STUDIES ON THE PRODUCTION AND APPLICATIONS OF BISPECIFIC ANTIBODIES

A dissertation submitted for the degree of

Doctor of Philosophy

by

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"Nothing would be done at all,
If a man waited until he could do it so well,
That no-one could find fault with it."

John Henry Cardinal Newman

DECLARATION

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

Signed: _____ Date: 8th Aug '96

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ABSTRACT

Studies on the Production and Applications of Bispecific Antibodies

The purpose of this project was to examine and optimise the production and application of bispecific antibodies. A bispecific antibody is an immunoglobulin-derived molecule which binds monovalently to two distinct antigens. It can be produced using somatic cell fusion techniques or by chemical manipulation of the parental immunoglobulin molecules.

Bispecific antibodies were produced from two different species of immunoglobulin, human immunoglobulin and mouse immunoglobulin or rabbit immunoglobulin and mouse immunoglobulin. These were characterised using high performance liquid chromatography, poly-acrylamide gel electrophoresis and enzyme-linked immunosorbent assays based on both parental species differences and double antigen specificity. The bispecific antibody was produced at 70% of total potential yield. Antigen specificity was retained but binding affinity was reduced by 30-50% compared to parental molecules.

A rabbit, polyclonal anti-alkaline phosphatase antibody was produced. This antibody was used, in conjunction with a murine, monoclonal anti-chronic lymphocytic leukaemia antibody (G12), to produce a bispecific antibody. The bispecific antibody was characterised, as before, following purification by double affinity chromatography. The bispecific antibody was used to develop a suspension and a plate enzyme-linked immunosorbent assay for the detection of the human leukaemic cell line, K562. These assays were used to detect chronic lymphocytic leukaemic cells in patient blood samples. A positive diagnosis was made in 80-90% of known chronic lymphocytic leukaemic patients. The same anti-chronic lymphocytic leukaemia X anti-alkaline phosphatase bispecific antibody was used in immunocytochemical studies of the same patients blood samples, with a positive response of 75-85%.

A second bispecific antibody was produced from the rabbit, polyclonal anti-alkaline phosphatase antibody and a rabbit, polyclonal anti-7-hydroxy coumarin antibody. This was characterised, as before, following purification by double affinity chromatography. The anti-7-hydroxy coumarin X anti-alkaline phosphatase bispecific antibody was used, with limited success, to develop an enzyme-linked immunosorbent assay for the quantitation of 7-hydroxy coumarin.

ABBREVIATIONS

α	Anti-
Å	Angstrom
Ab	Antibody
Abs	Absorbance
ADCC	Antibody-Dependent Cell-mediated Cytotoxicity
AFP	α-Fetoprotein
Ag	Antigen
ALL	Acute Lymphocytic Leukaemia
AP	Alkaline Phosphatase
AU	Absorbance Units
BAb	Bispecific Antibody
BCA	Bicinchoninic Acid
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
BF(ab')2	Bispecific F(ab')2
BSA	Bovine Serum Albumin
$C_{H/L}$	Constant region of an immunoglobulin heavy/light chain
CD	Cluster of Differentiation
CDR	Complimentarity Determining Region
CEA	Carcino-Embryonic Antigen
CLL	Chronic Lymphocytic Leukaemia
CML	Chronic Myelogenous Leukaemia
Da	Dalton (unit of atomic mass)
DMSO	Di-methyl Sulphoxide
DNA	Deoxyribonucleic Acid
dTNB	5,5' Dithio-bis-(2-nitro benzoic acid), Ellman's Reagent
EDTA	Ethylene Diamine Tetra Acetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
F(ab')2	Antigen Binding Fragment of an Ig
Fab'	Fragment obtained by reduction of F(ab')2
FACS	Fluorescence-Activated Cell Sorting
Fc'	Heavy chain segment from F(ab')2
FcγR	Leucocyte cell surface receptor
FITC	Fluorescein Isothiocyanate
HAMA	Human Anti-Mouse Antibody
HAT	Hypoxanthine, Aminopterin, Thymidine
hCG	Human Chorionic Gonadotrophin
HGPRT	Hypoxanthine Guanine Phosphoribosyl Transferase
HPLC	High Performance Liquid Chromatography

hr	Hours
HRP	Horse Radish Peroxidase
i.d.	Intra-Dermal
IFN	Interferon
Ig	Immunoglobulin
Il-2	Interleukin-2
Il-2R	Interleukin-2 Receptor
i.p.	Intra-Peritoneal
I.U.	International Units
kDa	Kilo Daltons (units of atomic mass)
LGL	Large Granular Lymphocyte
μ	Micro-
M	Molar
mM	Millimolar
MAb	Monoclonal Antibody
mg	Milligrams
MHC	Major Histocompatability Complex
min	Minutes
ml	Milliliters
MTX	Methotrexate
NK	Natural Killer cell
7OHC	7-Hydroxy Coumarin
o-PD	o-Phenylene Diamine
o-PDM	o-Phenylene DiMaleimide
OVA	Ovalbumin
PAGE	Poly Acrylamide Gel Electrophoresis
PBL	Peripheral Blood Lymphocytes
PBS	Phosphate Buffered Saline (0.1M, pH 7.4)
PA	Plasminogen Activator
RAC	Ricin A-Chain
RNA	Ribonucleic acid
Rt	Retention Time
SDS	Sodium Dodecyl Sulphate
SmIg	Surface Membrane Immunoglobulin
TcR	T-Cell Receptor
Tris	Tris[hydroxymethyl]aminomethane
TRITC	Tetramethyl Rhodamine Isothiocyanate
$V_{H/L}$	Variable region of an immunoglobulin heavy/light chain

CHAPTER 1

INTRODUCTION

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1.1 ANTIBODIES

1.1.1 The Immune Response:

When an animal encounters an antigen (Ag) either by infection or by deliberate injection, antibodies (Ab) are produced which appear in the serum. They are produced by the B-lymphocytes, primarily in the spleen. Structurally, Ab are proteins of the globulin class and, hence, are often referred to as immunoglobulins (Ig). In man, five classes of Ig have been identified on the basis of structural differences, not related to their specificity to Ag. These are; IgA, IgD, IgE, IgG and IgM (Table 1.1.1). After exposure to Ag, a period of 7-10 days elapses before specific Abs appear in the serum, reaching a peak at 14-21 days, after which levels fall. This response to the Ag is called the primary response, the Abs produced are generally of the IgM class. Following a second exposure to Ag, the levels of Ab in the serum begin to rise in 2-3 days and subsequently reach levels in excess of those achieved in the primary response. This accelerated response, termed the secondary response, is the result of stimulation of the Ab-forming cells of the immune system which had been sensitised by prior exposure to Ag. The Ab produced in the secondary response is generally IgG. IgG is quantitatively the most important serum Ig (Table 1.1.1) and its major function is to neutralize toxins, viruses and to bind to and opsonize bacteria (Steward, 1984; Abbas *et al.*, 1991).

The basic structure of the Ab consists of two light chains and two heavy chains and has a relative molecular mass of 150-160kDa (Fig. 1.1.1). Ab binds to Ag at a binding site located at the $-NH_2$ region of the light and heavy chains. Both heavy and light chains can be divided into variable and constant regions. Within the variable region there are certain areas which are highly conserved and provide a backbone for the structure. There are also hyper-variable areas called complementarity determining regions (CDR) which are involved in defining the binding specificity of the Ab. Ab fragments, e.g. $F(ab')_2$ and Fab', may be produced by enzymatic cleavage and by genetic engineering (Fig. 1.1.1). These can have advantages over the whole molecule in certain applications, particularly where reduced immunogenicity is important. The constant regions (Fc) of the Ab are involved in binding to cell surface receptors. They contain carbohydrates and have important physiological roles (Steward, 1984; Abbas *et al.*, 1991).

Antibody Type	Antibody Subtypes	% Total Serum Antibody	Molecular Weight (kDa)
IgA	IgA1	16	150, 300 or 400
	IgA2	3	150, 300 or 400
IgD	None	Trace	180
IgE	None	Trace	190
IgG	IgG1	47	150
	IgG2	16	150
	IgG3	5	150
	IgG4	3	150
IgM	None	8	950

Table 1.1.1:

Human antibody isotypes: the five classes of immunoglobulin and their relevant sub-classes, based on structural differences. The relative amount of each sub-class found in human serum is quoted as a percentage of total serum immunoglobulin. The molecular weight of the secretory form is also noted. (Multimeric forms of IgA exist which are associated via the tail piece region of the heavy chain.)

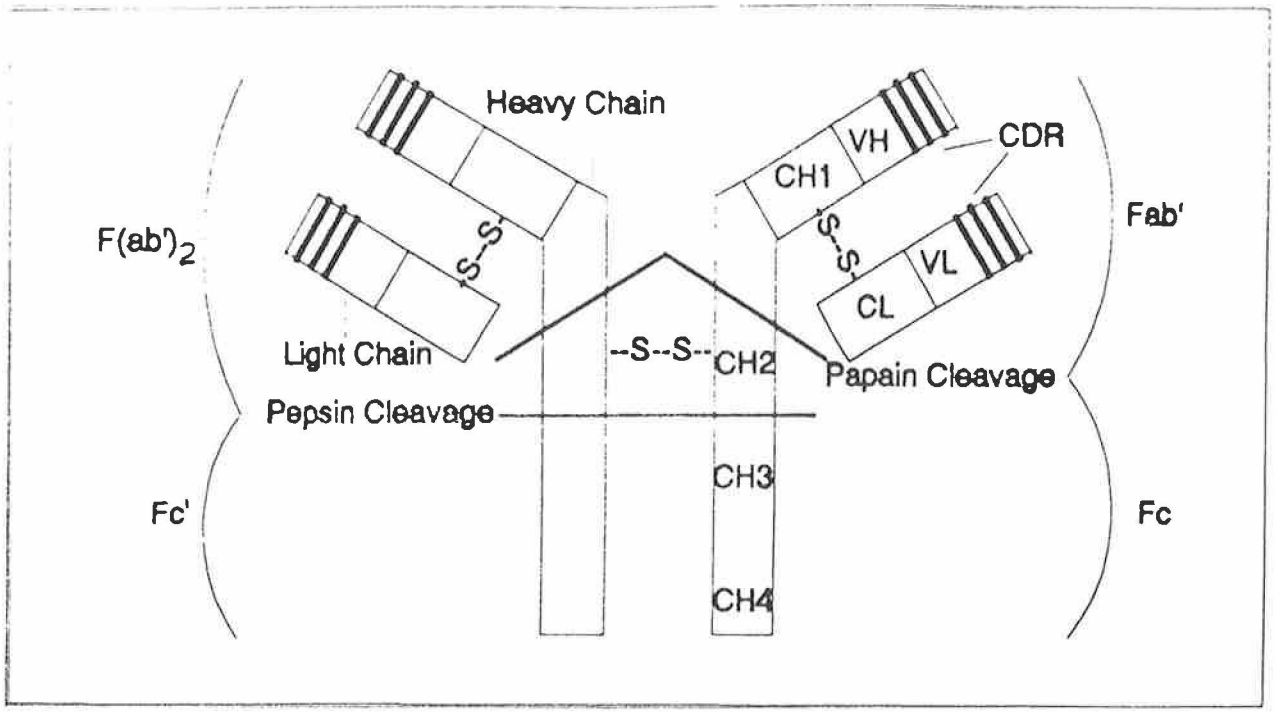


Figure 1.1.1:

The basic structure of an antibody. An antibody consists of heavy and light chains which are joined by disulphide linkages. The antibody can be further divided into variable (V) and constant (C) regions. CH_1, CH_2, CH_3 and CH_4 refer to various constant domains. The complimentarity determining regions (CDR) are the hyper-variable regions within the variable regions and are primarily responsible for antigen binding. The antibody can be broken down into fragments by treatment with the enzymes pepsin and papain. Two antigen binding fragments (Fab) of the IgG, containing one antigen binding site each, and one crystallisable fragment (Fc) are produced by papain digestion of the IgG. The antigen binding fragment ($F(ab')_2$) of the IgG, containing two antigen binding sites, is obtained by pepsin digestion of the IgG. The heavy chain fragments remaining after pepsin digestion are called Fc' fragments.

1.1.2 Antibody Production:

Ab of defined specificity can be produced by injecting an animal with the Ag of interest and subsequently collecting the blood. A molecule is defined as being immunogenic if it elicits a complete immune response in the host. Immunisation with a single Ag yields polyclonal antiserum, i.e. serum containing high titres of Ab specific to the Ag, but being produced by a number of B-cell clones. Some Ags fail to elicit a response and a particular Ag may provoke a much greater response than would another Ag. This is referred to as the antigenicity of the molecule. Proteins and large molecules are capable of eliciting good immune responses, with the aid of adjuvants (immune stimulants), but small peptides and other molecules with a relative molecular mass of less than 5kDa (often called haptens) are often too small to be immunogenic. Carrier proteins are frequently bound to these small molecules to increase their immunogenicity. Carrier proteins used include bovine serum albumin (BSA), keyhole limpet haemocyanin, thyroglobulin and many others. The use of adjuvants is often necessary in order to maximise the immune response to immunisation. Adjuvants non-specifically stimulate an immune response by causing local irritation leading to an inflammatory response. Large numbers of immune effector cells are attracted to the site of the injection. The Ag, which is emulsified in the adjuvant, is released slowly to give a prolonged exposure to the immune system, allowing a strong immune response to be generated. The most commonly used adjuvants are Freund's complete and incomplete adjuvants, (Foster, 1982; Steward, 1984; Abbas *et al.*, 1991; O'Kennedy *et al.*, 1994).

Although a number of species have been used, New Zealand white rabbits are probably the best animals to use for polyclonal Ab production. They are cheap, easy to handle for immunisation and bleeding purposes, and the housing required is a lot less than for larger animals. In general, the amount of Ag required to elicit an immune response is quite small. Typically an animal is immunised with 1 ml of a 0.1-4 mg/ml solution of Ag, made up in adjuvant, at intervals of 2-4 weeks. Ag may be injected at several sites; subcutaneously (sc), intramuscularly (im), intravenously (iv) or intradermally (id). Multi-site injections offer the advantage, over single-site injections, of presenting the Ag at several sites. Two weeks after the second immunisation the animal can be bled and the antiserum Ab levels measured. Antiserum Ab levels can be detected quantitatively by enzyme linked immunosorbent assay (ELISA) or qualitatively by immuno-histo-chemistry, immuno-diffusion and immuno-electrophoresis. If a satisfactory immune response has been obtained, the serum may be purified

for the specific Ab. Purification can be carried out in a number of ways, e.g. on affinity chromatography columns to which the Ag has been coupled. Milligram quantities of purified Ab may be obtained from a small volume (20ml) of serum. Polyclonal Ab production is often the most practical means of obtaining large quantities of Ab. They can be synthesised quickly and relatively cheaply and do not require the amount of time and level of expertise associated with the production of monoclonal antibodies, (Foster, 1982; Steward, 1984; Abbas *et al.*, 1991; O'Kennedy *et al.*, 1994).

1.1.3 Monoclonal Antibodies:

The technique of producing unlimited quantities of a single Ab specific for a particular antigenic determinant has revolutionised immunology. This technique is based on the fact that Ab from a given B-cell are homogenous in structure, specificity and affinity. Therefore, a monoclonal tumour derived from a B lymphocyte, called a myeloma, will produce only one Ab. Myeloma-derived homogenous Abs have proved invaluable in elucidating the structure of Ig proteins. However, most myelomas secrete Abs of unknown antigenic specificities, because these tumours arise spontaneously and it is not possible to predict the specificity of any randomly transformed clone of B-cells. Many attempts have been made to produce homogenous or monoclonal Abs of known specificity. Since normal B lymphocytes cannot grow indefinitely in culture, such attempts have focused on immortalising B-cells that produce a specific Ab. The first, and now generally used, technique for doing this was described by Kohler and Milstein in 1975. The method involves cell fusion, or somatic cell hybridisation between a normal Ab-producing B-cell and a non-Ab-secreting myeloma cell line. These cells can be fused using polyethylene glycol or by electrical methods. This is followed by selection of fused cells which secrete Ab of the desired specificity derived from the normal B-cell. Such fusion-derived, immortalized, Ab-producing cell lines are called hybridomas, and the Ab they produce are monoclonal antibodies (MAb), (Foster, 1982; Steward, 1984; Abbas *et al.*, 1991).

MAbs produced in this manner can be analysed and characterised in ways not possible with polyclonal Abs. Both heavy and light chains can be classified and sequenced, the nature and function of each of the variable and constant regions can be determined. Ag binding occurs at the point where the variable region of the heavy chain (V_H) lies adjacent to the variable region of the light chain (V_L). The actual Ag binding site is thought to be a cleft in the three-dimensional structure of the Ab lined by

loops of amino acid residues which form the hypervariable regions or complimentarity determining regions (CDR). X-ray crystallographic evidence suggests a canonical structural model for the CDRs. This model suggests that there exists a small repertoire of main-frame conformations, determined by a few residues in the amino acid sequence. Changes in the sequence at a few specific sites causes a shift in the loops, with respect to each other, by small but significant amounts. Each sequence change, or combination of changes, creates a different main-frame conformation, of which a limited number exist. Sequence changes at other sites cause variations in loop length and conformation. Differing patterns of loop sizes, combining with the effect of sequence diversity, alter the topography of the variable regions and generate a multitude of Ag specificities (Chotia *et al.*, 1989; Thornton, 1990; Williams *et al.*, 1990; Abbas *et al.*,1991; Roitt *et al.*, 1993).

Thus, while the framework residues are essential for the correct folding of the V region and the maintenance of the integrity of the binding site, only a small number of residues (10-12) actually interact with the Ag. CDRs form the Ag contact site and the chemical nature of the amino acids involved is citical in determining binding strength and specificity. Binding occurs with the formation of multiple non-covalent bonds, primarily hydrophobic interactions, but also hydrogen bonding, electrostatic attractions and Van der Waals forces. These bonds are weak individually, but can combine to give high energy binding. The strength of the bond formed is determined by the bond type and the distance between the amino acid residues involved. An Ag is said to be complimentary to an Ab if it has the correct 'shape', i.e. amino acid grouping, to fit the binding site. A high level of complimentarity would imply that a large number of non-covalent bonds form at once. These are then strong enough to overcome the steric repulsion created by the electron clouds around the two molecules. Variation from the ideal shape does not mean that binding cannot occur. Recent evidence suggests that Abs show conformational changes in response to Ag binding. This can occur through rearrangement of the side chains or CDR loops or through changes in the disposition of the V_H and V_L domains with respect to each other. X-ray crystallography of Fab fragments, both free and Ag bound, indicate a relative displacement of the variable domains on binding to Ag. It is thought that the flexible,linker region between the variable and constant domains can rotate to improve the 'fit' of the Ag at the binding site (Bhat *et al.*, 1990; Roitt *et al.*, 1993; Stanfield *et al.*, 1993).

The physico-chemical characteristics of the Ab-Ag interaction can be determined once a dynamic equilibrium has been established. The reaction of the Ab with Ag to produce a complex (Ab-Ag) can be represented as follows:

$$Ab + Ag \underset{k_d}{\overset{k_a}{\rightleftharpoons}} Ab\text{-}Ag$$

where k_a represents the association constant (or attractive forces) and k_d represents the dissociation constant (or repulsive forces). The tendency of an Ab to form stable complexes with Ag is called the affinity. The strength of this interaction is the sum of all the attractive and repulsive forces acting at the binding site, i.e. $k_a + k_d$. At low concentrations of Ab the formation of Ab-Ag complexes depends on the concentration of Ag and the strength of the interaction. The molar concentration of Ag which allows half the Ab concentration to complex is taken as a measure of the affinity of the interaction. The lower the concentration of Ag required to reach half-maximum occupancy of the Ab, the higher the affinity of the Ab for the Ag. The affinity constant is normally in the range 10^{-7}M to 10^{-11}M. This holds true for single binding sites. However, with multiple binding sites, the strength of the interaction increases geometrically with each binding site. A low affinity molecule may bind tightly to a multivalent Ab since multiple, low affinity interactions can combine to produce a single high strength bond. This is called the avidity of the interaction. (Abbas *et al.*, 1991; Roitt *et al.*, 1993) Thus, an exact measurement of Ab affinity is not always possible. However, measurement of the amount of Ag bound to Ab at equilibrium can give an index on which to compare the binding strength of a number of similar Abs. This can be particularly useful when screening a number of MAbs or MAb conjugates for use in immuno-diagnostics, immuno-therapeutics or immuno-assay development.

1.1.2 Bispecific Antibodies:

Murine, human and chimeric MAbs are currently being used for the treatment of autoimmune disease, graft rejection, infectious diseases, clotting disorders and cancer. Murine MAbs have been useful, but have been shown to have several drawbacks, particularly in the area of immunotherapy (Reading & Bator, 1988). One of the main limiting factors encountered with the use of MAbs in therapy is the development of human anti-mouse Ab (HAMA) responses. A number of attempts have been made to alleviate this problem. Human MAbs have been produced using human B-cells (Carroll *et al.*, 1990). Genetic engineering techniques have been used to develop chimeric MAbs, Abs having a murine variable region and a human constant region, and humanised MAb fragments which may also have therapeutic potential (O'Kennedy & Roben, 1991). The HAMA response is not completely eradicated, however, since anti-idiotypic and anti-allotypic responses are still possible. As toxin- and radionuclide- immunoconjugates (Fig. 1.1.2A), these MAbs have considerable potential, but problems associated with their toxicity have slowed their clinical applications (Songsivilia & Lachmann, 1990). Unconjugated MAbs have fewer side effects, but generally less biological activity *in vivo,* probably because most MAbs do not efficiently initiate host cellular or humoral effector mechanisms. For a MAb to be an effective therapeutic agent, it must bring together molecules or cells that mediate the desired biological effect. This might include facilitating the action of an enzyme such as plasminogen activator on its substrate, activating the human complement system to cause cell lysis or triggering molecules on leucocytes that are responsible for phagocytosis or cytolytic killing of a target cell. It has been suggested that this may be achieved, and many of the problems of MAbs overcome, by the use of a bispecific, monovalent Ab, i.e. a bispecific Ab (BAb). (Reading & Bator, 1988; Songsivilia & Lachmann, 1990; Fanger *et al.*, 1992; Nolan & O'Kennedy, 1992)

BAbs are antibody-derived molecules capable of monovalently binding to two distinct Ags. BAbs use the specificity of MAbs to join together the molecules or cells that mediate the desired effect. Such molecules can bind enzymes, therapeutic agents or radioactive labels to a target without the need for chemical modification (Fig. 1.1.2B). This is advantageous since the affinity and activity of both MAb and active agent may be altered by the chemical modifications required for conjugation. This concept was first introduced in 1961 (Nisonoff & Rivers, 1961) when polyclonal Abs were used to produce a fully functional BAb. The potential of BAbs went unnoticed for nearly twenty years.

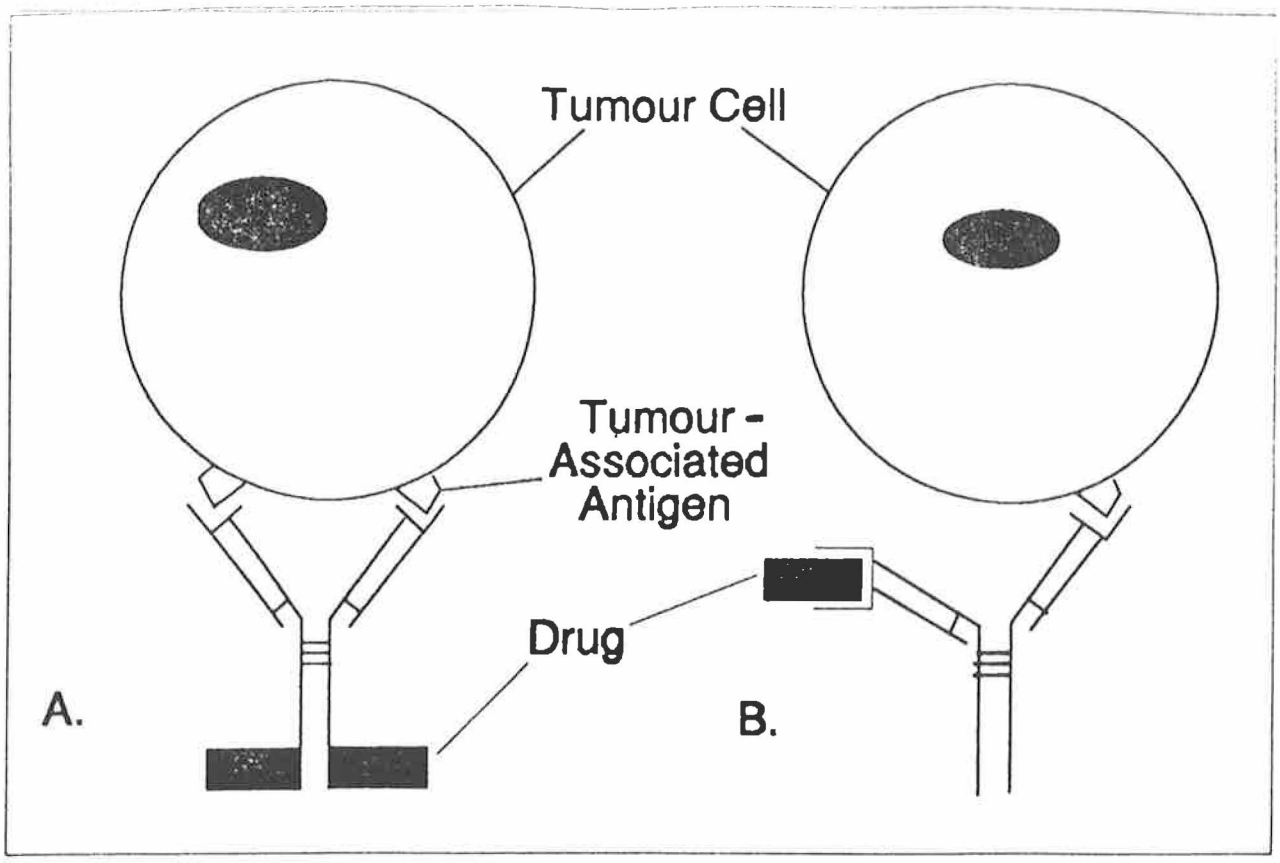


Figure 1.1.2:

An illustration of strategies for the use of antibodies in tumour targeting. (a) Monoclonal antibodies (i.e. bivalent IgG molecules specific for a tumour associated antigen) can be covalently bound to a drug molecule, and subsequently used to bring the drug into direct contact with the tumour cell. (b) Bispecific antibodies (i.e. IgG molecules which monovalently bind to two distinct antigens) can be used to bring a tumour-associated antigen and a drug molecule into close contact, without chemical modification of either the drug or BAb molecules.

The advent of myeloma technology (Kohler & Milstein, 1975) led to the production of hybrid-hybridomas (Reading, 1981). These provided a continuous source of BAbs of pre-determined specificity. Such BAbs could be isolated, tested and used for a variety of purposes, most particularly the immunotherapy of cancer. The toxicity associated with many MAb-drug conjugates can be reduced. BAbs can be administered, and allowed to localize at the desired site, prior to administration of the active agent. This allows the BAbs to wash out from non-specific binding sites, where it binds less strongly, so that the active agent is localised only at the desired site. These methods have an additional benefit. Both BAb and active agent are smaller than most immuno-conjugates, this makes them less immunogenic and increases penetration of these molecules to the desired site (Reading & Bator, 1988). BAbs can also be used to bind to trigger molecules on leucocytes such as T-cell receptors (TcR), thus focusing normal cellular immune defence mechanisms specifically to the tumour cell or infectious agent. For example, in antibody-dependent cellular cytotoxicity (ADCC), killing of the target cell is triggered when the Fc receptors for IgG (FcγR) on effector cells are cross-linked, leading to the directed release of cytotoxic molecules from the effector cell. However, many potentially useful MAbs are not of the appropriate isotype to mediate these effects and are inactive due to their inability to activate human complement and/or trigger FcγR on human cells. A BAb could be constructed to link a target cell to the FcγR on effector cells, thus triggering an ADCC response (Fanger *et al.*, 1992).

The potential applications of BAbs are not limited to immuno-therapeutics. Radionuclides can be localised for diagnosis as well as treatment (Somasundaram *et al.*, 1993). Enzymes and pro-drugs can be localized and activated *in situ* to reduce non-specific or systemic action (Branscome *et al.*, 1990; Fanger *et al.*, 1992). A range of novel enzyme immunoassays have been suggested which rely on the unique properties of BAbs (Paulus, 1985). BAbs now represent a potentially powerful new therapeutic and diagnostic tool. In the following pages the full potential of BAbs will be examined in more detail. In addition, the various methods for the production of BAbs will be discussed.

1.2 PRODUCTION OF BISPECIFIC ANTIBODIES

Since their inception, a number of methods have been used to produce BAbs, with varying degrees of success. BAbs have been created by fusion of Ab-producing cells, by chemical cross-linking or by molecular genetic approaches. Each of these approaches has limitations, and a major problem in establishing clinical trials with BAbs is the procurement of sufficient amounts of clinical-grade material necessary to carry out studies in their entirety. Thus, from a clinical standpoint, economical large-scale production of BAbs is vital to progress in this field.

1.2.1 Biological Production of Bispecific Antibodies:

The biological production of BAbs is based on the principles of somatic cell hybridization, first developed by Cotton and Milstein (1973). They demonstrated that the fusion of two Ig-secreting cell lines produces hybrid cells which secrete Ig of both parental types and of a hybrid type. This was of limited usefulness since the antigenic specificity of the Ab was unknown. This problem was overcome in 1975 when Kohler and Milstein fused antibody-producing lymphocytes with a myeloma cell line producing Abs specific to a given Ag. The potential of these developments was recognised by Reading 1980 (U.S. Patent: 4 474 893) and reported in 1981. BAbs were shown to arise via the fusion of two hybridomas to produce one quadroma, derived from four parental cells (Reading, 1981; Martinis *et al.*, 1982). A slightly diferent approach was used when a hybridoma with specificity for one Ag was fused with spleen cells from an animal immunised with the second Ag (Milstein & Cuello, 1984). These somatic hybrids are referred to as triomas since they derive from three parental cells.

Original production methods for BAbs are based largely on conventional hybridoma technology (Fig. 1.2.1). MAb-producing hybridomas are produced in the usual manner. Myeloma cell mutants lacking the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) were used. These fail to grow in medium containing hypoxanthine, aminopterin and thymidine (HAT), since both DNA biosynthetic pathways are blocked. Spleen cells contain HGPRT, but are incapable of continuous division. Thus, only hybridomas will survive in HAT medium, since they are formed by the fusion of a myeloma cell and a spleen cell and so produce HGPRT. Such cell lines are termed HAT-resistant. These hybridomas are commonly back selected for HAT sensitivity prior to further fusion.

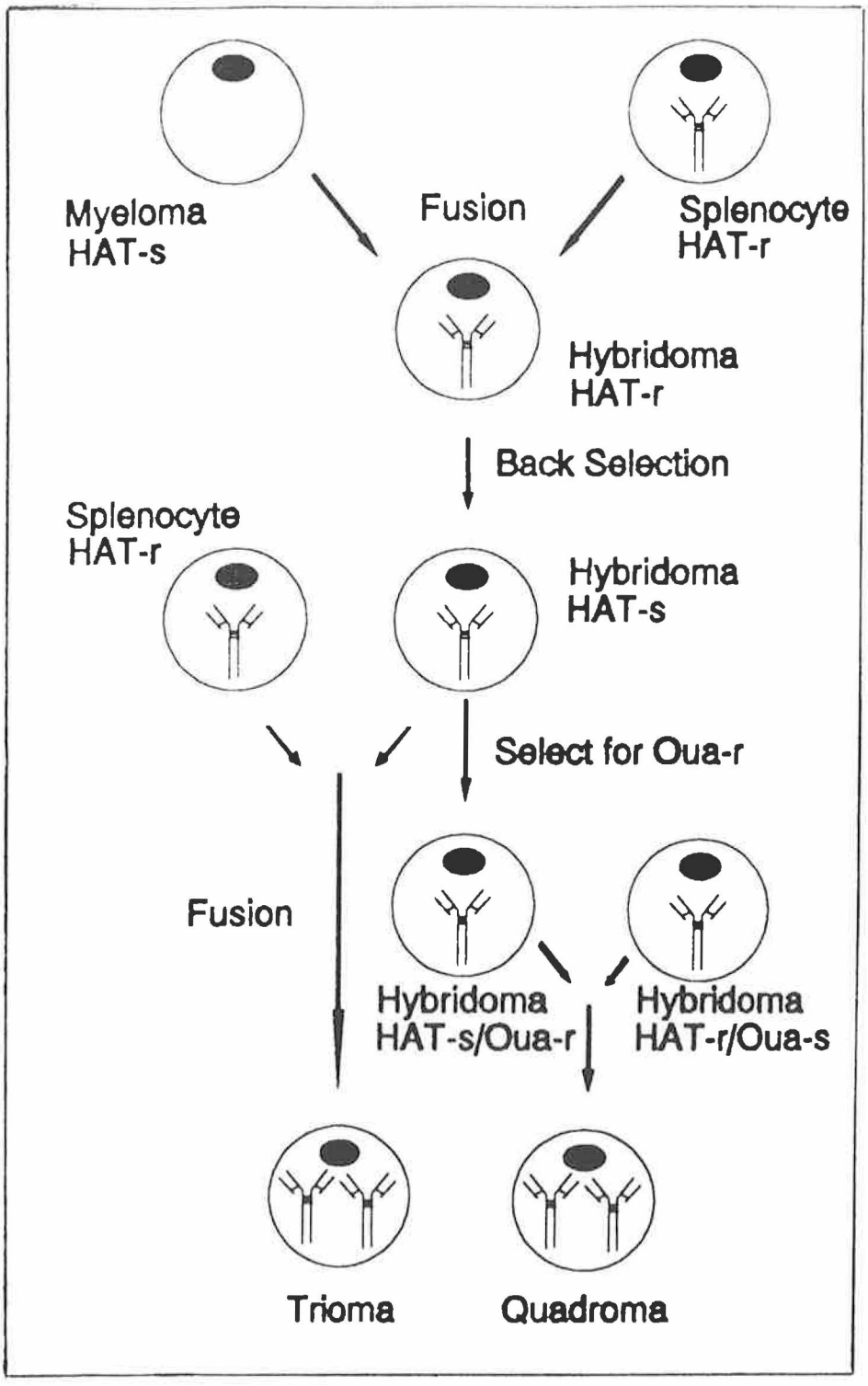


Figure 1.2.1:

A flow diagram illustrating the protocols used in the biological production of bispecific antibodies. Hybridomas are produced by somatic cell fusion and back selected, by growth in 8-azaguanine, for Hypoxanthine-Aminopterin-Thymidine medium (HAT) sensitivity. A trioma can then be produced from the hybridoma and a splenocyte, using the same fusion and selection procedures. Alternatively, the hybridoma is selected for ouabain resistance, by adding ouabain to the growth medium. This is then fused to a HAT-resistant/ouabain-sensitive hybridoma. The quadroma so produced is selected by growth in medium containing both HAT and ouabain. (-r = resistant; -s = sensitive)

HAT sensitivity is readily induced by growing the cells in increasing concentrations of 8-azaguanine in complete growth medium, thus inducing HGPRT deficiency. HAT sensitivity can similarly be induced using bromodeoxyuridine to induce thymidine kinase deficiency (Wong & Colvin, 1987; Urnovitz *et al.*, 1988). These back-selected hybridomas can be fused to Ab-secreting spleen cells from an animal immunised with a second Ag in the same manner as for hybridomas. Yield of BAb by this method has been shown to be approximately 34% of total Ig production (Wong & Colvin, 1987; Tada *et al.*, 1989).

Although there have been successes using triomas, many workers have concentrated on quadroma production. It is potentially easier to work with a population of cells producing Abs of defined Ag specificity and affinity, than with a heterogenous population of spleen cells producing uncharacterised Abs. Most workers use one hybridoma line maintained with HAT resistance. The other hybridoma is back-selected for HAT sensitivity, and is further modified for resistance to biological inhibitors or cytotoxic drugs. Of these, ouabain appears to be the most popular (Staerz & Bevan, 1986; Tiebout *et al.*, 1987; Reading & Bator, 1988), but the use of emetine (Suresh *et al.*, 1986), actinomycin (Chervonsky *et al.*, 1988) and neomycin (DeLau *et al.*, 1989) has also been reported. Thus, when a HAT-resistant-ouabain-sensitive hybridoma is fused with a HAT-sensitive-ouabain-resistant hybridoma, quadromas are produced which are both HAT and ouabain resistant (Fig. 1.2.1). Up to 90% of supernatants from growth-positive wells, so produced, were shown to contain both Ab activities. It was estimated that the actual yield of the desired hybrid was between 12.5% and 50% of total Ig production (Staerz & Bevan, 1986). Another possibility is to fuse two single mutant hybridomas, both of which are HAT sensitive - one due to HGPRT deficiency, the other due to tyrosine kinase deficiency (Wong & Colvin, 1987; Urnovitz *et al.*, 1988).

An alternative method for inducing resistance to compounds has been suggested. In certain instances, genomic markers are available for transfer into cell lines, so inducing resistance. Most of these revolve around the induction of resistance to geneticin. This gene, originating from *Escherichia coli*, would behave as a true dominant marker after introduction, independent of the degree of ploidy of the hybridoma involved. Retroviral transduction was found to be the most efficient method of gene transfer into cells growing in suspension (DeLau *et al.*, 1989). Similar techniques were used to induce hybridoma resistance to geneticin and ouabain (Reading & Bator, 1988) or to geneticin and

mycophenolic acid (Xiang *et al.*, 1992). Quadromas can then be selected for in medium containing both drugs.

Selection of quadromas initially depended entirely on the use of mutant phenotypes and biochemical selection. The isolation of these mutant phenotypes is time consuming and the addition of biochemical inhibitors may not be conducive to outgrowth of quadromas. A method has been developed involving the use of Percoll density gradient centrifugation and fluorescence-activated cell sorting (FACS), which largely avoid interference in cell biochemistry (Koolwijk *et al.*, 1988). In Percoll density gradient centrifugation cells pass through layered solutions of increasing density. Cells of different size/density stop at different solution density interfaces. When this was carried out following a fusion, production of quadromas was increased eight-fold in cells taken from the 1.082-1.075 g/ml density fraction. When FACS was used, hybridomas were labelled with either fluorescein isothiocyanate (FITC) or tetramethyl-rhodamine isothiocyanate (TRITC) via the membrane marker octadecylamine. Cells were fused to produce quadromas labelled with both FITC and TRITC. A double-gated flow cytometry system was set up. The first gate separated cells on the basis of axial light loss, or size, and right angle scatter, or internal structure. The second gates simultaneously detected green (FITC) and red (TRITC) fluorescence, seperating out double-positive cells. A single cell deposition system can be used to seed the double-positive cells into 96-well plates. This system appears to offer a fast efficient method of quadroma isolation with the results of the fusion (percentage of fused cells) immediately available. In addition, quadromas are maintained in optimal growth conditions after fusion. It was found, however, that sorting must be carried out within six hours after fusion. After this time the marker dyes begin to randomly exchange between non-fused parent hybridomas in suspension together, (Karawajew *et al.*, 1987; Koolwijk *et al.*, 1988; Jantscheff *et al.*, 1993).

Before use, quadroma products must first be isolated from the growth medium and characterised. The first step following fusion is the determination of antibody secretion in growth-positive wells. Detection of BAb has, thus far, depended on the use of double antigen enzyme-linked immunosorbent assay (ELISA) (Tiebout *et al.*, 1987; Takahashi & Fuller, 1988; Tada *et al.*, 1989) or affinity chromatography with each specific Ag (Corvalan & Smith, 1987). In 1984, Milstein and Cuello analysed the Ab mixture. Since Ab-producing cells have V and C regions of each chain attached to a single transcriptional unit, the expression of each chain is not altered. However, the individual light and heavy chains are themselves on

separate transcriptional units. During transcription, chains are synthesised through the membrane of the rough endoplasmic reticulum and released into the cisternal space where they are assembled. The expected molecular species resulting from random association of the two heavy and two light chains are shown (Fig. 1.2.2). It is difficult to predict the concentrations of each molecular species produced due to the differential rate of synthesis of the different chains, and the preferential association of homologous vs. heterologous pairs. *In vitro* studies have shown that under competitive re-association the homologous light-heavy chain prevails in 80% of cases. In addition, there appears to be a homologous heavy-heavy chain binding preference, resulting in a 70% prevalance of this combination (Milstein & Cuello, 1984). This would indicate that hybrids of a single sub-class have an advantage in that they are most likely to give best yields. However, the difference in electrophoretic or chromatographic behaviour of assymetric hybrids is advantageous for the fractionation of different molecular species and may compensate for the lower yield of bispecific hybrids (Xiang *et al.*, 1992).

Suitable quadromas are clonally expanded, either in culture or, more generally, as an ascitic tumour. Recently it has been suggested that the use of hollow fibre bioreactors could greatly increase yields (Gorter *et al.*, 1993). Ig can be purified from ascites fluid by dialysis against phosphate buffered saline (PBS) at pH 7.4 (Staerz & Bevan, 1986; Takahashi & Fuller, 1988), or by precipitation with 50% (w/v) saturated ammonium sulphate (Tada *et al.*, 1989). The Ab solution can then be passed through a protein A-Sepharose column, and the Igs eluted with acetate or citrate buffer in a pH gradient from pH 3.0-5.0. BAbs have been shown to elute in the fraction pH 4.0-4.5 (Kuppen *et al.*, 1993). The Ig can also be separated on a hydroxyl-appatite column by high performance liquid chromatography (HPLC), using increasing concentrations of sodium phosphate buffer (10-350 mM) (Staerz & Bevan, 1986; Barr *et al.*, 1989). Quadromas can be expected to produce three main types of functional Ig: the two parental Ab and the hybrid BAb, which elute in three separate fractions following HPLC. The degree of separation varies. Hybrids formed by Abs of different sub-class (e.g. IgG1 & IgG2b) are easier to separate than those formed by Abs of the same sub-class. The isolated Abs can be further characterised using iso-electric focusing or sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), (Milstein & Cuello, 1984).

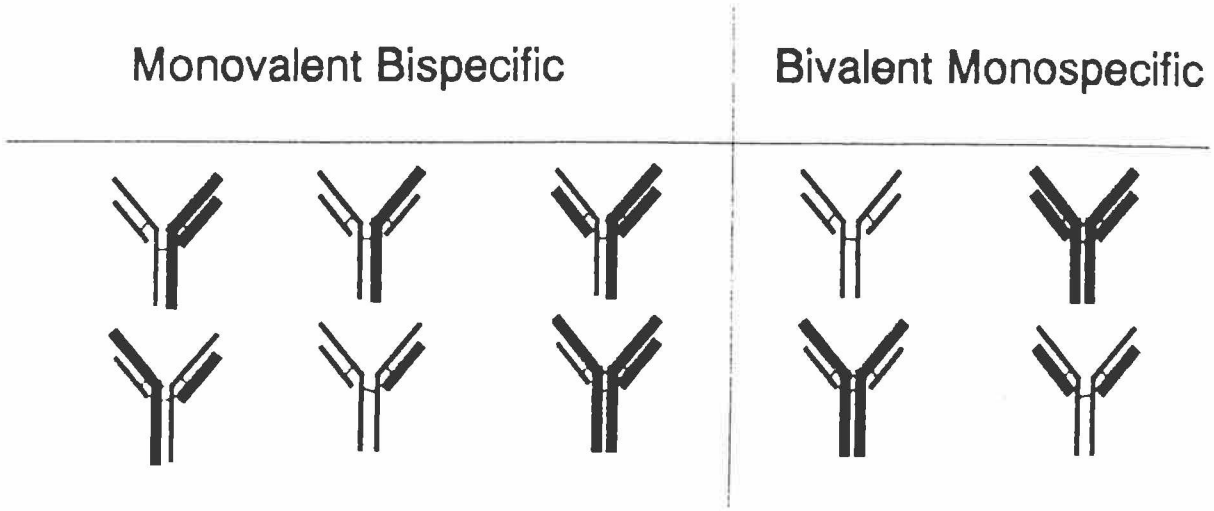


Figure 1.2.2:

Antibody production by quadromas and triomas. The expected molecular species arising from the random association of heavy and light chains. Native heavy-light combinations will produce the best three-dimensional configuration for high affinity binding. Other heavy-light chain combinations will have reduced affinity for the antigen.

1.2.2 Chemical Production of Bispecific Antibodies:

One of the major problems associated with the use of BAbs is the procurement of large quantities of the purified product. With biological production a mixture of both parental and bispecific Abs is produced, from which the BAb must be purified. For this reason several workers have used chemical means of production. These methods, with purified parental Abs as the starting material, are designed so that BAb forms the sole product which can be extracted in one step purification procedures. Such methods were first discussed as early as 1961 by Nisonoff and Rivers. Polyclonal rabbit Abs were digested with pepsin and mildly reduced to yield two univalent fragments (Fab' fragments). The univalent subunits were combined to give good yields of BAb by oxidation of the disulphide bonds. When a mixture of approximately equal amounts of Ab of two different specificities was used, the recombined product was precipitated only in the presence of an appropriate mixture of the two Ags. This suggested the presence of a large portion of Ab of dual specificity. Further work confirmed the presence of bispecific Ab fragments (Nisonoff & Mandy, 1962).

These methods were used by Brennan *et al.* (1985) in an attempt to create BAbs from two murine MAbs (Fig. 1.2.3). Limited pepsin hydrolysis was used to produce F(ab')2 fragments with an 80% yield. Complete reduction of the F(ab')2 to Fab' was obtained using 2-mercapto-ethylamine (1 mM) in sodium phosphate (0.1 M), with EDTA (1 mM). Attempts to regenerate F(ab')2 fragments gave poor results compared to those achieved with rabbit Abs. This is probably related to the three disulphide bonds that bridge the heavy chains in mouse IgG (only one exists in rabbit IgG), resulting in the formation of intra-chain disulphide bonds. Modifications to the procedure were made to prevent this recurring. Firstly, F(ab')2 fragments were reduced in the presence of the di-thiol complexing agent sodium arsenite, thus impeding intra-molecular disulphide bond formation (Brennan *et al.*, 1985). The successful use of dithiothreitol in this step has also be reported (Nitta *et al.*, 1989). In the second step, the thiols were stabilised as the dithio-nitro-benzoate (dTNB) derivative. One of the Fab'-TNB derivatives was then converted to the Fab'-SH by reduction with 2-mercaptoethylamine and was mixed with an equimolar amount of the other Fab'-TNB derivative to form the hybrid (Fig. 1.2.4). The absence of hybrid dimer formation, when either of the reaction partners was absent, suggested that the dimer formed in the complete mixture represented pure BAb. Following high-performance gel exclusion chromatography, the yield of bispecific F(ab')2 fragments was 50-70% of total potential yield.

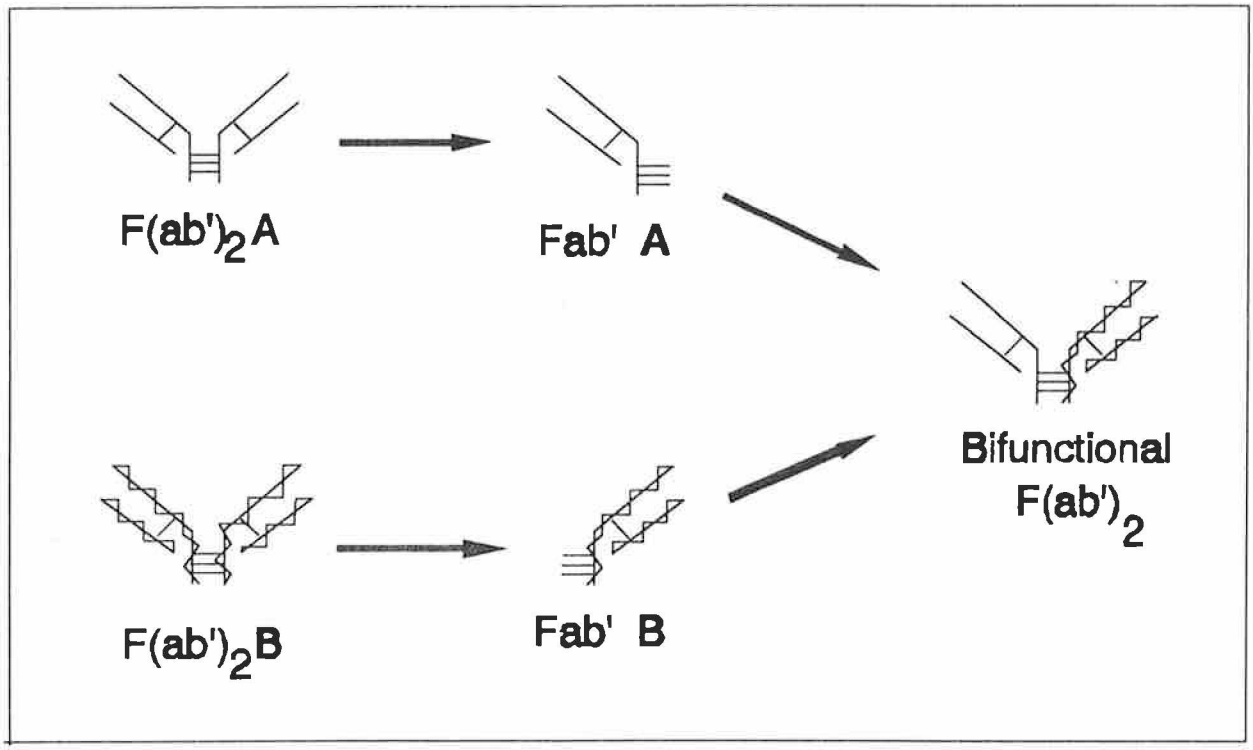
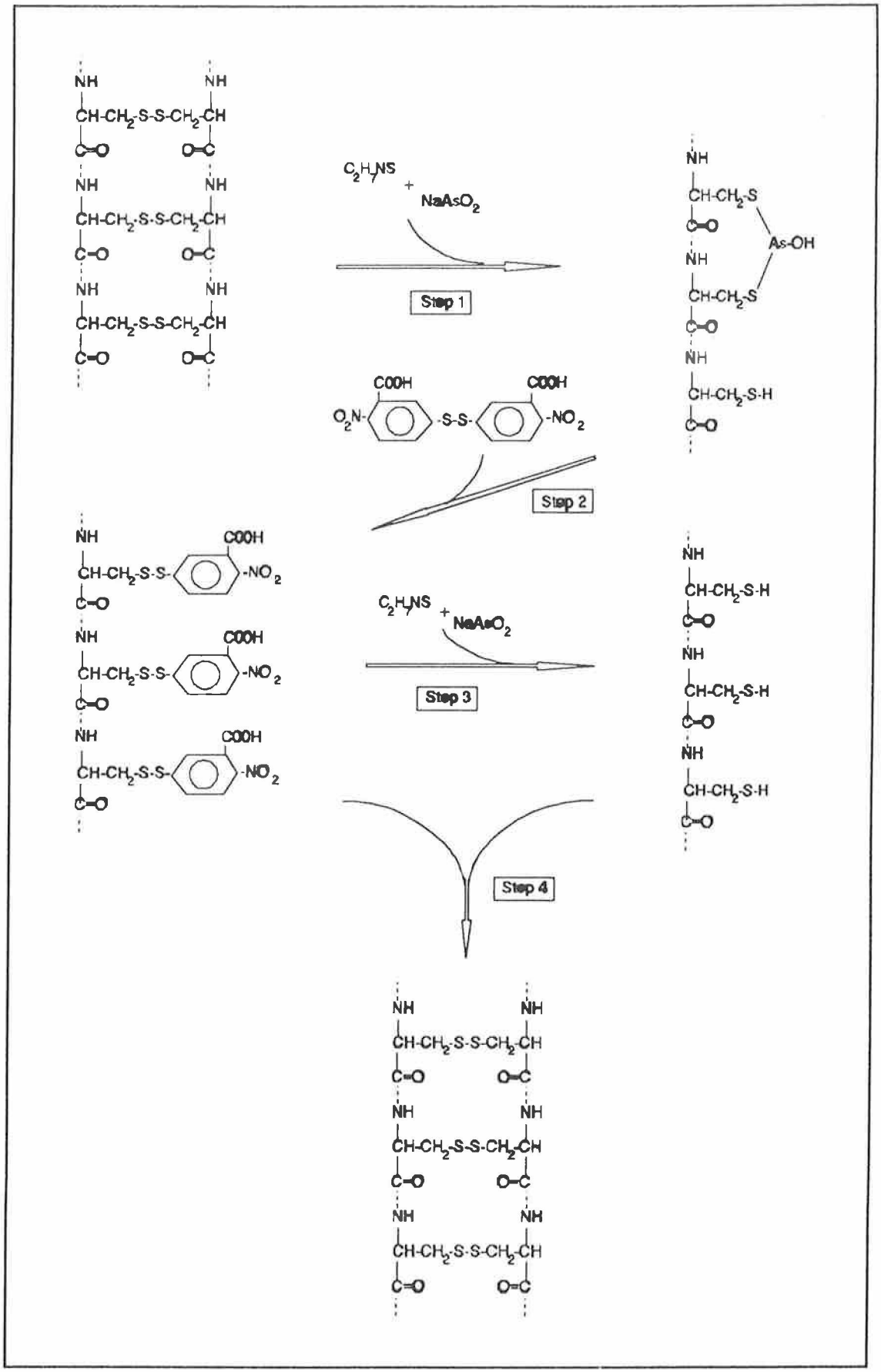


Figure 1.2.3:

Chemical production of bispecific antibodies. Using this method, the parental IgG are first subjected to a pepsin digestion to produce $F(ab')_2$ fragments. The disulphide bonds are reduced to the Fab' fragments, using 2-mercaptoethylamine, in the presence of sodium arsenite and Ellman's reagent. The two different Fab' are mixed in an equimolar ratio and recombined under appropriate conditions to produce the hybrid $F(ab')_2$ molecule (after Brennan *et al.*, 1985).

Figure 1.2.4:



Another important consideration was product quality. When an enzyme-linked immunoassay was used to compare the immunoreactivity of the original and the reconstituted $F(ab')_2$, no significant difference was found. However, 0.1-0.2 moles of residual free thiol per mole of $F(ab')_2$ was detected, indicating that not all disulphides were completely reformed. These observations and others suggested that the procedure led to some reduction and incomplete re-oxidation of the disulphide bond between heavy and light chains, without significantly affecting the integrity of the Ag-binding site. The bispecific nature of the $F(ab')_2$ fragments was illustrated by double antigen ELISA (Brennan *et al.*, 1985).

Other methods have been tried in which heterodimers of rabbit and mouse Abs have been produced (Glennie *et al.*, 1987). The protocol for this preparation falls into three main steps. Firstly, $F(ab')_2$ fragments were prepared and reduced for both Ab partners to give high yields of Fab'-SH fragments. Subsequently, all of the available thiol groups on one of the Ab were maleimidated with the bifunctional cross-linking reagent, o-phenylene dimaleimide (o-PDM). This step was carried out in a large molar excess of o-PDM to avoid possible intermolecular linking. Finally, the Fab'-SH and the Fab'-mal were combined under conditions favouring -SH and maleimide reaction while minimizing the re-oxidation of -SH groups, through low pH and the presence of EDTA. The final products were reduced and alkylated, prior to gel filtration to remove any $F(ab')_2$ which may have formed by re-oxidation of hinge region -SH groups. The two Ab combinations tried, mouse-mouse and mouse-rabbit, gave similar products, with the required $F(ab')_2$ species constituting 50-70% of the recovered material. Differential pre-labelling with radioactive isotopes showed that the purified $F(ab')_2$ contained almost equal amounts of the two Ab species, a result consistent with a 1:1 union. The nature of the linkage between the Fab' Ab partners was investigated using SDS-PAGE. In most molecules it appears that the heavy chain segments of the two Fab' fragments were coupled, probably as a result of thio-ether linkage between the hinge region -SH groups. The proposed typical structure is shown (Fig. 1.2.5). Only 0.84 maleimide groups per Fab' were detected. This suggested that two of the three hinge region SH groups present in the mouse Fab' are cross-linked intra-molecularly during maleimidation, so that just one is available to the rabbit or mouse Fab'-SH. Functionally, the BAb molecules were tested in three different systems, in which they behaved as would be predicted assuming that both Abs bound to their respective Ags simultaneously.

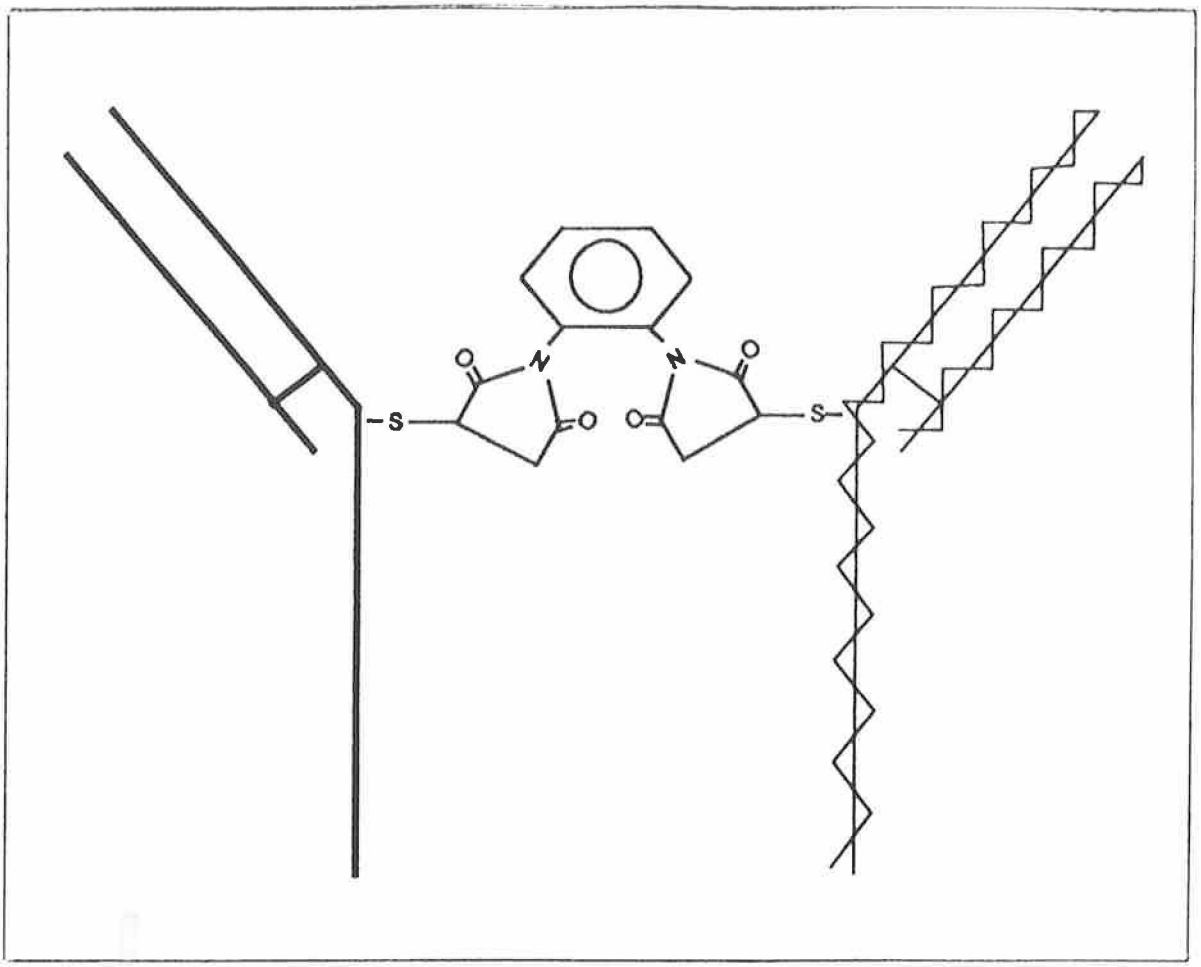


Figure 1.2.5:

Bispecific F(ab')2 fragment, the proposed typical structure, formed by the recombination of Fab' fragments using o-phenylene dimaleimide as a cross-linking agent, according to the methods of Glennie *et al.* (1987).

The protocols of both Brennan *et al.* (1985) and Glennie *et al.* (1987) describe rapid and efficient methods for the preparation of BAbs. Of the two, the former appears to offer the advantage of having the BAb as the sole product, removing the need for separation and purification of the products. Functional studies have demonstrated that bispecific $F(ab')_2$ heterodimers behave in a similar manner to that described for bispecific IgG, and may offer a number of advantages, both in their preparation and in their performance. The major advantage over bispecific IgG secreted from quadromas is the ease with which they can be produced and the eventual good yield of useful products.

1.2.3 Genetic Engineering of Bispecific Antibodies:

Molecular biology techniques have advanced to the point where designer genes are regularly transfected into *E. coli* and hybridomas in order to produce Ab libraries specific to a given Ag (O'Kennedy & Roben, 1991). There is no reason why this technology cannot be taken a step further in order to produce BAbs. The double transfection of DNA constructs of murine IgG genes into producer hybridoma cells has been carried out successfully (Songsivilai *et al.*, 1989; Lenz & Weidle, 1990). The products of this hybridoma will be murine, bispecific IgG. Because of the immunogenicity of the IgG molecule, when used in immunotherapy, there has been a move away from the use of murine IgG. It is now possible to produce humanised/chimeric MAbs. These are constructed when the gene of the variable region of a murine IgG and the gene for the constant region of a human IgG are co-transfected into the same cell. The genes for two of these molecules have been transfected into SP2 hybridomas, the products of which were found to be 10-20% bispecific IgG (Phelps *et al.*, 1990). Such molecules have been used in two phase radio-immunotherapy without inducing the immune response that occured with murine MAbs (Bruynck *et al.*, 1993).

An alternative to transfection of the whole gene has been to use only that part which codes for the Fab' fragment. The gene coding for a murine, hypervariable region has been transfected into *E. coli* along with the gene coding for human Fab'. This latter gene can be adjusted so that the Fab' produced contains only one free thiol group, thus preventing intra-chain disulphide bond formation. The secreted Fab' can be purified from the growth medium and chemically cross-linked to produce an active bispecific $F(ab')_2$ molecule (Rodrigues *et al.*, 1992; Shalaby *et al.*, 1992). As an alternative to chemical cross-linking, a molecular approach has been used to form BAbs through the use of "leucine zippers" (Kostelny *et al.*, 1992).

Leucine zippers are α-helices, approximately 30 amino acids long, with leucine every seventh residue. The leucine residues align on the outside of the helix and are available for binding with similar constructs. The leucine zipper region of transcription factor Jun or Fos were genetically fused to the genes for each Fab' molecule, and transfected into SP2 hybridoma cells. Effective BAbs were then created by mixing both constructs together. This method has the advantage of a greater tendency to produce the heterodimers of interest rather than unwanted homo-dimers. Such protocols could well be the methods of choice in the future of bispecific antibodies.

1.3 ANTIBODY APPLICATIONS

One of the primary objectives of immunotherapists over the last decade has been to improve the therapeutic ratio of cytotoxic agents in cancer patients by using the specificity inherent in MAbs. The administration of murine MAbs to humans has generated widely varying results, with rare "cures" and, in general, no firm conclusions as to their most efficacious use. It was initially envisaged that a BAb could be used to improve the efficacy of such treatments. The BAb would bind univalently to both the target cell and the therapeutic agent, thus localising large quantities of drug at the target site and increasing the effectiveness of the therapy. Since then, a wide variety of other uses have been postulated and tested for both MAbs and BAbs.

1.3.1 Chemotherapy:

Most of the therapeutic drugs available for treatment of cancer rely on uptake by rapidly dividing cells, a poor basis for selectivity since it will result in significant damage to normal cells. Tumour cells, though, differ not only in freedom from normal growth constraints, but also in an altered spectrum of gene expression. Where this results in expression of tumour-specific or tumour-associated Ags (TAA) at the cell surface, Abs recognising these may be considered for use as therapeutic agents. If tumour-specific MAbs are chemicaly linked to suitable drugs, toxins or radionuclides, then cytotoxic agents with a high degree of selectivity are formed. These immno-conjugates bind the tumour cells and are internalised and degraded by the lysosomes to release the active agent which then acts on the target - a particular enzyme, DNA or RNA. A number of TAAs have been identified and MAbs produced for these (Teh *et al.*, 1985). Several drugs (Pietersz, 1990), toxins (Spooner & Lord, 1990) and radionuclides (Britton *et al.*, 1991) have been attached to MAbs and their effects *in vitro* as well as *in vivo* in mice and humans have been studied.

One of the most formidable obstacles in the production and utilization of immuno-conjugates is the effect of chemical reactions on the activities of both the active agent and the MAb. Attempts to maximise the number of drug/toxin molecules conjugate per MAb molecule result in significant decreases in protein recovery due to precipitation. The binding activity of the MAb and the effectiveness of the active agent can both be drastically reduced. Despite these factors, initial *in vitro* studies were encouraging. However, *in vivo* testing showed mixed results. The failure

of immuno-conjugates, so far, to fulfill their potential may be attributed to a number of causes (Spooner & Lord, 1990). These include:

- failure of large conjugates (180kDa) to penetrate solid tumours;
- circulation of shed Ag competing for immuno-conjugate;
- escape of Ag-negative tumour variants;
- the nature and density of target Ag;
- non-specific binding of the constant region of the Ab to non-target cells, resulting in systemic toxicity;
- neutralization of therapeutic molecules by patients who are not severely immuno-suppressed;
- the development of human anti-mouse Ab responses limits the number of treatments possible for a patient;
- the appearance of side effects not predicted by animal models, e.g. fluid retention and muscular pain.

An alternative approach for the localization of agents is the use of BAbs. These molecules have the potential to overcome some of the problems associated with immuno-conjugates. The bispecific $F(ab')_2$ [$BF(ab')_2$] molecule is as effective as the whole molecule, but its smaller size makes it less immunogenic and increases penetration of the molecule to solid and inaccessable tumours. The monovalent binding of the BAb increases the density of Ab and, hence, active agent on the cell surface. The BAb can be administered prior to the active agent and allowed to localize to the target tissue. Non-specifically bound BAb will wash out quicker than specifically bound BAb; active agent administered after this will localize to the tumour site only. A number of BAbs have been produced and tested and their effectiveness compared to the parental MAbs and immuno-conjugates.

Several *in vitro* studies have been carried out using BAbs to target ricin, a toxin derived from the castor bean, to tumour cells. This compound, which consists of an A- and B-chain, is toxic only in the dimeric form. The B-chain is required to bind the complex to the galactose determinants on the cell surface. Only when it is in such close association with the cell surface does the A-chain enter the cell, resulting in inactivation of the ribosomes and shut down of protein synthesis. The A-chain alone is non-toxic and can be administered *in vivo* without effect. Using trioma technology, a BAb was produced which bound ricin A-chain (RAC) and a prostate-restricted Ag (Webb *et al.*, 1985). The BAb brings the

RAC into sufficiently close contact with the cell to allow it to enter and act in the usual manner (Fig. 1.3.1).

BAbs were also produced by chemically linking Fab' fragments from rabbit anti-RAC Ab and rabbit anti-human IgG Ab (Raso & Griffin, 1981). This introduced an anti-globulin step where the toxin is directed only towards those cells which carry cell surface human IgG. This approach has the disadvantage of being blocked by free IgG. The Abs were tested *in vitro* with RAC, human IgG and cancer cells and were shown to have retained both affinity and biological activity. There was some indication that univalent binding by the Abs increases the cell surface saturation with BAbs and, hence, increases cell killing by the toxin. Univalent binding of BAbs to tumour cells may also reduce modulation of the Ag at the cell surface (e.g. reducing levels of internalisation), leaving the Ag accessible for further treatments (Webb *et al.*, 1985). In addition, *in vitro* tests showed that protein synthesis was inhibited in BAb-bound cells at considerably lower ricin concentrations than in non-BAb-bound cells (Raso & Griffin, 1981). There was a 50% reduction of protein synthesis (measured by ^{14}C-labelled amino acid uptake) in those cells treated with BAb and RAC compared to controls (Webb *et al.*, 1985). These investigators also produced a BAb recognising a prostate-restricted Ag and pokeweed anti-viral protein, which was equally effective in similar cytotoxicity tests (Webb, 1988; Webb *et al.*, 1988).

More extensive BAb testing was performed using an anti-carcino-embryonic Ag (CEA)/anti-vinca alkaloid BAb (Corvalan *et al.*, 1987a&b; Corvalan *et al.*, 1988). BAbs selectively targeted vinblastine to CEA-bearing cells as shown by examination of histological sections of human colorectal cancer. An *in vivo* tumour model was set up using nude mice bearing established MAWI human tumour xenografts. Tritiated (^{3}H)-vinblastine distribution was measured in tumour, visceral organs and plasma following administration of the BAb (day 0) and the radio-labelled drug (day 14). Results indicated a ten-fold higher concentration of radio-labelled vinblastine in the tumour following BAb administration (Corvalan *et al.*, 1987a). The efficacy of the drug targeted with BAb was estimated in the same model using tumour volume, tumour weight and subsequent histology as guidelines. These tests showed that BAb-targeted drug is available in the active form and, for a given dose, is more effective at tumour supression than free drug, probably due to the increase in local concentration, mediated via the BAb (Corvalan *et al.*, 1987b). The drug was found to be most effective when administered simultaneously with the BAb (Corvalan *et al.*, 1988).

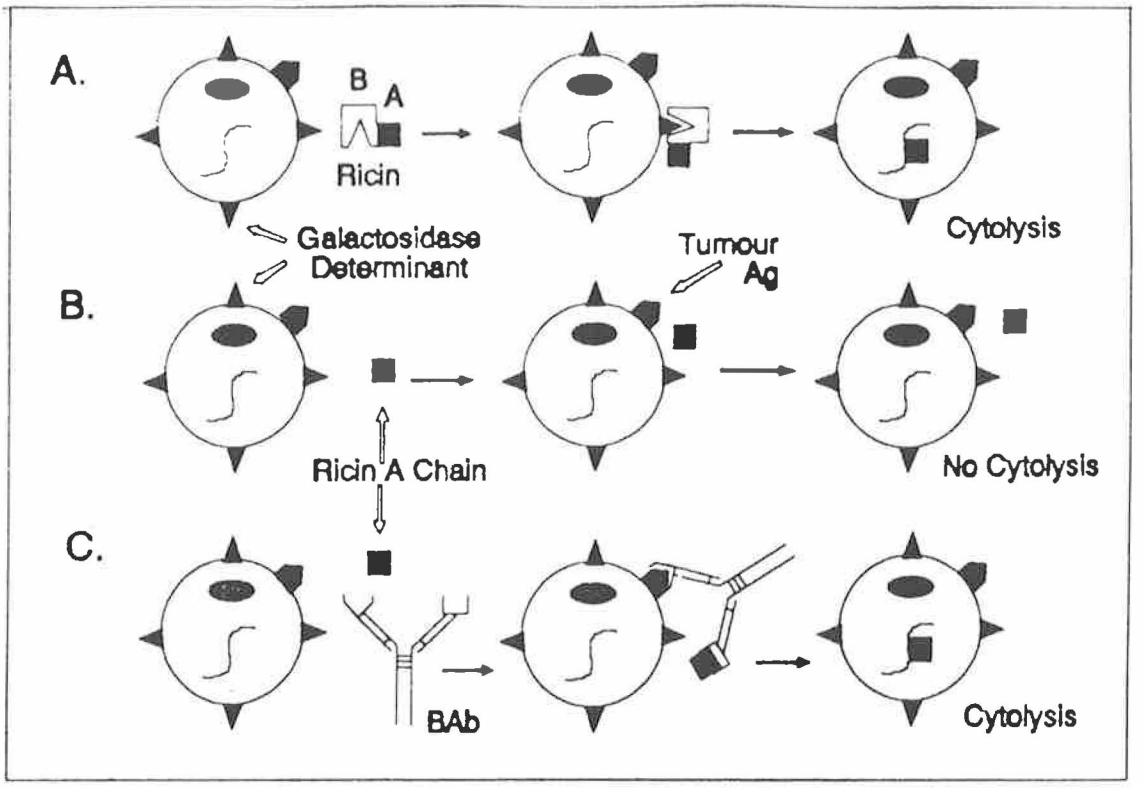


Figure 1.3.1:

Ricin chemotherapy. (A) Administration of the ricin complex leads to binding of the cell by the B-chain, the A-chain enters resulting in ribosome inactivation. (B) Administration of the ricin A-chain has no cytotoxic effect as it cannot enter the cell. (C) Administration of ricin A-chain and a bispecific antibody leads to binding of the cell and the A-chain by the bispecific antibody, the A-chain enters the cell resulting in ribosome inactivation.

This model also illustrates the efficiency of BAbs in a more realistic cancer model system. Both BAbs with drug and MAb-drug conjugates caused tumour suppression in freshly implanted tumour xenografts. However, in well established xenografts (3 weeks) significant suppression was obtained only following treatment with BAb and vinblastine (Corvalan *et al.*, 1987b). These MAWI human tumour xenografts, implanted in nu/nu mice, were in the exponential growth phase and constituted 2% of body weight. Maximum cytostasis was achieved only following the establishment of the optimum drug:BAb ratios. *In vivo* tumour suppression was sustained for two months after withdrawal of treatment. Histological examination indicated a reduction in the cellularity, but not size, of these tumours. In addition, it was shown that those tumour cells remaining after a two month lapse in treatment still express CEA on the cell surface. This would indicate the potential succcess of re-challenge with the same treatment protocol (Smith *et al.*, 1990).

BAbs to several other tumour and drug types have been produced from quadromas. These antibodies have been tested both *in vitro* and *in vivo*. A BAb was produced which bound to both an osteosarcoma cell line and a carrier protein (Pimm *et al.*, 1990). Methotrexate (MTX) was conjugated to the carrier protein. This resulted in a forty fold increase in MTX concentration at the cell surface when targeted with BAb, compared to a MTX-MAb conjugate. Not all this extra MTX is available or active, however, since similar rates of cell death were recorded in *in vitro* assays comparing BAb targeting with MTX-MAb conjugate targeting.

A BAb reacting with both a rat hepatoma cell line and the anthracycline drugs was produced (Tsukada *et al.*, 1989). The cell line was grafted into Donryu rats to set up a tumour model with micrometastases. This model was used to compare the therapeutic effectiveness of this BAb with that of a MAb-drug conjugate and controls. It was found that, in order to produce the same survival rates (60 days), one and a half times more drug was required with the MAb-drug conjugate than when the drug was administered and targeted with the BAb. Blood α-fetoprotein (AFP) levels were also lower following BAb therapy, indicating preferential activity of the BAb and drug over the MAb-drug conjugate.

Saporin, the ribosome-inactivating protein, has been targeted to lymphoma cells by the use of BAbs (French *et al.*, 1991). It was found that the use of polyclonal anti-saporin Ab in the production of BAbs, increased the potency of a given dose 10- to 20-fold. Best results were obtained when several anti-saporin MAbs were used synergistically in conjunction with an anti-CD22 MAb to produce several BAbs with similar affinities. (Clusters of

Differentiation (CD) are Ags on membrane proteins of human blood cells that define stages of differentiation of these cells.) Targeting via the CD22 increased internalisation of the drug and, hence, cytotoxicity by up to 1,000 times compared to targeting via CD19 or CD37. Use of pairs of BAbs with affinity for different epitopes on the same Ag gave a 10-fold increase in cytotoxicity compared to using a single BAb (Bonardi *et al.*, 1992; Bonardi *et al.*, 1993). This type of therapy has also been shown to be successful using BAbs targeted against CD7 and CD38 on the cell surface (Flavell *et al.*, 1992). It would appear that multiple cell surface Ag targeting alleviates the problem of small numbers of cells within the diseased population not expressing the target Ag. Such cells would not be targeted and destroyed if only one BAb was used. It also serves to increase the drug concentration at the target site.

1.3.2 Effector Cell Retargeting:

The administration of murine anti-TAA MAbs alone to humans has generated widely varying results. In cases in which MAb administration resulted in elimination of tumour cells, the most likely mechanisms for the destruction have been:

- regulatory - as in anti-idiotype treatment of lymphomas;
- resulting from immunisation - anti-anti-idiotype human Ab production, thought to recognise the original tumour epitope;
- indirect - based on Ab-dependent cell-mediated cytotoxicity (ADCC);
- directly - stimulating complement fixation that results in cell lysis.

Unfortunately, oncology patients' immune systems are often compromised from prior therapy, tumour burden, or both. Thus, the patient may be unable to benefit from MAb therapy via these mechanisms. Although some remissions have been observed, there is no convincing evidence of any cancer being cured exclusively by administration of MAbs (Reading & Bator, 1988). A more successful means of stimulating the patients immune response to a tumour has been achieved through the use of BAbs. In directed or redirected cytotoxicity, BAbs bind both to target cells (pathogens or tumours) and to cytotoxic trigger molecules on leucocytes such as TcR or FcγR. BAbs can thus be used to focus normal cellular immune defence mechanisms specifically to the tumour cell or infectious agent (Fig. 1.3.2). In the likely event that the patient's immune system has been depleted by disease or treatment, the cellular defence mechanisms can be augmented by the extra-systemic stimulation, and subsequent reinfusion, of effector cells.

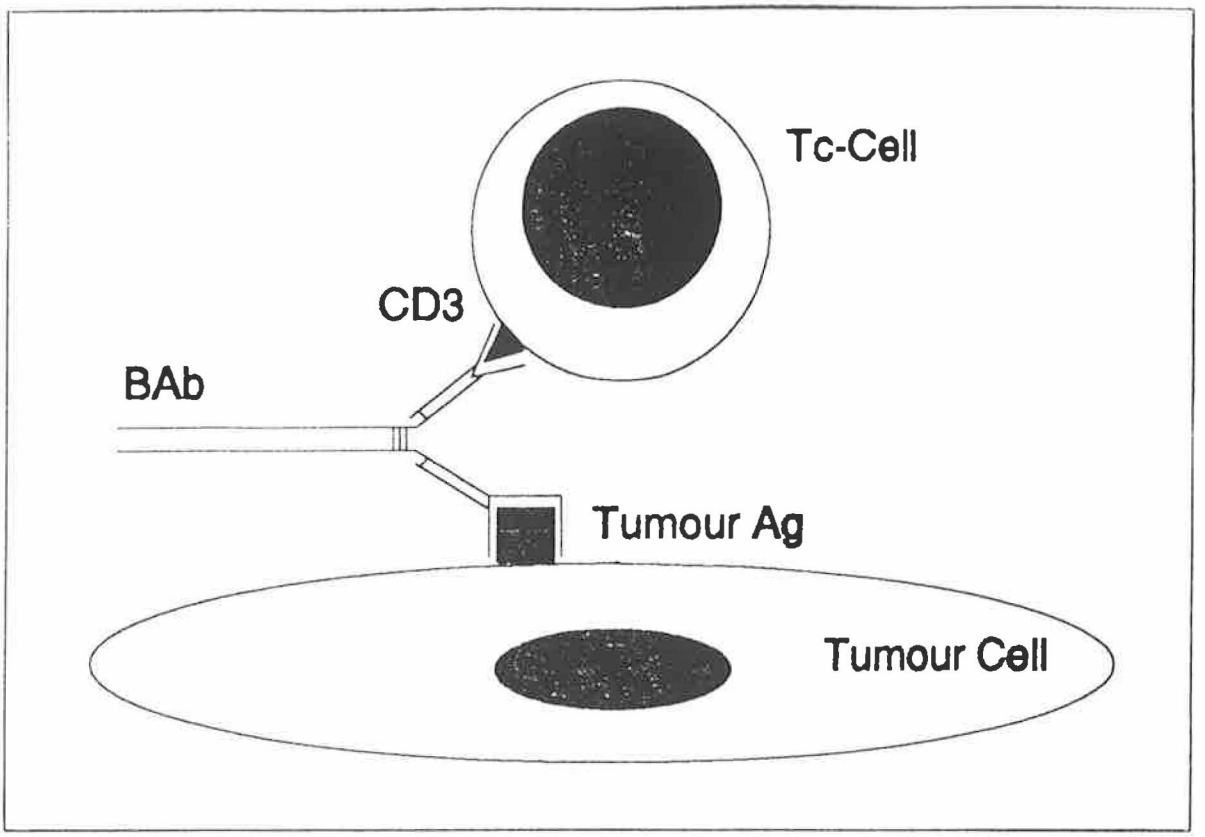


Figure 1.3.2:
Effector cell re-targeting. A bispecific antibody, which binds to a triggering molecule (CD3) on a cytotoxic T-cell and a tumour associated antigen, is used as a bridging agent to bring a cytotoxic T-cell in close contact with a tumour cell. Stimulation of the T-cell results in cytolysis of the tumour cell.

1.3.2a THE COMPONENTS OF THE SYSTEM:

It has been suggested that T-cells would be the most efficient cells to redirect with BAbs (Ferrini *et al.*, 1989a&b). These cells represent a homogenous cytolytic population and their high motility and unique homing properties increase their ability to reach target cells. The primary cytotoxic trigger complex on T-cells is the TcR complex, which consists of a non-covalent association of T_i heterodimers (α/β or γ/δ) with the CD3 molecular complex. The TcR is normally Ag-specific and major histo-compatability complex (MHC)-restricted. However, BAbs can react with this complex and initiate cytotoxicity that is not MHC-restricted, and can thus redirect the cytolytic action of virtually all available T-cells to the Ag of choice (Perez *et al.*, 1985). CD2 can also initiate T-cell mediated cytotoxicity. However, the engagement of two different epitopes on this molecule may, under some conditions, be required for initiation of cytotoxicity (Scott *et al.*, 1988). CD28, in conjunction with CD2 or CD3, provides a co-stimulatory signal that plays a major role in T-cell activation. Interaction of CD28 with its ligand provides an Ag derived co-stimulatory signal resulting in interleukin-2 (Il-2) production by human T-cells (Ferrini *et al.*, 1992). Other T-cell surface Ags that have been used in effector cell retargeting include CD4, CD8 (Wong *et al.*, 1989; Gorter *et al.*, 1992) and CD16 (Weiner *et al.*, 1993a&b).

Other effector cells of the immune system have also been retargeted using BAbs. FcγRs appear to be the only molecules on human myeloid cells capable of mediating ADCC of tumours. The only requirement for triggering of FcγR function appears to be receptor cross-linking. There are three structurally and functionally distinct classes of FcγR. FcγRI (CD64) is highly expressed only on monocytes and macrophages, and is a potent cytotoxic trigger on these cells. It has been used successfully as a target molecule in BAb-mediated effector cell retargeting (Chokri *et al.*, 1992; Deramount *et al.*, 1992). FcγRII (CD32) is a trigger molecule on monocytes, macrophages and eosinophils that can mediate target cell lysis and/or phagocytosis when used in conjunction with BAbs (Greenman *et al.*, 1991). It is also expressed on platelets and can trigger platelet aggregation, thus blocking BAbs from binding to potent effector cells. FcγRIII (CD16) is present on macrophages and the natural killer (NK) cell/large granular lymphocyte (LGL) population and can act as a cytotoxic trigger molecule for these cells. Resting cells targeted, via this molecule and a BAb, to tumour cells have been stimulated to active cytolysis of the target cells (DePalazzo *et al.*, 1990; Ferrini *et al.*, 1991; DePalazzo *et al.*, 1992a&b; Greenman *et al.*, 1992; Hsieh-ma *et al.*, 1992; Ferrini *et al.*, 1993).

Circulating lymphocytes from healthy donors are typically in a resting state and must be activated before they will perform cytolytic functions. Similarly, optimal cytotoxicity by BAbs may also require activation of the effector cells by immune cytokines, in addition to specific trigger molecules. The predominant cytokines involved are Il-2 and gamma-interferon (IFN-γ). Culture of effector cells in Il-2 conditioned medium for up to five weeks prior to treatment was shown to greatly enhance their cytolytic effect following targeting with BAbs (Nitta *et al.*, 1991; Chen *et al.*, 1992; Haagen *et al.*, 1992). In addition, *in vivo* studies in immuno-competent mice, with tumour implants, showed that when BAb and effector cells alone were administered 75% mortality occurred. However, when Il-2 was administered along with the effector cells and BAb mortality fell to 18% (Weiner, 1992). Pre-treatment of tumour cells with IFN-γ was shown to induce expression of intercellular adhesion molecule 1. This, in turn, enhances BAb-directed NK cell cytotoxicity (Azuma *et al.*, 1992). Cytotoxicity triggered by BAbs may also include the release of cytokines that are toxic to the target cell. This effect has been shown to have a significant role in cytolysis of target cells, as triggered by BAb binding, and may aid in the destruction of bystander, Ag-negative tumour cells (Qian *et al.*, 1991; Segal *et al.*, 1991).

1.3.2b *IN VITRO* STUDIES:

BAbs have been extremely useful in probing the functional properties of cell surface molecules and in defining the ability of the different FcγR to mediate cytotoxicity by the various effector cells, as well as the effect of cytokines on these activities. Overall, these studies indicate that the different classes of FcR have different capabilities for directing cellular cytotoxicity, and that their function seems to be dictated by the isoform of the receptor, the cell type on which it is displayed and the state of activation of the cell. BAbs have been used to study T-cell signalling pathways. Anti-CD3/anti-CD8 BAbs were shown to cause Il-2-dependent proliferation of these cells (DeLau *et al.*, 1992). It is speculated that Ag stimulation causes CD3 and CD8 to be expressed in close proximity, as mimicked by the BAb, which in turn facilitates Il-2-dependent proliferation. Similar studies have been carried out on the Il-2 receptor (Il-2R). A BAb binding to both α- and β-chains of the Il-2R can completely block Il-2 stimulation of T-cell proliferation (Francois *et al.*, 1993a&b). This has therapeutic potential in the prevention of allograft rejection.

The earliest *in vitro* studies examined the potential for therapy of BAb-directed effector cell retargeting, human T-cells were targeted to

tumour cell lines causing high levels of cytolysis (Lanzavecchia & Scheidegger, 1987; Clark *et al.*, 1988). Effector cell retargeting was also carried out using an anti-globulin step. Murine MAbs were bound to the tumour cells, a BAb was applied which was specific for mouse IgG and a T-cell Ag. This extra step increases the range of tumours to which a single BAb may be applied (Gilliland *et al.*, 1988). Subsequently, BAbs have been used to target cytotoxic cells to a number of cell lines, these include; murine B-cell lymphomas (Clark & Waldman, 1987; DeManet *et al.*, 1992), ovarian carcinoma (Canevari *et al.*, 1988; vanRavensway-Classen *et al.*, 1993), multi-drug resistant tumour cells (vanDijk *et al.*, 1989) and human immuno-deficiency virus (HIV)-infected cells (Okada *et al.*, 1992). Finally, BAbs have been used to detect the presence of activated T-cells, those expressing CD3 and/or Il-2R, and to target cytotoxic or NK cells to destroy them (Kaneko *et al.*, 1992; MacLean *et al.*, 1993). This has therapeutic potential in cases of granular lymphocyte proliferative disorders, auto-immune disease and graft rejection where immune-suppression therapy is indicated.

1.3.2c *IN VIVO* STUDIES:

BAb targeting of T-cell cytotoxicity has shown promise in several rodent model systems. These *in vitro* and animal model studies have provided direction for the initiation of human clinical trials. BF(ab')2 molecules were used to study targeting of T-cells to murine lymphoma cells grown as ascitic tumours in mice (Clark *et al.*, 1989). It was later shown that BAb and effector cells administered nine days after the tumour had been established, could initiate a complete cure, with no remissions recorded at day 150 (Brissinck *et al.*, 1991). This model also showed that T-cell to target cell binding was essential for activation of the T-cell, an important consideration if systemic activation of T-cells is to be avoided. A similar model was set up in immune-competent mice (Weiner & Hillstrom, 1991). This model illustrated that T-cells were more effective than macrophages, hence it is the BAb and not the Fc portion of the Ab that is responsible for target cell lysis. A subcutaneous xenograft of a solid tumour, human renal cell carcinoma, was examined by vanDijk *et al.* (1991). In this model the parental IgG and F(ab')2 were compared to the BF(ab')2 for their ability to localize to the tumour. The lowered affinity of the BF(ab')2 for the target, with respect to the parental molecules, had very little effect on the quantity of Ab localized to the tumour. A BF(ab')2, which has a similar size to a F(ab')2 molecule, was shown to have a higher tumour:non-tumour binding ratio than the larger IgG. This would indicate that localization to solid tumours depends more on size than affinity. Recently, a rat colon

carcinoma model has been developed which more closely resembles the human situation (Beun *et al.*, 1992; Beun *et al.*, 1993a&b). Here, the tumour-host relationship is weakly or non-immunogenic, liver metastases are common and spontaneous regression does not occur. The murine Abs used in therapy are xenogenic, which also mimics the clinical application. Significant lysis of the tumour was shown to occur following administration of effector T-lymphocytes, which had been pre-treated with an anti-T-cell/anti-colon carcinoma BAb. Some activation of resting T-cells by the BAb was also evident.

A murine model has been developed in which ovarian carcinoma implants were established in athymic mice (Mezzanzanica *et al.*, 1991a&b; Wunderlich *et al.*, 1992). This model showed that normal or activated lymphocytes administered, along with BAb, four days after the tumour cells were established, caused a significant reduction in tumour growth rates and doubled the host survival time. This model was developed for use in clinical trials using BAb and activated T-cells to treat patients with advanced ovarian carcinoma with some success (Pupa *et al.*, 1988; Mezzanzanica *et al.*, 1991a&b). A multicentre study of this treatment showed that significant tumour regression was accompanied, in some cases, by a marked local inflammatory response. In addition, some patients developed a HAMA reaction (Bolhuis *et al.*, 1992). A separate clinical study was carried out using a less specific BAbs, anti-CD3/anti-TAA (Kroesen *et al.*, 1993). This BAb will bind to cytotoxic T-cells and to a number of tumour types. The patients chosen for the trial all suffered from malignant ascitic tumours, arising from several primary tumour types. Lymphocytes were activated with Il-2 and administered at the tumour site along with the BAb (i.e. by intra-peritoneal injection (i.p.)). This treatment resulted in a significant local reduction in tumour load, accompanied by a strong inflammatory response. Since the inflammatory reaction does not occur in the absence of BAb, it is thought that T-cell to tumour cell cross-linking triggers local cytokine release, resulting in granulocyte influxes and inflammation.

1.3.3 Fibrinolytic Agents:

Plasminogen activators (PA) trigger the conversion of plasminogen to the fibrinolytic enzyme plasmin, which causes dissolution of fibrin-containing thrombi. These agents are used clinically following acute myocardial infarction, but in amounts that may result in severe bleeding as a side effect. It has been suggested that, if PAs could be focused to fibrin-containing clots widespread fibrinolysis, and the resultant haemorrhagic

side effects, could be prevented. BAbs were produced, by somatic cell fusion and by chemical coupling of Fab' fragments, which bound to fibrin and tissue plasminogen activator (tPA). In a solid phase assay, the BAb produced by cell fusion caused a 5-10-fold increase in tPA activity when they were applied together, compared to the tPA applied alone. This same BAb enhanced clot lysis *in vivo* approximately 2-fold in the rabbit jugular vein model of thrombolysis (Branscomb *et al.*, 1990). Similar results were obtained using chemically produced BF(ab')2s, which caused 10-fold more fibrinolysis *in vitro* than tPA alone. The smaller BF(ab')2 molecule may have the advantage in *in vivo* studies due to its increased diffusibility and reduced immunogenicity. Other studies have explored the use of BAbs to target urokinase-type plasminogen activator (uPA). The presence of BAb has increased the potency of tPA and uPA 10-20-fold in an *in vitro* assay, when compared to tPA or uPA alone (Bos & Nieuwenhuizen, 1991). In an *in vivo* hamster model of pulmonary embolism, the thrombolytic potency of recombinant uPA was enhanced 13-17-fold relative to that of uPA alone and 4-fold in the rabbit jugular vein thrombosis model (Kurokawa *et al.*, 1991). Neither of these models showed any major systemic activation of fibrinolysis or haemorrhagic side effects. The use of BAbs in this area has recently been reviewed (Bos & Nieuenhuizen, 1992).

1.3.4 Radioimmunoscintigraphy & Radioimmunoassay:

Radiolabelled MAbs have been used successfully in the imaging and treatment of some solid tumours. Immunotherapy depends on the absolute amount of Ab taken up by and remaining on or in the tumour, whether the Ab is linked to a toxin, radionuclide or chemotherapeutic agent. For radioimmunoscintigraphy it does not matter if only a fraction of the cells in a tumour mass bind the Ab. For radioimmuno-therapy, depending on the radionuclide, the majority of all the viable tumour cells must bind the Ab. Lack of a requirement for internalization and the beneficial effects of cross-irradiation are advantages of radio-immunotherapy over other immuno-conjugates. Access to the tumour site may be a limiting factor. For radioimmunoscintigraphy it does not matter if only the outer cells of the tumour mass take up the radiolabelled Ab because a signal is still obtained from the tumour. For radioimmuno-therapy penetration throughout the tumour is required. This is one of the reasons why radioimmunotherapy requires a large amount of Ab (approx. 40mg) whereas radioimmunoscintigraphy is generally successful with lesser amounts (approximately 0.2-2mg). The requirement for tumour penetration is all the more critical when radio-sensitivity is considered.

Tumour that is less well oxygenated is less radio-sensitive so the deeper parts of large tumours not only receive less radiolabelled Ab but the radiation they receive is less effective. (Haller, 1988; Britton *et al.*, 1991)

It has been suggested that diagnosis and treatment could be improved by the use of BAbs. A BAb has been produced which binds CEA and BLEDTA IV, an ^{111}In-benzyl EDTA derivative (Gridley *et al.*, 1988; King *et al.*, 1988). The ability of this BAb to localise BLEDTA IV in T380 human tumour xenografts in nude mice has been established (King *et al.*, 1988). Using the same model, it was shown that hyperthermia enhances localisation of BLEDTA IV to the BAb in the xenograft (Gridley *et al.*, 1991b). One day after BAb administration, the animals were heated to 41.5^{o}C for 45min. After a further two hours the BLEDTA IV was administered. Of the administered dose, 4.46% per gram of tumour was localised to this tumour in the hyperthermic mice, whereas only 1.44% per gram of tumour was found in the xenografts of non-hyperthermic mice (measured by auto-radiography and radioactivity readings of the excised tumours).

In clinical studies, patients with colorectal carcinoma or melanoma were injected first with BAb, then with radiolabelled hapten. This system allowed the BAb to accumulate in the tumour with "wash out" from other tissues before administration of the labelled hapten (Gridley *et al.*, 1988; LeDoussal *et al.*, 1990; Stickney *et al.*, 1991; LeDoussal *et al.*, 1992; Somasundaram *et al.*, 1993). Tumour was detected, within the liver and at other locations, using gamma(γ)-camera and immunoscintigraphy. Targeting of known lesions was 84%, and new lesions were discovered (Gridley *et al.*, 1988). Although localisation of radiolabel was reduced, the tumour:target cell ratio was increased, resulting in a three-fold increase in imaging efficiency when compared to labelled MAb (LeDoussal *et al.*, 1992). The BAb had a faster blood clearance and there was less uptake by the bone marrow and liver than the parent MAb, thus reducing side effects and non-specific binding (Somasundaram *et al.*, 1993). This would be advantageous, particularly in the event of this system being used for radio-immunotherapy. A side effect of such treatments is the immuno-modulation of leucocyte populations following BAb and radioactive hapten administration. The responsiveness of the patient's lymphocytes is markedly reduced. Conversely, the cytotoxic activity of NK-cells, including oxygen radical production, is increased (Gridley *et al.*, 1991a).

Therapeutic doses of targeted radiolabelled hapten must be higher than those used for diagnostic purposes, since deep penetration of solid tumours is required. The long half-life of labelled Ab results in high levels of irradiation of normal tissues. With BAb the radiolabelled hapten, which

is rapidly cleared by the kidneys, can be injected after the BAb has been localised in the tumour, so reducing damage to normal tissues (Britton *et al.*, 1991). Alternatively, the specificity of radiolabelled Abs could be improved. It has been shown that BAbs directed towards two diferent Ags on the target cell surface will increase the specificity of the BAb to that cell when compared with MAbs, which bivalently bind one Ag (Wong & Colvin, 1987).

1.3.5 Immunochemistry & Enzyme Immunoassay:

Traditionally, cell types and certain hormones and peptides have been visualised by the use of conventional immunochemistry or ELISA. The practice of immunochemistry originated with Albert H. Coons and his colleagues (Coons *et al.*, 1941 & 1955) who were the first to label an Ab with a fluorescent dye, and use it to identify an Ag in tissue sections. As a result of the absolute specificity of the Ab-Ag reaction, positive identification of tissue constituents was achieved and much of the uncertainty was removed from some aspects of histopathology. The first fluorescent dye to be attached to an antibody was fluorescein isocyanate, but fluorescein isothiocyanate (FITC) soon became the label of choice because the molecule is much easier to conjugate to the Ab. These compounds fluoresce when excited at a wavelength of 490nm and are easily visible against a non-fluorescent background. The first enzyme to be used was peroxidase (Avrameas & Uriel, 1966; Nakane & Pierce, 1966), other enzymes include alkaline phosphatase (Mason & Sammons, 1978) and glucose oxidase (Suffin *et al.*, 1979). The development of the enzyme-substrate colour reaction identifies the Ab binding site. Enzyme-labelled Abs have the advantage that an ordinary transmitted-light microscope can be used and the preparations are permanent. The development of the enzyme reaction is progressive and can be monitored and stopped when the 'signal-to-noise' ratio is adequate. The label:Ab ratio used in conjugation must be carefully calculated. Ab activity may be altered by an excess of label and un-conjugated label must be removed by dialysis or column chromatography before the Ab can be used. All Ab molecules must be labelled as any un-labelled Ab present will compete for binding sites with the labelled Ab and reduce the visibility of the final reaction.

In his original method Coons (Coons & Kaplan, 1950) used a one-step (direct) staining method in which the specific Ab was conjugated to FITC. The conjugated antiserum, diluted in PBS, was allowed to react with a tissue section and the unbound Ab was then washed off with PBS. The section was examined in an ultra-violet microscope and the site of

attachment of the Ab fluoresced apple green. The direct labelling method was subsequently adapted to the indirect immunofluorescence technique (Coons *et al.*, 1955) which is still widely used. In this method the primary Ab is not conjugated, but a second layer is added which consists of an Ab raised to the Ig of the species which donated the first Ab, e.g. goat anti-rabbit Ig, which is conjugated to FITC. The primary Ab, rabbit anti-Ag, which is bound to antigenic sites in the tissue section, then acts as an Ag for the second fluorescent Ab. The indirect method often produces an amplified signal, since two labelled anti-Ig molecules can bind to each primary Ab molecule. In addition, conjugation of a label to the primary Ab is entirely avoided, along with the damage to its reactivity which that entailed. The incubation time required to allow adequate binding of primary and secondary Ab ranges from 1 to 48 hours, depending on the activity and dilution of the Ab. Similar methods are applied with enzyme-labelled Ab. Other methods involved the use of a second Ag (hapten) as a label, visualised by a second Ab, the exploitation of the strong attraction between avidin and biotin, and numerous ways of improving the specificity and intensity of the final reaction and of carrying out multiple staining, (Polak & vanNoorden, 1987).

Immunochemistry is applied only to tissue components, and so is limited in application to histological and histo-pathological investigations. The development of enzyme immunoassays occurred with the realisation that Ag or Ab could be immobilised onto solid supports with retention of their immunological reactivity. This gave rise to the enzyme-linked immunosorbent assay (ELISA), for the quantitative measurement of Ag (Engvall & Perlmann, 1971; Van Weemen & Schurs, 1971). As a result, the possible applications of enzyme-labelled Ab were greatly increased. In its simplest form, ELISA technique closely resembles immunochemistry. The first step is the immobilisation of Ag to a solid phase, most commonly a polystyrene plate with 96 wells, each holding 150-250 µl. Non-specific binding sites are blocked using a high molecular weight protein (e.g. BSA). The second step is the binding of the primary Ab. This can be enzyme-labelled (direct method) or un-labelled (indirect method). Unbound Ab is washed away with PBS. Secondary or labelled Ab is then applied for the indirect method. This is often the preferred method, since one labelled Ab can be used in a number of different assays. In all cases, the amount of enzyme activity is detected by its conversion of a colourless substrate to a highly coloured product that can be visually or spectrophotometrically determined. Despite mechanisation and other improvements, this procedure can take from six to twenty four hours, depending on the

conditions and reagents in use. The use of BAbs could considerably shortened these procedures. Once the plate has been coated and blocked, BAb and enzyme can be added simultaneously, and the plate developed in the usual manner. Such techniques reduce the time for the assay without loss of specificity for Ag. One possible disadvantage of this technique is the loss of the amplification step available with the indirect method. This problem is less relevant in immunochemistry than in ELISA. BAbs also allow for the development of novel assay techniques not possible with labelled MAbs (Paulus, 1985; Nolan & O'Kennedy, 1993).

Hammerling *et al.* (1968) used chemically produced BAbs, with anti-IgG and anti-ferritin specificities, to locate surface Ags by electron microscopy. Biologically produced BAbs have been applied successfully to the immuno-chemical analysis of somatostatin and substance P, one arm of the Ab reacting with the peptide the other binding horse-radish peroxidase (HRP) (Suresh *et al.*, 1986). These same principles have been applied to ELISAs. Karawajew *et al.* (1988) have used BAbs as bridging reagents for ELISAs for human chorionic gonadotrophin (hCG) and AFP. A MAb, raised against hCG or AFP, is labelled with FITC. The BAb binds both the FITC-labelled MAb and HRP, and the ELISA is developed using any HRP substrate. The sensitivity was found to be similar to conventional ELISAs. An anti-hCG/anti-alkaline phosphatase IgM-like Ab has also been used to develop an assay for hCG with sufficient sensitivity to use in a pregnancy test (Behrsing *et al.*, 1992). A urease-based immunoassay for hCG has also been described which utilises BAb binding both urease and hCG. This assay system could detect 25 mIU/ml hCG (Takahashi & Fuller, 1988). Some BAbs have been shown to have greatly increased sensitivity compared with conventional systems. A BAb was produced reactive against both β-galactosidase and luteinising hormone (Bugari *et al.*, 1990). This had low reactivity with hCG which commonly interferes with luteinising hormone asays. When used in an ELISA, the BAb detected concentrations as low as 0.8 IU/ml. This compared well with conventional ELISA (2.0 IU/ml), radio-immunoassay (1.5 IU/ml) and immuno-radiometric assays (0.5 IU/ml). Other BAbs result in reduced sensitivity compared with conventional systems. An anti-Il-2/anti-HRP BAb was used to develop an ELISA which could detect Il-2 in the range 1.5-4.0 ng/ml. This compared un-favourably with standard methods which were capable of detecting 0.2 ng/ml (Stratieva-Taneeva *et al.*, 1993). The use of BAbs in ELISA should still be considered, however, since the simultaneous addition of reagents eliminates unnecessary washings and additions, thus reducing time required and increasing the reproducibility of the assay.

Apart from basing assays on conventional systems, there are many potential novel immunoassay formats. BAbs have been used to develop a homogeneous enzyme immunoassay system (Gorog *et al.*, 1989). Anti-CEA/anti-β-galactosidase BAbs were produced which were capable of protecting the enzyme from thermal denaturation. Having incubated CEA and β-galactosidase in the presence of BAb, heating the solution to 62°C for 1 hour destroyed the free enzyme. Under appropriate conditions, heat-resistant enzyme activity was shown to be proportional to the concentration of CEA in the range up to 75 ng/ml, the range likely to be of clinical significance. Other assays have been postulated but few so far have been achieved (Paulus, 1985). Enzyme immobilisation to a solid matrix, or multi-enzyme immobilization, by BAb has the potential to greatly enhance responses in enzyme-dependent assays. Similarly, with enzyme electrodes, the reaction time can be speeded up by using BAb to link the enzymes directly to the permeable membrane covering the electrode. Multi-enzyme sequences can be used to increase the analytical repertoire of the immunoassays and enzyme electrodes. With a protein analyte as a bridge, a pair of BAbs directed against different epitopes on an analyte can bring two indicator proteins into close proximity and thereby generate a measurable signal. Again a variety of detection systems are possible.

1.3.6 Summary:

Several MAbs have been produced and used, with varying degrees of success, in immuno-therapy and immuno-diagnosis. Their use has been curtailed, particularly in immuno-therapy, by problems which include the immunogenicity of the immuno-conjugate and loss of activity resulting from conjugation. Some of these problems may be overcome by the use of BAbs. BAbs are monovalent, bispecific, Ab-derived molecules. BAbs can be produced by both biological and chemical means and can be used to bind two separate Ags simultaneously. Production methods have improved greatly in recent years. BAbs have been tested both *in vitro* and *in vivo* and found to efficiently destroy targeted tumour cells. In many instances BAbs were found to have greater efficacy than the corresponding MAb conjugate. As well as tumour cells, other diseased tissues can be targeted by BAbs for therapy. The area of immuno-diagnostics has not been developed to the same extent as immuno-therapeutics. This project sets out to look at the chemical production of BAb. In particular, the use of BAbs in immuno-assays will be examined. It will be some time, however, before the full potential of BAbs as a powerful tool in both immuno-diagnostics and immuno-therapeutics is realised.

1.4 LEUKAEMIA

Tumours of haemopoietic tissue may involve lymphoid cells, myeloid cells or erythroid cells. They are mostly malignant sarcomata. Those arising from tissues that form leukocytes are called leukaemias and may be myeloid, lymphatic or monocytic. Each of these may arise in the chronic or the acute form. Human leukaemias were first recognised about 165 years ago, but a century passed before acute/chronic lymphocytic leukaemia and acute/chronic myelogenous leukaemia were clinically differentiated. The clonal nature of each of these disorders has since been established, (Bowman & Rand, 1984).

1.4.1 Acute Leukaemia:

Acute leukaemia is a general term for a group of malignant disorders of blood leukocytes. Age at onset of disease and certain morphological characteristics have significant implications for patient survival and responsiveness to chemotherapy. Acute lymphocytic leukaemia (ALL) is the predominant form of leukaemia in childhood and the most common form of cancer in children. The usual cell of origin appears to be a "null" lymphocyte. These children have a relatively good prognosis. A subset of patients with a poor prognosis have neoplastic disease expressing surface antigenic features of T-lymphocytes. A cytoplasmic enzyme expressed by normal thymocytes, terminal deoxycytidyl transferase, is also expressed in many cases of ALL. T-cell ALL also express high levels of the enzyme, adenosine deaminase. Cell mediated immunity to ALL can be demonstrated most readily when the patient enters remission. The median survival in childhood acute leukaemia is now approaching four years. Acute leukaemia in adults is predominantly of the myelocytic variety, although some cases of lymphoblastic leukaemia are also seen. Acute myelogenous leukaemia has been quite dificult to treat, with the median survival times in the range of three months. (Katzung, 1987)

1.4.2 Chronic Myelogenous Leukaemia:

Chronic myelogenous leukaemia (CML) is rare in childhood in western populations, but a small peak in incidence has been observed internationally in white males under age five. Incidence of the adult type CML begins to rise in adolescence among both sexes and increases steadily with age. A number of environmental factors, particularly radiation exposure, have been asociated with CML development. CML arises from a chromosomally abnormal hematopoietic stem cell. The Ph^1 chromosome

associated with CML was the first leukaemia chromosomal abnormality to be identified. The Ph^1 chromosome is a chromosome 22 that has lost its long arm due to a reciprocal translocation with chromosome 9. This combination produces a chimaeric messenger RNA, which translates into a larger protein than normal with *in vitro* tyrosine kinase activity. Several growth factor receptors are tyrosine kinase-linked and it is recognised that this abnormal protein can confer growth factor independence to several cell lines. Presence of the Ph^1 chromosome is indicative but not diagnostic of CML. Diagnosis of CML is generally not difficult to establish. However, mis-classification has been estimated to occur in approximately 10-15% of patients with this disorder. Some patients are incorrectly diagnosed as having one of the closely related myeloproliferative diseases such as polycytaemia vera, especially if they are among the 10% with Ph^1 chromosome-negative CML. Survival in CML is extremely variable but on average median survival is in the range 40-47 months, (Finch & Linet, 1992).

1.4.3 Chronic Lymphocytic Leukaemia:

Chronic lymphocytic leukaemia (CLL) is rare in persons under 30 years, but incidence increases dramatically with age for both sexes. CLL is the leukaemia type with the highest incidence among persons aged 50-55 years and older. About 95% of persons with CLL have the mature B-cell type, less than 5% have adult T-cell leukaemia an aggressive variant of CLL caused by the human T-cell lymphotropic virus type 1 (HTLV-I), while less than 2% have hairy cell leukaemia. CLL has been linked to exposure to acid-containing chemicals, aliphatic and chlorinated hydrocarbons and certain organic solvents. Links to other environmental factors remain tenuous and putative causative agents are not well established. Familial aggregation of CLL has been reported. An underlying inherited chromosome abnormality (e.g. of chromosome 12) or a high frequency of chromatid exchange figures could predispose to CLL, (Finch & Linet, 1992).

B-cell CLL is characterised by sustained lymphocytosis of greater than $10^{10}/l$ in peripheral blood with mature appearing lymphocytes and bone marrow lymphocytosis of at least 30% in bone marrow aspirates. In typical cases of CLL the cells are small with a narrow rim of cytoplasm. The nuclear chromatin is dense and nucleoli are not visible. Morphologically they resemble small resting B-cells. CLL cells express surface membrane Ig (SmIg), C3dr complement receptors and receptors for the Fc fraction of Ig. SmIg are constantly restricted to a single light chain, and frequently express IgM or both IgM and IgD. CLL B-cells also express several Ags, including

D-related human leukocyte Ag and Ags related to B-cells. Most cases of B-cell CLL appear to react with anti-CD19, CD20, CD24, CD37 and CD21 MAbs. About 60% of CLL are positive for CD23, whereas membrane positivity with CD22 is infrequent. CLL B-cells have been shown to express a 69 kDa glycoprotein that is not expressed by normal blood T and B lymphocytes, thymocyte-cultured T- and B-cell lymphoblastoid cell lines, or acute lymphoblastic leukaemia cells (Dighiero *et al.*, 1991). The G12 Ab, produced in our laboratory, has been shown to bind to a CLL specific, cell surface Ag of molecular weight 69.2 kDa. This Ab was shown to distinguish CLL cells from a range of other leukaemias and myelo-proliferative disorders (Lannon *et al.*, 1988). The G12 Ab was used in this project to develop a range of diagnostic tests for CLL which are quicker and require less expertise than traditional histopathological examination. It is proposed that these could form part of a battery of tests to determine the diagnosis and progress of the disease.

1.5 COUMARIN

Coumarin is a naturally occuring constituent of many plants and essential oils. It derives its name from the plant *Coumarouna odorata*. Coumarin is a member of a class of compounds called benzopyrones. These consist of fused benzene and α-pyrone rings. Coumarin has a molecular weight of 146.15 Da and exists as colourless, glistening, small plates with a pleasant and characteristic odour. It often occurs as an odourless complex conjugated to sugars and acids, from which it is released by the action of enzymes, acids or ultraviolet radiation.

1.5.1 The Action & Applications of Coumarin:

Coumarin compounds have been used to treat such diverse ailments as cancer, burns, brucellosis and rheumatic disease, and have been used as anti-spasmodics. Coumarin has a wide variety of uses in industry, mainly due to its strong fragrant odour. Coumarin has a number of natural and synthetic derivatives, but only a few are of economic importance. These include:

- 3,4-dihydroxycoumarin, used mainly in the perfume industry.
- 6-methylcoumarin, used as a flavour enhancer.
- 7-amino-4-methylcoumarin, used as a laser dye.
- 4-hydroxycoumarin, a precursor of dicoumarol and warfarin.
- amino-methylcoumarin acetic acid, a fluorescent label.
- 7-hydroxycoumarin, used in sunscreen and fluorescent brighteners.

Derivatives of 7-hydroxycoumarin (7-OHC) are also used as fluorigenic enzyme substrates (Cohen, 1979; Khaflan *et al.*, 1986; Egan *et al.*, 1990).

Coumarin is metabolised initially by a specific cytochrome P-450-linked mono-oxygenase enzyme system in liver microsomes, resulting in hydroxylation to form 7-OHC. The levels of this enzyme have been shown to be very high in human liver microsomes, although there exists a large inter-individual variability in the activity of this enzyme (Pelkonen *et al.*, 1985). The rate of 7-OHC formation during the first two hours after coumarin administration shows inter-individual variability, but is stable and reproducible for a given individual (Rautio *et al.*, 1992). Coumarin, having been metabolised to form 7-OHC, undergoes glucuronidation prior to excretion. Between 30% and 100% of an oral dose of coumarin was recovered within four hours of administration, as 7-OHC in the urine of volunteers (Rautio *et al.*, 1992). Coumarin is often considered a pro-drug due to its rapid metabolism to 7-OHC. This theory is partially substantiated, since coumarin has a short half-life and low bioavailability. Various *in*

vitro and *in vivo* studies have suggested that 7-OHC and not coumarin is the pharmacologically active agent. It has been shown that 7-OHC is a more potent cytostatic agent than coumarin. These effects are reversible (Marshall *et al.*, 1993). *In vitro* studies suggest that 7-OHC, and not coumarin, significantly increase the phagocytic activity of human neutrophil granulocytes, while reducing the motility of carcinoma cells, so reducing the possibility of metastasis (Rosskopf *et al.*, 1992; Zanker *et al.*, 1993). In clinical trials coumarin has been effective in the treatment of cancers, particularly melanoma and renal cell carcinoma. Significant prolongation of progression-free intervals and survival, with minimal toxicity, has been reported (reviewed by Marshall & Mohler, 1993). These trials are to be continued.

1.5.2 Detection of Coumarin & Coumarin Derivatives:

The ability to quickly and easily measure levels of coumarin and 7-OHC in a variety of samples is important for metabolic and activity studies of these drugs. A very wide range of techniques have been used to analyse coumarin and coumarin-related compounds. Two of the most commonly used techniques are HPLC and spectrofluorimetry. Less commonly used techniques include: paper chromatography, thin layer chromatography, gas chromatography, radio-labelling, polarography, luminescence and voltammetry (reviewed by Egan *et al.*, 1990). These techniques have been developed quantitatively and qualitatively for the determintion of coumarin and coumarin derivatives. They have been applied to a variety of samples including: urine, plasma, serum, blood, tissue honogenates, and plant material. Many of these methods require significant sample preparation prior to analysis. Many of these extraction procedures are time-consuming and cumbersome and, hence, prohibitive when handling large numbers of samples. A recently developed HPLC method provides a simplified method for the routine analysis of coumarin, 7-OHC and 7-OHC-glucuronide. The method involves a single solvent extraction procedure and utilises an internal standard. The method is accurate in the range 0.5-100 μg/ml and has been used successfully to monitor the urinary excretion of these compounds (Egan, 1993). A spectrophotofluorimetric method was also developed with the same linear range. This had the advantages of not needing prior solvent extraction of the samples, using very small sample volumes and allowing for the simultaneous determination of large numbers of samples, through the use of 96-well microtitre plates. The method had the disadvantages of only measuring 7-OHC and 7-OHC-glucuronide, and needing a fluorescence spectrometer with microtitre plate

capacity, an instrument not always readily available (Egan, 1993). A number of Ab based assays have recently been developed. A competitive ELISA was developed which could detect 7-OHC, in the range 5-100μg/ml, in un-extracted urine. The results obtained were shown to be consistent with those obtained by HPLC. The polyclonal Abs used were shown to be specific for a number of coumarin derivatives. These Abs were also used in the development of Ab-based electrochemical assays. The first is a non-competitive assay and requires solvent extraction of the urine samples. The Ab is bound to a glassy carbon electrode. Interaction of the Ab and 7-OHC causes measureable fluctuations in the current passing through the electrode. The second is a competitive assay in which 7-OHC is bound to the electrode. The 7-OHC in the sample must compete with the bound 7-OHC for binding of the Ab. Alterations in the current through the electrode, caused by Ab binding, allow the measurement of 7-OHC levels in the sample (Egan *et al.*, 1994). The need still exists for a rapid, simple assay, with minimum sample preparation and high sample capacity, for the routine determination of coumarin, 7-OHC and 7-OHC-glucuronide in patient samples. It is thought that an Ab-based approach will provide the answer to this problem, and that BAbs have potential in this area.

1.6 PROJECT OUTLINE

This project was set up to examine the chemical production and diagnostic application of bispecific antibodies. A number of bispecific antibodies were produced and tested, prior to the development of detection systems for chronic lymphocytic leukaemia and 7-hydroxy-coumarin. This involved a four-fold approach:

1. The technology for the chemical production of bispecific antibodies by two separate methods (Brennan *et al.*, 1985; Glennie *et al.*, 1987) was set up. It was shown, using HPLC, that both of these resulted in the desired product. These methods were compared for ease of use and level of production of bispecific antibody.

2. The bispecific antibodies produced by both methods were tested by a series of ELISAs. These were carried out to show that the molecules produced were hybrid molecules, which had retained their binding specificity. A second series of ELISAs was used to examine the extent to which the affinities of the bispecific antibodies were retained compared to the parental IgG and $F(ab')_2$ molecules.

3. A rabbit polyclonal anti-alkaline phosphatase antibody was produced and used, along with the murine monoclonal anti-chronic lymphocytic leukaemia antibody (G12), to produce a bispecific antibody. This bispecific antibody was tested for specificity and affinity by ELISA. Methods for the detection of chronic lymphocytic leukaemia cells were developed using the G12 X anti-alkaline phosphatase antibody. The first method was a conventional ELISA system, using a 96-well polystyrene plate as a solid support. The second method was a suspension ELISA, this was carried out in 1.8 ml conical centrifuge tubes. An immunohistochemical method was also developed.

4. The polyclonal anti-alkaline phosphatase antibody was used along with a polyclonal anti-7-hydroxy-coumarin antibody to produce a bispecific antibody. This bispecific antibody was tested for specificity and affinity by ELISA. A competitive ELISA was set up for the quantitative detection of 7-hydroxy-coumarin.

CHAPTER 2

MATERIALS

2.1 REAGENTS

The following reagents were supplied by Sigma Chemical Co., Poole, Dorset, England; alkaline phosphatase (bovine intestinal mucosa, affinity purified), ammonium sulphate, bovine serum albumin, 5-bromo-4-chloro-3-indolyl phosphate, di-methyl-sulphoxide, Ellman's reagent, ethylene-diamine-tetra-acetic acid (EDTA), Freund's incomplete adjuvant, L-glutaraldehyde, glycerol, glycine, horse radish peroxidase (affinity purified), mercapto-ethylamine, methylene-bis-acrylamide, pepsin, o-phenylene diamine, o-phenylene dimaleimide, poly-L-lysine, pristane, sodium acetate, sodium carbonate, sodium hydrogen carbonate and all antibodies not produced in house.

The following reagents were supplied by Riedel de Haen AG, Hannover, Germany: acetic acid, ammonium per sulphate, bromophenol blue, citric acid, di-sodium hydrogen phosphate, glycerine, hydrochloric acid, iodoacetic acid, mercaptoethanol, potassium di-hydrogen phosphate, sodium chloride, sodium di-hydrogen phosphate, tris[hydroxymethyl] aminomethane & Tween 20.

The following reagents were supplied by BDH Chemicals Ltd., Poole, Dorset, England: acrylamide, hydrogen peroxide 30%(w/v), potassium chloride, sodium azide, sodium dodecyl sulphate & tetra-methyl ethlyene di-amine (TEMED).

The following were supplied by Pierce Chemicals, 3747 North Meridian Road, P.O. Box 117, Rockford, Il 61105, USA: Bicinchoninic acid assay kit, IODOGEN, Protein A column kit.

The following were supplied by Pharmacia Fine Chemicals AB, P.O. Box 175, S-75104 Uppsala 1, Sweden: CNBr-Sepharose 4B, Sephacryl S-200, Sephadex G-25, G-75, G-100.

All organic solvents were of HPLC grade and were supplied by LabScan, Unit T26, Stillorgan Industrial Park, Co. Dublin. These were: acetic acid, acetone, aminomethyl propanol, chloroform, dimethyl formamide, ethanol, isopropanol & methanol.

All cell culture media, supplements, sera and disposable plastic-ware were supplied by Flow Laboratories, Woodcock Hill, Harefield, Herts., England.

Stains used in cytocentrifuge preparations for histological examination were purchased from Diagnostic Developments, Unit 2-4, 50A Everton Road, Berkdale, Southport, England.

2.2 EQUIPMENT

Item	Manufacturer	Supplier
Balance	OHau's Brainweigh B-100	1
Centrifuge	Heraeus, Labofuge GL	2
Cytospin	Heraeus, Sepatech	2
Homogeniser	Ultra Turrax, Kika-Werk	1
HPLC system	Beckman, System Gold	3
Incubators (CO_2)	Leec	4
Laminar flow cabinets	InterMed, Microflow pathfinder	4
Magnetic stirrer	Ikamag RCT	1
Microscopes;		
Fluorescence	Leitz Labrolux	2
Light	Nikon SE	2
Phase contrast	Nikon SE	2
Mini-gel Electrophoresis	LKB	5
Peristaltic pump P-1	Pharmacia	5
pH meter	Beckman, Zeromatic SS-3	1
Plate-Reader	Titertek Twinreaderplus	2
Spectrophotometer	UV/Visible, UV-160A, Shimadzu	4
Superspeed Centrifuge	Sorvall RC-5B, Refrigerated	4
Waterbath	Clinicon, Precitherm PFV	1

Suppliers:

1. Lennox, John F. Kennedy Dr., Naas Rd., Dublin 12.
2. Medlabs, Unit 1c, Stillorgan Industrial Pk., Stillorgan, Dublin 4.
3. B.M. Brownes Ltd., Sandyford Industrial Est., Sandyford, Co. Dublin.
4. Medical Supply Co., Unit 9, Santry Industrial Est., Santry, Dublin 9.
5. United Drug PLC., Belgard Rd., Dublin 24.

CHAPTER 3

METHODS

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3.1 CELL CULTURE METHODS

Four cell lines were used in this project: K562 is a human erythro-leukaemic cell line with a non-adherent growth pattern. NSO is a murine myeloma cell line with a non-adherent growth pattern. G12 is a murine hybridoma cell line, which secretes antibodies reactive with PBLs from patients suffering from chronic lymphocytic leukaemia and the cell line K562. G12 has a non-adherent growth pattern. NRK is a cell line derived from normal rat kidney cells, which has an adherent growth pattern.

3.1.1 Storage of Cell Lines:

Long term storage of cell lines was in cryotubes, in liquid nitrogen at -186°C. Thawing of cells was accomplished by immersion of the cryotube in a 37°C waterbath for 2 min. The cells were washed with Dulbecco's Modification of Eagle's Medium (DMEM) and centrifuged at 2,000 rpm for 10 min. The pelletted cells were resuspended in DMEM supplemented with 50% (v/v) Ham's F-12 medium, 10% (v/v) foetal calf serum (FCS), 2% (w/v) Hepe's buffer (1 M, pH 7.2), 1% (v/v) L-glutamine (200 mM) and 2% (v/v) penicillin (5000 IU/ml)/streptomycin (5,000 μg/ml). These were transferred to a 25 cm^2 sterile tissue culture flask, which was sealed and placed in an incubator at 37°C.

Cells to be frozen were suspended in the growth medium by gentle agitation of the culture flask. The cell suspension was decanted into a 30 ml sterile centrifuge tube and centrifuged at 2,000 rpm for 10 min. The cells from a 25 cm^2 flask were resuspended in 1.5 ml of FCS, supplemented with 7.5% (v/v) dimethylsulphoxide (DMSO), and transferred to a cryotube using a sterile pasteur pipette. The cryotubes were lowered into a container of liquid nitrogen so that the temperature of the cells was reduced at a rate of 1°C/min to -186°C. The cryotubes were then submerged in liquid nitrogen for long term storage.

3.1.2 Expansion of Cell Lines *In Vitro* :

All cell lines used were grown in either DMEM supplemented with 10% (v/v) FCS, 2% (v/v) Hepes buffer (1 M, pH 7.2), 2% (v/v) penicillin (5,000 IU/ml) / streptomycin (5,000 μg/ml) and 1% (v/v) L-glutamine (200 mM) (DMEM.S_{10}) or DMEM supplemented with 5% (v/v) FCS, 2% (v/v) Hepes buffer (1 M, pH 7.2), 2% (v/v) penicillin (5,000 IU/ml) / streptomycin (5,000 μg/ml) and 1% (v/v) L-glutamine (200 mM) (DMEM.S_5). The medium was changed whenever the pH fell below pH 7, this was indicated by the change in colour of the phenol red indicator in the medium from

red/pink to orange/yellow. For those cell lines which are non-adherent, the cells were suspended within the flask by gentle agitation. The cell suspension was decanted into a 30 ml sterile centrifuge tube and centrifuged for 10 min at 1,500 rpm. For adherent cell lines, the old medium was poured off, the cells were then exposed to trypsin (0.25% (w/v) in EDTA (0.2 mg/ml)) for 5-15 min at 37°C. The cells were suspended within the flask and centrifuged as above. The old medium, or trypsin solution, was poured off and the cells resuspended in fresh medium (DMEM.S_{10} or DMEM.S_5). The cell suspension was returned to sterile tissue culture flasks, which were sealed and placed in an incubator at 37°C.

3.1.3 Expansion of Cell Lines *In Vivo* :

Many murine cell lines can be grown as an ascitic tumor in the abdominal cavity of a mouse. In the case of hybridomas the resultant ascitic fluid is rich in antibody, secreted by the growing cells. Ascitic fluid production is greatly increased if an adjuvant is used to prime the mice prior to injection of the cells. Pristane (2,6,10,14 - tetraethyl-pentadecane) can be used for priming the mice. The mice were injected with 0.5 ml of pristane, i.p.. Seven or eight days later the mice were injected with 10^6 cells i.p.. An alternative to Pristane is Freund's incomplete adjuvant, 0.5 ml of which were administered i.p.. Twenty four hours later the mice were injected with 10^6 cells i.p.. The animals were left until abdominal distension occurred. Tapping can then be carried out if desired, i.e. ascitic fluid is removed from the abdominal cavity using a syringe. The animals were again left until abdomnal distension occurred, at this time the animals were sacrificed by cervical dislocation. The sacrificed animal was washed with alcohol and the ascitic fluid removed under sterile conditions and placed in a 30 ml sterile centrifuge tube. This was then centrifuged at 2,000 rpm for 10 min. The fatty layer was removed using a sterile pasteur pipette and discarded. The antibody-containing supernatant was poured into sterile vials and stored at -20°C. The pelletted cells were washed and grown as described previously.

3.1.4 Cell Counts & Viability Testing:

Cell counts were performed on an improved Neubauer Hemocytometer slide. Trypan blue was used routinely to determine cell viabilities (Wilson, 1986). One volume of cell suspension was added to an equal volume of stain (0.25%, w/v in PBS), left for one minute and examined within five minutes by light microscopy, using a phase contrast setting. Longer incubation periods lead to non-specific uptake of dye and

inaccurate results. Live cells excluded dye and remained white, while dead cells stained blue.

3.1.5 Mycoplasma Detection - Hoechst 33258 Fluorescence Assay:

Cell lines were regularly screened for contamination with mycoplasma using NRK cells and the DNA chelating agent bisbenzimid (Hoechst 33528). A vial of mycoplasma-free NRK was grown in antibiotic-free medium (DMEM.S5) for at least two passages. Cultures were then established on sterile glass coverslips in sterile petrie dishes at 10^4 cells/ml and grown overnight in antibiotic-free DMEM.S5 at 37°C, in an atmosphere maintained at 5% CO_2. Supernatant from cultures to be screened was applied to the prepared petrie dish and incubated for three days at 37°C, in an atmosphere maintained at 5% CO_2. Duplicate NRK cultures were fed with fresh medium and used as a negative control. The coverslips were washed three times in PBS, keeping the cells uppermost, and fixed for 6 min in methanol:acetone (1:1), at -20°C. The coverslip preparations were rinsed three times in PBS and stained for 10min in Hoechst 33258 (0.05 μg/ml in PBS). The excess stain was washed from the cells and the coverslips air-dried and mounted on clean slides. Slides were examined under a fluorescence microscope (Leitz Labrolux) using the 100X, oil-immersion lens. Uncontaminated cells fluoresce in the nucleus only, while contaminated cells show fluorescence in the cytoplasm as well as the nucleus.

3.2 ANTIBODY PRODUCTION

3.2.1 Polyclonal Antibody Production:

Polyclonal antibodies were raised by immunisation of a mature, male, New Zealand white rabbit. Prior to immunisation, the animal was bled from the marginal ear vein, to provide control serum. To 1 ml of Freund's incomplete adjuvant was added 100 μl alkaline phosphatase solution (10 mg/ml in PBS). The two layers were mixed to produce an emulsion (Kika-Werk, Ultra-Turrax homogeniser). A 1 ml sample was injected i.d., at various sites along the animals back. Booster immunisations were given at 28 day intervals. The animal was bled from the marginal ear vein on day 12 of each 28 day cycle. The cycles were continued until the antibody titre was sufficiently high (i.e. greater than 1:10,000), as determined by ELISA (Section 3.6).

3.2.2 Ammonium Sulphate Precipitation:

Immunoglobulins were precipitated from ascitic fluid, or culture supernatants in 50% saturated ammonium sulphate solution, according to the methods of Hudson and Hay (1980).

The ascitic fluid was centrifuged for 20 min at 15,000 rpm, at 4°C (Sorvall RC-5B refrigerated superspeed centrifuge). The supernatant was then filtered through glass wool. The solution was adjusted to pH 4.75 using HCl and left at 4°C for 1 hr. This was then centrifuged for 20 min at 15,000 rpm, at 4°C. The supernatant was adjusted to pH 7 using NaOH. An equal volume of saturated ammonium sulphate (pH 7.4, $(NH_4)_2SO_4$) was added dropwise to the stirring mixture and left at 4°C for 1 hr. The mixture was then centrifuged for 20 min at 15,000 rpm, at 4°C. The pellet was resuspended in 50% (w/v) $(NH_4)_2SO_4$ (pH 7.4) and centrifuged for 20 min at 15,000 rpm, at 4°C. The pellet was resuspended in the minimum amount of PBS for storage at -20°C.

3.3 AFFINITY CHROMATOGRAPHY

Affinity chromatography is a type of adsorption chromatography in which the molecule to be purified is specifically and reversibly adsorbed by a complementary binding substance (ligand) immobilised on an insoluble support (matrix). Ligands containing primary amino groups are readily coupled to polysaccharide matrices which have been activated by cyanogen bromide. For this reason CNBr-activated Sepharose is regularly used for the immobilisation of ligands, (Axen *et al.*, 1967; Cuatrecasas, *et al.*, 1968).

3.3.1 Coupling of Ligand:

The required amount of CNBr-activated Sepharose 4B (Pharmacia) was weighed out and suspended in 1 mM HCl (3.5 ml/g). The gel was transferred to a scintered glass filter (5 ml Gooch crucible) and washed with 1 mM HCl for about 15 min (200 ml/g). The gel was then washed with coupling buffer (0.1 M $NaHCO_3$, pH 8.3, containing 0.5 M NaCl), 5 ml of coupling buffer for every 5 g of gel. The ligand was dissolved in coupling buffer (2 mg/ml). The ligand and gel were combined in a stoppered vessel and the mixture rotated end-over-end for 2 hr at room temperature. The mixture was returned to the scintered glass filter and washed with coupling buffer (5 ml/g gel), to remove any excess ligand. Any remaining active groups were blocked by suspending the gel in coupling buffer with glycine, 0.2 M, and mixing gently for 1 hr at room temperature. The gel was then washed with three cycles of alternating pH, in the scintered glass filter. Each

cycle consisted of a wash with acetate buffer (0.1 M, pH 4), containing NaCl (0.5 M), followed by a wash with Tris buffer (0.1 M, pH 8) containing NaCl (0.5 M). The gel was suspended in PBS and a column poured in a 1 ml syringe, the outlet having been stopped with a plug of glass wool. The column was stored at 4-8^{o}C.

3.3.2 Use of Immunoadsorbent:

The column was washed with PBS (0.02 M, pH 7.1) to remove leached protein. Fractions were collected until the Abs_{280nm} was less than 0.03. The sample was applied slowly followed by PBS (0.02 M, pH 7.1) and the column washed as before, until collected fractions had Abs_{280nm} of less than 0.03. The PBS (0.02 M, pH 7.1) was run to the top of the gel before the dissociation buffer was applied (glycine/HCl, 0.1 M/0.1 M, pH 2.5). When 1 ml of dissociation buffer had run onto the column, the outlet was clamped for 15 min. The dissociation buffer was used to elute the sample into 1 ml fractions, each containing 100 µl of Tris-HCl (1 M, pH 10.5) to neutralise the pH. The fractions were monitored for protein by measuring the Abs_{280nm}.. The column was regenerated using three cycles of alternating pH, using acetate buffer (0.1 M, pH 4) with 0.5 M NaCl and Tris buffer (0.1 M, pH 8) with 0.5 M NaCl.

3.3.3 Protein A Chromatography:

Protein A is isolated from *Staphylococcus aureus* and shows strong, specific binding for the Fc part of the IgG molecule (Krovall *et al.*, 1970; Langone *et al.*, 1978). IgG can be purified from solution using Protein A as the ligand in affinity chromatography. Pre-packed Protein A Sepharose columns are available from a variety of suppliers (column volume of 1 ml or 5 ml); a similar protocol can be applied to each. The column was equilibrated with at least eight column volumes of binding buffer (Tris-HCl, 50 mM, pH 8.6). The sample was diluted with an equal volume of binding buffer, before being applied to the column. The column was allowed to stand for 15 min to allow adsorption of the IgG to the Protein A to occur. The column was washed with ten column volumes of binding buffer to remove unbound protein. To release the IgG the column was washed with five column volumes of elution buffer (Citrate buffer, 0.1 M, pH 4). Prepared collection tubes, each containing 50 µl of Tris-HCl (1 M, pH 9) to neutralise the elution buffer, were used to collect 1 ml fractions of elution buffer. The BCA protein assay was used to identify those fractions containing purified protein. These were pooled and stored at -20^{o}C. The

columns were washed and stored in 20% ethanol. They can be re-used up to twelve times.

3.3.4 Affi-T Chromatography:

Affi-T (Kem-en-Tec) is a matrix with an immobilised thiophilic ligand. Most proteins will bind to Affi-T at high concentrations of ammonium sulphate and will easily be eluted by gradually lowering the concentration of salt in the washing buffer (Lihme & Heegaard, 1991).

The matrix is supplied as a 50% aqueous suspension with 15 mM sodium azide. The gel was washed with distilled water and drained under vacuum in a scintered glass filter until cracks appeared in the gel bed. The dry gel was weighed out (5 g) and suspended in excess $(NH_4)_2SO_4$ (0.75 M). A 10 cm x 1 cm column was poured and washed with ten column volumes of $(NH_4)_2SO_4$ (0.75 M). The sample was adjusted to 0.75 M $(NH_4)_2SO_4$ and a 3 ml volume applied to the column. The column was washed with the same buffer until a baseline was reached ($Abs_{280nm} < 0.03$). Bound immunoglobulins were eluted in 0.05M Tris (pH 9) and 1 ml fractions collected. All protein-containing fractions were pooled and stored at -20°C for future use. The column was regenerated by washing with distilled water followed by 0.01 M sodium hydroxide containing 20% (v/v) isopropanol, followed by distilled water.

3.4 SIZE EXCLUSION CHROMATOGRAPHY

Size exclusion chromatography is a commonly used method for the separation of biological macromolecules (Porath & Flodin, 1959). The separation depends on the different abilities of the various sample molecules to enter pores in the stationary phase. Large molecules never enter the stationary phase and move through the chromatographic bed fastest. Smaller molecules, which enter the gel pores, move more slowly through the column, since they spend some time in the stationary phase. Molecules are, therefore, eluted in order of decreasing molecular size.

3.4.1 Low Pressure Chromatography:

Sephadex (Pharmacia) is a bead-formed gel of cross-linked dextran available in a variety of pore sizes and, hence, fractionation ranges. The required amount of dry Sephadex, of the desired grade, was weighed out and allowed to swell in excess PBS overnight at room temperature. The gel was then degassed under vacuum for 15 min. The gel was poured into the column (generally 15 cm x 1 cm) and allowed to settle while washing with

at least five column volumes of mobile phase. This was carried out using a peristaltic pump (P-1, Pharmacia) at a flow rate of 1 ml/min. The sample was applied in PBS, in a volume less than 1/10th that of the column, and eluted in 1 ml fractions which were monitored for protein.

3.4.2 High Performance Liquid Chromatography:

High performance liquid chromatography (HPLC) was carried out on a Protein-Pak 300SW series column (Waters). This was a 7.5 mm x 300 mm gel filtration column packed with a rigid hydrophillic porous silica gel (particle size 10 μm, pore size 300Å), with a fractionation range of 10,000-300,000 Da. The column was used in series with a 7.5 mm x 50 mm guard column packed with a similar silica gel (particle size 35μm, pore size 125Å). The guard column was used to protect the analytical column from becoming blocked by precipitate in the analyte solution or irreversible binding of analyte components to the silica. The columns were attached to a Beckman System Gold HPLC system. This consisted of; a Programmable Solvent Module 126, double-headed pump, a Programmable Detector Module 166, variable wavelength detector fitted with a flow through cell and an Autosampler 507. These were controlled by the System Gold (version 4) software package, loaded onto an IBM system/2 Model 50 Z personal computer.

Sodium phosphate buffer (0.1 M, pH 7.4), the mobile phase, was filtered, using 0.22 μm filters (Millipore), and degassed under vacuum, before being pumped through the column at 0.5 ml/min. The sample was made up to a concentration of 0.5-1 mg/ml in the mobile phase. A volume of 20-50 μl was injected onto the column either manually or using the autosampler. The elution of protein was detected at 280nm. Data was collected for a minimum of 30 min. The column was stored in sodium azide (0.05%, w/v), filtered and degassed as for the mobile phase. (Carty & O'Kennedy, 1988)

3.5 DETECTION & CONJUGATION METHODS

3.5.1 Bicinchoninic Acid Assay:

Bicinchoninic acid (BCA), in the form of its water-soluble sodium salt, is a sensitive, stable and highly specific reagent for the cuprous ion (Cu^{+}). Proteins in alkaline medium react with Cu^{++} to form Cu^{+}. Thus, proteins, particularly the amino acids cysteine, cystine, tryptophan and

tyrosine, are responsible for colour formation in protein samples when combined with BCA in alkaline conditions (Smith *et al.*, 1985).

The reagents for the BCA assay are available in kit form from various suppliers (Pierce & Sigma). Reagent A contains BCA in buffered, alkaline solution, reagent B contains copper sulphate, (4% w/v). The working solution was prepared by mixing A and B in the ratio - 50:1. A range of protein standards (0-1 mg/ml) were prepared from the stock BSA solution (2 mg/ml) supplied. To 20 parts working solution was added 1 part standard or unknown sample, (eg. 10 µl protein solution in 200 µl working solution in a microtitre plate). The samples were mixed by gentle aggitation and incubated for 30 min at 37°C. The Abs_{560nm} was determined on a Titretek Twin reader Plus plate reader. The protein concentration of the sample was determined from a standard curve.

3.5.2 Periodate Conjugation:

This method was used for the chemical conjugation of BSA to horse radish peroxidase (HRP). It is adapted from the method described in Tijssen and Kurstak (1984) for the coupling of glycoproteins, specifically HRP and IgG.

The HRP was activated by the addition of $NaIO_4$. This oxidises the enzyme's carbohydrates producing aldehyde and carboxyl groups. To 0.5 ml of HRP solution (1 mg/ml in 0.1 M $NaHCO_3$, pH 9.2) was added 0.5 ml of 8 mM $NaIO_4$. This was incubated at 20°C for 2 hr in the dark. Conjugation occurs when free, non-protonated, amino groups of the added BSA form Schiff bases with the aldehyde groups of the activated HRP. To the activated HRP was added 1 ml of BSA (1mg/ml in 0.1 M $NaHCO_3$, pH 9.2) and 5 mg Sephadex G-25. The Sephadex beads swell rapidly causing an apparent increase in protein concentration, thus accelerating the reaction. This was incubated at 20°C for 2 hr in the dark. The conjugate was subsequently stabilised by reduction with $NaBH_4$. $NaBH_4$ (0.1 ml of a 5 mg/ml solution in 0.1 M NaOH) was added to the conjugate mixture, with gentle aggitation. After 30 min in the dark at 20°C a further 0.2 ml of $NaBH_4$ solution was added. The mixture was incubated in the dark for 1 hr at 4°C. Excess reagents were removed by dialysis against PBS at 4°C in the dark.

3.5.3 Iodine Labelling:

The compound 1,3,4,6-tetrachloro-3α-6α-diphenylglycouril is supplied as a reagent for the iodination of proteins (IODO-GEN, Pierce). Virtually insoluble in water, IODO-GEN mediates rapid iodination at the

solid phase interface with aqueous solutions of iodide and proteins (Fraker & Speck, 1978). In this method, the exposed tyrosine residues of the IgG are iodinated.

Glass test tubes were washed and rinsed with a dry organic solvent, e.g. chloroform, and allowed to dry. To each test tube was added 100 µl of IODOGEN solution (0.1 mg/ml in chloroform). The solvent was evaporated under nitrogen gas. The test tubes were covered and stored desiccated overnight or until needed. The test tubes were rinsed with PBS to remove unbound IODOGEN. To each test tube was added 100 µl of IgG solution (1 mg/ml in PBS). NaI was added to a final concentration of 25 mM in each test tube. The tubes were incubated for 20 min at room temperature, with gentle aggitation. The reaction was terminated by removing the protein solution from the test tube. Excess iodine was removed by extensive dialysis against PBS.

3.5.4 Detection of Cold Iodine:

A micro-assay system based on the effect of the catalytic Sandell-Kolthoff reaction of iodide on the oxidation of arsenic (III) by cerium (IV) was used to measure iodine in solution and, hence, iodine conjugated compounds. This rapid assay uses small quantities of reagents, is suitable for use with a photometric microplate reader, can test many samples simultaneously and eliminates problems associated with the use of radiolabelled compounds to measure iodination, (O'Kennedy *et al.*, 1989).

Standards of KI were prepared in the range 0.001-0.1 µg/ml. To the wells of a 96-well plate were applied 100 µl of standard or sample, 60 µl of 75 mM arsenious acid and 25 µl of acidified ceric ammonium sulphate (0.1 M $Ce(SO_4)_2.2(NH_4)_2SO_4.2H_2O$: 1 M HNO_3, 1:2.57). The plate was incubated at room temperature and the absorbance read at 414nm every 5 min for 20 min.

3.6 ENZYME-LINKED IMMUNOSORBENT ASSAY

Enzyme-linked immunosorbent assays (ELISA) are methods for detecting antigens or antibodies utilizing enzyme-substrate reactions. The enzymes are usually coupled to antibodies (either to antibodies specific for the antigen or to anti-immunoglobulin). It is essential that both the enzymatic activity and the immunological reactivity of the conjugate are preserved. The amount of enzyme conjugate is detected by the enzymatic conversion of a colourless substrate to a highly coloured product that can be visually or photometrically determined. ELISA's are carried out in 96-well

microtitre plates, to which antibody or antigen can be adsorbed, (O'Kennedy, 1989).

3.6.1 Adsorption of Cells to ELISA Plates:

The PVC, 96-well microtitre plates (Nunc) were washed three times with PBS, containing 5% (w/v) Tween 20, then three times with PBS. To each well was added 125 µl of poly-L-lysine, 0.1% (w/v) in PBS. The plate was incubated for 1.5 hrs at 37°C. The plate was washed as before. A single cell suspension was prepared (5 x 10^6 cells/ml) from the relevant source material. This was applied to the plate at 50 µl/well. The plate was incubated for 1.5 hrs at 4°C. At this time 150 µl of L-glutaraldehyde (0.1% w/v in PBS) were added to each well. The plate was left for a further 15 min at 4°C. The plate was washed as before. Non-specific binding sites on the plate were blocked using 200 µl/well of a solution of 1% (w/v) BSA in PBS, the plate was left at 4°C overnight.

3.6.2 Adsorption of Proteins to ELISA Plates:

The PVC, 96-well micro-titre plates (Nunc) were washed three times with PBS, containing 5% (w/v) Tween 20, then three times with PBS. A solution of protein (10 µg/ml in 1 M carbonate/bicarbonate buffer pH 9.6) was applied to the plate, 100 µl in each well. The plate was incubated for 1hr at 37°C. The plate was washed as before. Non-specific binding sites on the plate were blocked using 200 µl/well of a solution of BSA (1% w/v in PBS). The plate was left at 4°C overnight.

3.6.2 Colour Development in ELISA:

The wells of a plate, to which protein or cells had been adsorbed, were washed three times with PBS, containing 5% (w/v) Tween 20, then three times with PBS. The primary antibody, with affinity for the adsorbed cells or protein, was applied, in suitable working dilution, at 100 µl per well. This was incubated for 1 hr at 37°C. The plate was washed as before. The secondary, or enzyme-labelled, antibody was applied, in suitable working dilution, at 100 µl per well. The most common enzyme label used was horse radish peroxidase (HRP), but alkaline phosphatase was also used. The plate was incubated for 1 hr at 37°C. The plate was washed as before. The substrate for the enzyme-catalysed, colour-producing reaction was prepared fresh every time. For HRP the substrate used was o-phenylenediamine (o-PD), 0.2 mg/ml in citrate buffer (0.1 M, pH 4.5), to which was added hydrogen peroxide (0.003%, w/v) immediately before use. This was applied to the plate at 100 µl per well and incubated for 30min at 37°C. Colour

development was estimated by measuring the absorbance of the wells at 405nm on a Titretek Twinreader Plus plate reader. For alkaline phosphatase, the substrate used was 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP), 1 mg/ml in amino methyl propanol buffer (pH 10.25). This was applied at 100 µl per well and incubated at room temperature for 30 min. Colour development was estimated by measuring the absorbance of the wells at 620nm on a Titretek Twinreader Plus plate reader.

3.7 ELECTROPHORESIS

Electrophoresis in polyacrylamide gels in the presence of the anionic detergent, sodium dodecyl sulphate (SDS), has proven to be a useful tool for the separation of protein subunits and the determination of their molecular weights. The slab gel procedure developed by Laemmli (1970) was used in a LKB mini-gel system (Pharmacia).

The most commonly used resolving gels were prepared according to Table 3.1, in Tris-HCl buffer (0.4 M, pH 8.8). The tetra-methyl-ethylene-diamine (TEMED) was added last as this starts the polymerisation reaction. The gel was poured between two glass plates and overlayed with water. The water layer excludes air thus speeding the polymerisation process, while the surface tension at the water/gel interface ensures a smooth surface to the top of the gel. When the gel had set the water was poured off. The resolving gel was then overlayed with the stacking gel, prepared according to Table 3.1, in Tris-HCl (0.05 M, pH 6.8). The TEMED was added last. The gel was allowed to set around a "comb". This created wells in the stacking gel for application of samples. The wells were washed out with electrode buffer before use.

Samples for electrophoresis were prepared by heating at 100°C for 2 min in sample buffer. This consisted of SDS (2%, w/v), glycerol (10%, v/v) and bromophenol blue (0.05%, w/v) in Tris-HCl buffer (0.08 M, pH 6.8). The prepared samples (10-30 µl) were applied to the wells in the stacking gel, under the electrode buffer. The electrode buffer (pH 8.3) was prepared containing Tris (25 mM), glycine (0.2 M) and SDS (0.1%, w/v).

Electrophoresis was performed at constant current (30 mA per mini-gel) until the dye front had traversed the gel. The dye was not allowed to run off the gel. The gel was removed from the glass plates and stained for 20 min with Coomassie brilliant blue (5%, w/v) in acetic acid : water : methanol (1:10:8, v/v/v). The same solvent mixture was used to destain the background until only the protein bands remained stained.

	High Mol. Wt. Proteins	General Purpose	Low Mol. Wt. Proteins	Stacking Gels
Polyacrylamide (w/v)	7.5%	10%	15%	3%
Methylene-bis-acrylamide (w/v)	0.2%	0.27%	0.4%	0.08%
Sodium dodecyl sulphate (w/v)	0.05%	0.05%	0.05%	0.08%
Ammonium per-sulphate (w/v)	0.05%	0.05%	0.05%	0.07%
TEMED (w/v)	0.1%	0.1%	0.1%	0.9%

Table 3.1 :

The components required for the preparation of gels for polyacrylamide gel electrophoresis. The resolving gels, those required for the separation of general protein mixtures or more specific gels for the separation of high molecular weight proteins or low molecular weight proteins, were prepared in Tris-HCl buffer (0.4 M, pH 8.8). The stacking gel was prepared in Tris-HCl buffer (0.05 M, pH 6.8).

3.8 BISPECIFIC ANTIBODY PRODUCTION

Bispecific antibodies were prepared by the breakdown and chemical re-association of purified antibodies. Two methods were used, both of which had the F(ab')2 fragment as the starting material.

3.8.1 F(ab')2 Fragment Production:

The antigen binding fragment (F(ab')2) is obtained from immunoglobulins by digestion with the enzyme pepsin at pH 4.0-4.5. The F(ab')2 fragment, which has a molecular weight of approximately 95 kDa, retains the antibody combining sites of the parent IgG molecule. Pepsin digests the IgG hinge region leaving the disulphide bridges intact. The heavy chain piece remaining is known as Fc'; it may become further degraded into smaller peptides. Pepsin digestion was carried out under two different sets of conditions.

In the first method (Brennan *et al.*, 1985), the IgG was suspended in PBS and adjusted to a final concentration of 20 mg/ml. This was dialysed against a 0.1 M sodium acetate solution for 3 hrs. The dialysate was adjusted to pH 4.5 by dropwise addition of acetic acid (glacial). The pepsin was added to this to a final concentration of 2 mg/100 mg IgG. The solution was incubated overnight at 37°C. The reaction tubes were centrifuged at 2,000 rpm for 10 min to remove any precipitate that may have formed. The solution was dialysed extensively against 1mM sodium phosphate buffer (pH 7.2) to inactivate the enzyme.

In the second method (Runge *et al.*, 1990), the IgG was suspended in 10 mM sodium phosphate buffer, with 0.15 M sodium chloride (pH 7.4), to a final concentration of 2 mg/ml. To 10 ml of IgG solution was added 1 ml of pepsin (3 mg/ml) and 1 ml of 1M sodium citrate (pH 2.75). The mixture was adjusted to pH 3.5. This was incubated for 2 hrs at 37°C. Stopping solution was added, consisting of 1 ml of 3 M Tris-HCl (pH 8.5). The solution was dialysed extensively against 1 mM sodium phosphate buffer (pH 7.2).

All antibodies and fragments were stored in PBS at 4°C for immediate use, or at -20°C for long term storage. Repeated freezing and thawing was avoided.

3.8.2 BF(ab')$_2$ Production I:

The first method examined was that of Brennan *et al.* (1985). The starting material was F(ab')$_2$ fragments in 1 mM sodium phosphate buffer (pH 7.2) at a concentration of 3 mg/ml. This was reduced to the Fab' fragment by the addition of 1 mM 2-mercaptoethylamine, 1 mM EDTA and 10 mM sodium arsenite. The solution was incubated at 25°C overnight. Solid 5,5'-dithio-bis(2-nitrobenzoic acid), (dTNB, Ellman's reagent), was added to a final concentration of 5 mM. The solution was incubated at 25°C for 3 hrs. Excess dTNB can be removed by extensive dialysis against 1 mM sodium phosphate (pH 7.2). The remaining protein constitutes Fab'-TNB fragments. The Fab'-TNB of one specificity was further reduced to the Fab'-SH fragment by the addition of 10 mM 2-mercaptoethylamine, 1 mM EDTA and 10 mM sodium arsenite, for 30 min at 25°C. Excess reagents were removed by dialysis as before.

BF(ab')$_2$ fragments were produced by combining equimolar amounts of Fab'-TNB and Fab'-SH, in 0.1 M sodium phosphate buffer with 1 mM EDTA. This was incubated for 16 hrs at 25°C. Addition of solid dTNB, 5 mM after this point prevents the formation of homologous pairs. Excess reagents were removed by extensive dialysis as before. The BF(ab')$_2$ was purified by double affinity chromatography and stored at 4°C in PBS.

3.8.3 BF(ab')$_2$ Production II:

The second method examined was that of Glennie *et al.* (1987). The starting material was F(ab')$_2$ fragments in 0.2 M Tris-HCl with 10 mM EDTA at a concentration of 2 mg/ml. This was reduced to the Fab' fragment by the addition of 20 mM 2-mercaptoethanol. The solution was incubated at 21°C for 30 min. Excess reagents were removed by extensive dialysis against 50 mM sodium acetate, pH 5.3, with 0.5 mM EDTA at 4°C. To the murine Fab'-SH fragment was added 1/2 volume of 12 mM o-phenylene dimaliemide in dimethyl-formamide. This was incubated at 4°C for 30 min to form the Fab'-mal fragment. Excess reagents were removed by dialysis as before.

BF(ab')$_2$ fragments were produced by combining Fab'-SH and Fab'-mal in a ratio of 1:1.3, at a concentration of 5 mg/ml. The mixture was adjusted to pH 8.0 using 1 M Tris-HCl and incubated for 18 hrs at 4°C. Homologous pairs were disrupted by the addition of 20 mM 2-mercapto-ethanol for 30 min at 30°C, followed by 25 mM iodoacetamide. Excess reagents were removed by dialysis against PBS. The BF(ab')$_2$ was purified by double affinity chromatography and stored at 4°C in PBS.

3.9 IMMUNOCYTOCHEMISTRY

3.9.1 Isolation of Mononuclear Cells:

Mononuclear cells form 0.06% of all blood cells, 50% of white blood cells. In order to increase the sensitivity of the immunodetection techniques in use, the mononuclear cells were isolated from venous peripheral blood samples.

The isolation of mononuclear cells from peripheral blood samples was carried out in a one step density centrifugation procedure. Histopaque-1077 (Sigma) is a solution of Ficoll (Pharmacia) and sodium diatrizoate, adjusted to a density of 1.077 $\pm$ 0.001 g/ml. Anti-coagulated venous blood can be layered onto Histopaque-1077. During centrifugation, erythrocytes and granulocytes are aggregated by Ficoll and rapidly sediment, whereas, lymphocytes and other mononuclear cells remain at the plasma/Histopaque-1077 interface, (Boyum, 1968).

Samples of venous peripheral blood were collected from healthy volunteers and leukaemic patients, in heparinised blood tubes. These were centrifuged at 1,000 rpm for 10 min and the plasma removed. The blood cells were resuspended in 5 ml PBS and layered onto 10 ml Histopaque-1077. The samples were centrifuged for 30 min at 2000 rpm. A pasteur pipette was used to aspirate the mononuclear cells from the interface, where they form a "buffy layer". The cell suspension was diluted with an equal volume of PBS and centrifuged at 1,000 rpm for 10 min. The pelletted lymphocytes were stored in liquid nitrogen, as described previously (Section 3.1.1).

3.9.2 Preparation of Slides:

A Heraeus Sepatech cytocentrifuge was used to centrifuge cells onto a clean glass slide, in a single cell layer. Single cell suspensions, of 5 x 10^5 cells/ml in PBS, were prepared. This was applied to the slides with 100 µl cell suspension in each receptacle. The slides were spun for 2 min at 1,000 rpm. Each sample was checked for morphological integrity by staining, using the Rapi-Diff triple stain set (Diagnostic Developments), and examination under a light microscope (Nikon SE). Slides of each sample were stored at 4°C for immunochemical examination.

3.9.3 Immunochemistry:

Immunocytochemistry is the use of labelled antibodies as specific reagents for the localisation of tissue constituents (antigens) *in situ*. Because of the absolute specificity of antibody-antigen interactions, positive

identification of tissue constituents can be achieved. As a result, much of the uncertainty has been removed from some aspects of histopathology, (Polak & vanNoorden, 1987). The indirect method of staining was adapted for use in this instance.

A $BF(ab')_2$ was produced from the G12, anti-CLL, Ab and an anti-alkaline phosphatase Ab. The $BF(ab')_2$ was diluted, in 1% (w/v) BSA in PBS, to a concentration of 10 μg/ml. This was applied to the prepared cytospins. These were left at room temperature, in a humidified chamber, for 2 hrs. The slides were then washed three times by immersion in PBS. Alkaline phosphatase (50 μg/ml in PBS) was applied to the cells and incubated for 30 min at room temperature, in a humidified chamber. The slides were washed three times by immersion in PBS. The substrate BCIP was prepared as for ELISAs (Section 3.6.3) and applied to the slides for 30 min at room temperature, in a humidified chamber. The slides were rinsed three times by immersion in PBS. The slides were mounted in glycerol jelly. This was prepared by mixing gelatine (1 g), glycerol (7 ml) and water (6 ml) and heating gently.

3.10 BIOLOGICAL BUFFERS

- Acetate-Acetic Buffer ;

Stock solutions of 0.6 M sodium acetate and 0.6 M acetic acid were prepared. To 435 ml acetic acid (0.6 M) was added 230 ml sodium acetate (0.6 M). The solution was adjusted to 1 L with distilled water. This gives a buffer of molarity 0.4, pH 4.0.

- Amino Methyl Propanol Buffer ;

150 mg $MgCl_2.6H_2O$, 0.1 ml Triton X-405 and 1 g NaN_3 were dissolved in 30 ml distilled water. This was added to 95.8 ml amino-methyl-propanol, while stirring. HCl (6 M) was used to adjust the pH of the solution to 10.25. The solution was left at 4°C overnight. The pH of the solution was checked and readjusted with HCl (6 M) if necessary, before making the solution up to 1 L with distilled water.

- Carbonate-Bicarbonate Buffer ;

Stock solutions of 1 M sodium carbonate and 1 M sodium bicarbonate were prepared. The carbonate and bicarbonate were mixed in proportions 1:9 (v/v). The solution was titrated to the correct pH using the stock

solutions; carbonate to raise the pH, bicarbonate to lower the pH. The buffer was diluted to the correct molarity using 0.14 M NaCl in water.

- Citrate Buffer ;

Stock solutions of 0.1 M citric acid and 0.1 M sodium dihydrogen phosphate were prepared. These solutions were titrated against each other to produce the correct pH. For a buffer below pH 5.0 the NaH_2PO_4 (0.1 M) was added to 100 ml of citric acid (0.1 M). For a buffer above pH 5.0 the citric acid was added to 100 ml of NaH_2PO_4 (0.1 M). This gives a 0.1 M buffer.

- Glycine-Hydrochloric acid Buffer ;

Stock solutions of 0.2 M glycine and 0.2 M HCl were prepared. Stock HCl was added to 500 ml of stock glycine until the correct pH was reached. The solution was made up to 1 L with distilled water. This gives a 0.1 M buffer.

-Phosphate Buffered Saline ;

8 g NaCl, 0.2 g KCl, 1.15 g Na_2PO_4 and 0.2 g KH_2PO_4 were dissolved in 500 ml of distilled water. This solution was made up to 1 L with distilled water. This results in a buffer of molarity 0.15, pH 7.4. The buffer can be adjusted to another pH if required by adding 6 M HCl or 6 M NaOH prior to adjusting the total volume to 1 L. Alternative molarities can be achieved by altering the final volume of the solution.

- Sodium Phosphate Buffer ;

Stock solutions of 0.5 M NaH_2PO_4 and 0.5 M Na_2HPO_4 were prepared. The two solutions were titrated to obtain the required pH and the final volume adjusted to give the required molarity. e.g. 100 ml of 0.5 M NaH_2PO_4 and 100 ml 0.5 M Na_2HPO_4, mixed together and made up to 1 L, result in a 0.1 M buffer of pH 7.5, the mobile phase used in HPLC.

- Tris-Hydrochloric acid Buffer ;

Stock solutions of tris[hydroxymethyl]aminomethane (1 M) and HCl (1 M) were prepared. The stock Tris was diluted with distilled water to give 500 ml of Tris solution at twice the required molarity. The solution was adjusted to the required pH with the stock HCl. The final volume was made up to 1 L with distilled water.

CHAPTER 4

RESULTS

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4.1 OPTIMISATION OF BISPECIFIC ANTIBODY PRODUCTION PROTOCOLS

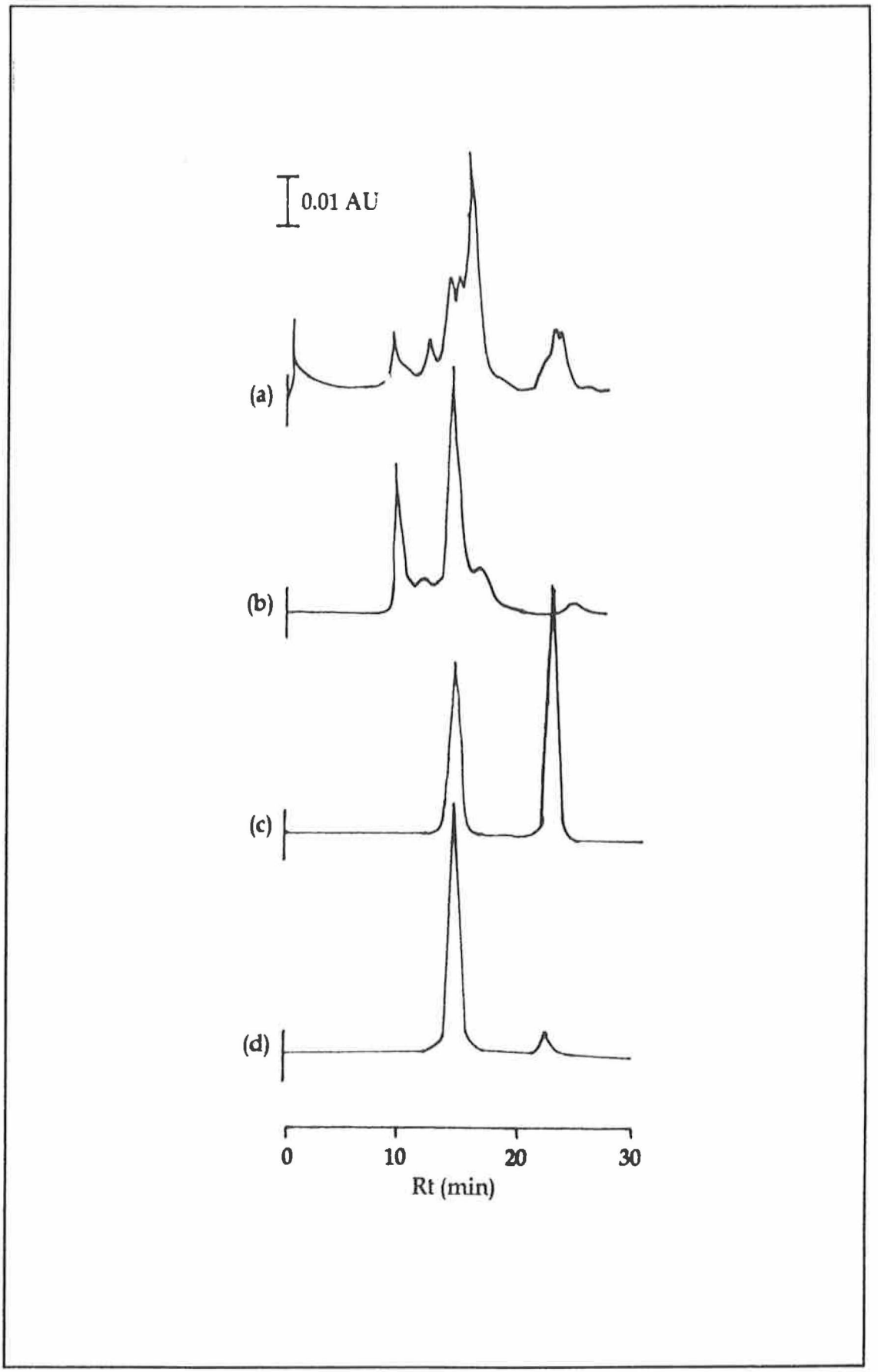
Two methods have been described for the production of $BF(ab')_2$s (Brennan *et al.*, 1985; Glennie *et al.*, 1987). Both of these methods require large quantities of IgG as the starting material. Methods for the purification of IgG from serum, growth medium or ascites fluid were examined. Subsequently, the $BF(ab')_2$ production protocol of Brennan *et al.* (1985) was examined and optimised.

4.1.1 Antibody Purification:

Ab was purified from ascites fluid by precipitation in 50% (w/v) saturated ammonium sulphate (Section 3.2.2). The Ab solution was checked for purity by HPLC (Section 3.4.2) and shown to contain a number of other proteins which may interfere in $BF(ab')_2$ production (Fig. 4.1.1). The precipitated Ab was further purified by affinity chromatography using a Protein A sepharose column (Section 3.3.3). The Ab purification procedure was optimised as follows.

A solution of 'pure' human IgG (Sigma) was made up in ddH_2O. This was diluted 1:1 with binding buffer, supplied with the Protein A Sepharose column (Pierce). The IgG was applied to the 1 x 2 cm column and washed onto the column with 15 ml binding buffer (Pierce). The eluate was collected in 1 ml fractions. The column was washed with 10 ml elution buffer (Pierce) and 1 ml fractions collected. The BCA assay (Section 3.5.1) was used to determine the presence of protein in each of the fractions and to quantitate the protein where present (Fig. 4.1.2), i.e. in fractions 16, 17 and 18, (Fig. 4.1.3). The protein concentration of the human IgG solution, which was applied to the Protein A column, was measured at the same time. Results indicated that, of the 12.8 mg IgG applied to the column, a total of 10.34 mg IgG were recovered, which was 80.8% of the total potential recovery. Following three separate determinations, the mean recovery was found to be 80.4 ± 1.1% of total possible recovery.

Figure 4.1.1:



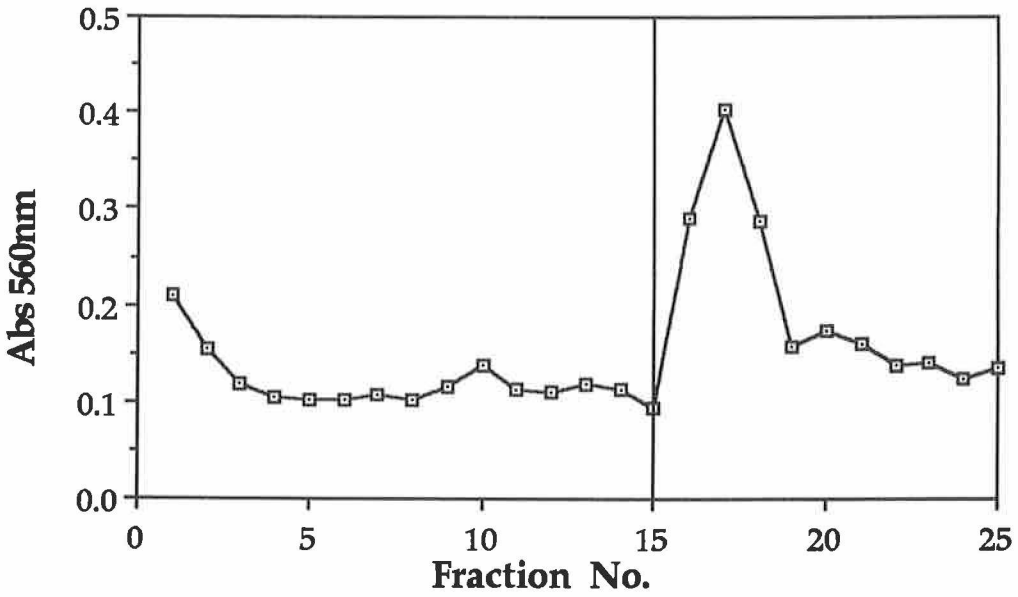


Figure 4.1.2:

The elution profile of 'pure' IgG through a Protein A Sepharose column (1 x 2 cm), 1 ml fractions were collected. The column was washed with binding buffer (Pierce), 15 fractions of which were collected. The buffer was changed to elution buffer (Pierce). A further 10 fractions were collected. The protein content of the fractions was determined by BCA assay (Abs_{560nm}). This illustrates that a small amount of protein elutes in the binding buffer, due to overloading of the column or impurities in the sample, while the bulk of the protein (IgG) elutes in fractions 16-18 (the first three fractions of elution buffer), which were pooled for future use.

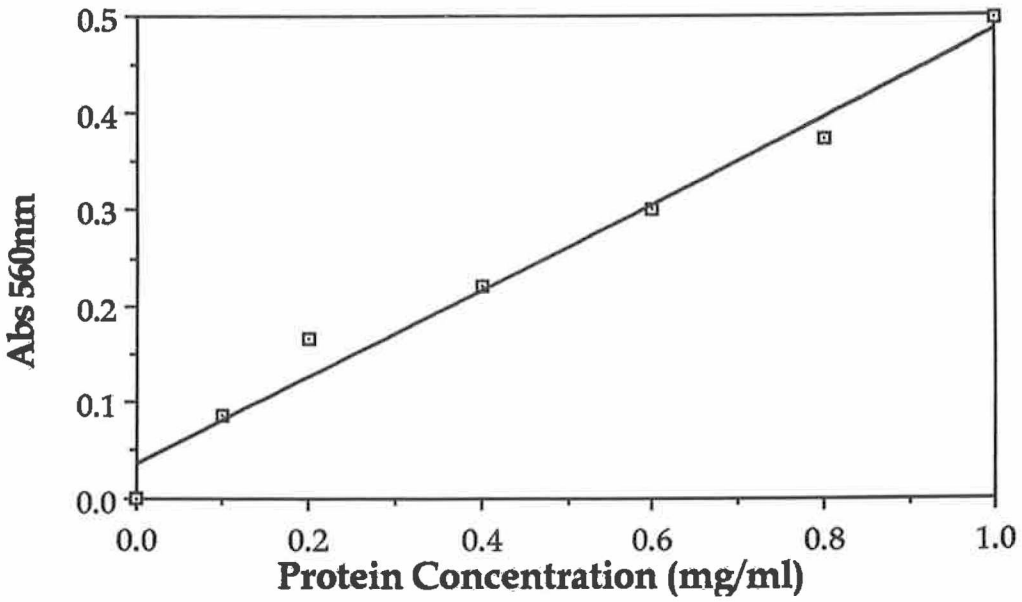


Figure 4.1.3:

The BCA assay (Section 3.5.1) was used to determine protein concentration. The graph shows a sample standard curve produced using the BCA assay and a range of BSA standards, with a regression coefficient (r) of 0.993.

In order to show that only IgG was eluting from the Protein A column, HPLC (Section 3.4.2) was carried out on the fractions which contained protein (Fig. 4.1.1c). It was found that human IgG has a retention time on HPLC of 14.4 ± 0.1 min. HPLC analysis (Section 3.4.2) of fractions 16, 17 and 18 showed two peaks, one at 14.3 ± 0.1 min, the other at 22 ± 0.1 min. The identity of the later peak was uncertain but there were two possibilities. One was that Protein A was leaching off the column and eluting along with the IgG. The other was that the elution buffer interferes with the HPLC analysis. In order to establish which of these was the case, the Protein A column was run four times more using different elution buffers; elution buffer (Pierce), 0.1 M citrate buffer, 0.1 M Glycine buffer and 0.1 M Tris-HCl buffer. The relevant fractions, 16-18, were assayed by HPLC. In all cases the three fractions contained the 22 min peak, as well as the 14.3 min IgG peak. In which case; (a) all the buffers cause Protein A to leach off the column, or (b) the buffers themselves cause a 22 min peak using this type of HPLC analysis. A set of blanks were run where buffer alone was injected onto the HPLC column. Each of the buffers produced a 22 min peak, although the height of the peak varied. The System Gold software was used to subtract the 'buffer peak' from the profile obtained from fraction 17 eluted from the Protein A column. The second peak was eliminated in all cases, indicating that the 22 min peak is a buffer peak, i.e. it occurs at the exclusion limit of the column (Fig. 4.1.4).

Purification of the IgG was completed by de-salting on a Sephadex G-75 column, from which IgG is totally excluded and elutes in the void volume. Fractions 17 and 18 from the Protein A column were pooled and a sample applied to the G-75 column (1x10 cm, ddH_2O). Up to fifteen 1ml fractions were collected and the BCA assay (Section 3.5.1) was carried out on each in order to construct an elution profile (Fig. 4.1.5). Those fractions containing protein were assayed for protein concentration using the BCA assay. From a total of 9.94 mg applied to the column 9.4 mg were recovered, which was 94.6% of total potential recovery. Following three separate determinations, the mean recovery was found to be 95.1 ± 0.9% of total potential recovery.

IgG purification by this protocol resulted in a recovery of 76.4% of total potential yield. Following three separate determinations, the mean recovery was found to be 76.5 ± 1% of total potential yield.

The purity of the IgG was confirmed using HPLC (Section 3.4.2) as shown (Fig. 4.1.1) and using polyacrylamide gel electrophoresis (PAGE) (Section 3.7) as shown (Fig. 4.1.6).

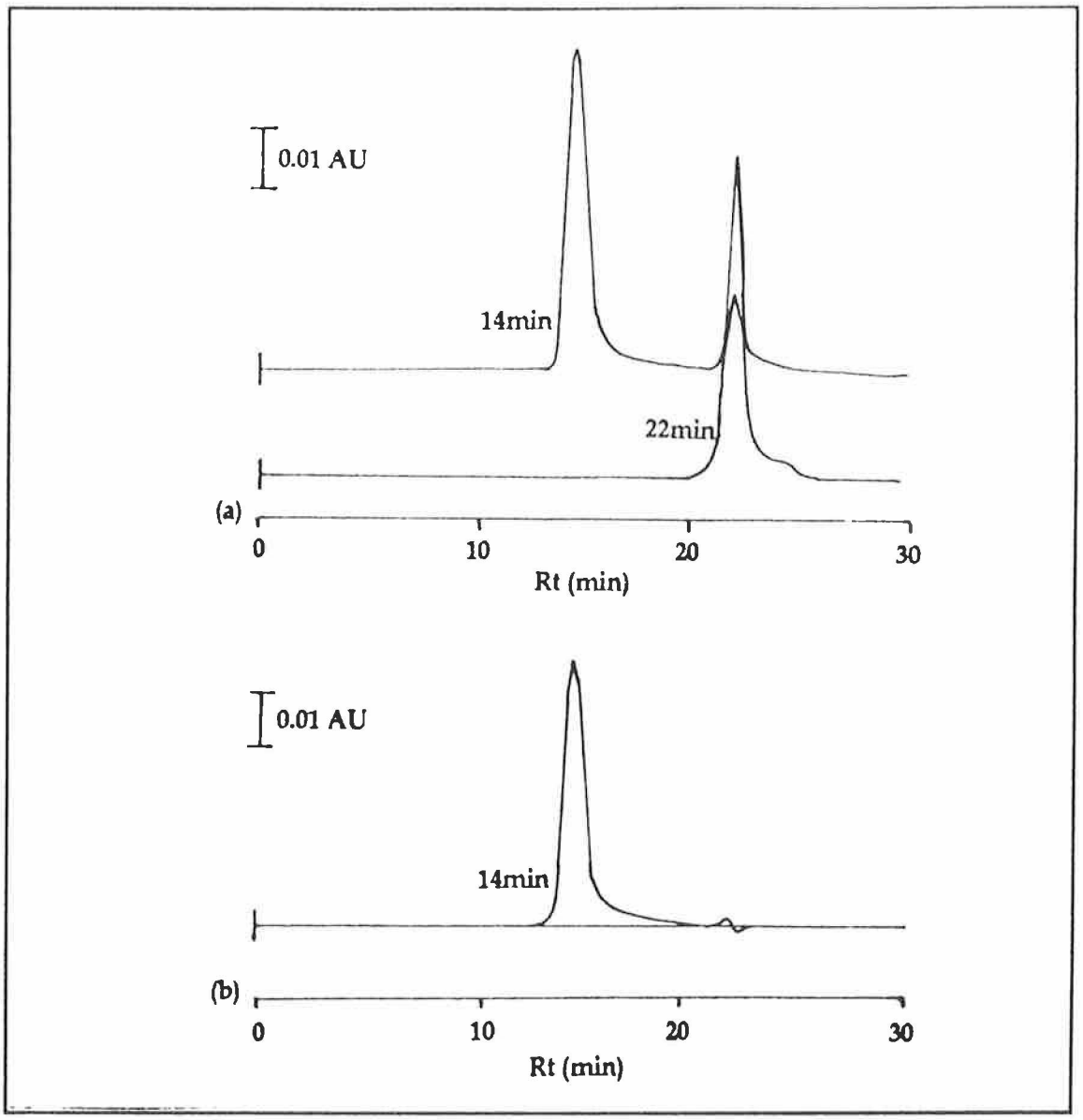


Figure 4.1.4:

The chromatograms produced following HPLC analysis of the IgG eluted from a Protein A column (14 min peak). The superimposed chromatograms (a) illustrate that the 22 min peak observed in the IgG sample corresponds to that produced when buffer alone is injected onto the column. This is confirmed when the system Gold software is used to subtract the buffer chromatogram from the IgG chromatogram, as the 22 min peak is erased (b). HPLC analysis was carried out on a Protein Pak 300SW size exclusion column, with a sodium phosphate buffer (0.1 M, pH 7.4) mobile phase, at a flow rate of 0.5 ml/min. The presence of protein was determined using a u.v./visible variable wavelength detector set at 280nm. (System Gold, Beckman) (AU = Absorbance Units)

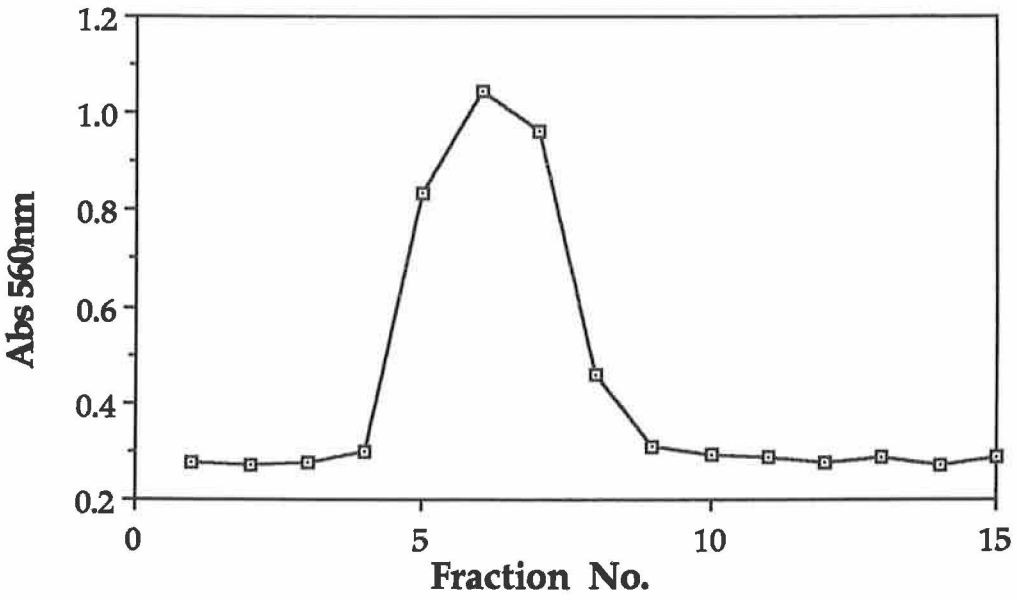


Figure 4.1.5:

The elution profile of pure IgG through a G-75 size exclusion column (1 x 10 cm), with a PBS (0.1 M, pH 7.4) mobile phase. Fifteen 1 ml fractions were collected. The protein content was determined by BCA assay. The bulk of the protein (IgG) elutes in fractions 5-8.

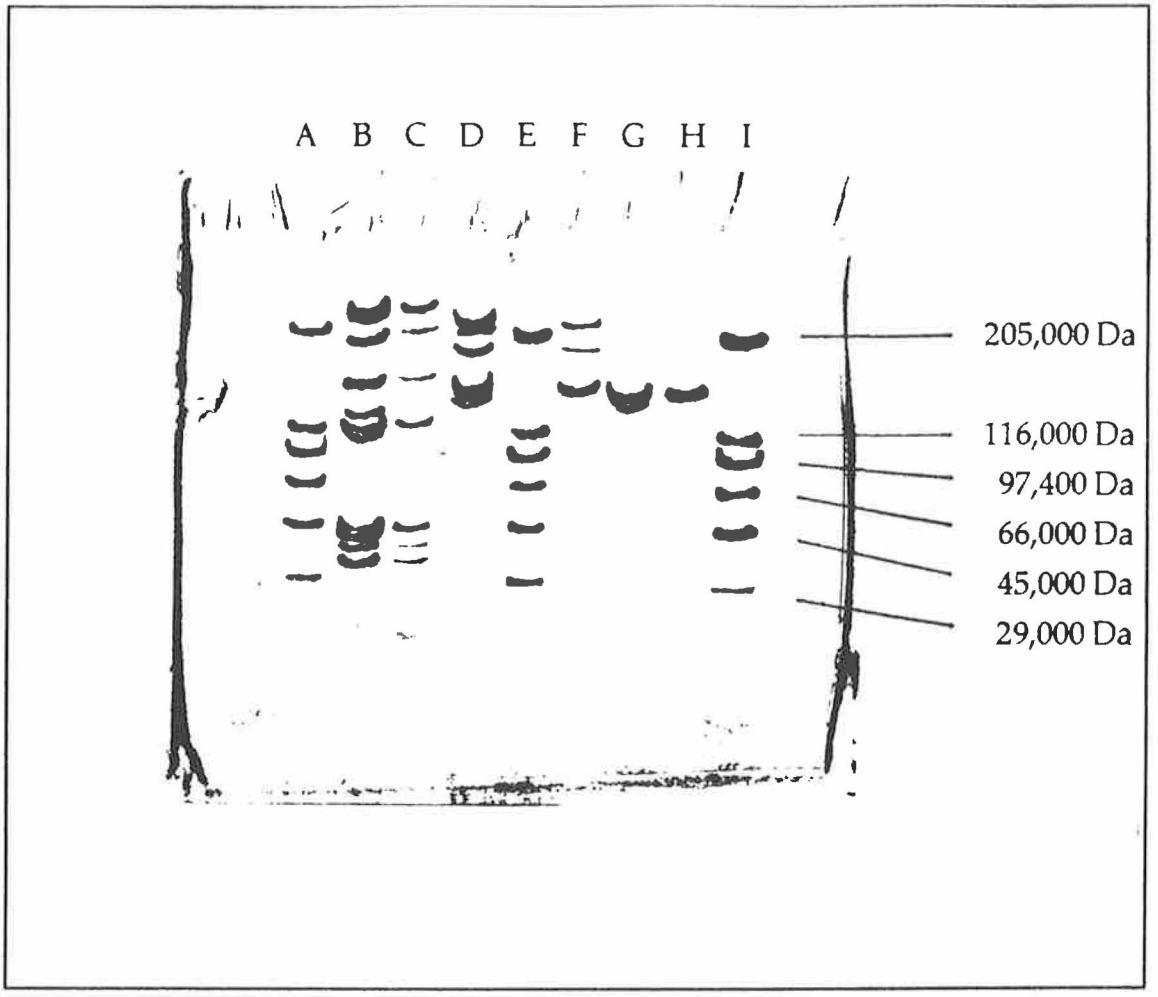


Figure 4.1.6:

This photograph shows the various stages of purification of IgG from ascites as determined by PAGE. Lanes A, E and I were loaded with 30 µl of a mixture of six proteins of known molecular weight (Sigma). Lanes B and C were loaded with 50 µl ascitic fluid diluted 1:50 and 1:100, respectively, with PBS (0.01 M, pH 7.4). These lanes show the large amount of protein of various sizes present in the ascitic fluid. Lanes D and F were loaded with 50 µl of the solution of IgG precipitated from the ascitic fluid using ammonium sulphate (50%, w/v), diluted 1:10 and 1:20, respectively, with PBS (0.01 M, pH 7.4). These lanes show an increase in the relative amount of a protein of the same size as IgG, although some impurities still remain. Lanes G and H were loaded with 50 µl of the protein containing eluate of the Protein A column, diluted 1:10 and 1:20, respectively, with PBS (0.01 M, pH 7.4). These lanes show that only one protein, of molecular weight 150 kDa approximately, is eluted from the Protein A column, i.e. pure IgG. PAGE was carried out using an LKB minigel system, with a polyacrylamide gel containing 10% SDS, run at a constant current of 25 mA and stained with Coomassie blue.

In an attempt to improve the yield of IgG, a thiophillic chromatography column (Affi-T) was set up (Section 3.3.4). A 150 µl sample of human IgG was applied to the Affi-T column. The column was washed with binding buffer and twenty 1.5 ml fractions were collected. The elution buffer was applied and a further twenty 1.5 ml fractions collected. The elution buffer contains Tris which interferes with the BCA reaction. Hence, the protein concentration of each fraction was estimated by measuring the Abs_{280nm} (Fig. 4.1.7). Results indicated that of the 0.975 mg applied to the column, 0.825 mg were recovered, which was 84.6% of total potential yield. Following three separate determinations, the mean recovery was found to be 84.1 ± 1.1% of total potential recovery. Buffer exchange was then carried out by extensive dialysis against 0.1 M sodium phosphate buffer (pH 7.4). Protein content, as estimated by Abs_{280nm}, was 0.825 mg before dialysis. A total of 0.785mg was recovered after dialysis, which was 95.1% of total potential yield. Following three separate determinations, the mean recovery was found to be 94.8 ± 0.7% of total potential recovery.

IgG purification by this protocol resulted in a recovery of 80.4% of total potential yield. Following three seperate determinations, the mean recovery was found to be 79.7 ± 0.9% of total potential recovery.

From the results (Table 4.1.1) it would appear that the second protocol, using Affi-T and dialysis, produces the greater yield. However, the Protein A column has a greater capacity to bind IgG, so that fewer column runs are necessary and losses are reduced. In light of the relatively large quantities of IgG required to produce $BF(ab')_2$, further purifications were carried out using the Protein A column and dialysis.

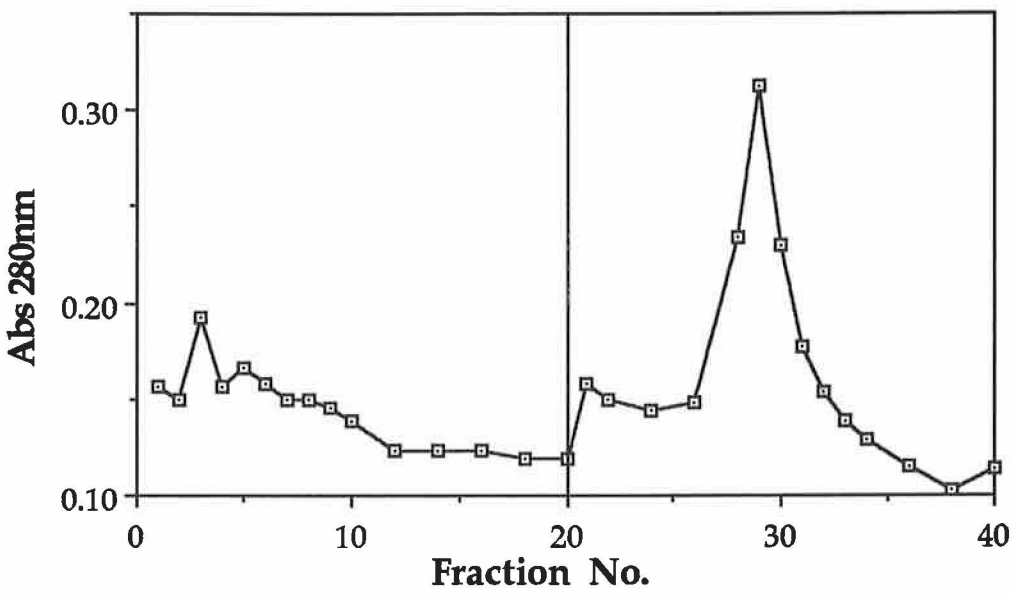


Figure 4.1.7:

The elution profile of 'pure' IgG through an Affi-T affinity column. Forty 1.5 ml fractions were collected, the buffer was changed, from ammonium sulphate (0.75 M) to Tris-HCl (0.05 M, pH 9), after 20 fractions. The protein content was determined by measuring the Abs_{280nm} of each fraction. This illustrates that a small amount of protein elutes in the binding buffer, possibly due to overloading of the column. The bulk of the IgG elutes in fractions 25-28 (fractions 5-8 of the elution buffer), which were pooled for future use.

Source	Total Protein (mg)	Total Protein (% of starting material)
Applied to Protein A	12.80	100
Recovered from Protein A	10.34	80.3
Applied to G-75	9.94	100
Recovered from G-75	9.40	94.6
Total Recovered	9.8	76.4
Applied to Affi-T	0.97	100
Recovered from Affi-T	0.82	84.6
Applied to Dialysis	0.82	100
Recovered from Dialysis	0.78	95.1
Total Recovered	0.78	80.4

Table 4.1.1:

The recovery of pure IgG from various purification protocols following the application of pure IgG. The IgG content was measured by estimating the protein concentration, using the BCA assay.

4.1.2 $F(ab')_2$ Production:

The $F(ab')_2$ fragment was readily produced using digestion with pepsin. Two different protocols were used. The procedure used by Brennan *et al.* (1985) involved an overnight digestion step (Section 3.8.1). An alternative procedure suggested by Runge *et al.* (1990) involved a 2 hr digestion step. Both methods proved equally successful at producing two similar peaks on analysis by HPLC (Section 3.4.2). The $F(ab')_2$ fragment eluted at 16.1 ± 0.2 min, while the Fc' fragment eluted at 22.5 ± 0.4 min (Fig. 4.1.8). The first procedure was chosen for future work since gentler conditions are used and it gives a more complete digestion.

The Fc' fragment should not interfere with future reactions. However, its presence may make interpretation of later chromatograms or PAGE gels difficult. Three methods were tried to separate the fragments.

The first method involved an AcA44 Ultrogel column, which was set up (1.5 x 30 cm) and equilibrated with 0.1 M sodium phosphate buffer, pH 7.4 (Section 3.4.2). A 0.5 ml sample of $F(ab')_2$ (10 mg/ml) was applied to the column and a total of fifty 1 ml fractions were collected. The Abs_{280nm} of each fraction was measured and an elution profile constructed. No visible peaks emerged in the profile (Fig. 4.1.9). This is possibly due to dilution of the sample beyond the detection limit. No separation could be determined. Thus, the AcA44 Ultrogel column is unsuitable for this separation.

The second method involved a Sephacryl S-200 column, which was set up (2.6 x 70 cm) and equilibrated with 0.15 M Tris-HCl buffer (pH 8) running at 0.4 ml/min (Section 3.4.2). A 0.5 ml sample of $F(ab')_2$ (10 mg/ml) was applied to the column and a total of one hundred 4 ml fractions were collected. The Abs_{280nm} of each fraction was measured and an elution profile constructed (Fig. 4.1.9). Again it appeared that the protein was diluted beyond the detection limit so that no separation could be carried out using the Sephacryl S-200 column.

The final method involved a Protein A affinity column, which was set up and equilibrated in binding buffer (Section 3.3.3). A 0.5 ml sample of $F(ab')_2$ (10 mg/ml) was applied to the column. Fifteen 1 ml fractions of binding buffer and five 1 ml fractions of elution buffer were collected. The Abs_{280nm} of each fraction was read and an elution profile constructed (Fig. 4.1.9). Two main peaks were present, these were both assayed by HPLC (Section 3.4.2) to determine what size of protein molecule was present. Two fractions (3 & 18) were assayed and found to contain the same sized protein with a retention time of 16.1 min (Fig. 4.1.10). It was concluded that the Protein A column used, in this instance, was unsuitable for the purpose intended.

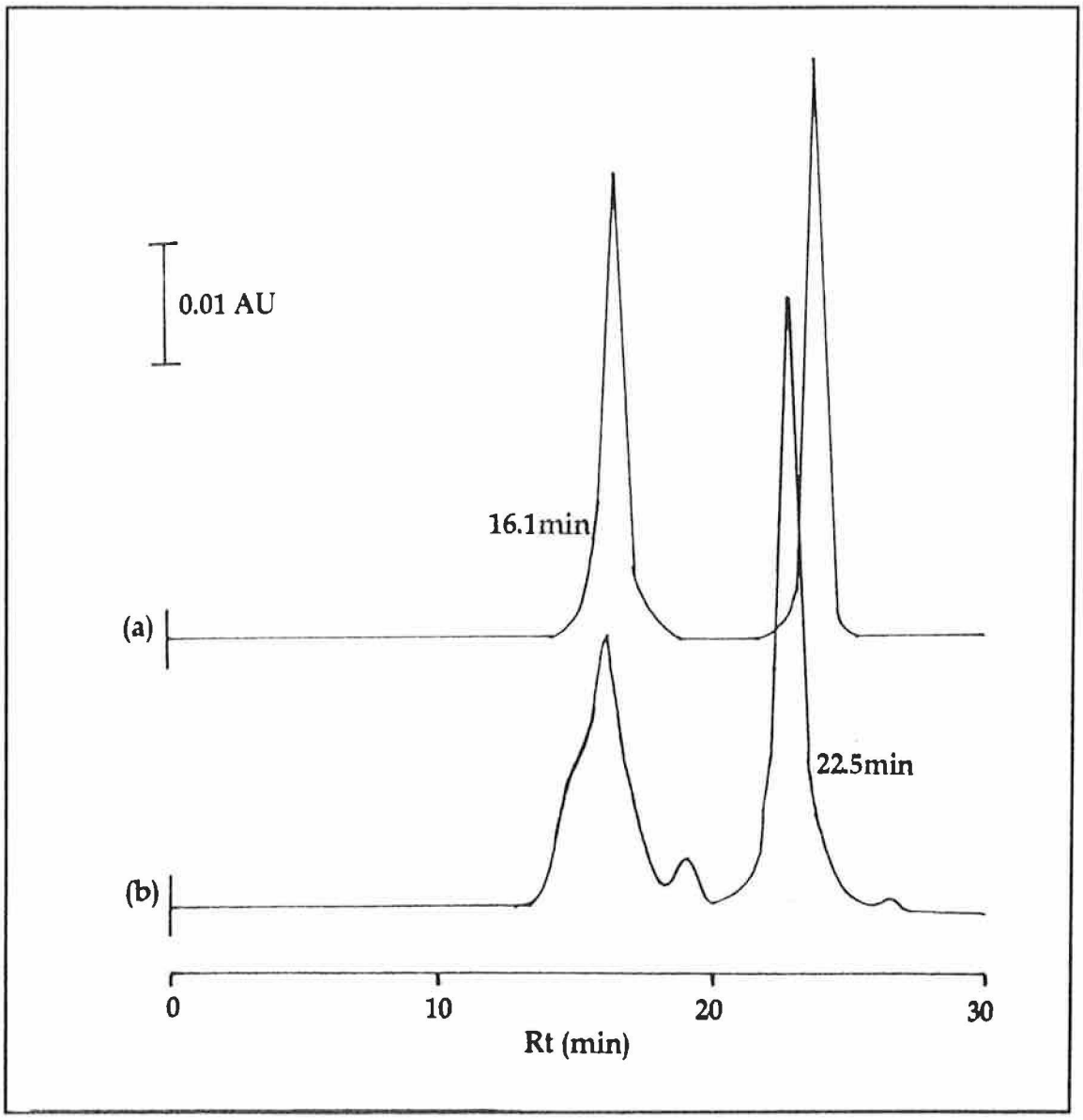


Figure 4.1.8:

The chromatograms produced following HPLC analysis of the $F(ab')_2$ fragments produced by two different pepsin digestion protocols. Both protocols showed two peaks, the $F(ab')_2$ fragment at 16.1 ± 0.2 min and the Fc' fragment at 22.5 ± 0.4 min. (a) Brennan *et al.* (1985). (b) Runge *et al.*, (1990). The latter contains two additional features: A shoulder on the 16.1 min peak indicating the presence of a heavier protein, i.e. undigested IgG. A third, smaller peak at 19 ± 0.1 min corresponds to Rt of pure pepsin. HPLC analysis was carried out on a Protein Pak 300SW size exclusion column, with a sodium phosphate buffer (0.1 M, pH 7.4) mobile phase, at a flow rate of 0.5 ml/min. The presence of protein was determined using a u.v./visible variable wavelength detector set at 280nm. (System Gold, Beckman) (AU = Absorbance Units)

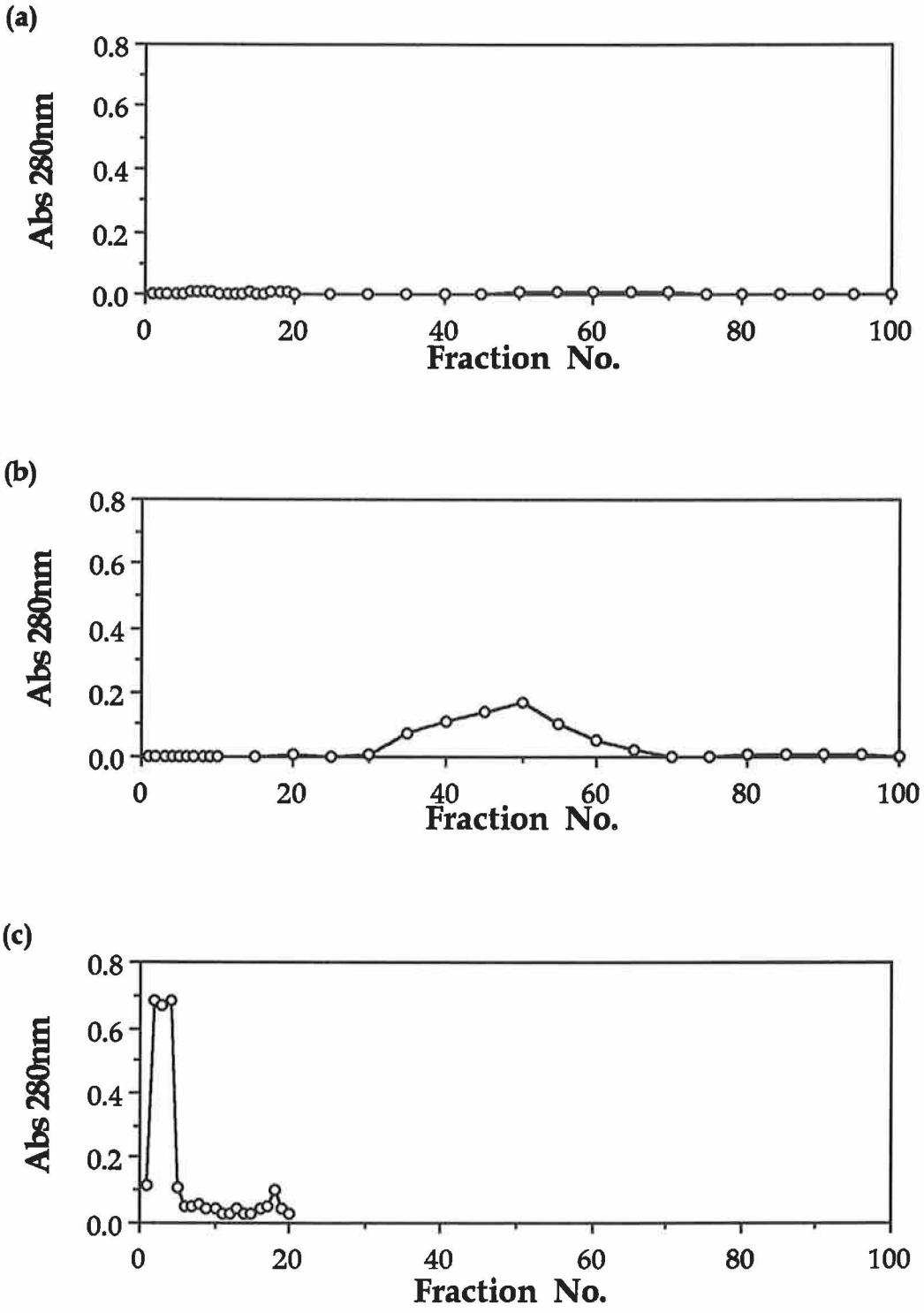


Figure 4.1.9:

The elution profiles obtained while 'purifying' the F(ab')2 fragment. (a) No protein could be detected following AcA44 chromatography. (b) No separation could be detected following Sephacryl S-200 chromatography. (c) Two peaks, one major and one minor, were detected following Protein A chromatography, these were further tested to determine their contents.

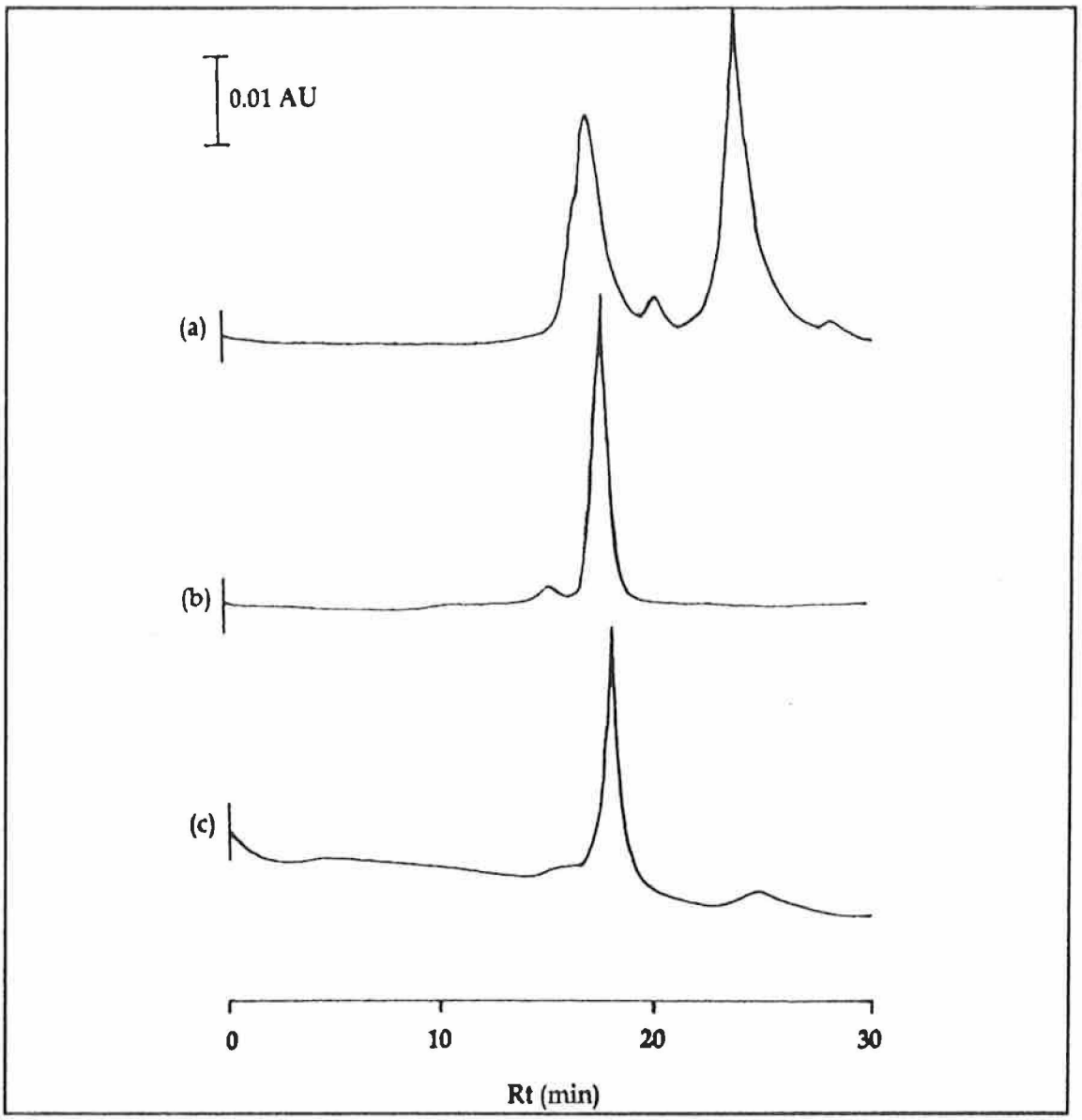


Figure 4.1.10:

IgG was subjected to pepsin digestion, the resulting mixture of F(ab')2 and Fc' was applied to a Protein A sepharose column (Pierce). The F(ab')2 should elute in the first fractions of binding buffer, Fc' should bind to the column until released by the binding buffer. HPLC analysis was carried out on the reaction mixture, containing F(ab')2 and Fc' (a). The third fraction of binding buffer (b) and the third fraction of elution buffer (c) from the Protein A column (Fig. 4.1.9) were also analysed by HPLC. The HPLC chromatograms obtained indicate that both fractions contain F(ab')2 only. Analysis was carried out using the Beckman System Gold HPLC in conjunction with a Protein Pak 300SW column. The sodium phosphate buffer (0.1 M, pH 7.4) mobile phase was run at a flow rate of 0.5 ml/min, the variable wavelength detector was set at 280nm for detection of protein. (AU = Absorbance Units)

4.1.3 Fab' Production:

The $F(ab')_2$ fragments were reduced to the Fab' fragments as described (Section 3.8.2). dTNB, which reacts with thiol groups, was added in order to prevent intrachain disulphide bond formation and stabilise the fragments. HPLC was used (Section 3.4.2) to show that the reduction had gone to completion and that the resulting molecule was approximately half the size of the original $F(ab')_2$ fragment. (Fig. 4.1.11)

It was essential to remove all the unreacted dTNB from the solution. This was primarily because dTNB interferes with later stages of $BF(ab')_2$ production. It was also necessary if the derivatisation of Fab' to Fab'-TNB was to be confirmed. This was carried out by extensive dialysis against 1 mM sodium phosphate buffer (pH 7.4).

A means of detecting dTNB in solution was required if conjugation was to be confirmed. dTNB is water soluble, producing a pale yellow solution. The absorbance (Abs) of the solution was measured between 200nm and 500nm, using a Shimadzu UV-160A uv-visible recording spectro-photometer. The λ_{max} was determined to be 325.5nm, (Fig. 4.1.12). This solution was allowed to react with cysteine for 3 hrs at 25°C in order to form Cys-TNB derivatives. The reaction mixture was dialysed extensively, against 1 mM sodium phosphate buffer (pH 7.4), to remove free dTNB. The λ_{max} of Cys-TNB was found to be 326.6nm, (Fig. 4.1.12). The λ_{max} of cysteine (λ_{max} 240nm) and human and murine $F(ab')_2$ (λ_{max} 271.9nm) was also measured, (Fig. 4.1.12). Thus, reading the Abs_{325nm} of these solutions gives an indication of the amount of dTNB (free and derivatised) present, without interference from any protein in solution. This was carried out for each step in the production of Fab'-TNB and Fab'-SH.

The Abs_{325nm} of the starting material, $F(ab')_2$, was measured. This was then reduced with mercaptoethylamine and allowed to react with dTNB (5 mM). The Abs_{325nm} rose considerably. The solution was dialysed extensively to remove free dTNB. The Abs_{325nm} of the post-dialysis solution did not fall significantly compared to the Abs_{325nm} of the reaction mixture, indicating that most of the dTNB has been bound to the protein. Further reduction of the protein by mercaptoethylamine to Fab'-SH released the dTNB into solution. The solution was dialysed extensively after which the Abs_{325nm} fell, indicating that the bound dTNB has been removed and only Fab'-SH remains. This final reduction was carried out on the human Fab'-TNB fragments only, to produce human Fab'-SH fragments. The murine fragments were retained as the Fab'-TNB. The above results are summarised in Table 4.1.2.

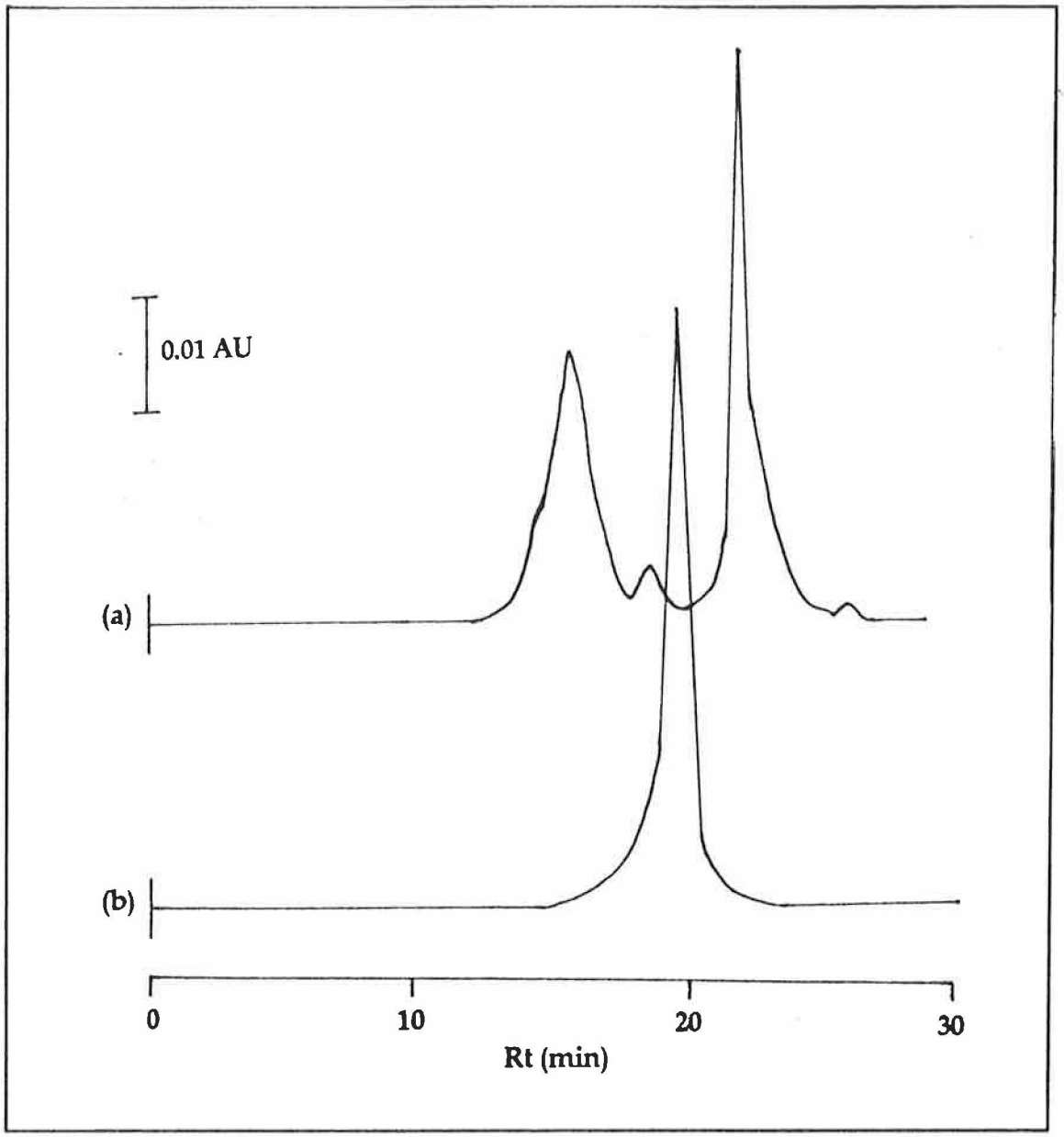


Figure 4.1.11:

IgG was subjected to pepsin digestion. This mixture containing, F(ab')2 and Fc', was analysed by HPLC (a). The mixture was reduced with mercaptoethylamine to the Fab'-SH fragment (b). The Fab'-SH has a retention time of 18.5 min, which is significantly longer than that of the F(ab')2, 16.1 min. This would indicate that the Fab'-SH fragments are considerably smaller than the F(ab')2. Analysis was carried out using the Beckman System Gold HPLC in conjunction with a Protein Pak 300SW column. The sodium phosphate buffer (0.1 M, pH7.4) mobile phase was run at a flow rate of 0.5 ml/min, the variable wavelength detector was set at 280nm for detection of protein. (AU = Absorbance Units)

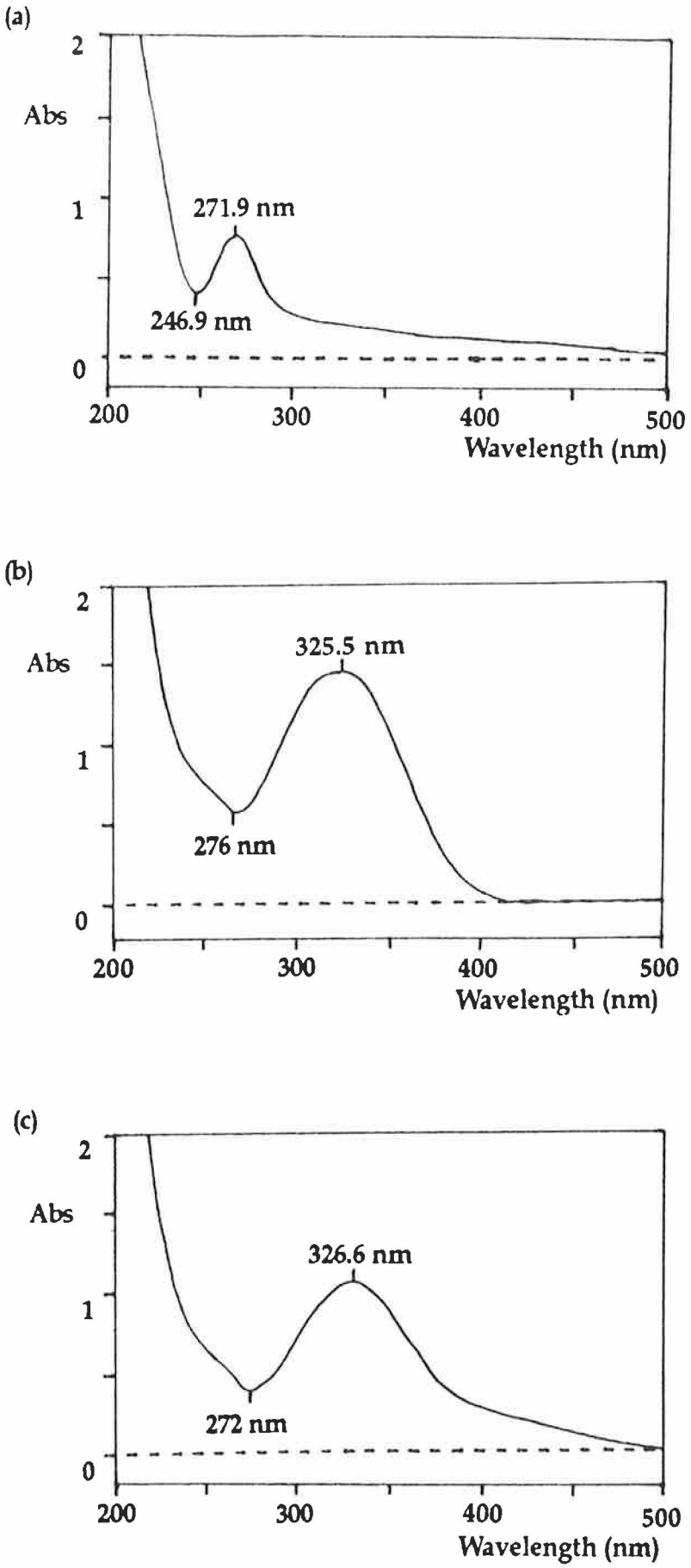


Figure 4.1.12:

The absorbance spectra of (a) protein (IgG), (b) dTNB and (c) cysteine-TNB derivatives, taken on a Shimadzu UV-160A recording spectrophotometer.

Solution	Abs_{325nm} ± SD
F(ab')2	0.166 ± 0.008
Fab'-TNB & free dTNB	1.314 ± 0.066
Fab'-TNB (post dialysis)	1.251± 0.063
Fab'-SH & free dTNB	1.222 ± 0.061
Fab'-SH (post dialysis)	0.255 ± 0.013

Table 4.1.2:

The derivatisation of antibody fragments by dTNB, and its removal, as illustrated by the measurement of the Abs_{325nm} of the solution. The F(ab')2 was reduced and allowed to react with dTNB. This caused the Abs_{325nm} to rise sharply. The Abs_{325nm} remains high after dialysis indicating that the dTNB has bound to the Fab' (Fab'-TNB). The Fab'-TNB is further reduced to the Fab-SH, releasing the dTNB. This is shown by the fall in Abs_{325nm} following dialysis. (SD = standard deviation, n = 3)

4.1.4 BF(ab')$_2$ Production:

The human Fab'-SH and the murine Fab'-TNB were combined according to the methods of Brennan *et al.* (1985). The mixture was subsequently analysed by HPLC (Section 3.4.2). The chromatogram showed the presence of large amounts of "Fab' sized" fragments and trace amounts of a larger fragment. The larger fragment had a similar Rt, and hence a similar size, to the F(ab')$_2$ fragments previously produced. It appeared that association of Fab'-TNB and Fab'-SH fragments to form F(ab')$_2$ fragments was limited (Fig. 4.1.13).

In order to increase the formation of F(ab')$_2$ the mixture was set up again, this time with the addition of 0.5% (w/v) hydrogen peroxide. It was hoped that the creation of oxidising conditions would increase the number of disulphide bonds formed and stabilise the resulting re-formed F(ab')$_2$. When this mixture was analysed by HPLC two peaks were evident (Fig. 4.1.13). In this case, the larger molecule formed 60% of the total protein present, as measured by peak area. The Rt of this peak was similar to that of the F(ab')$_2$ molecules previously produced, indicating that it may be a reformed F(ab')$_2$ fragment or a BF(ab')$_2$.

Five proteins, of known molecular weight, were applied to the HPLC (Fig. 4.1.14). From these results a standard curve of Rt vs. Log molecular weight was produced (Fig. 4.1.15). The various antibody fragments were also analysed on HPLC (Fig. 4.1.16) and the molecular weights calculated from the standard curve. The molecular weights of the standards were back calculated from the equation of the line. This indicated that the standard curve estimated the molecular weight to within 10% of the expected molecular weight. The molecular weight of the BF(ab')$_2$ was slightly lower than expected, i.e. less than the original F(ab')$_2$ fragment (Table 4.1.3). It was, however, twice the weight of a Fab' fragment. This would indicate that a F(ab')$_2$ size fragment was formed. It was necessary to determine if these fragments were formed by the combination of similar or dissimilar Fab' fragments.

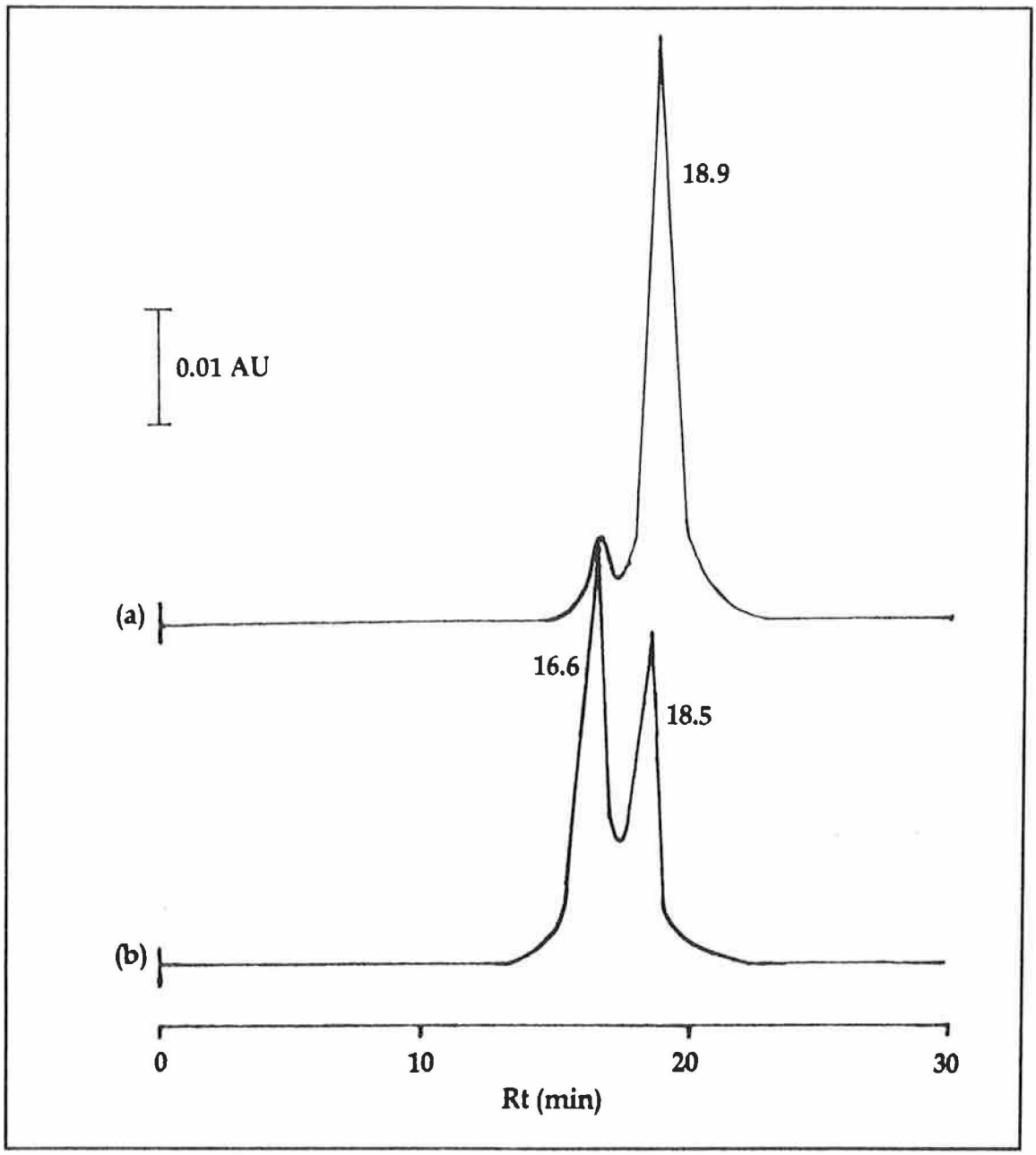


Figure 4.1.13:

Fab'-TNB and Fab'-SH fragments were combined to produce $BF(ab')_2$ fragments, and analysed by HPLC: (a) In the absence of H_2O_2, resulting in the production of small amounts of a $F(ab')_2$ sized molecule, Rt = 16.6 ± 0.3 min. (b) With the addition of 0.5% (w/v) H_2O_2, resulting in the production of increased quantities of the $F(ab')_2$ sized molecule, up to 75% of total potential yield was produced. Analysis was carried out using the Beckman System Gold HPLC in conjunction with a Protein Pak 300SW column. The sodium phosphate buffer (0.1 M, pH 7.4) mobile phase was run at a flow rate of 0.5 ml/min, the variable wavelength detector was set at 280nm for detection of protein. (AU = Absorbance Units)

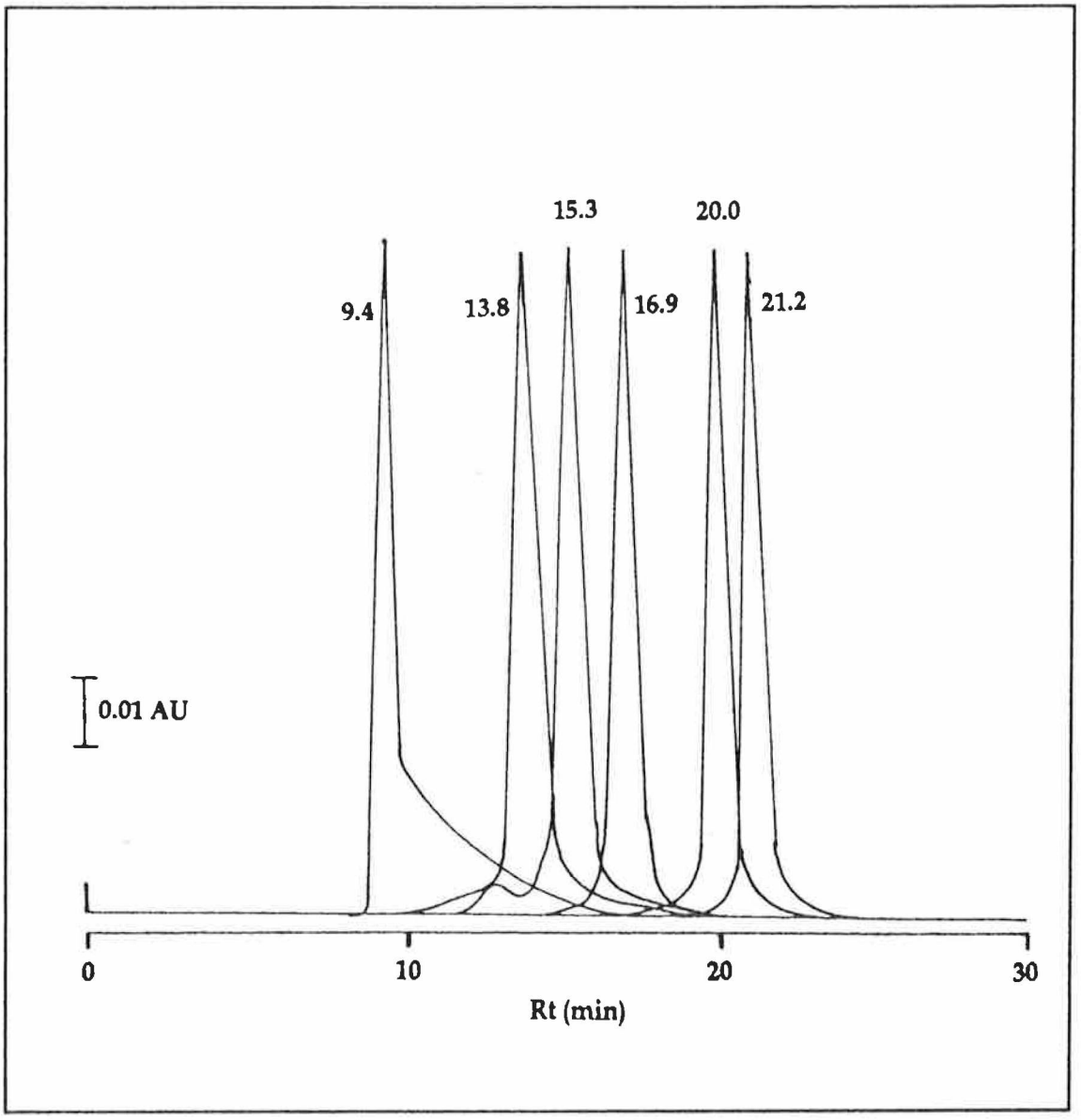


Figure 4.1.14:

The chromatograms produced as a result of the HPLC analysis of six proteins of known molecular weight The proteins elute in order of decreasing molecular weight: Blue Dextran, 2 x 10^6 Da, Rt = 9.4 min. β-Amylase, 2 x 10^5 Da, Rt = 13.8 min. Alcohol dehydrogenase, 1.5 x 10^5 Da, Rt = 15.3 min. Albumin, 6.6 x 10^4 Da, Rt = 16.9 min. Carbonic anhydrase, 2.9 x 10^4 Da, Rt = 20.0 min. Cytochrome C, 1.24 x 10^4 Da, Rt = 21.2 min. Analysis was carried out using the Beckman System Gold HPLC in conjunction with a Protein Pak 300SW column. The sodium phosphate buffer (0.1 M, pH 7.4) mobile phase was run at a flow rate of 0.5 ml/min, the variable wavelength detector was set at 280nm for detection of protein. (AU = Absorbance Units)

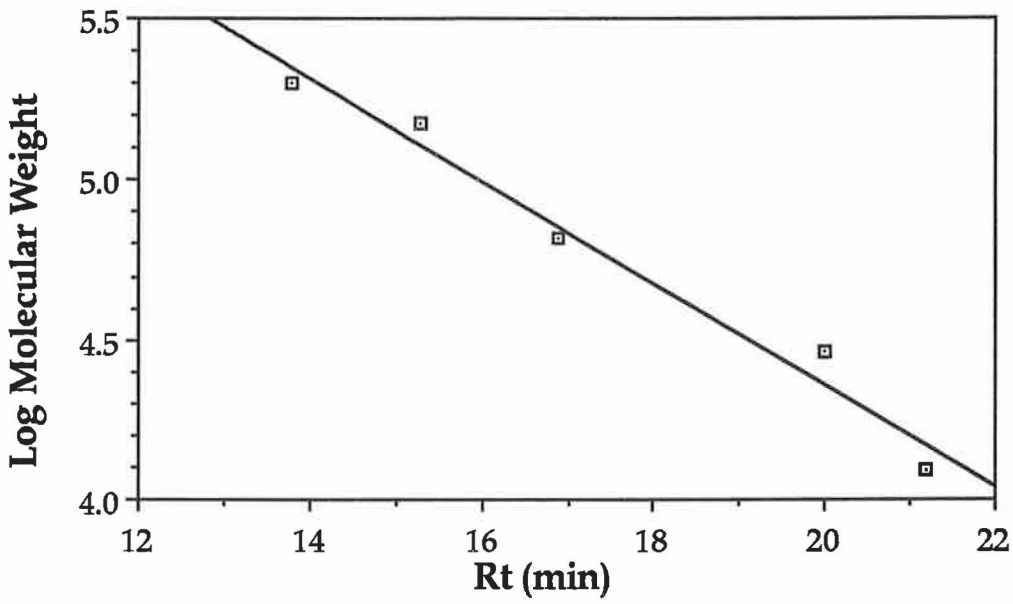


Figure 4.1.15:

The standard curve produced by plotting the retention times, from HPLC analysis, of proteins of known molecular weight against the Log molecular weight. (r = 0.988) Analysis was carried out using the Beckman System Gold HPLC in conjunction with a Protein Pak 300SW column. The sodium phosphate buffer (0.1 M, pH 7.4) mobile phase was run at a flow rate of 0.5 ml/min, the variable wavelength detector was set at 280nm for detection of protein.

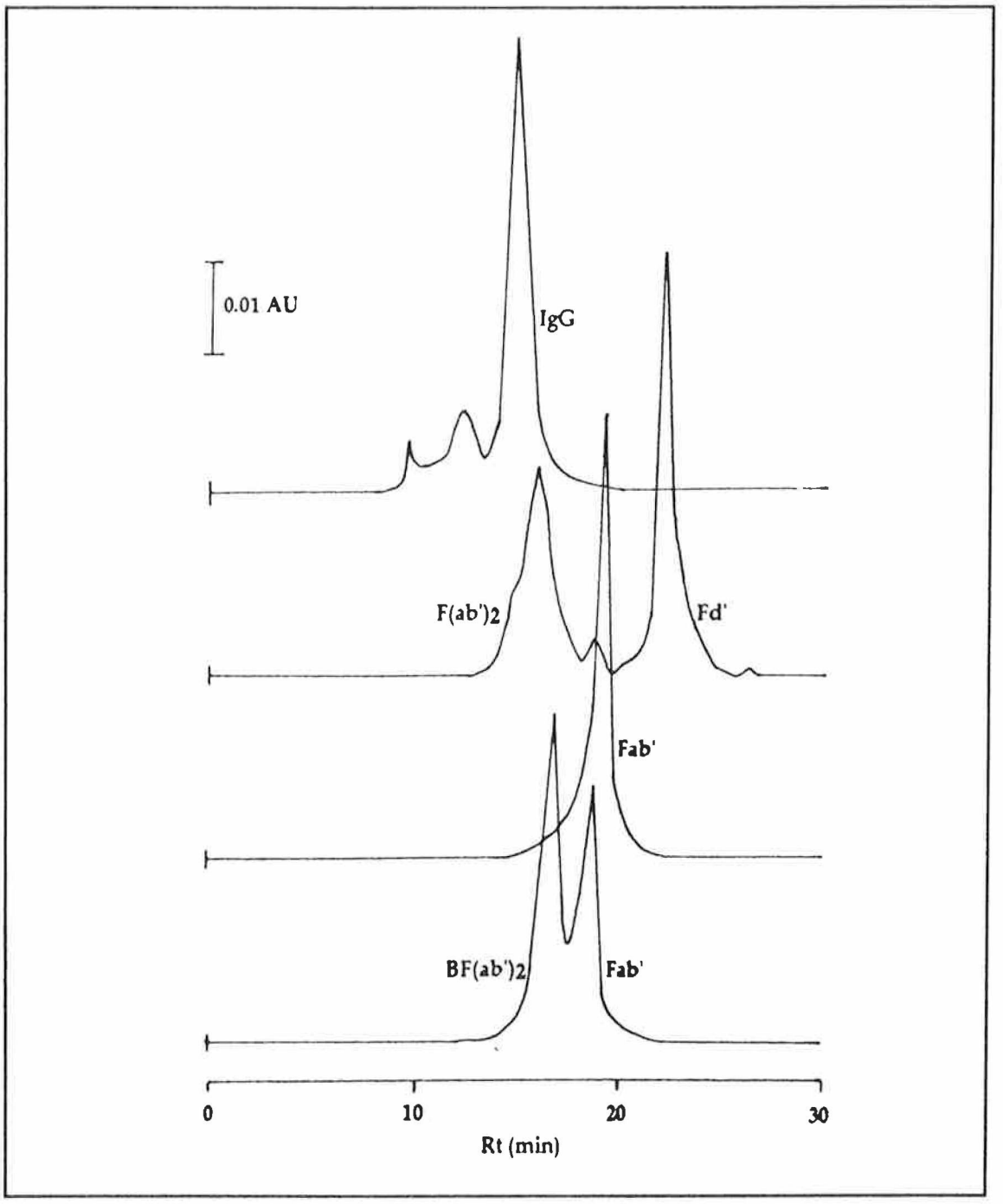


Figure 4.1.16:

The chromatograms produced following HPLC analysis of the antibody fragments formed during the production of $BF(ab')_2$, illustrating that the $BF(ab')_2$ has a similar retention time to the $F(ab')_2$ produced by pepsin digestion of the IgG. Analysis was carried out using the Beckman System Gold HPLC in conjunction with a Protein Pak 300SW column. The sodium phosphate buffer (0.1 M, pH 7.4) mobile phase was run at a flow rate of 0.5 ml/min, the variable wavelength detector was set at 280nm for detection of protein. (AU = Absorbance Units)

Protein	Rt ± SD (min)	Mean Molecular Weight
Blue Dextran	9.4 ± 0.3	2,000,000
β-Amylase	13.8 ± 0.5	200,000
Alcohol Dehydrogenase	15.3 ± 0.4	150,000
Albumin	16.9 ± 0.2	66,000
Carbonic Anhydrase	20.0 ± 0.3	29,000
Cytochrome C	21.2 ± 0.4	12,400
IgG	14.8 ± 0.3	156,500
F(ab')2	16.1 ± 0.2	96,600
Fab'-SH/Fab'-TNB	18.5 ± 0.4	40,100
BF(ab')2	16.6 ± 0.3	80,700

Table 4.1.3:

The results of HPLC analysis of standard proteins and antibody fragments derived from the production of BF(ab')2, according to the method of Brennan *et al.*. (1985). The retention time (Rt) of each protein was determined five times. A standard curve of Rt vs. Log molecular weight was constructed from the proteins of known molecular weight. The molecular weight of the antibody fragments was determined from the mean Rt.

A sandwich ELISA was developed to show that the molecule formed was in fact composed of a human Fab' and a murine Fab' linked together. The wells of a 96-well plate (Nunc) were coated with anti-mouse Fab' antibody. Non-specific binding sites were blocked with BSA. $BF(ab)_2$ was applied and the murine "half" of the molecule was allowed to bind. The secondary antibody was anti-human Fab' antibody labelled with HRP. This would only bind if the fragments contained a human Fab' "half" (Fig. 4.1.17). Suitable positive and negative controls were carried out (Table 4.1.4). The Abs_{414nm} of each well was determined. The positive controls had a mean reading of 0.597 ± 0.07 (n=4). The negative controls had a mean reading of 0.142 ± 0.03 (n=4). The test wells had a mean reading of 0.578 ± 0.03 (n=4). These results indicate that the $F(ab')_2$ sized molecule, produced in this procedure, was formed by linking a human Fab' fragment to a murine Fab' fragment, i.e. it is a $BF(ab')_2$ fragment. This ELISA was repeated five times, each of which confirmed that $BF(ab')_2$ had been produced.

A similar ELISA was used to determine the optimum concentration of hydrogen peroxide and the optimum incubation period for the production of $BF(ab')_2$. A series of vials were set up containing human Fab'-SH and murine Fab'-TNB, with H_2O_2 at concentrations of 0%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5% and 1.0% (w/v). These were incubated at 37°C. Samples were taken from each tube at 0 hr, 1 hr, 2 hr, 4 hr, 6 hr, 8 hr, and 24 hr intervals. After incubation samples were stored at 4°C. $BF(ab')_2$ production was estimated using the sandwich ELISA. The results indicate that good $BF(ab')_2$ production can be achieved by incubating the Fab' fragments at 37°C for 8 hrs with 0.5% (w/v) H_2O_2 (Fig. 4.1.18). There was very little increase in $BF(ab')_2$ production when the H_2O_2 concentration was doubled to 1% (w/v) or when the incubation period was increased to 24 hrs. To maximise $BF(ab')_2$ production, and for convenience, later $BF(ab')_2$ production runs were carried out in 0.5% (w/v) H_2O_2 and incubated overnight at 37°C.

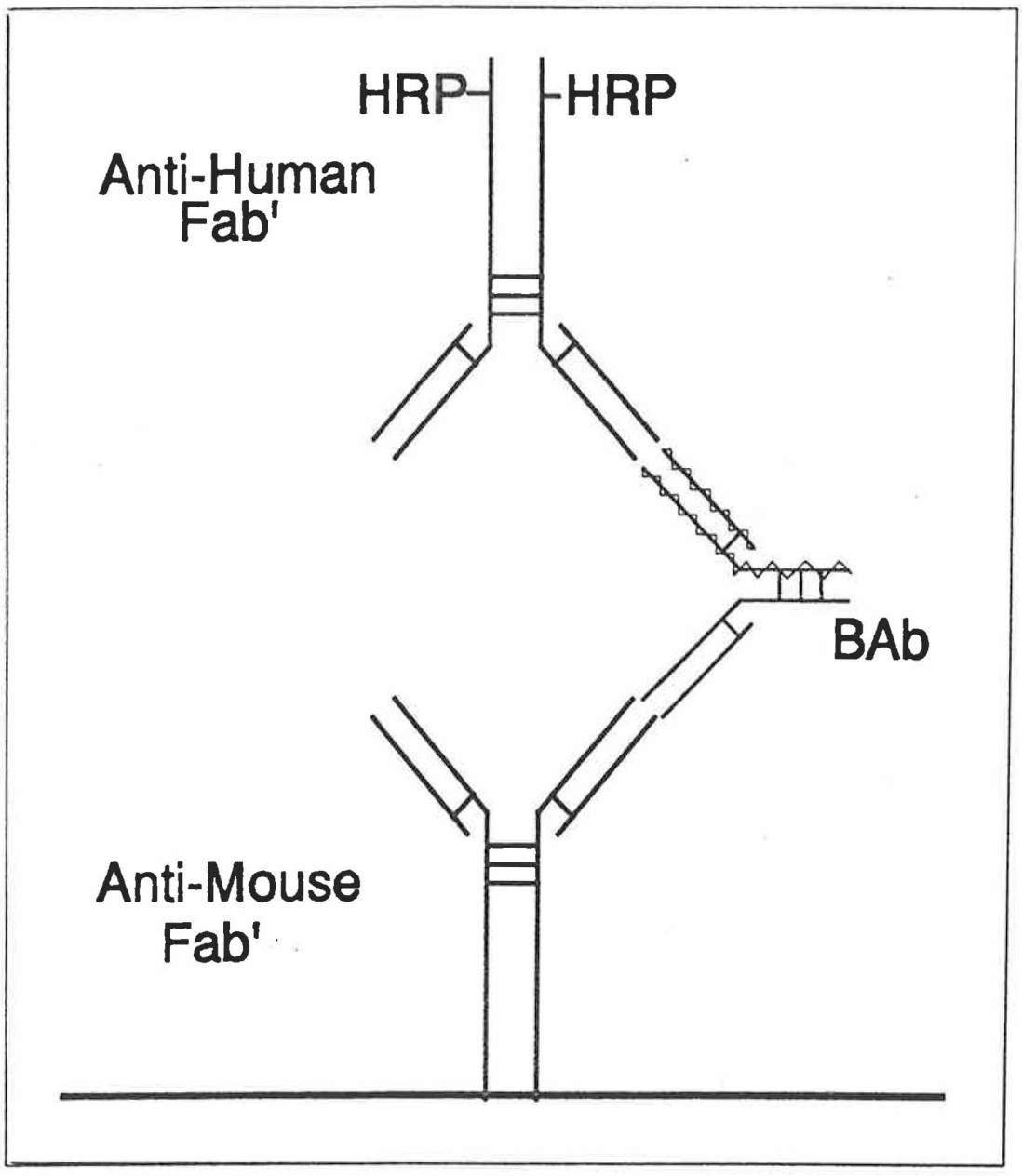


Figure 4.1.17:

An illustration of the ELISA used to determine if the BF(ab')2 produced was in fact formed from a murine Fab' and a human Fab'. The wells of a 96-well plate were coated with anti-mouse Fab' antibody. Non-specific binding sites were blocked with BSA. BF(ab)2 was applied and the murine "half" of the molecule was captured by the bound anti-mouse Fab' antibody. The secondary antibody was anti-human Fab' antibody labelled with HRP. This would only bind if the captured BF(ab')2 contained a human Fab' "half". Thus, on addition of the HRP substrate o-PD, colour will develop only in those wells containing BF(ab')2.

Table 4.1.4:

Primary Antibody	Second Antibody (Antigen)	HRP-Labelled Antibody	Mean Abs_{405nm} ± S.D.
α–Mouse IgG	Human IgG	α–Human IgG	0.153 ± 0.021
α-Mouse IgG	Human F(ab')2	α–Human IgG	0.103 ± 0.014
α-Mouse IgG	Human Fab'	α–Human IgG	0.114 ± 0.025
α-Mouse IgG	PBS	α–Human IgG	0.199 ± 0.033
α-Mouse IgG	PBS	α–Mouse IgG	0.114 ± 0.029
Human IgG	PBS	α–Mouse IgG	0.154 ± 0.031
Human F(ab')2	PBS	α–Mouse IgG	0.105 ± 0.018
Human Fab'	PBS	α–Mouse IgG	0.146 ± 0.031
Mouse IgG	PBS	α–Human IgG	0.179 ± 0.042
Mouse F(ab')2	PBS	α–Human IgG	0.141 ± 0.035
Mouse Fab'	PBS	α–Human IgG	0.151 ± 0.032
BSA	PBS	α–Mouse IgG	0.075 ± 0.027
BSA	PBS	α–Human IgG	0.077 ± 0.029
α-Mouse IgG	Mouse IgG	α–Mouse IgG	0.584 ± 0.049
α-Mouse IgG	Mouse F(ab')2	α–Mouse IgG	0.593 ± 0.031
α-Mouse IgG	Mouse Fab'	α–Mouse IgG	0.572 ± 0.040
Human IgG	PBS	α–Human IgG	0.627 ± 0.037
Human F(ab')2	PBS	α–Human IgG	0.640 ± 0.041
Human Fab'	PBS	α–Human IgG	0.640 ± 0.046
Mouse IgG	PBS	α–Mouse IgG	0.630 ± 0.040
Mouse F(ab')2	PBS	α–Mouse IgG	0.588 ± 0.036
Mouse Fab'	PBS	α–Mouse IgG	0.743 ± 0.043
α-Mouse IgG	BF(ab')2	α–Human IgG	0.587 ± 0.038
α-Human IgG	BF(ab')2	α–Mouse IgG	0.569 ± 0.032

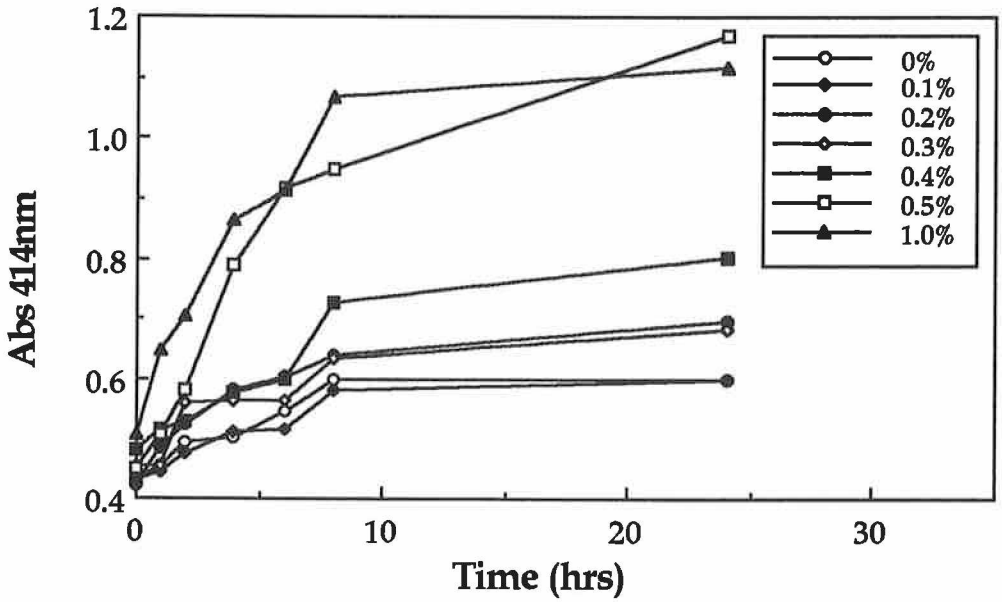


Figure 4.1.18:

A study was carried out to determine the optimum concentration of hydrogen peroxide required for the production of $BF(ab')_2$, as well as the optimum incubation period. The results would indicate that incubation of the Fab' fragments in 1.0% (w/v) H_2O_2, for 24 hrs, at 37°C, create optimum conditions for $BF(ab')_2$ production. However, the milder conditions of 0.5% (w/v) H_2O_2, incubated overnight (i.e. 16 hrs) at 37°C, are nearly as efficient and were chosen convenient conditions for $BF(ab')_2$ production.

4.1.5 Summary:

Large quantities of a murine IgG, G12, were purified from ascites fluid and used, in conjunction with human IgG, to examine the production of $BF(ab')_2$ according to the method of Brennan *et al.* (1985). From the studies in IgG purification it would appear that the Affi-T column produces the greater yield of IgG, but the Protein A column has a greater capacity to bind IgG. Thus, fewer column runs are necessary with the Protein A column and losses are reduced. In light of the relatively large quantities of IgG required to produce $BF(ab')_2$, purifications were carried out using the Protein A column. The purified IgG was subject to a pepsin digestion to produce an $F(ab')_2$ fragment. The method suggested by Brennan *et al.* (1985) was chosen since gentler conditions are used and it gives a more complete digestion than the other method tried (Runge *et al.*, 1990). Several methods were tested in an attempt to separate the $F(ab')_2$ and Fc' fragments (i.e. an AcA44 Ultrogel column, a Sephacryl S-200 column and a Protein A affinity column) but none were successful. The $F(ab')_2$ fragments were reduced to the Fab' fragment and bound to dTNB to prevent intrachain disulphide bond formation. The successful binding of dTNB was shown, as was the removal of dTNB from the human IgG molecules. The murine Fab'-TNB and the human Fab'-SH were allowed to re-associate according to the method of Brennan *et al.* (1985), the recombinant molecule produced formed less than 10% of the total protein present in solution, as measured by HPLC. The addition of 0.5% (w/v) H_2O_2 increased this yield to 60%. The HPLC analysis also indicated that the molecule formed is twice the weight of a Fab' fragment, but slightly lower than that of the original $F(ab')_2$ fragment. This may be due to the degree of error in the standard curve. Alternatively, it may be caused by the altered three dimensional structure of the fragments compared to the parental antibodies. This, as well as their molecular weight, effects the rate at which the fragments pass through the HPLC column. The results of the ELISA indicate that the $F(ab')_2$ sized molecule, produced in this procedure, was formed by linking a human Fab' fragment to a murine Fab' fragment, ie. it is a $BF(ab')_2$ fragment.

4.2 A MODEL SYSTEM FOR BISPECIFIC ANTIBODY APPLICATION

In the previous section (4.1) a method for the production of $BF(ab')_2$ was optimised. It was necessary to determine if the $BF(ab')_2$ produced retained the binding activity of the parental Igs, and, if so, to what extent that activity was retained. To this end a model system was developed for $BF(ab')_2$ application. Two IgGs were readily available, i.e. a polyclonal, rabbit anti-BSA Ab and a monoclonal murine anti-CLL Ab (G12). The cell line K562, to which G12 binds, was readily available. A detectable substrate for the anti-BSA Ab was required. A BSA-HRP conjugate was produced to fulfill this role. The model system was set up by coating an ELISA plate with K562 cells, applying the anti-BSA X G12 $BF(ab')_2$ and HRP-BSA conjugate and developing the colour using the HRP substrate o-PD.

4.2.1 Conjugation of Horse Radish Peroxidase to BSA:

The enzyme Horse Radish Peroxidase (HRP) was conjugated to the protein Bovine Serum Albumin (BSA) using the periodate method of conjugation (Section 3.5.2).

HPLC analysis was carried out on the 'HRP-BSA conjugate' and on HRP and BSA individually (Section 3.4.2). Monitoring the eluent at 280nm indicated that the HRP-BSA sample contained a protein which appeared to be larger than either HRP (45 kDa) or BSA (66 kDa) (Fig. 4.2.1). In order to confirm this a total of five injectons of each of HRP, BSA and HRP-BSA were made onto the HPLC. The mean Rt for HRP, the smallest protein was 17.76 ± 0.14 min. BSA is larger with a mean Rt of 16.36 ± 0.06 min. The mean Rt for the HRP-BSA peak was 15.90 ± 0.04 min. A one-tailed, paired Student t test was used to show that the Rt of BSA and the Rt of HRP are significantly longer than the Rt of HRP-BSA, within 99% confidence limits. This would indicate that a conjugated protein was produced which was significantly larger than the parent proteins.

During the HPLC analysis, fractions were collected at 1 min intervals. The relevant, protein-containing fractions were assayed for the presence of HRP. The HRP substrate o-PD was prepared as for ELISA. To the wells of a 96-well plate was added 100 µl substrate solution and 50 µl of each fraction from the HPLC analysis of BSA, HRP and HRP-BSA. After 30 min at room temperature, the Abs_{405nm} was determined. A plot of absorbance vs. fraction number (Fig. 4.2.2) showed that the HRP-BSA solution consisted of two HRP-containing peaks. These peaks corresponded in Rt with the HRP peak and the conjugated protein peak in the HRP-BSA solution.

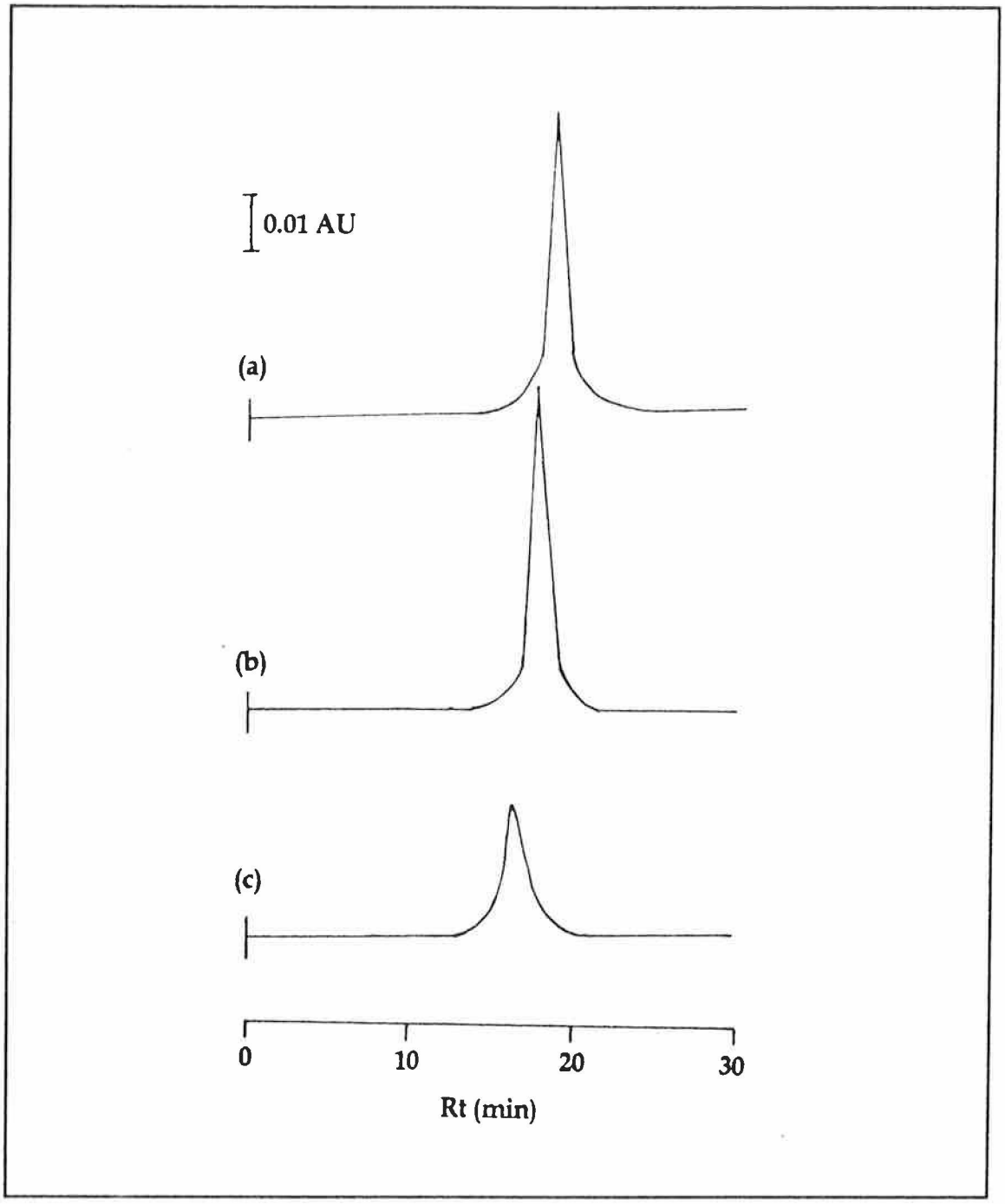


Figure 4.2.1:

The chromatograms produced following HPLC analysis of (a) HRP (Rt = 17.764 ± 0.141 min), (b) BSA (Rt = 16.363 ± 0.060 min) and (c) HRP-BSA conjugate (Rt = 15.905 ± 0.046 min), illustrating that the conjugate protein is significantly larger than either of the parent proteins. HPLC analysis was carried out on a Protein Pak 300SW size exclusion column, with a sodium phosphate (0.1 M, pH 7.4) mobile phase, run at a flow rate of 0.5 ml/min. The variable wavelength detector was set at 280nm for determination of protein. (System Gold, Beckman) (AU = Absorbance Unit)

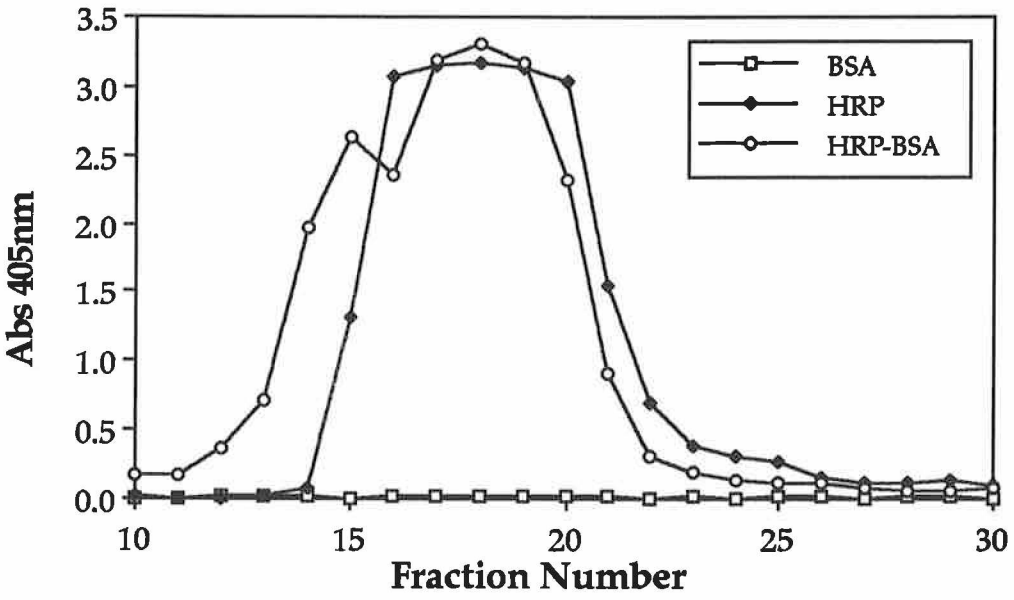


Figure 4.2.2:

HRP, BSA and HRP-BSA were each applied to HPLC (Fig. 4.2.1). Fractions of eluent were collected every minute (i.e. 0.5 ml each). Each fraction was analysed for the presence of active HRP, 50 µl of each fraction was added to 100 µl of o-PD in citrate buffer (0.1 M, pH 4.5). Colour development was measured at 405nm and was proportional to HRP activity. The elution profiles were constructed. The eluent from the HPLC analysis of BSA showed no HRP activity, as was expected. The eluent from the HPLC analysis of HRP showed a broad band of HRP activity, visible in fractions 16-20. The eluent from the HPLC analysis of HRP-BSA conjugate showed two peaks of HRP activity. The main peak of HRP activity occurs in fractions 17-19, corresponding to the peak seen in the HRP analysis. A minor peak of HRP activity occurs in fraction 14. This indicates the presence of a protein with HRP activity but with a greater molecular weight than HRP, i.e. a HRP-BSA conjugate.

This would indicate that, as a result of the conjugation procedure, a conjugated protein was produced which was larger than the parent proteins. One of the parent proteins, HRP, forms a part of the conjugated protein and retains its enzymatic activity.

The conjugated protein was purified from the HRP-BSA solution by the use of two affinity chromatography columns. In the first, the rabbit anti-HRP Ab was coupled to CNBr-activated Sepharose. In the second, the rabbit anti-BSA Ab was coupled to CNBr-activated sepharose (Section 3.3.1). The purpose of the former was to remove free BSA from solution, and of the latter to remove free HRP from solution. To the anti-HRP-column was applied HRP, BSA and HRP-BSA. The 1 ml fractions collected were assayed for protein using the BCA assay (Section 3.5.1), and for HRP as described above. Free BSA elutes in the wash buffer, while HRP and HRP conugates adsorb onto the column and elute only when dissociation buffer is applied (Fig. 4.2.3). To the anti-BSA-column was applied HRP, BSA and those fractions of HRP-BSA eluted from the first column which were shown to contain HRP. Fractions collected from this column were assayed for protein and HRP as before. Free HRP elutes in the wash buffer, while BSA and BSA conjugates adsorb onto the column and elute only when dissociation buffer is applied. It was shown that a protein, with HRP activity, elutes in the dissociation buffer from the second column (Fig.4.2.4). This could only be a conjugate protein having both BSA and HRP sub-units.

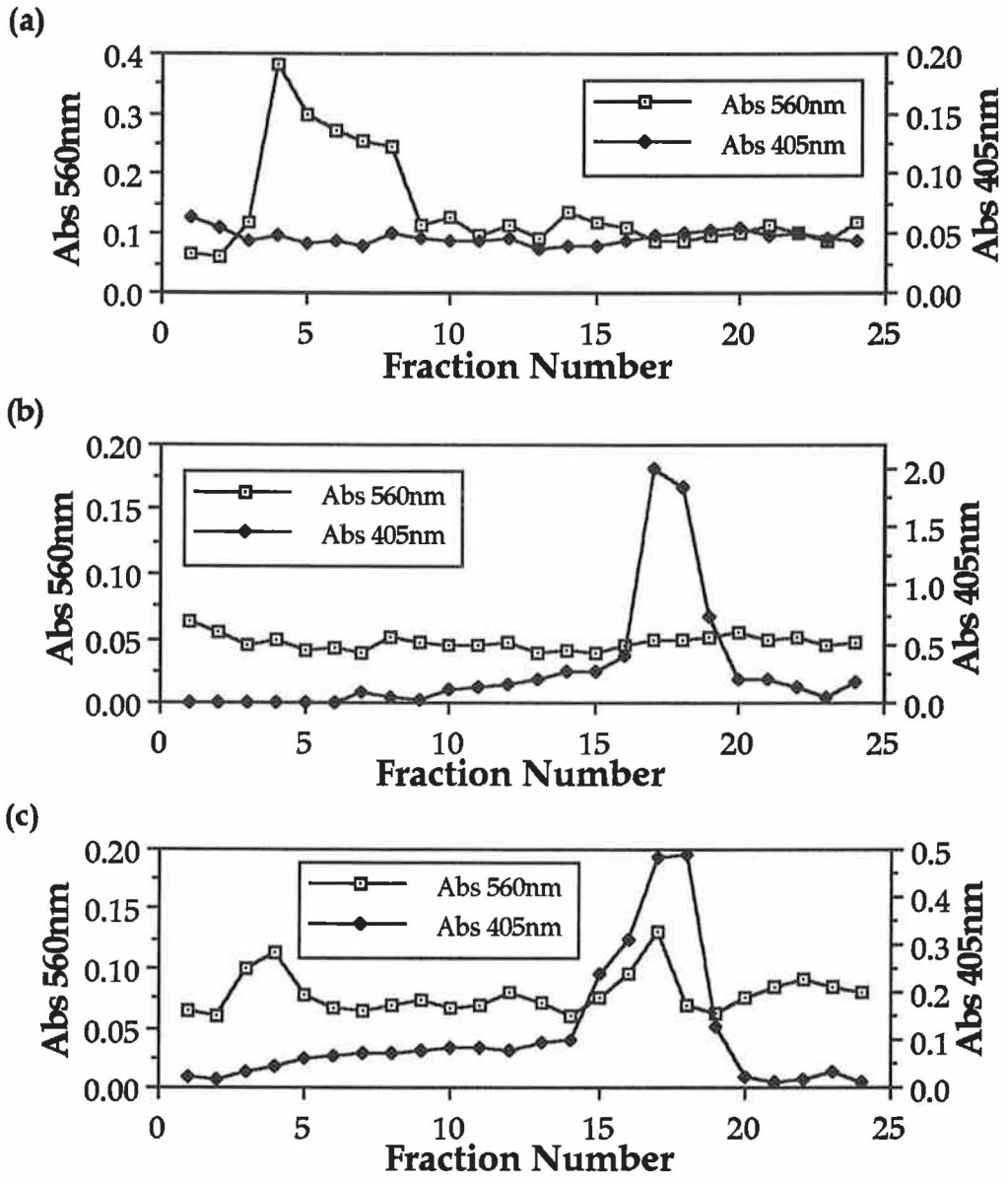


Figure 4.2.3:

Three proteins were applied to an affinity column, CNBr-activated Sepharose coupled to anti-HRP Ab (0.5 x 5 cm). The binding buffer was PBS (0.02 M, pH 7.1), of which 15 x 1 ml fractions were collected, and the elution buffer was 0.1 M glycine /HCl buffer (pH 2.5), of which 10 x 1 ml fractions were collected. Each fraction was assayed for protein, using the BCA assay (Abs_{560nm}), and active HRP, by testing for the conversion of the substrate o-PD to a measurable coloured product (Abs_{405nm}). BSA (a) did not bind to the column but eluted in the binding buffer (Abs_{560nm}), no HRP activity was detected (Abs_{405nm}). HRP (b) bound to the column and eluted in the elution buffer (Abs_{405nm}), the amount of protein present was below the limit of detection (Abs_{560nm}). In the sample containing HRP-BSA conjugate (c) all the HRP, free and conjugated, bound to the column and eluted in the elution buffer (Abs_{405nm}). Protein eluted in the binding buffer (Abs_{560nm}), representing free BSA, and in the elution buffer, representing conjugated BSA.

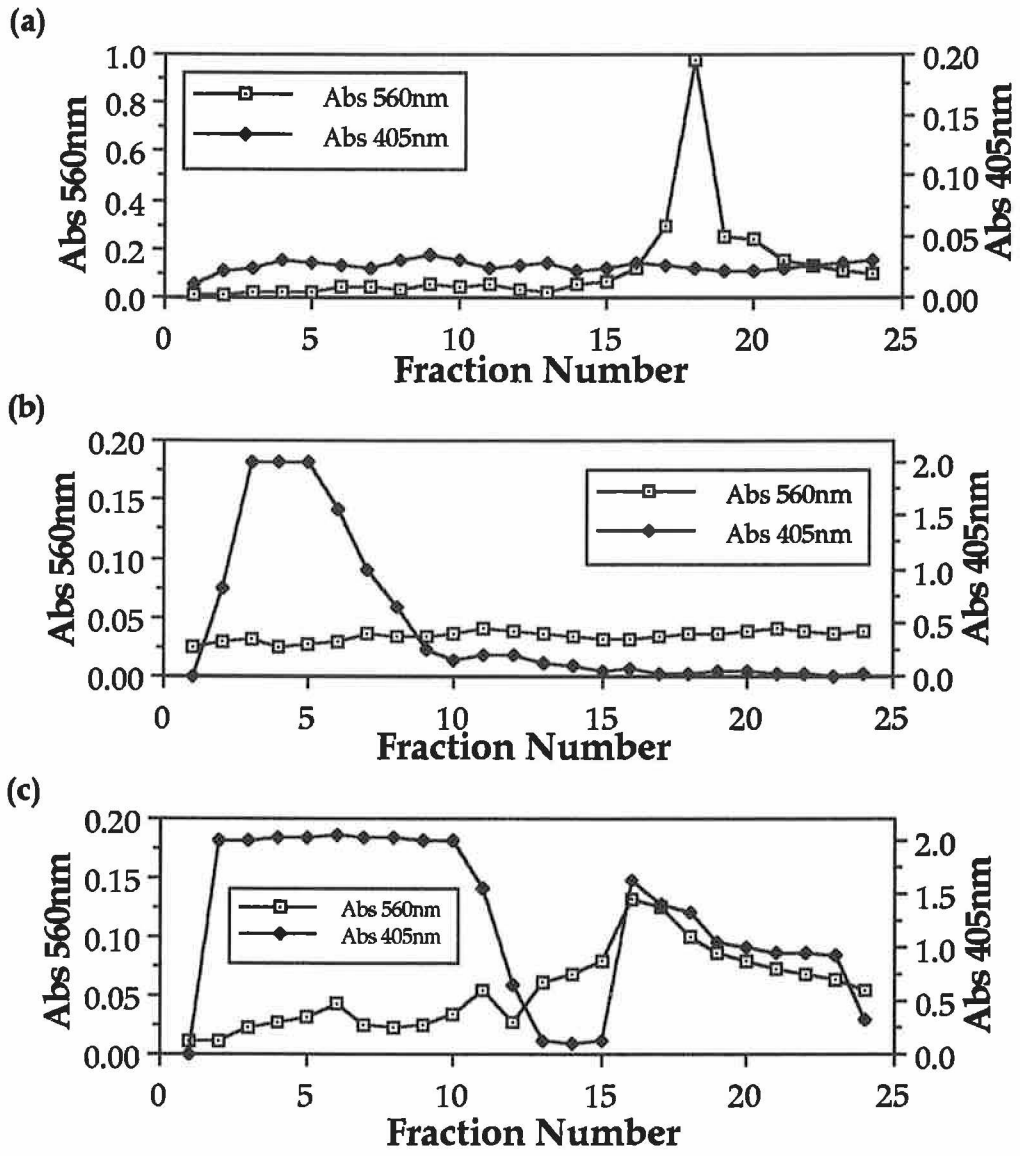


Figure 4.2.4:

Three proteins were applied to an affinity column, CNBr-activated Sepharose coupled to anti-BSA Ab (0.5 x 5 cm). The binding buffer was PBS (0.02 M, pH 7.1), of which 15 x 1 ml fractions were collected, and the elution buffer was 0.1 M glycine/HCl buffer (pH 2.5), of which 10 x 1 ml fractions were collected. Each fraction was assayed for protein, using the BCA assay (Abs_{560nm}), and active HRP, by testing for the conversion of the substrate o-PD to a measurable coloured product (Abs_{405nm}). BSA (a) bound to the column and eluted in the elution buffer (Abs_{560nm}), no HRP activity was detected (Abs_{405nm}). HRP (b) did not bind to the column and eluted in the binding buffer (Abs_{405nm}), the amount of protein present was below the limit of detection (Abs_{560nm}). In the sample containing HRP-BSA conjugate (c) free HRP eluted in the binding buffer (Abs_{405nm}), while that in the elution buffer, represented HRP conjugated to BSA. All the BSA bound to the column and eluted in the elution buffer (Abs_{560nm}). Fractions 16-18, in which the protein and HRP concentrations are highest, contain pure HRP-BSA conjugate.

4.2.2 Development of a Model ELISA System:

A BF(ab')$_2$ was produced from a rabbit, polyclonal anti-BSA Ab (Sigma) and a murine, monoclonal anti-CLL Ab (G12). A series of ELISAs were carried out to illustrate the bispecificity of the BF(ab')$_2$ produced and determine its activity compared to the parental IgGs. In the first of these it was shown that the BF(ab')$_2$ produced was in fact formed by the coming together of a murine Fab' and a rabbit Fab' (Fig. 4.2.5a). The plate was coated with an anti-mouse Fab' Ab and blocked with 1% (w/v) gelatin in PBS (0.1 M, pH 9) (Section 3.6.2). BF(ab')$_2$ was applied to the wells at a concentration of 0.1 mg/ml in PBS. A HRP-labelled goat anti-rabbit IgG Ab was then applied. The colour was developed in the plate using the HRP substrate o-PD (Section 3.6.3). The positive control wells were coated with rabbit IgG; these had a mean Abs_{405nm} of 0.418 ± 0.031. The negative control wells did not have BF(ab')$_2$ applied; these had a mean Abs_{405nm} of 0.050 ± 0.007. The absorbance of the test wells, mean of 0.350 ± 0.023, was considerably higher than for the negative control wells. This would indicate that a BF(ab')$_2$ had been formed.

The next two ELISAs were carried out to determine whether or not each seperate Fab' had retained binding activity for the specified Ags. In the first of these, the plate was coated with BSA and blocked with 1% (w/v) gelatin in PBS (0.1 M, pH 9) (Section 3.6.2). BF(ab')$_2$ was applied to the wells at a concentration of 0.1 mg/ml in PBS. A HRP-labelled goat anti-mouse IgG Ab was then applied (Fig. 4.2.5b). The colour was developed in the plate using the HRP substrate o-PD (Section 3.6.3). The positive control wells were coated with mouse IgG; these had a mean Abs_{405nm} of 0.328 ± 0.035. The negative control wells did not have BF(ab')$_2$ applied; these had a mean Abs_{405nm} of 0.041 ± 0.010. The absorbance of the test wells, mean of 0.286 ± 0.031, was considerably higher than for the negative control wells. This would indicate that the rabbit Fab' retains Ag binding activity when forming part of a BF(ab')$_2$. In the second ELISA, the plate was coated with K562 cells and blocked with 1% (w/v) gelatin in PBS (0.1 M, pH9) (Section 3.6.1). BF(ab')$_2$ was applied to the wells at a concentration of 0.1 mg/ml in PBS. A HRP-labelled goat anti-rabbit IgG Ab was then applied (Fig. 4.2.5c). The colour was developed in the plate using the HRP substrate o-PD (Section 3.6.3). The positive control wells were coated with rabbit IgG, these had a mean Abs_{405nm} of 0.298 ± 0.033. The negative control wells did not have BF(ab')$_2$ applied, these had a mean Abs_{405nm} of 0.037 ± 0.013. The absorbance of the test wells, mean of 0.259 ± 0.037, was considerably higher than for the negative control wells. This would indicate that the mouse Fab' retains Ag binding activity when forming part of a BF(ab')$_2$.

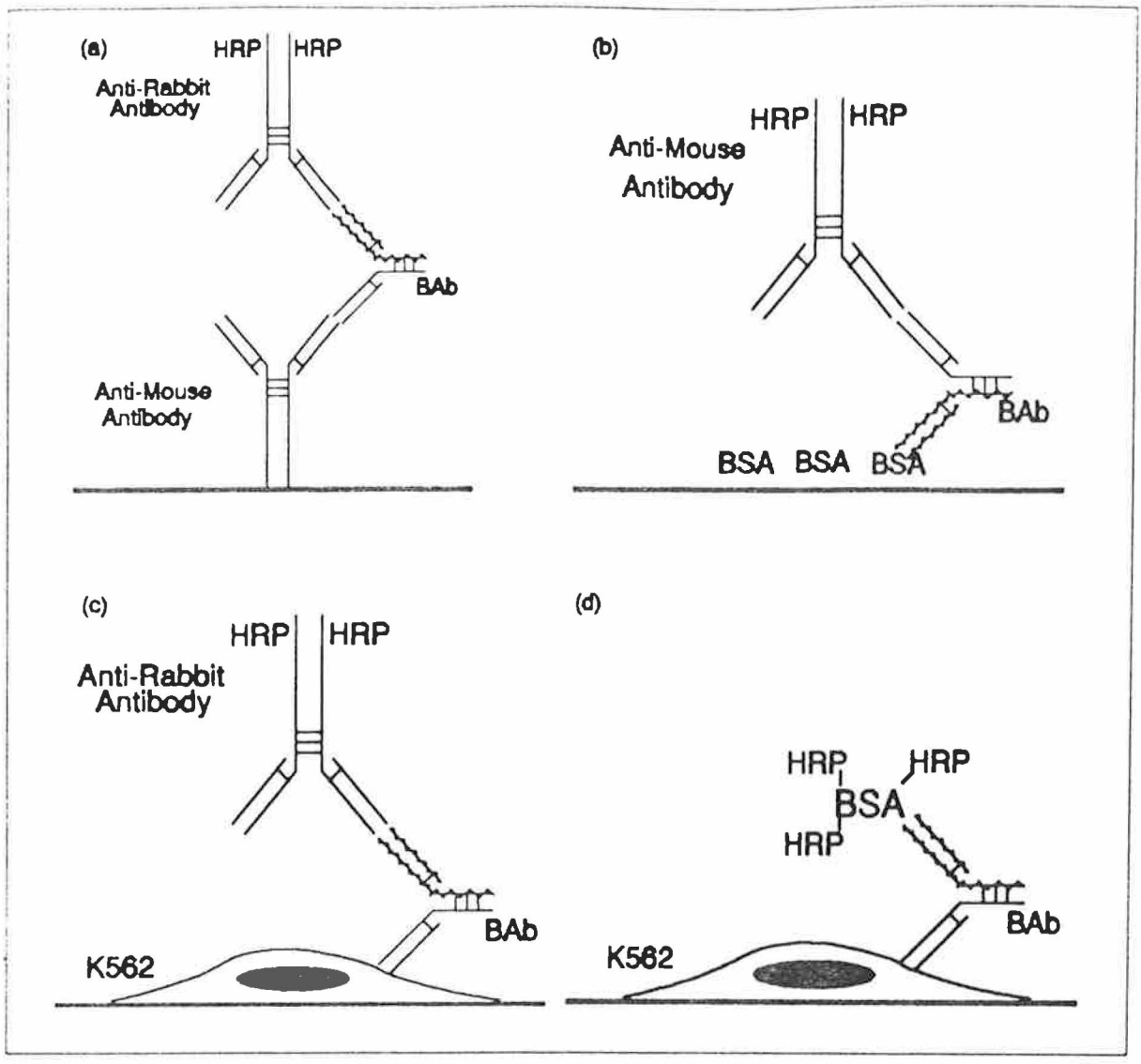


Figure 4.2.5:

An illustration of the series of ELISA formats used to assess the bifunctionality of the BF(ab')2. Each was run with suitable positive and negative controls and blank wells. The test wells were deemed to be positive if the absorbance was at least 150% of the negative control wells. Each of the illustrated test wells gave positive results in the ELISAs indicating that: (a) the BF(ab')2 is formed from two different Fab' fragments; (b) the rabbit anti-BSA Fab', which forms half of the BF(ab')2, retains its binding activity; (c) the murine anti-K562 Fab', which forms the other half of the BF(ab')2, retains its binding activity; (d) the BF(ab')2 can be used successfully in a one-step enzyme immunoassay for K562 cells.

The final ELISA utilizes the binding activity of both halves of the $BF(ab')_2$. The plate was coated with K562 cells and blocked with 1% (w/v) gelatin in 0.1 M PBS, pH 9 (Section 3.6.1). $BF(ab')_2$ was applied to the wells at a concentration of 0.1 mg/ml in PBS. The HRP-BSA conjugate was applied at the same time as the $BF(ab')_2$ at a concentration of 0.1 mg/ml in PBS (Fig. 4.2.5d). The colour was developed in the plate using the HRP substrate o-PD (Section 3.6.3). The positive control wells were developed with HRP-linked anti-rabbit IgG; these had a mean Abs_{405nm} of 0.214 ± 0.025. The negative control wells did not have $BF(ab')_2$ applied; these had a mean Abs_{405nm} of 0.045 ± 0.005. The absorbance of the test wells, mean of 0.134 ± 0.020, was considerably higher than for the negative control wells. This would indicate that both the mouse Fab' and the rabbit Fab' retain their Ag binding activity when forming part of a $BF(ab')_2$ and that the $BF(ab')_2$ can be used successfully in a one-step enzyme immunoassay for K562 cells.

4.2.3 Measurement of BF(ab')$_2$ Activity:

A series of ELISAs was carried out to determine if the BF(ab')$_2$ retained the binding affinity and specificity of the parental IgG molecules. The BF(ab')$_2$ to be tested was purified using double affinity chromatography. In the first column, an anti-rabbit Fab' Ab was coupled to CNBr-activated Sepharose. In the second column, an anti-mouse Fab' Ab was coupled to CNBr-activated sepharose (Section 3.3.1). The purpose of the former was to remove free murine Fab' from solution, and the purpose of the latter to remove free rabbit Fab' from solution.

The first ELISAs were set up to determine if the BF(ab')$_2$ had retained the specificity of the parental IgG molecules. A 96-well plate was coated with K562 cells and blocked with 1% (w/v) gelatin in PBS (0.1 M, pH 9) (Section 3.6.1). NSO cells, suspended in PBS, were diluted to give eight cell suspensions with a concentration range of 0-10^6 cells/ml. Each of these suspensions was applied in triplicate to wells of the plate. Murine IgG (G12) was applied to each of these wells at a concentration of 0.1 mg/ml in PBS. The cells and Ab fragments were incubated together for 1 hr at 37^{o}C, then washed out. A HRP-labelled goat anti-mouse Fab' Ab was then applied. The colour was developed in the plate using the HRP substrate o-PD (Section 3.6.3). This procedure was carried out simultaneously using G12 F(ab')$_2$, Fab' and BF(ab')$_2$. The specificity of each Ab fragment was determined by the degree to which the NSO cells interfere with the binding of the fragment to the adsorbed K562 cells. A graph was drawn of Abs_{405nm} vs. concentration of NSO cells (Fig. 4.2.6). This shows that the binding of the IgG is slightly affected by the presence of NSO cells. The Abs_{405nm} fell from 0.268 ± 0.014, in the absence of NSO cells, to 0.200 ± 0.018 in the presence of 10^6 cells/ml. The binding of the BF(ab')$_2$ was also affected by the presence of NSO cells. The Abs_{405nm} fell from 0.171 ± 0.012, in the absence of NSO cells, to 0.098 ± 0.013 in the presence of 10^6 cells/ml. Both the IgG and the BF(ab')$_2$ were inhibited to the same extent by the presence of the NSO cells. This would indicate that formation of the BF(ab')$_2$ does not affect the specificity of the Ab fragments used. This ELISA was carried out a further three times to confirm this result.

Another 96-well plate was coated with BSA and blocked with 1% (w/v) gelatin in PBS (0.1 M, pH 9) (Section 3.6.1). A serial dilution was carried out on a solution of ovalbumin (OVA) in PBS to give eight solutions with a concentration range of 0-10 mg/ml. Each of these dilutions was applied in triplicate to wells of the plate. Rabbit anti-BSA IgG was applied to each of these wells at a concentration of 0.1 mg/ml in PBS.

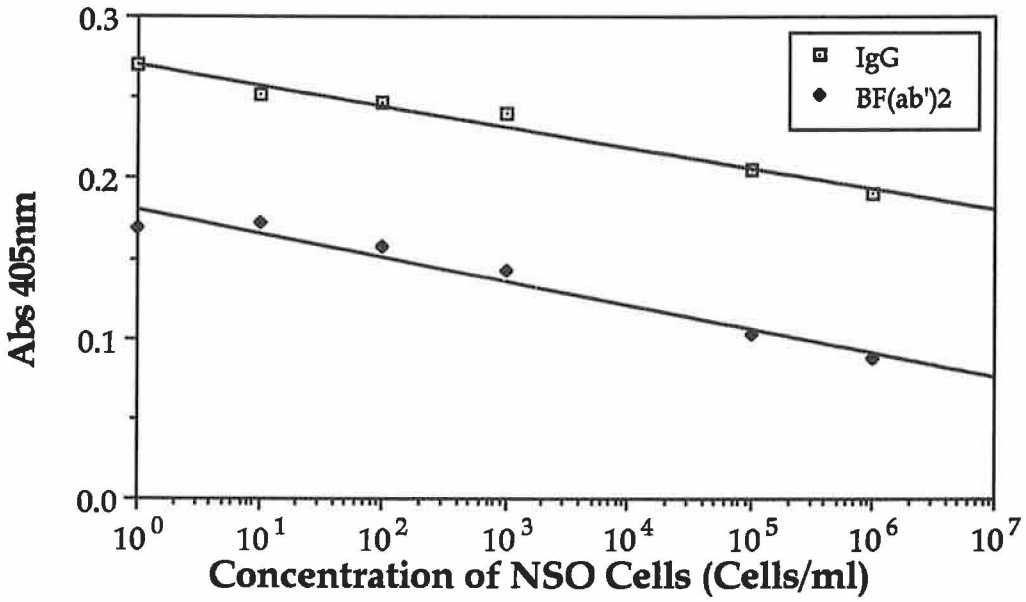


Figure 4.2.6:

The results of an ELISA to determine the extent to which NSO cells interfere with the the anti-K562 IgG's and BF(ab')2's ability to bind to a plate coated with K562. Large numbers of NSOs do cause some inhibition of binding. The extent of the inhibition is the same for both IgG and BF(ab')2.

The OVA and Ab fragments were incubated together for 1 hr at 37°C, then washed out. A HRP-labelled goat anti-rabbit Fab' Ab was then applied. The colour was developed in the plate using the HRP substrate o-PD (Section 3.6.3). This procedure was carried out simultaneously using rabbit anti-BSA $F(ab')_2$, Fab' and the $BF(ab')_2$. The specificity of each Ab fragment was determined by the degree to which the OVA interferes with the binding of the fragment to the adsorbed BSA. A graph was drawn of Abs_{405nm} vs. concentration of OVA (Fig. 4.2.7). This shows that the binding of the IgG is affected by the presence of OVA. The Abs_{405nm} fell from 0.542 ± 0.026, in the absence of OVA, to 0.321 ± 0.031 in the presence of 10 mg/ml. The binding of the $BF(ab')_2$ was also affected by the presence of OVA. The Abs_{405nm} fell from 0.487 ± 0.022, in the absence of OVA, to 0.271 ± 0.023 in the presence of 10 mg/ml. Both the IgG and the $BF(ab')_2$ were inhibited to the same extent by the presence of the OVA. This would indicate that formation of a $BF(ab')_2$ does not affect the specificity of the Ab fragments used. This ELISA was carried out a further three times to confirm this result.

From both these graphs (Figs. 4.2.6 & 4.2.7), it would appear that the binding affinity of the Fab'fragments involved in $BF(ab')_2$ production is somewhat reduced compared to the parental IgGs. In both cases the absorbances of the $BF(ab')_2$ fragment are lower than that of the IgG. The loss of binding affinity appears to be more pronounced for the murine than for the rabbit Fab'.

4.2.4 Summary:

A HRP-BSA conjugate was successfully produced, as shown by HPLC and double affinity chromatography. An anti-BSA X anti-K562 $BF(ab')_2$ was successfully produced, as shown by HPLC and ELISA. A series of ELISAs were used to show that both the Fab' fragments used in the production of the $BF(ab')_2$ retained the binding activity and specificity of the parental IgG, although some of the affinity is lost. The $BF(ab')_2$ was used successfully in a one-step enzyme immunoassay for K562 cells.

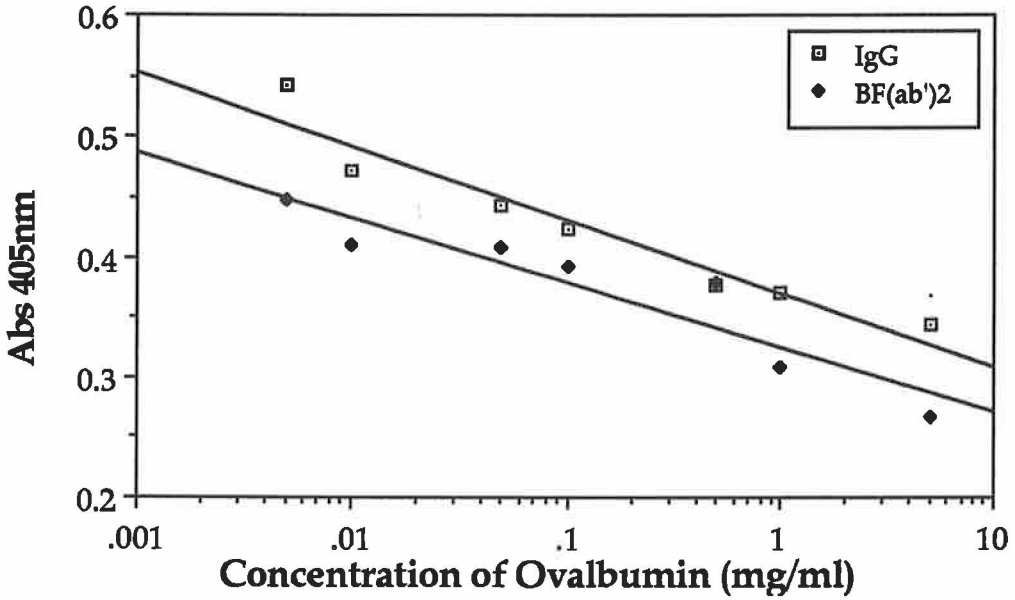


Figure 4.2.7:

The results of an ELISA to determine the extent to which ovalbumin interferes with the the anti-BSA IgG's and BF(ab')2's ability to bind to a plate coated with BSA. Large concentrations of ovalbumin do cause some inhibition of binding. The extent of the inhibition is the same with both IgG and BF(ab')2.

4.3 DETECTION OF CHRONIC LYMPHOCYTIC LEUKAEMIA USING BISPECIFIC ANTIBODIES

BF(ab')$_2$s have been used to develop a model system for the detection of the cell line K562. This system can be improved upon and applied to the detection of chronic lymphocytic leukaemia (CLL) in patient blood samples. A rabbit polyclonal anti-alkaline phosphatase (AP) Ab was produced to replace the anti-BSA Ab used previously. The anti-K562 Ab (G12) binds to B-CLL lymphocytes, via a 69.2 kDa cell surface Ag. A G12 X anti-AP BF(ab')$_2$ was produced which was used to develop two enzyme immuno-assays and an immuno-chemical method for the detection of CLL.

4.3.1 Production of an Anti-Alkaline Phosphatase Antibody:

Polyclonal antibodies were raised against the enzyme alkaline phosphatase (AP) in order to develop a detection system for CLL using BF(ab')$_2$s. The enzyme, molecular weight 100 kDa, is large enough to be immunogenic so that no conjugation or carrier proteins were required. An immunisation schedule was set up (Section 3.2.1). Following six cycles of immunisation an ELISA was set up to determine the serum titre. A serial dilution of the serum was carried out in the range $1:10^2$-$1:10^8$. AP was coated onto the 96-well plate and non-specific binding sites blocked with BSA (Section 3.6.2). The serial dilutions were applied in triplicate to the wells of the plate and allowed to bind for 1 hr at 37°C. The secondary Ab, HRP-labelled goat anti-rabbit IgG (Sigma), was applied after the plate had been washed. Colour was developed in the plates by the addition of the HRP substrate o-PD (Section 3.6.3). Maximum colour development occurred at the lowest dilution (1:100), with an Abs_{405nm} of 0.588 ± 0.029. The negative control, to which a 1:100 dilution of serum from an un-immunised rabbit was added, gave an Abs_{405nm} of 0.068 ± 0.015. The highest dilution of serum from the immunised rabbit to give a strong positive reaction was found to be $1:10^5$, with an Abs_{405nm} of 0.315 ± 0.018. Serum from this, and subsequent, immunisations were pooled and stored at -20°C prior to purification of the IgG.

4.3.2 ELISA Development:

A $BF(ab')_2$ was produced using the methods described, (Sections 3.8.2 & 3.8.3). The parental Abs were rabbit polyclonal anti-AP Ab and the murine, monoclonal anti-CLL Ab (G12) which also binds to the K562 cell line. A chequerboard ELISA was carried out to determine the optimum concentrations of $BF(ab')_2$ and AP required for the detection of the human leukaemic cell line, K562. The wells of a 96-well ELISA plate were coated with K562 cells and blocked with BSA (Section 3.6.1). In developing the ELISA, two variables were taken into consideration; concentration of $BF(ab')_2$ and concentration of AP. To each blocked and washed well was added 50 µl $BF(ab')_2$ (in the range 0-50 µg/ml) and 50 µl AP (in the range 0-500 µg/ml). (Fig. 4.3.1) These were incubated for 1 hr at 37°C. The plate was washed three times with PBS. Colour was developed in the wells using the AP substrate BCIP (Section 3.6.3). The lowest concentrations of AP and $BF(ab')_2$ to give a strongly positive result were chosen for future use. These were AP at 100 µg/ml and $BF(ab')_2$ at 1 µg/ml (Table 4.3.1). These concentrations, of AP and $BF(ab')_2$, were found to be optimum and were independent of the method of production of $BF(ab')_2$.

A suspension ELISA was also developed. This was carried out in 1.8 ml, conical centrifuge tubes. Non-specific binding sites on the plastic were blocked by filling the tubes with 1% (w/v) BSA in PBS, and storing them at 4°C for a minimum of 12 hr before use. K562 cells were harvested from culture vessels (Section 3.1.2) and suspended in PBS. In developing the ELISA, three variables were taken into consideration; concentration of K562 cells, concentration of $BF(ab')_2$ and concentration of AP. To each blocked and washed tube was added 200 µl K562 cells (in the range 10^4-10^8 cells/ml), 50 µl $BF(ab')_2$ (in the range 0-50 µg/ml) and 50 µl AP (in the range 0-500 µg/ml). These were incubated for 1 hr at 37°C. The cells were pelletted by centrifugation at 6,000 rpm for 3 min. The cells were washed with PBS and pelletted as before. This was repeated twice. The cells were re-suspended in 1 ml of the AP substrate BCIP, 1 mg/ml in amino methyl propanol buffer, and incubated for 30 min at 37°C (Section 3.6.3). The cells were pelletted as before and the Abs_{620nm} of the supernatants determined on a spectrophotometer (Table 4.3.2). Good results were achieved at a K562 concentration of 10^6 cells/ml, a $BF(ab')_2$ concentration of 50 µg/ml and an AP concentration of 50 µg/ml.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10	9	8	8	7	7	6	6	5	5	4	0
B	9	8	8	7	7	6	6	5	5	4	4	0
C	8	8	7	7	6	6	5	5	4	4	3	0
D	8	7	7	6	6	5	5	4	4	3	3	0
E	7	7	6	6	5	5	4	4	3	3	2	0
F	7	6	6	5	5	4	4	3	3	2	2	0
G	6	6	5	5	4	4	3	3	2	2	1	0
H	0	0	0	0	0	0	0	0	0	0	0	0

Figure 4.3.1:

A schematic diagram of a chequerboard ELISA plate. To each well in column 1 (1A-1H) was applied 50 μl of the highest concentration of $BF(ab')_2$ (2 mg/ml), i.e. PBS. To each well in column 12 (12A-12H) was applied 50 μl of the lowest concentration of $BF(ab')_2$ (0 mg/ml). To each well of the columns in between (2A-2H, 3A-3H, etc.) was applied 50 μl of one of a serial dilution of $BF(ab')_2$. In a similar manner, to each well of row A (A1-A12) was applied 50 μl of the highest concentration of AP (500 μg/ml). To each well in row H (H1-H12) was applied 50 μl of the lowest concentration of AP (0 μg/ml). To each well of the rows in between (B1-B12, C1-C12, etc.) was applied 50 μl of one of various dilutions of AP. When colour was developed in the wells, well H12 acted as a blank, since no AP or $BF(ab')_2$ were applied to this well (numbers 0-10 indicate colour intensity). Maximum colour development occured in well A1, since the maximum concentration of both AP and $BF(ab')_2$ were applied to this well. The colour development increased diagonally across the plate as the concentrations of AP and $BF(ab')_2$ increased. At some point in the plate (e.g. D5) a strong colour development is achieved using the minimum amount of reagents.

AP	$BF(ab')_2$ (μg/ml)											
μg/ ml	2×10^3	1×10^3	2×10^2	1×10^2	2×10^1	1×10^1	2×10^0	1×10^0	2×10^{-1}	1×10^{-1}	2×10^{-2}	0.0
500	0.28	0.26	0.26	0.25	0.23	0.23	0.21	0.20	0.19	0.11	0.09	0.03
100	0.25	0.25	0.24	0.22	0.21	0.20	0.20	**0.18**	0.15	0.09	0.07	0.02
50	0.26	0.24	0.19	0.17	0.16	0.16	0.15	0.13	0.10	0.07	0.05	0.02
10	0.23	0.17	0.15	0.13	0.12	0.11	0.11	0.09	0.06	0.04	0.04	0.03
5	0.13	0.11	0.09	0.08	0.07	0.07	0.08	0.06	0.04	0.03	0.03	0.01
1	0.07	0.05	0.04	0.04	0.04	0.03	0.04	0.03	0.02	0.01	0.01	0.02
0.5	0.02	0.02	0.02	0.01	0.02	0.01	0.01	0.00	0.01	0.00	0.01	0.01
0	0.01	0.01	0.02	0.01	0.02	0.01	0.00	0.01	0.00	0.01	0.01	0.00

Table 4.3.1:

The results (shown as Abs_{620nm}) of one of a series of five chequerboard ELISAs carried out to optimise the conditions to be used in an ELISA for the detection of K562 cells using $BF(ab')_2$. A 96-well plate was coated with K562 cells and blocked with BSA. Alkaline phosphatase and $BF(ab')_2$ were added simultaneously in the concentrations indicated. The lowest concentrations of alkaline phosphatase and $BF(ab')_2$ to consistently give a strongly positive result were chosen for future use. In this case, as in each of the ELISAs, these were alkaline phosphatase at 100 μg/ml and $BF(ab')_2$ at 1 μg/ml.

AP	BF(ab')2 (μg/ml)							
μg/ml	50	10	5	1	0.5	0.1	0.05	0.0
500	0.787 ± 0.139	0.730 ± 0.105	0.721 ± 0.093	0.646 ± 0.121	0.576 ± 0.089	0.537 ± 0.098	0.444 ± 0.079	0.036 ± 0.011
100	0.539 ± 0.031	0.398 ± 0.024	0.325 ± 0.018	0.256 ± 0.013	0.174 ± 0.009	0.122 ± 0.006	0.095 ± 0.006	0.049 ± 0.003
50	**0.253 ± 0.011**	0.147 ± 0.008	0.128 ± 0.007	0.135 ± 0.007	0.116 ± 0.005	0.091 ± 0.005	0.073 ± 0.004	0.054 ± 0.002
10	0.101 ± 0.005	0.096 ± 0.005	0.089 ± 0.005	0.075 ± 0.004	0.081 ± 0.003	0.069 ± 0.004	0.058 ± 0.003	0.055 ± 0.003
1	0.044 ± 0.002	0.063 ± 0.003	0.060 ± 0.003	0.057 ± 0.003	0.045 ± 0.002	0.038 ± 0.002	0.034 ± 0.002	0.039 ± 0.002
0	0.044 ± 0.003	0.059 ± 0.003	0.039 ± 0.002	0.063 ± 0.003	0.060 ± 0.003	0.043 ± 0.002	0.030 ± 0.001	0.008 ± 0.001

Table 4.3.2:

The results of one of a series of three suspension ELISAs carried out to optimise the conditions to be used in an ELISA for the detection of K562 cells using BF(ab')2. A number of 1.8 ml conical centrifuge tubes were blocked with BSA. K562 cells were added at a concentration of 10^6 cells/ml, along with alkaline phosphatase and BF(ab')2 in the concentrations indicated. Each combination was carried out in triplicate, the results are shown as Abs_{620nm} ± standard deviation. The tubes which contained 500 μg/ml alkaline phosphatase showed erratic colour development due to the formation of precipitate in the substrate solution. This assay was repeated three times with similar results. The lowest concentrations of alkaline phosphatase and BF(ab')2 to give a consistently strong positive result were chosen for future use. In this case, as in all three ELISAs, these were alkaline phosphatase at 50 μg/ml and BF(ab')2 at 50 μg/ml. The other two ELISAs in the series were carried out with K562 at 10^8 and 10^4 cells/ml. With the former, precipitate formed in the substrate of nearly all the tubes. In the latter, low absorbances were recorded even at the highest concentrations of alkaline phosphatase and BF(ab')2 (i.e. Abs_{620nm} of 0.098 ± 0.004).

4.3.3 Measurement of BF(ab')$_2$ Activity:

A BF(ab')$_2$ was produced using the methods described, (Sections 3.8.2 & 3.8.3). The parental Abs were rabbit polyclonal anti-AP Ab and the murine, monoclonal anti-CLL Ab (G12) which also binds to the K562 cell line. The presence of the BF(ab')$_2$ was determined by HPLC (Section 3.4.2) (Fig. 4.3.2), PAGE (Section 3.7) (Fig. 4.3.3) and ELISA. A 96-well plate was coated with goat anti-mouse IgG Ab and blocked with BSA (Section 3.6.2). After washing, the BF(ab')$_2$ were applied (10 μg/ml), for 1 hr at 37°C. The secondary Ab was HRP-labelled anti-rabbit IgG (Section 3.6.3). Colour was developed in the plate using the substrate o-PD. Test wells, containing BF(ab')$_2$ had a mean Abs_{405nm} of 0.437 ± 0.021 (n=5). This compared well with control wells, to which no BF(ab')$_2$ was added, which had a mean Abs_{405nm} of 0.084 ± 0.005 (n=5). The BF(ab')$_2$ was purified using two affinity chromatography steps (Section 3.3.2). The first column was prepared using CNBr-activated Sepharose and anti-rabbit IgG Ab, the second using CNBr-activated Sepharose and anti-mouse IgG Ab. The purified BF(ab')$_2$ were concentrated by dialysis on a sucrose bed and stored at 4°C, until their binding activity could be determined.

The specific activity of AP was determined, in order that the amount of AP bound to Ab fragment can be estimated. A serial dilution of AP was carried out in the range 0-1 mg/ml. To 25 μl of AP solution was added 0.5 ml of the substrate BCIP, 1 mg/ml in amino-methyl-propanol buffer, in 1.8 ml, conical centrifuge tubes. These were incubated for 30 min at 37°C. The Abs_{620nm} was then determined and a graph of Abs_{620nm} vs concentration (μg) constructed. The specific activity of the AP was calculated from the graph to be 1.868 AU/μg.

The binding activities of the parental IgG and F(ab')$_2$, the Fab'-B and BF(ab')$_2$-B (produced by the method of Brennan *et al.*, 1985) and the Fab'-G and BF(ab')$_2$-G (produced by the method of Glennie *et al.*, 1987) were determined against AP. This was carried out in 1.8 ml, conical centrifuge tubes. Non-specific binding sites on the plastic were blocked by filling the tubes with BSA (1% w/v, in PBS) and storing them at 4°C for a minimum of 12 hr before use. A serial dilution of AP was made up in the range 0-1 mg/ml. Each concentration was dispensed, in 50 μl aliquots, into twelve blocked and washed tubes. To each tube was added 25 μl of a 5 μg/ml solution of one of rabbit IgG, F(ab')$_2$, Fab'-B, Fab'-G, BF(ab')$_2$-B or BF(ab')$_2$-G. Each combination (i.e. AP concentration and Ig fragment) was carried out in duplicate. The tubes were incubated for 1 hr at 37°C.

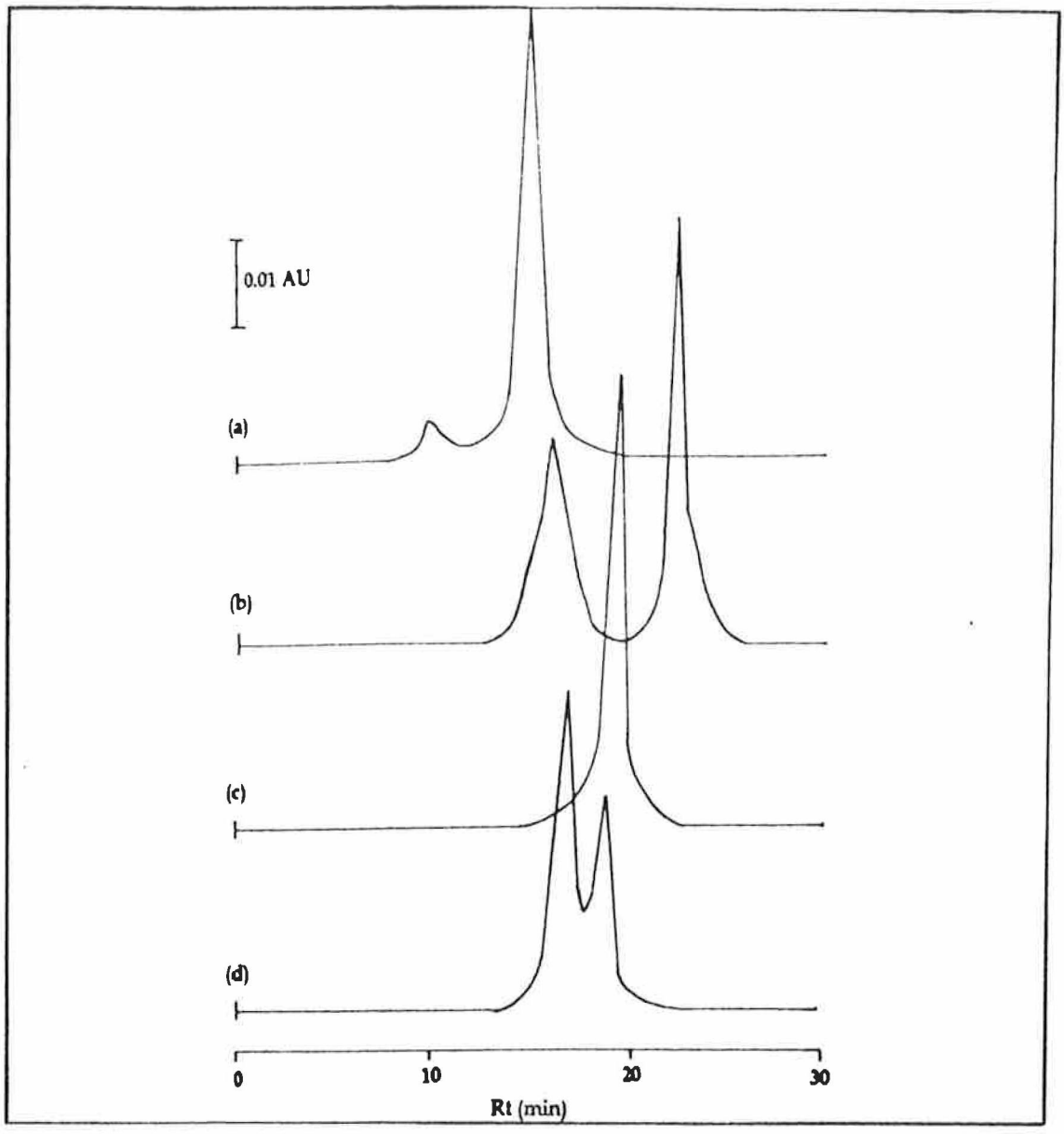


Figure 4.3.2:

The chromatograms produced following HPLC analysis of the antibody fragments formed during production of $BF(ab')_2$. The antibodies were applied to a Protein Pak 300SW size exclusion column in a sodium phosphate buffer (0.1 M, pH 7.4) mobile phase, 0.5 ml/min, and detected at 280nm. The samples were: (a) IgG with a retention time (Rt) of 14.8 min. (b) $F(ab')_2$ with a Rt of 16.2 min. (c) Fab' with a Rt of 18.7 min. (d) $BF(ab')_2$ with a Rt of 16.5 min. This would indicate that the $BF(ab')_2$ produced was of a similar size to the parental $F(ab')_2$. It will not be exactly the same since the $BF(ab')_2$ derives from two separate species of IgG.

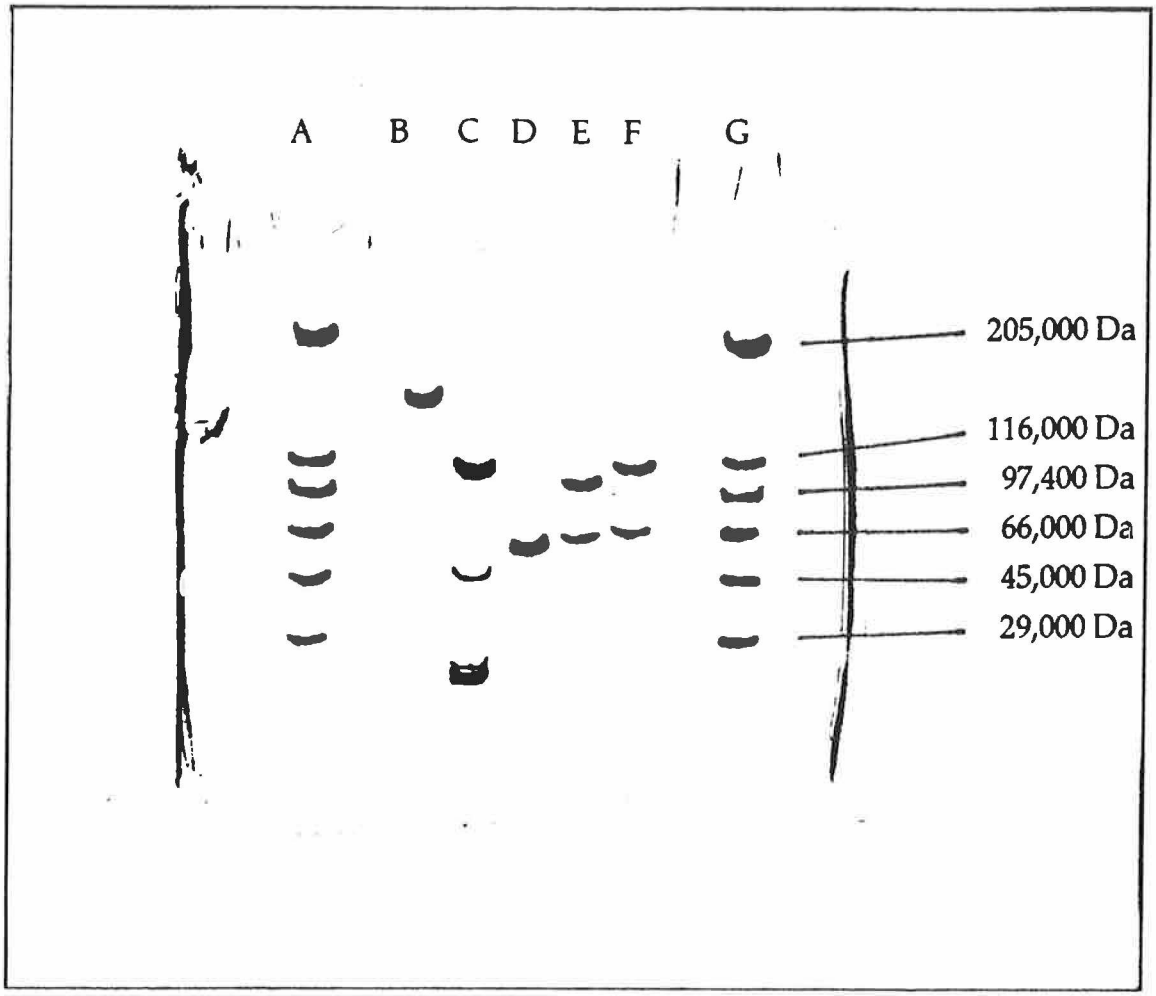


Figure 4.3.3:

A photograph of the gel produced following PAGE analysis of the antibody fragments formed during production of BF(ab')2s. The lanes contained: (a & g) molecular weight markers. (b) IgG. (c) F(ab')2. (d) Fab'. (e) BF(ab')2, produced by the method of Brennan *et al.* (1985). (f) BF(ab')2, produced by the method of Glennie *et al.* (1987). This would indicate that the BF(ab')2 produced was of a similar size to the parental F(ab')2, it will not be exactly the same since the BF(ab')2 derives from two different species of IgG.

To each tube was added 0.425 ml PBS and 0.5ml ammonium sulphate (100% w/v). The tubes were incubated for 15 min at 4°C and then centrifuged for 5 min at 10,000 rpm. In control studies, two tubes containing 2 mg/ml AP but no Ig were subjected to the same ammonium sulphate precipitation. These indicated that this procedure precipitated the Ig fragments and any bound AP, but not free AP. The proteins were resuspended in 500 ml BCIP (1mg/ml in amino methyl propanol buffer) and incubated for 30 min at 37°C. The Abs_{620nm} was determined for each solution. The results were used to determine the amount of AP bound in each tube. From this the amount of AP bound per mole of Ab fragment was calculated (Table 4.3.3). The results indicated that 25% of the binding activity of the parental IgG was lost following pepsin digestion. The binding activity of the Fab' fragments fell to 20-30% of the parental IgG. Some activity was restored on formation of the $BF(ab')_2$, returning to 43% of the parental IgG.

The binding activity of the same set of murine Ab fragments was determined against the cell line K562. The K562 cells were grown in culture in DMEM.S_5. These were suspended in PBS and a serial dilution carried out to give concentrations in the range $0\text{-}10^7$ cells/ml. These were dispensed in twelve 50 µl aliquots into BSA blocked 1.8 ml, conical centrifuge tubes. To each tube was added 25 µl of a 5 µg/ml solution of one of IgG, $F(ab')_2$, Fab'-B, Fab'-G, $BF(ab')_2$-B or $BF(ab')_2$-G. Each combination was carried out in duplicate. The tubes were incubated for 1 hr at 37°C. The cells were centrifuged for 5 min at 5,000 rpm, and suspended in PBS before being centrifuged again. The cells were resuspended in 200 µl of a solution of HRP linked-anti-mouse Fab' Ab, in PBS, for 1 hr at 37°C. The cells were centrifuged and washed as before. The cells were resuspended in 500 µl o-PD in citrate buffer (pH 4) and incubated at room temperature for 30min. The Abs_{405nm} was determined. In an ELISA where the Fab' fragments were bound to the wells of a 96-well plate, it was determined that the HRP-linked, anti-mouse Fab' Ab resulted in 0.321 AU/µg mouse Fab', while the HRP-linked, anti-rabbit Fab' Ab resulted in 0.167 AU/µg rabbit Fab'. This information was used to quantify the amount of Ab fragment bound to 10^6 cells. From this the average number of cells bound per mole of Ab fragment was determined (Table 4.3.4). The results indicated that 33% of the binding activity of the parental IgG was lost following pepsin digestion. The binding activity of the Fab' fell to 25% of the parental IgG. Following $BF(ab')_2$ production, binding activity was returned to 94% of that of the parental $F(ab')_2$.

Anti-AP	AP (μg)	AP/Ab (μg/μg)	AP/Ab (mol/mol)	Binding % of IgG	Binding % of F(ab')2
IgG	0.032	0.256	0.379	100.0	132.5
F(ab')2	0.037	0.296	0.286	75.5	100.0
Fab'-B	0.025	0.200	0.080	21.1	30.0
Fab'-G	0.038	0.304	0.122	32.2	42.7
BF(ab')2-B	0.025	0.200	0.161	42.5	56.3
BF(ab')2-G	0.026	0.204	0.165	43.4	57.5

Table 4.3.3:

The results of an ELISA used to measure the binding activity of the Ab fragments against alkaline phosphatase (AP). From the ELISA, the total amount of AP (μg) bound by each Ab fragment was calculated. This information was used to determine the amount of AP (μg) bound per μg Ab fragment. This was converted to a molar ratio. From this the binding activity of each Ab fragment was calculated as a percentage of the parental IgG and F(ab')2 binding activity. These results show that the BF(ab')2 retains 43% of the binding activity of the parental IgG and 57% of the binding activity of the parental F(ab')2. (Fab'-B= Fab' fragment produced using the method of Brennan *et al.*, 1985; Fab'-G= Fab' fragment produced using the method of Glennie *et al.*, 1987.)

Anti-K562	Ab (μg)	Ab (μmol/ 10^6 cells)	Cells x10^{12} / mol Ab	Binding % of IgG	Binding % of F(ab')2
IgG	0.588	3.7	0.27	100.0	150.0
F(ab')2	0.547	5.6	0.18	66.6	100.0
Fab'-B	0.572	14.2	0.07	25.9	38.9
Fab'-G	0.559	13.9	0.07	25.9	38.9
BF(ab')2-B	0.461	5.7	0.17	63.0	94.4
BF(ab')2-G	0.479	5.9	0.17	63.0	94.4

Table 4.3.4:

The results of an ELISA used to measure the binding activity of the Ab fragments against the leukaemic cell line K562. From the ELISA, the total amount of Ab (μg) bound by 10^6 cells was calculated. This information was used to determine the amount of Ab (μmol) bound to 10^6 cells. This was converted to number of cells per mole of Ab. From this the binding activity of each Ab fragment was calculated as a percentage of the parental IgG and F(ab')2 binding activity. These results show that the BF(ab')2 retains 63% of the binding activity of the parental IgG and 94.4% of the binding activity of the parental F(ab')2.

4.3.4 Assay of Clinical Samples by ELISA:

Two ELISAs, capable of differentiating a leukaemic cell line, K562, from non-leukaemic cell lines, i.e. NSO, EJ and NRK, had been developed. These assays were further tested to see if they could differentiate normal blood from that of leukaemic patients. Samples of venous peripheral blood were collected from patients known to be suffering from CLL (samples from the Mater Hospital) and from healthy volunteers (ABG, DCU), in heparinised blood tubes. The lymphocytes were isolated from thirteen of the twenty samples (Section 3.9.1). For a small, random number of samples both red and white blood cells were retained. The blood samples were coded and stored in liquid nitrogen.

On four different days, blood samples for the same set of patients were thawed and assayed by both ELISA protocols. The samples were tested in conjunction with positive controls (K562), negative controls (NSO & normal PBL) and a blank (BSA). The results of one days testing are shown in detail, along with overall results (Table 4.3.5). Any sample with Abs_{620nm} greater than 150% of the negative control samples was taken as a positive test result. Using the plate ELISA, seventeen of the twenty samples tested were consistently diagnosed as CLL samples. Following four repetitions, 84% of known CLL patients tested positive by this assay. Assay of isolated PBLs by this method resulted in the positive diagnosis of 84.6% of CLL patients. Assay of un-treated blood cells, i.e. red and white cells together, by this method resulted in the positive diagnosis of 82.1% of CLL patients. Using the suspension ELISA, twenty of the twenty samples tested were consistently diagnosed as CLL samples. Following four repetitions, 92.5% of known CLL patients tested positive by this assay. With the use of isolated PBLs the rate of diagnosis was 92.8%, which was not significantly different from the overall rate. Similarly, with the use of a mixed population of cells the rate of diagnosis, at 92.3%, remained close to the overall rate.

Table 4.3.5:

Sample	Cells	Plate ELISA	Positive/ 4 ELISAs	Susp. ELISA	Positive/ 4 ELISAs	Immuno-chemistry
K562	-	0.620±0.10	4	0.773	4	+++++
NSO	-	0.125±0.02	0	0.156	0	+
LC	W	0.116±0.03	1	0.147	0	+
BSA	-	0.084±0.01	0	0.022	0	-
JoB II	W	0.244±0.03	3	0.437	4	+++
NH	W	0.218±0.02	3	0.206	3	++
ToS II	W	0.216±0.02	3	0.402	3	++
ToS	W	0.182±0.01	2	0.395	3	++
PB	W	0.206±0.01	4	0.382	4	++++
ML II	W	0.234±0.03	4	0.409	4	+++
PG	W	0.200±0.02	3	0.418	4	+++
JH	W	0.228±0.01	4	0.500	4	++++
MS	W	0.208±0.02	4	0.428	4	++++
EG	W	0.202±0.02	3	0.487	3	+++
JoB III	W	0.244±0.01	4	0.467	4	+++
ML IV	W	0.212±0.02	4	0.623	4	+++
MoC II	W	0.208±0.02	3	0.678	4	++
PK	R/W	0.246±0.02	4	0.544	4	ND
JoB	R/W	0.226±0.02	4	0.531	4	ND
MoC	R/W	0.110±0.01	3	0.570	4	ND
JoB	R/W	0.234±0.02	4	0.554	4	ND
HH	R/W	0.204±0.01	3	0.564	4	ND
HH	R/W	0.200±0.02	2	0.564	3	ND
PmH	R/W	0.184±0.02	2	0.726	3	ND

4.3.5 Immunocytochemistry of Clinical Samples:

Cytospins were prepared from the same blood samples that were tested by ELISA (Section 3.9.2). This included CLL samples, K562 cells, NSO cells and normal PBL. Some slides were stained with Rapi-Diff stain (Section 3.9.2) to ensure structural integrity. The cultured cells appear to be more robust than the PBLs (Fig. 4.3.4). The remainder of the slides were subjected to immunocytochemical analysis (Section 3.9.3). A clear difference in staining intensity exists between the positive and negative controls (Fig. 4.3.5). Most of the CLL samples were strongly positive, 9 out of 13 graded +++ or higher (Fig. 4.3.6). A few CLL samples were only weakly positive (4 out of 13 graded only ++). The intensity of the staining varied greatly with CLL samples, in most instances this corresponded to the response of each particular sample to the ELISAs (Table 4.3.5).

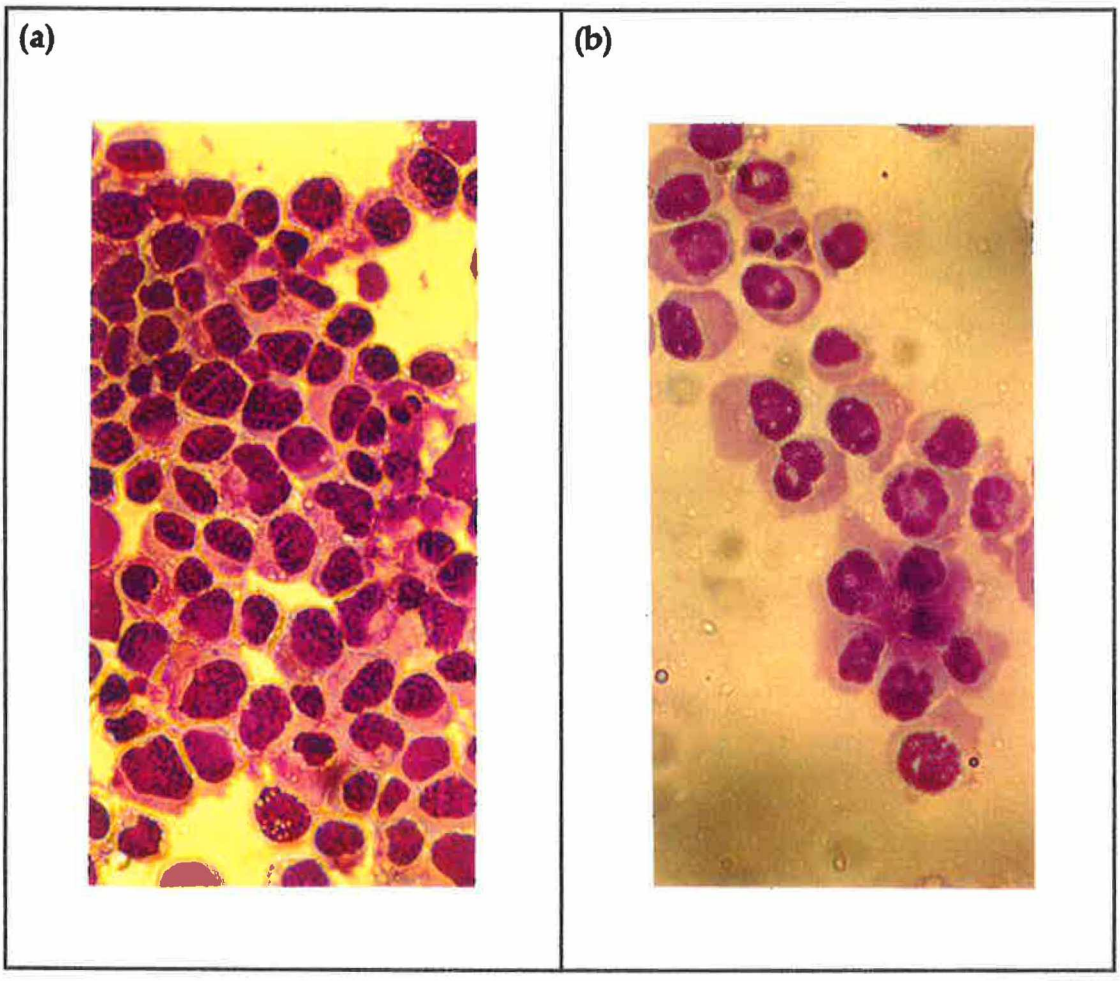


Figure 4.3.4:

Cytospin preparations: (a) K562 cells. (b) Lymphocytes from a patient suffering from CLL. Both slides were stained with Rapi-Diff stain, this colours the nucleus purple and the cytoplasm pink Although both slides were prepared using the same protocol, K562 cells adhere to the slide in higher density, and are less damaged, than the CLL cells.

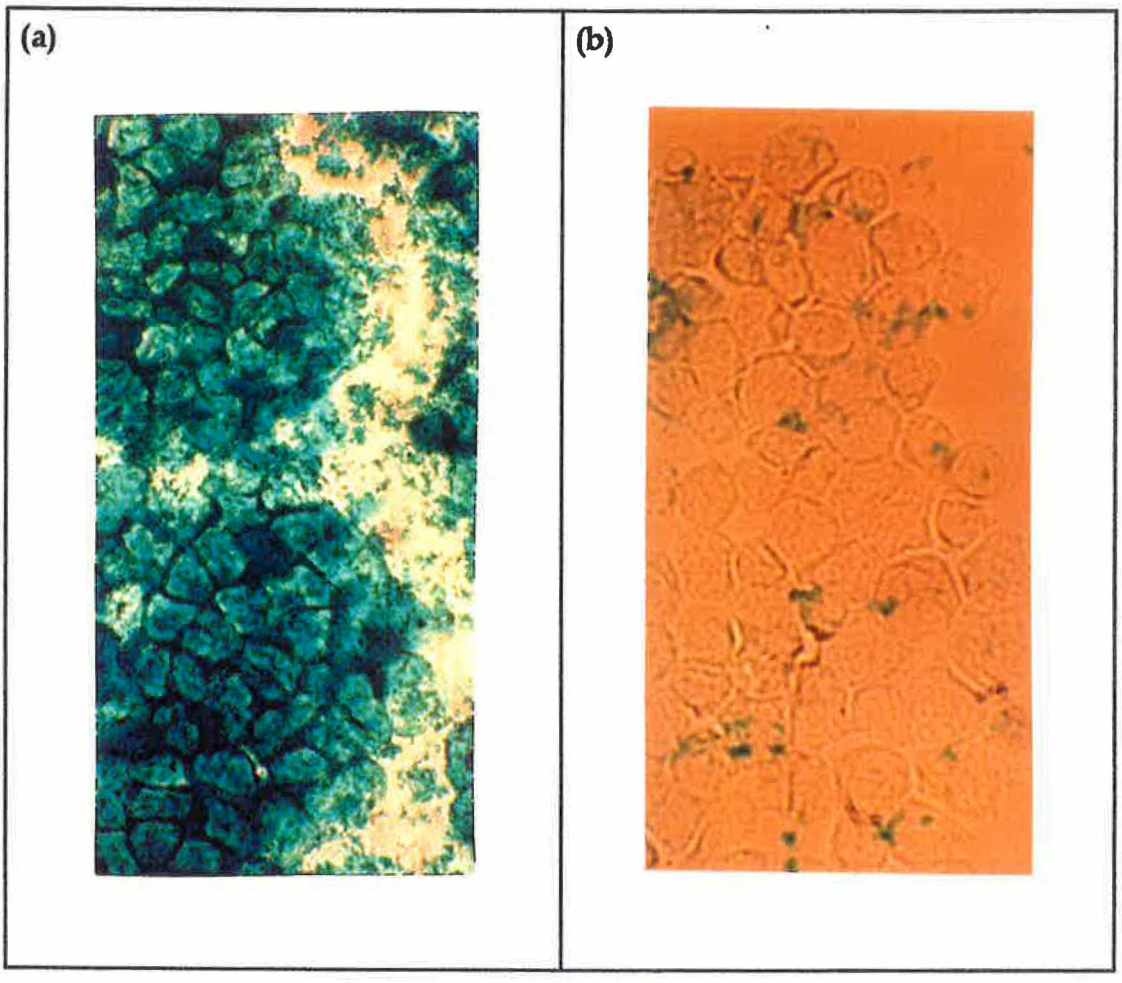


Figure 4.3.5:

Cytospin preparations stained using immunochemical techniques. Both slides were treated with an anti-CLL X anti alkaline phosphatase BAb (50μg/ml) and alkaline phosphatase (50μg/ml). Colour was developed in the slides using the substrate BCIP. (a) K562 cells, positive control, stain darkly blue (graded +++++). (b) NSO cells, negative control, do not stain (graded +).

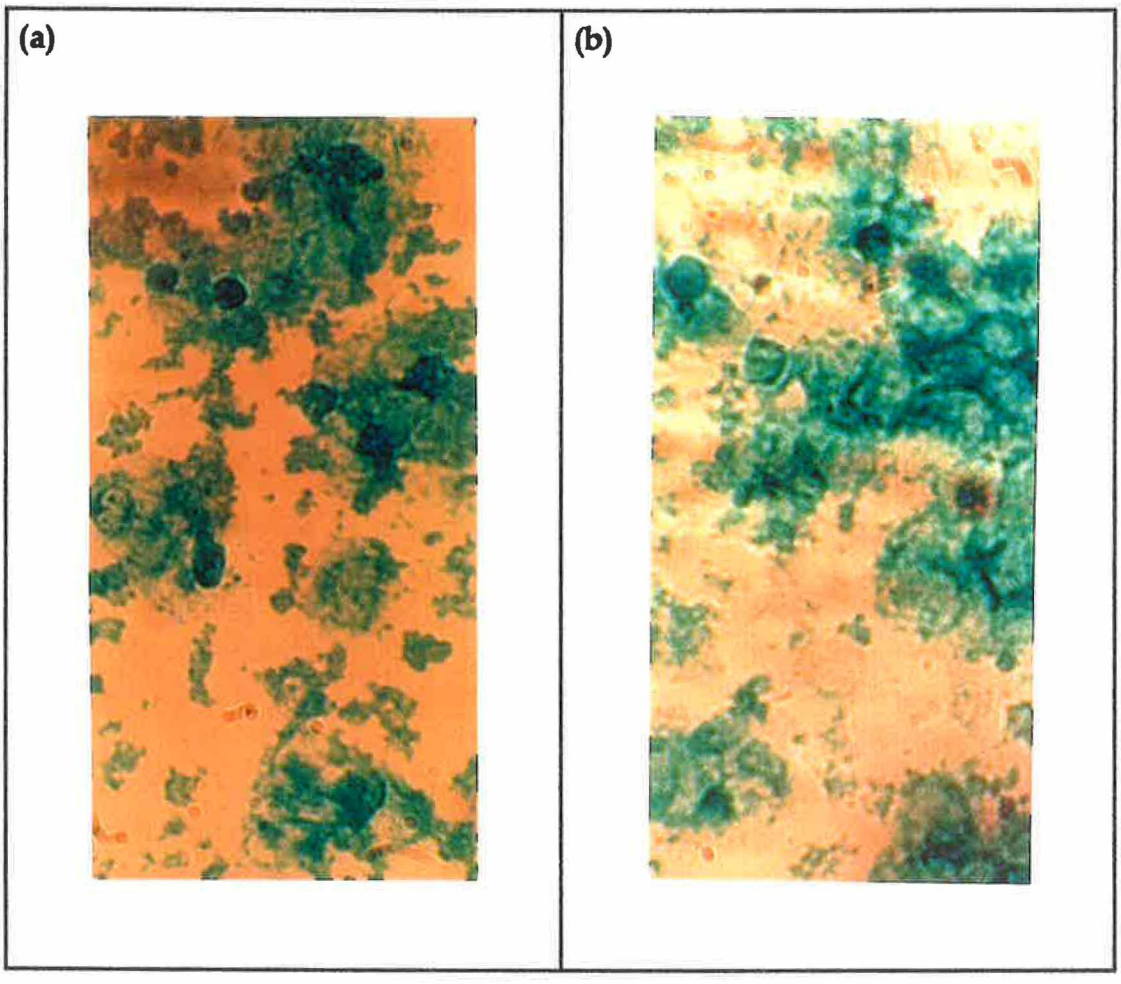


Figure 4.3.6:

Cytospin preparations of peripheral blood lymphocytes from CLL patients. Both slides were treated with an anti-CLL X anti-alkaline phosphatase BAb (50μg/ml) and alkaline phosphatase (50μg/ml). Colour was developed in the slides using the substrate BCIP. The strong blue staining indicates that both samples test positive for CLL. (a) Sample JOB, graded +++. (b) Sample EG, graded +++.

4.3.6 Summary:

The enzyme alkaline phosphase was used to raise polyclonal Abs in a rabbit. It has a molecular weight of 100 kDa and a specific activity of 1.868 AU/μg. An anti-AP X anti-CLL BF(ab')2 was produced and used to optimise a plate ELISA for the detection of CLL cells. Best results were achieved when AP and BF(ab')2 were added together at concentrations of 100 μg/ml AP and 1 μg/ml BF(ab')2, for 1 hr at 37°C. This concentration of BF(ab')2 was found to be optimum whether the BF(ab')2 was produced by the method of Brennan *et al.* (1985) or Glennie *et al.* (1987). A suspension ELISA was also developed to be carried out in 1.8 ml conical centrifuge tubes. Best results were achieved at a K562 concentration of 10^6 cells/ml, a BF(ab')2 concentration of 50 μg/ml and an AP concentration of 50 μg/ml.

An ELISA was carried out to estimate the binding activity of the rabbit anti-AP IgG, F(ab')2, Fab' and BF(ab')2. The results indicated that 25% of the binding activity of the parental IgG was lost following pepsin digestion. The binding activity of the Fab' fragments fell to 20-30% of the parental IgG. Some activity was restored on formation of the BF(ab')2, returning to 43% of the parental IgG. A second ELISA was carried out to estimate the binding activity of the mouse anti-CLL IgG, F(ab')2, Fab' and BF(ab')2. The results indicated that 33% of the binding activity of the parental IgG was lost following pepsin digestion. The binding activity of the Fab' fell to 25% of the parental IgG. Following BF(ab')2 production, binding activity was returned to 94% of that of the parental F(ab')2.

The ELISAs and an immunochemical method, which were developed using the BF(ab')2, were used to detect CLL cells in patient blood samples. 83.75% of known CLL patients tested positive using the plate ELISA. Assay of isolated PBLs by this method resulted in the positive diagnosis of 84.6% of CLL patients. Assay of un-treated blood cells, i.e. red and white cells together, by this method resulted in the positive diagnosis of 82.1% of CLL patients. 92.5% of known CLL patients tested positive using the suspension ELISA. The use of isolated PBLs did not significantly improve this rate of diagnosis, 92.8%. Similarly, the use of un-treated cells did not affect the rate of diagnosis, 92.3%. When immuno-chemistry was used, the intensity of the staining varied greatly with CLL samples, in most instances this reflected the results of the ELISAs.

4.4 DETECTION OF 7-HYDROXYCOUMARIN USING BISPECIFIC ANTIBODIES

An ELISA was developed for the detection of a cell line and diseased tissue, using a BF(ab')2. The following describes the production of a BF(ab')2 using the anti-alkaline phosphatase Ab and a rabbit polyclonal anti-7-hydroxy-coumarin (7-OHC) Ab. It was hoped that this BF(ab')2 could be used to develop an ELISA for the detection of 7-OHC in solution and in clinical samples.

4.4.1 Analysis of BF(ab')2 Production by HPLC:

A BF(ab')2 was produced from two rabbit polyclonal Abs. The first was the anti-alkaline phosphatase (AP) Ab, described previously (Section 4.3.1). The second was an anti-7-hydroxycoumarin (7OHC) Ab (donated by D. Bogan, DCU). This anti-serum had a titre of 1:5,000 when tested by competitive ELISA. The anti-AP and anti-7OHC Abs were used for the production of anti-AP X anti-7OHC BF(ab')2 using the methods of Brennan *et al.* (1985) (Section 3.8.2) and Glennie *et al.* (1987) (Section 3.8.3). HPLC analysis was carried out at each stage of BF(ab')2 production (Fig. 4.4.1). Analysis was carried out under the conditions described (Section 3.4.2) except that the flow rate used was 0.75 ml/min.

Five proteins, of known molecular weight, were applied to HPLC and a standard curve of Log molecular weight vs. Rt produced (Fig. 4.4.2). The moleular weights of the standards were back calculated from the equation of the line. This indicated that the standard curve estimated the molecular weight correct to within 12.6% of the expected molecular weight. The molecular weights of the various antibody fragments were also calculated from the standard curve (Table 4.4.1). The BF(ab')2 formed was found to be similar in size to the parental F(ab')2 fragments. This was confirmed by PAGE of the same fragments (Fig. 4.4.3). Using the method of Brennan *et al.* (1985) the BF(ab')2 formed 79% of the total protein present, as measured by peak area. Using the method of Glennie *et al.* (1987) the BF(ab')2 formed 51% of the total protein present, as measured by peak area. Both the BF(ab')2 produced were purified by affinity chromatography. The columns were prepared by binding the antigens, AP or 7OHC, to CNBr-activated sepharose (Section 3.3.1). The relevant BF(ab')2 containing fractions were pooled and concentrated in dialysis bags on a bed of sucrose. The BF(ab')2 were stored at 4°C for future use.

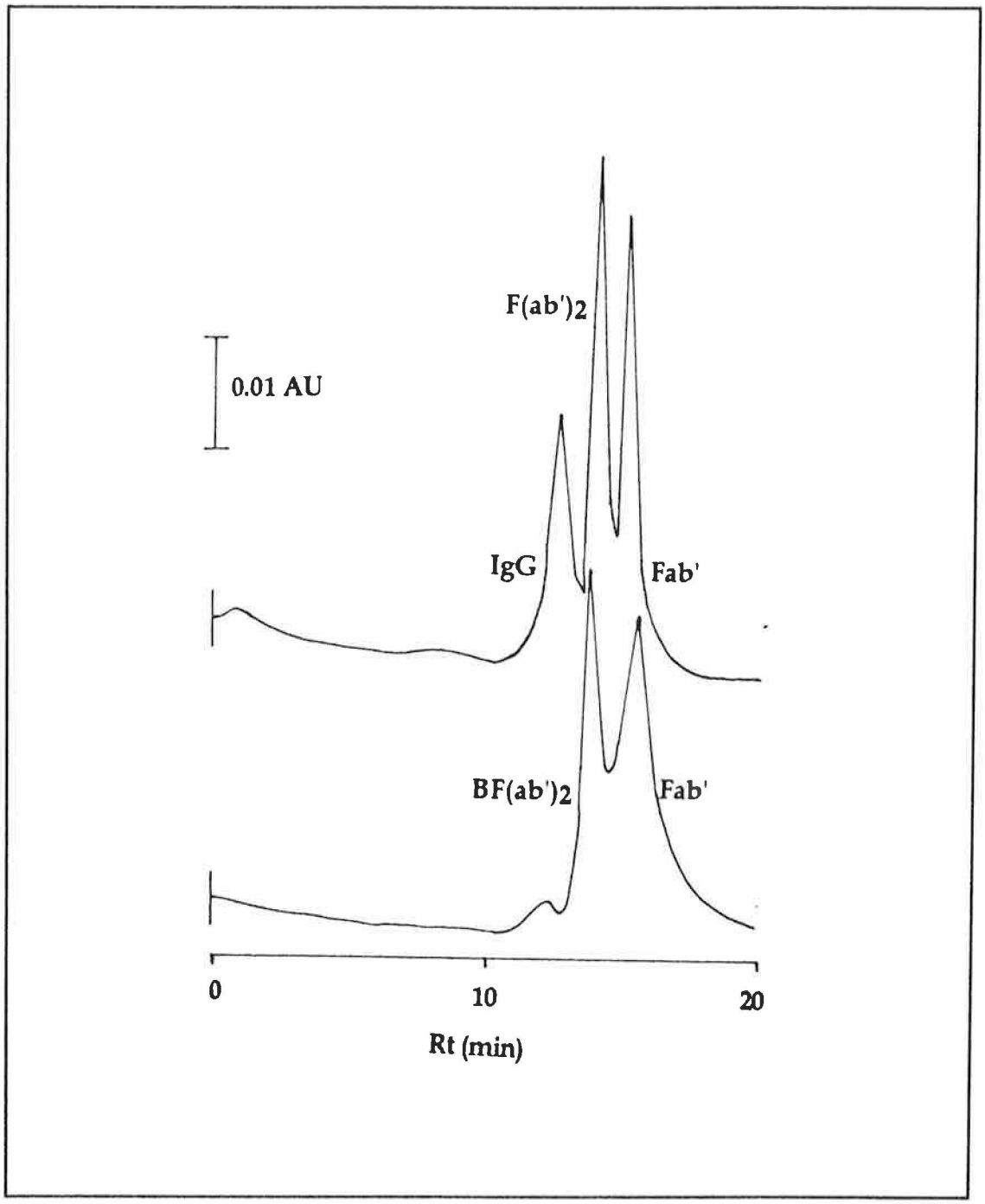


Figure 4.4.1:

The chromatograms produced following HPLC analysis of the Ab fragments produced using the method of Glennie *et al.* (1987) for the production of $BF(ab')_2$. HPLC was carried out on a Protein Pak 300SW series column, with 0.1 M sodium phosphate buffer (pH 7.4), at a flow rate of 0.75 ml/min.

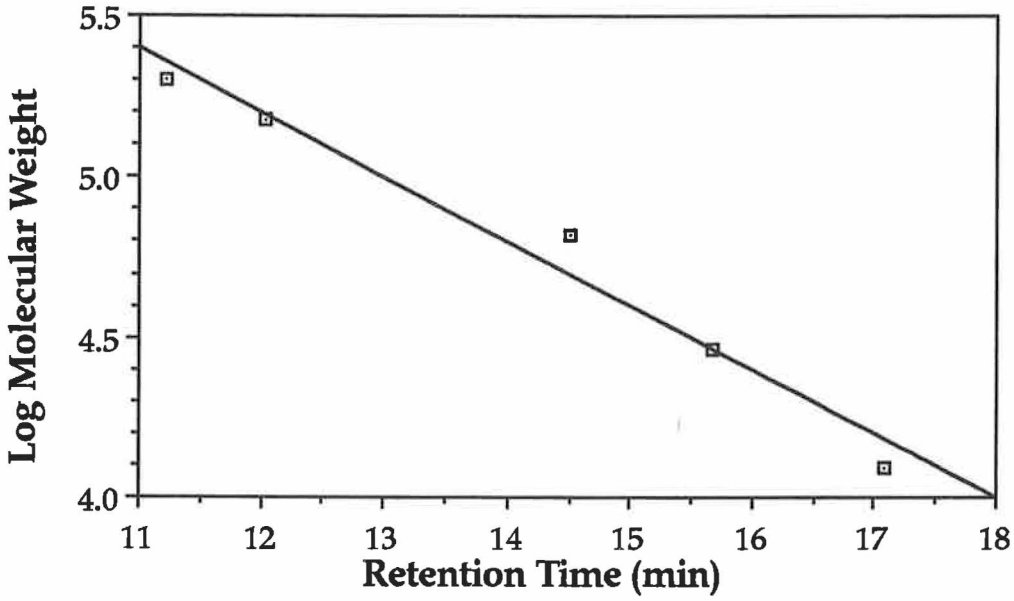


Figure 4.4.2:

The standard curve produced by plotting the retention times, from HPLC analysis, of proteins of known molecular weight against the log molecular weight (r = 0.987). HPLC was carried out on a Protein Pak 300SW series column, with 0.1 M sodium phosphate buffer (pH 7.4), at a flow rate of 0.75 ml/min. The proteins elute in order of decreasing molecular weight: β-Amylase, $2x10^5$ Da, Rt = 11.23 min. Alcohol dehydrogenase, $1.5x10^5$ Da, Rt = 12.04 min. Albumin, $6.6x10^4$ Da, Rt = 14.50 min. Carbonic anhydrase, $2.9x10^4$ Da, Rt = 15.68 min. Cytochrome c, $1.24x10^4$ Da, Rt = 17.07 min.

Protein	Rt (min)	Molecular Weight
Blue Dextran	7.65 ± 0.3	2,000,000
β-Amylase	11.23 ± 0.5	200,000
Alcohol Dehydrogenase	12.04 ± 0.4	150,000
Albumin	14.50 ± 0.2	66,000
Carbonic Anhydrase	15.68 ± 0.3	29,000
Cytochrome C	17.07 ± 0.4	12,400
IgG	12.42 ± 0.3	128,915
$F(ab')_2$	13.78 ± 0.2	68,549
Fab'-SH/Fab'-mal	14.97 ± 0.4	39,387
$BF(ab')_2$	13.83 ± 0.3	66,922

Table 4.4.1:

The results of HPLC analysis of standard proteins and antibody fragments derived from the production of $BF(ab')_2$s, according to the method of Glennie *et al.* (1987). HPLC was carried out on a Protein Pak 300SW series column, with 0.1 M sodium phosphate buffer (pH 7.4), at a flow rate of 0.75 ml/min. The Rt of each protein was determined five times. A standard curve of Log molecular weight vs. Rt was constructed from the proteins of known molecular weight. The molecular weight of the antibody fragments was determined from the mean Rt.

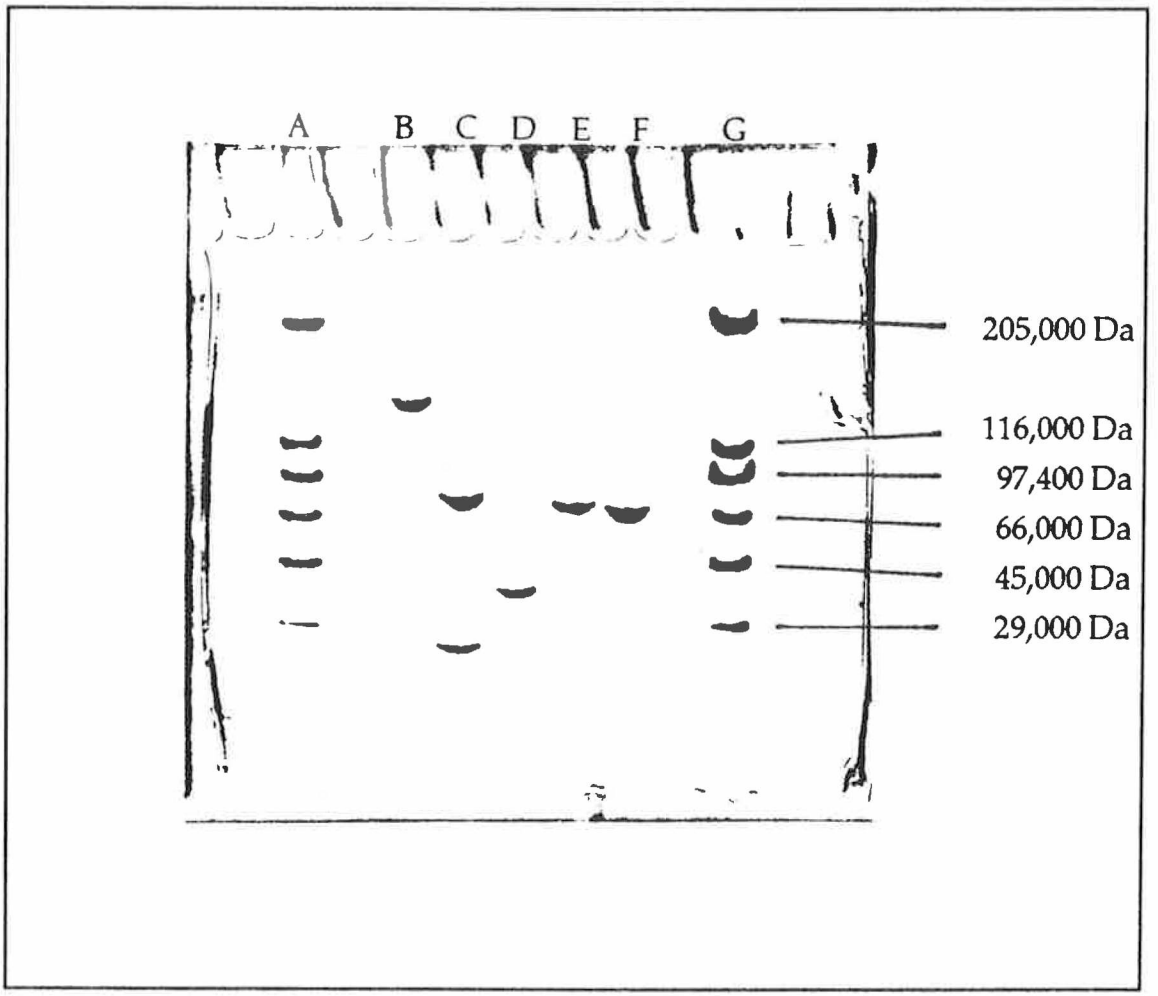


Figure 4.4.3:

A photograph of the gel produced following PAGE analysis of the antibody fragments formed during production of BF(ab')2. The lanes contained: (a & g) molecular weight markers. (b) IgG. (c) F(ab')2 and Fc'. (d) Fab'. (e) BF(ab')2, produced by the method of Brennan *et al.* (1985). (f) BF(ab')2, produced by the method of Glennie *et al.* (1987). This would indicate that the BF(ab')2 produced was of a similar size to the parental F(ab')2, approximately 70 kDa.

4.4.2 Measurement of BF(ab')2 Activity:

The IODOGEN reagent has been used to label Ab fragments with radioactive iodine prior to their use in BF(ab')2 production (Glennie *et al.*, 1987). IODOGEN was used to label the Ab fragments with non-radioactive iodine (Section 3.5.3). These were; the anti-7OHC IgG, F(ab')2, Fab'-B (from the method of Brennan *et al.*, 1985) and Fab'-G (from the method of Glennie *et al.*, 1987). The reaction mixtures were dialysed extensively against PBS to remove free iodine. The concentration of protein and iodine present in these samples was measured using the BCA assay (Section 3.5.1) and the Sandell-Kolthoff assay (Section 3.5.4), respectively. From this the amount of iodine bound to each Ab fragment was calculated (Table 4.4.2).

BF(ab')2s were produced using the methods of Brennan *et al.* (1985) and Glennie *et al.* (1987). One set of BF(ab')2s was produced using unlabelled anti-AP Fab' and iodine-labelled anti-7OHC Fab'. Another set of BF(ab')2s was produced using unlabelled anti-7OHC Fab' and iodine-labelled anti-AP Fab'. These BF(ab')2s, along with the iodine labelled Ab fragments, were used to determine approximate affinity values for the IgG, F(ab')2, Fab'-B, Fab'-G and BF(ab')2.

A 96-well plate was coated with 1 μg AP per well and blocked with BSA (Section 3.6.2). To each well was applied one of the following; iodine-labelled anti-AP IgG, iodine-labelled anti-AP F(ab')2, iodine-labelled anti-AP Fab'-B, iodine-labelled anti-AP Fab'-G, BF(ab')2s made by both methods from unlabelled anti-AP Fab' and iodine-labelled anti-7OHC Fab' (Fig. 4.4.4b). Each labelled Ab fragment or labelled BF(ab')2 was applied in the concentration range 0.001-100 μg/well and each concentration was applied to three wells. The amount of iodine bound in each well was measured, from a standard curve (Fig. 4.4.5), using the Sandell-Kolthoff assay. The quantity of Ab fragment bound to the plate was then calculated (Table 4.4.3).

Since 7OHC does not bind strongly to 96-well plates, the second 96-well plate was coated with a 10 μg/ml solution of 7OHC-BSA conjugate, in PBS, per well (donated by D. Bogan, DCU) and blocked with BSA (Section 3.6.2). To each well was applied one of the following; iodine-labelled anti-7OHC IgG, iodine-labelled anti-7OHC F(ab')2, iodine-labelled anti-7OHC Fab'-B, iodine-labelled anti-7OHC Fab'-G, BF(ab')2s made by both methods from unlabelled anti-7OHC Fab' and iodine-labelled anti-AP Fab' (Fig. 4.4.4a). Each labelled Ab fragment or labelled BF(ab')2 was applied in the concentration range 0.001-100 μg/well. Each concentration of Ab fragment was applied to three wells. The amount of iodine bound in each well was measured, from a standard curve (Fig. 4.4.5), using a Sandell-Kolthoff assay. The quantity of Ab fragment bound to the plate was calculated (Table 4.4.3).

Sample	Protein (mg/ml)	Iodine (μg/ml)	Iodine/Protein (μg/mg)
α-AP IgG	1.01	10.4	10
α-AP F(ab')2	1.25	9.7	8
α-AP Fab'-B	1.20	7.2	6
α-AP Fab'-G	1.29	9.3	7
α-7OHC IgG	1.01	10.1	10
α-7OHC F(ab')2	1.15	9.5	8
α-7OHC Fab'-B	0.74	8.6	12
α-7OHC Fab'-G	0.74	8.2	11

Table 4.4.2:

The BCA assay and Sandell-Kolthoff reaction were used to measure the amount of protein and iodine present in each sample. From this the level of non-radioactive iodine bound to each Ab fragment following iodination using the IODOGEN reagent was calculated. (α = anti-)

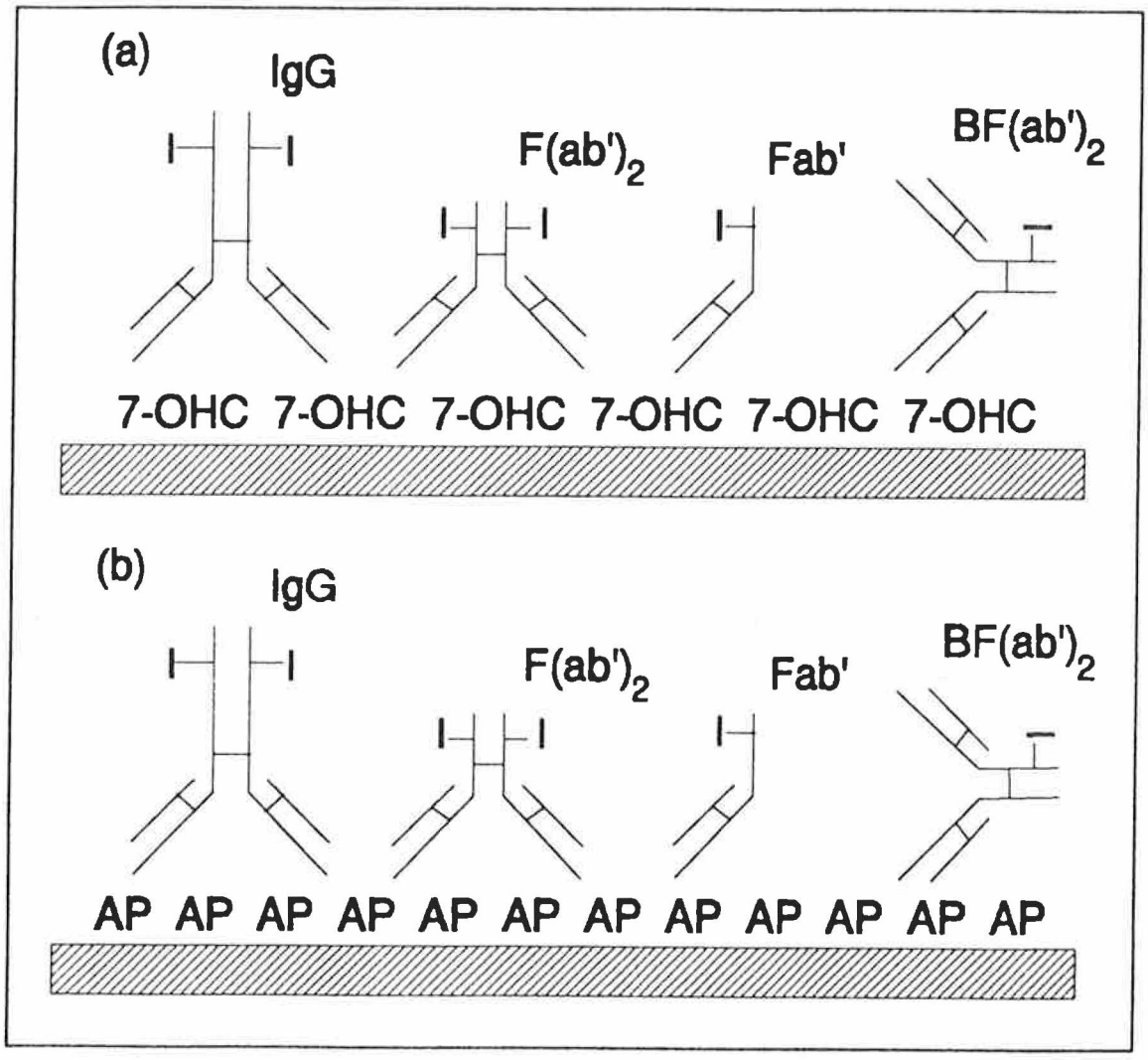


Figure 4.4.4:

An illustration of the ELISA set up to determine approximate affinity values for various IgG, F(ab')2, Fab' and BF(ab')2 fragments. (a) The anti-7-hydroxy-coumarin antibody was tested in a 96-well plate coated with 7-hydroxy-coumarin. To each well was applied one of the antibody fragments to be tested, which had been pre-labelled with iodine (I). In the case of the BF(ab')2 only the anti-alkaline phosphatase half of the molecule was labelled. (b) The anti-alkaline phosphatase antibody was tested in a 96-well plate coated with alkaline phosphatase. To each well was applied one of the antibody fragments to be tested, which had been pre-labelled with iodine (I). In the case of the BF(ab')2 only the anti-7-hydroxy-coumarin half of the molecule was labelled. The presence of iodine was detected using the Sandell-Kolthoff reaction. (AP = alkaline phosphatase, 7-OHC = 7-hydroxy coumarin, I = iodine)

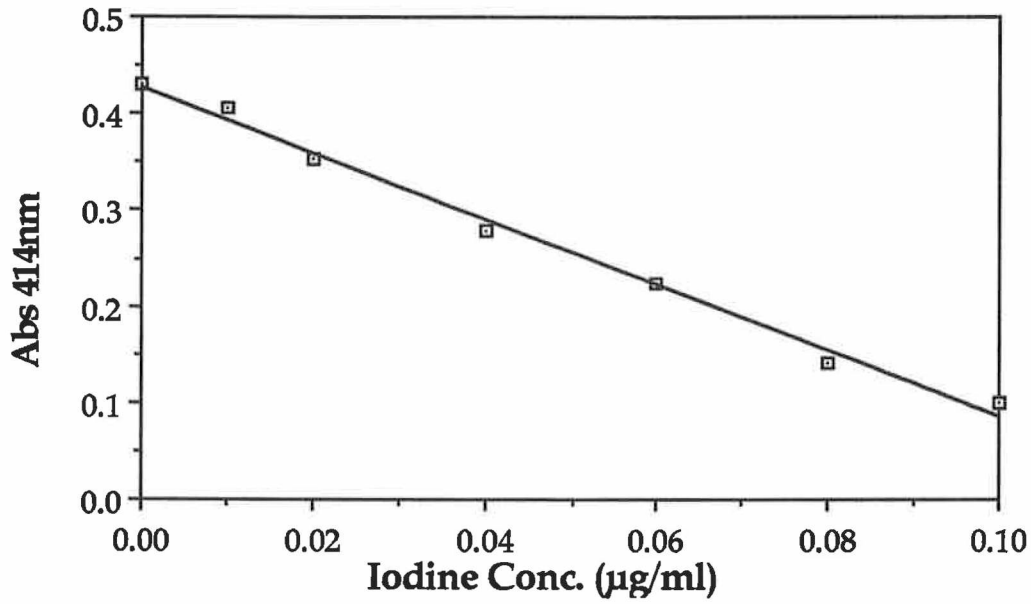


Figure 4.4.5:

A sample standard curve produced using the Sandell-Kolthoff reaction in a micro-assay and a range of KI standards, with a regression coefficient (r) of 0.996. From this the amount of iodine-conjugated Ab fragment bound to each plate was calculated.

Sample	Iodine (μg)	Ab (mg)	Ab (moles)	Ag/Ab (mol/mol)
α–AP IgG	0.83	0.083	5×10^{-10}	2.800
α–AP F(ab')2	0.71	0.089	12×10^{-10}	1.167
α–AP Fab'-B	0.88	0.143	36×10^{-10}	0.389
α–AP Fab'-G	0.85	0.121	31×10^{-10}	0.452
BF(ab')2-B	0.41	0.034	8×10^{-10}	1.750
BF(ab')2-G	0.55	0.050	12×10^{-10}	1.167
α–7OHC IgG	0.50	0.050	3×10^{-10}	4.667
α–7OHC F(ab')2	0.85	0.106	14×10^{-10}	1.000
α–7OHC Fab'-B	0.28	0.023	5×10^{-10}	2.800
α–7OHC Fab'-G	0.33	0.030	7×10^{-10}	2.000
BF(ab')2-B	0.89	0.148	37×10^{-10}	0.378
BF(ab')2-G	0.55	0.078	20×10^{-10}	0.700

Table 4.4.3:

The results of assays to determine the binding affinity of various Ab derivatives for their respective Ags. The plates were coated with a known amount of Ag and the Ab fragments allowed to bind. The amount of iodine in each well was measured and, hence, the amount of Ab fragment bound was calculated. The amount of Ag bound per mole of Ab was determined. The results indicate that much of the binding activity is lost following the pepsin digestion. Some further activity may be lost following reduction to the Fab' fragment, although this is not always the case. (α = anti-; -B = produced by the method fo Brennan *et al.*, 1985; -G = produced by the method of Glennie *et al.*, 1987)

4.4.3 Development of a Competitive ELISA for 7OHC:

A checkerboard ELISA was carried out to determine the optimum concentrations of $BF(ab')_2$ and AP required for the detection of 7OHC. The wells of a 96-well ELISA plate were coated with 7OHC-BSA conjugate and blocked with BSA (Section 3.6.2). To each column of wells (1-12) was applied increasing concentrations of $BF(ab')_2$ in the range 0-2 mg/ml, 50 µl/well. To each row of wells (A-H) was applied increasing concentrations of AP in the range 0-500 µg/ml, 50 µl/well. (Fig. 4.3.1) These were incubated for 2 hr at 37°C. The plate was washed three times in PBS. Colour was developed in the wells using the AP substrate BCIP (Section 3.6.3). The lowest concentrations of AP and $BF(ab')_2$ to give a strongly positive result were chosen for future use. These were AP at 100µg/ml and $BF(ab')_2$ at 20 µg/ml. These concentrations of $BF(ab')_2$ and AP were found to be optimum, independent of method of production.

A competitive ELISA was set up to produce a standard curve for 7OHC concentration. The plates were coated with 7OHC-BSA conjugate and blocked with BSA as before. To the eight wells of each column (1-12) was added one of a serial dilution of 7OHC, in the range 0-20 µg/ml, 50 µl per well. To each well was added 50 µl $BF(ab')_2$, at 20 µg/ml, and 50 µl AP, at 100 µg/ml. The plate was then incubated at 37°C for 2 hr or overnight. The plates were developed using BCIP as before. This was carried out using $BF(ab')_2$ produced by the method of Brennan *et al.* (1985) (Fig. 4.4.6, a & c) and those produced by the method of Glennie *et al.* (1987) (Fig. 4.4.7, a & c). In both cases the ELISAs were repeated five times and, although the standard curves produced were straight lines with good regression coefficients, the small difference in Abs_{620nm} between top and bottom standard (0.033 ± 0.003 AU) prohibited their use in 7OHC quantitation.

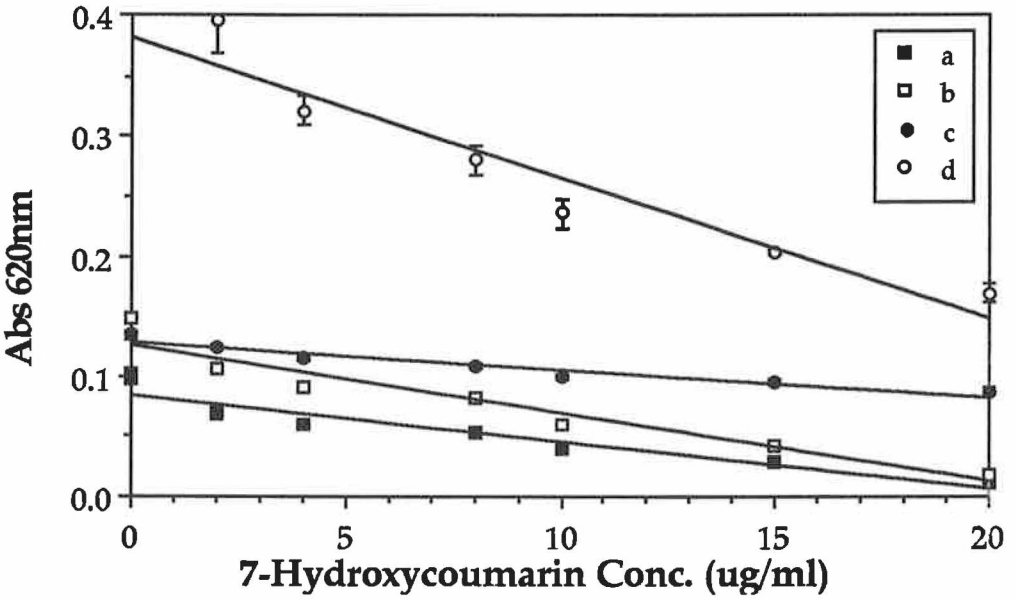


Figure 4.4.6:

Standard curves produced following the analysis by competitive ELISA of 7-hydroxycoumarin. ELISA plates were coated with 7-OHC-BSA conjugate. Standards of 7-OHC were incubated in the presence of $BF(ab')_2$ produced by the method of Brennan *et al.* (1985) and alkaline phosphatase (a) for 2 hr (r = 0.958) and (c) overnight (r = 0.964). The same standards and $BF(ab')_2$ were incubated without AP (b) for 2 hr (r = 0.959) and (d) overnight (r = 0.956), AP was subsequently applied for 1 hr. Each concentration of 7-OHC was measured in quadruplicate.

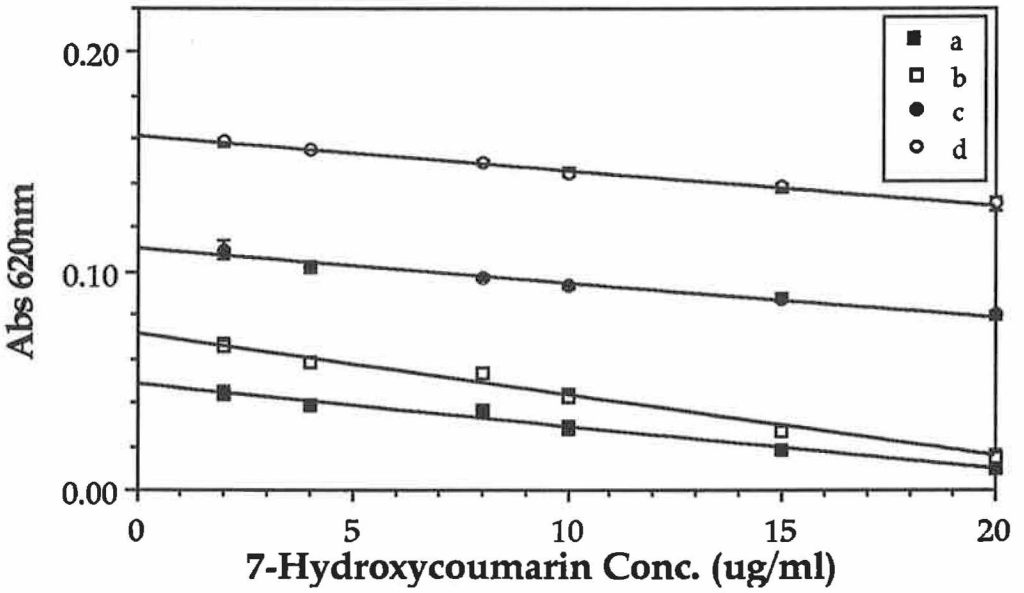


Figure 4.4.7:

Standard curves produced following the analysis by competitive ELISA of 7-hydroxycoumarin. ELISA plates were coated with 7-OHC-BSA conjugate. Standards of 7-OHC were incubated in the presence of BF(ab')2 produced by the method of Glennie *et al.* (1987) and alkaline phosphatase (a) for 2 hr (r = 0.991) and (c) overnight (r = 0.984). The same standards and BF(ab')2 were incubated without AP (b) for 2 hr (r = 0.991) and (d) overnight (r = 0.997), AP was subsequently applied for 1 hr. Each concentration of 7-OHC was measured in quadruplicate.

The competitive ELISA was repeated, with the exception that the AP was left out of the initial incubation. AP was applied as before but after the unbound $BF(ab')_2$ and 7OHC had been washed out. The plate was incubated for a further 1 hr at 37°C. Each ELISA was carried out five times. This was carried out using $BF(ab')_2$ produced by the method of Glennie *et al.* (1987) (Fig. 4.4.7, b & d), as before, the small difference in Abs_{620nm} between top and bottom standard (0.030 ± 0.004 AU) prohibit their use in 7OHC quantitation. This was also carried out using $BF(ab')_2$ produced by the method of Brennan *et al.* (1985) (Fig. 4.4.6, b & d). In this case, the plate which was incubated with $BF(ab')_2$ and 7OHC for 2 hr showed some improvement in the top to bottom standard Abs_{620nm} difference (0.100 ± 0.004 AU). The second plate, which was incubated with $BF(ab')_2$ and 7OHC overnight and with AP for 1 hr, had the greatest difference in Abs_{620nm} between top and bottom standard (0.230 ± 0.006 AU) and appears to offer the best route for development of the desired assay. However, since this protocol offers no significant improvement on previous methods, either in detection of 7-OHC or speed of the assay, it was not examined further.

4.4.4 Summary:

An anti-AP X anti-7OHC BF(ab')$_2$ was produced as shown by HPLC. Using the method of Brennan *et al.* (1985) the BF(ab')$_2$ formed 79% of the total protein present, as measured by peak area. Using the method of Glennie *et al.* (1987) the BF(ab')$_2$ formed 51% of the total protein present. IODOGEN was used to label the Ab fragments and the Sandell-Kolthoff reaction was used in a micro-assay to assess the binding activity of each of the labelled Ab fragments. The results indicate that much of the binding activity is lost following the pepsin digestion. Some further activity may be lost following reduction to the Fab' fragment, although this is not always the case.

In order to develop an ELISA for the detection of 7-OHC, a checkerboard ELISA was carried out to find the optimum concentrations of AP and BF(ab')$_2$. These were found to be AP at 100 μg/ml and BF(ab')$_2$ at 20 μg/ml. This concentration of BF(ab')$_2$ was found to be optimum whether the BF(ab')$_2$ was produced by the method of Brennan *et al.* (1985) or Glennie *et al.* (1987). These conditions were used in several competitive ELISAs to detect 7-OHC in the concentration range 0-20 μg/ml. The standard curves produced were straight lines with good regression co-efficients, the small difference in Abs_{620nm} between top and bottom standard (0.033 ± 0.003 AU) prohibited their use in 7OHC quantitation. One plate, which was incubated with BF(ab')$_2$ and 7OHC overnight and then with AP for 1 hr, had the greatest difference in Abs_{620nm} between top and bottom standard (0.230 ± 0.006 AU). This protocol was not developed since it offered no significant improvement on methods already available.

CHAPTER 5

DISCUSSION

Bispecific antibodies (BAb) are antibody-derived molecules capable of monovalently binding to two distinct antigens (Fig. 5.1). BAbs use the specificity of monoclonal antibodies (MAb) to join together molecules or cells to mediate a desired effect. Such molecules can bind enzymes, therapeutic agents or radioactive labels to a target without the need for chemical modification. MAbs must be chemically conjugated to these active agents, a procedure which may alter the affinity and/or activity of either the MAb or the active agent. This would indicate that BAbs may have several advantages over MAbs in diagnostic and therapeutic procedures (Section 1.1.2). In addition to replacing MAb conjugates, the unique properties of BAbs suggest the possibility of a range of novel immunoassays. These areas have been reviewed recently by a number of workers (Reading & Bator, 1988; Songsivilia & Lachmann, 1990; Fanger *et al.*, 1992; Nolan & O'Kennedy, 1992).

Since their inception, a number of methods have been used to produce BAbs, with varying degrees of success. BAbs have been created by the fusion of Ab-producing cells, by chemical cross-linking of two Abs or by molecular genetic approaches. Each of these approaches has its limitations, and a major problem in establishing trials with BAbs is the procurement of sufficient amounts of clinical-grade material necessary to carry out studies in their entirety. The production of hybrid-hybridomas is a time consuming and uncertain process, and, once produced, the high nuclear content of the cell causes a high level of genetic instability. Yields of BAb by this method have been shown to be approximately 34% of total Ig production (Wong & Colvin, 1987; Tada *et al.*, 1989). The BAb must then be extracted in a multi-step purification involving dialysis, salt precipitation, affinity chromatography and HPLC (Staerz & Bevan, 1986; Takahashi & Fuller, 1988; Barr *et al.*, 1989; Tada *et al.*, 1989). Chemical BAb production methods offer several advantages over biological BAb production, primarily, the speed and ease with which they can be carried out. The starting material for these procedures is purified IgG of defined specificity. Purification of the desired BAb is simplified since only IgG fragments are present in a buffered solution compared to hybrid-hybridoma products which must be isolated from growth medium or ascitic fluid. Most commonly, chemically produced BAbs are bispecific $F(ab')_2$ fragments [$BF(ab')_2$] (Brennan *et al.*, 1985; Glennie *et al.*, 1987), they can thus be separated from the larger parental IgG, and smaller Fab' fragments on the basis of size.

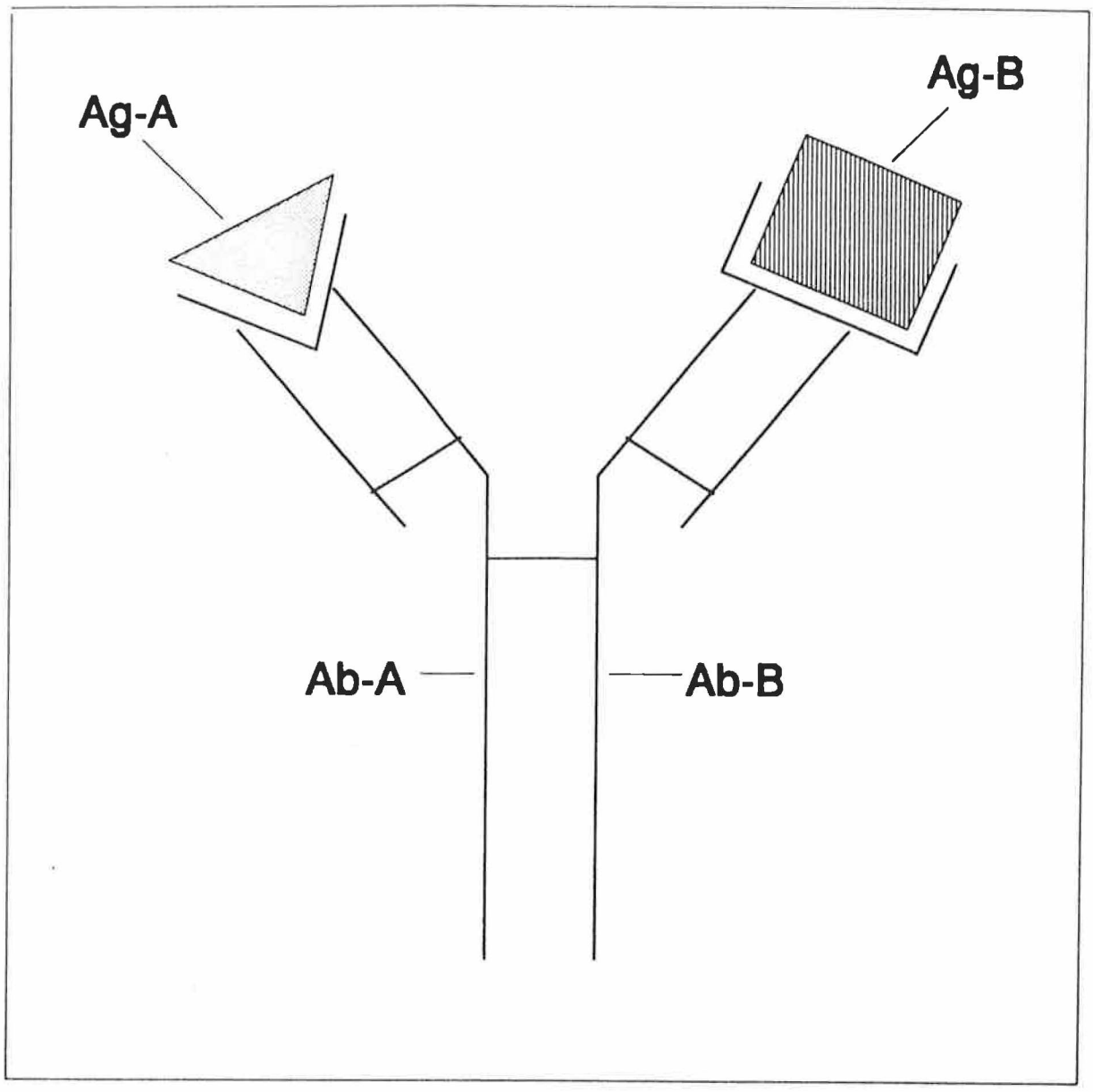


Figure 5.1:

A diagramatic representation of a bispecific antibody. Bispecific antibodies are antibody-derived molecules capable of monovalently binding to two distinct antigens. When produced by hybrid-hybridomas, bispecific antibodies are IgG sized molecules, consisting of one light and one heavy chain from each parental IgG. When produced chemically, bispecific antibodies are $F(ab')_2$ sized molecules, consisting of one light chain and part of the heavy chain (the section containing the variable region and hinge region) from each parental IgG.

Yields of BF(ab')$_2$ produced by chemical methods have been shown to be approximately 60% of total potential yields (Brennan *et al.*, 1985; Glennie *et al.*, 1987; Nitta *et al.*, 1989). Two protocols for the chemical production of BF(ab')$_2$s were examined. Of these the method of Brennan *et al.* (1985) appears to offer the advantage of having BF(ab')$_2$ as the sole product, compared to the method of Glennie *et al.* (1987) in which heterodimers occur. For this reason the method of Brennan *et al.* (1985) was examined initially.

Large quantities of a murine IgG, G12, were purified from ascitic fluid and used, in conjunction with human IgG (Sigma), for the production of a BAb. Precipitation of the Ig using ammonium sulphate is a quick and easy method of extracting Ig from solution (Section 3.2.2). However, the level of purity achieved by this method was insufficient and further purification steps were necessary (Section 4.1.1). From the studies on IgG purification, it would appear that the Affi-T column produces the greater yield of IgG, but the Protein A column has a greater capacity to bind IgG. Thus, fewer column runs are necessary with the Protein A column and losses are reduced. In light of the relatively large quantities of IgG required to produce BF(ab')$_2$s, purifications were carried out using the Protein A column. The purified IgG was subject to a pepsin digestion to produce an F(ab')$_2$ fragment. The method of Brennan *et al.* (1985) was chosen since it used gentler conditions and gave a more complete digestion than the method of Runge *et al.* (1990) (Section 4.1.2). The F(ab')$_2$ fragments were reduced to the Fab' fragment and conjugated to dTNB to prevent intrachain disulphide bond formation, (Fig. 5.2). The successful conjugation of dTNB was shown using spectrophotometric analysis, as was the removal of dTNB from the human IgG molecules (Section 4.1.3) (Fig. 5.3).

The murine Fab'-TNB and the human Fab'-SH were allowed to re-associate according to the method of Brennan *et al.* (1985). The recombinant molecule produced formed less than 10% of the total protein present in solution, as measured by HPLC (Section 4.1.4). This yield was improved by the addition of an oxidising agent, such as hydrogen peroxide, although this also increases the risk of homologous pairing of Fab' fragments. For this reason, an ELISA was carried out to determine which, of several, concentrations of H_2O_2 would maximise BF(ab')$_2$ production while minimising the amount of H_2O_2 required. The addition of 0.5% (w/v) H_2O_2 was found to increase the yield of BF(ab')$_2$ to 60% of total potential yield, as measured by HPLC (Section 4.1.4) (Fig. 5.3).

Preparation of Bispecific Antibodies (I)

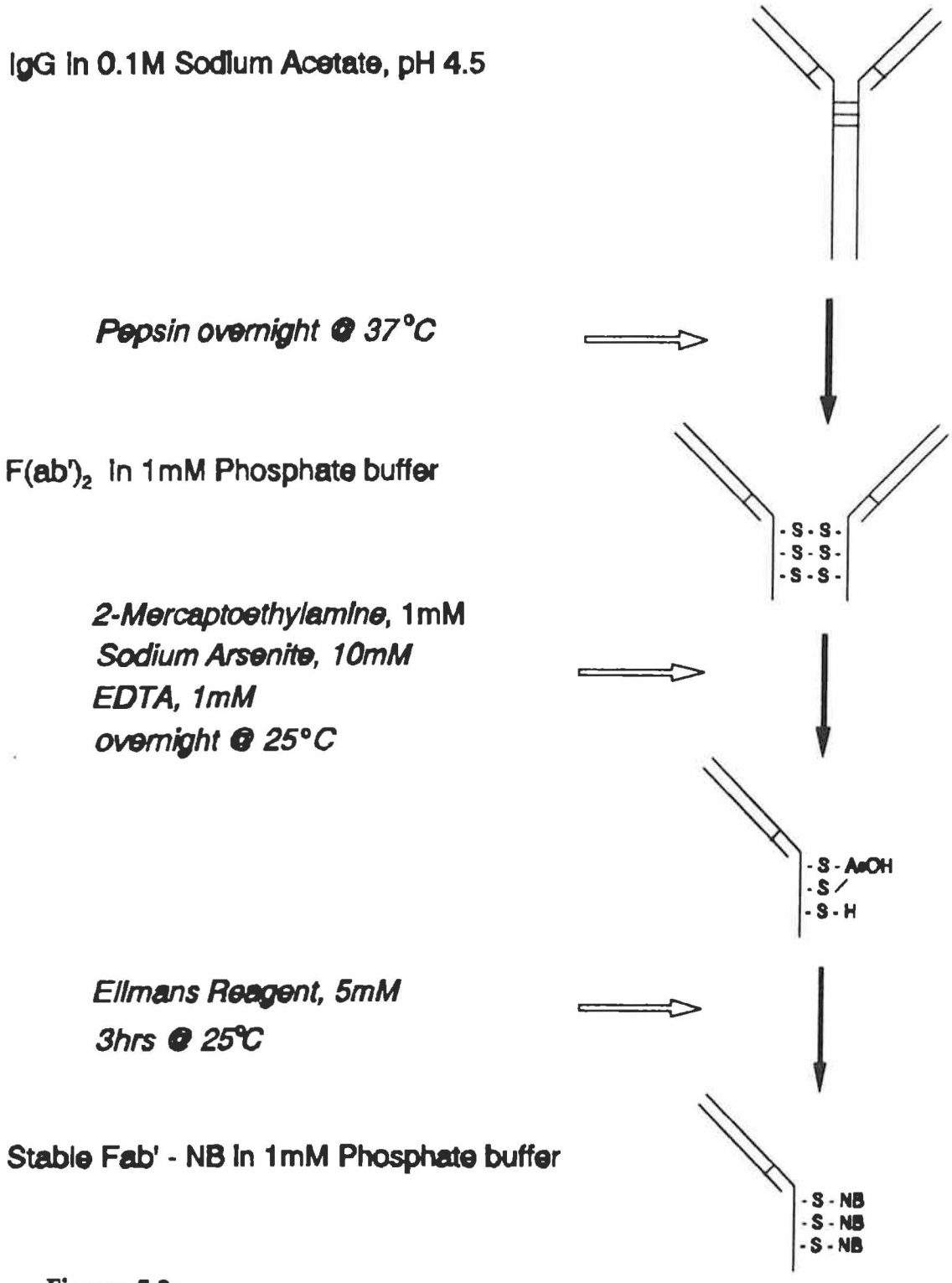


Figure 5.2:

An illustration of the first part of the production protocol for bispecific antibodies, which was adapted from the method of Brennan *et al.* (1985). The conditions given are those optimised for use in our laboratory.

Preparation of Bispecific Antibodies (II)

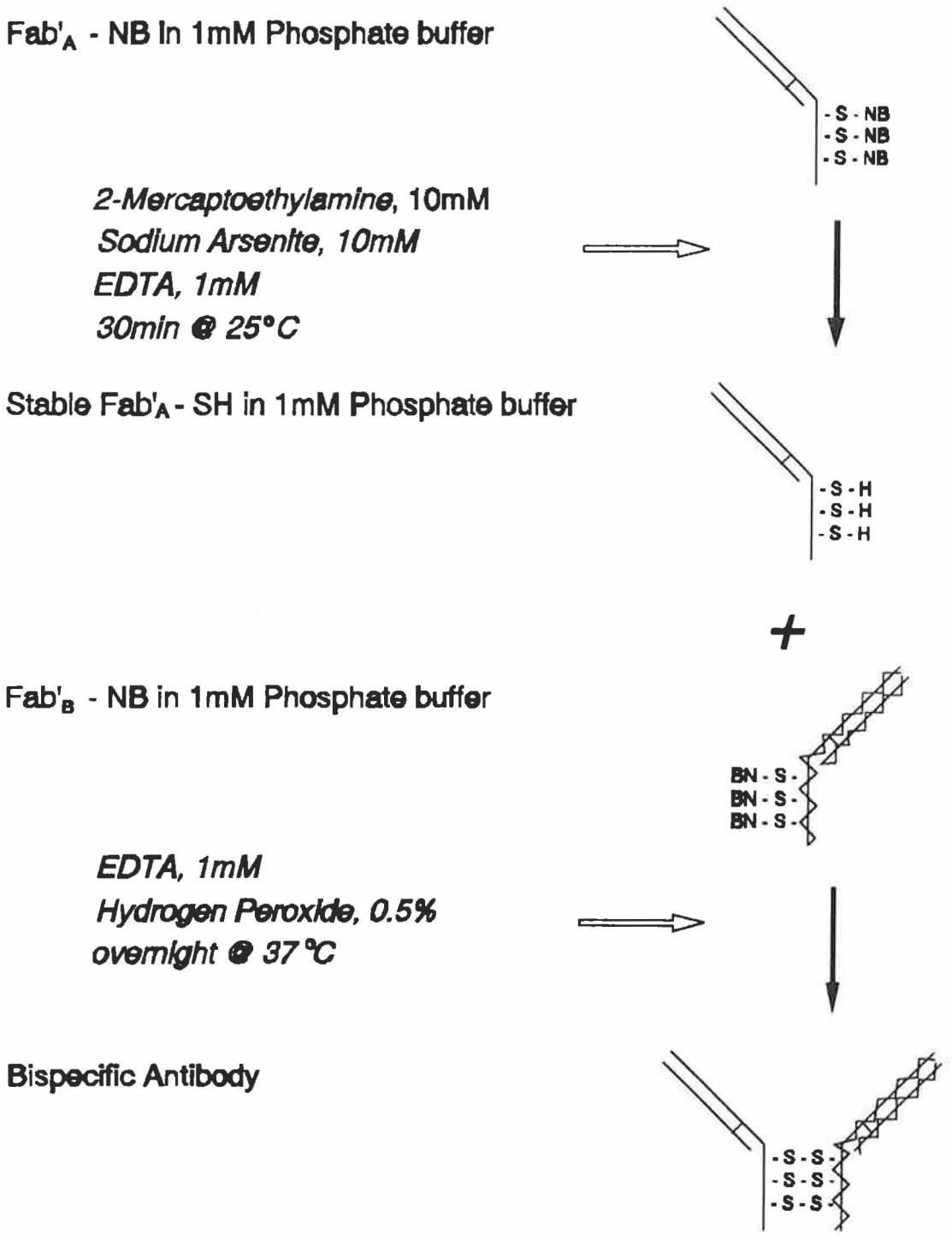


Figure 5.3:

An illustration of the second part of the production protocol for bispecific antibodies, which was adapted from the method of Brennan *et al.* (1985). The conditions given are those optimised for use in our laboratory.

The HPLC analysis also indicated that the molecule formed is twice the weight of a Fab' fragment, but slightly lower than that of the original F(ab')2 fragment. This may occur for two distinct reasons. Firstly, the species differences in parental IgG mean that any recombinant F(ab')2 cannot equal either of the parental molecules in size. Secondly, the method of analysis used produced a standard curve which estimated molecular weight correct to within 10% of actual molecular weight. Thus, the accuracy of molecular weight prediction by this method is reduced at very high molecular weights. In addition, proteins of the same molecular weight, but with different 3-dimensional structure, will differ somewhat in retention time on the Protein Pak 300SW column. When these factors are taken into consideration, the recombinant F(ab')2 produced falls well within the size parameters of a F(ab')2 fragment. This was confirmed by electrophoretic analysis of the various Ig fragments produced (Section 4.1.4). The results of the ELISA indicate that the 'F(ab')2-sized' molecule, produced in this procedure, was formed by linking a human Fab' fragment to a murine Fab' fragment, ie. it is a BF(ab')2 fragment.

It had been suggested that BF(ab')2 showed no loss of activity compared to the parental IgG (Glennie *et al.*, 1987). This was tested by the production of a functional BF(ab')2. A HRP-BSA conjugate was produced, as shown by HPLC and double affinity chromatography (Section 4.2.1). An anti-BSA X anti-K562 BF(ab')2 was successfully produced, as shown by HPLC and ELISA (Section 4.2.2). A series of ELISAs were used to show that both the Fab' fragments, used in the production of the BF(ab')2, maintained the binding activity and specificity of the parental IgG, although some of the affinity is lost. In a comparative test, the affinity of the anti-K562 Fab' appeared to fall by 70% compared to parental IgG, while that of the anti-BSA Fab' fell by approximately 50%. Much of this affinity was lost as a result of the pepsin digestion (Section 4.2.3), an indication of the importance of the Fc' fragment in stabilising the IgG molecule. The BF(ab')2 was used successfully in a one-step enzyme immunoassay for K562 cells which served as a model system for later trials.

Two further BAbs were produced and used to study the applicability of BAbs to enzyme immunoassay and immunochemical studies. A number of novel approaches have been suggested (Paulus, 1985; Gorog *et al.*, 1989). However, the main advantage of BAbs, at the moment, is in the improvement of conventional systems. Primarily, the simultaneous addition of reagents to an assay reduces the time required for the assay, while increasing the reproducibility (Suresh *et al.*, 1986; Gorog *et al.*, 1989; Tada *et al.*, 1989; Bugari *et al.*, 1990; Stratieva-Taneeva *et al.*, 1993). This

concept was put to the test. The enzyme alkaline phosphase (AP) was used to raise polyclonal Ab in a rabbit. The serum had an anti-AP titre of 1 in 10,000 (Section 4.3.1). AP has a molecular weight of 100 kDa and a specific activity of 1.868 AU/μg. An anti-AP X anti-CLL BF(ab')$_2$ was produced and used to optimise a microtitre-plate, based ELISA for the detection of CLL cells. Best results were achieved when AP and BF(ab')$_2$ were added together at concentrations of 100 μg/ml AP and 1 μg/ml BF(ab')$_2$, for 1 hr at 37^{o}C. These concentrations of BF(ab')$_2$ and AP were found to be optimum and independent of the method of production of the BF(ab')$_2$ (Section 4.3.2). A suspension ELISA was developed in 1.8 ml conical centrifuge tubes. These ELISAs are faster than plate ELISAs, since the cells do not have to be adsorbed onto PVC plates. Thus, the entire sample is analysed and not just those cells that 'stick' well to PVC. Their accuracy and usefulness in the detection of CLL was tested here. Best results were achieved at a K562 concentration of 10^6 cells/ml, a BF(ab')$_2$ concentration of 50 μg/ml and an AP concentration of 50 μg/ml (Section 4.3.2). Again these concentrations of BF(ab')$_2$ and AP were found to be optimal and independent of the method of production of the BF(ab')$_2$. For both the plate ELISA and the suspension ELISA the concentration of AP, in particular, and BF(ab')$_2$ were somewhat higher than desirable. However, since fairly small volumes of those particular reagents are used in the assays, these high concentrations were tolerated.

ELISAs using BAbs, that have been developed and reported, have used enzyme and BAb concentrations in the range 1-10 μg/ml (Gorog *et al.*, 1989; Tada *et al.*, 1989; Stratieva-Taneeva *et al.*, 1993). Each of these was for the detection of a soluble, rather than a membrane-bound, protein Ag (i.e. CEA, lymphotoxin and Il-2, respectively). Standard curves were produced using pure analyte-Ag to coat the 96-well plate. Thus, a known, high quantity of analyte-Ag is available for binding to the BAb, and low concentrations of BAb and developing enzyme will be sufficient to detect the presence of analyte-Ag. When the analyte-Ag is membrane-bound the entire cell must be adsorded onto the 96-well plate. Levels of expression of the analyte-Ag on the cell surface will vary greatly and the surface of the wells of the plate will be coated with large amounts of membrane lipo-proteins and non-antigenic membrane proteins as well as the analyte-Ag. With the relatively small amounts of analyte-Ag available for binding, larger quantities of BAb and developing enzyme are required for the detection of the analyte-Ag. There are no reports available with evidence of ELISAs, using BAbs, directed against cells or membrane-bound Ags. Thus,

there is no basis of comparison with which to determine the efficiency of the conditions used here.

An ELISA was carried out to estimate the binding activity of the rabbit anti-AP IgG, F(ab')2, Fab' and BF(ab')2. The results indicated that 25% of the binding activity of the parental IgG was lost following pepsin digestion. The binding activity of the Fab' fragments fell to 20-30% of the parental IgG. Some activity was restored on formation of the BF(ab')2, returning to 43% of the parental IgG (56% of the parental F(ab')2). A second ELISA was carried out to estimate the binding activity of the mouse anti-CLL IgG, F(ab')2, Fab' and BF(ab')2 The results indicated that 33% of the binding activity of the parental IgG was lost following pepsin digestion. The binding activity of the Fab' fell to 25% of the parental IgG. Following BF(ab')2 production, binding activity was returned to 63% of the parental IgG (94% of the parental F(ab')2). These alterations in binding activity are independent of method of production of BF(ab')2 used. The loss of activity appears to be greater for the rabbit, polyclonal, anti-AP Ab than for the mouse, monoclonal, anti-K562 Ab. This appears to contradict the earlier test, in which the same murine, anti-K562 Ab lost more of its binding activity than the rabbit, anti-BSA Ab. This would seem to indicate that effects on binding activity depend on the combination of parental Abs used, rather than on the method of production of the BF(ab')2. This makes it difficult to predict, in advance, the probable efficiency of a BF(ab')2.

It is interesting to note the effects of the manipulations involved in BAb production have on the various Ab fragments produced. The reduced activity of the F(ab')2, compared to the whole molecule, emphasises the stabilising role of the Fc' portion within the IgG molecule. The activity of the Fab' was less than half that of the parental F(ab')2. This was, in part, expected since the Fab' molecule has only one Ag-binding site compared to two on the F(ab')2 molecule. The recovery of this activity on formation of the BF(ab')2 molecule was not anticipated, although it was duplicated with BF(ab')2 produced later in this project, and with BAbs produced by other workers in this laboratory (Quinlan, 1994). It is possible that this is due to the conformational changes which occur when the two Fab' fragments are bound together. The fact that recovery of activity is incomplete, i.e. the activity of the BF(ab')2 does not equal that of the parental F(ab')2, is probably due to the added strains placed on the molecules when Fab' from two different species are joined together.

The ELISAs and an immunochemical method, which were developed using the BF(ab')2, were used to detect CLL cells in patient blood samples. 83.75% of known CLL patients tested positive using the plate ELISA (Section 4.3.4). Assay of isolated PBLs by this method resulted in the positive diagnosis of 84.6% of CLL patients. Assay of untreated blood cells, i.e. red and white cells together, by this method resulted in the positive diagnosis of 82.1% of CLL patients. 92.5% of known CLL patients tested positive using the suspension ELISA (Section 4.3.4). The use of isolated PBLs did not significantly improve this rate of diagnosis, (92.8%). Similarly, the use of untreated cells did not affect the rate of diagnosis, (92.3%). It was thought that use of isolated PBLs would concentrate the the number of Ag positive cells and, hence, amplify the signal, compared to a mixed population of red and white blood cells. This was shown not to be the case. Cell counts were carried out prior to each ELISA. Since these were counts of PBL population, rather than total cell counts, the same number of PBL were present in the assay whether the PBL are isolated or not. Since the G12 Ab does not bind to red blood cells, their presence does not interfere with the assay, or the results obtained. Thus, there was no significant difference in the rate of diagnosis of CLL when isolated PBLs were used, compared to when a mixed population of red and white blood cells were used.

An immuno-chemical method was developed, in which the BF(ab')2 and AP were applied simultaneously to a cytospin preparation of K562 cells or isolated lymphocytes (Section 3.9.3). When this was used, the intensity of the staining varied greatly with CLL samples; in most instances this corresponded to the response of each particular sample to the ELISAs (Section 4.3.5). This would indicate that the intensity of the staining is a function of the binding activity of the G12 part of the BF(ab')2. This may be related to expression of Ag on the cell surface, which can alter with progression of the disease and treatment regimen. This information was not available and correlations could not be made for the blood samples tested. All the slides were made with isolated PBL from the same patients assayed in the ELISAs. Of those samples examined, 9 out of 13 (69%) gave a strongly positive diagnosis, while 4 out of 13 (30%) gave a weakly positive diagnosis. All these slides showed a definite positive reaction compared to controls (normal PBL).

The rates of diagnosis achieved with the ELISAs and immuno-chemical method are consistent with incidence of the disease. About 90-95% of persons with CLL have the mature B-cell type, the remainder have T-cell, hairy cell or other less well defined types of leukaemia (Finch & Linet, 1992). CLL B-cells express a 69 kDa glycoprotein not expressed on

normal or other leukaemic blood cells. It is thought that this glycoprotein may be the Ag for the G12 Ab (Lannon *et al.*, 1988; Dighiero *et al.*, 1991). Thus, assays for CLL based on binding with G12, or G12 derived BF(ab')2s, will positively diagnose a maximum of 95% of patients. In addition to a symptomatic approach, diagnosis of CLL would normally involve detailed histopathological examination, including identification of the following: sustained lymphocytosis, lymphocytes with atypical morphology, chromosomal abnormality, surface membrane Ig and other cell surface markers (Dighiero *et al.*, 1991; Finch & Linet, 1992). The assays developed here are quicker and require less expertise than traditional histopathological techniques. They will require more strenuous testing, against larger numbers of fresh clinical samples, before their true value can be accurately predicted. It is proposed that these tests could then form part of a battery of tests to determine the diagnosis and progress of the disease.

A second BAb was produced in order to develop an assay for the drug, 7-hydroxycoumarin (7-OHC). A rabbit anti-AP X anti-7OHC BF(ab')2 was produced as shown by HPLC. Using the method of Brennan *et al.* (1985) the BF(ab')2 formed 79% of the total protein present, as measured by peak area. Using the method of Glennie *et al.* (1987) the BF(ab')2 formed 51% of the total protein present, as measured by peak area. (Section 4.4.1) The high yields in the former method make it the method of choice in this instance. They are a clear indication that using parental IgG from the same species improves Fab'-Fab' re-association, and, hence, overall yields. The HPLC method used in this analysis was changed slightly from that used previously. The flow rate of the mobile phase was increased in an attempt to sharpen the peaks and speed up analysis times. The retention times of the Ab fragments were shortened, but the chromatography was not significantly improved. The standard curve of molecular weight vs. retention time estimated molecular weight correct to within 12.6% of the actual molecular weight. This represented a decrease in accuracy compared to the earlier method.

IODOGEN was used to label the Ab fragments (Glennie *et al.*, 1987) and the Sandell-Kolthoff reaction was used in a micro-assay to assess the binding activity of each of the labelled Ab fragments (O'Kennedy *et al.*, 1989). The results indicate that much of the binding activity is lost following the pepsin digestion. Activity may fall by as much as 60% compared to parental IgG (Section 4.4.2). Some further activity may be lost following reduction to the Fab' fragment. In the case of the anti-AP Ab, the activity of the Fab' fell sharply compared to the parental IgG. Most of this was recovered on formation of the BF(ab')2, which had the same activity as

the parental F(ab')2. This same pattern, of loss and recovery of activity, occurs with all previous BF(ab')2 molecules. A different pattern occurs with the anti-7-OHC Ab. In this case, activity is lost following formation of the BF(ab')2 when compared to IgG, F(ab')2 or Fab'. This may indicate that the anti-7-OHC Ab is not as stable as those used previously and may not be entirely suitable for the types of manipulations necessary in BF(ab')2 production.

In order to develop an ELISA for the detection of 7-OHC, a checkerboard ELISA was carried out to find the optimum concentrations of AP and BF(ab')2 (Section 4.4.3). These were found to be AP at 100 μg/ml and BF(ab')2 at 20 μg/ml. These concentrations, of BF(ab')2 and AP, were found to be optimum, independent of the method of production of BF(ab')2. These conditions were used in several competitive ELISAs to detect 7-OHC in the concentration range 0-20 μg/ml. This was chosen as it is the range of most clinical significance. The standard curves produced were straight lines with good regression co-efficients. However, the small difference in Abs_{620nm} between top and bottom standard (0.033 ± 0.003 AU) prohibited their use in 7OHC quantitation. One plate, which was incubated with BF(ab')2 and 7OHC overnight and then with AP for 1 hr, had the greatest difference in Abs_{620nm} between top and bottom standard (0.230 ± 0.006 AU) and appears to offer the best route for development of the desired assay. The amount of AP or BF(ab')2 applied to the plate could be increased to improve the signal. An amplification system could be used, e.g. a tri-specific Ab which binds two AP for every 7-OHC. The most likely route for improving the assay, however, is to use a more suitable parental anti-7-OHC Ab. The Ab that was used had a high level of specificity, but a low titre (1:5,000) compared to the parental anti-AP Ab (1:100,000) and poor stability. The ELISA that was developed, using these anti-7-OHC and anti-AP Abs, for the detection of 7-OHC does not represent an improvement on detection techniques already available. It is neither as fast nor as accurate as conventional competitive ELISA or HPLC methods. These methods have been used to analyse large numbers of samples in the space of a day and are accurate in the concentration range 5-100 μg/ml (Egan, 1995). The assay developed using BF(ab')2 required overnight incubation and was useful in the narrower concentration range of 0-20 μg/ml only (Section 4.4.3).

Overall, the results of these studies indicate that BAbs can be extremely useful in development of immunoassays. They can greatly increase the speed with which the assays are carried out without any great loss of detection. This is, however entirely dependent on the parental IgG used. Some species of IgG may not be stable enough to withstand the

various reduction steps, resulting in loss of activity. This is possibly due to loss of the light chain, since in most cases the BF(ab')2 was somewhat lighter than the parental F(ab')2. A number of polyclonal Abs were used for two main reasons. Firstly, they are more easily produced than MAbs. Secondly, it is possible that the application of a number of Abs, with specificity for more than one epitope on the target Ag, may act in synergy to improve both the specificity and the signal to noise ratio of the assay. Polyclonal Abs contain large quantities of Abs, specific to several epitopes on the one Ag. Parental IgG from the same species will result in higher yields of BAb and, with less strain placed on the reformed molecule, should be more stable and of higher activity than those produced using IgG from different species. ELISAs and other novel immunoassays may be improved by the use of in-built amplification systems, such as the use of tri-specific Abs.

BAbs can be produced in high yield and purity with relative ease using the methods described. It is probable that, in the future, genetic manipulations will take over as a means for producing BAbs. Although currently yields are low by these methods, techniques are improving all the time. Such "designer" molecules offer many potential improvements over what is currently available, particularly in the field of immuno-therapeutics.

CHAPTER 6

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