Antibody and cytokine studies related to Chronic Lymphocytic Leukemia

A dissertation submitted for the degree of Ph.D.

by

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Declaration

I hereby declare that all the work reported in this thesis is entirely my own and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the context of the text.

Noel Quinlan

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Finally, I would like to thank my family especially my parents to whom this thesis is dedicated.

Abbrevations

APAAP	Alkaline-phosphatase anti-alkaline phosphatase
8-Aza	8-Azaguanine
BCA	Bichinchoninic acid
BCGF	B-cell growth factor
BSA	Bovine serum albumin
Bsab	Bispecific antibody
CD	Cluster of differentiation
C6CM	CM prepared from the glioma cell line C6
CLL	Chronic lymphocytic leukemia
CLL-DMEM	DMEM.S5 supplemented with 5% (v/v) CLL plasma
CLL-CM	CM obtained from culturing the PBLs of patients with CLL
CM	Conditioned medium
D	Dalton
DMSO	Dimethlysulphoxide
DTT	Dithiothrietol
DMEM	Dulbecco's modification of Eagle's medium
DMEM.S5	DMEM supplemented with 5% (v/v) FCS
ECGS	Endothelial cell growth supplement
EDTA	Ethylenediaminetetra-acetic acid
EMS	Ethyl methanosulphonate
ELISA	Enzyme-linked immunosorbant assay
ESGF	Ewing sarcoma growth factor
Fab'	Fab' antibody fragment
$F(ab'\gamma)_2$	$F(ab'\gamma)_2$ antibody fragment
FCS	Foetal calf serum
HAT	Hypoxanthine aminopterin thymidine
HAT-medium	DMEM.S5 supplemented with HAT
1X HAT	2.5mg/ml aminopterin, 0.0048mg/ml thymidine and 0.13mg/ml

	hypoxanthine
HECS	Human endothelial culture supernatant
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethane sulphonic acid])
HGF	Hybridoma growth factor
HGPRT	Hypoxanthine guanine phosphoribosyl transferase
HPLC	High pressure liquid chromatography
HRP	Horseradish peroxidase
HUCS	Human umbilical cord serum
HUVEC	Human umbilical vein endothelial cells
Ig	Immunoglobulin
IgG	Immunoglobulin class G
IgM	Immunoglobulin class M
IL	Interleukin
IL-6	Interleukin-6
I.p.	Intra-peritoneal
L-CM	CM prepared from the murine fibroblast cell line L-929
M	Molar
MAb	Monoclonal antibody
MCM	Macrophage conditioned medium
2-Me	2-Mercaptoethanol
mg	milligram
ml	millimeter
MTT	(3-[4-,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NaOH	Sodium hydroxide
N.D.	Not determined
NHP	Normal human plasma
NHP-DMEM	DMEM.S5 supplemented with 5% (v/v) NHP

NHP-CM	CM obtained prepared from PBLs of normal healthy volunteers
o-pd	O-phenylenediamine
o-pdm	O-phenylenedimaleimide
PAGE	Polyacrylamide gel electrophoresis
PAP	Peroxidase anti-peroxidase
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PBS/Tween	PBS containing 1% (v/v) Tween-20
PEG	Polyethylene glycol
rpm	revolutions per minute
S.D.	Standard deviation
SRBC	Sheep red blood cells
SDS	Sodium-dodecyl sulphate
T24-CM	CM prepared from the human bladder carcinoma cell line T24
TEMED	N,N,N^1,N^1-tetramethyl-ethylaminediamine
TNF	Tumor necrosis factor
TRIS	Tris(hydroxymethyl)methylamine
µl	microlitre
v/v	volume per volume
w/v	weight/volume

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Abstract

The aim of our work was to produce bispecific antibodies using biological and chemical methods. Bispecific antibodies recognise two antigens simultaneuosly. The bispecific antibodies produced were used in the development of novel ELISA and immunocytochemical techniques for the detection of antigens present on the peripheral blood cells of patients with Chronic Lymphocytic Leukemia, (CLL). Cytokine production in these patients was also determined.

Biological production involved the production of triomas which were formed by the fusion of hybridoma cells with immunized splenocytes. This method required back-selecting the hybridoma cells for HAT-sensitivity, a procedure which was performed firstly by growing the cells in increasing concentrations of 8-azaguanine and, secondly, by treatment of the hybridoma cells with the mutagenic reagent, ethyl methanesulfonate. The cells were then fused with horseradish peroxidase-immunized mouse and rabbit splenocytes.

The chemical method chosen involved the activation of thiol groups on Fab´ fragments of one antibody. These Fab´ fragments were reacted with the second reduced Fab´ fragment to form the heterogeneous $F(ab'\gamma)_2$ bispecific antibody.

Studies were also performed on the cytokine, interleukin-6 (IL-6). These included measurement of the concentration of IL-6 in the plasma and in conditioned medium (CM), prepared from the peripheral blood lymphocytes of patients with CLL. The IL-6 levels were measured by an ELISA and by a bioassay using an IL-6-dependent cell line. The levels of circulating IgG present in the plasma of these patients were also determined by ELISA, but did not correlate. The effects of feeder cell layers and various medium supplements, including both IL-6-CM and CLL plasma, on the growth of hybridoma cells were also studied.

The growth of hybridoma cells which are of B-cell origin under such conditions may give indications of how the various growth factors present in CLL plasma affect B-cell growth in these patients.

Chapter 1

Introduction

1.1. An introduction to Chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL), was first clinically described by Turk in 1902. CLL is a disease involving the progressive accumulation of immunologically non-functional lymphocytes (Dameshek, 1967). 95% of all CLL cases are of B-cell origin (Dighiero *et al.*, 1991). In western countries CLL is the most frequent chronic leukemia in middle and elderly life; there is a higher incidence of the disease in males than in females with a ratio of 1.5:1 (Finch and Linet, 1992).

Some links between CLL and occupations have been reported although CLL is the only leukemia not associated with an occupational exposure to radiation (Dighiero *et al.*, 1991). These occupations include soybean production, cattle raising and dairy production in the farming sector and exposure to herbicides. Rubber manufacturing workers and asbestos workers also have increased incidences of the disease. In a cooperative chromosomal study on CLL, 51/433 patients examined had a structural abberation affecting the 13q14 band (Dighiero *et al.*, 1991). This is the region where the retinoblastoma tumor suppressor gene has been mapped. Rearrangements in the 14q32 band of 41 patients and less frequent alterations of chromosomes 11, 6, 18, 3, 17, 7 and 8 were also detected. Persons with first degree relatives suffering from the disease have between 2-7 times greater expectancy of developing the disease than have persons without such relatives.

Although therapy can improve the quality of life among CLL patients, no cure has yet been found and the median survival period of 96 months has not been altered by therapy (Finch and Linet, 1992). The clinical course of CLL is variable. Patients presenting with elevated lymphocytes, asymptomatic lymphadenopathy or splenomegaly do not benefit from treatment as they will not die from their leukemia. Patients presenting with lymphoma-like symptoms or features of bone marrow failure (severe anemia and thrombocytopenia), have a less than 2 year median survival. The majority of patients are in an intermediate group who only require treatment several years after the initial diagnosis.

1.1.2. Clinical staging of Chronic Lymphocytic Leukemia

Rai *et al.* (1975) have described a clinical staging systems of patients with CLL which involves 5 stages, Stage 0 - Stage IV. Binet *et al.* (1981) have also described a clinical staging system which involves three patient groups: Stages A, B and C. Both staging systems take into account the clinical presentation of patients. In the case of Rai *et al.* (1975) these clinical presenting features include lymphocytosis, enlarged lymph nodes, hepatomegaly, splenomegaly, anemia and thrombocytopenia. The staging system of Binet *et al.* (1981) takes into account the numbers of sites affected by the disease. These sites are the cervical, axillary or inguinal lymph nodes, spleen and liver. Both staging systems are summarised in Table 1.1.2.

Staging	**Clinical symptoms**
Rai *et al.* (1975)	
Stage 0	Lymphocytotosis in blood and lymph nodes
Stage I	Lymphocytosis and enlarged lymph nodes
Stage II	Lymphocytosis and/or splenomegaly
Stage III	Lymphocytosis and anemia
Stage IV	Lymphocytosis and thrombocytopenia
Binet *et al.* (1981)	
Stage A	Less than three areas involved*, Hb $\geq$ 10g/L and platelets $\geq$ $100x10^9$/L
Stage B	At least three areas involved*, Hb $\geq$ 10g/L and platelets $\geq$ $100x10^9$/L
Stage C	Hb > 10g/L and platelets > $100x10^9$/L independent of the number of areas involved*

Table 1.1.2. The staging systems used for the diagnosis of patients with CLL.

* The 5 areas used in the staging system of Binet *et al.* (1981) are the cervical, axillary and inguinal lymph nodes, spleen and liver.

Hb :- Haemoglobin.

Other prognostic factors include rapid lymphocyte count doubling times and diffuse patterns of infiltration, both of which carry poor prognosis. Patients with a normal karyotype fare better than those with an abnormal one (Dighiero *et al.*, 1991).

1.1.3. Phenotypic characterization of Chronic Lymphocytic Leukemia

The phenotypic characteristics of B-CLL have been well established (Dighiero *et al.*, 1991). CLL cells express surface membrane Ig, C3dr complement receptors and receptors for the Fc portion of Ig. Monoclonal antibodies against CD19, CD20, CD24, CD37, and CD21 are reactive with CLL cells. About 60% of B-CLL are positive for CD23. CLL B-lymphocytes are usually CD5-positive. Phenotypic alterations may occur over time (Patrick *et al.*, 1987). This could be an important feature of the biology of the disease. If clinical course and phenotypic profile can be correlated then treatment may be suitably tailored (Patrick *et al.*, 1987). These workers have detected phenotypic changes in the expression of CD20 and CD21.

1.1.4. Therapy in Chronic Lymphocytic Leukemia

Therapy in CLL has been reviewed extensively (Robertson and Keating, 1991). Chemotherapy involves treatment with an alkylating agent such as chloroambucil, alone or combined with a corticosteroid. The French cooperative group on CLL (1986), studied the efficacy of several drug regimens for the treatment of CLL. The patients were treated according to their clinical staging. Stage A patients (good prognosis), received either no treatment or chlorambucil. Stage B patients (intermediate prognosis), received either chlorambucil or COP (cyclophosphamide, vincristine and prednisone). Stage C patients (poor prognosis) received either COP or CHOP (COP plus doxorubicin). The survival rates were similar in stage A and B patients. In the final group, stage C, the 2 year survival rate was 44% for COP-treated patients and 77% for CHOP-treated patients. The effectivness of the CHOP regimen in stage B patients is now being determined. The potential of α-interferon as a biological agent for use in CLL has been studied (Foon, 1989), and was shown to be of no benefit to CLL patients with advanced CLL but maybe of greater use in untreated patients at an early stage of disease.

One of the clinical symptoms associated with CLL is hypogammaglobulinemia

(Cooperative group on CLL, 1988). Given the association of hypogammaglobulinemia with CLL intravenous IgG treatment has been used in the treatment of patients with CLL. As the disease progresses the most common cause of mortality and morbidity is due to infection and this may be a consequence of the decrease in normal IgG levels.

Splenectomy is often a chosen form of therapy when symptoms such as autoimmune haemolytic anemia or thrombocytopenia and massive painful splenomegaly exist. Ferrant *et al.* (1986) reported that splenectomized CLL patients did not have increased survival periods.

1.1.5. Chronic Lymphocytic Leukemia in relation to the work described in this thesis

This work is divided into three areas. Firstly, bispecific antibodies were produced for use in the development of ELISAs and immunocytochemical techniques for the detection of antigenic determinants present on the peripheral blood cells of patients with CLL. Secondly, production of the cytokine, interleukin-6, was examined in these patients. This was correlated with levels of IgG present in the circulating plasma as determined by ELISA. Thirdly, and finally, given that patients with CLL have been reported to produce various cytokines (Schena *et al.*,1991), the plasma of these patients was examined for its ability to promote hybridoma cell growth at the clonal stage.

These research areas have been more fully discussed in Sections 1.2, 1.3 and 1.4.

1.2. An introduction to bispecific antibodies

Bispecific antibodies (bsabs) have dual specificity for two distinct antigens. This enables them to be used in a variety of ways. For example, cancer cells can be targeted for lysis (Fanger and Guyre, 1991), drugs and toxins can be localised to cells (Flavell *et al.*, 1991) and enzyme immunoassays with one arm directed against an enzyme and the second arm against a tumour cell can be developed (Görög *et al.*,1989; Nolan and O'Kennedy, 1990; Takahashi *et al.*, 1991).

Synthesis of bsabs can be divided into three main categories: biological, chemical, and genetic (Nolan and O'Kennedy, 1990; O'Kennedy and Roben, 1991; Kostenly *et al.*, 1992). Biological production involves manipulation of standard somatic cell hybridization techniques to produce quadromas and triomas. Chemical production involves modifying two distinct antibodies. Finally, a method using "leucine zippers" has been developed to generate genetically engineered bispecific antibodies (Kostenly *et al.*, 1991). Leucine zippers are stretches of amino acids containing leucine residues at every seventh position.

1.2.1. Production of bispecific antibodies

1.2.1.1. Biological production

The general structure of an antibody is outlined in Figure 1.2.1.1.(a). This is a bivalent monospecific molecule which binds to a single antigen via its two antigen binding regions. An antibody consists of two light (L) and heavy (H) chains which are joined together by disulphide bonds [Figure 1.2.2.1.(a)]. The binding site of the antibody is situated at the amino terminal end, termed the variable (V) region of the L and H chains. The constant (C) region of the antibody is relatively conserved within a given species and may have a number of effector functions such as complement fixation and Fc receptor binding. In the germ line cells an antibody sequence is encoded in multiple gene segments scattered along a chromosome. When a lymphocyte is formed, recombination of these gene segments takes place to form a complete gene. Mutations also occur ensuring a high variability of amino acids at the amino terminal region (Tonegawa, 1983).

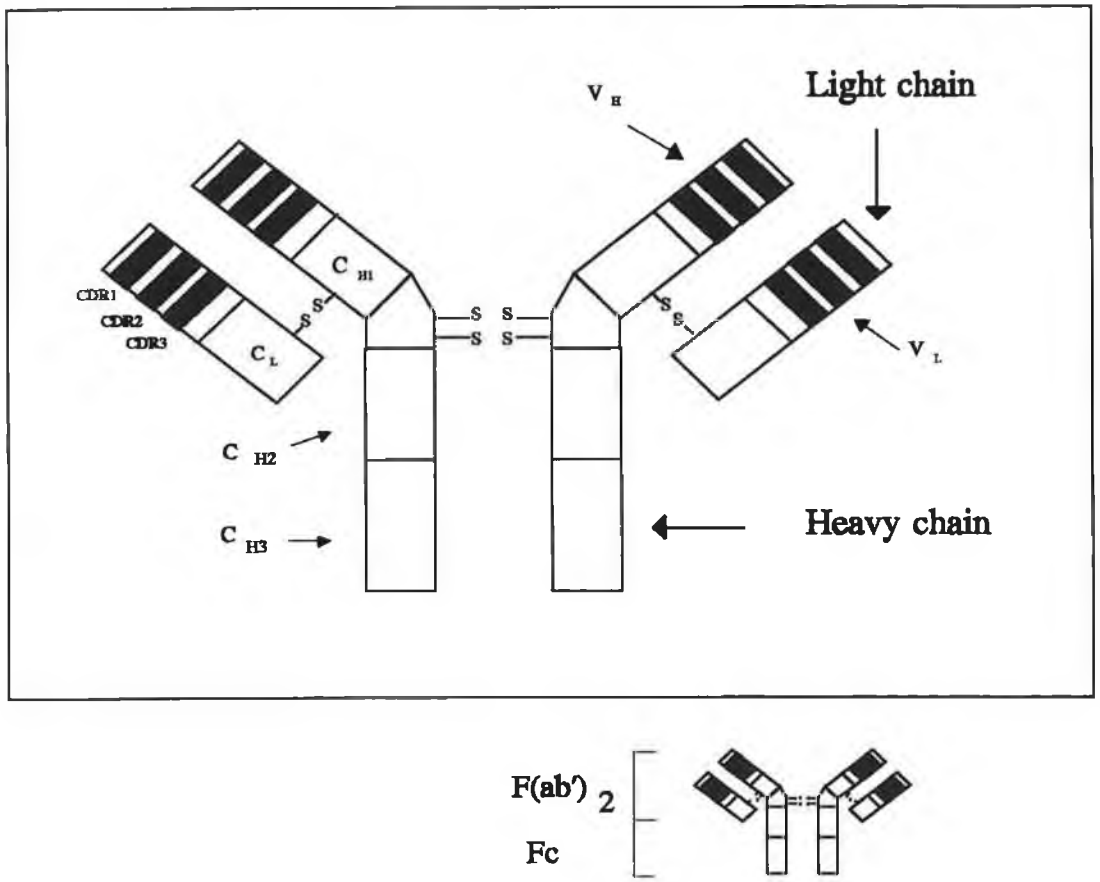


Figure 1.2.1.1.(a).

An antibody consists of four polypeptide chains. These consist of two identical light (L) chains and two identical heavy (H) chains. These chains are held together by a combination of noncovalent interactions and covalent disulphide bonds. The L chain consists of a single variable (V_L) and a single constant (C_L) region. The H chain consists of a single V_H and three distinct C ($C_{H1,2,3}$) regions. The L and H chain V regions consist of a highly conserved framework structure linked by three hypervariable loops known as the complementary-determining regions (CDR 1,2 and 3). The V region binds antigen whereas the C region controls effector functions such as complement activation. Proteolytic digestion of the antibody with the enzyme pepsin yields Fab and $F(ab\gamma')_2$ antibody fragments.

Biological production of bsabs involves manipulation of the somatic cell hybridization techniques first developed by Cotton and Milstein (1973) and Kohler and Milstein (1975). Reading *et al.* (1981) reported on the fusion of hybridomas to form quadromas which secrete bsabs [Figure 1.2.1.1.(b)]. Hybridomas may also be fused directly to antibody-producing splenocytes to form triomas [Figure 1.2.1.1.(b)]. One of the obstacles encountered during biological production of bsabs is selecting out hybridoma cells for use in cell fusions. Monoclonal antibody (mAb) production involves selecting for fused cells that grow in medium containing hypoxanthine, aminopterin and thymidine (HAT). The principle behind this approach is that the parent myeloma cell line is HAT-sensitive (HAT-s) and the parent lymphocytes are HAT-resistant (HAT-r). Cells that are HAT-s lack enzymes such as hypoxanthine guanine phosphoribosyltransferase (HGPRT) and thymidine kinase (TK), and, therefore, cannot chemically synthesise DNA and RNA when exposed to aminopterin. The parent lymphocytes contain these enzymes and are, thus, HAT-r. However, they cannot live long in culture.

To allow for selection of quadromas and triomas in HAT-containing medium hybridomas must be back-selected for HAT-sensitivity prior to cell fusion. This can be carried out in a number of ways. The cells may be grown in medium containing drugs such as 8-aza, 6-thioguanine and 5-bromodeoxyuridine, but, these methods can be long and tedious. Orikasa *et al.* (1985) have described a method for obtaining 8-aza-resistant leukemic and myeloid cell lines. The cells were cultured in RPMI medium containing 8-aza and 15% FCS and large amounts of amino acids and vitamins. The large amounts of amino acids and vitamins increase by about 15 times the chances of obtaining mutant cell lines. The correlation of amino acid levels with increased mutation levels is not known. One possibility is that the 8-aza results in the enhancement of the *de novo* purine synthesis by suppressing the salvage pathway resulting in a gradual loss of HGPRT activity. Kontsekova *et al.* (1991) have described a method which speeds up considerably the induction of HAT-resistance using 8-aza. This involves growing the cells in soft agar supplemented with 20μg/ml of 8-aza.

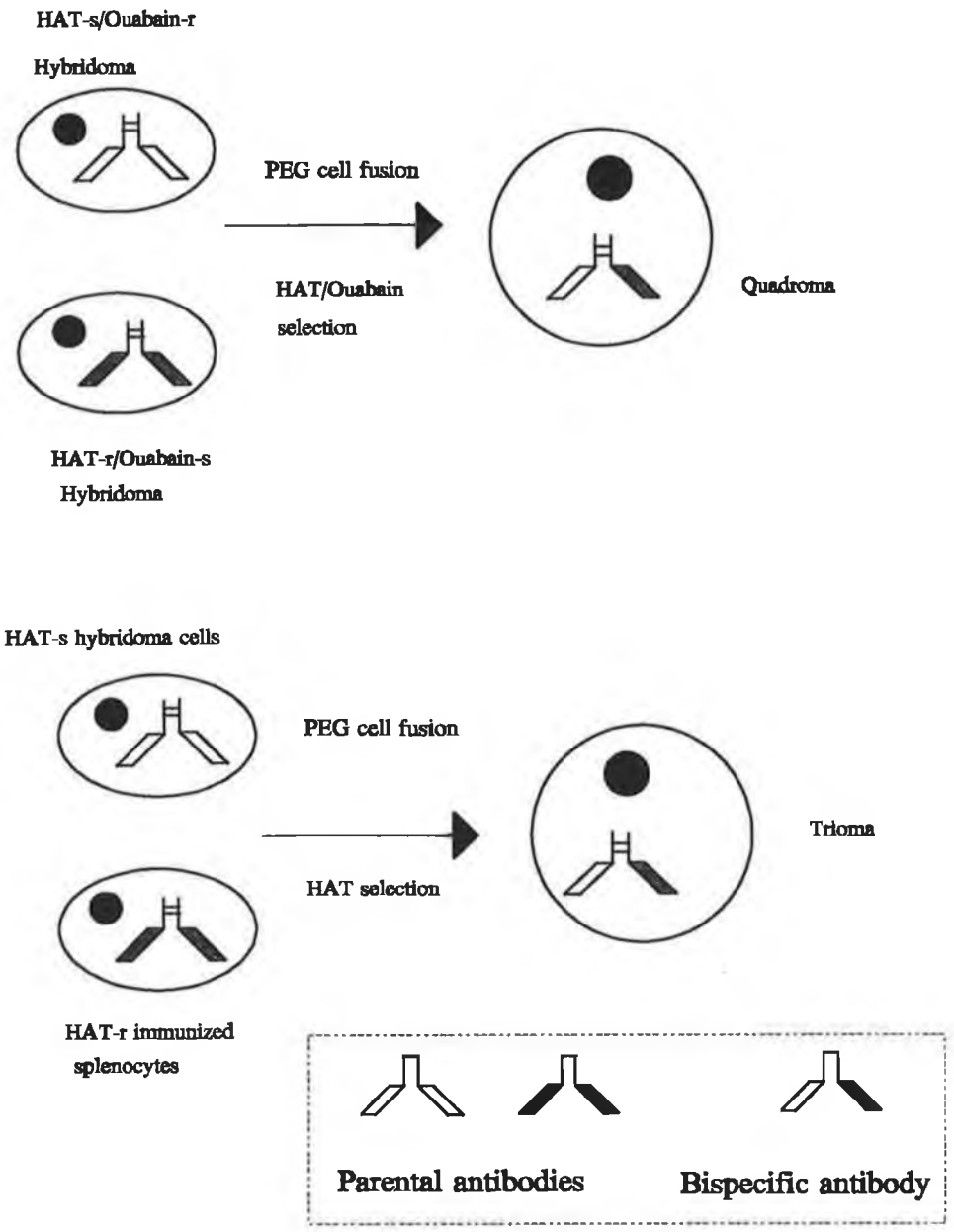


Figure 1.2.1.1.(b).

Biological production of bispecific antibodies can be achieved by the fusion of two hybridomas to form quadromas or by the fusion of one hybridoma directly to immunized splenocytes to form triomas. It is necessary that the hybridomas carry appropriate selection markers. In the examples given above for production of bsabs with quadromas one hybridoma is HAT-s/Ouabain-r and the second is HAT-r/Ouabain-s. In the second example of bsab production by triomas, HAT-r immunized splenocytes are fused with HAT-s hybridoma cells.

HAT-sensitivity can also be induced using mutagenic reagents such as ethyl methanesulphonate (EMS) or X-irradiation. Other properties such as resistance or sensitivity to drugs, for example, ouabain (Staerz and Bevan, 1986), emetine, actinomycin-D (Suresh *et al.*, 1986) and neomycin (De Lau *et al.*, 1989) may be used in selection procedures. The development of cells resistant to neomycin is performed by transfecting the cells with the bacterial neomycin resistance gene (Moran *et al.*, 1990).

Karawajew *et al.* (1987) have developed a method to aid selection of hybrid-hybridomas. Prior to fusion the hybridomas were labeled with the fluorescent markers fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC). This enabled hybrid-hybridomas which were heterofluorescent to be detected and selected by FACS. Shi *et al.* (1991) labeled two hybridomas internally with two fluorescent dyes, rhodamine 123 and hydroethidine, and the hybrid-hybridomas, showing dual fluorescence were separated by cell sorting. Viability assays on labelled cells indicated little kill due to the dye treatment. Recently a variation on these methods has been devised (Jantscheff *et al.*, 1993). This involves staining a HAT-s anti-CEA hybridoma with the TRITC. These cells are then fused with a second HAT-r anti-HRP hybridoma. Fluorescing cells were separated by FACS. These would include HAT-s parent anti-CEA cells and the desired hybrid-hybridomas. Selection of these cells in HAT-containing medium results in the outgrowth of only HAT-r hybrid-hybridomas.

Hudson *et al.* (1987) have studied immunoglobulin chain recombinations formed after fusion of anti-digoxin hybridomas. A cell line was formed which contained H chains derived from the first antibody (ab-1) and L chains derived from the second antibody (ab-2). The recombined antibody (bsab) expressed altered antigenic specificity dominated by the H chain donor and retaining the idiotype of ab-1. De Lau *et al.* (1991) reports on an absence of preferential homologous H/L chain recombination in fusions involving hybrid hybridomas. Milstein and Cuello (1984) have shown, using *in vitro* studies, that under conditions of competitive reassociation, the homologous L-H chain prevail in 80% of cases. Smith *et al.* (1992) have examined the light and heavy chain recombinations of a hybrid hybridoma called 28.19.8 developed by fusing an anti-CEA hybridoma and anti-vinca alkaloid (anti-VDS) splenocytes [Figure

1.2.1.1.(c)]. This hybrid-hybridoma has been shown to secrete monoclonal anti-CEA and anti-vinca alkaloids antibodies as well as the bsab. Purified ascitic fluid contained four antibody populations three of which are immunoreactive. The four fractions were monoclonal anti-CEA and anti-vinca alkaloids antibodies, the bsab and finally an immunoglobulin fraction with no antigen specificity. Of the ten possible combinations of heavy and light chains seven exist as immunoreactive forms, suggesting that the other three combinations exist as the inactive form [Figure 1.2.1.1.(c)]. The bsab was shown by FPLC to be monomeric. SDS-PAGE analysis revealed the presence of one H chain band and two L chain bands. Isotyping revealed that IgG1 and IgG2a heavy chains from the anti-CEA and anti-vinca alkaloid were present. The anti-CEA and anti-VDS fractions both showed negligble cross reactivity for the other. It was expected that the anti-CEA fraction would consist soley of IgG1 and the anti-vinca alkaloid of IgG2a. Isotype analysis, however, revealed that the anti-CEA had some anti-vinca alkaloid lymphocyte IgG2a heavy and light chains. These *Trans* H and L chains in the anti-CEA population cannot be linked as they would then exhibit anti-vinca alkaloid activity. Analysis reveals that the anti-CEA fraction consists of IgG with no free heavy or light chains. Therefore, anti-vinca alkaloid H chains and anti-CEA L chains must be linked through disulphide bonds as must the anti-vinca alkaloid light chain and the anti-CEA heavy chain. Thus, three possible assemblies of chains exist. The reverse situation would represent the anti-vinca alkaloid fraction. Recent reports suggest that H chains alone can result in antigenic binding capabilities (Collet *et al.*, 1992). Referring back to the work of Smith *et al.* (1992) this would suggest that all recombined antibodies with H chains of both species and, therefore, 4 and not just 1 of their recombined antibodies could be bsabs [Figure 1.2.1.1.(c)]. The antibody products formed when a murine hybridoma secreting IgM antibody to calf alkaline phosphatase and a hybridoma secreting IgA antibody to human chorionic gonadoprotein were fused have been reported (Behrsing *et al.*, 1992). The results indicate that the bsab are IgM-like polymers or monospecific IgG-like antibodies. Only the IgM-like species had bsab activity.

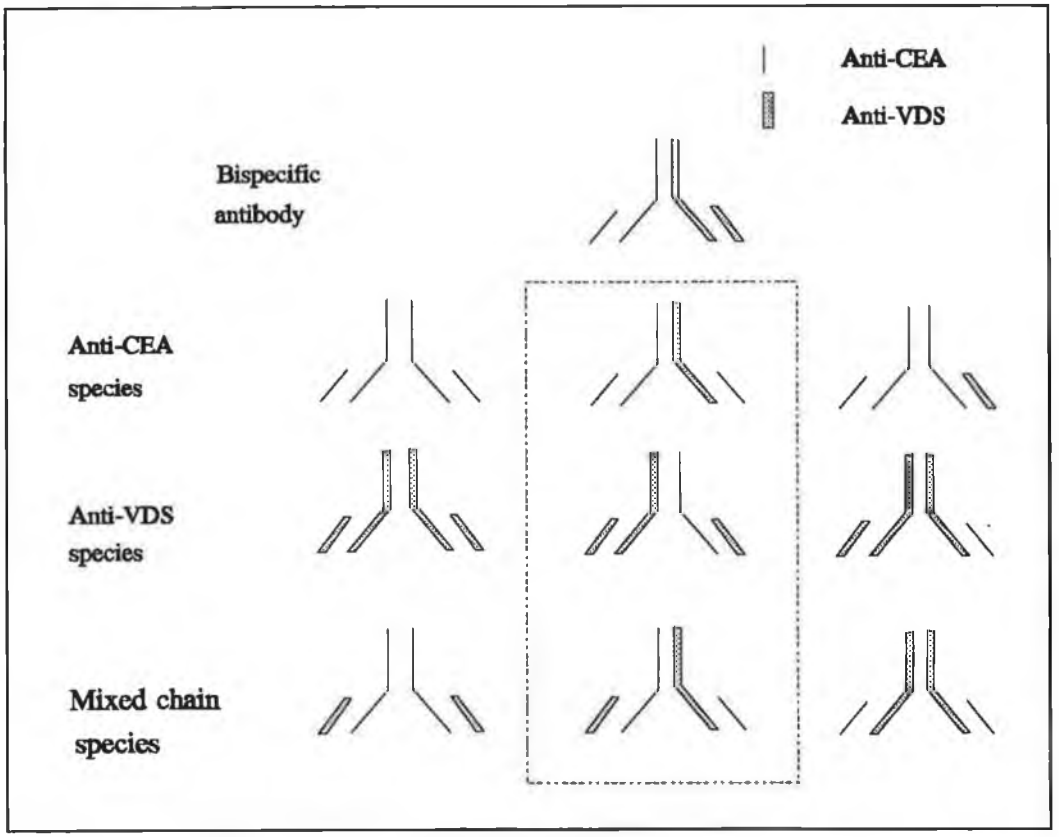


Figure 1.2.1.1.(c).

The possible heavy and light chain species obtained by Smith *et al.*, (1992). Four antibody populations were obtained; these were the bispecific antibody, anti-CEA antibodies, anti-VDS antibodies and mixed chain species. It has now been shown that the H chain alone can bind antigen (Sastry *et al.*, 1989; Collet *et al.*, 1992). This infers that recombined antibodies containing the H chain of both parental antibodies (boxed species) may have bispecific antibody activity.

The feasibility of producing large amounts of bsab by growing a quadroma in a hollow fibre bioreactor has been examined (Gorter *et al.,* 1993). The relative production of bsab was greater using this method than either of ascitic fluid or cell culture supernatant obtained using conventional flasks.

1.2.1.2. Chemical production of bispecific antibodies

As early as 1962 Nisonoff and Mandy demonstrated that random re-oxidation of the hinge region -SH group of two separate Fab′γ yielded both heterodimer and homodimer $F(ab'\gamma)_2$. Some workers have modified the method of protein thiolation developed by Carlsson *et al.* (1978) to chemically crosslink whole antibody molecules forming heteroconjugates. Briefly, parental IgG is thiolated with the heterobifunctional reagent SPDP resulting in pyridyl disulfide (PD) antibodies. One of the IgG-PD species thus formed is reduced with dithiothreitol (DTT). The IgG-PD and IgG-DTT are then mixed to form heteroconjugates. These heteroconjugates can be formed relatively quickly and separated from unreacted components by size exclusion. This method of crosslinking whole antibody molecules has been used successfully by a number of workers who developed heteroconjugates which lyse tumour cells such as ovarian cancer cells (Mezzanzanica *et al.*, 1988) , murine and human tumour cell lines (Perez *et al.*, 1985; Perez *et al.*, 1986; Titus *et al.*, 1987a), B-cell lymphoma cells (Liu *et al.*, 1985) and virally (HIV-1) infected cells (Zarling *et al.*, 1988). Alternatively, the antibodies may be chopped up and recombined to form bsabs (Brennan *et al.*, 1985; Glennie *et al.*, 1987). Brennan *et al.* (1985) reported a method which involves treatment of parental antibodies with pepsin, yielding $F(ab'\gamma)_2$ fragments. Reduction of the $F(ab'\gamma)_2$ fragments to Fab fragments is carried out using 2-mercaptoethylamine. Further reaction of the Fab fragments with Ellman's reagent yields Fab-TNB derivatives. One of the Fab-TNB is then reconverted to the Fab-thiol by further reduction with 2-mercaptoethylamine. Equal amounts of Fab-thiol and Fab-TNB are reacted to form the bsab. In the approach of Glennie *et al.* (1987) $F(ab'\gamma)_2$ fragments formed by peptic digests were reduced to Fab_{SH} with 2-mercaptoethanol. One Fab_{SH} species was alkylated with o-phenylenedimaleimide to yield Fab_{MAL} which has free maleimide groups. The Fab_{MAL} and Fab_{SH} were then reacted together to form bsab (Figure 1.2.1.2.) Both of these methods prevent any parental antibody recombinations.

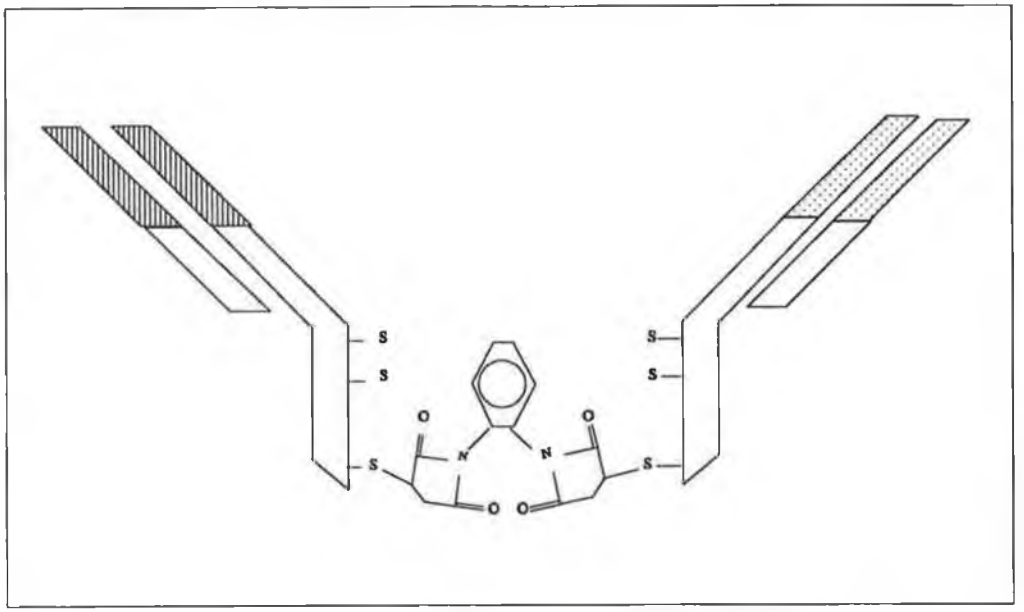


Figure 1.2.1.2.

The structure of the $F(ab'\gamma)_2$ bispecific antibody described by Glennie *et al.* (1987). The bispecific antibody is formed via thioether-linked Fab′ fragments. The protocol can be divided into three steps. $F(ab'\gamma)_2$ were first prepared using pepsin and reduced to Fab′γSH. Secondly, all the SH groups on one species were maleimidated with the bifunctional cross-linking agent *o*-phenylenedimaleimide yielding Fab′γMAL, and thirdly, Fab′γSH and Fab′γMAL were reacted under conditions favouring reactions between-SH and maleimide groups. These conditions are low pH and the inclusion of EDTA.

The method of Glennie *et al.* (1987) has been used successfully by a number of researchers, for delivery of an immunotoxin (saporin) to tumour cells (Flavell *et al.*, 1991; Flavell *et al.*, 1992; French *et al.*, 1992) and for targeting T-lymphocytes to tumour cells (Mezzanzanica *et al.*, 1991). Tutt *et al.* (1991) reported on a method for the formation of trispecific antibodies used to redirect resting cytotoxic T- cells to lymphoblastoid B-cell lines.

1.2.1.3. Genetic production of bsabs

A genetic method for the production of bsab fragments has recently been described (Kostelny *et al.*, 1992). This involves producing $F(ab'\gamma)_2$ antibody fragments directly by gene expression and using leucine zippers to generate the bispecific antibody fragments. A leucine zipper is a stretch of 35 amino acids containing leucine residues at every seventh position (Abel and Maniatis, 1989). These sequences are found in a number of DNA binding proteins. The regions used for bsab formation are obtained from the *Fos* and *Jun* protein products. These zipper regions are genetically fused to each of the Fab genes and, when expressed, result in Fab fragments complete with a zipper region. The two antibody species required in the bsab can then be simply mixed *in vitro* to result in heterodimer formation. The bsabs can also be obtained by co-expressing the Fab-Jun and Fab-Fos proteins in one cell line. The *in vitro* method is preferred due to the greater purity of the final product. Chimeric derivatives have also been made. Anderson *et al.* (1992) have used the method of Fc addition to form chimeric derivatives. Here the Fc portion of normal human Ig is linked by thioether bonds to proteins with suitable sulfhydryl groups. The Fc portion is linked via its hinge region and is therefore able to recruit effector molecules. These workers have made chimeric derivatives consisting of Fab derived from murine IgG and the Fc portion of normal human IgG.

O'Kennedy and Roben (1991) also report on the possibilties of using genetic engineering in the development of bsabs. These bsabs would be made up predominantly of the heavy chain variable regions of the antigen binding regions of both parental antibodies.

1.2.2. Applications of bispecific antibodies

1.2.2.1 Drug targeting

Corvalan and co-workers have shown that a bsab, targeting vinca alkaloids to the carcinoembryonic antigen (CEA) present on established MAW 1 human colorectal xenografts in nude mice, was capable of reducing both tumour growth and volume (Corvalan *et al.,* 1987a; Corvalan *et al* 1987b; Corvalan *et al.,* 1988; Corvalan and Smith 1987 & Apelgren *et al.,* 1988). Histological studies revealed that the remaining cells had altered morphology and cell organisation (Corvalan *et al.,* 1987a). One of the advantages of using bsabs to target tumour cells with toxins is that they eliminate the need for chemical conjugation of toxin to antibody, a process which may cause inactivation of the toxin (Tazzari *et al.,* 1993). A variety of toxins have been targeted to cells using bsabs (Figure 1.2.2.1.). These include ricin, pokeweed antiviral protein, cytostatic VDS (vinblastine and vindesine), anthracycline (Nolan and O'Kennedy, 1990) and saporin (Glennie *et al.,* 1988). Raso and Griffin (1981) have targeted Ig-expressing cells with a chemically made bsab consisting of anti-ricin and anti-human IgG binding regions. Protein synthesis in these cells was inhibited. Toxicity in non-Ig bearing cells was not augmented. Circulating Ig was capable of blocking the bsab effect.

Glennie *et al. (*1988) investigated the toxic effect of a bsab with specificty for L2C leukemic guinea pig cells and saporin. Concentrations of saporin which had previously failed to elicit a cytotoxic effect were now found to be cytotoxic. Flavell *et al.* (1991) demonstrated the cytotoxic effect of bsab used to redirect saporin to an acute T cell leukemic CD7-positive cell line HSB-2. Combinations of bsab have also been used to deliver saporin to T-ALL tumour cell lines (Flavell *et al.,* 1992). The principle behind this concept is that within any tumour cell population not all cells will be positive for a specific antigen and may thus escape potential therapeutic bsabs. Anti-saporin crossed with anti-CD7 and anti-CD38 were used singly and in combination and the relative cytotoxicities were assesed. It was shown, firstly, that combination therapy exhibited increased cytotoxicity and that this was more than just an additive effect and, secondly, that the rate of protein inactivation in targeted cells was enhanced.

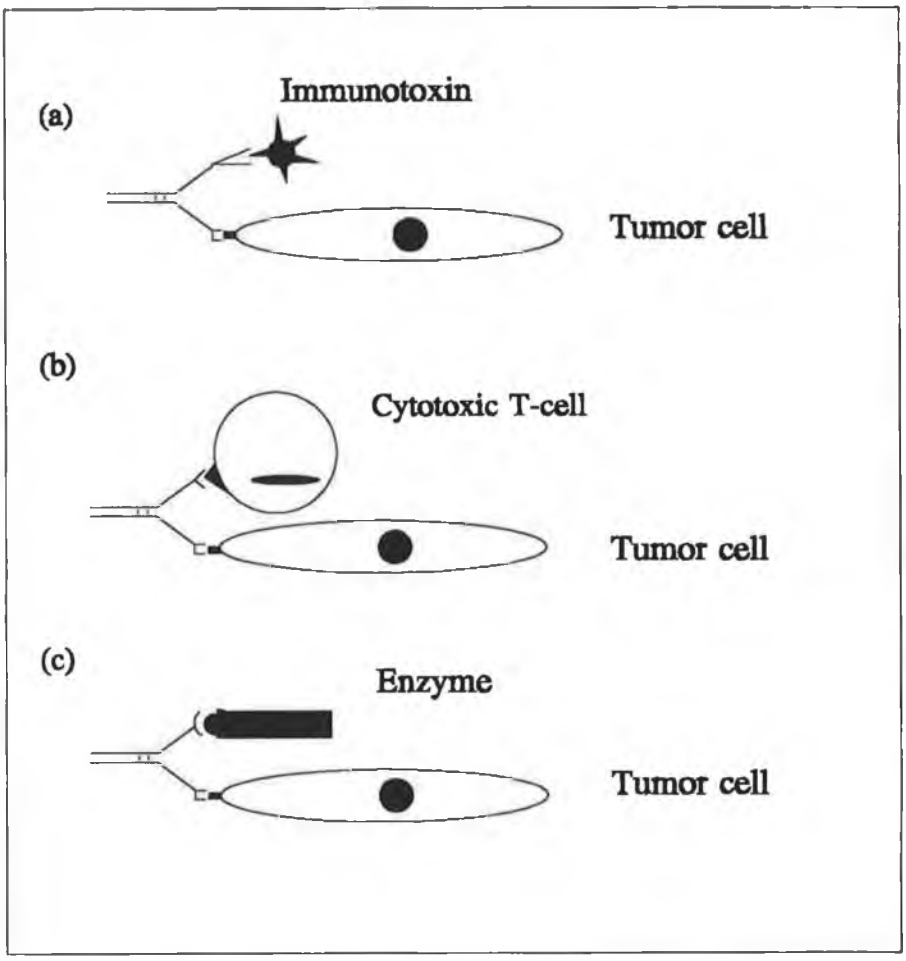


Figure 1.2.2.1.

Bispecific antibodies have been used to (a) direct immunotoxins to target cells and (b) target cells using a redirected effector cell. This cell could be a cytotoxic T cell, a natural killer cell or any cell capable of inducing cell death. It is vital that the bispecific antibody activates a molecule on the effector cell which can induce cell death. An example is the CD3 antigen on cytotoxic T cells. Another use for bispecific antibodies is in immunoassays (c) with one arm recognising a tumor cell and the second recognising an enzyme whose activity can be detected visually or spectrophometrically using a suitable substrate that is converted into a coloured product.

The CD25 antigen, which is present on activated lymphoid cells and leukemic and lymphoma cells with an activated membrane phenotype, has been used as a target molecule by Tazzari *et al.,* (1993). These have made a bsab using biological methods which can bind CD25 positive cells and saporin. This antibody has been shown to enhance the toxicity of free saporin against these CD25-positive cells. Embelton *et al.* (1991) used a combination of anti-ricin A chain, anti-CEA bsab and evaluated the cytotoxic effect of this bsab on a gastric tumour cell line, MKN45, which expresses large amounts of CEA. The cytotoxic effect of ricin A was observed at concentrations which would not normally be cytoxic. The inclusion of the ricin toxin B chain potentiated the cytotoxic effect of the ricin A chain.

1.2.2.2. Cell lysis

Fc receptors, FcγR, are present on a variety of cells. These act as cytotoxic trigger molecules when activated [Figure 1.2.2.1.]. CD3 complexed with αβ and γδ receptors is present on cytotoxic T cells. FcγRI (CD64) is present on monocytes, macrophage and INF-γ-activated PMN cells. FcγRII (CD32) is present on monocytes, macrophage, PMN and eosinophils and, finally, FcγRIII (CD16) is present on macrophages, NK cells and large granular lymphocytes as a transmembrane molecule and acts as a cytotoxic trigger molecule. On PMN's, however, FcγRIII is linked via phosphatidylinositol glycan and does not mediate cytotoxicity. Bsabs have been made with one arm against these receptors or effector cells and a second arm against a target cell such as a tumour cell. Normal functioning of lymphocytes and monocytes requires accessory molecules which are involved in migration, phagocytosis and extracellular cytotoxicity. These may be important in bsab-mediated cytotoxicity. Examples are the leucocyte function antigen (LFA-1) and CD56 (neural cell adhesion molecule N-CAM) which are accessory molecules in bsab-mediated cytotoxicity by myeloid cells and large granular lymphocytes, respectively. The purpose of these accessory molecules could be as a protection to the triggering of cytotoxicity unless the the target cell is bound. In addition, to trigger molecules (CD3 and FcγR) and accessory molecules, the cytokines may be involved in the activation of effector cells. For example IL-2, IL-4 and IL-7 are involved in T cell activation (Fanger and Guyre, 1991).

Heteroconjugates have been used to redirect cytotoxic T cells to lymphoma cells (Lui *et al.,* 1985). These consisted of anti-CD3 crossed with an anti-B-cell lymphoma idiotype. Heteroconjugates have been used to successfully mediate lysis of xenogenic tumour cells and chicken erythrocytes (Perez *et al.,* 1985). These workers have also mediated lysis in a variety of human tumour cell lines including lymphoblastoid cells, a Reed-Sternberg cell line, small cell lung cancer cell lines, lung and colon adenocarcinoma cell lines and a lung carcinoma cell line (Perez *et al.,* 1986) using redirected cytotoxic T cells. Heteroconjugates capable of targeting tumour cells with natural killer cells (anti-CD16) have proven successful in the lysis of fresh human melanoma cells and are capable of preventing tumour growth *in vivo* (Titus *et al.,* 1987*a*). Titus *et al. (*1987*b*) have demonstrated that heteroconjugates targeting a human subcutaneous colon adenocarcinoma tumour in nude mice with cytotoxic T cells via the CD3 T cell receptor can prevent tumour growth. Zarling *et al. (*1988) reported that heteroconjugates consisting of anti-CD3 crossed with anti-HIV and anti-CD16 crossed with anti-HIV were used to target cytotoxic T cells and large granular lymphocytes to HIV-infected cells to induce cell lysis. Lysis was found to be augmented by pre-treatment of peripheral blood lymphocytes with anti-CD3 or IL-2. Mezzanzanica *et al. (*1988) also used heteroconjugates successfully to target human ovarian carcinoma cell lines for lysis by cytotoxic T cells. Nitta *et al.* (1990) have reported a clinical trial in which patients were treated with heteroconjugates consisiting of crosslinked anti-CD3 and anti-glioma mAB which redirected lymphokine-activated killer cells to the tumour site. Treatment of patients with LAK cells alone proved to be as effective as conventional treatment (i.e. surgery, radiotherapy, chemotherapy). However, treatment with the heteroconjugates was even more successful than any of the conventional methods already listed.

$F(ab'\gamma)_2$ molecules have also been used to redirect effector cells. Athymic mice bearing human ovarian carcinoma cells had prolonged survival time when treated with anti-CD3 cross-linked with anti-tumour cell $F(ab'\gamma)_2$ (Mezzanzanica *et al.,* 1991). Greenman *et al. (*1991) have created $F(ab'\gamma)_2$ bsabs with one arm recognising chick red blood cells (CRBC) and the second recognising the FcγRII (CDw32) present on monocytes. These bsabs were as efficient as monoclonal and polyclonal anti-CRBC in inducing lysis in CRBC's. Addition of the Fc portion of human antibody *in vitro*

at a concentration equivalent to that found in serum was capable of inhibiting the activity of the monoclonal and polyclonal anti-CRBC. This is due to the competition between the added Fc with the Fc present on the CRBC for the antibody. This inhibition of lytic ability was not evident in the bsab system. This would be a beneficial property if the bsab was to be used *in vivo*. This also highlights how subtle changes in the bsab binding capabilities can have beneficial effects not present in the parental mAbs. There now exist trispecific antibodies, $F(ab'\gamma)_3$ which are capable of activating T cells with two arms and retargeting them with a third (Tutt *et al.*, 1991).

Bonardi *et al.* (1993) have created a panel of $F(ab'\gamma)_2$ with specificities for saporin and various B-cell antigens present on Raji and Daudi Burkitt's lymphoma cell lines. The antigens chosen were CD19, CD22, CD37 and μ-chain or κ-chain. When the tumour cells were targeted using these bsabs only the anti-saporin-anti-CD22 caused a significant cell kill, with a 1000 fold decrease in the amount of saporin required to achieve an IC_{50}. Bsab recognising either μ or κ chain and saporin resulted in about a 10 fold increase in saporin toxicity. The final two bsabs which recognised saporin and either CD19 or CD37 both failed to enhance toxicity significantly. These results are not due to the lack of bsab binding to the cells as work with ^{125}I-saporin indicates that the most toxic bsab is not the one which binds most abundantly to the cell surface. For example, the most abundant of the antigens present on the cells here is CD37 and, as expected, the bsab recognising CD37 and saporin is the one which binds most saporin to the cell surface. However, what seems to be most important is the ability of the bsab, when bound, to be internalized by processes such as endocytosis and/or intracellular routing of the bsab-complexes. In the example described above bsab recognising CD22 was the most efficient at accumulating saporin inside the targeted cells and was thus found to be the most cytotoxic.

Biologically formed bsabs have also been used in cytolysis. Staerz and Bevan (1986) have used a hybrid antibody with one arm recognising an allotypic determinant on the T-cell receptor and the second Thy-1.1. Tumour cells expressing the Thy-1.1 antigen are lysed by cytotoxic T cells. Lanzavecchia and Scheidegger (1987) used biologically made bsabs to target cytotoxic T cells to a variety of target cells. The bsabs formed had one arm against the CD3 T cell antigen and a second against any one of the

following: HLA antigens, human Ig, *Toxoplasma gondii* and an ovary carcinoma-associated antigen. Van Dijk *et al.* (1989) have demonstrated the lysis of a human renal cell carcinoma (RCC) cell line with cloned CD3-positive lymphocytes using a biologically formed bsab with specificities for the CD3 T cell antigen and G250 RCC-associated antigen. Bsab reacting with tumour cells and a non-activating T cell receptor failed to induce tumour cell lysis. Weiner and Hillstrom (1991) have also made bsabs biologically. These bsabs with specificities for CD3 and a tumour-associated antigen were superior in their lytic ability than were monospecific anti-idiotype antibodies. This work was performed on a murine B-cell lymphoma animal system. Hseih-Ma *et al.* (1992) have formed a bsab with specificities for the human C-*erb*B-2 protooncogene product and the FcγRIII (CD16). This biologically formed bsab and a chemically formed bsab with the same specificities were capable of lysing C-*erb*B-2 positive cells using CD16 positive mononuclear cells. Neither of the parental antibodies had this capability.

The method of "Fc addition" has been used to form chimeric derivatives which can be used to recruit effector molecules, such as complement and Fc receptor-bearing cells (Anderson *et al.*, 1992). Murine IgG Fab′ fragments which are capable of recognising antigens present on neoplastic lymphoid cells were combined to the Fc portion of human IgG to form $FabFc_2$ and FabFc (murine Fab and human Fc). $FabFc_2$ was the most successful in its ability to induce complement lysis and ADCC of guinea pig L2C leukemia; this may be due to its dual ability to bind C1q and Fc-receptor cells. Treatment and remission of human B-cell lymphoma required a plasma concentration of 50μg/ml of chimeric derivatized-antibody.

Shalaby *et al.* (1992) have used genetic engineering techniques to create bsabs with dual specificities for the T cell antigen CD3 and the phosphoglycoprotein HER2/$p185^{HER2}$. This antigen is overexpressed on breast and ovarian carcinomas as their development progresses. Here a bsab $F(ab'\gamma)_2$ with a humanised arm against the phosphoglycoprotein and a second against CD3 was formed. The anti-CD3 was derived from a hybridoma. The antigen binding region of the anti-CD3 antibody was installed in the context of human variable region framework residues. Anti-tumour and anti-CD3 Fab fragments were recommbined chemically forming a fully humanised $F(ab'\gamma)_2$ bsab. These can be used *in vitro* to lyse breast tumour cell lines bearing the

phosophoglycoprotein. The Fabs are chemically coupled after being excreted from *E. coli.*

Titus *et al.* (1987 a & b) have demonstrated the anti-tumour effect of redirected peripheral blood T cells, and NK cells, on subcutaneous tumours in nude mice. Recently they have reported on the production of a bsab which identified a CD8-negative T cell subset and tumour cells. This bsab was incapable of inducing tumour cell lysis *in vitro* but was capable of inhibiting subcutaneous tumour growth in mice (Segal *et al.,* 1991). Certain elements in the *in vivo* system prevent tumour growth and are absent from the *in vitro* system. The reasons for the diverging results *in vivo* and *in vitro* may be due to two different processes: (1) cytolysis which involves only targeted cells (Titus *et al.,* 1987 a & b) and (2) tumour growth inhibition which involves bystander cells in lysis (Segal *et al.,* 1991). These bystander cells may be absent from the *in vitro* system.

1.2.2.3. Immunoassays

One of the most powerful antibody-based analytical techniques is ELISA. This involves the detection of an antigen using an enzyme-labeled antibody. Bsabs which are capable of recognising an enzyme and an appropriate antigen can perform this procedure in a single step [Figure 1.2.2.1.]. Given the large potential that bsabs have in this research area it is surprising that their study with respect to ELISAs has been somewhat overshadowed by their uses therapeutically. Diagnostically ELISAs and immunocytochemical techniques can be used to detect proteins (antigens) in diseases where bodily fluids and biopsies are available for analysis. Suresh *et al.* (1986) made a biologically-derived bsab which detected peroxidase and substance P. The bsab was used in a one step immunoassay and was found to be superior to the peroxidase anti-peroxidase system in identifying substance P. Karawajew *et al.* (1988) has also made bsabs biologically with specificities for peroxidase and FITC. This bsab system can be used as a bridging molecule between various FITC-labeled proteins and peroxidase. α-Fetoprotein (AFP) and human chorionicgonadotropin (hCG) have been detected in this manner and the levels of detection are equivalent to standard assays used for their detection.

Bsabs reacting with CEA and galactosidase have been made and used in the

development of an homogeneous enzyme immunoassay (Görög *et al.*, 1989). This approach utilizes the ability of the anti-galactosidase antibody to protect the native enzyme from thermal degradation. On addition of bsab and enzyme any non-bound enzyme can be denatured at 62^0C. Substrate for the enzyme can then be added. Using optimal conditions, CEA concentrations up to 75ng/ml can be detected. This represents the concentration range of CEA which is of clinical significance. Kenisberg *et al.* (1991) have made bsabs biologically with dual specificity for mouse β growth factor (NGF) and HRP. The bsab, in combination with parental anti-NGF, can be used in an homogeneous sandwich enzyme immunoassay to quantify NGF. Takahashi *et al.* (1991) have developed several bsabs using biological methods for use in urease-based enzyme immunoassays. The second arm of the bsab generally recognised hCG. The assays developed using the bsabs were capable of detecting 25mIU/ml hCG, a concentration equivalent to that detected by conventional monoclonal antibody based immunoassays. HPLC purification of the bsabs enhanced detection of hCG by bsab to as low as 15mIU/ml. This is probably due to the removal of contaminating antibodies which would compete with the bsab for binding of the antigens.

Bsabs have been used to determine the concentrations of cytokines. Tada *et al.* (1989) used a biologically produced bsab capable of measuring human lymphotoxin (TNF-β). The bsab recognised human lymphotoxin and HRP. This was used in a one step ELISA which could detect between 1-100U/ml of human lymphotoxin. This correlated well with the concentration as determined using a bioassay. The ELISA using bsab could be completed in 2 hours whereas the conventional indirect ELISA was completed in 3-4 hours and a bioassay used took 3 days to complete. Stratieva-Taneeva *et al.* (1993) developed a bsab capable of measuring the concentration of human interleukin-2 (hIL-2) using a biologically made bsab which recognised both HRP and hIL-2. When applied to direct and competitive ELISAs this bsab could detect between 1.5 - 4ng/ml of IL-2 (the hIL-2 was prepared in PBS).

1.2.2.4. Miscellaneous uses of bispecific antibodies

Bruynk *et al.* (1993) have made humanized bsabs with dual specificities for CEA and a radiolabeled chelate DPTA-^{90}Y for use in two phase radiotherapy. The bsab had a humanized anti-CEA arm and a chimerized anti-DPTA-Y arm. Its production involved

double transfection of the corresponding genes into BHK cells. Bosslet *et al.* (1991) have studied the potential use of a biologically formed bsab in two phase radioimmunotherapy. One arm of the bsab recognises a tumour-associated antigen and the second recognises the radiolabelled chelate DPTA-Y. Whether or not the bsab has the same affinity as does the parental anti-tumour antibody is still under investigation. Snider *et al.* (1992) have used biologically formed bsabs to immunize mice. No adjuvant is required in this immunization protocol. The bsab recognised anti-class II MHC molecule with one arm and hen egg lysozyme (HEL) with the other. Several bsab were used for immunization each recognising a different epitope on the HEL. The antibody response in the mice is, therefore, determined by the epitope specificity of the anti-HEL arm, and will be against a new epitope. A subsequent single immunization with HEL did not effect the epitope response. However, further immunizations with HEL did induce an antibody response against new HEL epitopes. The Fc portion was not required in these studies for proper functioning of the bsab. $F(ab'\gamma)_2$ bsab fragments were as effective as whole bsabs in causing the anti-HEL response.

Stickney *et al.* (1991) have studied a bsab system for the delivery of imaging or therapeutic agents to tumour cells (metastatic adenocarcinoma of the colon). Initially the patients were treated with bsab which detected the tumour cell and a hapten. The bsab is localised at the tumour site. After 4 days the patients were treated with a hapten tagged with a radioisotype. This is bound to the pre-localized bsab. Twenty lesions were imaged and 8 new lesions were identified.

1.2.3. Future prospects for bsabs

Bsabs have thus far been used in immunotherapy, redirecting of effector cells or toxins to target sites and in immunoassays (Görög *et al.*,1989; Nolan and O'Kennedy, 1990; Takahashi *et al.*, 1991). One of the most advantageous features of bsabs is that they function in their native state and once made no chemical alterations in their structure are required. This is not the case with mABs for use in immunotherapy and immunoassays which must be chemically modified prior to use. Bsabs allow for one step systems whereby both antigens are linked by the one molecule thus negating the use of secondary antibodies labelled with enzymes and immunotoxins. They also allow

for accumulation of the immunotoxin or enzyme close to the cell surface. Appyling bsabs to immunotherapy requires that the antibodies be humanised, otherwise the risk of a host antibody response is increased. Use of bsabs in immunoassays, however, is not hampered by such restrictions and the potential applications of bsabs in this field should increase with further research.

In the work described in this thesis bsabs have been made with specificities for HRP and antigens present on Chronic Lymphocytic Leukemic cells. These were used to develop novel immunoenzymatic techniques.

1.3. An introduction to IL-6

The cytokines are a group of regulatory proteins which can be produced by almost all nucleated cells in the body. These include lymphocytes monocytes epithelial cells, keratinocytes and osteocytes. Hormones differ from cytokines in that they are produced by specific organs rather than a multitude of cells (Clemens, 1991a). They are present in greater quantities than are cytokines in the circulatory system and have a more constant and evident role in haemostasis. Cytokines have pleiotropic effects on haematopoietic and non-haematopoietic cells. Cells which respond to cytokines must possess the appropriate receptor for the particular cytokine. The cytokines act within a network and very often one cytokine may stimulate/inhibit another (Clemens, 1991b). A complete list of the cytokines is given in Table 1.3. and their mode of action is outlined in Figure 1.3.

This section deals with the role of interleukin-6 (IL-6) in the normal physiology and the pathophysiology of a number of diseases. IL-6 is a multifunctional cytokine which is produced by a wide variety of cells including both lymphoid and non-lymphoid cells. IL-6 is involved in regulating the final differentiation of B-cells into antibody-producing cells (Kishimoto, 1989), the acute phase response (Gauldie *et al.,* 1987; Nijstein *et al.,* 1987) and haematopoeisis (Hirano, 1991a). Murguchi *et al.* (1981) first identified human IL-6 in the supernatants of mitogen or antigen-stimulated peripheral mononuclear cells as a factor which induced immunoglobulin production in EBV-transformed cell lines or in normal B-cells which had been treated with *Staphylococcus aureus* Cowan 1 (SAC). IL-6 was identified independently by a number of groups before the protein's nucleotide sequence was known and hence it was known by a variety of names including interferon-β2 (May *et al.,* 1986), 26kDa protein (Haegeman *et al.,* 1986), B-cell stimulatory factor-2 (Hirano *et al.,* 1986), hybridoma/plasmacytoma growth factor (Aarden *et al.,* 1985; Nordan and Potter, 1986) and hepatocyte stimulating factor (Gauldie *et al.,* 1987). In the following section the structure of IL-6 and its role in disease are discussed.

Class	Example
Interferon (IFN)	IFN-α, IFN-β, IFN-γ.
Interleukins (IL)	IL-1 - IL-12.
Colony stimulating factors (CSF)	Colony stimulating factors (CSF) granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF).
Tumour necrosis factors (TNF)	Tumour necrosis factors (TNF) TNF-α, TNF-β.

Table 1.3. Classes of cytokines.

1.3.1. Structure of IL-6

IL-6 is a glycoprotein with a molecular mass of 21-28kDa. Human IL-6 consists of 212 amino acids including a hydrophobic signal sequence of 28 amino acid residues (Hirano, 1991). Murine (Van Snick *et al.,* 1988) and rat (Northemann *et al.,* 1989) IL-6 have been cloned and have been shown to consist of 211 amino acid residues which includes a signal sequence of 24 amino acid residues.

When compared to the cDNA sequence of other cytokines it has been shown that only the sequence of G-CSF bears any homology with that of IL-6 (Hirano *et al.,* 1986). It was shown that the positions of the four cysteine residues of IL-6 are similar to those of human and mouse G-CSF. This may lead to a similarity in tertiary structure and there may also be a functional homology. The role of the cysteine residues in the protein are not fully understood. Jamblou *et al.* (1988) replaced the cysteine residues with serines and no effect on the biological action of the protein was observed, indicating that the cysteine residues are not essential for protein function.

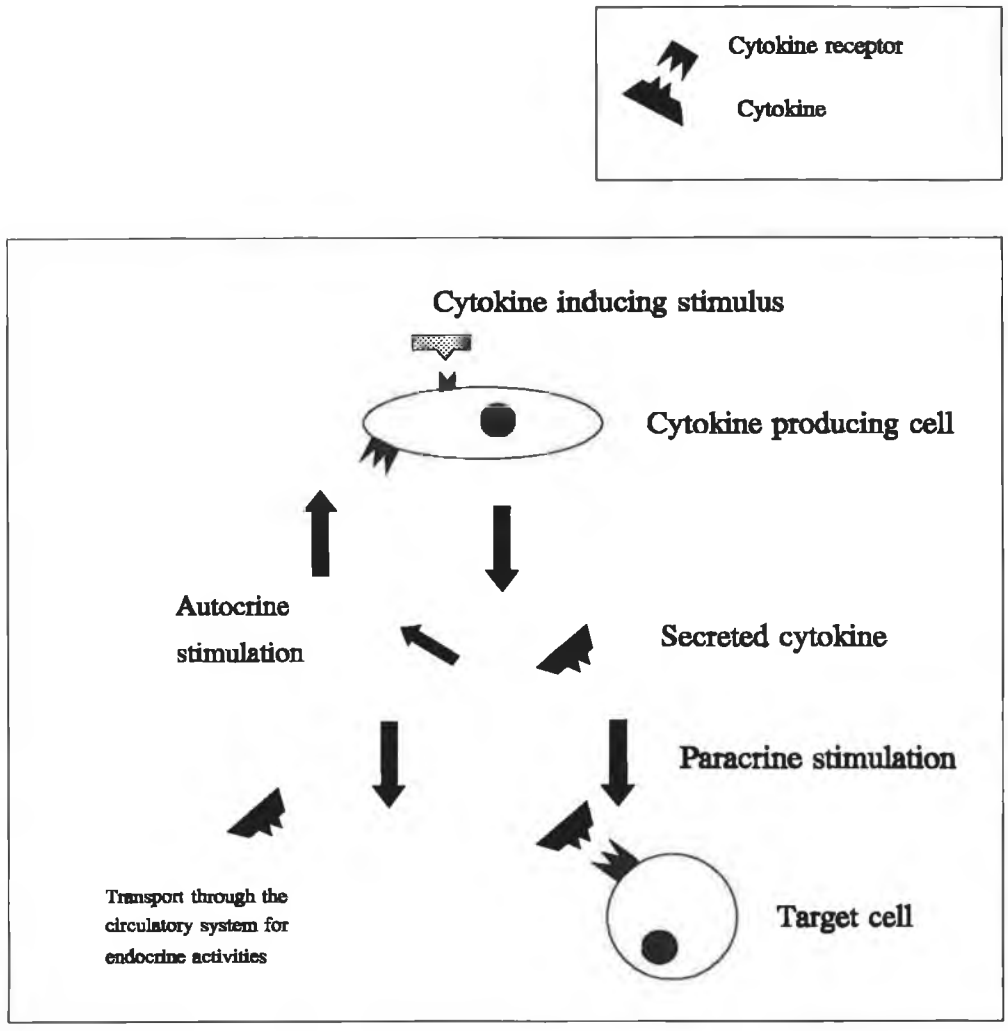


Figure 1.3.

The cytokines have three modes of operation. These are autocrine, paracrine and endocrine. Autocrine stimulation results when a cell secreting a cytokine has a receptor for that cytokine and is therefore self-stimulated. In the case of paracrine stimulation a cytokine secreted by cell stimulates a cell which is in close proximity. In the final case of endocrine stimulation cytokines may be be released into the circulatory system and stimulate cells over relatively large distances.

1.3.2. IL-6 and haematopoiesis

The involvement of IL-6 in the differentiation of activated B-cells into antibody-producing cells is described in Figure 1.3.2. Ikebuchi *et al.* (1987) were the first to report that the proliferation of murine multipotential haematopoietic progenitors requires the synergystic action of IL-3 and IL-6. The combination of IL-3 and IL-6 causes a decrease in the time that blast cell colony forming cells spend in the G_0 phase of cell division. Bodine *et al.* (1989) have reported that IL-3, in conjunction with IL-6, has a proliferative effect on primitive bone marrow progenitor cells. Chiu *et al.* (1988) demonstrated that survival and proliferation of a myeloid cell line was supported by IL-6. IL-6 production in stromal cells was found to be induced by IL-1 giving rise to the possibility that the involvement of IL-1 in haematopoeisis could be due to its induction of IL-6. Recently, proliferation of NK cells has been linked with IL-6, which has been shown to induce a small proliferative effect on these cells (Rabinowich *et al.*, 1993). The role of IL-6 in the maturation of megakaryocytes has been studied by Ishibashi *et al.* (1989) who showed that it acted directly in augmenting maturation in these cells. The observed effects include a physical change in cell size, an increase in acetylcholinesterase activity and a change in the DNA content. IL-6 also acts synergistically with IL-3 in the promotion of growth in megakaryocytes (Ishibashi *et al.*, 1989).

IL-6 is capable of inducing differentiation in myeloid leukemic cells (Sachs, 1987). Various authors have reported similar findings. For example, Onozaki *et al.* (1989) reported that IL-6 acts synergistically with IL-1 in the induction of differentiation in some human and mouse myeloid leukemic cells into macrophage-like cells. Chiu and Lee (1989) reported that M1 mouse myeloid leukemic cells when treated with IL-6 alone are induced to terminally differentiate into macrophages. The observed changes include an inhibition of M1 cell growth and the induction of phagocytic activity which is associated with macrophages.

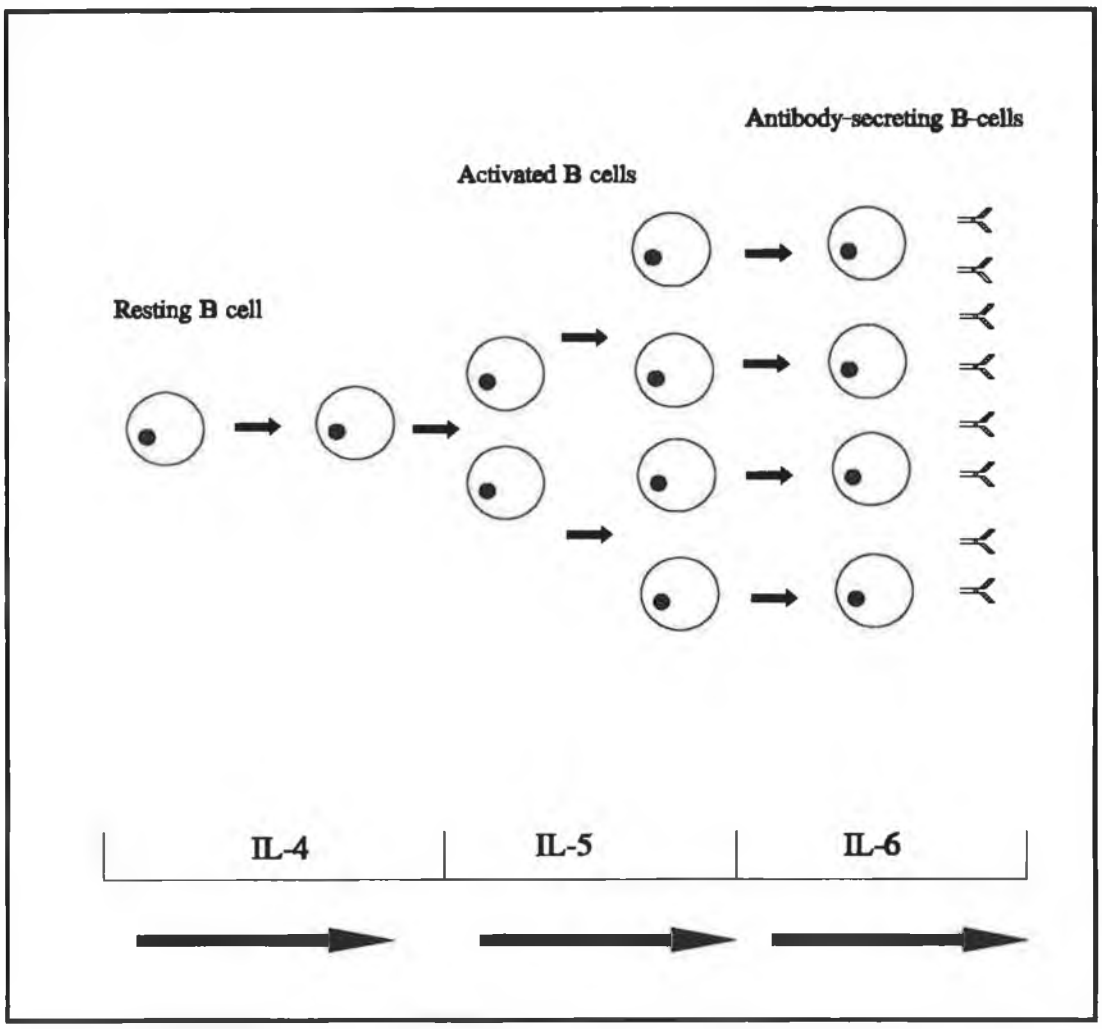


Figure 1.3.2.

Differentiation and proliferation of B-cells requires the involvement of various cytokines at different stages. Interleukin-4 is required for early activation of resting B-cells, interleukin-5, is then involved in the proliferation of these activated B-cells. Finally, interleukin-6 causes differentiation of activated B-cells into antibody-secreting cells.

1.3.3. IL-6 and the acute phase response

The acute phase response is a systemic reaction to inflammation or tissue injury. It is characterized by leukocytosis, fever, increased vascular permeability and alterations in plasma metal and steroid concentrations, together with increased levels of acute phase proteins (Hirano, 1991 b). Nijsten *et al.* (1987) examined IL-6 levels and acute phase proteins in serum from patients with severe burns and concluded that IL-6 plays a causal role in the acute phase response. Human umbilical vein endothelial cells (HUVEC) synthesise IL-6. This synthesis can be stimulated by treatment of the cells with the inflammatory mediators IL-1, TNF-α, TNF-β (lymphotoxin) and LPS (Jirik *et al.,* 1989). In contrast, transforming growth factor-β which regulates endothelial cell growth and biosynthetic functions, did not detectably stimulate IL-6. This suggests that HUVEC selectively respond to inflammatory mediators to increase synthesis of IL-6 again indicating that IL-6 contributes significantly to the inflammatory response. Hypergammaglobulinemia is also associated with inflammation and this too could be mediated by the action of IL-6 in the differentiation of B lymphocytes.

Biologically active IL-6 can be induced in cancer patients treated with rTNF (Jablons *et al.,* 1989). IL-6 induction decreased after serial daily doses of rTNF. Serum levels of acute phase plasma proteins and corticosteroids rose in response to rTNF administration. This could in turn be due to the increase in IL-6. M^{c}Intosh *et al.* (1989) have also reported that TNF induces IL-6 production in tumour-bearing mice.

1.3.4. IL-6 and disease

1.3.4.1. The haematopoietic leukemias

Certain haematopoeitic leukemias have been shown to have altered IL-6 levels (Table 1.3.4.1.).

Disease	IL-6 activity	Observed effect of IL-6 on tumour growth or symptoms
B-CLL ALL, (Biondi *et al.*, 1989)	+ -	Not reported
CLL, ProLL,HCL ALL, lymphoma (Freeman *et al.*, 1989)	- +	Not reported
Lymphoma (Yee *et al.*, 1989)	+	Possibly an autocrine growth factor for tumour cells
Lymphoma (Kurzrock *et al.*, 1993)	+	May induce B symptoms
ATL (Sawada *et al.*, 1990)	+	Not autocrine
Multiple myeloma (Kawano *et al.*, 1988)	+	Possibly an autocrine growth factor for tumour cells

Table 1.3.4.1. Involvement of IL-6 in haematopoietic diseases.

CLL, Chronic lymphocytic leukemia; ALL, Acute lymphoblastic leukemia; ProLL, Prolymphocytic leukemia; HCL, Hairy cell leukemia; ATL, Adult T cell leukemia

"+" :- IL-6 detected,

"-" :- IL-6 not detected.

Biondi *et al.* (1989) investigated IL-6 production in B-cell chronic lymphocytic leukemia (B-CLL). B-CLL cells constitutively express IL-6 mRNA and secrete a biologically active protein. Six of the 11 B-CLL tested were positive for mRNA expression. Conditioned medium collected from freshly isolated B-CLL cells (B-CLL-CM) stimulated the growth of an IL-6-dependent cell line 7TD1. Three out of five B-

CLL-CM tested showed IL-6 activity. These results correlated with the mRNA work. In 10 cases of acute lymphoblastic leukemia (ALL) no increase in IL-6 mRNA was found. Schena *et al.* (1991) examined cytokine production by normal and malignant B-cells. Normal B-cells from different stages of activation and proliferation were examined. These consisted of germinal centre B-blasts (GC-B) and mantle B-cells from tonsils. For comparison of malignant B-lymphocytes with their closest normal counterpart they chose normal CD5-positive B-lymphocytes from cord blood and malignant CD5-positive lymphocytes from a patient with B-CLL. The cytokines were analysed using Northern and Western blotting techniques. Among normal B-cells "mantle B-cells" (M-B) are the most active cytokine producers. They were found to secrete IL-1α, IL-6, TNF-α and TGF-β. This pattern of cytokine production is the same in B-CLL cells. The normal equivalent of B-CLL, CD5-positive B lymphocytes from cord blood, only produce TGF-β. GC-B produce IL-2, TNF-α and TGF. TGF-β is the only cytokine produced by all cell types examined and, therefore, its production in B-CLL cells may be expected. The relevance of the cytokines produced by B-CLLs is as yet unknown but it is suggested that IL-1α, TNF-α and IL-6 may be responsible for some of the sytemic manifestations of the disease such as fever and wasting (Schena *et al.,* 1991). The relationship between cytokine production and the progressive failure of polyclonal B-cell and T-cell function in CLL has yet to be evaluated. An opposite effect is seen in AIDS where elevated IL-6 levels are thought to be associated with polyclonal B-cell activation (Nakajima *et al.,* 1989; Breen *et al.,* 1990).

Freeman *et al.* (1988) reported that the IL-6 gene is expressed in anti-Ig-activated and neoplastic B-cells. After activation with anti-Ig, normal splenic B-cells expressed IL-6 mRNA with levels reaching a peak at 4 hours and declining thereafter. The expression of IL-6 mRNA was examined in various B-cell neoplasms. 11/25 patients with non-Hodgkins B-cell lymphoma and 4/4 patients with plasma cell leukemias expressed IL-6 mRNA in their neoplastic B-cells. Of 19 B-cell leukemias, only one, an ALL, was found to express IL-6 mRNA. IL-6 mRNA was not detected in B-CLL, prolymphocytic leukemia (ProLL) and hairy cell leukemia (HCL) neoplastic cells. Yee *et al.* (1989) established 14 cell lines from patients with non-Hodgkins lymphoma. IL-6 mRNA and the secreted protein were detected in two of the cell lines, OCI-LY3 and

OCI-LY12. It was found that the addition of anti-rIL-6 antibody had an inhibitory effect on their growth indicating a possible autocrine role for IL-6. Kurzrock *et al.* (1993) have also reported that a significant number of patients with lymphomas have elevated IL-6 levels. "B-symptoms" which are also known as night sweats, are a clinical manifestation of lymphoma disease and are associated with the increase in serum IL-6 concentrations. The survival period of patients with relapsed or advanced Hodgkin's disease is decreased if serum IL-6 concentration is greater or equal to 22pg/ml.

The ability of adult T cell leukemia (ATL) which is causally associated with the human T-cell lymphotropic virus (HTLV-1) to secrete IL-6 or proliferate in response to it was studied by Sawada *et al.* (1990). Neither control nor ATL sera had detectable IL-6 levels. However, conditioned medium collected from growing the cells *in vitro* was found to have detectable levels of IL-6. Conditioned medium from 4/6 patients examined had elevated IL-6 levels. Exogenous addition of IL-6 and anti-IL-6 did not effect ATL proliferation indicating that tumour growth is not stimulated by IL-6 acting in an autocrine manner.

Myeloma cells freshly isolated from patients produce IL-6 and express its receptor (Kawano *et al.*, 1988). On treatment with anti-IL-6 antibodies *in vitro* the myeloma cell growth is inhibited. This is direct evidence that an autocrine loop is operating in oncogenesis of human myelomas. The predictive significance of IL-6 and neopterin levels in patients suffering from multiple myeloma has been investigated (Reibnegger *et al.*, 1991). High concentrations of both these molecules are indicative of a poor prognosis, but the predictive strength of neopterin exceeds that of IL-6.

1.3.4.2. Non-haematopoietic malignancies

Non-haematopoeitic tumours have also been reported to secrete altered levels of IL-6 (Table 1.3.4.2.). Blay *et al.* (1992) reported that 48% of patients with metastatic renal cell carcinoma have detectable levels of IL-6 in their serum, whereas only 11% of the normal adults tested had detectable levels. The IL-6 levels of expression correlated with increased serum levels of C-reactive protein (CRP). These patients were observed to give poor response to IL-2 treatment and had shorter survival rates.

Disease	IL-6 activity	Observed effect of IL-6 on tumour growth or symptoms
Renal cell carcinoma (Blay *et al.*, 1992)	+	May induce the acute phase response
Ovarian carcinoma (Erroi *et al.*, 1989)	+	May induce the acute phase response
Ovarian cacinoma (Watson *et al.*, 1990)	+	Not an autocrine factor for ovarian carcinoma cell lines
Glioblastoma (Van Meir *et al.*, 1990)	+	May induce the acute phase response
Prostrate carcinoma cell lines (Seigall *et al.*, 1990)	+	Possibly an autocrine growth factor for tumour cells

Table 1.3.4.2. Involvement of IL-6 in non-haemopoietic malignancies.

"+" :- IL-6 detected,

"-" IL-6 not detected.

Mullen *et al.* (1992) transduced fibrosacroma tumour cells with the IL-6 gene. When injected subcutaneously into mice these tumours exhibited reduced tumourigenicity, increased immunogenicity and reduced metastatic potential. Suppression of subcutaneous tumour growth is not seen when using nude or irradiated mice thus implicating T-cell-dependent and radiosensitive host contribution to the observed effect.

Erroi *et al.* (1989) investigated IL-1 and IL-6 production in tumour-associated macrophages (TAM) isolated from ascitic fluid and solid human ovarian carcinomas. TAM were found to release IL-1 when stimulated with LPS. However, IL-6 production was detected *in vitro* without deliberate stimulation. IL-6 has also been detected in ovarian carcinoma ascitic fluid. This IL-6 may account for the levels of liver-derived acute phase proteins associated with malignancies of these carcinomas. Watson *et al.* (1990) has reported that 3/4 ovarian carcinoma cell lines tested constitutively produce IL-6. Immunoperoxidase staining reveal that >98% of the cells produce some IL-6. The levels of IL-6 may be modulated by other inflammatory cytokines such as TNF-α, IL-1β and interferon-γ. IL-6 is not an autocrine factor here

as neither exogenous IL-6 or anti-IL-6 affect the cell growth. IL-6 levels were also increased in ascitic fluid of ovarian cancer patients and in the supernatants of primary cultures from freshly excised ovarian tumours.

IL-6 release by glioblastoma patients has also been investigated (Van Meir *et al.*, 1990). IL-6 was analyzed in supernatants from glioblastoma cell lines, in cerebospinal fluid, in cyst fluids and in the tumoural tissues of glioblastoma patients. IL-6 levels in conditioned medium from cultured cells contained significant amounts of IL-6. IL-6 mRNA was also detected in glioblastoma cell lines. IL-6 was detected in patient body fluids and IL-6 mRNA was detectable in solid frozen tumours. Immunohistochemical analysis revealed that IL-6 is produced by GFAP-positive cells in glioblastoma, thus ruling out the possibility that IL-6 is being produced solely by T-cells. Monocytes and macrophages, fibroblasts, microglial cells, astrocytes and endothelial cells all of which are present in glioma tissue, are known IL-6 secreting cells. The clinical consequence of an increase in IL-6 in these patients is still not known. It may be responsible for the acute phase response and the elevated immune complexes often associated with glioblastoma patients.

Siegall *et al.* (1990) have provided data to indicate that IL-6 and its receptor may play an important role in prostate cancer. Three prostate carcinoma cell lines were treated with a chimeric toxin composed of IL-6 and *Pseudomas* endotoxin. Under such conditions all three cell lines died. This indicates the presence of the IL-6 receptor on the cells. Supernatants from the cell lines also had detectable levels of IL-6. These results indicate that an autocrine loop similar to that observed in multiple myeloma (Kawano *et al.*, 1988) may be in operation here. Pekarek *et al.* (1993) analyzed the cytokine mRNA profile of a range of human sarcoma cell lines and murine UV-induced sarcomas. The results obtained indicate that the cytokine profile can differ in tumours of the same type and even in variants of parental tumours.

1.3.4.3. Polyclonal B-cell abnormalities

Polyclonal B-cell abnormalities or autoimmune diseases have also been reported to have altered levels of IL-6 associated with their development (Table 1.3.4.3.).

Disease	IL-6 activity	Role of IL-6 in disease
Cardiac myxoma (Hirano *et al.*, 1987)	+	May be involved in autoantibody production
AIDS (Breen *et al.*, 1990)	+	May be involved in polyclonal B-cell activation
AIDS Kaposi sarcoma-derived cells (Miles *et al.*, 1990)	+	Possibly an autocrine growth factor for these cells
Psoriasis (Grossman *et al.*, 1989)	+	Not reported
Mesangial proliferative glomerulonephritis (Horii *et al.*, 1989)	+	Possibly an autocrine growth factor for these cells
Acute bacterial infection (Helgfoot *et al.*, 1989)	+	May induce the acute phase response

Table 1.3.4.3. Involvement of IL-6 in polyclonal B-cell abnormalities, proliferative diseases and infection.

"+" :- IL-6 detected,

"-" :- IL-6 not detected.

Cardiac myxoma is a benign intra-atrial tumour. Hirano *et al.* (1987) reported that these cells constitutively produce IL-6. This may be important in the production of autoantibodies which are a feature of the disease. In Castleman's disease activated B-cells in the germinal centre of hyperblastic lymph nodes were also found to constitutively produce IL-6 (Yoshizaki *et al.*, 1989).

Exposure of peripheral blood monocyte cells (PBMC) from healthy donors to the human immunodeficiency virus (HIV) leads to the production of IL-6 mRNA and the secreted protein (Nakajima *et al.*, 1989). Breen *et al.* (1990) detected IL-6 levels in the circulating plasma, and IL-6 mRNA in PBMC obtained from HIV infected donors. PBMC from these infected donors cultured without IL-6 activators constitutively produced elevated levels of IL-6. Levels of acute phase protein (which is induced by

IL-6) were also increased in HIV-infected donors. The increase in IL-6 levels in HIV infected patients may contribute to the polyclonal B-cell activation as seen in HIV and AIDS infection. IL-6 and the IL-6 receptor are produced by Kaposi sarcoma-derived cells of AIDS patients and IL-6 is required for the optimal proliferation of these cells (Miles *et al.*, 1990). There may be an autocrine loop involving IL-6 in the oncogenesis of AIDS-Kaposi sarcoma-derived cells. This is the case in multiple myeloma as discussed in section 1.3.4.2. IL-6 may therefore be important in the development of Kaposi sarcoma *in vivo*.

1.3.4.4. Infections

Fong *et al.* (1989) examined the appearance of circulating IL-6 in the plasma of healthy volunteers who received intravenous injections of endotoxin. Intravenous administration of endotoxin produced a rise in circulating immunoreactive IL-6 levels. An increase in circulating C-reactive protein was associated with this IL-6 rise. IL-6 is, therefore, likely to be one of the endogenous mediators triggered during bacterial infection by of the human host. Hellfgoot *et al.* (1989) have detected elevated levels of biologically active IL-6 in the body fluids of patients with acute bacterial infections. The levels of IL-6 are sufficiently high as to elicit enhanced synthesis of acute phase plasma proteins by hepatocytes. The proposed involvment of IL-6 during infections is outlined in Table 1.3.4.3.

1.3.4.5. Proliferative diseases

Elevated levels of IL-6 mRNA and protein in epidermal and dermal cells have been detected in psoriatic plaques (Grossman *et al.*, 1989). IL-6 levels were also elevated in the plasma of these patients, probably due to the enhanced production of IL-6 in psoriatic lesions. IL-6 was found to stimulate the proliferation of human keratinocytes in culture. This may be an important factor for other cutaneous disorders characterized by epidermal hyperplasia. The overproduction of IL-6 may contribute to the pathophysiology of the disease.

IL-6 may be an autocrine factor for rat mesangial cells (Horii *et al.*, 1989). rIL-6 was found to induce *in vitro* growth of rat mesangial cells and IL-6 activity was detected in supernatants obtained from cultured mesangial cells. IL-6 mRNA was also detected

in the cultured mesangial cells. 50% of urine samples from patients suffering with mesangial proliferative glomerulonephritis (PGN) had significant levels of IL-6 whereas urine from other primary glomerular diseases or healthy volunteers had no detectable IL-6 activity. There was also a relationship between the levels of urine IL-6 and the progressive stage of PGN. Immunohistochemical staining using an anti-IL-6 monoclonal antibody showed that mesangial cells in the affected glomeruli of PGN patients produced IL-6. This was not the case in normal kidney mesangial cells or mesangial cells from patients with membraneous nephropathy or minimal change nephrotic syndrome. In summary "deregulated production of IL-6 is involved in PGN and measurement of urine IL-6 is helpful for the differential diagnosis of PGN as well as for monitoring the progression of the disease" (Horii *et al.*, 1989). The involvment of IL-6 in proliferative diseases is summarized in Table 1.3.4.3.

1.3.5. IL-6 and immunotherapy

As discussed in the previous section IL-6 is associated with many diseased states. Whether its presence is pathological or therapeutic is of crucial importance if it is to be used in immunotherapy. IL-6 is reported to be an autocrine growth factor for many diseases including multiple myeloma (Kawano *et al.*, 1988), non-Hodgkins lymphoma (Yee *et al.*, 1989), Kaposi sarcoma (Miles *et al.*, 1990), and ovarian carcinoma (Watson *et al.*, 1990). M^{c}Intosh *et al.* (1989) reported that an increase in tumour burden was associated with an increase in IL-6. Various other investigators have reported constitutive expression of IL-6 in B-CLL (Biondi *et al.*, 1989; Schena *et al.*, 1991). A feature of B-CLL is the failure of polyclonal B-cell activation (hypogammaglobulinemia). Breen *et al.* (1990) have suggested that the stimulation of IL-6 production in monocytes by HIV may be associated with the polyclonal B-cell activation as observed in AIDS patients (hypergammaglobulinemia). This a clear example of elevated IL-6 levels in different diseases leading to different/opposite *in vivo* responses. Mulé *et al.* (1990) examined the therapeutic efficacy of IL-6 on established tumours in mice. Metastases from four tumour types examined were significantly reduced when treated with recombinant IL-6 alone. IL-6 treatment did not exhibit the usual toxic side effects observed with recombinant IL-2 treatment. Also unlike recombinant IL-2 treatment, no vascular leak syndrome was observed. The

mechanisms by which the anti-tumour effect occurs does not appear to be through the direct effect of recombinant IL-6 on the tumour since host immunosuppression eliminates successful therapy. Recombinant IL-6 may activate secondary cytokines which are directly/indirectly involved in tumour regression. Administration of low doses of IL-6 together with subtherapeutic doses of TNF to mice bearing an established weakly immunogenic syngenic tumour, at a subcutaneous site, resulted in marked tumour regression and higher cure rates. A possible mechanism for this anti-tumour IL-6 effect has been suggested by Rabinowich *et al.* (1993). This involves the upregulation of β_1 and β_2 integrins and the intracellular adhesion molecule ICAM-1. Coupled to this is the ability of IL-6 to allow binding of NK cells to the extracellular matrix. IL-6 has been shown to have an inhibitory effect on certain breast carcinoma and leukemic/lymphoma cell lines (Chen *et al.,* 1988; Danforth *et al.,* 1993).

Ohe *et al.* (1993) reported that Lewis lung carcinoma cells transfected with IL-6 cDNA caused a state similar to cachexia when transplanted into mice. The survival of these mice was shorter than that of mice transplanted with Lewis lung carcinoma cells alone. No difference in tumour growth and metastasis was observed. However, Mullen *et al.* (1992) has shown that fibrosarcoma cells, when transduced with the IL-6 gene, exhibited reduced tumourigenicity increased immunogenicity, and reduced metastatic ability in mice.

Serve *et al.* (1991) examined the effects of recombinant human IL-6 (rhIL-6) on the growth of 26 different cell lines derived from non-haematopoietic cells (solid tumours). The growth of the human malignant cell lines was not affected by the rhIL-6. The tumours examined here were of an advanced nature and, therefore, may not reflect the continual growth and development of the tumour. Lu *et al.* (1992) highlights the importance of the time, during tumour development, at which growth factors are measured. They report that IL-6 can act as an inhibitor in the early stages of melanoma development (metastatically incompetent) but not on advanced progression (metastatically competent). IL-6 may act as a growth regulator of other non-haematopoietic human solid tumours depending on the stage to which the disease has progressed.

Clearly an important point to keep in mind when assessing the potential of IL-6, or any other cytokine, as an immunotherapeutic molecule, is the fact that tumour

development at different stages of development may respond differently (inhibitory/stimulatory) to the same growth factor (Schawarz *et al.*, 1988; Lu *et al.*, 1992) and given the diverse nature of cytokine production in similar tumours (Pekarek *et al.*, 1993) it is important to know and essential to understand the cytokine profile of the tumour at a given time. Only when all of this infomation is available should cytokine therapy be contemplated.

In the work described in this thesis studies on the production and possible roles of IL-6 in CLL were investigated.

1.4. Introduction to hybridoma cell growth

The steps involved in hybridoma technology are represented schematically in Figure 1.4. The medium conditions are crucial if efficient fusion is to be accomplished. For example at post-fusion the cells are often seeded in medium containing various cytokines such as IL-6 which is reported to increase the numbers of antibody-positive hybrids formed. The following section critically examines the conditions affecting hybridoma growth post-fusion.

1.4.1. Feeder cell layers and conditioned medium (CM)

The use of conditioned medium (CM), or feeder layers is important for the successful growth of hybridoma cells at certain stages such as post fusion or at the low cell densities encountered in cloning procedures. At these stages the fused cells are quite vunerable and may therefore require the extra growth properties supplied by these factors. These factors would include the cytokines in B-cell growth and differentiation such as IL-4, IL-5 and IL-6. To a large extent CM and commercial preparations have all but replaced feeder cells. This is mainly due to the fact that the growth factors/cytokines secreted by feeder cell layers have been identified and, thus, may be replaced by defined growth factors. IL-6 or hybridoma growth factor (HGF), is one such factor and has been shown to increase the proportion of antibody-producing hybridomas post fusion (Bazin and Lemieux, 1989), indicating that IL-6 is involved in differentiation of hybridomas into antibody-producing cells. According to Sugasawara (1988) (i) the cloning efficiency of IL-6-dependent hybridomas increases with the addition of IL-6 and (ii), the total number of hybridomas (antibody positive and negative), post fusion are increased in the presence of IL-6 indicating that IL-6 is involved in differentiation and proliferation of hybridomas. Harris *et al.* (1992) have reported on an improved myeloma fusion system for the generation of a large number of hybridomas against specific antigens. This involves a retroviral infectant of Sp2/0 cells that constitutively expresses recombinant murine IL-6 (Sp2/mIL-6) as fusion partner. The results obtained show that yields of both Ig-secreting hybridomas and antigen-specific monoclonal antibodies were increased 3-15 fold and 5-9 fold, respectively.

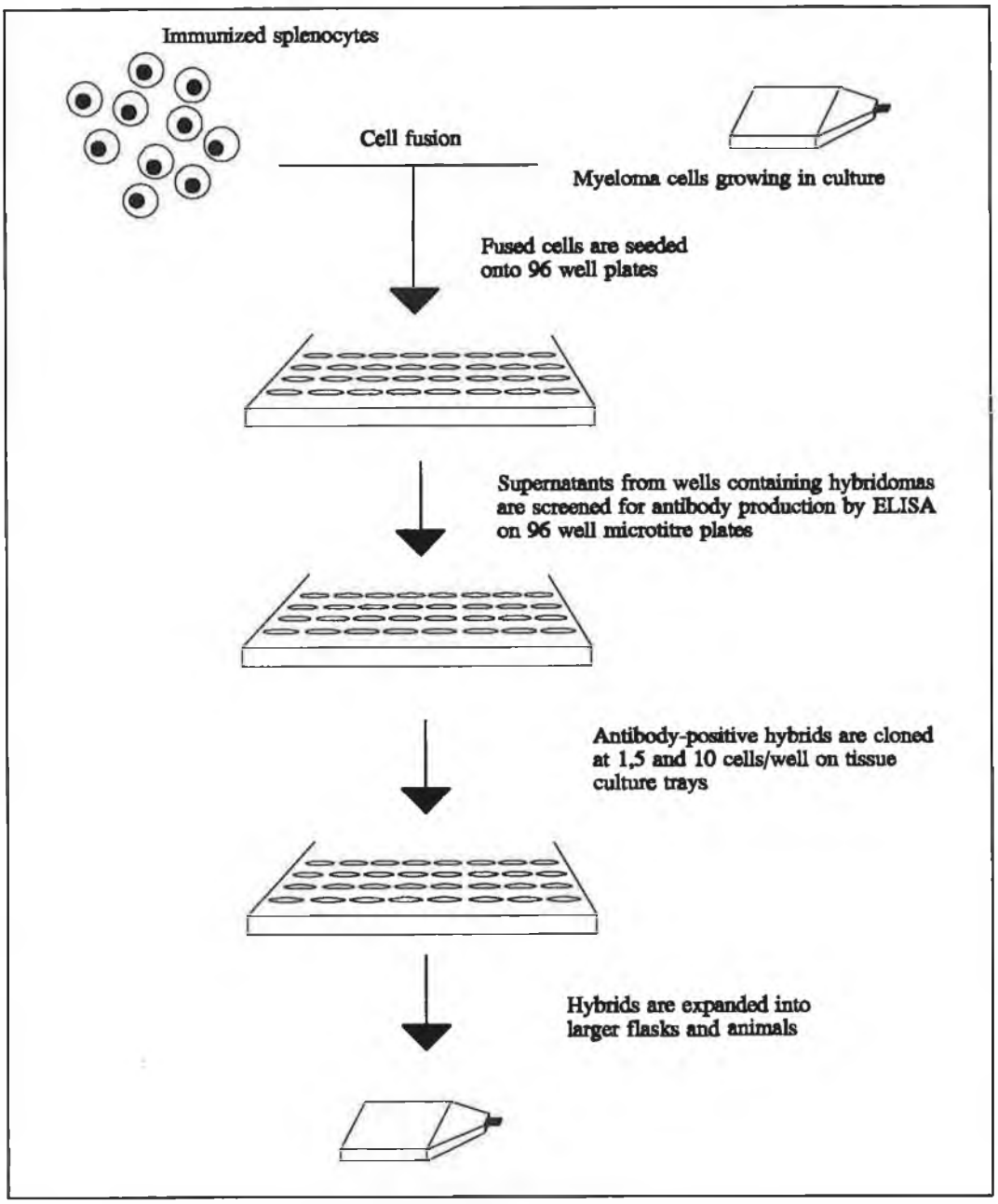


Figure 1.4.

Monoclonal antibody production involves the fusion of splenocytes from an immunized animal with myeloma cells using polyethylene glycol which promotes membrane fusion. Fused cells containing genetic material from both the myeloma and splenocyte cells are selected and screened for specific antibody production. Newly formed hybridoma cells can be quite unstable and at this stage the choice of growth factors present in the culture medium may increase the chances of hybridoma survival. Our work involved studies on the growth of hybridomas at the clonal stage.

The hybridomas generated with this system are comparable to those obtained using conventional systems as regards growth rates, stability and Ig-production.

IL-6 is not involved in the proliferation of activated B-cells but rather it induces final maturation of B-cells into antibody-producing cells (Kishimoto, 1989). IL-6 is, however, involved in the proliferation of myeloma cells (Kishimoto, 1989). Since hybridomas are of B-cell and myeloma cell origin this may account for the growth dependence of freshly fused hybridomas on IL-6/HGF.

Various authors have described a wide range of feeder cells and CM used in hybridoma technology (Pintus *et al.,* 1983; Sugasawara *et al.,* 1985; Butcher *et al.,* 1988; Long *et al.,* 1988; King and Sartorelli 1989; Perrson and Lerner, 1990). Pintus *et al. (*1983) compared endothelial cell growth supplement (ECGS), human endothelial culture supernatant (HECS), and murine peritoneal cells for their ability to support hybridomas during various growth stages and on mAB production. Both ECGS and HECS proved superior to mouse peritoneal cells. A 1.5-4 fold increase was obtained when fused cells were cloned in ECGS compared to feeder cells. Westerwouldt *et al.* (1983), examined the growth promoting ability of human umbilical cord serum (HUCS), HECS, and other B-cell promoters. The numbers of antibody-producing hybrids post fusion and the cloning efficiency were examined. The growth-promoting ability of HUCS proved to be the best. The number of antibody producing hybrids remained constant with all media examined with the exception of FCS. HECS was also a potent hybridoma growth medium though not as effective as HUCS.

The effect of macrophage-conditioned medium (MCM) on hybridoma colony formation and cloning efficiency experiments has been examined (Sugasawara *et al.,* 1985). MCM was found to act as a growth promoter for hybridomas (hybridomas were made using the P3X63-AG8.653 myeloma). The MCM used was obtained from primary mouse peritoneal macrophages and from 3 continuous macrophage cell lines. It is not reported if other cell lines produce MCM, or, whether hybridomas derived from different myeloma cell lines respond to MCM. ECGS was found to be ineffective as a hybridoma growth stimulator in the work described by these investigators. No comparison of MCM versus feeder cells was made for either hybridoma colony formation or cloning efficiency, but it is reported that previous experience of using feeder cells indicated that they were similar or slightly less effective than MCM, but

that outgrowth of hybridomas was interfered with by growth of macrophages, and exhaustion of medium by feeder cells. The macrophage product(s) responsible for stimulating hybridoma growth has not been identified. Ransom *et al.* (1986) used ECGS as a growth supplement for hybridomas. Their findings showed that when freshly fused cells were plated out in ECGS-supplemented medium the numbers of hybridomas formed exceeded those formed using mouse peritoneal cells. An increase in the number of fibroblastic type cells in the culture was associated with this increase in hybridoma numbers. This could be due to the growth enhancing properties of ECGS on splenic tissue in particular connective tissue and these cells may also aid hybridoma growth. ECGS was also found to enhance the cloning efficiency of hybridomas. Walker *et al.* (1986) has reported that CM prepared from the murine fibroblast cell line, L-929 (L-CM), acted as a growth promoter for B-cell hybridomas and increased the proportion of antibody-producing hybrids. In ten fusions those grown in L-CM had a significantly higher number of hybrids formed compared to peritoneal exudate cells (PEC). The percentage of antibody-secreting clones was also increased. L-CM also enhanced the cloning efficiency of the hybridomas as well as increasing the number of antibody-secreting clones. Van Mourik and Zeijemaker (1986) have demonstrated that culture medium supplemented with HUCS or normal human serum did not support the growth of hybridomas during cell fusions, whereas HGF and HECS did. All four supplements, however, were capable of supporting the growth of established hybridomas during cloning procedures suggesting that growth conditions are less stringent at this stage.

Butcher *et al.,* (1988), compared several growth systems for their efficiency at supporting hybridoma growth post fusion, during cloning and after recovery from liquid nitrogen. They found that mitomycin C-treated 3T3/A31 cells used as a feeder layer were the most effective and reliable system for supporting cell growth at these stages. A period of 3-5 days was required before the hybridoma cells were added. Macrophage cells could be as effective as the 3T3/A31 cells but were less reliable, a lot depending on the donor mouse. ECGS inhibited the growth-promoting capacity of both macrophages and 3T3/A31 cells.

Long *et al.* (1988) have examined eight different cell line feeder layers and two conditioned media for their abilty to increase the establishment efficiency of newly

formed hybridoma cells. The feeder layers consisted of three human fibroblast cell lines, IMR-90, MRC-5 and WI-38, NIH-3T3 cells, Vero African green monkey kidney cells, a clone of the Hela epitheloid cell line and murine macrophage and spleen cells. The human lung diploid fibroblastic cells, Vero African green monkey kidney cells, MRC-5-CM and medium containing ECGS, were all found to enhance the establishment of newly formed hybridomas. Murine splenocytes and macrophages and the Hela cell clone did not exhibit this effect and gave similar results to control medium. The irradiated lung fibroblast cells were found to be the most efficient at supporting hybridoma proliferation.

King and Sartorelli (1989) have compared macrophage and thymocyte feeder cells and soluble growth factors, Ewing sarcoma growth factor (ESGF) and ECGS, for the cloning of hybridomas. ESGF was comparable to feeder layers in its ability to support cloning of hybridomas. ECGS did not support the clonal growth of hybridomas examined. This was in agreement with the work of Butcher *et al.* (1988) but contrasts with the results of Pintus *et al.* (1983).

Medium supplemented with Sp2/0 ascitic fluid, as an alternative to FCS, added post fusion has been shown to enhance the cloning efficiency of hybridomas at least 2-fold (Stewart and Fuller, 1989). The number of antigen-specific antibody-secreting hybridomas was increased 5-fold in the eight fusions. Some of the advantages in the use of Sp2/0 ascites includes the ease in which it can be made and the small variation in batches. The growth factor(s) responsible is/are unknown. Supplementing fusion medium with 5% normal mouse serum has been reported to increase the number of monoclonal antibody-producing murine hybridomas by 7-10 fold (Perrson and Lerner, 1990). The cause of the beneficial effect is unknown. Raybould *et al.,* (1988) reported that production of antibody-secreting rabbit-murine hybridomas requires the use of rabbit supplemented serum. Without rabbit serum no antibody-producing hybrids were formed. These two sets of results are indicate that serum from the species of the cells used in fusions aid hybridoma growth. Gomathi *et al.* (1991) reported on the use of CM from the glioma cell line C6 (C6CM) for the growth of mouse hybridomas. They found that C6CM improved both hybridoma growth and cloning efficiency. The growth-promoting effect was observed on both the 3B3 hybridoma cells which were derived in the presence of C6CM and on the hybridoma, 3B6, which was derived

without C6CM. C6CM was found to have no effect on the growth of the myeloma cell line used. However, the survival time for spleen cells was increased. The factor(s) involved is(are) not well described, but it is suggested that the factor(s) is novel in so far as they say it is reported here for the first time. Sanjeev (1992) indicates that IL-6 is the factor and that this should have been further investigated by the initial reporters. Sharma (1992) claims that the IL-6 dependent cell line 7TD1 was used to test C6CM for IL-6 activity and that they are currently assaying the C6 cells for IL-6 mRNA.

1.4.2. Alternative strategies used in hybridoma growth

Bell *et al* . (1983) used a hanging drop method for cloning hybridomas. The concentration of FCS used also affected the cloning efficiency. 30% FCS in complete medium was found to be the most efficient. Civin and Banquerigo (1983) have described a method for cloning of hybridoma cells in ultra-low gelation temperature agarose. The advantages of this agarose method over the liquid systems given are that firstly it reduces incubator space and, secondly, the volumes required in the liquid systems are reduced. Unlike semi-solid systems, in which it may be difficult to break up cell clusters for transfer to liquid culture systems, the medium used here avoids gelation at room temperature for long periods. Davis (1986) has used semi-solid medium for the growth of hybridomas. This method has several advantages. Clonal competition is eliminated and this ensures that relevant antibody-secreting hybridomas are not overgrown by faster growing non-antibody-secreting hybridomas. A large number of hybridomas can be isolated from a single fusion and almost every colony examined will be a clone. This can be explained statistically in that the probability of clones which average 0.75mm in diameter overlapping on a plate 35mm in diameter is 4%. Given that each each colony can be regarded as a clone the necessity to clone is greatly reduced. The time scale involved in the fusion is reduced due to the elimination of cloning and feeding stages. One of the most advantageous properties listed is the fact that overgrowth with fibroblast cells is prevented because the petri dishes used are non-tissue grade. Nowadays, given the range of growth factor products available on the market which have replaced feeder cells, this is no longer a problem. Disadvantages include the increased workload due to the large numbers of hybridomas to be screened and problems associated with picking hybridomas for further culture.

1.4.3. Human feeder layer systems

Brodin *et al.* (1983) have reported on the use of human peripheral blood monocytes as a feeder layer for use in the growth of human hybridoma, myeloma and lymphoma clones. In liquid cultures human monocytes and mouse thymocytes were superior to rat and mouse peritoneal macrophages in their ability to enhance cloning efficiency. The macrophage may be exhibiting some sort of phagocytic activity on the cloned tumour (hybridoma, myeloma and lymphoma) cells. CM obtained from the monocytes or the tumour cells did not enhance the cloning efficiency. In semi-solid medium feeder cell layers of monocytes, mouse thymocytes and macrophage all exhibited enhanced cloning efficiency. B-CLL lymphocytes have also been used as feeder cells to clone human heterohybridoma cell lines (Sanchez *et al.*, 1991). The B-CLL cells were examined for the presence of CD5, CD21, CD3 and CD19 by immunoflourescence. Peripheral blood cells which were both CD5 and CD21 negative were selected for several reasons. Firstly, since CD21 is in fact the EBV receptor and the heterohybridomas are derived from EBV-infected cell lines this prevented feeder cells from being infected by any EBV occasionally released by the hybrids. Secondly, since a majority of CD5-positive lymphocytes are involved in the production of polyreactive antibodies *in vivo* this may interfere with the detection of antibody secretion by the heterohybridomas. An added advantage of B-CLL cells as feeder cells is that given their limited survival time *in vitro* they do not require irradiation prior to use.

There have been mixed reports as to whether or not IL-6 levels are increased in patients with CLL (Freeman *et al.*, 1988; Biondi *et al.*, 1989; Schena *et al.*, 1991; Aguilar-Santelises *et al.*, 1992). B-CLL have been shown also to produce BCGF (Kawamura *et al.*, 1986), a factor with IL-1 type activity (Uggla *et al.*, 1987) and IL-α, TNF-α and TGF-β (Schena *et al.*, 1991). Given the wide range of cytokines produced by these patients the possibility of using CLL plasma as a growth supplement, CLL-DMEM, for murine hybridomas was examined. CLL-DMEM was found to increase the cloning efficiency of the hybridomas examined. This discovery may allow for a better understanding of how the cytokines are effecting these patients *in vivo*, in particular the effects on B-cells since hybridomas are of B-cell origin.

Aims

Aims of the project

Various aspects of the disease Chronic Lymphocytic Leukemia (CLL) were examined. The potential of biologically and chemically produced bispecific antibodies for the detection of CLL cells and CLL related antigens using novel immunocytochemical and ELISA systems was assessed. The bispecific antibodies recognised both peroxidase and an antigen present on CLL cell. The hybridoma G12 secreting the anti-CLL antibody was produced and characterized previously in our laboratory. It was found to recognise a determinant on the cells with a MW of 69,200D. The antigen was shown to disappear subsequent to chemotherapy suggesting a correlation between disease stage and antigen expression. The antibody may therefore have be of value diagnostically if used to monitor presence of the antigen. The bsabs developed here allow for rapid detection of the antigen in single step system.

Aspects of the role of interleukin-6 (IL-6) in CLL were investigated. Among the normal functions of IL-6 is the final differentiation of activated B-cells into antibody-producing cells. It has been reported that IL-6 levels are raised in patients with CLL. The clinical manifestations of this have yet to be elucidated. One of the clinical symptoms of CLL is hypogammaglobulinemia and this may be associated with the increased susceptibility of these patients to bacterial infections which are a major cause of morbidity and mortality. IL-6 levels in the plasma of patients with CLL and CM obtained from growing peripheral blood lymphocytes of these patients in culture were determined. IgG levels in these plasma samples were also examined. Both sets of results were correlated. If elevated IL-6 is associated with normal IgG levels then these patients may have an improved prognosis. IL-6 may then have an immunotherapeutic potential.

The growth promoting ability of CLL plasma on hybridoma cells at the clonal stage was compared to conventional methods of hybridoma cell cloning such as cell feeder layers and to a novel growth medium developed in our laboratory. Cytokines levels have been well studied in patients with CLL. The role of these cytokines in the progression of the disease is not fully understood. Hybridomas are of B-cell origin and the effects of the plasma on the growth of hybridomas may mimic the effect of these cytokines *in vivo* on B-cell development in patients with the disease.

Chapter 2

Materials and Methods

2.1. Materials

All cell culture media, foetal calf serum, L-glutamine, penicillin-streptomycin solutions were obtained from Flow Laboratories, Irvine, Scotland. Cell culture vessels were obtained from Costar, Cambridge, MA, 02140, USA. Chemicals were of Analar grade and were obtained from the 'Sigma Chemical Co.', Poole Dorset, England; Riedel de Häen, AG, Seelze, Hannover, Germany and BDH Chemicals Ltd., Poole, Dorset, England.

Details of specific kits and antibodies are listed in Tables 2.1.1 and 2.1.2.
Cell lines used in the course of the work are listed in Table 2.1.3.

Materials	Supplier
Human recombinant IL-6,	Boehringer Mannheim, Hanover, Germany.
Enzyme conjugated antibodies Alkaline phosphatase-conjugated goat anti-mouse whole antibody Alkaline phosphatase-conjugated goat anti-mouse whole IgM, Alkaline-phosphatase conjugated goat anti-rabbit whole IgG, Peroxidase conjugated goat anti-mouse IgG,	Sigma Chemical Co., Poole, Dorset, England.
Monoclonal antibodies Goat anti-human IL-6 antibody Rabbit anti-human IL-6 antibody,	British Bio-technology Products Limited, 4-10 The Quadrant, Barton Lane, Abingdon, Oxon, OX143YS. SeraLab, Crawley Down, Sussex, RH104FF, UK.
Dako PAP kit System 40,	Dakopatts Limited, 16 Manor Courtyard, Hughunden Avenue, High Wycombe, Bucks., HP13 5RE.
Mouse monoclonal antibody isotyping reagents,	Sigma Chemical Co., Poole, Dorset, England.
Goat anti-mouse IgG,	Sigma Chemical Co., Poole, Dorset, England.

Table 2.1.1. Specific antibodies, antibody kits and recombinant proteins used and their sources.

Materials	Supplier
Hoechst 33258,	Calbiochem, Behring Diagnostics, La Jolla, CA 92037, U.S.A.
Polyethylene-glycol 1500,	Boehringer Mannheim, Hanover, Germany.
Rapi-diff II, Triple stain set for histochemical staining of cells,	Diagnostic Developments, Southport, UK.
PD-10 Sephadex columns G-25M, Ficoll-Paque,	Pharmacia, Uppsala, Sweden.
Ultragel AcA44,	LBK, IBF-Pharmindustries, Reactifs, France.
Bicinchoninic acid protein assay kit,	Pierce Chemical Co., Rockford, Illinois, 61105, U.S.A.

Table 2.1.2. Miscellaneous reagents used in the course of the work and their sources.

Cell line	Cell type	Cat. No.	Source
SP2/0	Mouse myeloma		Flow Laboratories, Irvine, Scotland.
Ep16 14E5 7P41 OKT8 LoVo K562 NRK	Mouse hybridoma Mouse hybridoma Mouse hybridoma Mouse hybridoma Human colon adenocarcinoma Human erythroleukemia Normal rat kidney	 CRL 8014 CCL 229 CCL 243 CRL 6509	American Tissue Culture Collection, 12301 Park Lawn Drive, Rockville, Maryland, 20861, USA.
G12 E5 H9 H12	Mouse hybridoma Mouse hybridoma Mouse hybridoma Mouse hybridoma		Produced by Dr. B. Lannon at DCU
EJ-138	Human bladder carcinoma	8511412	ECACC, PHLS, Porton Down, Salisbury, Wiltshire, SP4 OJG, UK.
B9	IL-6-dependent mouse hybridoma		Dr. L. Aarden, Red Cross, Amsterdam, Netherlands.
T24	Human bladder carcinoma		Dr. J. Tager, University of Amsterdam, 1105 AZ, Netherlands.
GCCM	Human glioblastoma		ECACC, PHLS, Porton Down, Salisbury, Wiltshire, SP4, OJG, UK.
HL60	Human promyelocytic leukemia		Dr. T. Cotter, St. Patricks College, Maynooth.

2.1.3. Cell lines used during the project.

2.2. Equipment

2.2.1. Centrifugation

A Heraeus Christ Labofuge was used for centrifugation of universal tubes (1-25mls) and centrifugation tubes (1-50mls). Smaller volumes were centrifuged on a Heraeus Biofuge A using eppendorf containers (1-1.5mls). Cytosmears were prepared on a Hereaus Sepetech cytocentrifuge. For centrifugation of large volumes (up to 200mls), at higher speeds a Sorvall centrifuge was used.

2.2.2. Cell culture

Asceptic cell culture techniques were undertaken in a Holten Laminar Air flow cabinet, HB 2448K. Procedures involving mutagenic agents were performed in a Cytogard Laminar Air cabinet, (Gelman Sciences). Cells were incubated in a humid 5% CO_2 athmosphere at 37^0C in a Jouan EG 115 IR incubator.

Long term storage cells were cryopreserved in a cryocontainer, (Union Carbide LR-30A).

Cells were visualised on a Nikon phase contrast ELWD 0.3 microscope.

Single cell suspensions of splenocytes were obtained using a Cell Dissociation Sieve/Tissue Grinder kit.

2.2.3. Electrophoresis

Electrophoresis was perfomed on a Biorad vertical electrophoresis system.

2.3. Tissue culture methods

2.3.1. Suspension cultures

Sp2/0, G12, Ep16, 14E5, OKT8, 7p41, B9, K562 and HL60 cells were cultured in Dulbecco's Modification of Eagle's Medium, (DMEM), containing 5% FCS (v/v), L-glutamine (2mM), Hepes (1mM) and sodium pyruvate (2mM). B9 cells which are interleukin-6-dependent (IL-6-dependent) are further supplemented with 5% T24 (v/v) supernatant. T24 cells produce interleukin-6 (IL-6). All cultures with the exception of the B9 cell line were seeded in $25cm^3$ and $75cm^3$ culture flasks. B9 cells were cultured in 24 and 96 well culture plates. All cell lines were incubated in a humid, 5% CO_2 atmosphere at 37^0C.

2.3.2. Adherent cell cultures

T24, LoVo, GCCM and EJ cells were cultured in DMEM containing 5% FCS (v/v), L-glutamine (2mM), Hepes (1mM) and sodium pyruvate (2mM). All cultures were seeded in $25cm^3$ and $75cm^3$ culture flasks. For passaging adherent cell lines it is necessary to trypsinise the cells. Firstly, the culture medium is decanted. The culture vessel is then flushed out with serum-free DMEM. This has a twofold effect in that it ensures all FCS, which could potentially interfere with trypsin activity, is removed and secondly, any non-adherent dead cells are removed. 5-10mls of the trypsin:EDTA solution, [trypsin 0.25% (w/v) with 0.02% EDTA in sterile PBS], was added to the flasks. The culture was then incubated at 37^0C for 10 minutes. The cell suspension was then added to a universal container containing 5mls of complete culture medium and centrifuged at 2000rpm for 10 minutes. Cells were resuspended in the appropriate amount of culture medium and seeded into culture vessels. All cell lines were incubated in a humid, 5% CO_2 atmosphere at 37^0C.

T24 cells were used as a source of IL-6. IL-6 is required for the growth of B9 cells.

2.3.3. Cell counts

Cell counts were performed on an improved Neubauer Haemocytometer slide using the Trypan blue stain. A 1:1 ratio of the cell suspension and stain was prepared. This was examined after 2-5 minutes by light microscopy. Live cells exclude the stain and maintain their integrity while dead cells stain blue.

2.3.4. Storage of cell lines

Stocks of cells were maintained in the liquid phase of liquid nitrogen in Cryocontainers, (Union Carbide). Washed cells were resuspended dropwise in 1ml ice-cold FCS supplemented with 5% (v/v) dimethylsulfoxide and transferred to sterile cryotubes. The cryotubes were lowered slowly into the gas phase and eventually into the liquid phase of liquid nitrogen.

Cells were recovered from liquid nitrogen by thawing the cells rapidly at 37^0C and then washing in complete medium. The cells were centrifuged at 2000rpm for ten minutes and resuspended in complete medium.

2.3.5. Mycoplasma detection - Hoechst 33258 fluorescent assay

Mycoplasma was detected using the Hoechst stain. The method used was that first described by Chen, (1977) and modified by Carroll *et al.*, (1988). Bisbenzimid, (Hoechst 33258) is a DNA interchelator which detects mycoplasma in cellular cytoplasm. $5x10^3$ mycoplasma free-NRK cells were cultured overnight in 1ml of complete medium on coverslips. Culture supernatant was removed from the test cell line and added to the NRK cells which were incubated for a further 3-4 days. The coverslips were then washed 3 times in PBS and fixed for 6 minutes in methanol:acetone (1:1) solution at -20^0C. The coverslips were then washed 3 times in PBS and incubated for 10 minutes with the Hoechst 33258 stain. The coverslips were washed again to remove unused stain and mounted on fresh slides. Samples were stored in the dark until viewed under UV light on a Nikon fluorescence microscope at 100X magnification with oil immersion and a B2 combination filter.

2.3.6. HAT sensitivity testing of myeloma cell lines

Sp2/0 cells were plated in complete medium supplemented with $1x10^4$ M 8-azaguanine (8-aza) for 3 days. HAT-sensitive (HAT-s) cells can grow in this medium but HAT-resistant (HAT-r) cells die. If the cells are found to be HAT-s they are grown in 8-aza-free medium for 1 week prior to use in cell fusions.

2.3.7. Antibody production by somatic cell fusions

2.3.7.1. Immunization

A 1ml solution of HRP at a concentration of 40µg/ml was prepared in PBS pH 7.2. The volume was brought to 2mls with Complete Freund's Adjuvant and emulsified using a Kikawerk Ultra-Turrax homogeniser. Balb/C mice were immunized intraperitoneally with 1ml of this emulsion. With the exception of the first immunization Incomplete Freund's was used at all times. Complete Freund's Adjuvant contains mycobacterial artifacts which act as an immune stimulant. Immunizations were performed fortnightly until a satisfactory antibody titre was detected in the mouse serum. The mice were bled via the tail vein. The blood was allowed to clot at 4^0C for between 10-20 minutes before being centrifuged at 2000rpm for 5 minutes in a Biofuge. Serum was screened by ELISA for anti-HRP antibody levels as described

in Section 2.8.7. The final immunization was given 3-5 days prior to cell fusion.

2.3.7.2 Isolation of immune splenocytes

The spleen was removed aseptically and the splenocytes were dissociated on a sterile cell dissociation sieve. The resulting cell suspension was washed 3 times in serum free DMEM. Red blood cells were lysed using Gey's haemolytic solution (Mishell and Shiigi, 1981). This involved resuspending the pellet in 5mls of Gey's haemolytic solution and incubating at 37^0C for 5-10 minutes. The cell suspension was then centrifuged for 10 minutes at 2000rpm. The remaining lymphocytes were washed 3 times in DMEM before fusion as described in Section 2.3.7.7.

2.3.7.3. LINO medium for use during *in vitro* immunizations

When preparing LINO medium it is important that the proteins are added before the lipids. A 5% (w/v) solution of BSA was prepared in Iscove's Modification of Eagle's Medium, (IMEM). To 50mls of the medium was added 2mls each of a 5% (w/v) solution of bovine insulin made up in 0.01N HCl, a 0.25% (w/v) solution of human transferrin prepared in sterile PBS and 2mls of a 1:1000 dilution of ethanolamine prepared in sterile 0.15M PBS, pH 7.2. 0.2mls each of 0.5% (w/v) solutions of linoleic acid palmitic acid and oleic acid each prepared in absolute ethanol were then added. The medium was filter sterilized and brought to 1 litre with IMEM. 50ml aliquots of this medium were stored at -20^0C until required for use. Just before use L-glutamine (2mM) and 50µM 2-mercaptoethanol were added.

2.3.7.4. Thymocyte conditioned medium, (TCM) for use during *in vitro* immunizations

Five female 3-4 week old Balb/c mice were sacrificed. A thymonectomy was performed on each mouse and the pooled thymocytes were dispersed by flushing with serum-free DMEM using a 21-G needle. The thymocytes were washed twice more using serum-free DMEM and for a final time in LINO. The cells were resuspended to give a final cell concentration of $5x10^6$/ml dispensed in 25ml aliquots into $75cm^3$ culture flasks and incubated in a humid, 5% CO_2 atmosphere at 37^0C for 48 hours. The supernatant was decanted and centrifuged at 2000rpm for 10 minutes, filter

sterilized and stored at -20^{0}C for until required for use.

2.3.7.5. *In vitro* immunization of rabbit splenocytes

The method used was that described by Ossendorp *et al.*, (1986). This method uses a serum-free medium called LINO as described in Section 2.3.7.3. (Yssel *et al.*, 1984) in which the splenocytes are cultured. Immunization medium consisted of an equal volume of LINO and TCM (LINO/TCM). Splenocytes from a New Zealand white male rabbit which had been immunized *in vivo* were isolated as described in Section 2.3.7.2. and stored in liquid nitrogen. The cells were washed in serum-free DMEM. The resulting pellet was washed for a final time in LINO/TCM medium. The cells were resuspended at a concentration of $5x10^6$ cells/ml in immunization medium, (LINO/TCM), which contained 5µg/ml of HRP and dispensed in 2ml lots into the wells of a 6 well culture tray which was then incubated in a humid, 5% CO_2 atmosphere at 37^{0}C for 72 hours. After this time period the cells were harvested for cell fusion. The concentration of antigen used depends on its antigenicity and this must be optimized for each different antigen.

2.3.7.6. Induction of HAT-sensitivity in hybridoma cell lines to be used in the production of triomas

HAT-sensitivity can be induced by continuous growth of hybridoma cells in increasing concentrations of toxic guanine analogues such as 8-aza, (0-20µg/ml), or by mutagenising the cells and subsequently selecting for new phenotypes using selection agents such as 8-aza.

The action of 8-aza is mediated through the enzyme hypoxanthine guanine phospho-ribosyl transferase, (HGPRT), whereby preformed purines or purine analogues are taken up by the so-called "scavenger pathways". Cells having a defective enzyme or no enzyme are resistant because they do not incorporate the toxic analogue into their nucleic acid.

The first method chosen to induce HAT-sensitivity was to grow hybridoma cells in increasing concentrations of 8-aza. Cells capable of growth in complete medium containing 20µg/ml 8-aza are considered HAT-s. Hybridoma cells were seeded at

$1x10^3$ cells/well in 24 well plates in complete medium supplemented with various concentrations of 8-aza. The cells were initially seeded in low concentrations of 8-aza, (0-10µg/ml). After 3-6 days the cells growing in the highest concentrations of 8-aza were removed and seeded in fresh medium containing increased concentrations of 8-aza, (up to 20µg/ml). If at this stage cells were found to be growing in 20µg/ml of 8-aza they were examined for HAT-sensitivity HAT-sensitivity as described in Section 2.3.6., and if HAT-s they were ready to fuse to immunized splenocytes for the formation of trioma cells.

HAT-sensitivity was also induced in hybridoma cells using the mutagenic reagent ethyl methanosulphate (EMS). Mutagenesis allows new and desirable phenotypes to exist. New phenotypes were selected from the mutagenised cells using 8-aza as a selective agent. Firstly, a kill curve using EMS was established for the particular cell line. This was to determine the concentration of EMS which caused a 50% kill of the cells. This concentration is arbitrary and allows for the out-growth of new phenotypes. $2x10^4$ cells were seeded into 24 well culture plates in complete medium containing a range of concentrations of EMS (0-200µg EMS/ml of complete medium) and incubated in a humid, 5% CO_2 atmosphere at 37^0C for between 16-24 hours. The cells were washed 3 times using sterile 0.15 M PBS, pH 7.2 and re-seeded in fresh culture medium. After between 3-6 days cell counts were performed as described in Section 2.3.3. and the 50% kill concentration was determined. Secondly, a kill curve using the selection agent 8-aza, was established. To ensure that HAT-s hybridoma cells are formed the concentration of 8-aza resulting in a 100% kill of the wild-type hybridoma cells was required. Surviving hybridoma cell are therefore HAT-s. Cells were seeded at $1x10^3$ cells/well in 24 well plates in complete medium supplemented with 8-aza ranging in concentration from 0-30µg/ml.

Once the concentrations of EMS resulting in a 50% kill and that of 8-aza resulting in a 100% kill of hybridoma cells were established the experiment to induce HAT-sensitvity was performed. The cells were mutagenised using the concentration of EMS resulting in a 50% cell kill and HAT-s cells are selected using 8-aza at a concentration determined to be that which results in a 100% cell kill of wild type hybridoma cells.

2.3.7.7. Production of triomas by somatic cell fusions

G12 hybridoma cells which were rendered HAT-s and tested for the presence of mycoplasma were maintained in the mid-log phase of growth for 7 days prior to fusion. These cells were fused to HRP-immunized mouse and rabbit splenocytes. On the day of cell fusion the cells were washed 3 times in serum free DMEM. Immunized spleen cells were depleted of RBC as described in Section 2.3.7.2. Both cell populations were counted and mixed at a ratio of 5 spleen cells to 1 myeloma cell and centrifuged at 3000rpm for 10 minutes in a 50ml centrifuge tube. The supernatant was decanted. It is important at this stage to aspirate all remaining medium from the centrifuge tube. This avoids diluting further the PEG-1500 which is to be added subsequently. The cells were fused using PEG-1500 at 37^0C. One ml of PEG-1500 was added with a gentle swirling motion to the pelleted cells over a 1 minute time period. This swirling motion was maintained for a further 1 minute. Three mls of serum free DMEM at 37^0C was added dropwise over the next 3 minutes. This was followed directly by a further 8 mls over the next 3 minutes. The cells were pelleted at 1000rpm for 2-3 minutes and resuspended in DMEM.S5 supplemented with 5% T24 supernatant at a cell concentration of $1x10^6$/ml. The cell suspension was plated into 96 well culture trays, (200µl/well). On the day after the cell fusion, 100µl of culture medium was removed gently and replaced with 100µl of culture medium containing 2x HAT. The cells are fed in a similar manner during the next couple of weeks with the HAT concentration being reduced to 1x. At this stage any non-fused myeloma cells are dead due to the aminopterin in the medium and non-fused lymphocytes die naturally. The cells can therefore be weaned off HAT. Over the next 2 weeks the HAT is replaced with HT. After a similar time period the HT can be removed altogether.

G12 HAT-s for use in cell fusions involving rabbit splenocytes were grown in complete medium supplemented with 5% normal rabbit serum. This was also added to the fusion medium. Monoclonal anti-HRP antibodies were produced in a similar manner using immunized mouse splenocytes and Sp2/0 myeloma.

2.3.8. Cloning of hybridomas by limiting dilutions

Hybridoma cells must be cloned out to ensure monoclonality. This method was also

used in cloning efficiency experiments on hybridomas.

Only hybridoma cell lines in the exponential growth phase were chosen. The cell concentration was brought to $1x10^6$/ml in serum free DMEM. A 1:1000 dilution was prepared in serum free DMEM. This was accomplished in two steps (a 1:100 dilution followed by a 1:10 dilution) to reduce error. This preparation contained 1 cell/µl. 100µl of this suspension was taken and resuspended in 20mls of complete medium and dispensed in 200µl volumes into the wells of a 96 well culture tray. The culture trays were incubated in a humid, 5% CO_2 athmosphere at 37^0C. After 1-2 weeks newly formed clones were visible to the naked eye. Wells containing single clones were grown to confluency and then transferred to larger culture vessels. After 2-3 months hybridomas are recloned to ensure continued monoclonality.

2.3.9. Preparation of spleen feeder cells from Balb/c mice

The spleen were isolated as described in Section 2.3.7.2. The cells were brought to a final cell concentration of $1x10^6$/ml in complete medium and dispensed in 100µl volumes into the wells of a 96 well culture tray. The culture trays were incubated in a humid, 5% CO_2 atmosphere at 37^0C for up to 5 days before the hybridoma cells, which were being cloned, were added at a concentration of 1 cell/well, bringing the final volume of medium/well to 200µl.

2.3.10. Preparation of macrophage feeder cells from Balb/c mice

8-12 week old mice were sacrificed. The abdominal skin was cut exposing the peritoneal cavity. 10ml of serum-free DMEM was injected gently into the peritoneal cavity. It is vital that the intestine which is a potential source of infection is not damaged. The abdominal region was massaged to disperse the DMEM throughout the peritoneal cavity. The macrophage cell-suspension was removed by inserting a 21-G needle into the peritoneal cavity. The cell suspension was removed and washed twice in serum-free DMEM. The cells were then treated similarly to those in Section 2.3.9.

2.3.11. Preparation of thymocyte feeder cells from Balb/c mice

3-4 week old mice were sacrificed. A thymonectomy was performed and the

thymocytes were dispersed by flushing with serum-free medium using a 21-G needle. The thymocytes were washed twice using serum free DMEM. The cells were then treated similarly to those in Section 2.3.9.

2.3.12. T24-conditioned medium, (T24-CM)

T24 cells were cultured as described in Section 2.3.1. After the second passage supernatant was collected from the cells, centrifuged at 2000rpm for 10 minutes and filter sterilized. This was used immediately or stored at -20^{0}C. T24-CM was used to supplement DMEM.S5 at a concentration of 5% (v/v).

2.3.13. CLL and normal human plasma

10ml aliquots of blood were collected by venipuncture in heparinised tubes from patients with CLL or from normal healthy volunteers. The blood was transferred aseptically to sterile centrifuge tubes and centrifuged at 2000rpm for 10 minutes. The plasma was removed, filter sterilized and used immediately or stored at -20^{0}C for future use.

2.3.14. Isolation of mononuclear cells from the heparinised whole blood

Leucocytes were removed from plasma of patients with B-CLL and healthy volunteers by density gradient centrifugation over Ficoll-Paque (Boyum, A., 1968). Heparinized whole blood was diluted 1:1 with 0.15M PBS, pH 7.2. Two parts of this mixture was then layered over 1 part of Ficoll-Paque in a 50ml centrifuge tube and centrifuged at 2000rpm for 30 minutes. The red blood cells form a pellet and the white blood cells form a buffy layer between the plasma and the lymphoprep. This layer can be removed with a pasteur pipette and washed twice in 0.15M PBS, pH 7.2. These cells may be used immediately or frozen down in liquid N_2.

2.3.15. Preparation of conditioned from normal and Chronic Lymphocytic Leukemic, (CLL), peripheral blood cells

Peripheral blood cells were isolated as described in Section 2.3.15. The cells were brought to a final concentration of 1x10^6/ml in DMEM.S5 and cultured in 25 cm^3 and 75cm^3 culture flasks as described in Section 2.3.1. After 48, hours the culture

supernatant was collected from the cells, centrifuged at 2000rpm for 10 minutes and filter sterilized. This was used immediately or stored at -20^{0}C for future use.

2.4. Production of polyclonal anti-HRP antibodies in a New Zealand White male rabbit

Prior to the immunization protocol, normal rabbit serum was obtained from the rabbit by bleeding from the marginal ear vein. This was used as a negative control during subsequent ELISA's and protein assays.

A 1mg/ml solution of HRP was prepared in 0.15M PBS, pH 7.2. The volume was adjusted to 2mls with Complete Freund's Adjuvant and emulsified as described in Section 2.3.7.1. The rabbit was immunized intradermally along the back with 1ml of this emulsion. The animal was boosted on day 14, and subsequently on 5 occasions at two weekly intervals. After the second immunization the animal was bled via the marginal ear vein prior to further boosts. The serum was processed as described in Section 2.3.7.1. and assayed for specific antibody production by ELISA. The rabbit was boosted until a sufficient titre, (1:100,000), was obtained.

2.5. Purification of antibodies

Antibodies were separated from whole rabbit serum or ascitic fluid according to the method described by Hudson and Hay (1980). Inorganic salts are used to reduce the dipole forces that exist between the surface amino acids and the solvent. These forces are necessary for solubility of the proteins and when reduced allow for non-denatured precipitation of the protein.

Saturated ammonium sulphate solution was prepared by the addition of ammonium sulphate (max. 800g/l) to warm distilled water with continual stirring until no more could be brought into solution. This can be stored for several months at 4^{0}C. IgG is precipitated out using a 50% SAS precipitation solution. Serum which had been separated from whole blood, as previously described, was placed on an ice bath and the pH altered to between 4.4 and 5.1 using 0.01N HCL. The solution was centrifuged at 15,000rpm for 20 minutes at 4^{0}C. All remaining steps were also carried out on an ice bath. SAS was added dropwise, with constant stirring, to the serum until a 1:1, (v/v), ratio was reached. The sample was stirred for a further 1 hour on the ice bath

followed by centrifugation at 10,000rpm in a Sorvall centrifuge for 30 minutes at 4^0C. The pellet was dissolved in the minimum volume of the desired buffer. To increase purity the pellet can be dissolved in 0.15M PBS, pH 7.2, and a second 50% precipitation can be carried out as before. The protein concentration of the pellet was estimated using the Bicinchonininic acid assay, (BCA), as described in Section 2.6. The SAS-purified sample can be further purified using affinity chromatography.

2.5.1. Affinity purification of antibodies using Protein A columns

This method utilises the ability of the Fc portion of IgG to bind to protein A. Protein A is a bacterial cell wall component produced by several strains of *Staphylococcus aureus*. Protein A affinity chromatography columns can be prepared by coupling protein A to a solid support such as agarose. Commercial 1ml protein A, (Immunopure, Pierce) columns were used. The column was equilibrated using 5 column volumes of Immunopure IgG binding buffer (Pierce Chem. Co.). A 1mg/ml solution of antibody was made up in Immunopure binding buffer and applied to the column. The antibody solution was allowed to run completely through the column and washed with 10-15 column volumes of Immunopure binding buffer and the fractions collected were assayed continuously for unbound protein using the BCA microassay, (Section 2.6). The bound protein was eluted with 3-5 column volumes of 0.1M glycine buffer, pH 2-3 or 0.1M sodium citrate buffer, pH 3.5. 500µl fractions were collected into 1ml eppendorfs containing 50µl of 1M Tris, pH 7.5, which readjusted the pH to the proper physiological level of 7.3. The eluted protein was detected by determining the absorbance at 280nm on a spectrophotometer. This method was used because the presence of Tris buffer at certain concentrations, interferes with the BCA assay described in Section 2.6.

2.6. Bicinchoninic acid, (BCA), protein assay

This is a colorimetric assay which makes use of the various oxidation states of Cu (Smith *et al.*, 1985). Under alkaline conditions Cu ions react with proteins and are converted from the Cu^{++} to the Cu^{+} oxidative state, a coloured product is thus formed. The reagents supplied for the assay are reagent A which is an alkaline bicarbonate buffer and reagent B, a 4% (w/v) copper sulphate solution. The BCA working solution

was made by mixing 50 parts reagent A with 1 part reagent B. This was stable for up to 1 week at room temperature. A range of protein standards were prepared in 0.15M PBS, pH 7.2, (0-1mg/ml) from a stock solution of 5mg/ml BSA. 10µl of protein standard or unknown protein solution were added, in duplicate, to the wells of a 96 well microassay plate. This was followed by the addition of 190µl of the BCA working solution. The plate was incubated for 30 minutes at 37^{0}C and the absorbance was measured at 562nm using a Titertek Multiscan plate reader.

2.7. Chemical production of bispecific antibodies

Affinity-purified antibodies were used for the production of bispecific antibodies. Polyclonal and monoclonal IgG molecules at concentration between 10-15mg/ml in 0.1M sodium acetate buffer, pH 4.2, were incubated with 0.3mg/ml pepsin. Polyclonal antibodies were incubated for 18 hours and monoclonal antibodies for 7 hours yielding $F(ab'\gamma)_2$ fragments. The $F(ab'\gamma)_2$ were purified from the digest mixture by gel filtration using a 1ml Ultrogel AcA44, (LBK), column equilibrated with 0.2M Tris-HCl, pH 8.0, containing 10mM EDTA. $Fab'\gamma_{SH}$ fragments were prepared by reduction of the $F(ab'\gamma)_2$ fragments with 20mM 2-mercaptoethanol for 30 minutes at 30^{0}C. Both $Fab'\gamma_{SH}$ were cooled to 4^{0}C and run through a Sephadex G-25 column equilibrated with 50mM sodium acetate, pH 5.3, containing 0.5mM EDTA. The temperature was maintained at 4^{0}C throughout the remainder of the experiment. The mouse antibody was treated with a half volume of 12mM *o*-phenylenedimaleimide, (*o*-PDM) dissolved in chilled dimethylformamide for 30 minutes. The resulting $Fab'\gamma_{MAL}$ was separated from contaminating molecules on a Sephadex G-25 column equilibrated with 50mM sodium acetate, pH 5.3, containing 0.5mM EDTA. Both Fab'γ were mixed at a molar ratio of $Fab'\gamma_{MAL}$:$Fab'\gamma_{SH}$ 1:1.3 and incubated for 18 hours at 4^{0}C. The pH was adjusted to 8 with 1M Tris-HCL, pH 8.0. Treatment of the mixture with 2-Me at a final concentration of 20mM and 25mM iodoacetamide for 30 minutes at 30^{0}C removes unwanted products. The bispecific $F(ab'\gamma)_2$ were isolated from unwanted reactants by gel filtration using a 1ml Ultrogel AcA44, (LBK), column equilibrated with 0.2M Tris-HCL, pH 8.0, containing 10mM EDTA.

2.8. ELISAs

2.8.1. ELISA for the detection of mouse IgG in cell culture supernatant

Supernatants were tested for antibody production after 2-3 weeks of growth in complete medium.

96 well microtitre plates were coated overnight at 4^0C or for two hours at room temperature (RT) with 100µl of a 10µg/ml solution of goat anti-mouse IgG in carbonate/bicarbonate (0.03M $NaHCO_3$, 0.01M Na_2CO_3), buffer, pH 9.6. The plates were then washed five times with 0.15M PBS, pH 7.2, containing 0.05% (v/v) Tween 20, and once in 0.15M PBS, pH 7.2, (the plates were washed after each subsequent incubation step in a similar manner). The plates were then blocked for 1 hour at 37^0C with a 2% (w/v) solution of BSA made up in 0.15M PBS, pH 7.2. In the next step, supernatant was removed from wells with clones of hybridomas and transferred to the 96 well microtitre plate and incubated for two hours at RT. Antibody presence was detected using a 1:5000 dilution of alkaline phosphatase-conjugated goat anti-mouse antibody made up in 0.15M PBS, pH 7.2, which was incubated on the plate for two hours at RT. A colour product was obtained by the addition of 100µl of substrate [1mg/ml solution of 5-bromo-4-chloro-3-indolyl phosphate, (BCIP), in 2-amino-2-methyl-1-propanol, (AMP), buffer pH 9.5]. Absorbance was read at 620nm using a Titretek Multiscan automatic plate reader.

2.8.2. ELISA for the detection of mouse IgM in cell culture supernatant

96 well microtitre plates were coated overnight at 4^0C or for two hours at RT, with supernatant from wells containing clones of the hybridoma Ep16. The plates were then washed five times with 0.15M PBS, pH 7.2 containing 0.05% (v/v) Tween 20, and once in 0.15M PBS, pH 7.2, (the plates were washed after each subsequent incubation in a similar manner). The plates were then blocked with a 2% (w/v) solution of BSA made up in 0.15M PBS, pH 7.2. Antibody presence was detected using a 1:3000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgM made up in 0.15M PBS pH 7.2, which was incubated for two hours at RT, and detected as described in Section 2.8.1.

2.8.3. ELISA for the detection of IL-6

96 well microtitre plates were coated overnight at 4^0C, or at RT for two hours, with 100µl of a 10µg/ml solution of goat anti-human IL-6 in carbonate/bicarbonate (0.03M $NaHCO_3$, $0.01Na_2CO_3$) buffer, pH 9.6. The plates were then washed five times with 0.15M PBS, pH 7.2 containing 0.05% (v/v) Tween 20, and once in PBS, pH 7.2, (the plates were washed in a similar manner after each incubation step). The plates were then blocked with a 2% (w/v) solution of BSA, made up in 0.15M PBS, pH 7.2. Samples consisting of neat CLL plasma, neat normal human plasma, T24 supernatant diluted 1:2 in 0.15M PBS, pH 7.2 and human recombinant IL-6 ranging in concentration from 0-10µg/ml in 0.15M PBS, pH 7.2, were added and incubated for two hours at RT. Rabbit anti-IL-6 antibody was then added at a concentration of 10µg/ml in 0.15M PBS, pH 7.2, and incubated for a further two hours at room temperature. A 1:5000 dilution of alkaline-phosphatase conjugated goat anti-rabbit IgG made up in 0.15M PBS, pH 7.2 was then added and incubated for two hours at RT and detected as described in Section 2.8.1.

2.8.4. Binding of cells to 96 well microtitre plates. These were used to determine the binding specificities of antibodies by ELISA (2.8.5.)

(i) Non-adherent cells

96 well microtitre plates were coated for 1 hour at 37^0C with 100µl of a 0.1% (w/v) solution of poly-L-lysine. This solution may be reused up to 10 times and on removal is stored at 4^0C. The plates were then washed five times with 0.15M PBS, pH 7.2 containing 0.05% (v/v) Tween 20, and once in PBS, pH 7.2, (all subsequent washings were carried out in similar manner). 50µl of a $5x10^5$ cell/ml suspension was added to each well. The plates were then incubated at 4^0C for 1 hour. 50µl of 0.05% glutaraldehyde, (GA), which had been precooled to 4^0C was then added directly without washing to each well to give a final concentration of 0.025%. The GA was added very gently to avoid disruption of the bound cells. The plates were incubated for a further 10 minutes at 4^0C and washed as before. Residual GA was blocked by incubation of the plates with 100µl/well of a 100mM glycine solution made up in PBS for 30 minutes at 37^0C and washed as before. The plates were then blocked with a 2% (w/v) solution of BSA, made up in 0.15M PBS, pH 7.2 containing 0.1% (w/v) sodium

azide. These plates were used immediately or stored at 4^0C for up to 6 months.

(ii) Adherent cells

Adherent cells could be grown directly onto 96 well tissue culture trays and the method is similar to (i) from the addition of GA onwards.

The ELISA's used in (i) and (ii) were carried out as outlined in Section 2.8.5.

2.8.5. ELISA used for detection of antigenic determinants on cells bound to 96 well microtitre plates

96 well microtitre plates were coated with cells as previously described, Section 2.8.4. The plates were then washed five times with 0.15M PBS, pH 7.2 containing 0.05% (v/v) Tween 20, and once in PBS, pH 7.2, (the plates were washed in a similar manner after each incubation step). The plates were then blocked with a 2% (w/v) solution of BSA, made up in 0.15M PBS, pH 7.2. Samples consisting of supernatants from hybridomas were added to the plate and incubated for two hours at RT. Antibody presence was detected using a 1:5000 dilution of alkaline phosphatase-conjugated goat anti-mouse antibody made up in 0.15M PBS, pH 7.2, which was incubated on the plate for two hours at RT. A colour product was obtained by the addition of 100µl of substrate to each well. The substrate consisted of a 1mg/ml solution of 5-bromo-4-chloro-3-indolyl phosphate, (BCIP), in 2-amino-2-methyl-1-propanol, (AMP), buffer pH 9.5. Absorbance was read at 620nm as described in Section 2.8.1.

2.8.6. Isotype analysis of antibody

Monoclonal antibodies were isotyped using a Sigma ELISA-based kit (Table 2.1.1).

2.8.7. ELISA for the detection of anti-HRP antibodies

Serum from immunized animals was screened for antibody production by ELISA.

96 well microtitre plates were coated overnight at 4^0C, or at RT for two hours, with 100µl of a 10µg/ml solution of HRP in carbonate/bicarbonate (0.03M $NaHCO_3$, 0.01M Na_2CO_3) buffer, pH 9.6. The plates were then washed five times with 0.15M PBS, pH 7.2 containing 0.05% (v/v) Tween 20, and once in PBS, pH 7.2, (the plates were washed in a similar manner after each incubation step). The plates were then blocked with a 2% (w/v) solution of BSA, made up in 0.15M PBS, pH 7.2. Samples consisting

of dilutions of immunized serum and normal rabbit serum were made up in 0.15M PBS pH 7.2, added to the plate and incubated for two hours at RT. A 1:5000 dilution of alkaline-phosphatase conjugated goat anti-rabbit IgG made up in 0.15M PBS, pH 7.2, was then added, incubated for two hours at RT and detected as described previously in Section 2.8.1.

2.8.8. ELISA for the detection of bispecific antibody activity using cells bound to ELISA micotitre plates

96 well microtitre plates were coated with cells as previously described in Section 2.8.4. The plates were then washed five times with 0.15M PBS, pH 7.2 containing 0.05% (v/v) Tween 20, and once in PBS, pH 7.2, (the plates were washed in a similar manner after each incubation step). The plates were then blocked with a 2% (w/v) solution of BSA, made up in 0.15M PBS, pH 7.2. Samples consisting of dilutions of $F(ab'\gamma)_2$ bispecific antibody or whole G12 and anti-HRP $F(ab'\gamma)_2$ molecules were made up in 0.15M PBS pH 7.2 added to the plate and incubated for two hours at RT. A 10µg/ml solution of HRP, made up in 0.15M PBS, pH 7.2 was then added and incubated for two hours at RT and detected as described previously in Section 2.8.1. This ELISA was also used to detect bispecific antibody production in the supernatants of triomas.

2.8.9. ELISA for the detection of rabbit and mouse Fab' fragments on the bispecific antibody

96 well microtitre plates were coated overnight at 4^0C, or at RT for two hours, with 100µl of a 10µg/ml solution of anti-mouse IgG in carbonate/bicarbonate, (0.03M $NaHCO_3$, 0.01M Na_2CO_3) buffer, pH 9.6. The plates were then washed five times with 0.15M PBS, pH 7.2 containing 0.05% (v/v) Tween 20, and once in PBS, pH 7.2, (the plates were washed in a similar manner after each incubation step). The plates were then blocked with a 2% (w/v) solution of BSA, made up in 0.15M PBS, pH 7.2. Samples consisting of dilutions of $F(ab'\gamma)_2$ bispecific antibody or whole G12 antibody and anti-HRP $F(ab'\gamma)_2$ molecules were made up to a concentration of 10µg/ml in 0.15M PBS, pH 7.2, added to the plate and incubated for two hours at RT. A 1:5000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG, made up in 0.15M

PBS, pH 7.2, was then added, incubated for two hours at RT and detected as described previously (Section 2.8.1.).

This ELISA did not detect bsab activity but does detect the bsab mouse and rabbit Fab' regions.

2.8.10. ELISA for the detection of human IgG in plasma

96 well microtitre plates were coated overnight at 4^0C or for two hours at room temperature (RT) with 100µl of a 10µg/ml solution of goat anti-human IgG in carbonate/bicarbonate, (0.03M $NaHCO_3$, 0.01M Na_2CO_3), buffer, pH 9.6. The plates were then washed five times with 0.15M PBS, pH 7.2 containing 0.05% (v/v) Tween 20, and once in 0.15M PBS, pH 7.2, (the plates were washed after each subsequent incubation step in a similar manner). The plates were then blocked for 1 hour at 37^0C with a 2% (w/v) solution of BSA made up in 0.15M PBS, pH 7.2. In the next step samples were added to the wells in duplicate. These consisted of commercial human IgG (0-10µg/ml) normal human plasma and plasma from patients with B-CLL. Plasma samples were diluted 1:5000 in the case of normal human plasma and 1:1000 in the case of B-CLL patient plasma in 0.15M PBS, pH 7.2. The plates were incubated for two hours at RT. Antibody presence was detected using a 1:5000 dilution of alkaline phosphatase-conjugated goat anti-human antibody made up in 0.15M PBS, pH 7.2, which was incubated on the plate for two hours at RT. A coloured product was obtained and detected as described in Section 2.8.1.

2.9. Histochemical staining of cells on cytospin smears

Morphological staining was performed on HL60, K562, T24, GCCM, CLL cells and normal human peripheral blood cells. 100µl of a $0.5x10^5$/ml cell suspension made up in 0.15M PBS, pH 7.2 was centrifuged at 500rpm for 2-3 minutes onto a clean glass slide, using a Heraeus Sepatech cytocentrifuge. The cells were allowed to air-dry for 20 minutes and stained immediately or stored at -20^0C for up to 6 months. Cells which had been stored at -20^0C were allowed to thaw at RT for 30 minutes prior to use. The stains used were methylene blue and eosin, (Rapi-diff II, Triple stain set, Diagnostic Developments). Stained cells were mounted with coverslips using DPX mountant, (BDH).

2.9.1. Immunocytochemical staining of cells using bispecific antibody

Cytocentrifuged samples were removed from the -20°C freezer and allowed to air-dry at room temperature. The cells were fixed in acetone, absolute alcohol and paraformaldehyde at a ratio of 19:19:2 for 30 seconds, and washed in a Tris buffered bath consisting of 0.05M Tris buffer, pH 7.6, for 5 minutes. After fixation the preparations must be kept moist at all times. The slides were removed from the Tris-buffered bath and the area around the smear was dried with a tissue. The slides were incubated for 5 minutes at room temperature with a 3% (v/v) solution of hydrogen peroxide made up in methanol. This step blocks endogenous peroxidase activity. To inhibit the non-specific binding of protein the specimens were incubated for 20 minutes at room temperature with a 2% (w/v) solution of BSA, made up in 0.15M PBS, pH 7.2. Excess blocking reagent was tapped off carefully. The samples were washed as before, in a Tris-buffered bath for 5 minutes. The bispecific antibody, at a concentration of 10µg/ml in 0.15M PBS, pH 7.2, was applied to the specimens and the slides were then incubated for 30 minutes at room temperature. The slides were washed as before in a Tris-buffered bath for 5 minutes. The specimens were then incubated for 30 minutes at room temperature with a 10µg/ml solution of HRP, made up in 0.15M PBS, pH 7.2. The slides were washed as before and an insoluble product was developed using a 0.066% (w/v) solution of 3,3'-di-aminobenzidine, (DAB), made up in 0.05M Tris-buffered saline. The specimens were counterstained for 1 minute using a 2% (w/v) solution of methylene green made up in ultrapure water. Stained cells were mounted with coverslips using DPX mountant as described in Section 2.9.

2.9.2. Immunocytochemical staining of cells using a HRP-labelled anti-mouse IgG secondary antibody to detect G12 whole antibody

The specimens were treated in a similar manner to that described in Section 2.9.1. Dilutions of the G12 purified antibody ranging from 1-20µg/ml were made up in 0.15M PBS, pH 7.2, and applied to the specimens and the slides were then incubated for 30 minutes at room temperature. The slides were washed as before in a Tris-buffered bath for 5 minutes. The specimens were then incubated for 30 minutes at room temperature with goat anti-mouse IgG peroxidase-conjugated antibody, made up in 0.15M PBS, pH 7.2. The slides were washed as before and an insoluble product

was developed using DAB as described in Section 2.9.1. The specimens were counterstained using a 2% (w/v) solution of methylene green made up in ultrapure water. Stained cells were mounted with coverslips using DPX mountant as described in Section 2.9.

2.9.3. Immunocytochemical staining of cells using Dako PAP kit

All reagents with the exception of the primary antibody were supplied in the kit. The specimens were treated in a similar manner to that described in Section 2.9.1. Dilutions of the G12 purified antibody ranging from 1µg-20µg/ml were made up in 0.15M PBS, pH 7.2, and applied to the specimens and the slides were then incubated for 30 minutes at room temperature. The slides were washed as before in a Tris buffered bath for 5 minutes. The specimens were then incubated for 30 minutes at room temperature with two drops of anti-mouse IgG and washed as before in a Tris buffered bath for 5 minutes. The specimens were then incubated with peroxidase anti-peroxidase complex. The slides were washed as before and an insoluble product was developed using DAB. The specimens were counterstained using a 2% (w/v) solution of methylene green made up in ultrapure water. Stained cells were mounted with coverslips using DPX mountant as described in Section 2.9.

2.10. MTT bioassay for IL-6

$1x10^4$ B9 cells were cultured on 96 well culture trays for 2-3 days in complete medium supplemented with 5% (v/v) T24-CM (diluted a further 1:100 in complete medium) CLL-DMEM, NHP-DMEM, rhIL-6 (0-10pg/ml in complete medium), or complete medium, (blank). On day 2/3 a 5mg/ml solution of MTT was made up in 0.15M PBS, pH 7.2 and filter sterilized. 20µl of this solution was added to each well and the plates were incubated for 4 hours at 37^0C. 100µl of 10% (w/v) sodium dodecyl sulphate, (SDS), SDS-0.01 N HCl was added to each well and incubated for a further 4 hours at 37^0C, (until all the crystals were dissolved). Absorbance was read at 560nm using a Titertek Multiscan automatic plate reader.

CLL-CM and NHP-CM were also examined for IL-6 activity.

2.11. SDS-polyacrylamide gel electrophoresis

Antibody fragments were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a modified procedure to that described by Laemmli (1970). Proteins were separated on a resolving gel containing 10% (w/v) acrylamide, 0.33% (w/v) bis-acrylamide, 0.75M Tris, 0.2% (w/v) SDS, 0.1% (v/v) TEMED and 0.1% (w/v) ammonium persulphate, pH 8.8. A stacking gel consisting of 3.5% (w/v) acrylamide, 0.08% (w/v) bis-acrylamide, 0.25% (w/v) Tris, 0.2% (w/v) SDS and 0.08% TEMED, 0.08% (w/v) ammonium persulphate pH 6.8 was used, 0.25% (w/v). The electrode buffer contained 0.025M Tris, 0.192M glycine and 0.1% (w/v) SDS prepared in distilled water. Samples for electrophoresis were dissolved at a concentration of 1mg/ml in solubilization buffer consisting of 0.062M Tris, 2.0% (w/v) SDS, 10% (w/v) glycerol and 0.001% (w/v) bromophenol blue. 0.5% (v/v) 2-mercaptoethanol was used when reducing conditions were required. The samples were boiled for between 2-3 minutes. 10-20µl of these solutions were applied to the gel. The gel was electrophoresed in electrode buffer, at 100mA using a Biorad vertical electrophoresis system. Gels were stained for 30 minutes in 0.05% (w/v) Comassie Brilliant blue in acetic acid:water:methanol (1:10:8) and destained overnight in the same solvent system.

2.12. HPLC analysis of proteins

A 20µl volume of protein diluted to 1mg/ml in 0.15M PBS, pH 7.2, was applied to a Waters Protein PAK 300 SW, (7.8x300µM) column which was pre-equilibrated with 0.1M phosphate, pH 7.2. A flow rate of 0.5 ml/min used and protein was monitored by determining absorbance at 280nm.

Chapter 3

Production of Bispecific Antibodies

3.1. Production of bispecific antibodies

As outlined in the introduction (Section 1.2.) bsabs can be made biologically, chemically and genetically. We have used both biological and chemical methods to synthesise bsabs with dual specificities for HRP and CLL cells. The bsabs have been used in the development of ELISAs and immunocytochemical techniques. The methods employed are outlined briefly in the following section. Triomas were made biologically by the fusion of HRP-immunized rabbit and mouse splenocytes with HAT-s G12 cells. G12 hybridoma cells secrete antibodies reactive with CLL cells. The antigen recognised by the G12 antibody has a MW of 69,200D. HAT-sensitivity was induced by mutagenesis using ethyl methanosulfonate (EMS), or, alternatively, by growing the cells in increasing concentrations of 8-aza. All efforts to induce HAT-sensitivity in E5, another hybridoma cell line secreting antibodies reactive with CLL cells, were unsuccessful.

Both hybridoma cell lines E5 and G12 also produce antibody reacting with the K562 cell line and, therefore, this cell line was chosen for routine assaying of mAb activity. Supernatants from the triomas formed were examined by ELISAs specific for the K562 cell line and HRP individually and for bispecificity to both antigens.

Bsab were also synthesised by chemical methods (Glennie *et al.*, 1987). This involves chopping up the parent antibodies to form Fab′ fragments which were then reconstituted to form bispecific $F(ab'\gamma)_2$.

The principle of using bsab as a detection system as opposed to more conventional ELISAs is outlined in Figure 3.1. A single bsab is capable of linking the cell and the detector molecule, HRP. Theoretically this could be achieved with the HRP pre-bound to the bsab followed by its addition to the cell or the the bsab could be added to the cell in its native state and followed by addition of the HRP. In the system described here the former approach proved more successful. This compares favourably with conventional detection systems which require enzyme-conjugated anti-tumour antibodies or peroxidase anti-peroxidase, PAP, and alkaline phosphatase anti-alkaline phosphatase, APAAP, amplification systems.

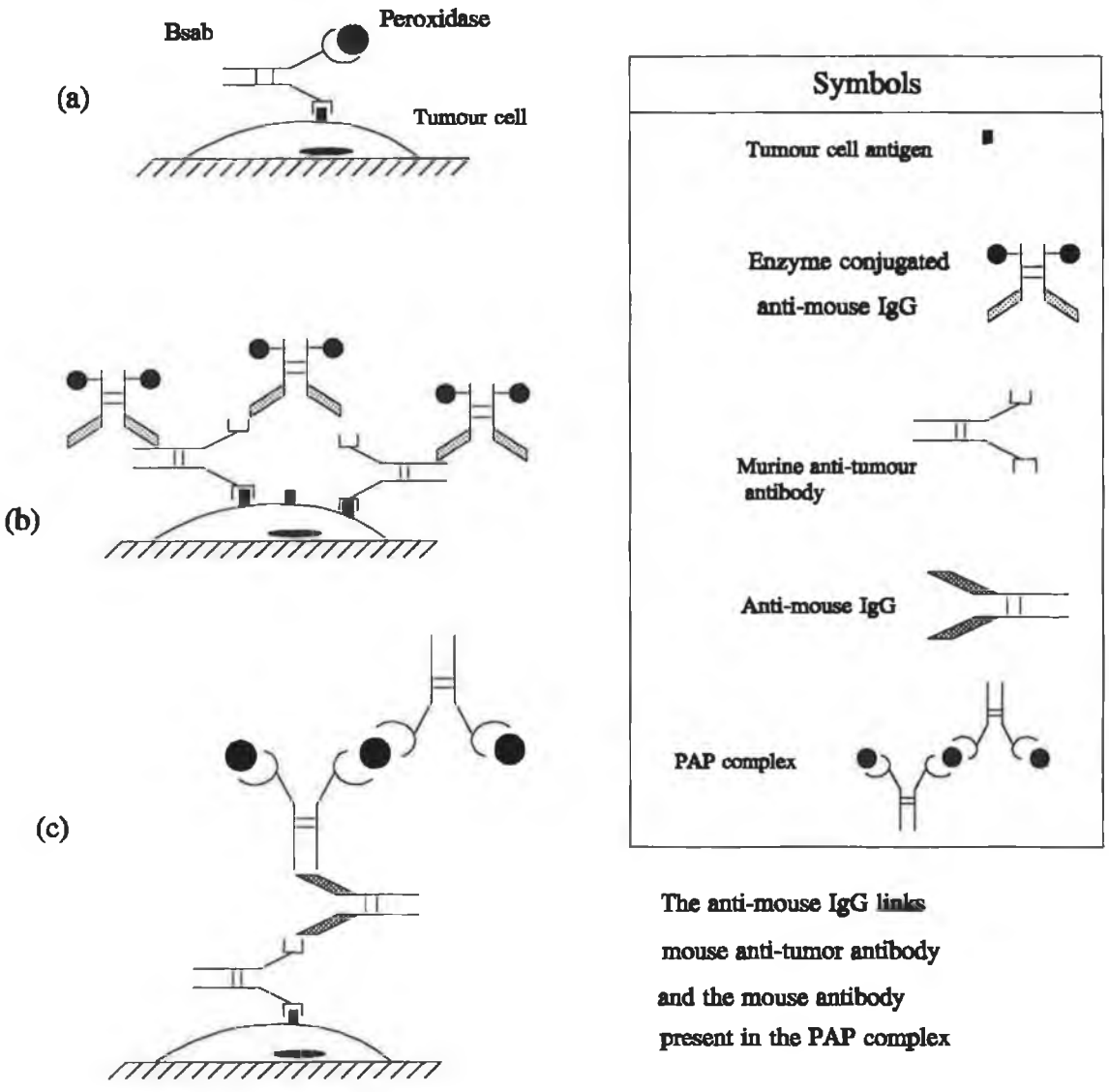


Figure 3.1.

The above diagram depicts the methods used to detect antigenic determinants on cells. The bispecific antibody detection system as represented in (a) has been used successfully in ELISA and immunohistrochemistry. A more conventional system using an enzyme-conjugated secondary antibody which is capable of binding to the anti-tumor antibody is represented in (b). The final system (c) represents the peroxidase anti-peroxidase complex which is used routinely as an amplification system during immunohistochemical procedures.

3.2. Biological production

Biological production of bsabs involves manipulation of the somatic cell hybridization techniques first developed by Cotton and Milstein (1973) and Kohler and Milstein (1975). Reading *et al.* (1981) reported on the fusion of hybridomas to form quadromas which secrete bispecific antibodies. The method chosen in our work was to fuse hybridomas directly to antibody producing splenocytes to form triomas. One of the first obstacles to be overcome using this method was the selection of hybridoma cells for use in cell fusions. The 2 methods chosen were either to grow the hybridomas in medium containing the drug 8-aza or to mutagenise the cells using EMS. Efforts to produce a quadroma (fusion of the G12 hybridoma with a hybridoma cell line secreting anti-HRP antibodies) were hampered by an outbreak of the mouse hepatitis virus (MHV) in the animal house facility. The hybridomas producing anti-HRP antibodies (IgG class) came in contact with infected animals when ascitic fluid was being produced and all further aspects of this work were halted.

Various other properties which are used in the selection of hybridomas for use in the formation of triomas and quadromas involve the induction of sensitivity or resistance in the hybridomas to drugs (Staerz and Bevan, 1986; Suresh *et al.*, 1986; De Lau *et al.*, 1989). Other properties such as the labelling of the hybridomas with fluorescent markers have also been applied (Karawajew *et al.*, 1987; Shi *et al.*, 1991; Jantscheff *et al.*, 1993). These methods have been discussed more fully in the introduction (Section 1.2).

3.2.1. Induction of HAT-sensitivity in E5 and G12 hybridoma cells using 8-azaguanine

In order to induce HAT-sensitivity in the hybridomas G12 and E5 the cells were grown in progressively increasing concentrations of 8-aza (1µg-20µg/ml) made up in complete medium.

Initially the E5 cells were seeded in 24 well culture trays in 0µg/ml, 2µg/ml and 4µg/ml 8-aza in complete growth medium [Table 3.2.1.(a)]. Each drug concentration was carried out in duplicate. Cells were counted on days 4 and 10. Cells growing in 2µg/ml 8-aza were seeded on day 4 in higher concentrations of the drug. The cells

were seeded in complete medium containing the following concentrations of 8-aza, 0µg/ml, 4µg/ml, 6µg/ml and 8µg/ml [Table 3.2.1.(b)]. On day 8 only cells seeded in 0 and 4µg/ml of drug were viable. Cells growing in 4µg/ml 8-aza were taken and seeded in higher concentrations of drug. The concentrations chosen on this occasion were 0µg/ml, 4µg/ml, 6µg/ml and 8µg/ml [Table 3.2.1.(c)]. On day 16 cells growing in 8µg/ml of 8-aza were seeded in higher concentrations of the drug. Up to 20µg/ml of the drug was used on this occasion [Table 3.2.1.(d)]. On day 30 cells were found to grow in all of the chosen concentrations (0, 12, 16 and 20µg/ml 8-aza). When examined for HAT-sensitivity [Table 3.2.1.(e)] the cells were still HAT-r.

The experiment was repeated using fresh E5 cells. On day 0 cells were seeded in 24 well culture trays containing 0, 2, 4, 6, 8 and 10µg/ml 8-aza [Table 3.2.1.(f)]. After 10 days cells in the highest concentration were dead. The cells growing in 6µg/ml 8-aza were then taken and seeded in 0, 6, 10, 12, 14 and 16µg/ml 8-aza [Table 3.2.1.(g)]. On day 20 the plates were examined and all wells had viable cells. Cells growing in the highest concentration of 8-aza, 16µg/ml 8-aza, were taken and grown in 0, 16, 20 24, 28 and 32µg/ml of 8-aza [Table 3.2.1.(h)]. Cells growing in 24µg/ml of the drug were examined for HAT-sensitivity on day 30 as shown in [Figure 3.2.1.(i)]. All cells were still HAT-r.

Induction of HAT-sensitivity in G12 cells proved to be far easier. On day 0 the cells were seeded in 0, 8, 14, and 20µg/ml 8-aza [Table 3.2.1.(j)] in complete medium. After 20 days only cells growing in 0 and 8µg/ml 8-aza were viable. Cells growing in 8µg/ml 8-aza were taken and seeded in 0, 12, 16 and 20µg/ml 8-aza in complete growth medium [Table 3.2.1.(k)]. On day 30 cells were growing in all wells. The cells growing in 20µg/ml 8-aza were HAT-s [Table 3.2.1.(l)] and maintained their ability to react with K562 cells [Table 3.2.1.(m)].

3.2.2. Induction of HAT-sensitivity in G12 cells using EMS

HAT-sensitivity was also induced in the hybridoma G12 using the mutagen EMS. The concentrations of EMS causing a 50% kill of G12 cells were established. G12 cells were seeded out in duplicates at a concentration of $1x10^4$cells/well on 24 well culture trays. The concentration of EMS ranged from 0-180µg/ml of complete medium. The

concentration resulting in a 50% kill of the cells was 20µg EMS/ml of complete medium [Table 3.2.2.(a)].

The concentration of the selection agent (8-aza) required to cause a 100% kill of wild type hybridoma cells was also established. The cells were seeded at a concentration of $1x10^4$ cells/well on 24 well plates in duplicates in complete medium containing a range of concentrations of 8-aza (0-20µg/ml). The concentration of 8-aza resulting in a 100% kill of hybridoma cells was 16µg 8-aza/ml of complete medium [Table 3.2.2.(b)].

Once the concentrations of EMS resulting in a 50% kill and that of 8-aza resulting in a 100% kill of hybridoma cells were established the cells were mutagenised in complete medium containing 20µg/ml EMS [Table 3.2.2.(c)] and new HAT-s phenotypes were selected with 16µg/ml of 8-aza. The concentration of 8-aza was adjusted to 16µg/ml slowly over the next 11 days [Tables 3.2.2.(d,e, & f)]. These cells were then examined for their ability to grow in HAT-containing medium [Table 3.2.2.(g)]. The G12 cells were HAT-s and the antibody secreted by these cells maintained its ability to bind K562 cells [Table 3.2.2.(h)].

3.2.3. Somatic cell fusions

These involved the fusion of HAT-s G12 cells with rabbit and mouse splenocytes which secreted anti-HRP antibodies. All newly formed triomas were examined by 3 separate ELISAs specific for anti-K562 activity, anti-HRP activity and bsab activity. Five of these involved the fusion of HAT-s G12 cells and *in vitro* immunized rabbit anti-HRP splenocytes. The *in vitro* immunization was necessary because the rabbit had been given a final *in vivo* immunization two weeks prior to being sacrificed which maximised the levels of antiserum to HRP but resulted in splenocytes which were in the wrong phase for antibody production. The G12 HAT-s cells were grown for a week prior to cell fusion in medium containing 3% FCS and 3% rabbit serum. The cells were fused at a 5:1 ratio of splenocytes to G12 HAT-s. The numbers of triomas formed are shown in [Table 3.2.3.(a)]. The triomas formed here were found to grow extremely slowly and the supernatants could not therefore be tested for antibody production until day 34. 3.5% of triomas formed produced antibody reactive with HRP

but a large majority (95%) only exhibited reactivity with the K562 cell line. No bsab producing triomas were formed.

Four of the final 8 cell fusions involved fusion of G12 HAT-s cells in which HAT-sensitivity had been induced using 8-aza and the final 4 involved the fusion of mutagenised G12 cells [Table 3.2.3.(b & c)] respectively. All of these 8 fusions involved immunized mouse splenocytes. In the first 4 fusions 2.8% of the total number of triomas formed secreted antibody reactive with HRP and 95% of the triomas secreted antibody reactive with the K562 cell line. In the final 4 fusions the the figures were similar in that 2.6% of the total number of triomas produced against against HRP and 95% were against the K562 cell line. No bsab was detected in any of the 8 cell fusions.

	E5 cell growth in a range of concentrations of 8-aza in DMEM.S5. The cells were seeded at $3x10^4$/well		
Day no.	0µg/ml 8-aza	2µg/ml 8-aza	4µg/ml 8-aza
0	$3x10^4$	$3x10^4$	$3x10^4$
4	$5x10^4$	$1x10^4$	$0.8x10^4$
10	$8x10^4$	$4x10^4$	$3x10^4$

Table 3.2.1.(a). Induction of HAT-sensitivity was performed by growing the cells in increasing concentrations of 8-aza. Initially E5 cells were seeded at $3x10^4$ cells/well in varying concentrations of 8-aza.

Cell counts were performed on day 4 and it is observed that cells growing in medium containing 8-aza experience an initial cell kill followed by recovery.

On day 4 cells from the wells containing 2µg/ml 8-aza were removed and seeded in higher concentrations of 8-aza [Table 3.2.1.(b)]

	E5 cell growth in a range of concentrations of 8-aza in DMEM.S5. The cells were seeded at $1x10^3$/well			
Day no.	0µg/ml 8-aza	4µg/ml 8-aza	6µg/ml 8-aza	8µg/ml 8-aza
4	$1x10^3$	$1x10^3$	$1x10^3$	$1x10^3$
8	$4x10^3$	$2x10^3$	dead	dead

Table 3.2.1.(b). E5 cells which had been growing in 2µg/ml 8-aza [Table 3.2.1.(a)] were seeded at $1x10^3$ cells/well in up to 8µg/ml 8-aza.

On day 8 cells from the wells containing 4µg/ml 8-aza were removed and seeded in higher concentrations of 8-aza [Table 3.2.1.(c)]. Cells growing in 6 and 8µg/ml 8-aza have died.

	E5 cell growth in a range of concentrations of 8-aza in DMEM.S5. The cells were seeded at $1x10^3$/well			
Day no.	0µg/ml 8-aza	4µg/ml 8-aza	6µg/ml 8-aza	8µg/ml 8-aza
10	$1x10^3$	$1x10^3$	$1x10^3$	$1x10^3$
16	$1x10^4$	$8x10^3$	$5x10^3$	$1x10^3$

Table 3.2.1.(c). E5 cells which had been growing in 4µg/ml 8-aza [Table 3.2.1.(b)] were seeded at $1x10^3$ cells/well in up to 8µg/ml 8-aza.

On day 16 cells were viable in all of the chosen concentrations of 8-aza. Cells were taken from the wells containing 8µg/ml 8-aza and seeded in higher concentrations of 8-aza [Table 3.2.1.(d)].

	E5 cell growth in a range of concentrations of 8-aza in DMEM.S5. The cells were seeded at $1x10^3$/well			
Day no.	0µg/ml 8-aza	12µg/ml 8-aza	16µg/ml	20µg/ml 8-aza
16	$1x10^3$	$1x10^3$	$1x10^3$	$1x10^3$
20	$4x10^3$	$0.3x10^3$	$0.6x10^3$	$0.4x10^3$
25	$7x10^3$	$3x10^3$	$3x10^3$	$0.7x10^3$
30	$1x10^4$	$2.3x10^3$	$4x10^3$	$2x10^3$

Table 3.2.1.(d). E5 cells which had been growing in 8µg/ml 8-aza [Table 3.2.1.(c)] were seeded at $1x10^3$ cells/well in up to 20µg/ml 8-aza. Cell counts were recorded on days 20 and 25 and all wells had cell viability.

On day 30 cells from the wells containing 20µg/ml 8-aza were grown in HAT-supplemented medium [Table 3.2.1.(e)].

	E5 cell growth in a range of concentrations of HAT-supplemented medium. The cells were seeded at $2x10^3$/well		
Day no.	0x HAT	2x HAT	4x HAT
30	$2x10^3$	$2x10^3$	$2x10^3$
35	$4x10^3$	$3x10^3$	$3x10^3$
39	$8x10^3$	$6x10^3$	$7x10^3$

Table 3.2.1.(e). Growth of E5 cells seeded at $2x10^3$ cells/well in varying concentrations of HAT. The cells were expected to be HAT-s as they grew in medium containing up to 20µg/ml of 8-azaguanine. On examination, however, the cells were found to be HAT-r. The experiment was repeated [Table 3.2.1.(f)].

1x HAT =2.2mg/ml aminopterin, 0.0048mg/ml thymidine and 0.13mg/ml hypoxanthine.

	E5 cell growth in a range of concentrations of 8-aza in DMEM.S5. The cells were seeded at $2x10^4$/well					
Day no.	0µg/ml 8-aza	2µg/ml 8-aza	4µg/ml 8-aza	6µg/ml 8-aza	8µg/ml 8-aza	10µg/ml 8-aza
0	$2x10^4$	$2x10^4$	$2x10^4$	$2x10^4$	$2x10^4$	$2x10^4$
10	$1x10^5$	$5x10^4$	$3x10^4$	$1x10^4$	dead	dead

Table 3.2.1.(f). Fresh E5 cells were seeded at $2x10^4$ cells/well in varying concentrations of 8-aza.

On day 10 cells from the wells containing 6µg/ml 8-aza were removed and seeded in higher concentrations of 8-aza [Table 3.2.1.(g)].

	E5 cell growth in a range of concentrations of 8-aza in DMEM.S5. The cells were seeded at $1x10^3$/well.					
Day no.	0µg/ml 8-aza	6µg/ml 8-aza	10µg/ml 8-aza	12µg/ml 8-aza	14µg/ml 8-aza	16µg/ml 8-aza
10	$1x10^3$	$1x10^3$	$1x10^3$	$1x10^3$	$1x10^3$	$1x10^3$
20	$1x10^4$	$6x10^3$	$6x10^3$	$6x10^3$	$4x10^3$	$2x10^3$

Table 3.2.1.(g). E5 cells which had been growing in 6µg/ml 8-aza [Table 3.2.1.(f)] were seeded at $1x10^3$ cells/well in up to 16µg/ml 8-aza.
On day 20 cells from the wells containing 16µg/ml 8-aza were removed and seeded in higher concentrations of 8-aza [Table 3.2.1.(h)].

	E5 cell growth in a range of concentrations of 8-aza in DMEM.S5. Cells were seeded at $4x10^2$/well.					
Day no.	0µg/ml 8-aza	16µg/ml 8-aza	20µg/ml 8-aza	24µg/ml 8-aza	28µg/ml 8-aza	32µg/ml 8-aza
20	$4x10^2$	$4x10^2$	$4x10^2$	$4x10^2$	$4x10^2$	$4x10^2$
30	$1x10^4$	$1x10^3$	$5x10^2$	$5x10^2$	dead	dead

Table 3.2.1.(h). E5 cells which had been growing in 16µg/ml 8-aza [Table 3.2.1.(g)] were seeded at $4x10^2$ cells/well in up to 32µg/ml 8-aza. On day 30 cell counts revealed the presence in wells containing up to 24µg/ml of 8-aza.
On day 30 cells from the wells containing 24µg/ml 8-aza were grown in HAT-supplemented medium [Table 3.2.1.(i)].

	E5 cell growth in a range of concentrations of HAT-supplemented medium. Cells were seeded at $0.8x10^2$ /well.					
Day no.	0x HAT	1x HAT	2x HAT	4x HAT	6x HAT	8x HAT
30	$0.8x10^2$	$0.8x10^2$	$0.8x10^2$	$0.8x10^2$	$0.8x10^2$	$0.8x10^2$
40	$1x10^3$	$1x10^3$	$1x10^3$	$9x10^2$	$7x10^2$	$9x10^2$

Table 3.2.1.(i). Growth of E5 cells seeded at $0.8x10^2$ cells/well in varying concentrations of HAT. The cells were expected to be HAT-s as they grew in medium containing up to 24µg/ml of 8-azaguanine. On examination, however, the cells were found to be HAT-r.

1x HAT =2.2mg/ml aminopterin, 0.0048mg/ml thymidine and 0.13mg/ml hypoxanthine.

	G12 cell growth in a range of concentrations of 8-aza in DMEM.S5. Cells were seeded at $2x10^3$/well.			
Day no.	0µg/ml 8-aza	8µg/ml 8-aza	14µg/ml 8-aza	20µg/ml 8-aza
0	$2x10^3$	$2x10^3$	$2x10^3$	$2x10^3$
10	$1x10^4$	$2x10^3$	$2x10^3$	$0.8x10^3$
20	$5x10^4$	$1.1x10^3$	dead	dead
30	$1x10^4$	$4x10^3$	dead	dead

Table 3.2.1.(j). Induction of HAT-sensitivity in G12 cells was performed by growing the cells in increasing concentrations of 8-aza. Initially G12 cells seeded at $2x10^3$ cells/well in varying concentrations of 8-aza.

On day 20 cells from the wells containing 8µg/ml 8-aza were removed and seeded in higher concentrations of 8-aza [Table 3.2.1.(k)].

	G12 cell growth in a range of concentrations of 8-aza in DMEM.S5. Cells were seeded at $0.5x10^3$/well.			
Day no.	0µg/ml 8-aza	8-µg/ml 8-aza	16µg/ml 8-aza	20µg/ml 8-aza
20	$0.5x10^3$	$0.5x10^3$	$0.5x10^3$	$0.5x10^3$
30	Confluent	$7x10^3$	$6x10^3$	$4x10^3$

Table 3.2.1.(k). G12 cells which had been growing in 8µg/ml 8-aza [Table 3.2.1.(j)] were seeded at $0.5x10^3$ cells/well in up to 20µg/ml 8-aza.

On day 30 cells from the wells containing 20µg/ml 8-aza were grown in HAT-supplemented medium [Table 3.2.1.(l)].

	Cell growth in a range of concentrations of HAT-supplemented medium			
Day no.	0x HAT	2x HAT	4x HAT	8x HAT
30	$1x10^3$	$1x10^3$	$1x10^3$	$1x10^3$
35	$4x10^3$	dead	dead	dead

Table 3.2.1.(l). Growth of G12 cells seeded at $1x10^3$ cells/well in varying concentrations of HAT. On day 35 the cells growing in HAT-supplemented medium were all dead indicating that the cells are HAT-s.

1x HAT =2.2mg/ml aminopterin, 0.0048mg/ml thymidine and 0.13mg/ml hypoxanthine.

Sample	OD ± SD @ 560nm
G12 HAT-s	0.740 ± 0.023
OKT8	0.000 ± 0.000
DMEM.S5	0.000 ± 0.000
PBS (blank)	0.000 ± 0.000

Table 3.2.1.(m). Supernatant from HAT-s G12 cells was examined by ELISA to detect reactivity of the antibody secreted by G12 cells with the K562 cell line. Supernatants from the hybridoma OKT8 was also examined. Samples were tested as neat supernatant.

SD - Standard deviation, n = 3.

Conc EMS (µg/ml complete medium)	Cell no. ($x10^4$)	
	Day 1	Day 4
0	5	10.00
10	5	7.5
20	5	5.0
40	5	0.00
60	5	0.00
80	5	0.00
100	5	0.00
120	5	0.00
140	5	0.00
160	5	0.00
180	5	0.00

Table 3.2.2.(a). The concentration of the mutagenic agent EMS causing a 50% kill of G12 cells was established by growing the cells in a range of concentrations of the mutagen (0-180µg/ml) in complete medium. The concentation causing a 50% kill was 20µg/ml of EMS. New phenotypes were selected using 8-aza.

Conc. 8-azaguanine (µg/ml of complete medium)	Cell no. ($x10^4$)
0	1.00
2	0.80
4	0.48
6	0.41
8	0.32
10	0.30
12	0.19
14	0.20
16*	0.00
18	0.00
20	0.00

Table 3.2.2.(b). The concentration of 8-aza which caused a 100% kill of wild type G12 cells was established. G12 cells were grown in complete medium containing 0-20µg/ml 8-aza. G12 cells which had been mutagenised with EMS were selected for new HAT-s phenotypes using 8-aza at a concentration which causes a 100% kill of wild-type cells.

* 16µg/ml of 8-aza caused a 100% kill of wild type G12 hybridoma cells.

	Cell number	
Conc. EMS (µg/ml complete medium)	Day 1	Day 4
0	$2x10^3$	$4.00x10^3$
15	$2x10^3$	$2.50x10^3$
20	$2x10^3$	$1.90x10^3$

Table 3.2.2.(c). G12 cells were mutagenised using EMS. G12 cells were seeded in 24 well plates in duplicates containing 0, 15 & 20µg/ml EMS. The concentration of EMS resulting in a 50% kill of hybridoma cells was 20µg/ml of EMS in complete medium. After 3 days the cells in all the wells were counted. Cells growing in 20µg/ml 8-aza were selected for HAT-sensitivity using 8-aza [Table 3.2.2.(d-f)].

	Cell growth in a range of concentrations of 8-aza		
Day no.	0µg/ml 8-aza	2µg/ml 8-aza	4µg/ml 8-aza
3	$2x10^3$	$2x10^3$	$2x10^3$
6	$4x10^4$	$2.8x10^3$	$2.5x10^3$ *

Table 3.2.2.(d). Three days after mutagenesis the G12 cells were seeded at $2x10^3$cells/well in varying concentrations of the selection agent 8-aza.

* On day 6 cells were taken from the wells containing 4µg/ml 8-aza and seeded in higher concentrations of 8-aza [Table 3.2.2.(e)].

	Cell growth in a range of concentrations of 8-aza		
Day no.	0µg/ml 8-aza	4µg/ml 8-aza	8µg/ml 8-aza
6	$5x10^2$	$5x10^2$	$5x10^2$
10	$1x10^3$	$7x10^2$	$7x10^2$

Table 3.2.2.(e). The mutagenised G12 cells were seeded in up to 8µg/ml of 8-aza. On day 10 cells were removed from the wells with 8µg/ml of 8-aza and seeded at $5x10^2$cells/well in up to 16µg/ml of 8-aza.

Cells were taken from these wells and seeded in up to 16µg/ml of 8-aza. This is the concentration of 8-aza which causes a 100% kill of hybridoma cells and, therefore, only the newly formed phenotypes which are 8-aza-resistant should suvive [Table 3.2.2.(g)].

	Cell growth in a range of concentrations of 8-aza		
Day no.	0µg/ml 8-aza	8µg/ml 8-aza	16µg/ml 8-aza
10	$2x10^2$	$2x10^2$	$2x10^2$
14	$5x10^2$	$4x10^2$	$3x10^2$

Table 3.2.2.(g). The G12 cells were seeded in up to 16µg/ml of 8-aza. This is the concentration of 8-aza which causes a 100% kill of hybridoma cells and, therefore, only the newly formed phenotypes which are 8-aza-resistant should survive.

On day 14 cells were removed from the wells with 16µg/ml of 8-aza and grown in HAT-supplemented medium.

	Cell growth in a range of concentrations of HAT-supplemented medium			
Day no.	0x HAT	2x HAT	4x HAT	8x HAT
14	$6x10^2$	$6x10^2$	$6x10^2$	$6x10^2$
20	$1x10^3$	dead	dead	dead

Table 3.2.2.(g). Mutagenised cells which had been selected for HAT-sensitivity using 8-aza were examined for their ability to grow in HAT-containing medium. The cells were seeded at $6x10^2$ cells/well in varying concentrations of HAT. On day 20 the cells were examined for viability and were all dead and, therefore, HAT-s. These cells were now ready for to be used in cell fusions to form triomas.
1x HAT =2.2mg/ml aminopterin, 0.0048mg/ml thymidine and 0.13mg/ml hypoxanthine

Sample	OD $\pm$ SD @ 560nm
G12 HAT-s (mutagenesis)	0.610 $\pm$ 0.020
OKT8	0.000 $\pm$ 0.000
DMEM.S5	0.000 $\pm$ 0.000
PBS (blank)	0.000 $\pm$ 0.000

Table 3.2.2.(h). Supernatant from the mutagenised HAT-s G12 cells were examined by ELISA to detect reactivity of the antibody secreted by G12 cells with the K562 cell line. Supernatant from the OKT8 hybridoma was also examined. Samples were tested as neat supernatant.
SD - Standard deviation, n = 3.

Fusion number	No. hybrids/480 wells	HRP +ve	K562 +ve	HRP/K562 +ve
Fusion 1	114	2	111	0
Fusion 2	93	3	90	0
Fusion 3	118	3	114	0
Fusion 4	68	3	64	0
Fusion 5	130	8	120	0
Total	523/2400 wells	19/523 triomas	499/523 triomas	0/523 triomas

Table 3.2.3.(a). HAT-s G12 hybridoma cells were fused with rabbit splenocytes secreting anti-HRP antibodies. The G12 cells used were rendered HAT-s using 8-aza. Specific antibody was detected using three ELISAs two of which detected both parental antibodies and a third which detected the bsab (Section 2.8). The total numbers of triomas formed and their various reactivities are given above. No bsab was detected in any of the 5 cell fusions.

Fusion number	No. hybrids/480 wells	HRP +ve	K562 +ve	HRP/K562 +ve
Fusion 1	106	3	99	0
Fusion 2	112	4	105	0
Fusion 3	81	4	77	0
Fusion 4	88	0	87	0
Total	387/1920 wells	11/387 triomas	368/387 triomas	0/387 triomas

Table 3.2.3.(b). HAT-s G12 hybridoma cells were fused with mouse splenocytes secreting anti-HRP antibodies. The G12 cells used were rendered HAT-s using 8-aza. Specific antibody was detected using three ELISAs two of which detected both parental antibodies and a third which detected the bsab (Section 2.8.). The total numbers of triomas formed and their various reactivities are given above. No bsab was detected in any of the 4 cell fusions.

Fusion number	No. hybrids/480 wells	HRP +ve	K562 +ve	HRP/K562 +ve
Fusion 1	119	3	111	0
Fusion 2	84	1	81	0
Fusion 3	73	2	70	0
Fusion 4	74	3	71	0
Total	350/1920 triomas	9/350 triomas	333/350 triomas	0/350 triomas

Table 3.2.3.(c). HAT-s G12 hybridoma cells were fused with mouse splenocytes secreting anti-HRP antibodies. The G12 cells used were rendered HAT-s using EMS. Specific antibody was detected using three ELISAs two of which detected both parental antibodies and a third which detected the bsab (Section 2.8.). The total numbers of triomas formed and their various reactivities are given above. No bsab was detected in any of the 4 cell fusions.

3.3. Chemical production of bsabs

Bsabs have been made chemically using the method of Glennie *et al.* (1987). $F(ab'\gamma)_2$ fragments formed by peptic digests of both parental antibodies were reduced to FabSH with 2-mercaptoethanol. One FabSH species was maleimidated with the bifunctional cross-linking agent *o*-phenylenedimaleimide to yield FabMAL which has free maleimide groups. The FabMAL and FabSH were then reacted together to form bsab. The method prevents any parental antibody recombinations. As already outlined in the introduction (Section 1.1) the method of Glennie *et al.* (1987) has been used successfully by a number of researchers, for delivery of the immunotoxin saporin to tumour cells (Flavell *et al.*, 1991; Flavell *et al.*, 1992; French *et al.*, 1992) and for targeting T-lymphocytes to tumour cells (Mezzanzanica *et al.*, 1991).

3.3.1. Production and purification of antiserum to HRP

Polyclonal anti-HRP antisera were produced in a New Zealand White male rabbit. A titre of 1:100,000 was achieved using a HRP-specific ELISA [Table 3.3.1.]. The antisera was purified by $(NH_4)_2SO_4$ precipitation and affinity chromatography using a 1ml Protein A column.

3.3.2. Characterization of anti-HRP antiserum by ELISA

The antisera was examined for cross reactivity with a range of other enzymes [Table 3.3.2.]. No cross reactivity of anti-HRP antibody with hexokinase, β-galactosidase or lactate dehydrogenase was observed.

3.3.3. Characterization of anti-HRP antiserum by HPLC

Whole normal rabbit serum, antiserum to HRP and various purified protein fractions were analysed by HPLC. The PAP complex formed on reacting the anti-HRP and HRP was also examined by HPLC. The retention times of a range of protein standards are shown in [Table 3.3.3.] and these allowed for the identification of the proteins present in various samples.

HPLC chromatographic profiles of $(NH_4)_2SO_4$ purified antiserum to HRP [Figure 3.3.3.(a)] revealed a major peak at 15 minutes and a smaller peak at 9 minutes. These represent IgG and IgM, respectively. Further purification on a Protein A column again

shows a large peak at 15 minutes and a lesser peak at 22 minutes. The latter could be due to the leaching of protein A from the column [Figure 3.3.3.(b)]. The HPLC chromatographic profile of antiserum incubated with HRP [Figure 3.3.3.(d)] revealed a peak with a retention time of 12 minutes. This is representive of the PAP complex which has a molecular weight of approximately 432,000 daltons. This compares favorably with the individual retention times of peroxidase and the purified antibody, which are 17 minutes and 15 minutes, respectively [Figures 3.3.3.(a & c)]. The chromatographic profile of peroxidase incubated with normal rabbit serum was performed and no shifts in the parental peaks were observed [Figure 3.3.3.(e)].

3.3.4. Characterization of anti-HRP antiserum by SDS electrophoresis

SDS-Page analysis under non-reducing conditions was used to detect purity of the protein A purified antisera [Figure 3.3.4.]. A single large band with a MW of approximately 150KD representative of IgG is present.

Titre	OD $\pm$ SD @ 620nm
1:1000 antiserum	1.300 $\pm$ 0.049
1:10,000 antiserum	1.276 $\pm$ 0.076
1:100,000 antiserum	0.539 $\pm$ 0.253
1:10 Normal rabbit serum	0.221 $\pm$ 0.210
PBS (blank)	0.000 $\pm$ 0.000

Table 3.3.1. The titre of anti-HRP antiserum was assessed by ELISA. A sufficient signal was obtained using a titre of 1:100,000. All serum dilutions were performed in 0.15M PBS, pH7.2.

SD - Standard deviation, n = 3.

	OD ± SD @ 620nm			
Sample	HRP	LDH	Hexokinase	β-Gal
1:10,000 anti-HRP	0.806 ± 0.020	0.000	0.000	0.000
1:100 normal rabbit serum	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
PBS	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000

Table 3.3.2. Cross-reactivity of rabbit anti-HRP antibodies with various enzymes. LDH:- lactate dehydrogenase, β-Gal:- β-Galactosidase. Antiserum dilutions were made up in 0.15M PBS, pH7.2.
SD - Standard deviation, n = 3.

Protein	Molecular weight	Retention time (mins)
IgM	900,000	9
IgG	150,000	15
HRP	44,000	18

Table 3.3.3. The retention times of a range of commercial proteins were determined by HPLC. The column used was a 10μM Protein Pak SW 300 column with a mobile phase of 0.1M phosphate buffer, pH 7.0, with a flow rate of 0.5ml/min. Absorbance due to proteins was detected at 280nm.

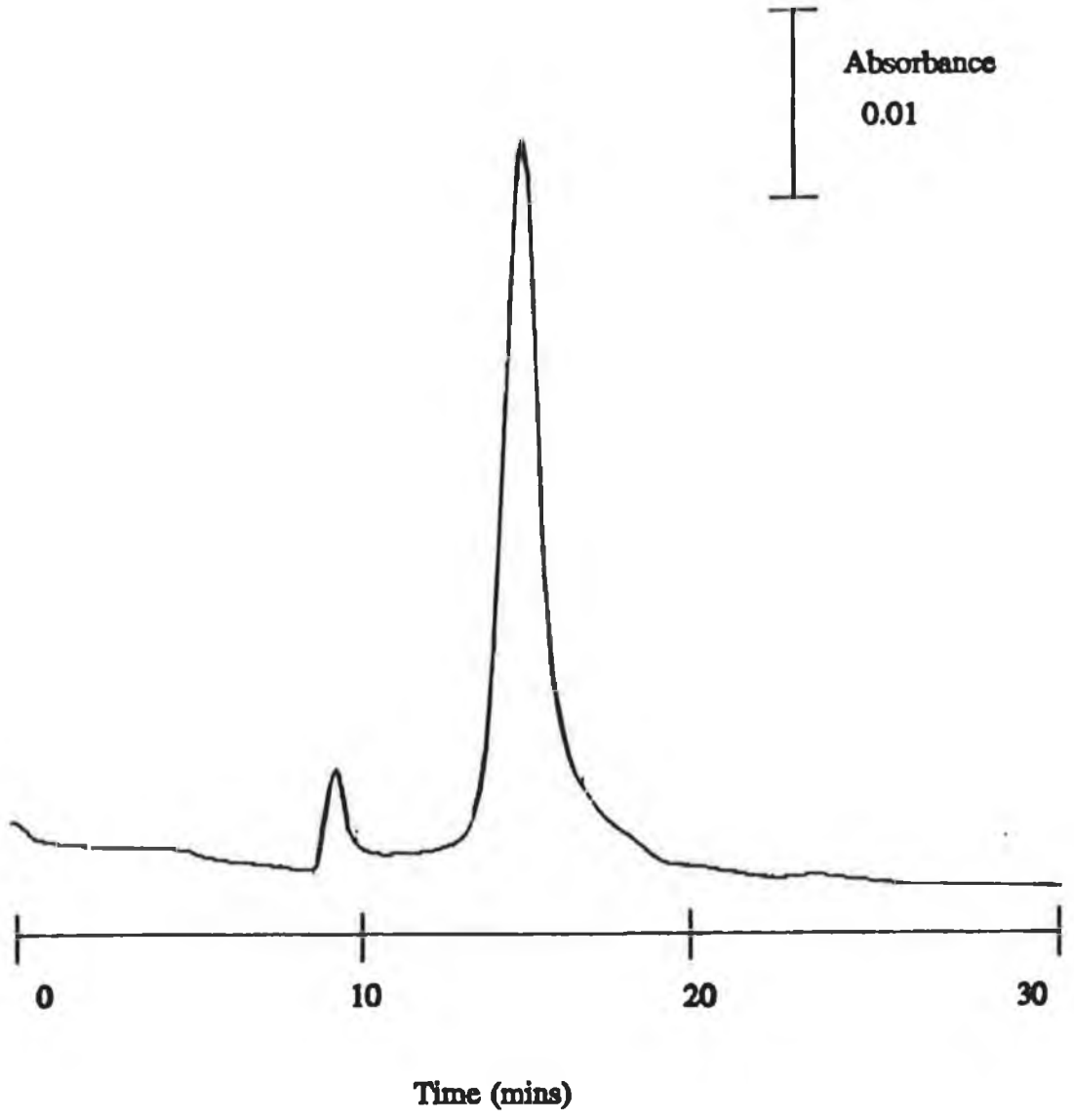


Figure 3.3.3.(a).

HPLC chromatogram of rabbit antisera to HRP (1mg/ml), after $(NH_4)_2SO_4$ precipitation. The column used was a 10µM Protein Pak SW 300 column with a mobile phase of 0.1M phosphate buffer, pH 7.0, and flow rate of 0.5ml/min. Absorbance due to protein was detected at 280nm. Two peaks were detected. One peak had retention time of 15mins and was IgG, the second protein peak with a retention time of 9 mins was that of IgM.

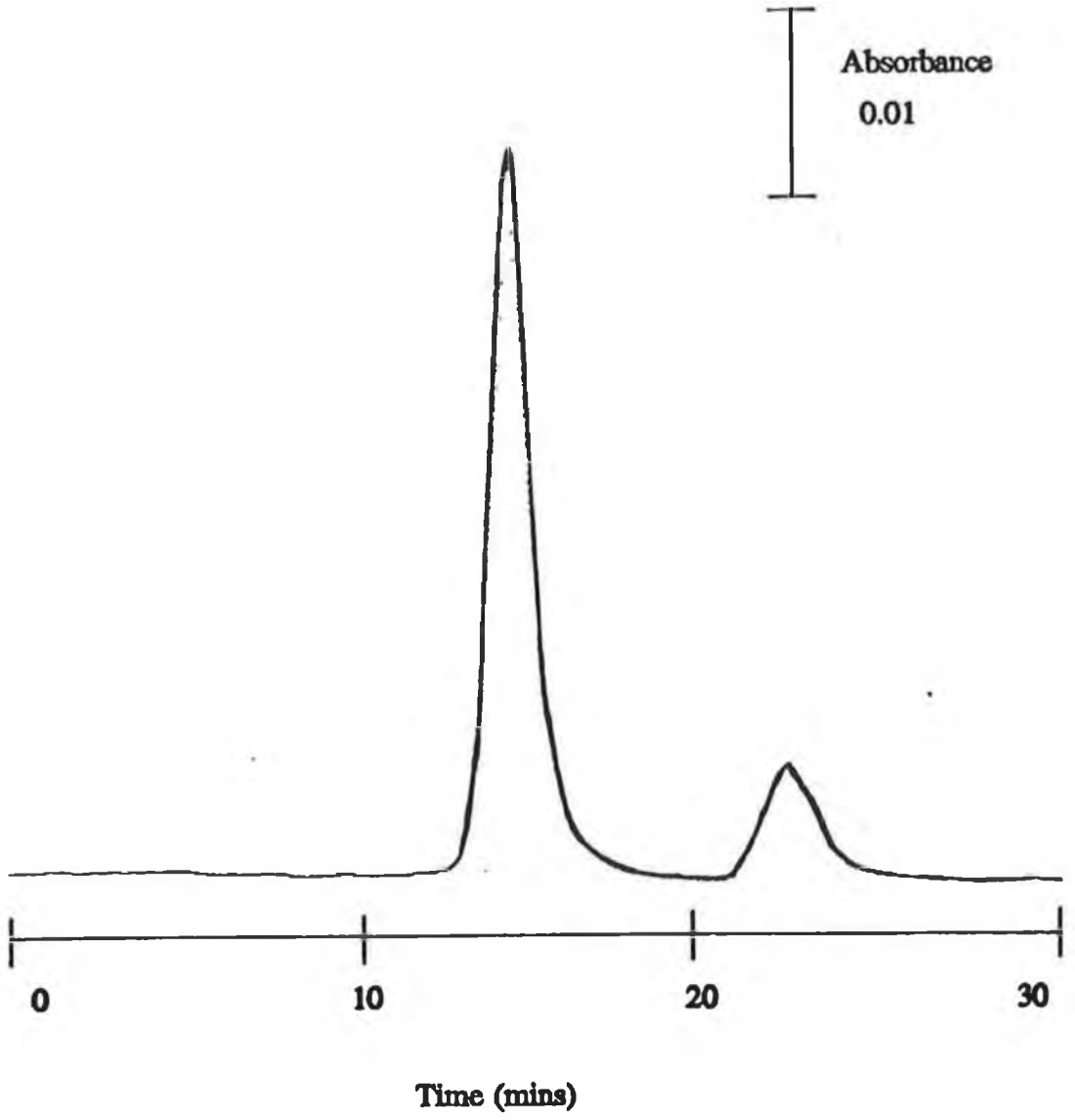


Figure 3.3.3.(b).

HPLC chromatogram of antisera to HRP after purification on a Protein A affinity column. The column used was a 10μM Protein Pak SW 300 column with a mobile phase of 0.1M phosphate buffer, pH 7.0, and a flow rate of 0.5ml/min. Absorbance due to protein was detected at 280nm. Two peaks were detected. One peak had a retention time of 15mins and was IgG, the protein peak with a retention time of 22mins could be that of protein A which has leached from the column.

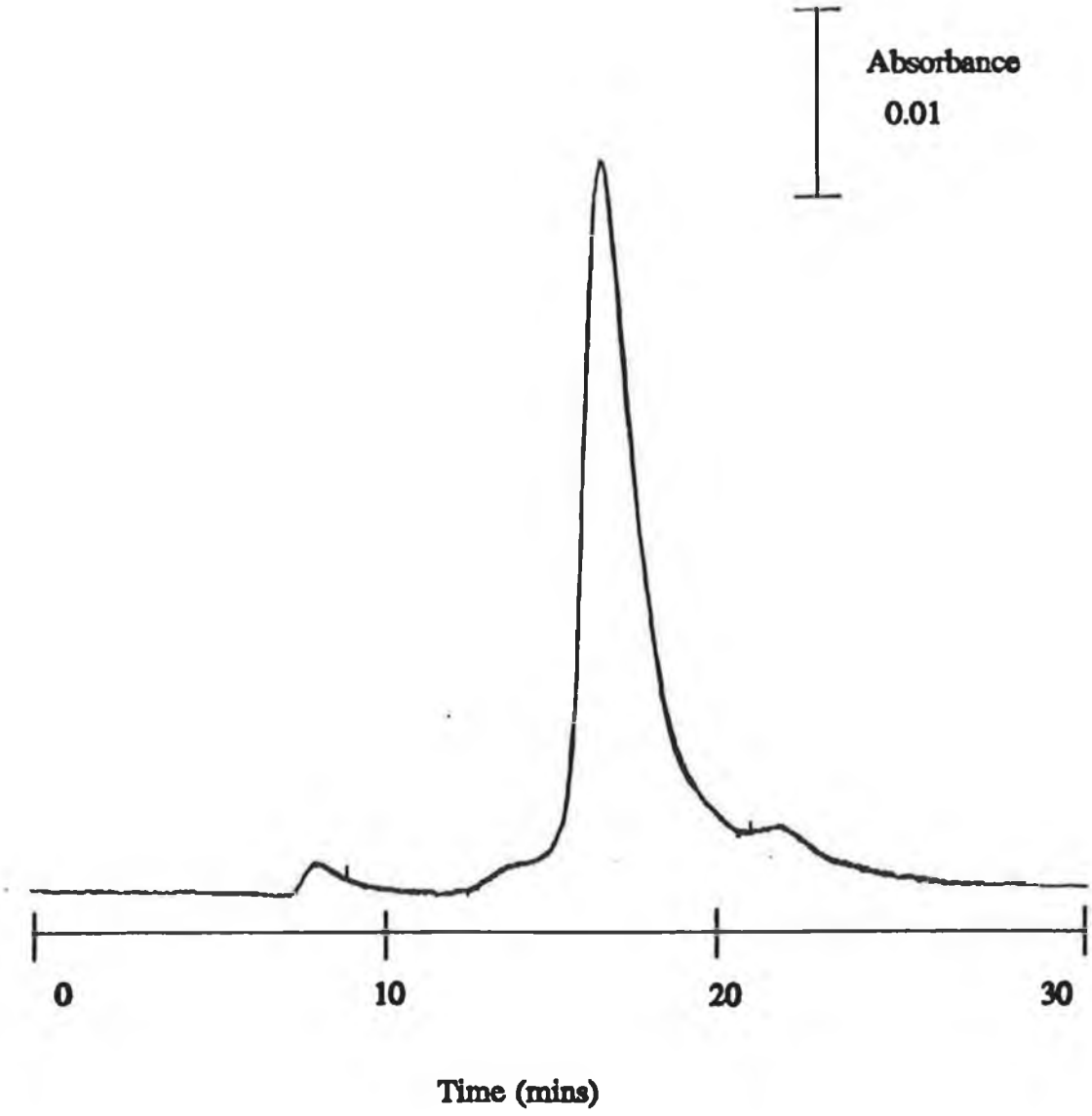


Figure 3.3.3.(c).

HPLC chromatogram of HRP, (1mg/ml). The column used was a 10μM Protein Pak SW 300 column with a mobile phase of 0.1M phosphate buffer, pH 7.0, and a flow rate of 0.5ml/min. Absorbance due to protein was detected at 280nm. A large protein peak is present with a retention time of approximately 17mins.

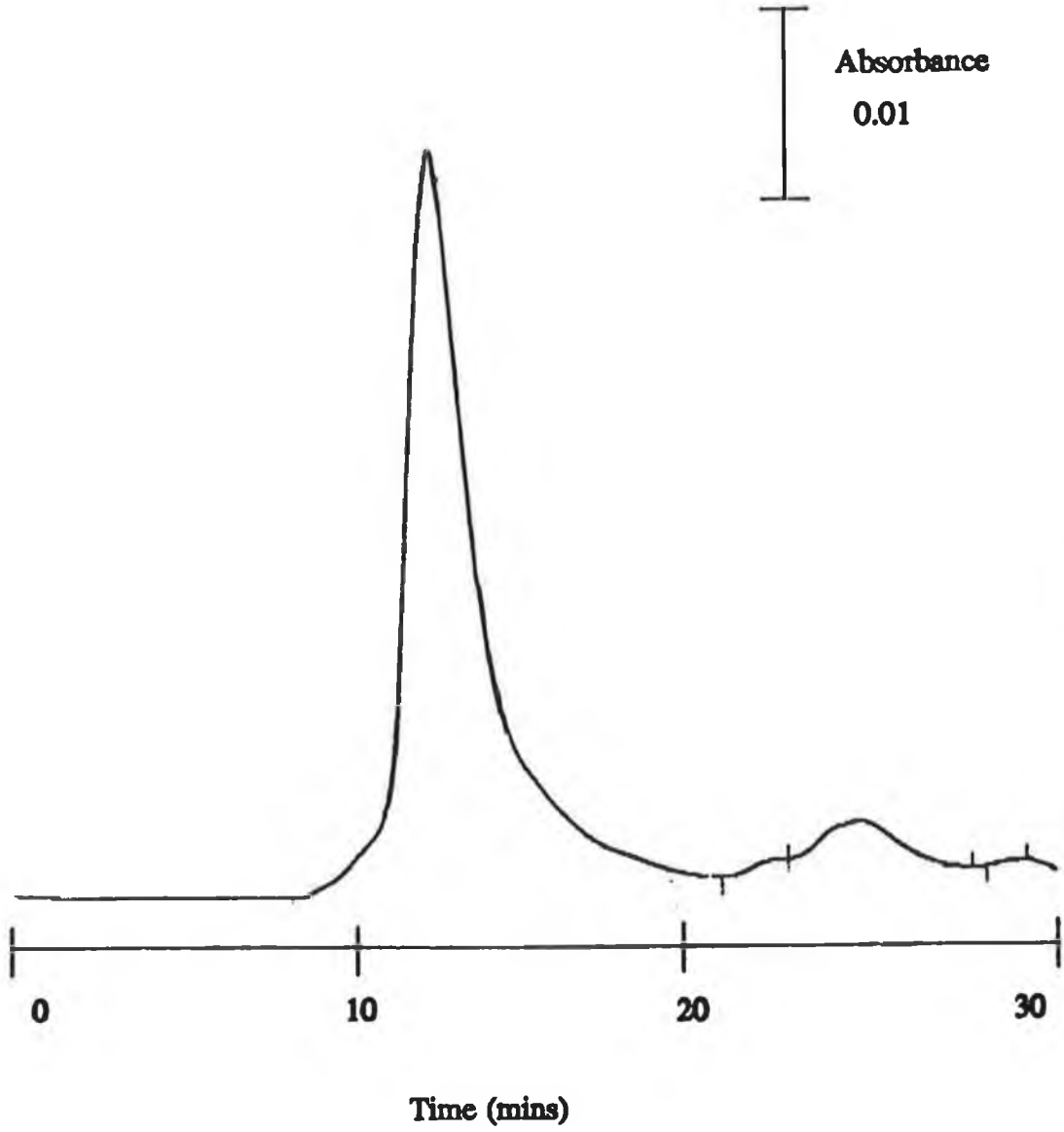


Figure 3.3.3.(d).

HPLC chromatogram of affinity-purified rabbit antisera to HRP, (1mg/ml), incubated with HRP, (1mg/ml), for 30mins at 37^{0}C. The column used was a 10µM Protein Pak SW 300 column with a mobile phase of 0.1M phosphate buffer, pH 7.0, and a flow rate of 0.5ml/min. Absorbance due to protein was detected at 280nm. One major peak was detected with a retention time of 12mins. This is representative of the peroxidase anti-peroxidase complex which has a MW of 432,000D (MWs: IgG = 150,000D, HRP = 44,000D). Smaller broad peaks are present with a retention time of 25mins and could be representative of denatured proteins.

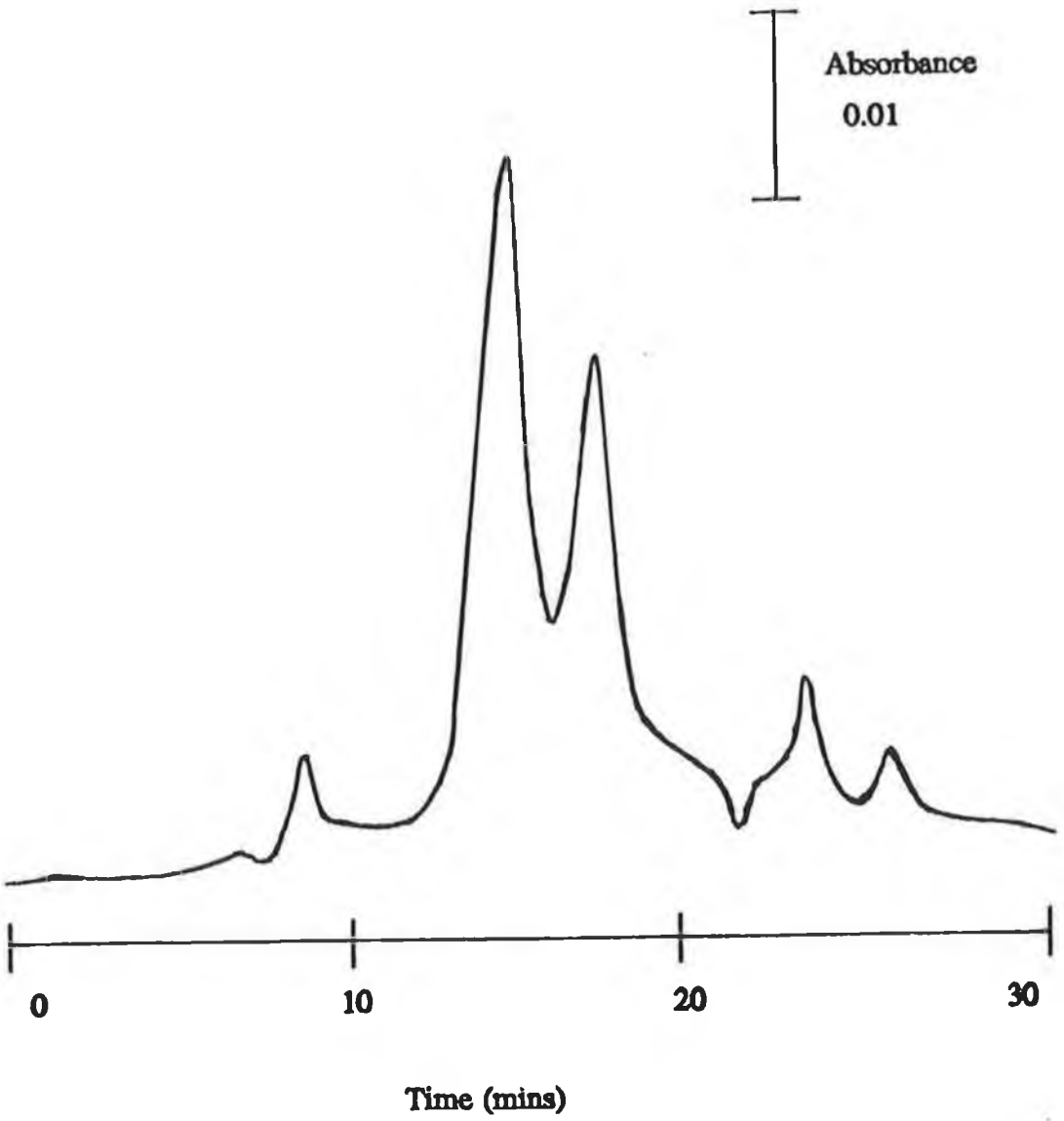


Figure 3.3.3.(e).

HPLC chromatogram of normal rabbit serum, (1mg/ml), incubated with HRP, (1mg/ml), for 30mins at 37°C. The column used was a 10µM Protein Pak SW 300 column with a mobile phase of 0.1M phosphate buffer, pH 7.0, and a flow rate of 0.5ml/min. Absorbance due to proteins was detected at 280nm. This chromatogram should be compared to those obtained for HRP (Figure 3.3.3.c), and for HRP incubated with rabbit antisera to HRP. The protein peaks of interest are present at 15mins and 17mins. These are representative of normal rabbit IgG and HRP. No change in the retention was observed, hence, no interaction of HRP and normal rabbit IgG has taken place. Other smaller protein peaks which are present are from the rabbit serum.

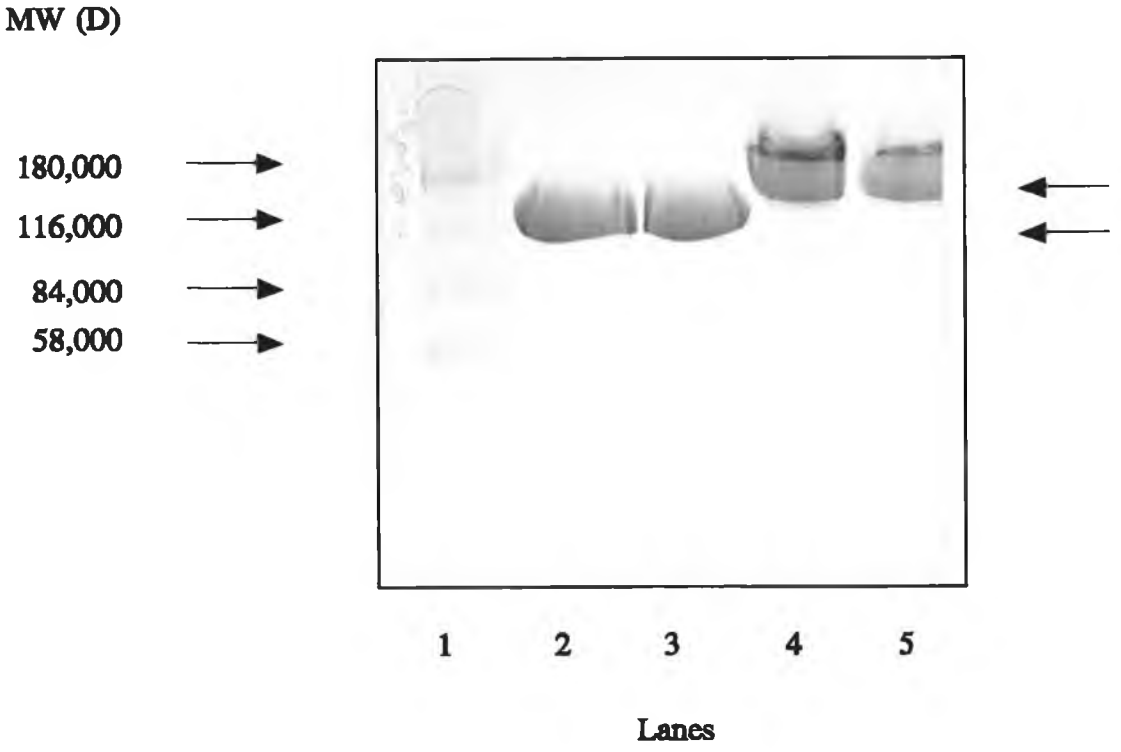


Figure 3.3.4.

SDS-Page electrophoresis of purified antibodies (MW: 150,000D) and F(ab'γ)$_2$ fragments (MW: 100,000). Lane 1 contains molecular weight markers. Lanes 2 and 4 are the anti-HRP F(ab'γ)$_2$ and anti-HRP whole antibody molecules respectively and lanes 3 and 5 are the G12-F(ab'γ)$_2$ and G12 whole antibody molecules, respectively. Electrophoresis was performed under non-reducing conditions.

3.3.5. G12 antibody production and purification

G12 monoclonal antibody was purified from ascites by $(NH_4)_2SO_4$ precipitation and affinity chromatography using a 1ml protein A column.

3.3.6. Characterization of G12 antibody by ELISA

The reactivities of the mAbs G12 and E5 against various cell lines and human cancers was examined by ELISA [Tables 3.3.6.(a & b)]. Both G12 and E5 were found to be reactive with the K562 and GCCM cell lines [Table 3.3.6.(a)]. No reactivity was detected with the T24 and HL60 cell lines. H9 and H12 are hybridomas which also secrete antibody reactive with CLL cells were negative on all cell lines examined. All 4 antibodies reacted positively with six CLL samples when examined by ELISA [Table 3.3.6.(b)].

3.3.7. Characterization of G12 antibody by HPLC

HPLC analysis was also carried out on the $(NH_4)_2SO_4$ and protein A purified fractions [Figures 3.3.7.(a & b)].

HPLC chromatographic profiles of $(NH_4)_2SO_4$ purified ascitic fluid containing antibody [Figure 3.3.7.(a)] revealed a major peak at 15 minutes and a smaller peak at 9 minutes, these represent IgG and IgM, respectively. Further purification on a protein A column shows a single large peak at 15 minutes [Figure 3.3.7.(b)]. A smaller peak at 23 minutes could be due denatured proteins or protein which has leached from the column.

3.3.8. Characterization of G12 antibody by SDS-electrophoresis

SDS-Page analysis under non-reducing conditions was used to detect purity of the protein A purified G12 mAB [Figure 3.3.4.]. A single large band with a MW of approximately 150,000D is present. This would be representative of IgG.

	OD ± SD @ 560nm			
Sample	K562	G-CCM	T24	HL60
G12	0.580 ± 0.020	0.270 ± 0.001	0.000 ± 0.000	0.000 ± 0.000
E5	0.403 ± 0.037	0.285 ± 0.001	0.000 ± 0.000	0.000 ± 0.000
H9	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
H12	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
DMEM.S5	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
PBS	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000

Table 3.3.6.(a). Reactivity of a panel of the antibodies produced by the hybridomas G12, E5, H9 and H12 with various cell lines (K562, G-CCM, T24 & HL60). The ELISAs were performed on 96 well microtitre plates to which the cell lines had been prebound as described in Section 2.8.4. The samples were examined as neat supernatants.

SD - Standard deviation, n = 3.

	OD ± SD @ 620nm				
Cell type	G12	E5	H9	H12	DMEM.S5
CLL 1	0.420 ± 0.004	0.355 ± 0.013	0.238 ± 0.012	0.203 ± 0.003	0.000 ± 0.000
CLL 2	0.308 ± 0.003	0.180 ± 0.002	0.200 ± 0.002	0.207 ± 0.007	0.000 ± 0.000
CLL 3	0.210 ± 0.002	0.362 ± 0.001	0.260 ± 0.015	0.209 ± 0.002	0.000 ± 0.000
CLL 4	0.398 ± 0.020	0.184 ± 0.007	0.230 ± 0.004	0.220 ± 0.001	0.000 ± 0.000
CLL 5	0.333 ± 0.006	0.227 ± 0.006	0.220 ± 0.007	0.200 ± 0.003	0.000 ± 0.000
CLL 6	0.410 ± 0.013	0.250 ± 0.006	0.230 ± 0.005	0.210 ± 0.001	0.000 ± 0.000

Table 3.3.6.(b). Reactivity of the antibodies produced by the hybridomas G12, E5, H9 and H12 with CLL cells by ELISA. The ELISAs were performed on 96 well microtitre plates to which the CLL cells had been pre-bound as described in Section 2.8.4. The samples were examined as neat supernatants.

SD - Standard deviation, n = 3.

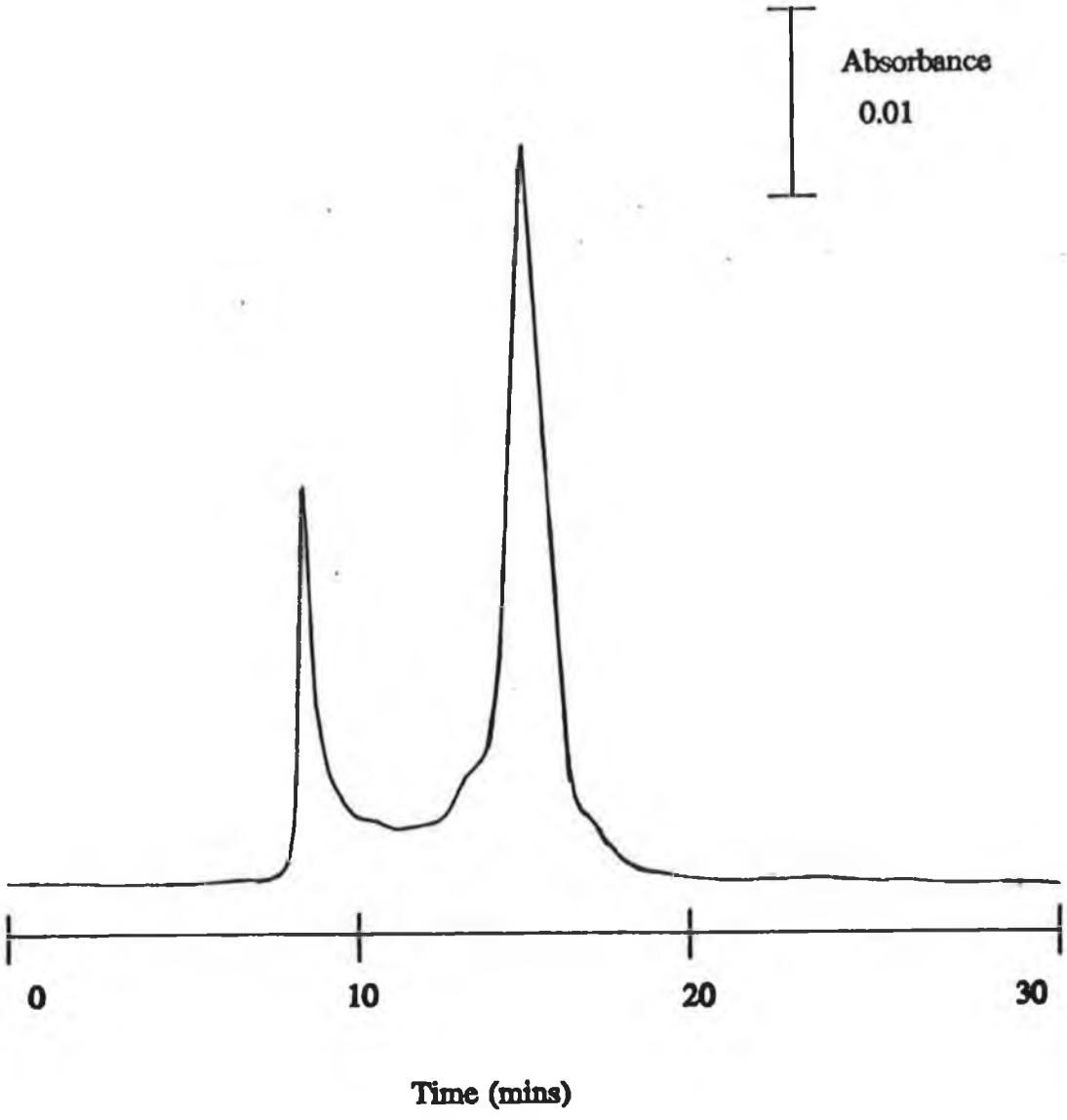


Figure 3.3.7.(a).

HPLC chromatogram G12 ascitic fluid, (1mg/ml) after $(NH_4)_2SO_4$ precipitation. The column used was a 10μM Protein Pak SW 300 column with a mobile phase of 0.1M phosphate buffer, pH 7.0, and a flow rate of 0.5ml/min. Absorbance due to proteins was detected at 280nm. Two peaks were detected. An IgG protein peak is present with a retention time of approximately 15 minutes. The smaller protein peak eluted in the void volume with a retention time of 9 minutes is that of IgM.

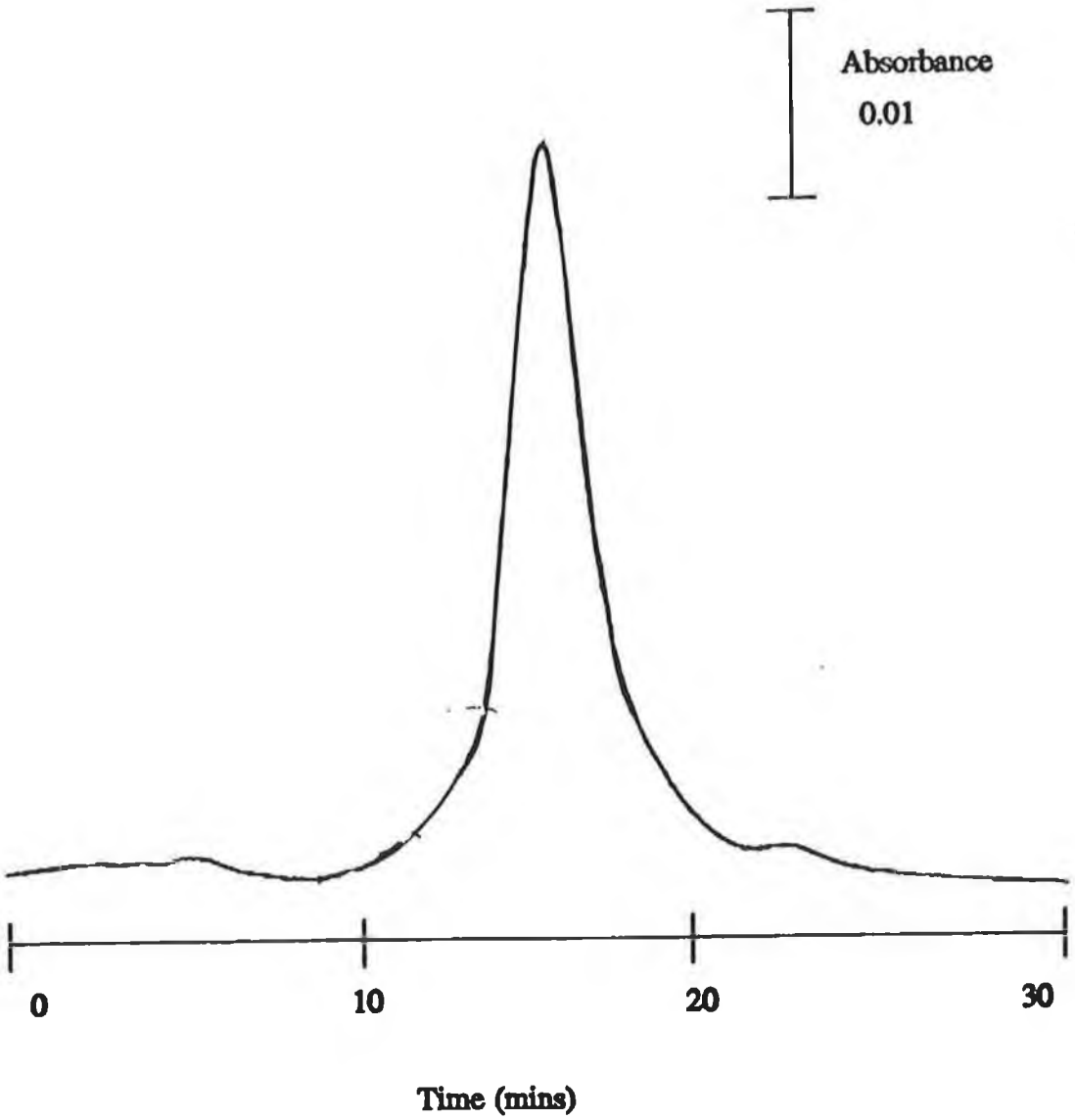


Figure 3.3.7.(b).

HPLC chromatogram of G12 ascitic fluid, (1mg/ml), after purification on a Protein A affinity column. The column used was a 10µM Protein Pak SW 300 column with a mobile phase of 0.1M phosphate buffer, pH 7.0, and a flow rate of 0.5ml/min. Absorbance due to proteins was detected at 280nm. The protein peak with a retention time of 15mins is that of IgG. A small protein peak with a retention time of 23mins could be that of denatured proteins or that of protein A which has leached from the column.

3.4. Reconstitution of Fab′ fragments to form $F(ab'\gamma)_2$

Fab′ fragments were made from purified G12 mAB and anti-HRP antiserum as described in Section 2.7. These were reconstituted to form bsab using the method of Glennie *et al.* (1987).

These $F(ab'\gamma)_2$ bsabs were characterized by ELISA, immunocytochemistry, SDS-electrophoresis and HPLC.

3.4.1. Characterization of $F(ab'\gamma)_2$ bsab by ELISA

The bsab and the whole antibody secreted by the hybridoma G12 exhibited similar binding patterns when examined by ELISA [Table 3.4.1.(a)]. Of the cell lines examined both G12 and bsab reacted with K562 and GCCM. Both also reacted with 7 CLL samples and were negative for 3 normal peripheral blood samples. The ELISA used to detect reactivity of G12 mAB is outlined in [Figure 3.1.(b)] and that used to detect reactivity of bsab is outlined in [Figure 3.1.(a)].

The bsab was also analysed by an ELISA which detected mouse and rabbit IgG since the bsab had Fab′ fragments derived from both sources [Table 3.4.1.(b)].

3.4.2. Characterization of $F(ab'\gamma)_2$ by HPLC

HPLC chromatographic profiles of both G12 and anti-HRP Fab′ fragments revealed a single peak with a retention time of 22 minutes in both cases [Figures 3.4.2.(a & b)]. The reconstituted $F(ab'\gamma)_2$ was present as a large peak at 16 minutes [Figure 3.4.2.(c)]. A smaller peak at 22 minutes is probably unreacted Fab′, fragments.

3.4.3. Characterization of $F(ab'\gamma)_2$ bsab by SDS electrophoresis

SDS-Page analysis under non-reducing conditions was used to detect purity and MW of the $F(ab'\gamma)_2$ reconstituted bsab [Figure 3.4.3.].

3.4.4. Characterization of $F(ab'\gamma)_2$ bsab by immunocytochemistry

K562, T24, HL60 and CLL were stained histochemically using methylene blue and eosin [Figures 3.4.4.(a and b)]. The cells were also examined immunocytochemically for antigenic reactivity using the bsab. In all 6 CLL samples were tested. HL60, K562, T24 and normal lymphocytes all tested negative whereas all 6 CLL samples were

positive [Figure 3.4.4.(c and d)]. Immunocytochemical staining was also performed using firstly a commercial Dako PAP kit [Table 2.1.1.] and secondly a HRP-conjugated goat anti-mouse IgG antibody. In the latter 2 cases whole G12 antibody was used as a primary binding antibody to the immobilized cells [Figure 3.1.(b & c)]. Neither of the latter 2 methods were capable of detecting antigen using immumocytochemical techniques.

	Samples: OD ± SD @ 405nm			
Cell type	DMEM.S5	PBS	G12	$F(ab'\gamma)_2$
K562	0.000	0.000	0.390 ± 0.004	0.297 ± 0.023
GCCM	0.000	0.000	0.218 ± 0.017	0.343 ± 0.028
CLL 1	0.000	0.000	0.440 ± 0.007	0.260 ± 0.015
CLL 2	0.000	0.000	0.197 ± 0.015	0.157 ± 0.023
CLL 3	0.000	0.000	0.152 ± 0.016	0.370 ± 0.010
CLL 4	0.000	0.000	0.342 ± 0.017	0.369 ± 0.020
CLL 5	0.000	0.000	0.145 ± 0.012	0.203 ± 0.020
CLL 6	0.000	0.000	0.236 ± 0.001	0.327 ± 0.020
CLL 7	0.000	0.000	0.404 ± 0.006	0.321 ± 0.004
PBL 1*	0.000	0.000	0.000	0.000
PBL 2*	0.000	0.000	0.000	0.000
PBL 3*	0.000	0.000	0.000	0.000

Table 3.4.1.(a). Reactivity of G12 whole antibody and $F(ab\gamma')_2$ bsab with 7 CLL samples, normal peripheral blood lymphocytes and various cell lines. Both G12 whole antibody and $F(ab\gamma')_2$ were used at a concentration of 10µg/ml in PBS. The ELISAs used to detect the G12 whole antibody and the bsab are outlined in Sections 2.8.5. and 2.8.8.

SD - Standard deviation, n = 3.

* :- Normal peripheral blood lymphocytes

Sample	OD ± SD @ 620nm
Bsab $F(ab'\gamma)_2$	0.322 ± 0.002
G12 antibody	0.000
Rabbit anti-HRP antibody	0.000
PBS (blank)	0.000

Table 3.4.1.(b). Chemically formed bsabs were made using rabbit and mouse IgG Fab′ fragments. An ELISA detecting rabbit and mouse Fab′ simultaneously was performed to verify that both of these arms were in fact present in the newly formed bsab. Anti-mouse IgG was immobilized onto a 96 well microtitre plate. The bsab was then added. The bsab was detected using an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody.

SD - Standard deviation, n = 3.

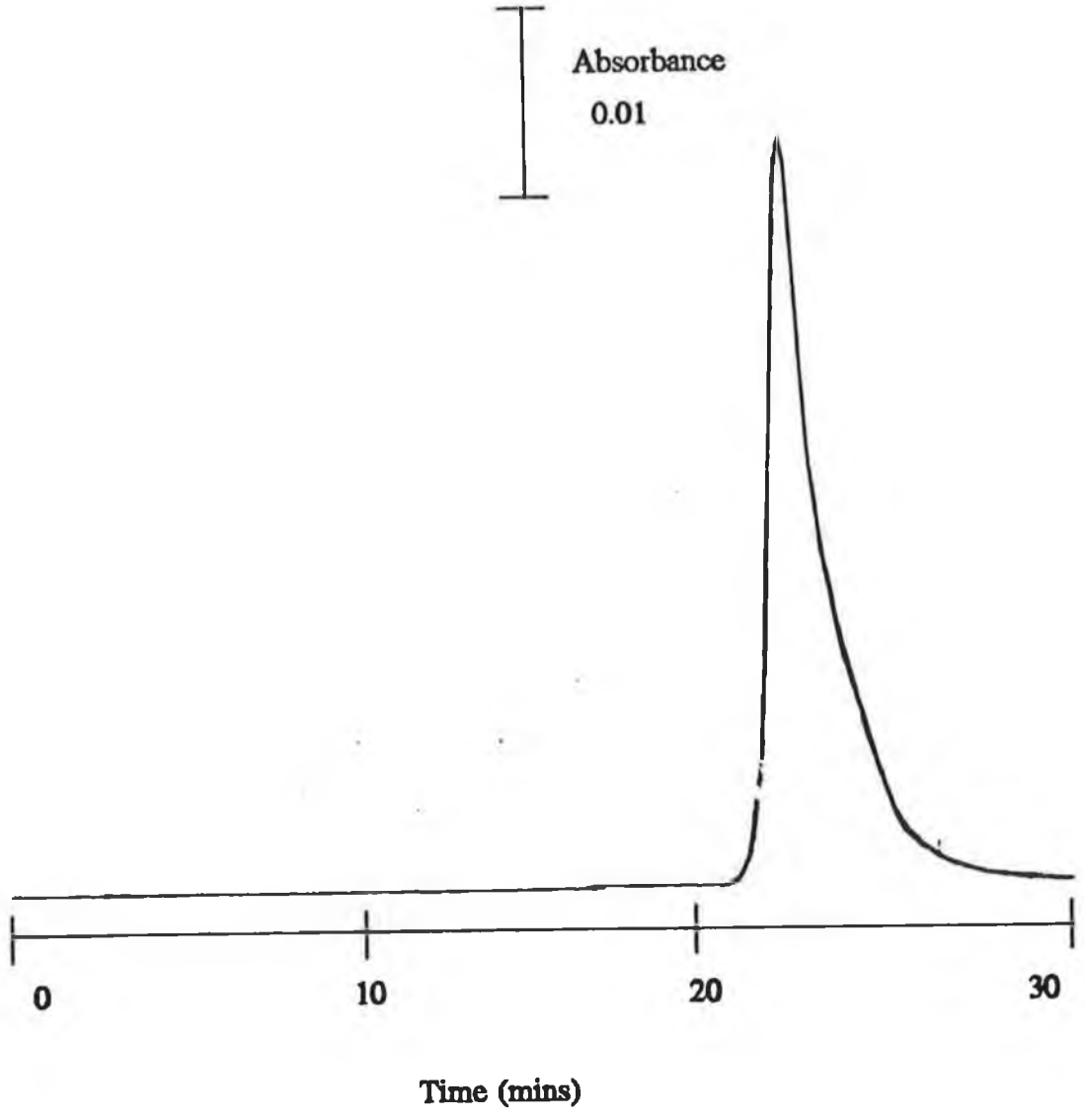


Figure 3.4.2.(a).

HPLC chromatogram of rabbit anti-HRP antibody Fab' fragment. The column used was a 10μM Protein Pak SW 300 column with a mobile phase of 0.1M phosphate buffer, pH 7.0, and a flow rate of 0.5ml/min. Absorbance due to proteins was detected at 280nm. A protein peak with a retention time of 22 minutes is present. This corresponds to the Fab' molecule.

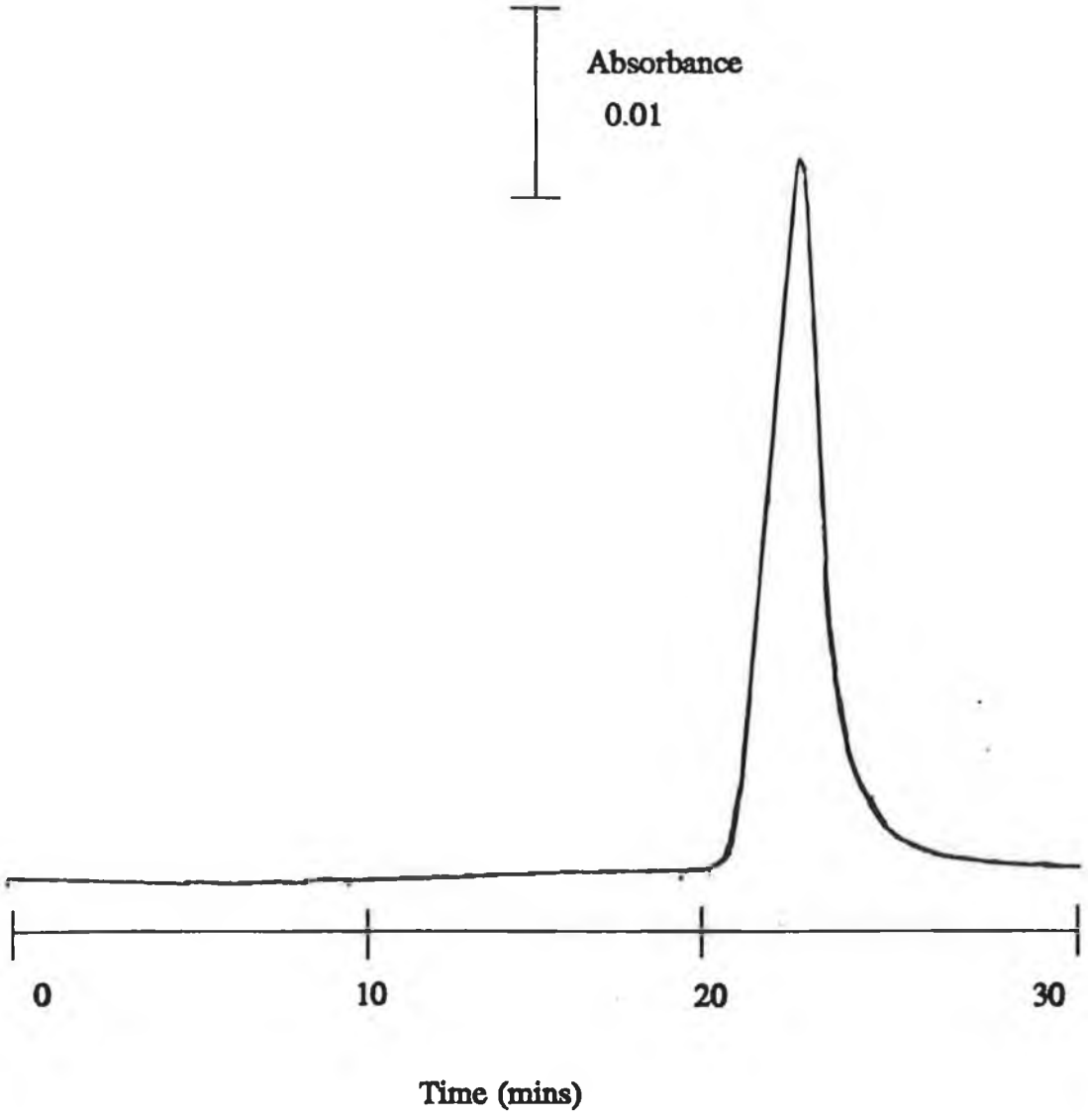


Figure 3.4.2.(b).

HPLC chromatogram of G12 antibody Fab' fragment. The column used was a 10μM Protein Pak SW 300 column with a mobile phase of 0.1M phosphate buffer, pH 7.0, and a flow rate of 0.5ml/min. Proteins were detected at 280nm. A protein peak with a retention time of 22 minutes is present. This corresponds to the Fab' molecule.

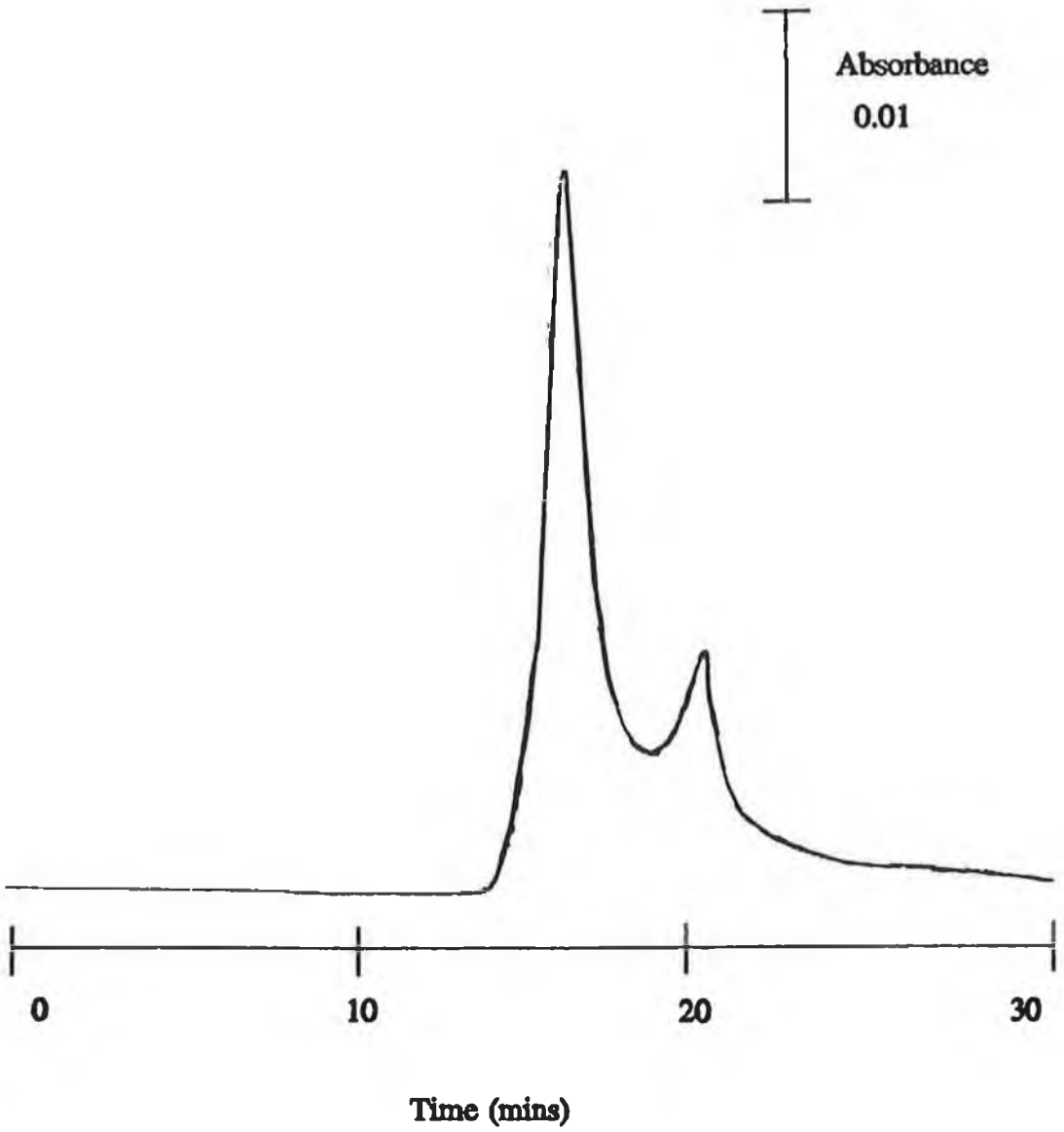


Figure 3.4.3.

HPLC chromatogram of the bsab $F(ab'\gamma)_2$ formed during reconstitution of rabbit anti-HRP and G12 antibody Fab' fragments using a 10µM Protein Pak SW 300 column with a mobile phase of 0.1M phosphate buffer, pH 7.0, and a flow rate of 0.5ml/min. Proteins were detected at 280nm. Protein peaks with Rts of approximately 16 minutes and 22 minutes are present. The peak at 16 minutes is thought to be that of the bsab which would have a molecular weight of approximately 100kD and the second with a retention time of 22 minutes that of unreacted Fab' fragments (See discussion section 3.4.5.)

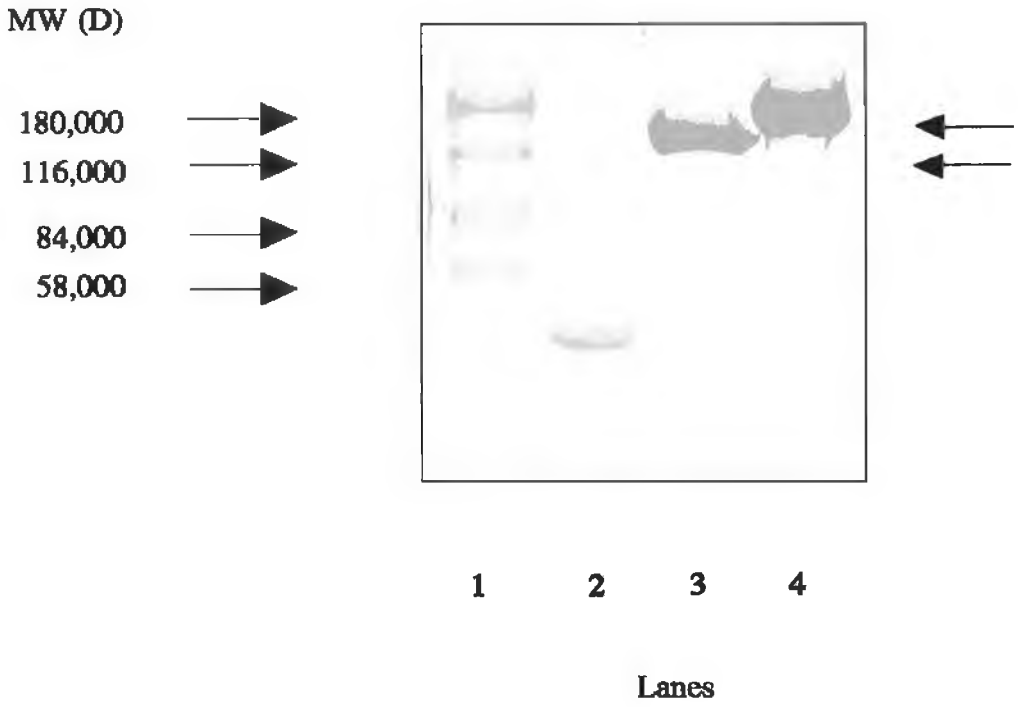
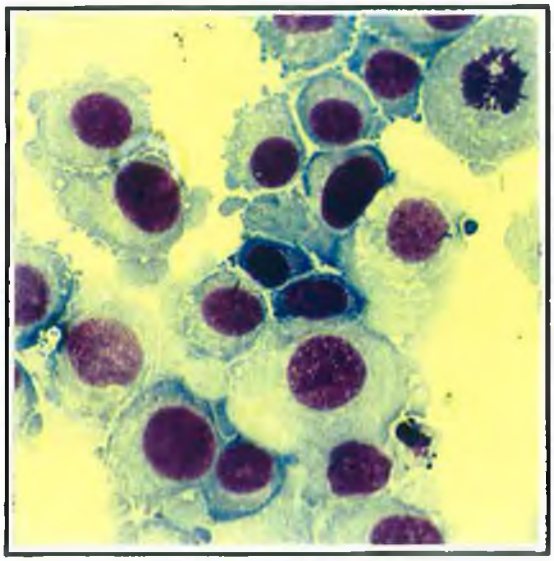


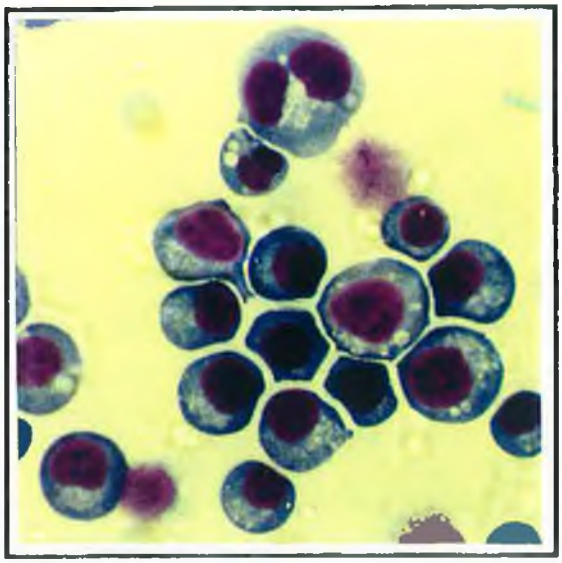
Figure 3.4.3.

SDS-Page electrophoresis of HRP (lane 2, MW: 44,000D), reconstituted $F(ab'\gamma)_2$ bispecific antibody (lane 3, MW: 100,000) and purified anti-HRP antiserum (lane 4, MW: 100,000). Lane 1 contains molecular weight markers. Electrophoresis was performed under non-reducing conditions.

(a)



(b)



(c)

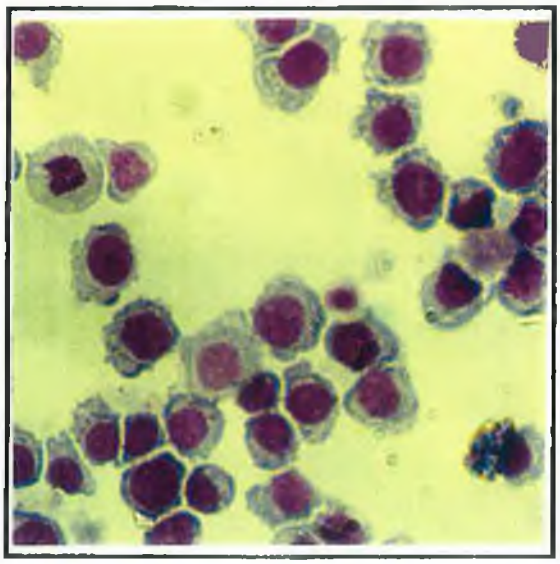


Figure 3.4.4.(a).

Histochemical staining of (a) K562, (b) T24 and (c) HL6O cytocentrifuged cell preparations using methylene blue and eosin as described in Section 2.9. (Magnification x 40).

(a) (b)

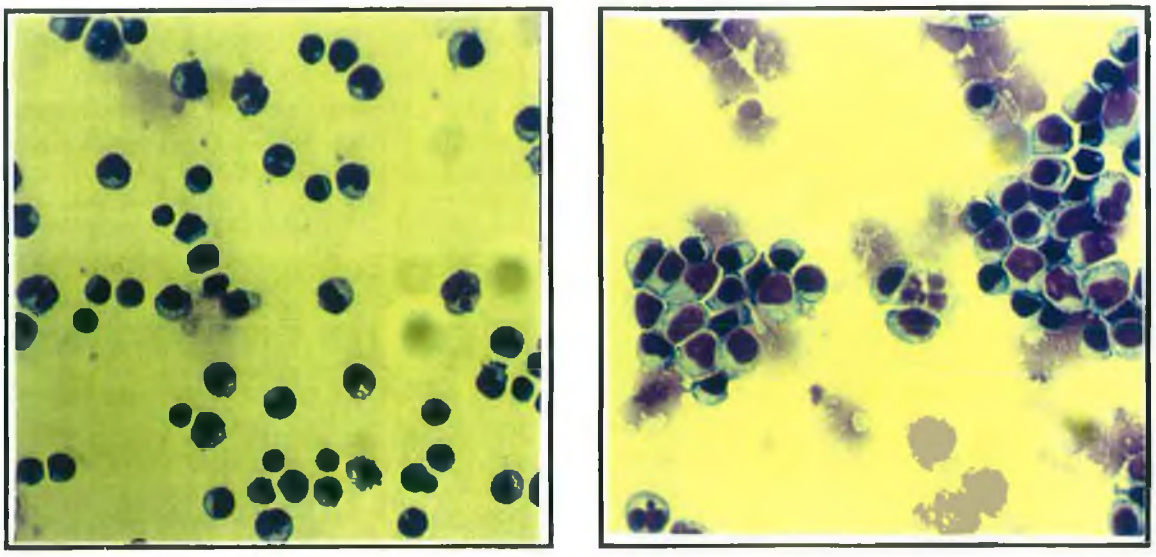


Figure 3.4.4.(b).

Histochemical staining of cytocentrifuged cell preparations from the peripheral blood lymphocytes of (a) normal healthy volunteers and (b) patients with CLL using methylene blue and eosin as described in Section 2.9.

(Magnification x 40).

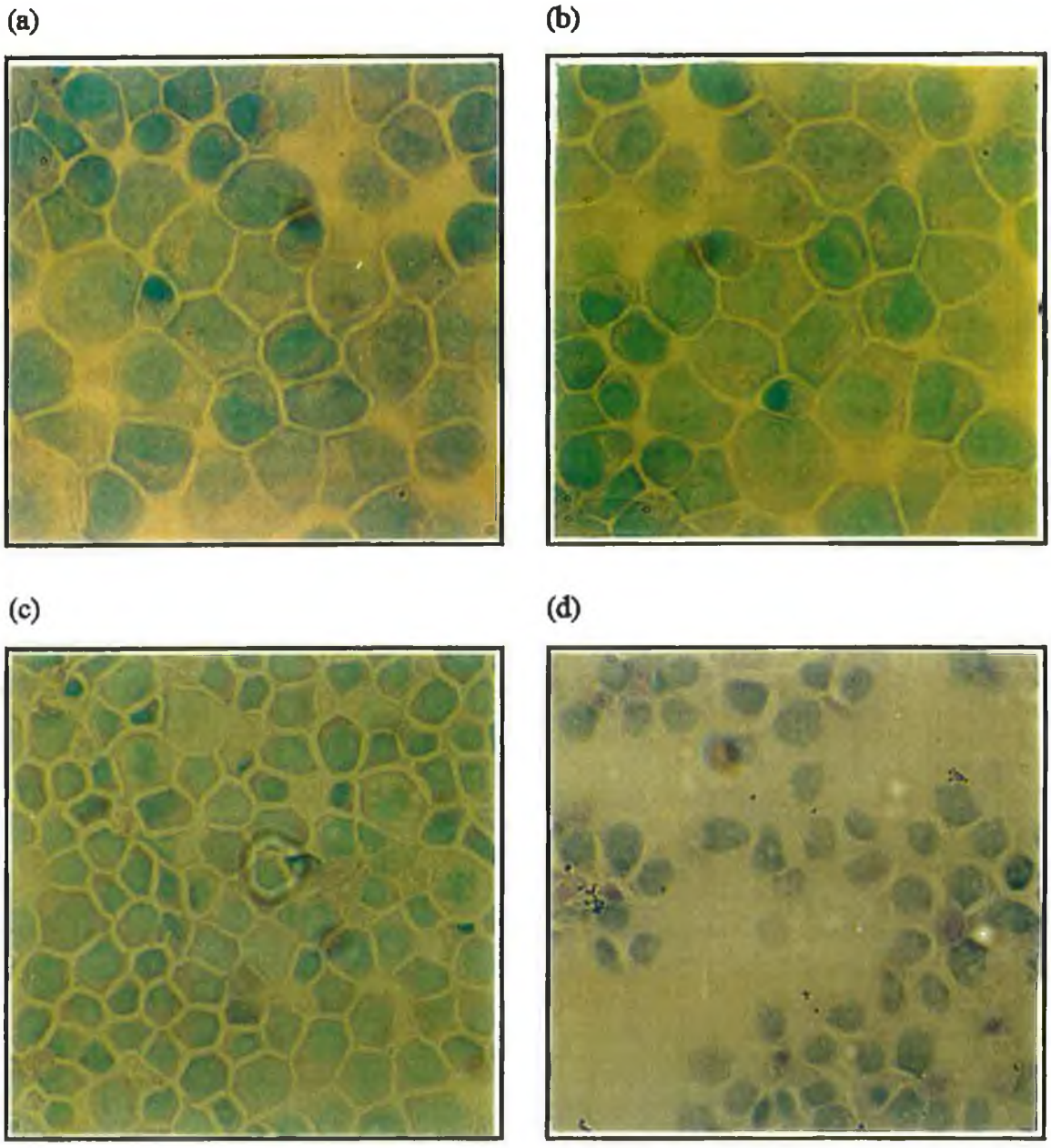


Figure 3.4.4.(c).

Immunohistochemical staining of (a) K562, (b) T24, (c) HL60 and (d) normal peripheral blood lymphocyte (PBL) cytocentrifuged cell preparations using chemically synthesised 'G12 anti-CLL crossed with anti-HRP' bsab counterstained with methylene green as described in Section 2.9.1. All samples are negative.
(Magnification x 40).

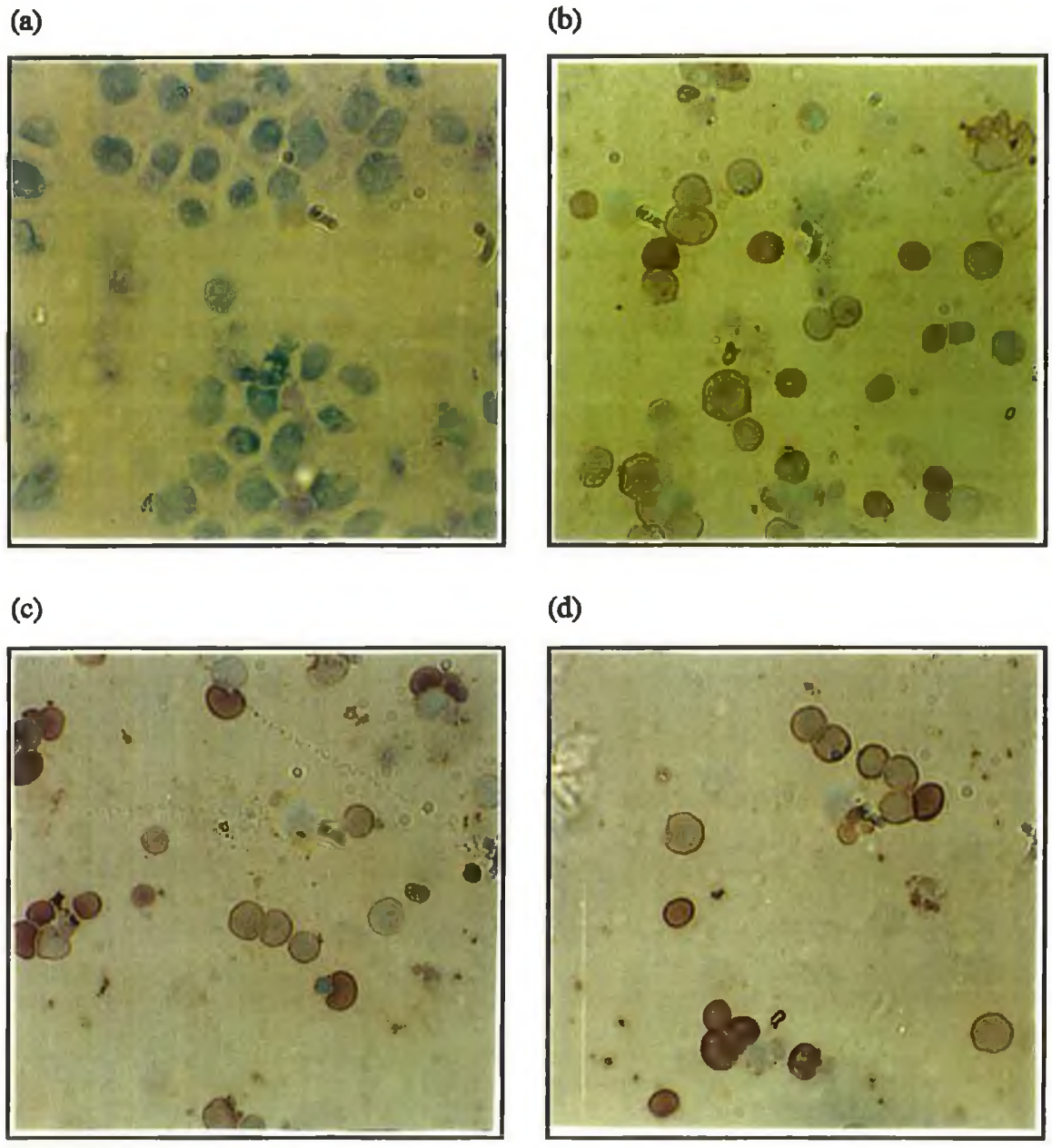


Figure 3.4.4.(d).

Immunohistochemical staining of cytocentrifuged cell preparations from (a) the peripheral blood lymphocytes of normal healthy volunteers and (b, c & d) the peripheral blood lymphocytes of 3 patients with CLL using chemically synthesised 'G12 anti-CLL crossed with anti-HRP' bsab counter stained with methylene green as described in Section 2.9.1. Positive cells stain brown. The CLL samples stain positively with the bsab.

(Magnification x 40).

3.4.5. Discussion

Biological production of triomas was very time consuming. Production of hybridomas using normal somatic cell fusion involves raising a sufficient titre in the immunized animal, a procedure which in itself is highly variable with regard to time (Baumgarten 1992). These splenocytes are then fused to myeloma cells. Newly formed hybridomas are then assayed for specific antibody production. The time scale involved in the biological production of bsabs is increased by the need to back select for HAT-sensitivity in the hybridomas cells. Two methods were used to back select for HAT-sensitivity. Firstly, the hybridoma cells were grown in increasing concentrations of 8-aza and secondly the cells were mutagenised using EMS and new phenotypes were selected using 8-aza as a selection agent. The fusion frequencies of HAT-s G12 cells with immunized splenocytes was quite satisfactory. The resulting antibodies secreted from the triomas were either anti-K562 or anti-HRP parental antibodies. No bsabs were formed.

Newly formed triomas can potentially secrete 10 reassociated antibody types (Nolan and O'Kennedy 1991). The relative quantities of each of these ten is dependent on whether reassociation is random or non-random. The exact nature of heavy and light chain reassociation has been well studied. Milstein and Cuello (1984) demonstrated that among antibodies secreted by hybrid-hybridomas there was a preferential association of homologous heavy and light chains. This would be advantageous to the production of bsabs. De Lau *et al.* (1989) on the other hand reported that the homologous reassociation of heavy and light chains of 8 quadromas examined was favoured only in a minority of cases. Generally heavy and light chain reassociation was random. One of the quadromas produced was developed from parental hybridomas secreting antibodies to bovine IgG and HRP. Analysis of the secreted antibody species revealed that reassociation of the anti-HRP light chain with heavy chains was absolutely heterologous. Reassociation of the bovine anti-IgG light chain showed no such constraints. No anti-HRP antibody activity and therefore no bsab activity was detected. The implication of these results is that when antibodies are produced by B-cells the reassociation of homologous chains is not a property which is selected. This would not favour bsab production.

Given that the majority of triomas formed in the work described here secrete

antibodies reactive with the K562 cell line it can be taken that the reassociation of heavy and light chains is such that the binding pattern of newly formed antibodies is dominated predominantly by heavy and light chains from the G12 hybridoma. It has been demonstrated that the heavy chain alone can bind antigen (Collet *et al.,* 1989; Sastry *et al.*,1992). Therefore any reassociated antibody with an anti-K562/CLL heavy chain could bind the antigen. Less stable reassociated antibodies such as the anti-HRP antibodies may be overgrown at an early stage post-fusion by more stable triomas secreting anti-K562 antibodies.

The triomas formed by the fusion of mouse HAT-s G12 cells and rabbit splenocytes (secreting antibodies to HRP) are of particular interest because the resultant cells are chimeric in nature. It was required that these splenocytes receive an *in vitro* immunization. This was necessary because the splenocytes had been isolated 2 weeks subsequent to the final *in vivo* immunization which facilitated maximum antiserum levels to HRP but meant that the splenocytes were in the incorrect growth phase for antibody production by fusion. The *in vitro* immunization system used was developed previously in our laboratory for application to a murine system and its use here is the first occasion it has been applied to the development of an immune response in rabbits. The fusion of rabbit splenocytes with mouse myeloma or hybridoma cells can be more advantageous in some cases than the fusion of mouse splenocytes with mouse myeloma/hybridoma cells. Mice may not always be the most suitable animal in which to stimulate an immune response. Hyperimmunization of rabbits is often more practical, therefore, the chimeric mouse-rabbit system is better in these cases for mAb production. Due to the difficulties in raising an immune response in mice to group A *Streptococcus* Raybould and Takahashi (1988) developed rabbit-mouse hybridomas using the splenocytes of a hyperimmunized rabbit. The resultant chimeric cells secreted rabbit antibodies to group A *Streptococcus*. Given that the trioma produced in our work is of such a nature their exists the potential that this system could be used to produce bsabs in cases where a mouse antibody response is either not possible or difficult to obtain. The anti-HRP antiserum obtained from the rabbit was used for the production of bsabs using chemical procedures. Therefore, the use of rabbits in the course of this work allows for the production of bsabs firstly by chemical methods using the anti-HRP antiserum and secondly by biological methods using the isolated

splenocytes in somatic cell fusions to form triomas. The splenocytes may be used immediately or stored in liquid nitrogen indefinitely. It may be necessary that the splenocytes receive an *in vitro* immunization depending on when the animal received its final antigenic boost.

Another feature of the mouse-rabbit triomas is that they required normal rabbit serum to be included in the growth medium post fusion. It was observed that this altered the morphology of the fused cells to one of a fibroblastic type-cell. When parental G12 cells were grown in medium supplemented with normal rabbit serum they too exhibited this altered morphology. The triomas grew extremely slowly and were more unstable than mouse-mouse triomas.

To facilitate bsab production by triomas it may be necessary in the future to optimise the growth conditions used to culture the cells. The next chapter deals with the influence of the growth conditions on hybridoma growth at the clonal stage. Perhaps more defined growth medium would allow for production of triomas which secrete stable bsab.

Chemical methods proved more successful in the production of bsabs. A bsab was readily formed and was fully functional for both antigens as determined by ELISA and immunocytochemistry. Antiserum to HRP was produced in a New Zealand White male rabbit (Cahill *et al.,* In press). A titre of 1:100,000 was achieved. The antibody produced by the hybridoma G12 was obtained from ascitic fluid. Both antibodies were affinity purified on Protein A columns prior to use. The resultant bsab was stable for up to six months when stored at 4^0C. Stability of the bsab may be dependent on the relative stabilities of the parental antibodies both of which were very stable.

The bsab proved more efficient than the whole anti-tumour antibody when applied to immunocytochemical techniques. Immunocytochemical staining of cells using the whole G12 antibody in a PAP kit and in a direct stain using an anti-mouse IgG enzyme-conjugated antibody proved unsuccessful. The reactivity of the bsab was slightly altered when used in the immunocytochemical system as opposed to the ELISA. Whereas in the ELISA the bsab reacted with the K562 and GCCM cell lines as well as CLL cells, in the immunocytochemical system the cross-reativity with the human tumour cell lines was eliminated, only reactivity with the CLL cells was obtained. Optimal functioning required that the HRP be added after the bsab was

reacted with the cells. Addition of the bsab with pre-bound HRP resulted in poor binding of the bsab complex. Milstein and Cuello (1984) reported that bsabs used in immunocytochemical techniques had reduced non-specific binding compared to more conventional immunoenzymatic techniques. These authors also highlight the importance of penetration of the bsab. For example, their bsab which detected both HRP and extrahypothallmic somatostatin was incapable of functioning when the bsab was applied to samples with the pre-bound HRP. When the bsab was added first followed by addition of the HRP staining was obtained. The size of the bsab was 150,000D and that of the bsab with HRP bound would be approximately 190,000D. This difference in MW was enough to interfere with binding of the bsab. This manipulation of procedure would not be possible in conventional systems where the functional agents are conjugated to antibodies. In the present study the MWs of the $F(ab'\gamma)_2$ bsab and Fab' were analyzed using HPLC and SDS polyacrylamide gel electrophoretic techniques. The approximate MWs would be in the region of 100,000D and 50,000D for $F(ab'\gamma)_2$ bsab and Fab', respectively. SDS-electrophoresis revealed that the molecular weigth of the $F(ab'\gamma)_2$ bsab was approximately 100,000D as expected. HPLC analysis revealed that the retention times obtained for $F(ab'\gamma)_2$ bsab and Fab' fragments were 16 and 22 minutes respectively. This is unusual because the retention time of HRP which has a MW of 44,000D is 17 minutes. The cause of such an observation could be due to the loss of the light from the $F(ab'\gamma)_2$ bsab and Fab' fragments. Although this would alter the MW the functioning of the bsab would not neccessarily be altered because as already discussed the light chain may not be required for antigenic binding (Collet *et al.*, 1989; Sastry *et al.*, 1992). The various reduction steps involved in the chemical production of bsabs could reduce the stability of the thiol bonds linking the light and heavy chains. Loss of the light chains may actually increase binding of bsab to antigen because the smaller molecule can penetrate antigen more readily (Milstein and Cuello 1984).

Some of the obvious drawbacks in the chemical production of bsabs are the large amounts of parent antibodies required for their synthesis and the fact that, unlike biologically made bsabs, a continuous supply of bsab is not readily available. Some of their advantages include the fact that their production method allows for purification by size, this would not be the case with biologically made bsabs where

up to 10 different heavy and light chain rearrangements are possible and all newly formed antibodies are of equivalent size. Purification of bsabs would therefore involve at least two affinity purification procedures. The large amounts of antibodies required during chemical procedures can be overcome if facilities are available which allow for the production of large quantities of antibodies such as animal houses where ascites can be raised in mice and polyclonal antiserum developed in rabbits and large cell culture facilties for the production of antibody in cell culture supernatants.

The use of bsabs *in vitro* for ELISA and immunocytochemical techniques is quite promising. This is mainly due to the fact that once they are made they can be used in their native states. Their is no need to humanize them as is the case for bsabs with potential therapeutic applications.

There are several advantages of bsabs over mAbs. Bsabs work in a single step so reducing the time required to perform the assay. The dependency on and cost of, commercial labelled antibodies are eliminated. Biologically produced bsabs are active in their native state. No chemical alterations are required once the bsabs are made in order to bind the functional agent and the target cell. This is important as chemical modification may reduce the activity of the antibody or the functional agent which is being coupled. Although production of the bsab made here involved chemical modification of both parental antibodies these modifications were very site-specific. For example, the pepsin digestion of parental antibodies was followed by the reduction of thiol groups to yield the Fab′ fragments. These modifications were not near the antigen binding site and, therefore, the antigen binding capability of the bsab should be unaffected. Conjugation of enzymes to whole antibodies is usually performed using accessible lysine residues and is therefore less specific than the conjugation procedure used to produce bsabs.

Bsabs do not require that the functional agent be bound prior to their administration. This allows pre-localization of the bsab at the target site prior to the addition of the functional agent. This may be of benefit in situations where penetration of the bsab/antibody to the antigic site hindered. This has been outlined in more detail above (Milstein and Cuello, 1984). An added advantage of the bsab produced here was that it performed in both ELISA and immunocytochemical based systems. Although

ELISAs are very useful for determining antigenic determinants on cell surfaces the result is obtained as an absorbance measurement spectrophotometrically. Immunocytochemistry on the other hand allows for the microscopic visualisation of samples. These samples can be stored indefinitely.

Potentially, bsabs could find applications in the area of biosensors. A biosensor consists of a biological material that is responsive to the property or substance being sensed, in contact with a suitable transducing element that is needed to convert the signal sensed into a signal that can be easily processed. The specific and sensitive nature of antibody-antigen interaction provides the biosensing mechanism of immunosensors. The transducing mechanisms can be be based on for example electochemical and optical detection. Bsabs with one arm specific for an enzyme which produces electroactive product can be utilised in electrochemical immunosensors (Boitieux *et al.*, 1984). Difficulties which may arise in these systems include failure of the antibody-enzyme to penetrate the antigenic determinant due to the sheer bulk of the complex and difficulties in conjugating the enzyme to the antibodies. In such cases the use of bsabs with one reactive with an enzyme could alleviate such problems.

Chapter 4

Optimal growth conditions
for hybridoma cells

4. Growth patterns of hybridomas at the clonal stage

4.1 Introduction

Hybridoma growth is usually aided by the presence of exogenous growth factors or feeder layers in the culture medium. One such factor is interleukin-6 (IL-6) which is also known as hybridoma growth factor (HGF). Various hybridoma cloning products are available commercially. For example 'Sigma Chemical Company' have on offer conditioned medium (CM) from Giant Cell Tumour, EL4-IL2 and J774A.1 cells and hybridoma enhancing supplements with macrophage-like and thymoma cell origins. IL-6 is essential for differentiation of activated B-cells into antibody-producing cells, but it is not involved in the growth/proliferation of B-cells (Kishimoto, 1989).

Factors such as B-cell growth factor (BCGF) are involved in the actual growth/proliferation of activated B-cells. IL-6, is, however, a growth factor required for myeloma/plasmacytoma cells (Kishimoto, 1989). Whether or not myeloma cells used routinely in cell fusions are IL-6-dependent may be dependent on the growth medium used in individual laboratories. According to Sugasawara (1988) (i) the cloning efficiency of IL-6 dependent hybridomas increases with the addition of IL-6 to basal medium and (ii) the total number hybridomas (antibody-positive and negative) post fusion are increased in the presence of IL-6, indicating that the IL-6 is involved in differentiation and proliferation of hybridomas. Bazin and Lemieux (1989) observed that IL-6 has a much more beneficial effect on the overall number of antibody-producing hybridomas post fusion rather than on the overall number of hybridomas formed. This indicates that IL-6 is involved in differentiation rather than proliferation of B-cell hybridomas. These reporters go on to state that the actual number of hybridomas formed may depend on the FCS batch. Aarden *et al.* (1985) report that IL-6 dependency may be induced. They suggest that during fusions hybridomas become HGF-dependent but may be readily selected for IL-6 independency in post fusion cloning steps.

Various authors have reported on the uses of feeder layers and CM in hybridoma technology (Butcher *et al.*, 1983; Pintus *et al.*, 1983; Sugasawara *et al* 1985; Long *et al.*, 1988; King and Sartorelli, 1989; Perrson and Lerner, 1990). Their work and that of others on this research topic is summarised in Table 4.1.

Growth factor	Increased levels of Ig +ve hybridomas	Increased cloning efficiency	References
IL-6/HGF	+	NR	Bazin & Lemieux (1989)
IL-6	+	+	Sugasawara (1988)
IL-6 secreting myeloma cells	+	NR	Harris *et al.* (1992)
ECGS & HECS	NR	+	Pintus *et al.* (1983)
HUCS	+	+	Westerwouldt *et al.* (1983)
MCM ECGS	NR NR	+ -	Sugasawara *et al.* (1985)
L-CM (L-929 cell line)	NR	+	Walker *et al.* (1986)
HUCS & Human plasma HGF & HECS	- +	+ +	Van Mourik & Zeijlmaker (1986)
3T3/A31 feeder cells	NR	+	Butcher *et al.* (1988)
IRM-90, MRC-5 & Vero feeder cells & ECGS-CM & MRC-5-CM. Hela, splenocytes & macrophages feeder cells	+ -	NR NR	Long *et al.* (1988)
ESGF ECGS	NR	+ -	King & Sartorelli (1989)
Sp2/0 ascitic fluid w/o FCS	NR	+	Stewart & Fuller (1989)
Normal mouse serum	+	NR	Perrson & Lerner (1990)
CM-C6 (Glioma cell line)	NR	+	Gomathi *et al.* (1991)

Table 4.1. Examples of the effects on hybridoma growth of various conditioned medium, feeder cell layers and other supplements.

"+" ;- Positive effect, "-" :- Negative effect,

ECGS; Endothelial cell growth supplement, ESGF; Ewing sarcoma growth supplement,
HECS; Human endothelial culture supernatant, HUCS; Human umbilical cord serum,
MCM; Macrophage conditioned medium,

IMR-90 & MRC-5; Human fibroblast cell lines,
Vero; African green monkey kidney cells,
Hela; A clone of the epitheloid cell line,
C6; A glioma cell line.
NR; Not reported.

In the work described here two aspects of hybridoma cell growth were examined. Firstly medium conditioned with recombinant human IL-6 (rhIL-6-CM) supernatant from a known IL-6-secreting cell line, T24 (T24-CM) or feeder layers were compared for their ability to enhance the cloning efficiency of established hybridomas. Antibody production in established clones was also examined.

In a second set of experiments media supplemented with human plasma obtained from healthy volunteers and from patients with chronic lymphocytic leukemia, CLL, were examined for their ability to enhance hybridoma clonal growth. As before supernatants from the newly formed clones were examined for their ability to produce antibody.

CLL is a haematological neoplasm characterized by clonal proliferation of B-cells (Gale and Foon, 1987) which have a mature appearance but are functionally immature. There have been contradictory reports as to whether or not patients suffering from CLL produce increased levels of IL-6 (Freeman *et al.,* 1988; Biondi *et al.,* 1989; Schena *et al.,* 1991; Aguilar-Santelises *et al.,* 1992). If so, then culture medium conditioned with CLL plasma/serum, should increase the cloning efficiency of hybridomas.

Hybridomas are of B-cell origin. Therefore, how these cells respond to treatment with CLL plasma may mimic how the various cytokines present in plasma of patients with CLL effects the growth of their B-cells.

4.2. Hybridoma growth in IL-6-supplemented medium

CM and feeder layers were compared for their ability to enhance the cloning efficiency of 3 established hybridomas, G12, 14E5 and 7P41. Basal medium was conditioned with supernatant from the IL-6-secreting cell line, T24, at the following concentrations, 1.0%, 2.5%, 5% and 10% (v/v). When compared to conventional feeder layers of macrophages, thymocytes and splenocytes the numbers of clones formed by growing cells in T24-CM was not affected. The levels of IL-6 present in T24 supernatant was measured by an ELISA specific for IL-6 and MTT assay on IL-6-dependent B9 cells. Antibody production was detected in supernatants by ELISA and was found to be unaltered by any of the CM or feeder layers.

One of the hybridomas, G12, was taken and grown in a range of concentrations of rhIL-6. No increase in the numbers of clones formed was observed.

4.2.1. Results

Feeder layers or T24-CM did not increase the cloning efficiency of hybridomas compared to basal medium [Table 4.2.1.(a)], indicating that the cloning efficiency of the hybridomas was not influenced by the presence of IL-6 or feeder layers in the medium. The results given are the total number of clones/288 wells as the cloning was carried out on 3 occasions. In the case of the hybridoma 7P41 the results ranged from 56/288 using 5% T24-CM to 78/288 using macrophages as feeder layers. A similar pattern emerged for the other 2 hybridomas. For example, the cloning efficiency of the G12 hybridoma ranged from 88/288 to 103/288 in the case of 1% T24-CM and 2.5% T24-CM, respectively. For the hybridoma 14E5 the results ranged from 82/288 to 107/288 using 5% T24-CM and macrophages, respectively. Supernatants were taken from the clones and examined for antibody production by ELISA [Table 4.2.1.(b)]. No effect was observed on the numbers of mAb-producing clones in any of the medium or feeder layers examined. The concentration of IL-6 in T24 supernatant as determined by ELISA and MTT assays (both of which were specific for IL-6) was found to be approximately 15ng/ml [Figures 4.2.1.(a & b)]. Using this data it is possible to work out the concentration of IL-6 in all the T24-CM ranges.

The hybridoma G12 was taken and cloned at 1 cell/well using medium conditioned with recombinant human IL-6 (rh-IL-6) ranging from 50-700pg/ml. No effect on the numbers of clones formed was observed [Table 4.2.1.(c)].

It is interesting to note that although no increase was observed in cloning efficiency using rhIL-6-CM as compared to basal medium, the total number of clones formed in this set of cloning experiments [Table 4.2.1.(c)] far exceeds those formed in the first set of cloning results [Table 4.2.1.(a)]. For example, the numbers of clones formed using rhIL-6-CM ranged from 114/288 to 138/288 using 500pg and 100pg/ml rhIL-6-CM, respectively. When G12 was cloned in T24-CM the numbers of clones ranged from 88/288 using 1% T24-CM to 103/288 using 5% T24-CM [Table 4.2.1.(a)]. This highlights the variablity which is associated with hybridoma growth at the clonal stage

Supplement	No.of clones of 7P41/288 wells	No. of clones of G12/288 wells	No. of clones of 14E5/288 wells
Thymocytes	69	103	101
Macrophage	78	98	107
Splenocytes	66	101	ND
1% T24 (150pg/ml) *	68	88	93
2.5% T24 (375pg/ml) *	60	103	99
5% T24 (750pg/ml) *	56	93	82
10% T24 (1500pg/ml) *	63	102	90
5% FCS	66	95	86

Table 4.2.1.(a). Cloning of the hybridomas 7P41, G12 and 14E5 at 1 cell/well. The hybridomas were cloned out on 3 separate occasions. Therefore, the results given above represent the total number of clones formed in 288 wells. Supernatants were taken from the first set of cloning experiments and tested for antibody production by ELISA [Table 4.2.1.(b)].

* The concentrations of IL-6 in T24 supernatant listed above represents the concentration in whole medium.

Supplement	No. of 7P41 clones secreting mAB	No. of G12 clones secreting mAB	No. of 14E5 clones secreting mAB
Thymocytes	54/69	67/103	89/101
Macrophage	56/78	61/98	86/107
Splenocytes	50/66	65/101	ND
1% T24	50/68	63/88	77/93
2.5% T24	46/60	72/103	84/99
5% T24	43/56	68/93	74/82
10% T24	50/63	63/102	77/90
5% FCS	50/66	67/95	72/86

Table 4.2.1.(b). Supernatants from the first set of cloning experiments were taken and examined for the presence of mAB by ELISA.

Supplement	No of clones/288 wells
50pg IL-6/ml *	136
100pg IL-6/ml *	138
200pg IL-6/ml *	127
300pg IL-6/ml *	135
400pg IL-6/ml *	121
500pg IL-6/ml *	114
600pg IL-6/ml *	121
700pg IL-6/ml *	122
5% FCS	136

Table 4.2.1.(c). The hybridoma, G12, was then taken and cloned at 1 cell/well in medium conditioned with a range of concentrations of rhIL-6.

* The concentrations of IL-6 listed above represent the concentration in whole medium.

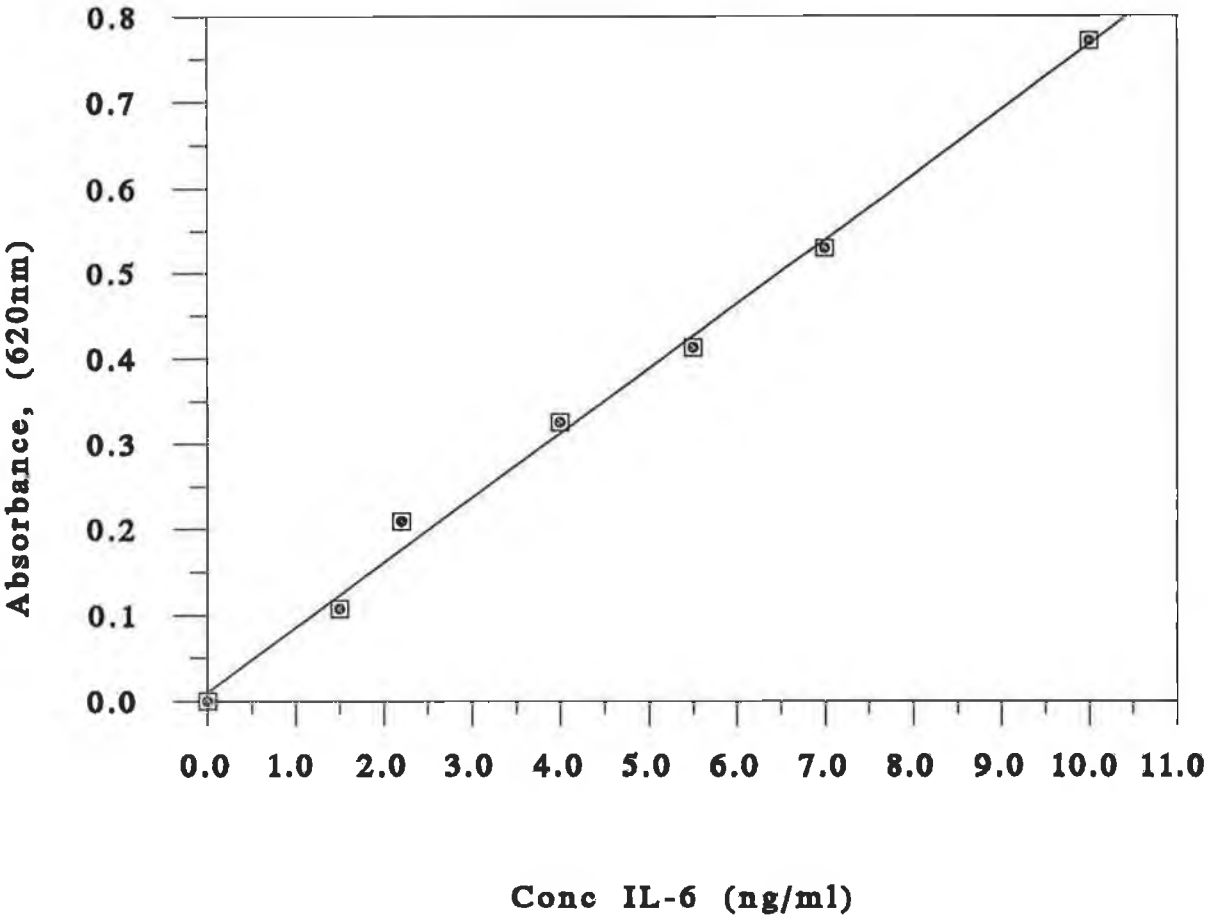


Figure 4.2.1.(a).

Standard curve for the determination of interleukin-6 (IL-6) using an ELISA specific for human IL-6 (Regression coefficient = 0.999). This was used to determine IL-6 levels in normal human plasma, plasma of patients with Chronic Lymphocytic Leukemia (CLL) and T24-conditioned medium prepared from the T24 cell line.

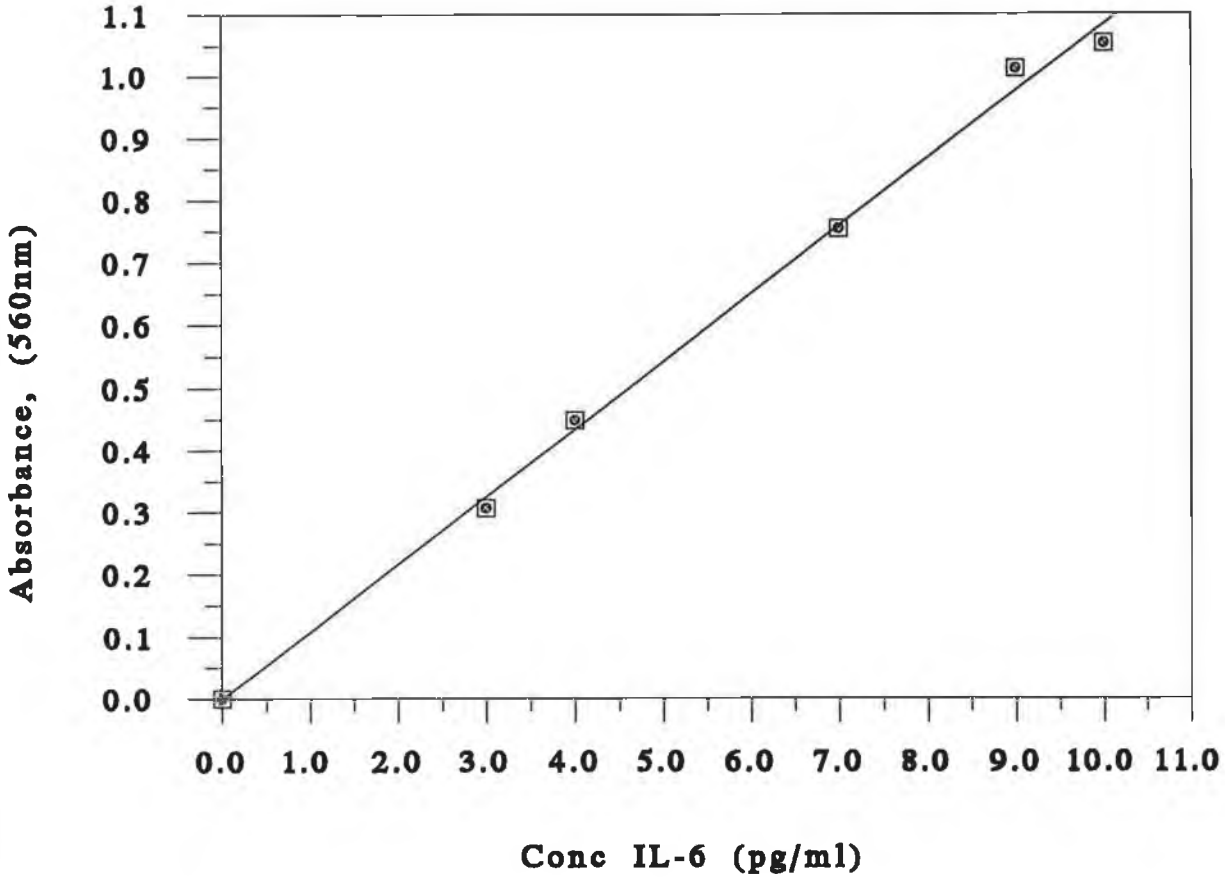


Figure 4.2.1.(b).

Standard curve for the determination of interleukin-6 (IL-6) using an MTT-based bioassay the IL-6 dependent cell line B9 (Regression coefficient = 0.999). This was used to determine IL-6 levels in normal human plasma, plasma of patients with Chronic Lymphocytic Leukemia (CLL) and T24-conditioned medium prepared from the T24 cell line.

4.3 Hybridoma growth in plasma-supplemented medium

5% T24-CM, plasma-supplemented medium and feeder layers were compared for their ability to increase the cloning efficiency of established hybridomas. Basal medium, consisting of DMEM+5% Foetal Calf Serum (FCS) was supplemented with 5% (V/V) T24 supernatant, 5% T24-CM normal human plasma (NHP-DMEM) and plasma from patients with chronic lymphocytic leukemia (CLL-DMEM). When compared to conventional feeder layers of macrophages, thymocytes, splenocytes and to feeder CLL cells and 5% T24-CM it was discovered that the numbers of clones formed by growing hybridomas in CLL-DMEM was greatly enhanced. NHP-DMEM also enhanced the cloning efficiency. CM and plasma supplemented medium were examined for the presence of IL-6, which is also known as hybridoma growth factor (HGF). Of the eight CLL plasma samples examined only two had elevated IL-6 levels. Antibody production in established clones was examined by ELISA (Sections 2.8.1. and 2.8.2.) and to determine if antibody production was affected by the cloning media used. It was found that both the total numbers of clones produced and the number of antibody-producing clones were much higher using CLL-DMEM.

4.3.1. Results

The results indicate that basal medium conditioned with CLL plasma gave better results than conventional feeder layers NHP-DMEM and 5% T24-CM in hybridoma cloning work. In all eight CLL plasma samples were used as a supplement to basal medium, 4 in the first set of cloning experiments, CLL-DMEM A-D [Table 4.3.1.(a)], and 4 in the second set of cloning experiments, CLL-DMEM E-H [Table 4.3.1.(c)]. All CLL-DMEM enhanced the cloning efficiency of the hybridomas [Tables 4.3.1.(a & c)]. Each cloning efficiency experiment was carried on three separate occasions. Therefore, the results in each case are given as the total numbers of clones in 288 wells.

In the first set of cloning experiments [Table 4.3.2.(a)], using the OKT8 hybridoma, CLL-DMEM was 4-5 fold more efficient for cloning than conventional feeder layers, 4 fold more efficient than 5% T24-CM and 2.4 fold more efficient than NHP-DMEM. For the hybridoma 14E5, CLL-DMEM was 4-5 fold more efficient than feeder layers and 5% T24-CM, and 2-3 fold more efficient than NHP-DMEM and, finally, for the

hybridoma Ep16, CLL-DMEM was 6 fold more efficient than feeder layers, 5 fold more efficient than 5% T24-CM and 1.6 fold more efficient than NHP-DMEM. Monoclonal antibody levels were measured in supernatants from all the established clones in the first set of cloning experiments. Due to the increase in the numbers of clones formed using CLL-DMEM, a corresponding increase in the number of antibody-producing clones is also observed [Table 4.3.1.(b)].

In the second set of cloning experiments a similar pattern emerged [Table 4.3.1.(c)]. CLL-DMEM was 3 fold more efficient for cloning 14E5 chybridoma cells than feeder layers and 5% T24-CM and two fold more efficient than NHP-DMEM. For the hybridoma Ep16 CLL-DMEM was 4-5 fold more efficient than feeder layers, 3-4 fold more efficient than 5% T24-CM and two fold as efficient as NHP-DMEM. Finally, for the hybridoma 7p41, CLL-DMEM was 3-4 fold more efficient than feeder layers, 3 fold more efficient than 5% T24-CM and 1.5 times more efficient than NHP-DMEM.

The use of CLL cells as a feeder layer did not increase cloning efficiency [Table 4.3.1.(a)]. The feeder cells used were obtained from a patient who was found to have increased IL-6 levels (CLL plasma A). Sanchez *et al.* (1991) have reported enhanced cloning efficiency of heterohybridomas using CLL feeder cells, concluding that this may be due to an overall increase in secretion levels of several cytokines . All of the samples were examined for IL-6 levels using, firstly, an MTT-based bioassay on IL-6-dependent B9 cells [Figure 4.2.1.(a)], and secondly, an ELISA for rhIL-6 with a working range of 0.1-20ng/ml [Figure 4.2.1.(b)]. Of the 8 CLL samples two, CLL-A and CLL-D, tested postive for IL-6 in the MTT assay and the ELISA. CLL-A had 1.3ng/ml IL-6 as determined by the MTT assay [Figure 4.2.1.(a)] and 1.5ng/ml IL-6 as determined by ELISA [Figure 4.2.1.(b)]. CLL-D had 0.6ng/ml of IL-6 as determined by MTT assay [Figure 4.2.1.(a)], and 0.8ng/ml as determined by ELISA [Figure 4.2.1.(b)]. T24 supernatant was found to secrete approximately 15ng/ml IL-6 by the MTT assay [Figure 4.2.1.(a)] and 15.6ng/ml IL-6 by ELISA [Figure 4.2.1.(b)]. Newly formed clones obtained using CLL-DMEM and basal medium are shown in Figure 4.3.1.(a).

CLONING MEDIUM	No. of clones of OKT8	No. of clones of 14E5	No. of clones of Ep16
5% FCS	42	31	18
10%FCS	37	36	15
Splenocytes	36	48	18
Macrophage	45	30	12
Thymocytes	48	42	18
5% T24-CM	50	39	20
5% CLL serum	156	153	104
CLL-DMEM A	204	237	96
CLL-DMEM B	189	171	101
CLL-DMEM C	201	189	92
CLL-DMEM D	258	216	90
CLL feeder *	45	27	12
Normal human plasma	81	69	57

Table 4.3.1.(a). Results of cloning efficiency experiments with the hybridomas OKT8, 14E5 and Ep16. Each cell line was cloned on 3 separate occasions. The numbers listed are the total numbers of clones growing in 288 wells. Supernatant from all clones formed here were examined for antibody production by ELISA [see Table 4.3.1. (c)]. CLL feeder * :- Here CLL cells were used as a feeder layer.

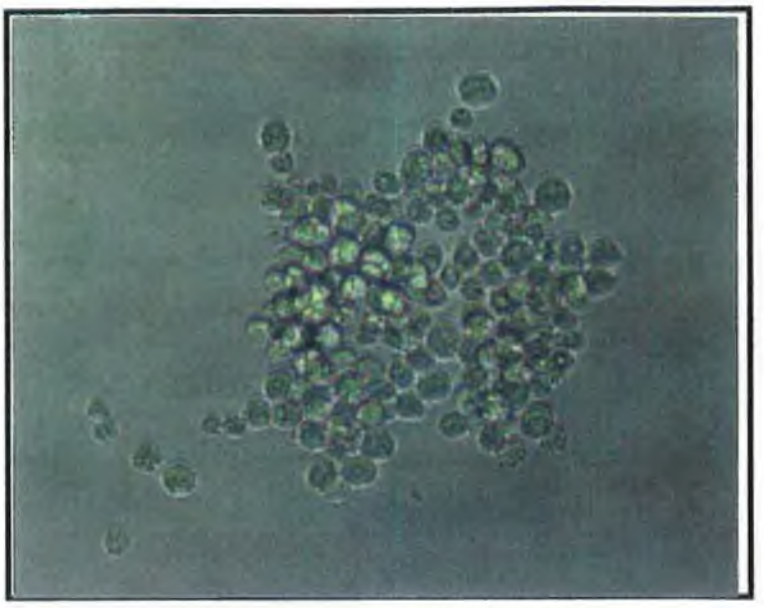
Sample	No. of OKT8 clones secreting mAB	No. of 14E5 clones secreting mAB	No. of Ep16 clones secreting mAB
5% FCS	33/42	22/31	10/13
10% FCS	30/37	28/36	12/15
Splenocytes	29/36	38/48	14/18
Macrophage	36/45	20/30	11/12
Thymocytes	40/48	27/42	14/18
T24-CM	38/50	26/39	16/20
CLL-DMEM A	153/204	154/237	78/96
CLL-DMEM B	147/189	117/171	79/101
CLL-DMEM C	161/201	138/189	69/92
CLL-DMEM D	217/258	162/216	75/90
CLL feeder cells	35/45	21/27	10/12
Normal numan plasma	61/81	50/69	48/57

Table 4.3.1.(b). The supernatants from all the clones (Table 4.3.1.a) formed were removed and assayed for antibody production by ELISA. As can be seen from the above data the higher numbers of clones formed using CLL-DMEM causes a corresponding increase in the numbers of antibody-producing clones.

CLONING MEDIUM	No. of clones of 14E5	No. of clones of Ep16	No. of clones of 7p41
5% FCS	35	18	25
10% FCS	38	21	29
Splenocytes	47	22	27
Macrophage	39	12	30
Thymocytes	42	22	25
5% T24-CM	44	25	31
CLL-DMEM E	111	89	90
CLL-DMEM F	190	120	122
CLL-DMEM G	108	61	90
CLL-DMEM H	140	106	82
Normal human plasma	68	43	62

Table 4.3.1.(c). Results of cloning efficiency experiments on the hybridoma 14E5, Ep16 and 7p41. Each cell line was cloned on 3 seperate occasions. The numbers listed are the total numbers of clones growing in 288 wells.

(a)



(b)

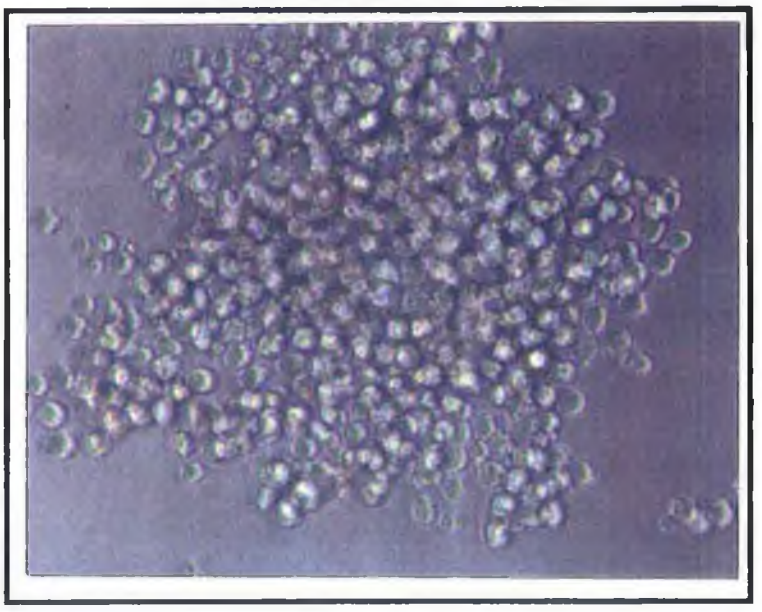


Figure 4.3.1.(a).

Newly formed clones of hybridoma cells seeded at a cell concentration of 1 cell/well in growth medium. The cells were grown in (a) DMEM.S5 and (b) DMEM.S5 supplemented with 5% CLL plasma.

4.4. Discussion

The use of CM or feeder cells, which are essential aspects of hybridoma cell culture, have been discussed fully in the Introduction (Section 1.4) and are outlined in Table 4.1. CM has some clear advantages over the use of feeder cell layers. For example, the risk of contamination is reduced because the CM has either been grown in the laboratory and contamination can be checked prior to its use or the CM may be acquired commercially in which case the product would be for cell culture use only and checked for contamination. Very often the growth factors which are secreted by a cell line used as a source of CM are known. For example the T24 cell line secretes IL-6. In addition the requirement for animal use is eliminated as are the possibilities of infections derived from the animal feeder cell systems.

Some of the advantages of using IL-6 in the growth of hybridomas include increasing the proportion of antibody-producing hybridomas post fusion (Bazin and Lemieux, 1989) and increasing the cloning efficiency of IL-6-dependent hybridomas (Sugasawara, 1988).

In the present study two aspects of hybridoma cell growth were examined. Firstly rhIL-6-CM, T24-CM, and feeder layers were compared and examined for their ability to enhance the cloning efficiency of hybridomas and to support antibody production. A range of concentrations of rhIL-6 and T24 supernatants were used. The presence of IL-6 was not a growth requirement for the hybridomas examined here. Antibody production in established clones was also found to be constant. In a second series of experiments, T24-CM, feeder layers and plasma-supplemented medium were examined and compared for their ability to enhance the cloning efficiency of hybridomas and to support antibody production.

DMEM supplemented with plasma from patients with Chronic Lymphocytic Leukemia (CLL) [CLL-DMEM] was found to increase the cloning efficiency of the hybridomas examined here (Quinlan and O'Kennedy *In press*). This is the first reported instance of such an effect and it may give clues as to how normal haematopoietic controls in patients with CLL are effected by aberrant cytokine production. The samples were examined by ELISA and an MTT assay for IL-6 and 2/8 were found to have elevated levels. This correlates with the first set of cloning experiments which indicated that the cloning efficiency of the hybridomas was IL-6-independent. The superiority of

CLL-DMEM in cloning efficiency experiments could be due to an overall increase in cytokines in these patients. There have been mixed reports as to whether on not IL-6 levels are increased in patients with CLL (Freeman *et al.,* 1988; Biondi *et al.,* 1989; Schena *et al.,* 1991; Aguilar-Santelises *et al.,* 1992). B-CLL have been shown to produce a range of cytokines. Kawamura *et al.* (1986) suggested that BCGF is a factor with a possible autocrine nature in B-CLL. Uggla *et al.* (1987) reported the spontaneous production of an IL-1-'like' growth factor by CLL cells and Schena *et al.* (1991) reported that B-CLL produce IL-1α, IL-6, TNF-α and TGF-β which are consistent with the cytokines produced by normal mantle B-cells. It is conceivable that an increase in these and other cytokines which act on normal B-cells may be responsible for the growth promoting effect of the CLL plasma on the B-cell hybridomas. BCGF is needed for the normal growth of activated B-cells and this may fit the description of the factor enhancing the hybridomas used here.

When studying the effects of IL-6 on hybridoma cell growth a crucial factor may be the fact that IL-6-dependent and non-dependent hybridomas exist. This lends itself to the possibility of similar growth factors giving diverging results in different laboratories when used in cloning experiments, as can be seen in the case of ECGS. ECGS was found in some cases to be beneficial as a hybridoma growth factor (Long *et al.,* 1988; Pintus *et al.,* 1983) and in other cases to be ineffective (Butcher *et al .,* 1983; King and Sartorelli, 1989; Sugasawara *et al.,* 1985). Freshly fused hybridomas are perhaps open to more influences in the medium than at any other stage. The new genetic material in a fused cell can be unstable and, therefore, dependency on growth factors may be induced here. Van Mourik and Zeijlemaker (1986) have demonstrated that culture medium supplemented with human umbilical cord serum and normal human serum did not promote the growth of hybridomas during cell fusions, whereas HGF and human endothelial cell supernatant did. All four supplements, however, were capable of supporting the growth of established hybridomas during cloning procedures indicating that growth conditions at this stage are less stringent.

As IL-6 is reported to increase the numbers of antibody-producing hybridomas post fusion, (Bazin and Lemieux, 1989) it is advisable to include it as a supplement in the culture medium at this stage. As a result these hybridomas may subsequently become dependent on IL-6 and it is therefore essential that IL-6 be included in the culture

medium at this crucial stage of hybridoma growth post fusion.

In summary, the hybridomas examined here grow independently of IL-6. However, factors present in CLL plasma, and to a lesser extent NHP, are able to significantly enhance the cloning efficiency of hybridomas. We have only examined IL-6 as a possible growth factor involved in this process due to its reported beneficial effects on hybridomas (Sugasawara, 1988; Bazin and Lemieux, 1989). Other cytokines, perhaps in combination with IL-6, may be responsible for the observed effects. The anticoagulant tubes into which plasma is collected may be contaminated with endotoxin which may stimulate B-cells to secrete various cytokines (Riches *et al.*, 1992). B-cells from both normal volunteers and CLL patients examined here could be stimulated to secrete cytokines in such a manner. The effect could be greatly amplified in patients with CLL due to the large numbers of circulating B-cells. A very important factor to consider when developing novel growth medium is the actual effect on the cellular function. Here the cells under investigation are hybridomas. It is therefore of vital importance that the plasma does not interfere with antibody production. Antibody production was detected by ELISA. Antibody production was not affected by the CLL plasma. However, given the increase in the total numbers of clones formed a similar increase in the number of antibody producing clones was obtained. This would be of greatest importance in the first cloning step post-fusion where each newly formed clone may secrete a unique antibody.

An important point to be taken from the results presented here is that if the optimum growth conditions can be identified for a particular hybridoma then this can greatly enhance the growth of the hybridoma cells. At large cell numbers this may not be of great importance but during times when the cells are under stress such as after recovery from liquid nitrogen or during the low cell numbers encountered during cloning procedures the microenvironment seems to influence the capacity of a cell to survive and at this stage the growth conditions are of greatest importance.

Given the commercial availability of recombinant cytokines further studies investigating the effects of the various cytokines present in CLL plasma on hybridoma cell growth both indvidually and in various combinations may indicate exactly which cytokines are responsible for the observed stimulatory effects. Highly defined novel

hybriboma growth factors could thus be identified which be of high commercial value. In the work described here the effect of CLL plasma on hybridoma cell growth was examined. It is possible that the CLL plasma may act in a similar way on cell lines derived from various other cell types.

How these results relate to the progression of the disease is of particular interest since the clinical implications of elevated cytokine levels in these patients is unclear. Hybridomas are of B-cell origin. Therefore, the growth promoting ability of the plasma on hybridomas may be mimicked *in vivo* on cells of B-cell origin. Given that the cytokines act on wide range of cell types further studies *in vitro* on different cell types may indicate how the cytokines affect the progression of the disease.

Chapter 5

Correlation of Interleukin-6 and Hypogammaglobulinemia in Chronic Lymphocytic Leukemia

5.1. Correlation of IL-6 and hypogammaglobulinemia in CLL

The importance of cytokines in CLL has recently been highlighted at the "5th International Workshop on Chronic Lymphocytic Leukemia", (Montserrat *et al.*, 1992). There have been mixed reports as to whether on not IL-6 levels are increased in patients with CLL, (Freeman *et al.*, 1988; Biondi *et al.*, 1989; Schena *et al.*, 1991; Aguilar-Santelises *et al.*, 1992). B-CLL have been shown to produce a range of cytokines including BCGF which Kawamura *et al.*, (1986) have suggested is a factor with a possible autocrine nature in B-CLL. Uggla *et al.*, (1987) reported the spontaneous production of an IL-1 growth factor by CLL cells and Schena *et al.*, (1991) reported that B-CLL cells produce IL-1α, IL-6, TNF-α and TGF-β which are consistent with the cytokines produced by normal mantle B cells.

IL-6 is a multifunctional cytokine which is produced by a wide variety of cells including both lymphoid and non-lymphoid cells. IL-6 is involved in the regulation of immune responses, final differentiation of B cells into antibody-producing cells (Kishimoto, 1989), acute phase responses (Gauldie *et al.*, 1987; Nijstein *et al.*, 1987) and haematopoeisis (Hirano, 1991). The biology of IL-6 has been reviewed comprehensively in the introduction, (Section 1.3).

One of the clinical symptoms associated with CLL is hypogammaglobulinemia (Coperative Group on CLL, 1988). This may be a factor in some of the clinical symptoms of the disease such as the infections which are a major cause of morbidity and mortality in these patients. Correlations between the concentrations of IL-6 and IgG have been identified in several diseases. These include AIDS (Breen *et al.*, 1990) and rheumatoid arthritis (Hermann *et al.*, 1989) and are discussed in more detail in Section 5.4.

There were two main aims in the work described in this chapter. Firstly, the production of IL-6 by patients with B-CLL was examined. IL-6 levels in plasma and conditioned medium (CM), obtained from growing the cells in culture medium for 48 hours, were assayed. The assays used were the MTT assay using IL-6-dependent B9 cells and an ELISA specific for human IL-6. Of eleven patients examined IL-6 was detected in the plasma and CM of five.

Secondly, the plasma were examined by ELISA for the presence of IgG. The

relationship between IL-6 and IgG levels in these patients may indicate if patients with elevated IL-6 levels have normal or raised IgG levels. This could represent a sub-group of patients better able to fight infections. Alternatively raised IL-6 may be part of a cytokine network responsible for some of the clinical aspects of the disease.

5.2. IL-6 levels in CLL plasma

IL-6 levels in plasma from patients with CLL were determined by ELISA, [Figure 5.2.(a)], and an MTT-based bioassay using IL-6 dependent B9 cells [Figure 5.2.(b)]. Of 11 patients tested 5 were found to be positive for IL-6 in both systems [Table 5.2.]. The levels detected ranged from 2.0 - 3.3ng/ml in the ELISA system and 1.86 - 3.1ng/ml in the MTT bioassay. A good correlation between both assays was obtained. IL-6 was not detected in any of 3 normal human plasma samples examined by both systems.

5.2.1. IL-6 levels in CLL-CM

Conditioned Medium (CM) was obtained by growing peripheral blood lymphocytes (PBL), obtained from patients with CLL cells (CLL-CM), and from normal healthy volunteers (PBL-CM), as described in Section 2.3.15. It was tested for IL-6 activity by ELISA and MTT bioassay, as described in Section 5.2. The levels of IL-6 measured in CLL-CM correlated with those levels detected in CLL plasma [Section 5.2]. The levels of IL-6 detected in CLL-CM ranged from 3.0 - 4.2ng/ml by ELISA and 3.1 - 3.9ng/ml by MTT assay [Table 5.2.1.]. IL-6 was not detected using either system in DMEM.S5 or PBL-CM.

5.3. IgG levels in plasma

Plasma samples from the 11 patients with B-CLL were also examined for the presence of circulating IgG using an ELISA specific for human IgG (Figure 5.3.). IgG was not detected in any of the CLL plasma samples examined. The 3 normal human plasma samples all had detectable IgG levels, (Table 5.3), ranging from 8-18mg/ml.

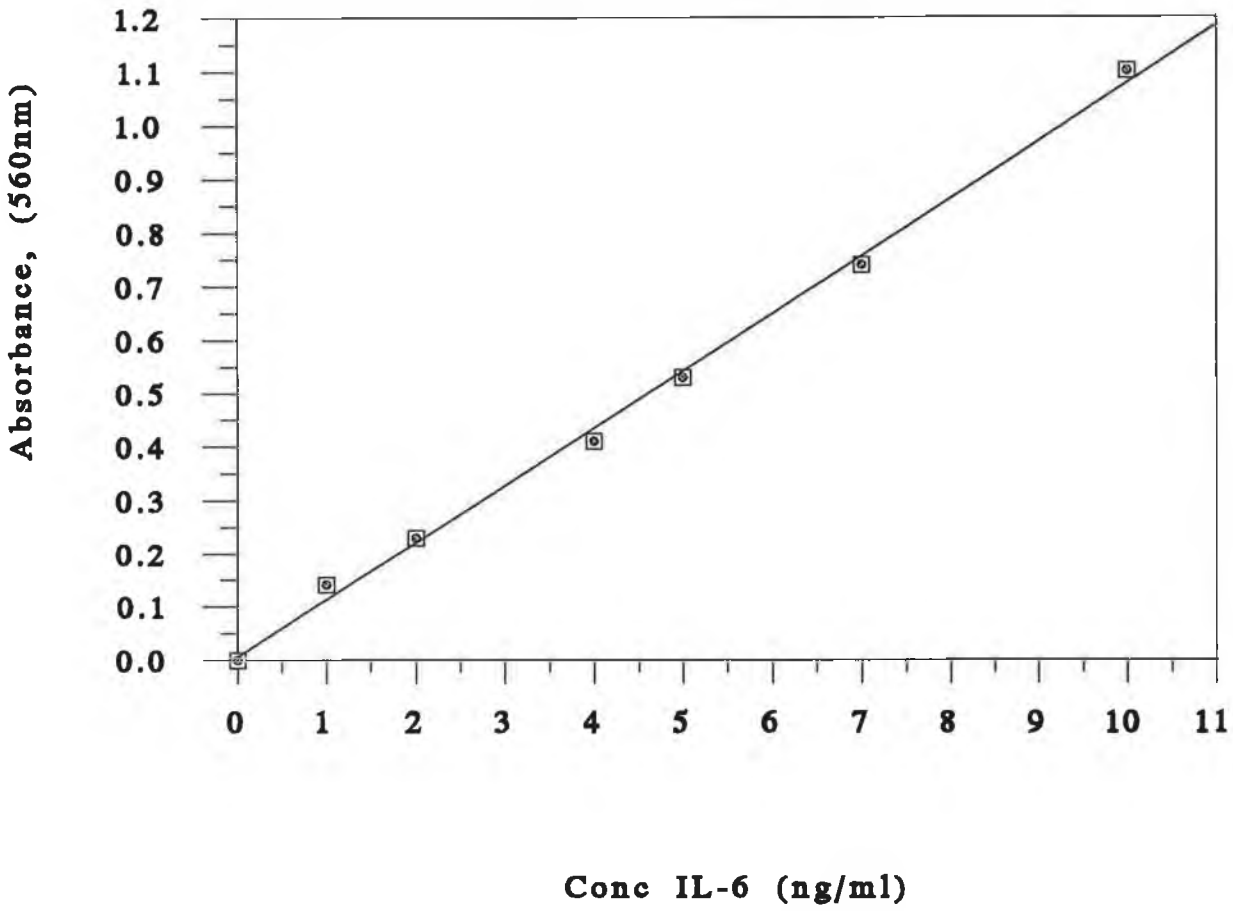


Figure 5.2.1.(a).

Standard curve for the determination of interleukin-6 (IL-6) using an ELISA specific for human IL-6 (Regression coefficient = 0.997). This was used to determine IL-6 levels in normal human plasma, plasma of patients with Chronic Lymphocytic Leukemia (CLL) and conditioned medium (CM) obtained from the growth of both normal human peripheral blood lymphocytes and peripheral blood lymphocytes from patients with CLL. Undiluted CLL plasma samples and CM were analyzed.

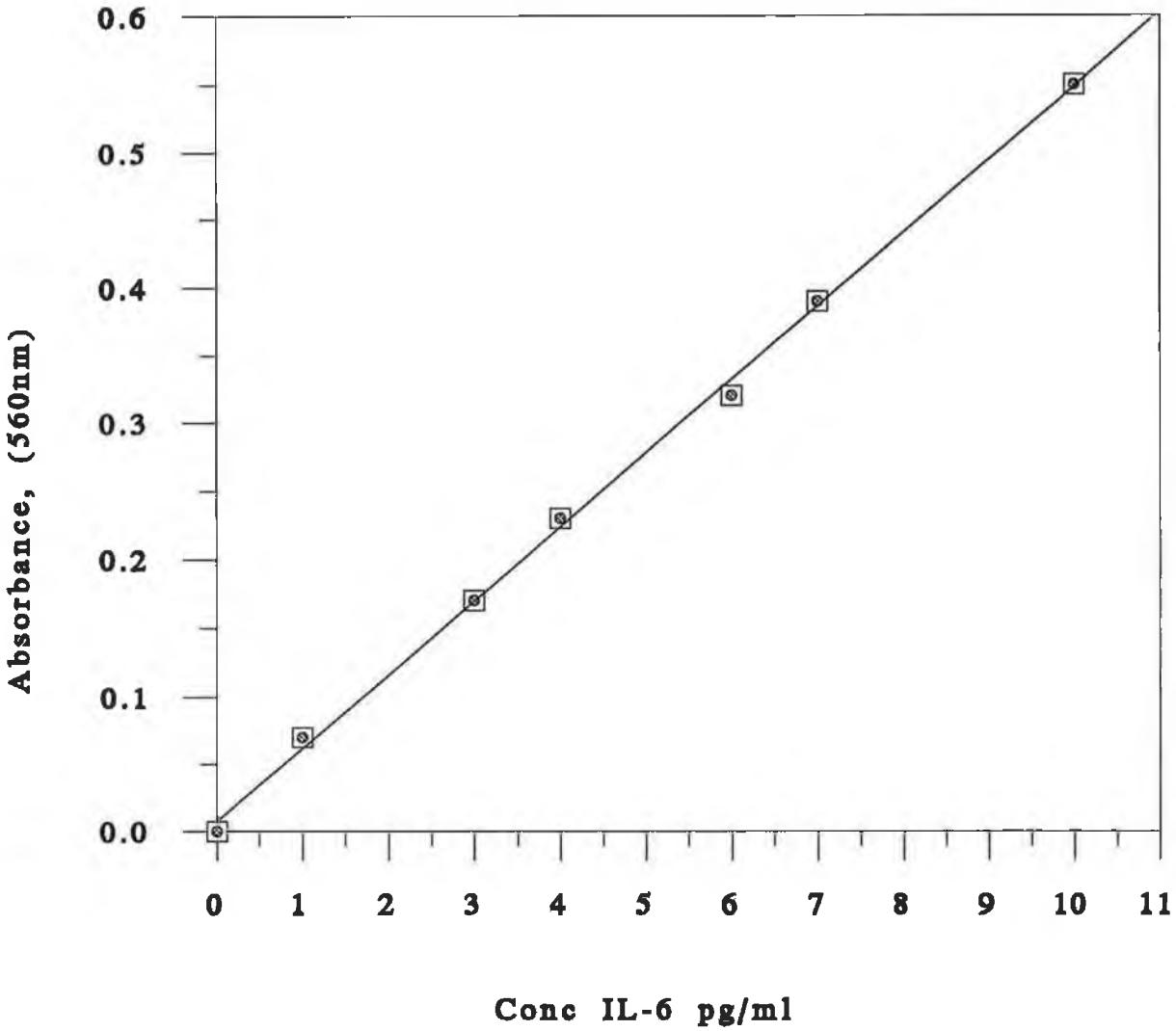


Figure 5.2.1.(b).

Standard curve for the determination of interleukin-6 (IL-6) using an MTT-based bioassay with an IL-6-dependent cell line B9 (Regression coefficient = 0.999). This was used to determine IL-6 levels in normal human plasma, plasma of patients with Chronic Lymphocytic Leukemia (CLL) and conditioned medium (CM) obtained from the growth of both normal human peripheral blood lymphocytes and peripheral blood lymphocytes from patients with CLL.

Sample	Conc as determined by ELISA (ng/ml)	Conc as determined by MTT assay (ng/ml)
CLL-1	3.1	2.7
CLL-2	3.3	3.1
CLL-3	2.9	3.0
CLL-4	0.0	0.0
CLL-5	0.0	0.0
CLL-6	2.0	1.86
CLL-7	0.0	0.0
CLL-8	0.0	0.0
CLL-9	2.3	2.1
CLL-10	0.0	0.0
CLL-11	0.0	0.0
NHP 1	0.0	0.0
NHP 2	0.0	0.0
NHP 3	0.0	0.0
DMEM.S5	0.0	0.0

Table 5.2. The concentration of IL-6 in normal human plasma and in the plasma of patients with CLL were determined by an ELISA and an MTT-based bioassay both of which were specific for IL-6. Each sample was assayed on three occasions.

Sample	Conc as determined by ELISA (ng/ml)	Conc as determined by MTT assay (ng/ml)
CLL-1	4.1	3.63
CLL-2	4.2	3.9
CLL-3	4.2	3.8
CLL-4	0.0	0.0
CLL-5	0.0	0.0
CLL-6	3.0	2.9
CLL-7	0.0	0.0
CLL-8	0.0	0.0
CLL-9	3.5	3.1
CLL-10	0.0	0.0
CLL-11	0.0	0.0
NHP 1	0.0	0.0
NHP 2	0.0	0.0
NHP 3	0.0	0.0
DMEMS5	0.0	0.0

Table 5.2.1. The concentration of IL-6 in conditioned medium obtained from the growth of peripheral blood lymphocytes from normal healthy volunteers and from patients with B-CLL in complete medium for 48 hours were determined by an ELISA an an MTT-based bioassay, both of which were specific for IL-6. Each sample was assayed on three occasions.

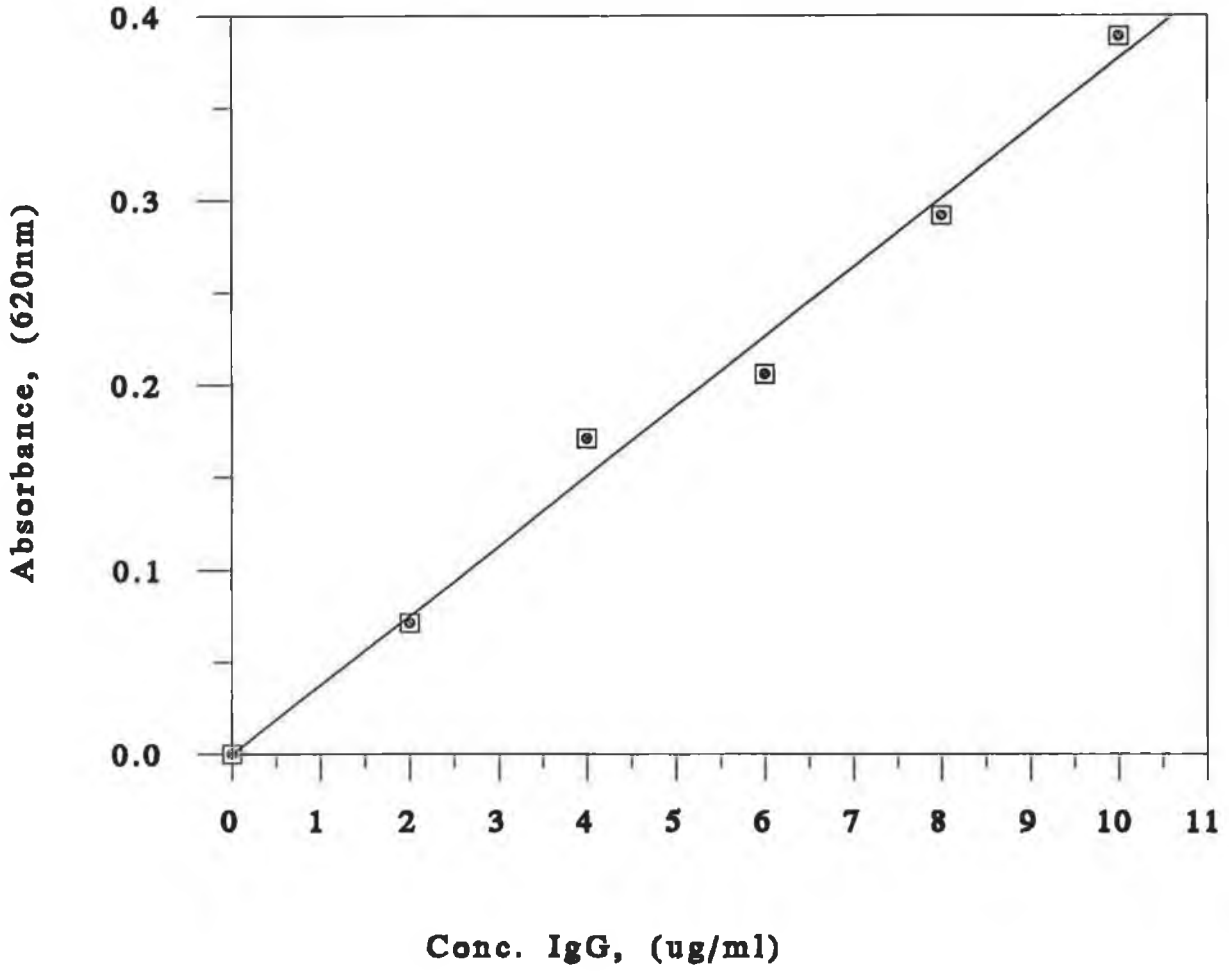


Figure 5.2.2.

Standard curve for the determination of human immunoglobulin-G (IgG) [Regression coefficient = 0.989]. IgG levels were detected in normal human plasma and in the plasma of patients with Chronic Lymphocytic Leukemia (CLL).

Sample	Conc IgG (mg/ml)
CLL 1	0.0
CLL 2	0.0
CLL 3	0.0
CLL 4	0.0
CLL 5	0.0
CLL 6	0.0
CLL 7	0.0
CLL 8	0.0
CLL 9	0.0
CLL 10	0.0
CLL 11	0.0
NHP 1	18.5
NHP 2	15
NHP 3	8
PBS (blank)	0.0

Table 5.3. The concentrations of IgG in normal human plasma (NHP) and plasma from patients with CLL were determined by an ELISA. Plasma samples were diluted 1:1000 in the case of CLL samples and 1:5000 in the case of NHP. All dilutions were made in 0.15M PBS, pH 7.2. Each sample was assayed on three occasions.

5.4. Discussion

In the work described here the concentrations of interleukin-6 (IL-6) and immunoglobulin-G (IgG) in the plasma of patients with Chronic Lymphocytic Leukemia (CLL) were examined. IL-6 is a crucial element in host-mediated responses to disease through its roles in the induction of both the acute phase response and final differentiation of B-cells into antibody-producing cells. IL-6 has been implicated in certain diseases to be responsible for the polyclonal B-cell activation responsible for such symptoms as the production of auto-antibodies and hypergammaglobulinemia. This may be the case in AIDS (Breen *et al.,* 1990) and cardiac myxoma (Hirano *et al.,* 1987). IL-6 has also been detected in the synovial fluid of patients with rheumatoid arthritis [RA] which is also an auto-immune disorder (Bhardwaj *et al.,* 1989; Hirano 1991). It has been demonstrated that the concentration of IL-6 and IgG in the synovial fluid of these patients is correlated. The association of IL-6 with various diseased states including CLL has been discussed fully in Section 1.3.

One of the clinical symptoms associated with CLL is hypogammaglobulinemia and one of the chosen methods of treatment is with the administration of intravenous (i.v.) IgG (Helenius *et al.,* 1992). However, this, is a very costly form of therapy and, therefore, its use has been limited to a select number of patients. To review their policy on prophylatic treatment with i.v. IgG in patients with CLL Helenius *et al.,* (1992) analyzed infection history, serum-IgG (s-IgG) levels and the disease stage of 146 patients. S-IgG levels were available for 98 patients. 55% were hypogammaglobulinemic and 56% had presented with at least one serious infection. Severe infections tended to accumulate in patients with subnormal s-IgG and advanced disease stages, and these patients would benifit most from i.v. IgG prophylaxis. The Cooperative Group on CLL, (1988), performed a double-blind trial in which 84 patients, who were judged to be at increased risk of infections, were chosen to receive i.v. IgG or placebo. Eligible patients had hypogammaglobulinemia, a history of infection or both. To determine which patients benefited most from i.v. IgG treatment several factors were determined prior to treatment. These were infection history, age, sex, disease stage and duration, previous therapy (including splenectomy), haemoglobulin level, neutrophil and platelet counts and s-IgG level. Patients receiving i.v. IgG had significantly fewer bacterial infections and a longer time period before

succumbing to their first serious bacterial infection. There was no significant difference between both groups in the incidence of non-bacterial infections, or, in the number of patients remaining infection-free. The group conclude that selected CLL patients with particular risk from infection may benefit from i.v. treatment. Viral and fungal infections which are thought to be less dependent on antibody responses were unaffected in both groups. The fact that bacterial infection was not completely eliminated using i.v. IgG highlights the fact that other factors also contribute to the risk of bacterial infection in these patients. These factors include T-cell dysfunction, NK dysfunction, defects in complement, neutropenia (often caused by cytotoxic therapy), monocytopenia and immunosuppressive therapy. Initial s-IgG levels did not correlate with the incidence of infection. The American Society of Hospital Pharmacists (1992), suggest that specific IgG could be more important than whole s-IgG levels. These workers go on to point out that the identification of specific subgroups of CLL which could benifit from from i.v. treatment is also desirable.

One such subgroup could be those patients with elevated IL-6 levels. Given the reported increased IL-6 levels in some patients with CLL it was investigated whether the levels of IL-6 and IgG in these patients correlated. The results presented in this chapter indicate that the concentration of IL-6 and IgG present in the plasma of patients with CLL are not correlated. IL-6 was detected in the plasma and CM in 5 of 11 CLL patients examined by ELISA and an MTT based bioassay both of which were specific for IL-6. None of the plasma samples examined had detectable IgG levels as determined by ELISA. Given that IL-6 was detected in the CM it is probable that the neoplastic lymphocyte cells are responsible for some if not all of the observed IL-6 over-production.

The reasons for the elevated levels of IL-6 in CLL are still unclear. Under normal conditions IL-6 controls the immune response, the acute phase response and haematopoiesis. Patients with CLL have a high incidence of infections. During infection the acute phase response is induced (Helgfoot *et al.,* 1989). This could be one explanation for the elevated IL-6 levels. It is unlikely that the elevated IL-6 are involved in the antibody responses because no correlation between IL-6 and IgG was detected. The IL-6 may be involved in the autocrine growth of the tumour cells or in

the production of auto-antibodies.

The role of IL-6 in various diseased states have been reported. These include:

(1) the autocrine stimulation of tumour cells (Kawano *et al.,* 1988; Yee *et al.,* 1989 and Seigall *et al.,* 1990), AIDS Kaposi sarcoma-derived cells (Miles *et al.,* 1990) and cells associated with mesangial proliferative glomerulonephritis (Blay *et al.,* 1992),

(2) the induction of the acute phase response associated with glioblastoma (Van Meir *et al.,* 1990), ovarian carcinoma (Erroi *et al.,* 1989 and Watson *et al.,* 1990), renal cell carcinoma (Blay *et al.,* 1992) and acute bacterial infections (Helgfoot *et al.,* 1989),

(3) the production of auto-antibodies in patients with cardiac myxoma (Hirano *et al.,* 1987),

(4) the polyclonal activation of B-cells associated with AIDS (Breen *et al.,* 1990) and

(5) the induction of B-symptoms associated with lymphoma (Kurzrock *et al.,* 1993).

In the course of this work IL-6 measurements were taken from single blood samples. It is conceivable that the levels of IL-6 and other cytokines change as the clinical course of the disease changes. Therefore, further studies could be conducted to investigate the cytokine profile of patients over a significant time period.

In the previous chapter it was reported that plasma from patients with CLL could stimulate the growth of hybridoma cells at the clonal stage. It was also shown that this was due to the cocktail of cytokines present in the plasma rather than the activities of IL-6 alone. Since hybribomas are of B-cell origin it is conceivable that the plasma causes a similar effect *in vivo*. It is possible that IL-6 is functioning as part of a cytokine network which could be responsible for some of the clinical symptoms experienced by these patients.

As a research area the field of cytokine study is still relatively new (Durum 1991). A majority of the studies performed thus far have been performed using individual cytokines. Now that a lot of the groundwork is established the role of cytokines in normal systems is under intense investigation. The action of the cytokines *in vivo* has

been described as contextual (Sporn and Roberts, 1988). In a living organism the cytokines are influenced by such factors as other cytokines, hormones and prostaglandins. During haematopoiesis cells are influenced by networks of these factors rather than by the action of the factors individually. Cytokines must, therefore, be studied as mixtures rather than individually. Understanding how these networks operate under normal conditions is central to assessing how they may operate in disease states. During a disease it is certain that production of various cytokines occurs. The consequence of this for the host depends on whether this production is normal or abnormal under the given conditions. For example, in the case of multiple myeloma (Kawano *et al.*, 1988) an autocrine loop may operate whereby tumour cell growth requires IL-6 which in turn has been produced by the cells. Another example is the involvement of IL-6 in RA. IL-6 has been detected in the synovial fluid of patients with RA (Bhardwaj *et al.*, 1989; Hirano, 1991c). Associated with this is an increase in the concentration of IgG in the synovial fluid which may be due to polyclonal B-cell activation (Hermann *et al.*, 1989). IL-6 has been linked to various pathogenic aspects of the disease. These include the high frequency of EBV-positive B-cells associated with RA. IL-6 has been shown to be a growth factor for EBV-transformed cell lines (Tosato *et al.*, 1988). In patients with Felty's syndrome (RA associated with enlagement of the spleen) increases in serum levels of IL-6 are associated with aggravation of the arthritic condition. The involvement of IL-6 in both multiple myeloma and RA would seem to be indicative of worsening prognosis.

In times of disease IL-6 plays a vital role the regulation of defence mechanisms through its involvement in the acute phase response, the induction of the antibody response and haematopoiesis (Figure 5.4.). Some of the cytokine networks involving IL-6 have been well documented. It has been demonstrated that IL-3 and IL-6 act synergistically to induce the proliferation of murine pluri-potential haematopoietic progenitors (Ikebuchi *et al.*, 1987). IL-1 has been shown to induce IL-6 production in stromal cells (Chiu *et al.*, 1988), the resultant IL-6 can then exert its effect on haemotopoietic stem cells.

IL-6 acts on the hypothalamo-pituitary axis causing an increase in cortisol. Cortisol production has the dual functions of enhancing induction of the acute phase proteins in liver cells by IL-6 and of inhibiting IL-6 gene expression. Both IL-1 and TNF

stimulate IL-6 synthesis and are, in turn, inhibited by IL-6. These complex networks control host response mechanisms. Progression of the various diseased states described above may involve the aberrant functioning of these networks.

Under normal circumstances differentiation and proliferation of activated B-cells into antibody-secreting cells is involves IL-4, IL-5 and IL-6 (Figure 1.3.2.). IL-4 and IL-5 control early activation and proliferation of B-cells. Final differentiation of activated B-cells into antibody-secreting cells is controlled by IL-6.

IL-6 has been shown to induce Ig production in EBV transformed B-cell lines (Teranishi *et al.*, 1982; Yoshizaki *et al.*, 1982) and IgM, IgA and IgG production in B-cells which have been activated with either *Staphylococcus aureus* Cowan I or pokeweed mitogen but not in non-activated B-cells (Muraguchi *et al.*, 1988). Antibody production in the latter case can be inhibited by anti-IL-6 antibodies indicating the essential role of IL-6. IL-6 has also been shown to induce the production of antibodies to polysaccharides in humans (Ambrosino *et al.*, 1990) and to the influenza A virus in murine B-cells (Hilbert *et al.*, 1989). A breakdown in the cytokine network controlling antibody production may be responsible for the hypogammaglobulinemia observed in patients with CLL.

CLL involves the clonal proliferation of a mature appearing but functionally immature B-cell (Gale and Foon 1987). The initial cell giving rise to CLL may not always have the same profile with regards to the cytokines being secreted. As the neoplasm develops (increase in cell numbers) the concentrations of cytokines would increase. The cytokine profile may also alter in response to the various cytokines which are now circulating. This scenario would mean that the cytokine profile would be due entirely to the cytokine profile of the B-cell giving rise to the neoplasm. Under normal conditions the levels of cytokines produced by this B-cell would be minuscule but on transformation to the neoplastic cell and subsequent proliferation the cytokine profile could alter dramatically. Neoplastic cell growth in individual cases of CLL may be dependent on the specific cytokines secreted at initial stages of the neoplasm. Treatment of these patients with functional agents which block cytokine production such as anti-cytokine antibodies could be of therapeutic value. This would explain why only some patients have elevated cytokine levels. It would also indicate that some, but not all, patients could have autocrine tumours.

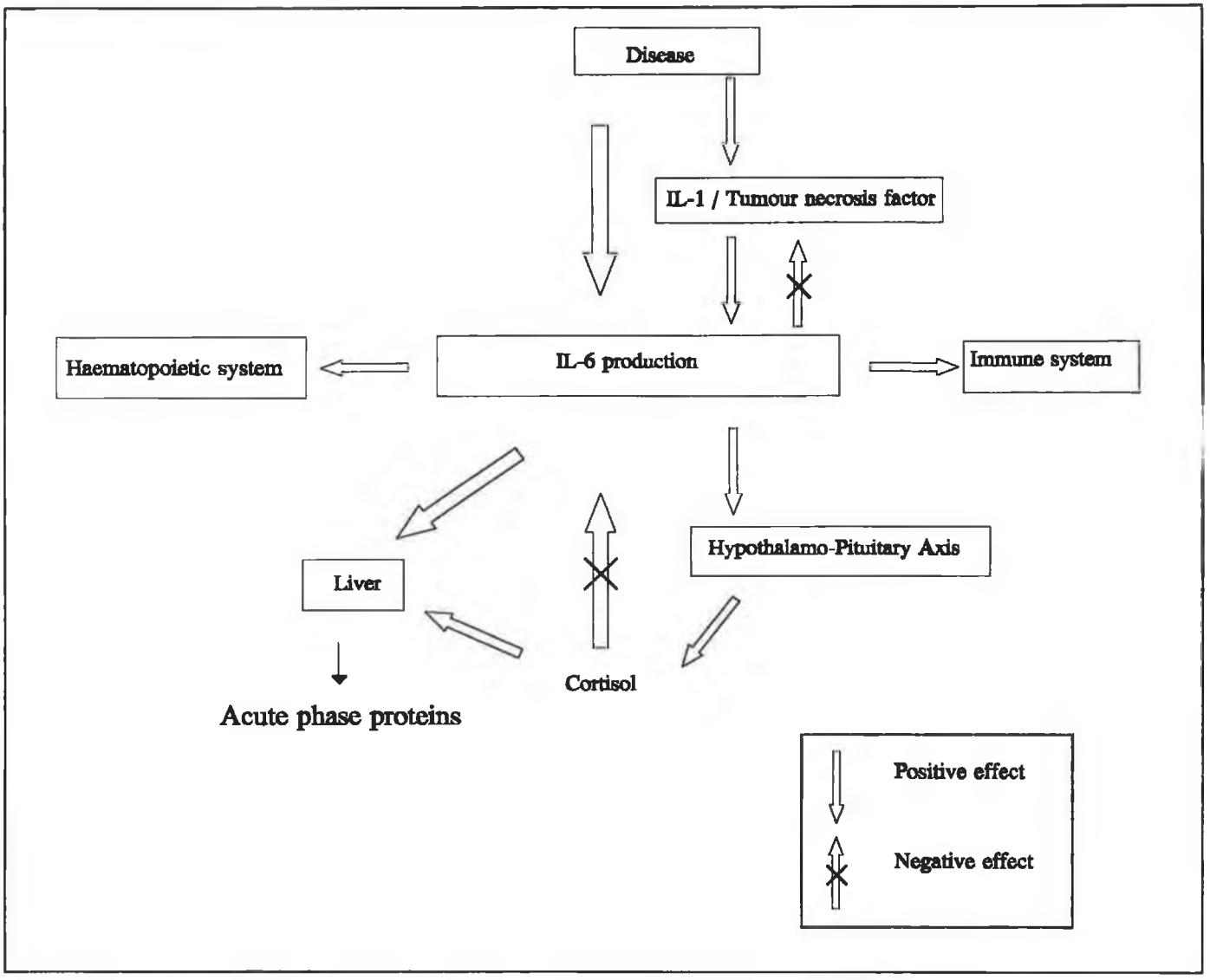


Figure 5.4.

The role of interleukin-6 (IL-6) as part of the host response mechanism to disease is represented diagrammatically above. The network begins with the induction of IL-6 production by disease, IL-1 or tumour necrosis factor. IL-6 then induces cortisol production by the hypothalamo-pituitary axis. Cortisol has the dual role of firstly, enhancing acute phase protein production by liver cells in conjunction with IL-6 and secondly of inhibiting the expression of the gene encoding IL-6.

Chapter 6

Conclusions

6. Conclusions

Bsabs were made using chemical methods. These recognised determinants on CLL cells and the enzyme HRP simultaneously. ELISA and immunocytochemical techniques were developed using these bsabs. The binding pattern of the bsab with various cell lines and CLL cells was similar to that of the parental anti-tumour antibody (G12) when examined by ELISA. When applied to immunocytochemical techniques the binding patterns of the bsab were altered. No reactivity was detected with the cell lines examined. However, reactivity of the bsab with the CLL cells remained similar to that observed during the ELISAs. Immunocytochemical staining using the parental G12 antibody was also performed using a commercial PAP amplification kit and a HRP-labelled goat anti-mouse antibody. Neither of these systems were successful in detecting antigenic determinants on the cell lines or the CLL cells. This would indicate that the bsab is more appropriate for use in such techniques. The observed alterations in the binding capabilities of antibodies between systems are not uncommon. The antibodies used in this work (HRP antiserum and the antibody secreted by the hybridoma G12) were initially assayed by ELISAs. Often the antibodies will not be suited to other forms of detection such as is the case here. The bsab maintained its ability to recognise the antigenic determinants present on the CLL cells indicating that the modifications to the G12 and anti-HRP antibodies may reduce cross-reactivity in the immunocytochemical system.

Somatic cell fusion techniques were used to fuse the G12 hybridoma cell line with rabbit and mouse splenocytes which secreted antibodies reactive with HRP. Two methods were used to induce HAT-sensitivity in the G12 hybridoma. Firstly the cells were grown in increasing concentrations of 8-aza and secondly the cells were mutagenised using EMS and new phenotypes were subsequently selected using 8-aza. These cells were then fused to either mouse or rabbit splenocytes both of which secreted antibodies reactive with HRP. Antibody production by newly formed triomas was examined by ELISA. Individual triomas secreted antibodies with parental binding patterns (anti-K562 or anti-HRP) only. No bsab was detected. Given the workload, cost and uncertainty involved in the production of antibodies using somatic cell fusion techniques it is important that biological methods of production be persued only if essential for the future of the work.

The mouse-rabbit chimeric trioma may be of benefit in situations where rabbits give better antibody responses than mice to a particular antigen. For example, in the case of group A *Streptococcus* antibody responses are more superior in rabbits compared to mice (Raybould and Takahashi 1988). These authors have successfully produced mAb secreting chimeric cells by fusing hyperimmunized rabbit splenocytes and mouse myeloma cells. We have successfully fused mouse hybridoma cells and rabbit splenocytes. This type of system may be used in the future to produce bsabs which have proven difficult to make using the conventional mouse-mouse system. It was also possible to generate an immune response in the rabbit splenocytes using an *in vitro* immunization system. This could be of benefit in the case of weakly immunogenic antigens.

To date the main interest in bsabs has been as therapeutic agents either to redirect effector cells or toxins to tumour cells. Less work has been performed on their use in immunoassays. However, the therapeutic use of bsabs encounters such obstacles as host anti-foreign antibody responses. No such problems exist for bsabs used in immunoassays.

Studies on the production of the cytokine IL-6 by patients with CLL were also performed. These included measuring IL-6 levels in plasma and CM (prepared from the PBL) of patients with CLL by ELISA and by an MTT-based bioassay using IL-6 dependent cells. Five of eleven patients had detectable levels of IL-6 as determined using both systems. The significance of IL-6 in CLL is still unclear. One of the clinical manifestations of the disease is hypogammaglobulinemia and this in turn may lead to the development of other clinical symptoms such as the increased numbers of infections experienced by these patients. Given that IL-6 is involved in the final differentiation of B-cells into antibody-secreting cells the possibility that patients with raised IL-6 levels had normal IgG levels (which could indicate better prognosis due to less infection) was examined. IgG presence in the plasma was determined by ELISA. All of the samples examined were hypogammaglobulinemic and, therefore, no correlation between elevated IL-6 levels and normal antibody production was recorded. Lack of patient data rendered it impossible to correlate the relevance, if any, between disease status and IL-6 levels. Discovering the significance of IL-6 in CLL

may require that all the cytokines with reported elevated levels in CLL be examined together and not in isolation. This is highlighted in the final line of research of this thesis where the cytokines present in CLL operate in a network to stimulate hybridoma cell growth. This is outlined in more detail below.

The final area of research studied was the conditions required for optimal hybridoma cell growth at the clonal stage. IL-6, which is also known as HGF, was examined for its ability to enhance the cloning efficiency of the hybridomas examined. The T24 cell line was used as a source of IL-6 (T24-CM). T24-CM, conventional feeder layers and basal medium all had similar cloning efficencies. CM was also prepared using a range of concentrations rhIL-6 and no effect was observed on the cloning efficiency.

It was found that the cloning efficiency of hybridomas was enhanced using culture medium supplemented with CLL plasma. The levels of IL-6 in the plasma were measured by ELISA and IL-6 dependent MTT-bioassay. Of eight plasma samples examined only two had detectable levels of IL-6 indicating that IL-6 is not responsible for the observed enhanced cloning efficiency. This is in agreement with the initial results using T24-CM and rhIL-6. A likely scenario is that an increase in several cytokines causes enhanced clonal growth. Elevated levels of BCGF, IL-1, TNF-α and TGF-β have been reported in patients with CLL. Further studies on the exact nature of the cytokines which cause the increase in cloning efficiency should be possible given the availability of recombinant cytokines. These could then be used for the development of novel growth medium supplements. The implications of these results on patients with CLL are interesting. Hybridomas are of B-cell origin and given the enhanced growth of the hybridomas examined here it is quite feasable that alterations in the concentration of these cytokines *in vivo* have similar effects on B-cell growth. Treatment of hybridomas with IL-6 alone did not affect affect their growth. Therefore, it is likely that the cytokines are acting in a network. This network could affect normal (non)-haematopoietic development. The cytokines control the development of a wide range of cell types and further studies examining the effects of CLL plasma on cell lines of various origins could help to elucidate how these cytokines are influencing course of the disease.

List of Publications

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O'Kennedy, R., Bator, J., Reading, C.L., Nolan, O., **Quinlan, N.,** Cahill, D. and Thomas, M.W. Recent novel applications of antibodies in analysis: Production and applications of iodinated conjugates for use in antibody screening, isolation and detection procedures. The Analyst, (*In press*).

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