptor

mediated lysis (Mezzanzanica et al., 1988). Similarly a bispecific hybrid antibody containing a IgG2b/IgG2c mixed Fc region was ineffective in ADCC but showed a higher titre in mediating killing of tumour targets than a similar bispecific antibody containing a fully functional IgG2b/IgG2b Fc region (Clark and Waldmann, 1987). These data suggests that bispecific antibodies constructed from Fab fragments are likely to exhibit a better therapeutic ratio of target cell to effector cell cytotoxicity.

Most studies on cytotoxicity by CTLs in the presence of bispecific antibody involved *in vitro* assays with CTL clones which were maintained in culture in the presence of IL-2 or freshly isolated PBL pre-incubated with IL-2 before addition to the target cells. There have been only limited studies involving *in vivo* determination of the therapeutic potential of these derivatives. Staerz and Bevan (1986) injected Thy 1.1. positive tumour cells into irradiated non-congenic hosts followed two days later with control antibodies or a bispecific hybrid hybridoma product that reacted with Thy 1.1. and an allotypic determinant found on approximately 25% of murine T-cell receptor β-chains. Later the same day the mice were injected with host spleen cells, pre-activated using mixed lymphocyte culture with B6 spleen cells. Irradiated B6 cells were injected i.p. on day 9 followed by antibody injections on day 12. With this elaborate regime 17/20 control mice died by day 33 the remaining mice within these groups being well at day 68, whilst in the group receiving the bispecific IgG 75% were alive at day 68. The requirement for exposure of T-cells to exogenous IL-2 in order to destroy tumour cells *in vivo* has been examined within a nude mouse model of tumour growth (Titus et al., 1987a). In this model human adenocarcinoma cells were mixed with human PBL coated with an appropriate bispecific antibody reactive with the tumour and CD3 and injected s/c into nude mice. It was shown that the increased lytic activity induced by IL-2 in an *in vitro* assay was not apparent using the Winn-like assay. In addition the importance of CD4 positive cells in tumour growth inhibition was demonstrated.

In the present study an attempt was made to construct a bispecific antibody using the rat anti-mouse CD3 monoclonal

antibody KT3 (Tomonari, 1988) and the A31 anti-idiotypic monoclonal antibody Mc39-16 in order to recruit murine T-cells to the A31 tumour.

6.2. PREPARATION OF KT3 X Mc39-16 BISPECIFIC ANTIBODY

The KT3 immunoglobulin was grown within a hollow fibre bioreactor as described in section 2.6. Culture supernatant from the bioreactor (7 litres) was concentrated using an amicon chamber as described in section 3.6. to a final volume of 600 ml. The immunoglobulin was purified by an ammonium sulphate precipitation as described in 2.9. and affinity chromatography as described in 2.14. The eluted KT3 containing peak (230 mg) was aliquoted and further purified by size exclusion chromatograpy on a 5 cm x 85 cm AcA 34 (LKB) column equilibirated in 0.1 M sodium acetate pH 7.5. Peaks from the individual AcA 34 runs that gave significant immunofluorescence on CBA splenocytes were pooled (90 mg) and analysed by SDS-PAGE and HPLC and found to give an IgG profile essentially free of contaminating proteins.
The procedure used to construct the Mc39-16 x KT3 derivative (designated anti-A31 x anti-CD3) was as described in 2.28. using 14 mg of KT3 $F(ab')_2$ and 12 mg of Mc39-16 $F(ab')_2$ with the former being reacted with the oPDM. After the final reduction, alkylation and concentration within an amicon chamber a fine precipitate was noticed which was removed by filtration through a 0.22 µm filter. The HPLC profile of the reduced bispecific preparation before the final AcA 44 chromatograpy separation is shown in Figure 6.1. The final yield of bispecific product was 2 mg.

6.3. CONFIRMATION OF BISPECIFIC NATURE OF DERIVATIVE BY FLOW CYTOMETRY.

As the light chain of KT3 was known to be lamda (Tomonari, 1988) and a mouse anti-kappa chain specific monoclonal antibody (M35-9F10; Tenovus in house) had clearly shown Mc39-16 to possess kappa chain specificity it was possible to use this difference to confirm the bispecific nature of the derivative. If mouse splenocytes were taken and reacted with the derivative followed by M35-9F10-FITC a positive signal by flow cytometry would indicate the presence

of both the CD3 binding component and the kappa light chain from Mc39-16.

The result in Figures 6.2a. and 6.2b. show that Mc39-16 x KT3 was capable of binding to both murine T-cells and the A31 tumour clearly demonstrating the bispecific nature of the product.

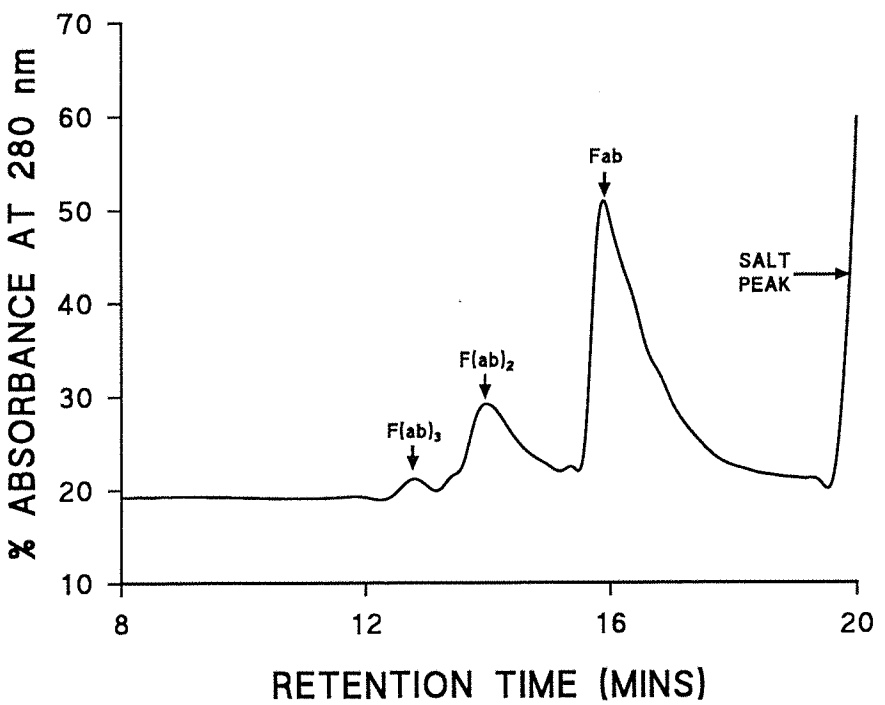


Figure 6.1. HPLC profile of bispecific antibody preparation anti-A31 x anti-CD3 after final reduction but before separation by AcA44 chromatography.

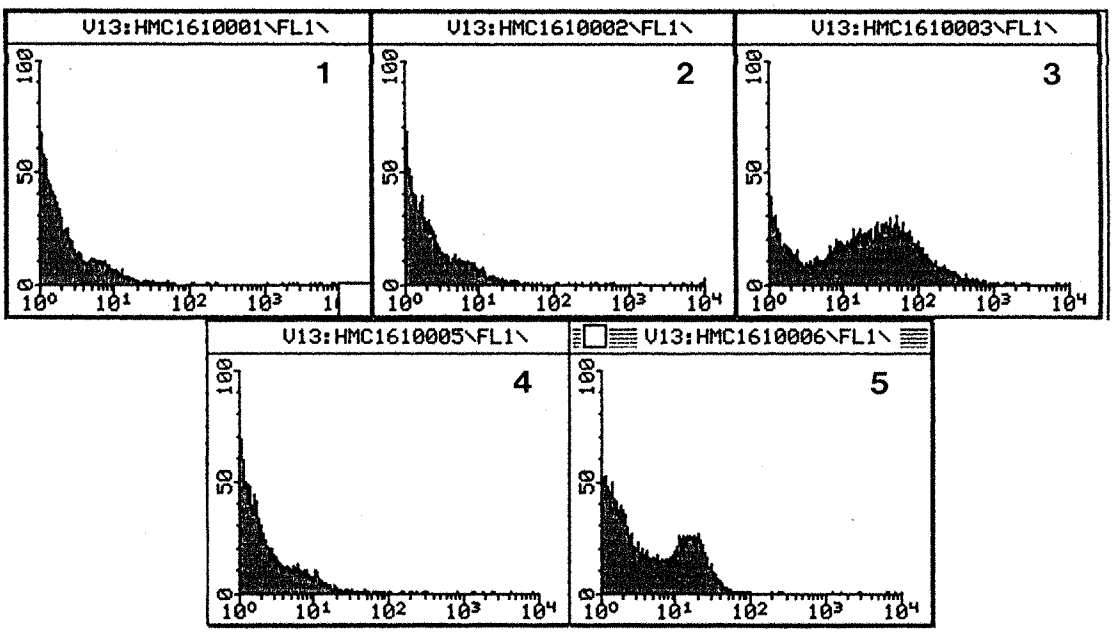


Figure 6.2a Flow cytometry analysis of Mc39-16 x KT3 bispecific derivative using CBA splenocytes. After addition of primary antibody all cells were stained with M35-9F10-FITC conjugate (anti-rat kappa chain: Tenovus in house). Primary antibodies used were (1) PBS control (2) Mc39-16 IgG (anti-A31 idiotype) (3) Mc39-12 (anti-Murine Fcμ) (4) KT3 IgG (anti-murine CD3 (5) Mc39-16 x KT3 (bispecific derivative).

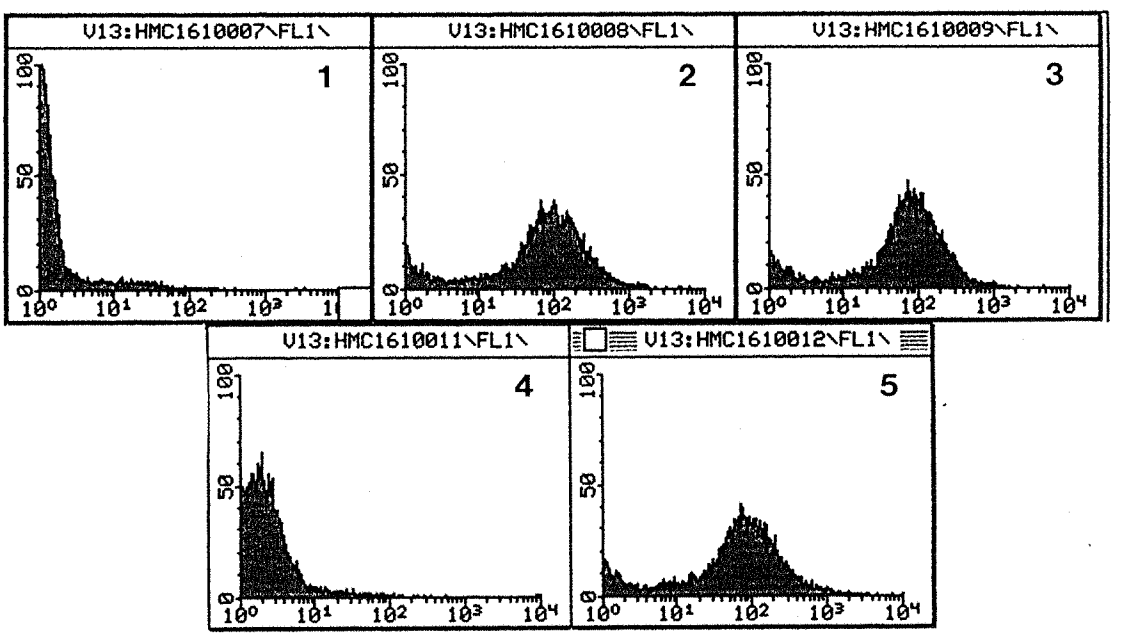


Figure 6.2b Flow cytometry analysis of Mc39-16 x KT3 bispecific derivative using A31 tumour cells. After addition of primary antibody all cells were stained with M35-9F10-FITC conjugate (anti-rat kappa chain: Tenovus in house). Primary antibodies used were (1) PBS control (2) Mc39-16 IgG (anti-A31 idiotype) (3) Mc39-12 (anti-Murine Fcμ) (4) KT3 IgG (anti-murine CD3 (5) Mc39-16 x KT3 (bispecific derivative).

6.4. Mc39-16 x KT3 IN THERAPY

Having established the bispecific nature of the derivative its therapeutic potency was examined by immunotherapy of the A31 tumour *in vivo*.

Twenty-two groups, each containing five mice, were set-up and each animal received a specific number of A31 cells by an i.p. injection as described in Table 6.1. Twenty-four hours later therapy was initiated by an i.p. injection of 5 μg of the appropriate antibody also as itemised in Table 6.1. To maintain the presence of the antibodies within the circulation the treatments were repeated 2 and 5 days later and the animals monitored for signs of illness.

6.4.1. RESULTS

The results of this therapy are shown in Figures 6.3a. and 6.3b. Figure 6.3b. shows that the control antibodies or antibody mixtures gave marginal increases in survival over animals given tumour alone. The only notable prolongation was seen in mice given 5×10^2 tumour cells following treatment with Mc39-16 $F(ab')_2$ or a mixture of the latter with KT3 $F(ab')_2$. It is possible that this slight increase in survival was the result of contamination of the Mc39-16 $F(ab')_2$ preparation with intact Mc39-16 IgG. The control antibodies and antibody mixtures shown in Figure 6.3b. were also injected into animals receiving a larger tumour inoculation (5×10^5 cells) with which the slight prolongation in survival with Mc39-16 $F(ab')_2$ was completely lost (data not shown). At this larger inoculum all control antibodies and and antibody mixtures were grouped tightly together and did not extend beyond thirty days post tumour inoculation and were therefore essentially without therapeutic benefit.

TABLE 6.1. Mc39-16 x KT3 THERAPY TREATMENT GROUPS

GROUP	NUMBER OF CELLS	TREATMENT
1	5×10^2	PBS
2	5×10^5	PBS
3	5×10^2	anti-CD3 IgG
4	5×10^5	anti-CD3 IgG
5	5×10^2	anti-CD3 $F(ab')_2$
6	5×10^5	anti-CD3 $F(ab')_2$
7	5×10^2	anti-A31 IgG
8	5×10^3	anti-A31 IgG
9	2.5×10^4	anti-A31 IgG
10	5×10^4	anti-A31 IgG
11	5×10^5	anti-A31 IgG
12	5×10^2	anti-A31 $F(ab')_2$
13	5×10^5	anti-A31 $F(ab')_2$
14	5×10^2	anti-A31 x anti-SAP3
15	5×10^5	anti-A31 x anti-SAP3
16	5×10^2	anti-A31 x anti-CD3
17	5×10^3	anti-A31 x anti-CD3
18	2.5×10^4	anti-A31 x anti-CD3
19	5×10^4	anti-A31 x anti-CD3
20	5×10^5	anti-A31 x anti-CD3
21	5×10^2	anti-A31 $F(ab')_2$ + anti-CD3 $F(ab')_2$
22	5×10^5	anti-A31 $F(ab')_2$ + anti-CD3 $F(ab')_2$

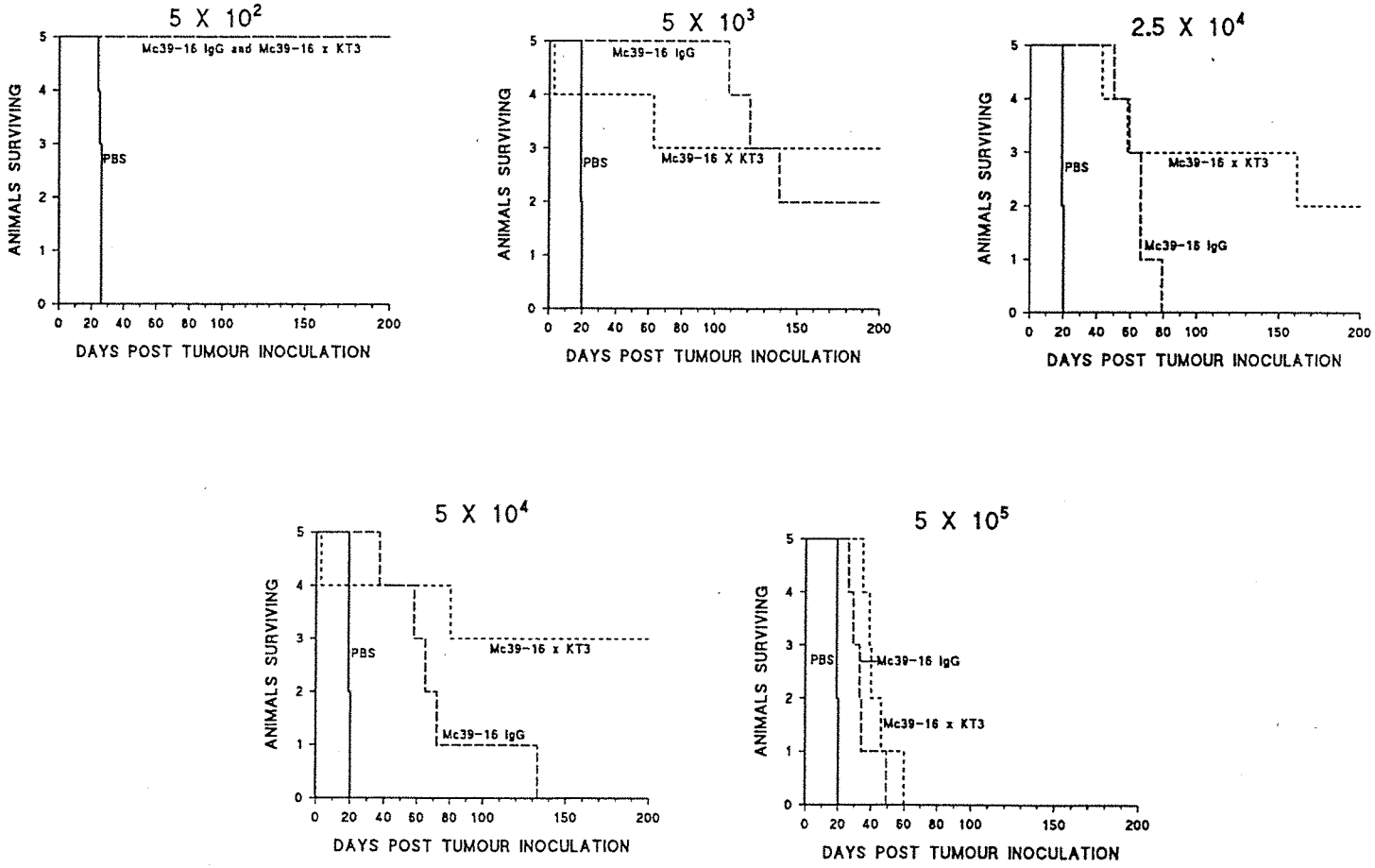


Figure 6.3a Immunotherapy of A31 tumour with Mc39-16 x KT3 bispecific antibody. Each individual graph represents the therapy observed at a particular cell inoculum. Each mouse received 5 μg of antibody i.p. at 1, 3, and 5 days post tumour inoculation.

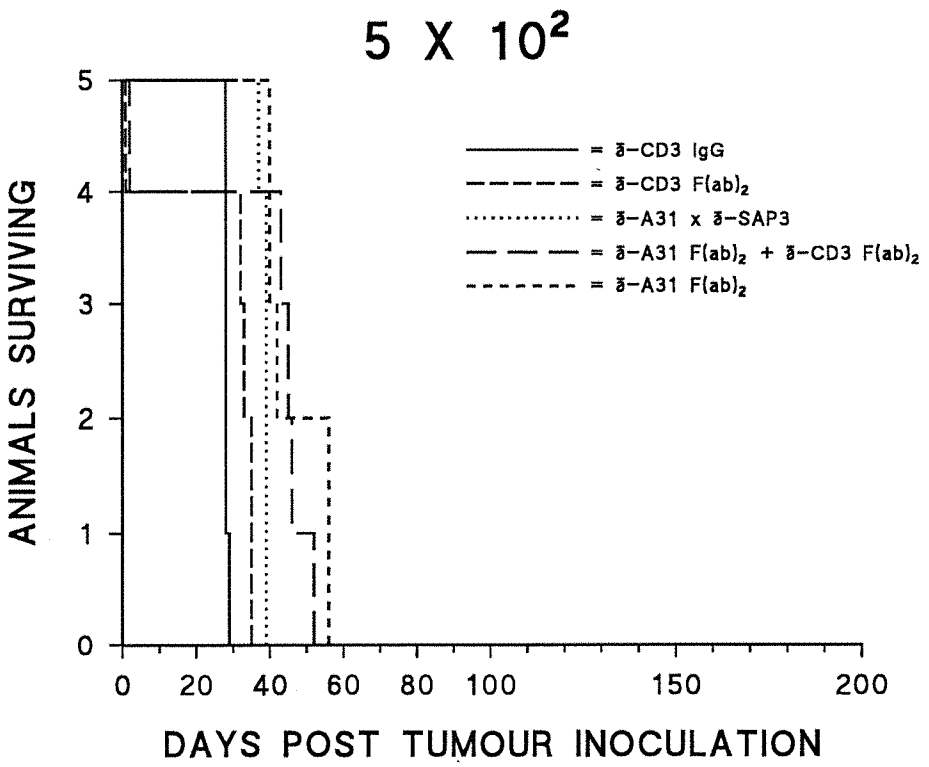


Figure 6.3b. Immunotherapy of A31 tumour with Mc39-16 x KT3 bispecific antibody. Graph represents the therapy observed with control antibody mixtures at a defined A31 cell number inoculum. Each mouse received 5 μg of antibody i.p. at 1, 3, and 5 days post tumour inoculation.

Both Mc39-16 IgG and the bispecific derivative Mc39-16 x KT3 gave prolonged survival to mice given 5 x 10^4 tumour cells or less. Figure 6.3a shows all animals given 5 x 10^2 tumour cells and 50% of those receiving 5 x 10^3 cells survived beyound 200 days. Interestingly, at tumour inoculations five and ten fold higher than this only the bispecific derivative was able to show significant protection, with long-term survivors in animals receiving both 2.5 x 10^4 and 5 x 10^4 tumour cells. However, above this level of tumour inoculation neither derivative show significant protection.

Tumour cells emerging from therapy were examined by flow cytometry essentially as described in 2.18. with the exceptions that a mouse anti-rat Fcγ specific FITC was used (1:40 dilution in PBS; Stratech Scientific Ltd, Luton, Beds, U.K.). Typical profiles are shown in Figure 6.4a. and 6.4b. In all cases the flow cytometric analysis indicated that the animals had succumbed to A31 tumour which still retained the idiotope recognised by Mc39-16. Although staining, with KT3 (anti-CD3), of spleen cells isolated from the final mouse to succumb in group 20 suggested that there may have been a minor population of $CD3^+$ cells remaining within the tissue, (compare Fig.6.4a. (1) with Fig 6.4a. (4), Figure 6.4b. shows a similar population within the PBS control indicating that this is probably not of any consequence.

6.5. MODULATION OF SURFACE IDIOTYPIC IgM BY MC39-16 X KT3.

6.5.1. INTRODUCTION

The possible presence of aggregates in our bispecific preparations was a concern as these might not only reduce the serum half-life of the material but would also have other physiological effects on tumour therapy. These aggregates may cross-link T-cell receptor complexes before delivery of the bispecific antibody to the tumour cell surface or by modulation from the tumour surface after cross-linking the membrane immunoglobulin. Determination of the presence of complexes within the therapeutic antibody solution was therefore examined by monitoring the degree of modulation of surface IgM from the surface of A31 cells by flow cytometry.

6.5.2. RESULTS

The method employed to determine modulation of surface immunoglobulin has been described in 2.18.1. The percentage reduction in maximum fluorescence with time for Mc39-16 IgG and Mc39-16 x KT3 shown in Figure 6.5. From the modulation studies a control antibody having reactivity towards the idiotype carried on BCL_1 cells and the saporin recruiting bispecific antibody Mc39-16 x DB7-18 induced minimal modulation of surface IgM of A31 cells. The bispecific antibody Mc39-16 x SI-1 induced modulation, but this did not

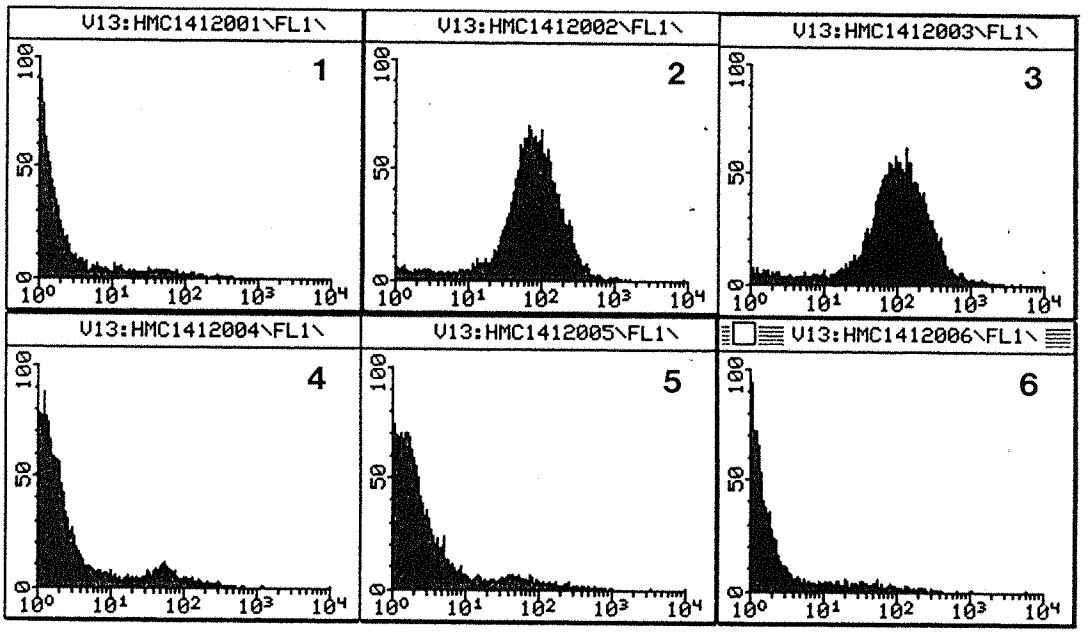


Figure 6.4a Flow cytometer analysis of spleen cells isolated from final mouse to succumb in group 20 (5×10^5 tumour cell inoculum with Mc39-16 x KT3 therapy). Primary antibodies used for staining were (1) PBS control (2) Mc39-12 (3) Mc39-16 (4) KT3 (5) anti-L_3T_4 (6) M35-9F10.

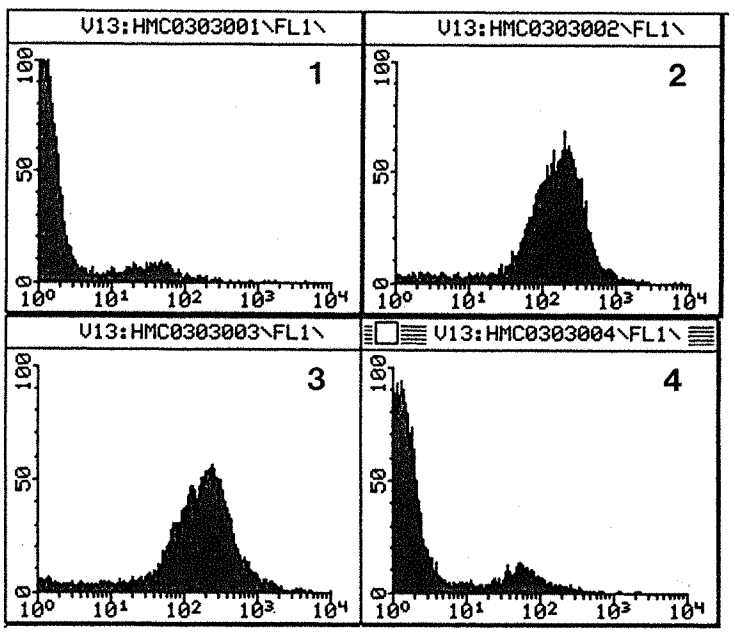


Figure 6.4b Flow cytometer analysis of spleen cells isolated from final mouse to succumb in group 8 (5×10^3 tumour cell inoculum with Mc39-16 IgG therapy). Primary antibodies used for staining were (1) PBS control (2) Mc39-12 (3) Mc39-16 (4) KT3.

exceed a reduction below 80% of the initial fluoresence even after four hours exposure. The T-cell recruiting bispecific antibody Mc39-16 x KT3 did produce significant modulation such that at four hours only one quarter of the initial surface fluoresence remained. This degree of modulation was only eclipsed by that induced by the A31 anti-idiotypic monoclonal antibody Mc39-16.

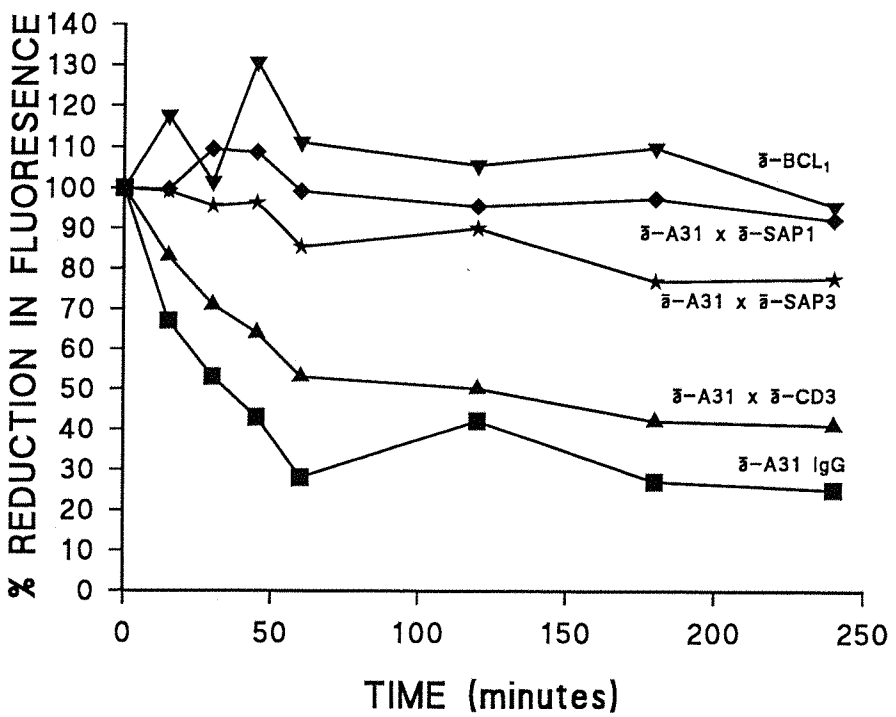


Figure 6.5. Modulation of A31 surface IgM by anti-idiotypic monoclonal antibody and Mc39-16 x KT3 bispecific antibody. The mean fluoresence observed at a particular time point is expressed as a percentage of the initial mean fluorescence observed at time 0.

6.6. DISCUSSION

A number of experimental therapies using T-cell recruiting bispecific antibodies have appeared in the literature in recent years.

In a similar study Damanet and colleagues (1991) used the murine 38C13 B-cell lymphoma as a model test system. As in the present study an anti-idiotype x anti-CD3 bispecific antibody was used, however this was isolated from a hybrid hybridoma. The bispecific antibody in that study therefore contained a Fc region composed of disparate heavy chains with some activity *in vivo*, but not *in vitro*, suggestive of a limited ADCC towards the tumour. The mice received i.p. injections of the tumour cells (range 1000-5000) followed 48 hours later by the appropriate control or specific therapy.

The efficacy of the anti-Id x anti-CD3 bispecific antibody was shown to be both dose and tumour load sensitive. Long term survival (> 60 days) of animals given a single i.v. injection of bispecific antibody after tumour cell inoculation of 1×10^3, 2.5×10^3, or 5×10^3 being 80, 50 and 40% respectively. Interestingly, as mentioned above, protection was also afforded by a bispecific antibody that had anti-id binding but lacked anti-CD3 activity (50% protection at 1×10^3 and 2.5×10^3 tumour cell inoculation). The lack of protection given by this non-CD3 binding antibody to a mouse strain with disfunction in macrophage function and FcR-γ dependent ADCC was used to show that a certain degree of ADCC was possible by these bispecific antibodies despite the mis-matched Fc region. Allogeneic immunisation of mice was used to generate activated T-cells *in vivo* prior to injection of tumour cells. This approach led to an increase in protection over that observed with naive, unstimulated animals. Approximately 70% of the mice survived a tumour cell inoculum of 5×10^3 and, providing two immunisations were given, 50% of mice survived an i.p. injection of 1×10^4 cells following treatment with the bispecific antibody.

The same murine lymphoma model has been used by a second group (Weiner and Hillstrom, 1991) who once again used hybrid hybridoma technology to prepare an anti-idiotype x anti-CD3 IgG bispecific antibody with the anti-CD3 component being a

hamster IgG. Therapeutic conditions were chosen such that use of the anti-idiotypic monoclonal antibody IgG was ineffective. Experiments were initiated by injection of 5 x 10^3 tumour cells i.p. on day 0 followed 48 hours later with 10 μg of the appropriate antibody. All untreated control mice succumbed within 35 days and all but one animal receiving anti-idiotypic antibody alone died within the same period. Treatment with a mixture of anti-idiotypic monoclonal antibody plus anti-CD3 monoclonal antibody did produced a significant increase in survival, 10% survival at 47 days, with one mouse out of fifteen surviving significantly longer. The specific anti-idiotype x anti-CD3 bispecific antibody gave 11 out of 15 mice long term survival, >50 days. Interestingly, whereas tumour aspirates from mice succumbing to therapy after treatment with anti-idiotypic IgG or various mixtures of antibodies showed the presence of idiotypic positive tumour cells, 50% of tumours emerging in mice following bispecific antibody therapy revealed no surface idiotype, i.e. antigen negative variants. No bystander killing was observed during these *in vivo* studies.

A different murine tumour was used by Brissinck and co-workers (1991) to investigate the therapeutic potential of anti-id x anti-CD3 bispecific antibody. In this case the tumour was BCL_1 but once again the bispecific antibody component was the product of hybridoma x hybridoma fusion. An important difference in this report was that *in vivo* assessment of anti-tumour activity involved a delay of 8 days between tumour inoculation and administration of the therapeutic antibody. At low tumour loads (5 x 10^2 cells/mouse) a control antibody functional for anti-id but incapable of binding CD3 gave some protection, once again suggesting that a bi-isotypically mismatched antibody can be active in ADCC at least to a limited extent. This protection was lost at higher tumour loads at which point clear therapeutic benefit from the bispecific antibody was seen, indeed 60% of animals given 5 x 10^4 tumour cells survived longer than 150 days. An important finding was that although dormant tumour could be found in long term survivors treated with the mismatched antibody no such dormant tumour could be found in those animals treated with the bispecific antibody

indicating that the latter mice had been cured of the tumour. This result might indicate that the bispecific antibody was responsible for bystander killing (Tite and Janeway, 1984; Ochi et al., 1987), not seen with conventional anti-idiotypic antibody, which prevents the survival of dormant cells.

In light of these studies the investigation into the therapeutic potential of anti-idiotype x anti-CD3 within the A31 model had some important differences. In the present study oPDM was also used to construct anti-id x anti-CD3 bispecific antibodies from the Fab fragments of each antibody. The anti-CD3 component was the monoclonal antibody KT3 which had not been previously used as a component of a bispecific antibody but was known to be able to induce activation and cytolytic activity in murine T-cells (Tomonari, 1988), therefore these initial studies did not involve prior *in vitro* or *in vivo* activation of the T-cell population. The bispecific antibody component was unlikely to contain an Fc region therefore any significant role for ADCC in the therapy could be discounted. The bispecific arms were held by a thioether bond rather than the disulphide bonds found in hybrid hybridoma products. From the immunotoxin and other therapies it appears that the A31 tumour does not show a high propensity to produce idiotypic variants even when placed under a high selection pressure. This implies that the entire tumour population should be targeted by the derivative. As the immunotoxin therapy has shown there can be a fine-cut off between a curable tumour load and a tumour mass that cannot be cleared, it was important to perform this therapy against a range of initial tumour cell numbers.

In view of our general experience preparing bispecific antibodies (Glennie et al., 1993), the final yield of bispecific antibody in the current work was disappointing being only 7-8% of the total Fab. The factors contributing to this were the low efficiency of the conjugation, as demonstrated by the HPLC profile, evidence of aggregation upon concentration of the reaction mixture within the Amicon chamber and a severe cut made from the AcA 44 peak to ensure a clean product. The final derivative did however demonstrate reactivity with normal PBL isolated from CBA mice and also with the A31 tumour as shown by flow cytometry. In testing

the derivative for its ability to protect mice inoculated with the A31 tumour twenty-four hours previously it was important to assess the effects of control mixtures of antibodies. With a tumour load of 5 x 10^5 cells all the animals succumbed between nineteen and twenty-nine days when injected with PBS or control antibodies, however at a tumour load of 5 x 10^2 cells greater variation was seen. In particular the bispecific antibody Mc39-16 x SI-1 extended the survival of the mice from 24-26 days to approximately 39 days. However the greatest protection within the control groups was seen with Mc39-16 $F(ab')_2$ or a mixture of Mc39-16 $F(ab)_2$ + KT3 $F(ab)_2$ with the last animals succumbing within these groups at 56 and 52 days respectively. It is almost certainly the case that this protection at such a low tumour cell inoculum was due to the presence of a small amount of contaminating Mc39-16 IgG within the $F(ab')_2$ preparation. In the test groups the Mc39-16 IgG gave long term protection to a proportion of the mice given 5 x 10^3 tumour cells and protected all mice given 2 x 10^2 tumour cells. However above a tumour cell inoculum of 2.5 x 10^4 Mc39-16 IgG became incapable of protecting the mice. In contrast the bispecific antibody derivative showed protection at double this tumour burden. There was some variation in protection observed, with more animals surviving in the 5 x 10^4 cell group than the 2.5 x 10^4 cell group, however with small sample sizes such variation is not unexpected within a bioassay. Finally neither the anti-Idiotypic IgG or bispecific antibody derivative could protect at a cell inoculum of 5 x 10^5 although once again the bispecific antibody derivative increased the survival of the animals slightly more than Mc39-16 IgG.

Flow cytometry of tumour cells isolated from mice succumbing at day 60 and 139 post tumour cell inoculation indicated a normal surface phenotype including expression of surface idiotypic IgM making it unlikely that these animals fell prey to tumour variants. Surface modulation studies confirmed that the saporin recruiting bispecific derivatives described in chapter 5 did not cause significant modulation of surface IgM, however the T-cell recruiting derivative was capable of such modulation. The possibility exists that this

modulation was caused by cross-linking of the bound bispecific antibody via T-cells present within the splenocyte preparation, however as a terminal A31 spleen was used the number of T-cells present would be extremely low making this possibility unlikely. It is more probable, considering the precipitate observed during the conjugate formation, that the modulation was caused by the presence of complexes within the bispecific preparation. If this was the case then the therapeutic potential of the derivative may have been compromised. Despite this the overall therapeutic results are similar to those of most of the other groups described above taking into consideration different tumour loads and whether or not pre-activation of T-cells was used. The exception is that result reported by Brissinck and colleagues (1991) with an initial tumour load of 5×10^4, a delay of eight days before commencement of therapy and a single i.v. injection of 5 µg of derivative making this an overall outstanding therapeutic result. However this latter result is complicated by the possibility of ADCC having played a role in the overall effectiveness of the therapy. The present study may have also been complicated by the presence of Fc within the bispecific antibody preparation, however at a tumour load of 5×10^4 this would have had a minor effect leaving the bispecific antibody, anti-idiotype x anti-CD3, responsible for clearance of tumour.

CHAPTER 7

SUMMARY AND CONCLUSIONS

A31 is a murine lymphoma which has been in existence for many years but which has only been studied in any detail over the last few years (Cobb et al, 1986). This work has identified A31 as a B cell tumour resembling lymphoblastic lymphoma in man. Cytology showed the tumour to express IgMκ on the surface (more recently lower levels of surface IgD have also been reported; see Dyke et al., 1991) and secretion profiles demonstrated it to be a low secreting tumour.

It was found that LPS gave significant stimulation (as measured by ^{3}H-thymidine incorporation) up to an LPS concentration of 125 μg/ml, above this value there was reduced incorporation of ^{3}H-thymidine. In immunotherapy experiments, irradiated A31 tumour cells were not immunogenic when inoculated into mice and this treatment gave no protection. Information on the histopathology of animals injected with tumour 20 and 27-52 days previously was also given. At 20 days infiltration of tumour within the B cell regions of spleen, Peyer's patches and lymph nodes was found as was invasion of liver tissue. At 27-52 days splenic architecture was obliterated and Peyer's patches and lymph nodes were packed with tumour and extensive neoplasm was seen within the liver. Data on passive protection using whole body X-radiation or treatment with cyclophosphamide and vincristine sulphate has been reported (Cobb et al., 1986). Animals had been injected with 10^5 tumour cells prior to treatment and the results suggested that cyclophosphamide was the most effective therapy, extending survival of the animals to 72 days post tumour inoculation compared to 36 days for control animals. The only other data available on active immunization as a mode of protection *in vivo*, (Dyke et al., 1991), showed complete protection of mice immunised with A31IgM-KLH and challenged with 5×10^5 tumour cells. Persistence of protection was shown to extend to at least 170 days post-immunisation. In this study the presence of dormant A31 tumour cells was found in three-fifths of animals surviving between 112-246 days post tumour challange. Protection against tumour challange could also be initiated

by the transfer of immunised mouse serum but not by transfer of immune spleen cells. The transferred serum was protective providing at least 50% of the cell-associated immunoglobulin was bound by antibody showing a primary role for the latter in this system.

Targeting of T-helper cells (Th cells) by utilisation of the antigen presentation activity of A31 tumour cells has also been examined as a therapeutic procedure (Montgomery et al., 1992). In this study purified protein derivative (PPD) of tuberculin was delivered to the A31 tumour using the rat anti-idiotypic monoclonal antibody Mc39-16. After internalisation and processing the PPD was re-expressed on the tumour cell surface in the context of MHC class II molecules which gives stabilised membrane targets for PPD-specific Th cells. Despite the secretion of TNF by the latter, A31 cells were resistant to Th-mediated cytotoxicity although the T cells did activate macrophages which in turn were cytostatic towards the tumour.

This dissertation extends these studies to include passive protection of tumour-bearing mice with anti-idiotypic antibodies and antibody derivatives designed to kill A31 tumour cells by delivering ribosomal inactivating protein or cytotoxic T cells.

7.1. ISOLATION OF A31 IDIOTYPIC IGM AND ANTI-IDIOTYPIC MONOCLONAL ANTIBODIES.

The recovery of idiotypic IgM by 'rescue' fusions (Levy and Dilley, 1978) in which non-immortalised B cells are fused to immortalized plasmacytoma cells is a well tried technique to obtain usable quantities of immunoglobulin from low secreting human B cells (Hatzubai et al., 1981; Abrams et al., 1984., Giardina et al., 1985). Human rescue fusions can on occasions give reasonable levels of immunoglobulin but they usually secrete low levels of idiotypic IgM and have the additional problem of the instability of the heterohybrids leading to loss of chromosomes, frequently involving preferential loss of the human component (Croce et al., 1980). The 'rescue' fusion of A31 cells to NS-1 cells should have lacked some of these problems as both cell sources were of murine origin, albeit one being CBA and the other BALB/c.

A clone (Mc22 D5) that was stable in culture was isolated and produced 19s IgM, however transfer of cells to pristane primed mice, including animals that had been immunosurpressed, failed to generate ascites containing idiotypic IgM. The reason(s) for this instability when grown *in vivo* are not known, but the inability to maintain clones which secrete immunoglobulin suggests that non-secreting variants have a significant growth advantage and may out-grow the original hybridoma. It is unlikely that the A31 idiotypic IgM itself is the cause of this problem as a rescue hybridoma has recently been isolated that does produce A31 IgM within the peritoneal cavity of non-surpressed, pristane primed F1 mice (Dyke et al., 1991). Here again, however, vigorous cloning was required to prevent the outgrowth of non-secreting clones. Syngeneic anti-idiotypic monoclonal antibodies could not be isolated although detection of such antibodies within the serum of immunised mice indicates a response had been made. In contrast, isolation of rat anti-A31 idiotypic monoclonal antibodies was relatively straightforward.

The main problem with these derivatives was the isolation of large quantities of immunoglobulin. The use of nude mice is not recommended due to the cost and special facilities required for their care together with the fact that the ascities from individual animals must be screened as not all the mice produce antibody-containing ascities. Similarly extreme immunosurpressive regimes with conventional mice or rats can give ascities containing the anti-idiotypic rat immunoglobulin but once again careful screening is required to pool only antibody-containing material. Recent advances in bioreactor systems, such as hollow-fibre and 'fluidised' bed micro-carrier reactors, have given our laboratory a viable means of producing large amounts of both rescued IgM and rat anti-idiotypic monoclonal antibodies for future studies.

The rat anti-idiotypic monoclonal antibody chosen for further studies (Mc39-16) was shown to have a functional association constant for A31 cells of approximately 3.8×10^8 M^{-1}. It should be emphasised that binding of bivalent antibody to bivalent surface IgM does not meet the criteria used in determining intrinsic affinities as seen with haptan

binding to antibody in free solution. The mobility of the antigen within the membrane, orientation of the epitopes i.e. in terms of membrane bound immunoglobulin the segmental flexibility between the Fab' 'arms', whether monogamous or bigamous binding predominates and interaction between bound and free antibody can all affect the assumptions used in the ideal situation (Elliott et al., 1987; Larsson, 1989). The plotting of data as a Scatchard plot, although commonly used, can lead to mis-interpretation in some circumstances. The most likely problem is the fitting of the data to a straight line when this is not warranted (Klotz, 1985). As this latter report explains, results can be checked by using a plot of molecules bound as a function of free antibody concentration, which should give a S-shaped curve with a definite saturation point. The data from the present study fulfils this criteria indicating that the saturation point observed was genuine (section 3.10).

7.2. CHARACTERISATION OF THE A31 TUMOUR

From the Scatchard plot the total number of A31 IgM molecules at the surface of the cell is approximately 3.8×10^5. This is the first formal determination of this feature of A31 cells. Despite this high level of surface expression, the level of idiotypic IgM in the serum of terminally ill animals was shown to be relatively low. Idiotypic IgM levels are unlikely to be a major factor in the present study where most therapies were initiated twenty-four hours after tumour cell inoculation. However, in situations where it might be required to give therapy to animals with established tumour, low secretion might be a major advantage of A31 over other experimental lymphomas (Brissinck et al., 1991).

Immunohistology of the lymphoma suggests, as with other murine lymphomas, the tumour preferentially localises to the spleen, initially locating to the marginal zone before invading the B-cell areas. The tumour cells remain localised within the B-cell region for approximately ten days by which time tumour infiltration of the red pulp and T cell areas is evident. This initial localisation to the B-cell areas presumably involves the interaction of specific homing receptors on the tumour and addressins within the B-cell area

of the peripheral white pulp. Although considerable knowledge has been gained about the homing of lymphocytes via high endothelial venules (HEV) to peripheral lymph node, or mucosal surfaces and inflamed endothelial surfaces (Springer, 1990; Larson and Springer, 1990; Shimizu et al., 1992) little is currently known regarding the homing of B lymphocytes to the spleen. Some evidence suggests complement receptors may be important in initial localisation of B lymphocytes to this site (Kraal et al., 1985). The importance of sialic acid and complex N-linked oligosaccharides in the homing of lymphoma cells to the spleen has also been suggested (Schaaf-Lafontaine et al., 1985).

Whilst the growth and homing of A31 within the murine spleen does not follow the description of human lymphoma given by van-Krieken and colleagues (1989), it does parallel the description of lymphoma invasion of human spleen given by Falk (1991). The author of this latter report investigated 450 splenectomy specimens of malignant lymphoma and described the histology patterns seen in terms of the classification of the neoplasm. The ablation of the splenic architecture by A31 is consistent with aggressive high grade non-Hodgkin's lymphoma as described by Falk, however we must always remember that A31 has been passaged through many animals and it is unwise to draw close comparison with any human counterparts.

7.3. THERAPY OF A31 WITH ANTI-IDIOTYPIC IgG

The rat anti-A31 idiotypic monoclonal antibody Mc39-16 gave significant protection to tumour bearing mice. Comparison of the protection provided by intact IgG anti-idiotype (Fig 5.7.) with that given by the $F(ab')_2$ fragment (therapy group 13; 6.4.1.) indicates that this protection was Fc mediated. At low tumour loads of 5×10^2 and 5×10^3 three separate injections of 5 µg Mc39-16 IgG/mouse was capable of giving long term survival to 100% or 40% of mice respectively. No antibody treatment protected animals given a tumour load of 2.5×10^4 cells. Similarly when mice were given 5×10^5 tumour cells multiple injections of 100 µg of therapeutic antibody also could not induce long term survival. An interesting finding in these studies was that

tumour emerging after antibody treatment, designated A31-LTS, was more resistant to subsequent therapy than the original wild-type tumour. This pattern of tumour escape may reflect either the level of variants present within the population or the distribution of tumour to privileged sites with increasing tumour load. The available data cannot distinguish between these two possibilities, nor at present can the nature of the partial resistance exhibited by A31-LTS cells be explained although this may reside in metabolic changes within the cell rather than changes in surface phenotype.

The transfer of various tissues from Mc39-16 IgG treated A31-LTS inoculated mice produced a particularly interesting finding with the identification of dormant tumour within an animal, surviving over 300 days, that had received bone marrow tissue from an animal that had been given anti-idiotypic therapy. Isolation of this tumour was by transfer of splenic tissues from the latter animal to naive mice. The endpoint dilution excision assay is a very sensitive 'all or nothing' determination of minimal residual disease (Hagenbeek, 1987). It does not, in the way used here, quantify the actual number of tumour cells present within each tissue. The only statement that can be made is that, under the conditions used for this experiment, tumour cells were present in all sites examined in untreated animals and also within all sites in treated animals. This statement is further qualified by the comment that within the blood of some treated animals and the bone marrow of all treated animals the tumour was eradicated (some bone marrows only) or held in a dormant state broken only by transfer to naive recipients.

Dormancy is an important issue in the treatment of human neoplasms and has been well documented (Hadfield, 1954; Gordon-Taylor, 1959; Wheelock and Brodovsky, 1982,1983; Israel, 1990; Meltzer, 1990). A number of mechanisms can be responsible for establishment of the dormant state including: avascularity and sequestration; constitutive dependency for growth factors such as found with oestrogen-dependent carcinoma which remains latent until administration of oestrogen (Nobel and Hoover, 1975) or the dependence of SJL/FCC lymphomas on the presence of T_H cells for

proliferation (Alisauskas et al., 1990); and immunological restraint (reviewed by Wheelock and Robinson, 1983). In the present study avascularity is unlikely to be the cause as the tumour would not be present as a large nodule but as single cells within the spleen of animals receiving the transferred tissue. Sequestration could be a possible mechanism if, for instance, the antibody treated cells relocated in the spleen at a site that was not optimal for growth. Dependency on growth factors is unlikely as the tumour is located in an organ that it normally invades quite readily. The most likely explanation is some form of immunological restraint induced by the anti-idiotypic monoclonal antibody that is broken upon disaggregation of the splenic tissue. In the present study it would appear likely that the constraint imposed on the A31 tumour by the anti-idiotypic antibody was still present 6 days after administration of the therapy and that this control was passaged with the tumour. It is not known whether this involved the presence of the anti-idiotypic antibody and/or cells recruited to the tumour. Certainly the presence of antibody 300 days later in the recipient animals is most unlikely therefore the control at this stage may be due to the tumour cells being in G_0 or to a balance between tumour cell proliferation and destruction. Disruption of the splenic environment thus creates a signal that breaks the cells away from G_0 or allows escape of the tumour away from cytostatic/cytolytic cellular effectors.

It has recently been shown that immunisation of mice with A31 IgM prior to tumour inoculation also leads to tumour dormancy (Dyke et al., 1991). In this study the establishment of dormancy could also be initiated by the transfer of immunised mouse serum prior to tumour challenge but not by transfer of spleen cells. The transferred serum was protective providing at least 50% of the cell-associated surface IgM was bound by antibody showing a primary role for the latter in this system. However it was not shown, within the present study, whether the antibody alone was responsible for initiating the dormant state or if ADCC was involved. Further work is required on this aspect of immune therapy of the A31 tumour.

7.4. RIBOSOMAL INACTIVATION OF A31 CELL

Two different RIP delivery systems were examined for their ability to kill A31 cells, saporin covalently linked to anti-idiotypic monoclonal antibody (immunotoxin) and non-covalently associated saporin-bispecific antibody derivatives. The immunotoxin proved to be extremely effective at eliminating the tumour *in vivo* such that a single, 0.1 μg, dose was capable of curing mice given 5 x 10^5 tumour cells twenty-four hours previously. However, increasing the immunotoxin dose to cope with higher tumour loads caused lethal hepatic toxicity. In spite of the univalent nature of the binding of the bispecific antibody anti-A31 x anti-SAP1, (Mc39-16 x DB7-18), it achieved a similar IC_{50} leucine-incorporation inhibition value to the immunotoxin suggesting that the bispecific antibody must be as, or more, efficient at delivery of saporin to the cytosol of tumour cells. Despite the encouraging IC_{50} results the bispecific antibody was virtually inactive when tested *in vivo*. Injection of 10 μg of bispecific antibody with 1 μg of saporin gaving only marginally better survival to the animals (42 days post tumour inoculation) compared to 2 μg of Mc39-16 IgG (90% of animals dying by day 34). In view of other studies showing bispecific antibody delivering saporin very efficiently *in vitro* or *in vivo*, this result is very surprising. For the tumour to kill all the animals within this time period requires a large number of the tumour cells to escaped the therapy. Even when tumour cells were treated *in vitro*, at bispecific and saporin concentrations that achieved maximum leucine inhibition, followed by transfer of the cells to mice the latter did not survive beyond 40 days. Insensitivity of detection of ^{3}H-leucine incorporation means that a threshold exists such that at least 1 x 10^4 tumour cells must be present before detectable levels of leucine incorporation can be measured.

There are two possible explanations for these data: (1) A significant proportion of the A31 tumour cell population must be resistant to the bispecific/saporin combination but not the immunotoxin delivery of saporin. As the surface immunoglobulin of cells isolated from the spleen of a mouse succumbing to tumour after treatment with bispecific antibody

and saporin appeared normal any variation within this population conferring resistance must reside at a latter stage than the surface binding, possibly reflecting differences in intra-cellular trafficking; (2) The intoxication of tumour cells by the saporin loaded bispecific antibody must be reversible e.g. if 90% of all ribosomes in all tumour cells are inactivated the ^{3}H-leucine incorporation assay would indicate maximal inhibition but if the saporin was itself inactivated before all the ribosomes could be damaged then the cells could recover from this sub-lethal attack. If this hypothesis is true then the immunotoxin must protect its saporin from a similar fate.

The occurrence of a particular mutation that confers resistance to attack varies depending on the antigen involved. One of the highest frequencies of variation is found for those changes involving immunoglobulin genes. For instance the frequency of heavy chain loss within the murine myeloma cell line MPC-11 has been reported to be as high as 1.1×10^{-3} per cell per generation (Coffino et al., 1971; Baumal et al., 1973) with figures of 2×10^{-4} for the P3 cell line (Cotton et al., 1973). Obviously immunoglobulin genes have an unusually high rate of mutation. Drug resistance markers are probably more representitive of more normal genetic mutations and within the MPC-11 cell line these have been found to occur with a frequency of less than 10^{-6} (Baumal et al., 1973).

In a study in which loss of surface markers from a fibrosarcoma were identified using monoclonal cytolytic T cell probes it was stated that the frequency of variants having lost a single marker was too low to detect within a freshly isolated population but rose to 10^{-4} after 2-months in culture (Wortzel et al., 1983). Closer association to the present study can be found in an analysis of the occurrence of variants showing resistance to ricin within Chinese Hamster Ovary cells. Four separate variants were found, three exhibited recessive phenotypes occurring at a frequency of 10^{-5} to 10^{-6} while the forth was a dominant phenotype occurring at a frequency of less than 3.9×10^{-8}. The nature of the resistance within these cells probably resides within structural changes in the ribosomal proteins and/or RNA

(Sallustio and Stanley, 1990).

It is known that 100 A31 tumour cells will kill animals within approximately 55 days (Dyke et al., 1991). Assuming a doubling in cell numbers in the twenty-four hours between injection of A31 tumour and the administration of bispecific antibody, the frequency of resistant cells would have to approach the level seen for immunoglobulin variants, approximately 10^{-4}, in order for the animals to succumb in forty days. Although this is not impossible it does seem unlikely, however further work in examination of the saporin sensitivity of tumour cells recovered after such therapies is needed to exclude or confirm this possibility.

The second possibility is that of recovery of intoxicated cells, a mechanism that must also take into account the lethal effect of the immunotoxin. There is very limited evidence of such a phenomenon, with the only previous report involving the comparison of leucine incorporation inhibition and cell kill with immunotoxins containing ricin or diphtheria toxin (Sung et al., 1991). The authors reported a situation in which diphtheria immunotoxin gave a significantly higher inhibition of leucine incorporation than the ricin immunotoxin yet in a limiting dilution assay achieved a significantly lower cell kill i.e. the kinetics of protein synthesis inactivation did not correlate with killing efficiencies. No definitive explanation was given for these results although the authors suggest that either the intoxication was reversible or that significant amounts of the diphtheria toxin were degraded within the cell.

Any difference in the sensitivity of saporin probably reflects differences in the processing of the immunotoxin or bispecific antibodies through the cell. The most obvious difference between the two derivatives is in the nature of the binding to the cell surface, the immunotoxin being divalent whilst the bispecific is univalent. Evidence for differential internalisation between divalent and univalent ligands has been found (Metezeau et al., 1984). Murine B-splenocytes were examined for their ability to internalise either IgG anti-mouse immunoglobulin or Fab' anti-mouse immunoglobulin. The rate of internalisation of the IgG was the same as for the Fab' fragment and although the latter did

not clear as much surface immunoglobulin as the former they were of the same order of magnitude. Interestingly, whereas the IgG cross-linked surface immunoglobulin was found within an acidic intracellular environment after internalisation the Fab' bound material was not, in addition more of the Fab' fragment was released from the cell after endocytosis than was the case with the bound IgG. Various biochemical inhibitors of cell processes were found to inhibit the internalisation of both ligands to a similar degree. These data were used by the authors to suggest that internalisation of Fab' and IgG ligands depends on similar processes but after entry the routes taken by the two differ, with the majority of the IgG being directed to a lysosomal pathway and the Fab' being recycled to the surface. It was not suggested that these routes were mutually exclusive but rather a balance between both pathways existed. In a similar study Myers and Vitetta (1989) compared the intracellular degradation of antigen binding to surface Ig with the degradation of divalent anti-Ig. Their results suggested that antigen was directed principally to endosomes were rapid degradation occurred and fragments were rapidly returned to the cell surface. In contrast divalent anti-Ig caused more extensive cross-linking, and the internalised complexes were routed via endosomes to a lysosomal compartment, the overall process was slower but the fragments being formed were smaller due to more complete digestion. Routing to and degradation of immunotoxins within a lysosomal compartment may account for the large number of toxin molecules that need to be delivered to the cell to ensure destruction of that cell.

Further support for this view comes from a number of studies within different cell types of the toxicity of ricin immunotoxins directed to different cell surface markers. The effectiveness of ricin A-chain immunotoxins against human T cells has been shown to depend on the surface antigen utilised as a target. Immunotoxins binding to CD5, CD3, and CD2 were all equally effective within reticulocyte lysate assays and were endocytosed at the same rate, however the CD2 immunotoxin was significantly less toxic to whole cells than the CD5 and CD3 ITs. Ultrastructural studies indicated that

the CD2 immunotoxin was delivered to the lysosomal compartment more quickly than either the CD5 or CD3 derivatives (Press et al., 1986). A similar finding has been reported for anti-transferrin receptor and anti-CD33 immunotoxins targeted to myeloid leukaemia cells were the former IT eliminated the tumour cells and the latter IT did not. Once again it was suggested that the more rapid degradation of the anti-CD33 immunotoxin within lysosomal compartment was responsible for the difference in potency (Engert et al., 1991).

Epitope specificity can also play an important role in intracellular trafficking of toxins. A ricin immunotoxin with specificity for Fd of B cell surface IgD was less cytotoxic than an anti-Fc ricin immunotoxin. The difference did not reside in surface cross-linking or rate of internalisation but the less effective IT became as cytotoxic as the anti-Fc IT in the presence of chloroquine (May et al., 1991) suggesting that the epitope specificity may influence intracellular processing of the ITs. Epitopes had previously been shown to be important in the proteolytic processing of antigen in B lymphoblastoid cells (Davidson and Watts, 1989). Fragmentation patterns of processed antigen were shown to be both cell line and epitope specific and modifiable by the binding of Fab to further epitopes before endocytosis of the antigen.

All the evidence presented above can be used to proffer a hypothesis for the discrepancy between the leucine incorporation inhibition assay data and the *in vivo* results observed with the bispecific antibodies and immunotoxin. The divalent binding of the immunotoxin delivers more toxin to the cell surface of A31 cells than the bispecific antibody for a given external concentration of RIP. However the nature of this binding causes more of the IT to be routed to lysosomes were degradation occurs whereas, on the other hand, the bispecific antibody is mainly routed through an endosomal pathway giving a more efficient delivery to the cytosol. A possible explanation for the poor kill achieved with the bispecific antibody may reside in the actual degradation of the conjugates. The fragmentation of the saporin attached non-covalently to the bispecific antibody is likely to be

different to that of the saporin in the immunotoxin, analogous to the epitope fragmentation mentioned above. This fragmentation could occur within the same intracellular site for both conjugates i.e. the endosomal pathway or may be at different sites, endosomal for bispecific and lysosomal for immunotoxin. In either case saporin entering the cytosol as a immunotoxin fragment may be associated with protective peptide(s) derived from the immunoglobulin component protecting the toxin from inactivation within the cytosol and leading to total inactivation of all ribosomes within the cell. The saporin attached to the bispecific antibody may also enter the cytosol associated with peptide fragments but in this case they would be non-protective and although saporin inactivation of ribosomes is rapid enough for inactivation of most of the ribosomes within the tumour cell the saporin itself could be inactivated before total enzymic cleavage of all ribosomes occurs. The high pI of saporin would make non-covalent association of peptides likely and the random position of disulphide linkage of toxin within the immunotoxin would give a higher chance of some saporin molecules receiving a protective peptide.

Rather than proposing that differences in the susceptibility of saporin to inactivation account for the potency of the IT and the lack of potency of the bispecific antibody the same observed results could be accounted for by a relative change in the susceptibility of the ribosomes to inactivation. Cross-linking of the surface immunoglobulin of A31 cells by the IT could induce calcium flux within the cells. Elevation of calcium within cells has been shown to induce phosphorylation of elongation factor II thereby causing temporary inhibition of translation. It is thought that this may cause the disappearance of short-lived repressors leading to activation of genes and a change in the status of the cell (Ryazanov and Spirin, 1990). It is also known that in the presence of EF2 and GTP, when the former is bound to the ribosome, there is partial protection of the ribosome from the action of ricin A-chain (Fernandez-Puentes et al., 1976). A 'window of opportunity' may therefore exist after stimulation of surface IgM by the divalent anti-idiotype were the ribosomes are more susceptible to

inactivation by the RIP.

7.5. RECRUITMENT OF T CELLS TO THE A31 TUMOUR

As explained in the introduction there are a number of problems associated with the destruction of solid tumours using antibodies to passively deliver toxic molecules to the cells. Engagement and activation of effector cells has the potential to overcome these limitations by setting up an on-going immunological attack at the site of the neoplasm with the possible additional benefit of bystander killing and/or cytostasis of antigen-negative tumour cells. Delivery of ribosomal inactivating toxins to tumour cells relies on internalisation of the derivative to achieve access to the cytosol, in contrast a derivative that has specificity for effector cell surface antigens requires to be maintained in a stable state at the tumour cell surface to ensure interaction between circulating effector cells and the tumour. It is in fulfilling this latter criteria that the anti-CD3 x anti-idiotype derivative constructed in the present study may have failed. The reasons for the formation of aggregates during the concentration step of bispecific construction are unknown but as the anti-idiotype Fab had been used in construction of the anti-saporin bispecific antibodies without problems the difficulty may reside with the KT3 component. Priority exists for the reproducible construction of anti-A31 x anti-CD3 bispecific antibodies free of aggregation. With this reagent further parameters of the bispecific antibody directed killing of A31 tumour cells can be examined. Foremost amongst these will be the effect of IL-2 activation and the role of ADCC on the therapeutic quotient. The precise mode of activation via IL-2 is an important consideration. Administration of the cytokine at some time after targeting of the T cells to the tumour by the bispecific antibody is likely to be the most effective procedure to avoid complete or partial down regulation of T-lymphocyte mediated cytotoxicity (Weil-Hillman et al., 1991). The A31 model may be an excellent system to investigate the exact timing of this combined therapy. The excellent therapy demonstrated by Brissinck and colleagues (1991) gives some evidence that ADCC reactions in combination with T-cell directed killing may

give augmented tumour clearance. In the present study the presence of contaminating Fc within $F(ab')_2$ preparations may account for the latters ability to shown some protection *in vivo* at low tumour inoculations (fig 6.3b.). The presence of contaminating IgG would allow the recruitment of NK cells via the latters FcR (CD16). As we have gained more experence in the prepartion of multispecific antibody derivatives it has become clear that these preparations often contain trace amounts of contaminating IgG (Glennie et al., 1993). It should be remembered however that in the present study each therapy contained control antibodies, including IgG or $F(ab')_2$ preparations, and that any contaminating IgG within the bispecific preparations would have been minuscule compared to the levels used in the controls. Therefore, although the problem of contaminating Fc is now recognised, and eliminated by the use of anti-Fc chromatography columns, in the present study the bispecific antibodies out performed the controls by such a margin that any effect of contaiminating IgG would have been slight. This aspect, including the balance between increased tumour killing and inappropriate T cell activation via the Fc region (Jung et al., 1991), can be examined by the isolation of intact bispecific antibodies from hybrid hybridoma fusions. With purified A31 anti-idiotypic and anti-CD3 antibodies available it should be possible to isolate anti-idiotypic antibodies reactive with each of these components making the isolation of the bispecific monoclonal antibody relatively straightforward.

Bispecific antibody technology can also extend the target cell killing via anti-Fc receptor antibodies as originated by Karpovsky and colleagues (1984). This approach has a particular advantage in that monoclonal antibodies can be chosen that bind to the Fc receptor via an epitope not involved in binding to IgG in serum which would under normal circumstances act as a block (Fanger et al., 1989), or that due to a high association constant can successfully compete with serum immunoglobulin (Greenman et al., 1991). The different classes of Fcγ receptors and the different cell types that these receptors are found on provide rich ground to cultivate different therapies based on bispecific antibody

technologies. Which Fc receptor to utilise and the type of effector cell to target to the tumour must be chosen with care. There is no point in recruiting neutrophils through CD16 as the PIG linkage is inefficient at transmitting a signal to initiate tumour cell killing (Shen et al., 1989; Fanger et al., 1989), the same surface epitope can however mediate efficient tumour cell lysis when LGL/NK cells are recruited (Titus et al., 1987b; Lanier et al., 1988; de Palazzo et al., 1990; Greenman et al., 1992). Similarly human monocytes and macrophages can lyse tumour cells, usually represented experimentally by self-directed lysis of anti-FcR secreting hybridoma cells, through FcRγI and FcRγII (Shen et al., 1989; Greenman et al., 1991). One recent study that examined the killing of the same tumour cell population, guinea pig L_2C cells, by recruitment of effector cells through the different Fc receptors suggests however that tumour cell lysis mediated by bispecific antibodies specific for CD16 of LGL/NK cells were considerably more effective than similar antibodies specific for CD32 and CD64 of monocytes (Greenman et al., 1992). Neutrophils incapable of killing tumour cells through CD16 become effective killers through CD32 and CD64 after augmentation with IFN-γ or GM-CSF (Graziano and Fanger, 1987; Graziano et al., 1989; Shen et al., 1989). Similarly heteroantibody-mediated killing of chick erythrocytes by human monocytes, neutrophils and myeloid cell lines via CD32 and CD64 is increased by IFN-γ, although detectable elevation in expression of these markers was not seen (Fanger et al., 1989). The role of cytokines in activation/enhancement of the various effector cells therefore also requires careful evaluation in experimental models.

7.6. FUTURE WORK

Low immunoglobulin secretion and lack of emergence of tumour variants should ensure that the A31 tumour continues to be a useful model for immunotherapeutic approaches to B cell lymphoma in the future. Further investigations are required to ascertain the mechanisms behind the dormant state with this tumour. Examination of the microenvironment surrounding the dormant cells may give an indication as to

the nature of the restraint held on these cells or selective elimination of various cell lineages can be examined in an attempt to induce tumour outgrowth. Determination of the reasons behind the discrepancy between the ^{3}H-leucine inhibition assay and therapeutic efficacy of the bispecific antibodies will be of particular interest. The first priority is to determine whether this is due to the existence of a variant tumour population or differences in cellular processing of the IT and bispecific antibodies. If the latter turns out to be the case then detailed electron microscopic tracking of the two derivatives through the cell may prove useful. Further work is required to improve the anti-idiotype x anti-CD3 derivatives specifically the elimination of complexed material and the examination of the role of cytokines in the enhancement of the therapeutic benefit.

A further approach to tumour therapy for which the A31 tumour may be suitable should also be mentioned. This involves the construction of a chimeric antibody with two Fc regions joined to antibody Fabs. The construct can be univalent, bivalent, or bispecific and may promote enhanced binding of effector cells to tumour targets (Stevenson et al., 1989).

APPENDIX 1

EQUILIBRIUM BINDING EQUATION

The following derivation of the equilibrium binding equation was kindly supplied by Dr. T. Elliott to whom I am most grateful.

The quantitative relationship of the interaction between antibody and antigen at equilibrium is usually represented by the expression:

$$Ab + Ag \rightleftarrows AbAg$$

At equilibrium, the forward rate of reaction equals the rate of dissociation of antibody-antigen complexes. Thus, applying the law of mass action:

$$k_a[Ab][Ag] = k_d[AbAg] \qquad (1)$$

where k_a and k_d are the forward and reverse rate constants respectively. Hence:

$$\frac{k_a}{k_d} = \frac{[AbAg]}{[Ab][Ag]} = K_A \qquad (2)$$

where K_A = the equilibrium, or association constant for the reaction.

In dealing with reactions with cell-surface antigens, it is impossible to measure free antigen in terms of molar concentration since local concentrations may vary according to antigen distribution on the cell surface. The following treatment (Dower et al., 1981) has been used during this investigation which dispenses with the need for molar concentration terms for cell surface antigen, and instead expresses bound antibody as the number of antibody molecules which are bound per cell at equilibrium, r.

r is defined thus:

$$r = ro \left(\frac{[Ab_B]}{[Ag_o]}\right) \qquad (3)$$

where r_o is the total number of antigenic determinants per cell, $[Ab_B]$ is the concentration of bound antibody and $[Ag_o]$ is the cell surface concentration of antigen. The last two of these parameters are immeasurable since they relate to concentrations at the cell surface. they can, however be rewritten:

$$[Ag_o] = [Ag] + [AbAg] \qquad (4a)$$

$$[Ab_B] = [AbAg] \qquad (4b)$$

When the expression for [AbAg] is substituted for equation 2:

$$[Ag_o] = [Ag] + K_A[Ab][Ag] \qquad (5a)$$

$$[Ab_B] = K_A[Ab][Ag] \qquad (5b)$$

so, substituting 5a and 5b into equation 3:

$$r = r_o\left(\frac{K_A[Ab][Ag]}{[Ag] + K_A[Ab][Ag]}\right)$$

i.e.

$$r = \frac{r_o K_A[Ab]}{1 + K_A[Ab]} \qquad (6)$$

We are left with a term which no longer relies upon immeasurable parameters of cell surface concentrations while enabling the calculation of K_A and r_o from experimental derived data. It can be seen that by rearranging equation 6 thus:

$$\frac{r}{[Ab]} = r_o K_A - K_A r \qquad (7)$$

a plot of r/[Ab] versus r should give a straight line with gradient of $-K_A$, and extrapolation to r/[Ab] = 0 will yield the number of antigenic determinants per cell, r_o. This is a form of the widely used Scatchard plot.

Implicit in this treatment is that the antibody-antigen reaction is best characterised by the expression $Ab+Ag \rightleftarrows AbAg$ which, though being a reasonable interpretation of the interaction of Fab with antigen (where K_A represents intrinsic affinity), is a gross simplification of the interaction of bivalent antibody with antigen which is best described by the linked equilibria $Ab+Ag \rightleftarrows AbAg+Ag \rightleftarrows AbAg_2$. Here, K_A or functional affinity is a composite term which relates not only to intrinsic affinity but also to the association rate and dissociation rate constants for the second binding step. Dower et al., (1984) have developed a form of equation 6 in which the terms K_{A1} and K_{A2} appear separately, and has been used to explain deviations from linearity when data derived from bivalently binding antibody is plotted according to equation 7. Briefly, since:

$$K_{A1} = \frac{[AbAg]}{[Ab][Ag]} \quad \text{and} \quad K_{A2} = \frac{[AbAg_2]}{[AbAg][Ag]} \qquad (8a)\ \&\ (8b)$$

then $$[AbAg] = K_{A1}[Ab][Ag] \qquad (9a)$$

and $$[AbAg_2] = K_{A2}[AbAg][Ag]$$

$$= K_{A1}K_{A2}[Ab][Ag]^2 \qquad (9b)$$

Hence equivalent expressions of 5a and 5b become:

$$[Ag_o] = [Ag] + K_{A1}[Ab][Ag] + K_{A1}K_{A2}[Ab][Ag]^2 \qquad (10a)$$

and $$[Ab_B] = K_{A1}[Ab][Ag] + K_{A1}K_{A2}[Ab][Ag]^2 \qquad (10b)$$

so, substituting equations 10a and 10b into the basic binding equation 6:

$$r = r_o \left(\frac{K_{A1}[Ab][Ag] + K_{A1}K_{A2}[Ab][Ag]^2}{[Ag] + K_{A1}[Ab][Ag] + K_{A1}K_{A2}[Ab][Ag]^2} \right)$$

i.e.

$$r = r_o K_{A1}[Ab] \left(\frac{1 + K_{A2}[Ag]}{1 + K_{A1}[Ab] + K_{A1}K_{A2}[Ab][Ag]} \right) \qquad (11)$$

A Scatchard plot thus gives a line with gradient $-K_{A1}(1+K_{A2}[Ag])$ and an x-axis intercept r_o. Hence affinities derived from Scatchard plots are functional affinities, and in addition to intrinsic affinity K_{A1}, contain the term $K_{A2}[Ag]$: a dimensionless factor (since K values are measured in M^{-1} and [Ag] in M) called the enhancement factor. Alone, the enhancement factor is immeasurable using the Scatchard linear transform. A decrease in [Ag] either by increased occupancy or by local spatial distribution will effect a decrease in $K_{A2}[Ag]$, thus lowering the functional affinity of bivalent antibody. The effect will be seen as a negatively curved Scatchard plot when decreased [Ag] is due to increased occupancy. Where curved plots are obtained, average gradients are measured over the most linear portion of the plot.

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