THE HUMORAL IMMUNE RESPONSE OF PATIENTS RECEIVING RADIOLABELLED MURINE MONOCLONAL ANTIBODIES FOR THE TREATMENT OF MALIGNANT NEOPLASIA.

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ABSTRACT

The *in vivo* use of murine monoclonal antibodies (with specificity for tumour-associated antigen) in cancer therapy leads to a humoral immune response to the administered xenogeneic protein. The generation, specificity, immunoregulation and clinical significance of this response has been studied.

Serum from patients prior to the administration of murine monoclonal antibodies, and from healthy controls (blood donors) contained antibodies which bound to constant antigenic determinants located on the Fc portion of murine and human IgG. These pre-existing antibodies were mainly of the IgM class and were shown to be rheumatoid factors.

After a single therapeutic dose of between 2 and 10 milligrams of murine monoclonal antibody, the majority of patients studied developed an elevated anti-murine immunoglobulin response. This elevated response was also directed against antigenic determinants located on the Fc portion of the murine immunoglobulin. No significant response to the $F(ab')_2$ region was detectable. However, patients receiving two or more administrations of the same murine monoclonal antibody developed both IgM and IgG antibodies to the $F(ab')_2$ portion of murine IgG. A component of this anti-$F(ab')_2$ reactivity was directed to an antigenic determinant either in or very near the combining site (paratope) of the administered murine monoclonal antibody, and inhibited their binding to the tumour associated antigen *in*

vitro. Thus anti-Id^1 antibodies are generated.

Patients who had developed an anti-Id^1 response subsequently developed serum antibodies with specificity for the tumour-associated antigen, i.e. the same specificity as that of the original administered monoclonal antibody. These could either be anti-anti-idiotypic (anti-Id^2) antibodies, or antibodies generated to shed tumour antigen from cells targeted and destroyed by the radiolabelled murine monoclonal antibodies.

CONTENTS

LIST OF ABBREVIATIONS

$F(ab')_2$	Antibody fragment binding
Fc	Antibody fragment crystalizable
Ig	Immunoglobulin
PBS	Phosphate buffered saline
ELISA	Enzyme-linked immunosorbant assay
RIA	Radioimmunoassay
SDS-PAGE	Sodium dodecylsulphate Polyacrylamide gel electrophoresis
MFGI	Milk Fat Globule
id	idiotypic

The term "tumour associated" antigen is used throughout this thesis to descibe an antigen that, although present at low levels on normal tissue, is expressed at much higher levels on tumour tissue.

The term "tumour associated" monoclonal antibody is used to desribe a monoclonal antibody with specificity for a "tumour associated " antigen.

The term "tumour specific" antigen is used to descibe an antigen that is present on tumour cells, but not on any normal tissues.

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I dedicate this thesis to my

wife, Maria and my two children,

Giovanni and Santina.

CHAPTER 1

GENERAL INTRODUCTION

1.1 GENERATION OF HUMORAL IMMUNITY

The ability of the body to respond to infectious agents has been known for centuries. Voltaire in 1733 wrote of a chinese custom, practised since the fifteenth century, of prophylactic induction of smallpox infection by taking by the nose, dried powders of smallpox crusts (J.H. Humphrey and R.G. White, 1971). In 1798 Edward Jenner published his first memoir "An enquiry into the causes and effects of the Variolae Vacciniae", previously having embarked on the experiment in which James Phipps was inoculated with cowpox material with the intent on inducing immunity to smallpox.

In 1885 Louis Pasteur injected a boy, Joseph Meister, who had severe and multiple bites from a rabid dog, with a spinal cord emulsion which contained the attenuated virus responsible, in its unattenuated form, for causing the human disease, hydrophobia or rabies. Joseph Meister received thirteen injections, survived, and later became the gate-porter of the Pasteur Institute in Paris. Pasteur had hence shown that vaccination, a term introduced by Pasteur, in honour of Jenner, could induce responses which resulted in the survival from known lethal diseases.

Emil Von Behring in 1890 demonstrated the neutralization of diptheria organisms in a test tube, by the serum of animals with diptheria. He showed that the response to disease, at least in

part, was due to the action of neutralizing humoral ("in the body's fluids") factors. He called these factors antibodies. In 1897 Paul Ehrlich formulated the theory that antibodies are made by specific cells. Ehrlich realised that antibody interactions with "antigen", a substance that reacts with antibody, were specific and followed basic laws of chemistry.

These early studies, together with those of numerous other investigators since, have led to an indepth knowledge of what is now called, humoral immunity. Humoral immunity includes those processes mediated by antibodies, a class of proteins that circulate in the blood and specifically bind foreign antigens that induce them.

Initial contact with antigen leads to what is termed the primary immune response, where the antibody producing cells, B-cells, produce antibody predominantly of the class IgM. On subsequent challenge with the same antigen a more rapid response usually occurs. The predominant class of antibody produced in this secondary immune response is IgG. The change in class of receptor and secreted immunoglobulin, so called "class switching", takes place during differentiation and occurs through rearrangement of immunoglobulin genes.

The range of antigen-binding specificities seen in the IgG response to a particular antigenic determinant is greater than that seen in the early IgM antibodies. This may result, in part, from point mutations that arise during class switching. Some of

these mutations occur in the region of the immunoglobulin gene that codes for the antigen binding site. Such mutations result in a broader range of antibody specificity and affinity.

The reason for the secondary immune response being both more rapid and heightened is that during the primary immune response some activated B-cells do not mature into plasma cells. Some revert back to small lymphocytes, called memory cells. These memory cells are more easily activated on subsequent challenge by the same antigen.

Although the majority of antigens will on subsequent challenge result in a secondary immune response where the predominant class of antibody produced is IgG, a minority of antigens will not. These antigens which have simple repeating antigenic determinants, such as dextrans and polysaccharides, are referred to as thymus independent (TI) antigens; many of them possess the ability, at high concentrations, to activate B cell clones other than those specific for that antigen, this is, polyclonal B cell activation.

Antigen is normally encountered by antigen presenting cells, which include dendritic cells, circulating monocytes and tissue bound macrophages. The antigen is internalised, processed, and presented on the antigen presenting cell's surface. In conjunction with MHC class II, the antigen is then recognised by an MHC class II restricted cell, for example, the CD4 positive T-helper cell.

T-helper cells, on binding to the antigen - MHC class II complex then secrete a number of factors. Some factors elevate T-helper cell activity, others attract and stimulate B-cells to proliferate. Some of the T-cell derived factors are known to influence antibody class expression.

Immunoglobulin class switching is therefore thought to be under the control of T-cell factors. Support for this hypothesis is evidenced by the fact that the secondary response to T-independent antigens resembles the primary response. It is weak and almost entirely confined to IgM production whereas, as mentioned above, the response to T-dependent antigens is more rapid and heightened, and involves class switching from IgM to IgG.

1.2 ANTIBODY STRUCTURE

Immunoglobulins (antibodies) are a group of glycoproteins detectable in the serum and tissue fluids of all mammals. Five distinct classes of the immunoglobulin molecule are recognised. These are IgG, IgA, IgM, IgD and IgE as shown together with other characteristics of human immunoglobulins in Table 1.

IgG accounts for between 70 and 75 percent of the total immunoglobulin pool in normal human serum. Its molecular weight is 146,000 and it has a sedimentation coefficient of 7S. Within the IgG class, in humans, there are four subclasses, IgG1, IgG2, IgG3 and IgG4. These four subclasses differ only slightly with

respect to their heavy chains, classed $\gamma 1$, $\gamma 2$, $\gamma 3$ and $\gamma 4$, and are found in the proportions of 66, 23, 7 and 4 per cent respectively.

IgA accounts for between 15 and 20 per cent of human serum immunoglobulins and occurs as the basic four chain monomer in man. In most mammals IgA in serum is polymeric, occurring mostly as a dimer, with a sedementation coefficient of 11S and a molecular weight of 385,000.

IgM which accounts for about 10 per cent of the Ig pool has a pentameric structure. The individual heavy chains in this pentameric structure have a molecular weight of 65,000. The whole molecule has a molecular weight of 970,000. IgD accounts for less than 1 per cent of the total Ig pool and occurs mainly as a membrane bound molecule on circulating B lymphocytes.

IgE is found only as a trace serum protein and bound to the membrane of both basophils and mast cells (via Fc receptors). Although the classes of immunoglobulin differ from each other with respect to their size, carbohydrate content and amino acid composition, they all have the same basic four chain polypeptide structure, as shown in Figure 1. Of the four polypeptide chains, two are light chains and two are heavy chains, linked together by disulphide bonds.

The two light chains, each with a molecular weight of 25,000, are common to all classes of immunoglobulin. The heavy chains,

unlike the light chains are structurally distinct for each class or subclass of immunoglobulin and have molecular weights of between 50,000 and 77,000. The light chains can be of two types, called Kappa and lambda, which are distinguishable by their amino acid sequence. A given antibody molecule will have either two Kappa or two lambda light chains; hybrid molecules do not exist naturally.

The antibody molecule is made up of a number of domains. At the N-terminus (Figure 1) there are four variable domains, one on each of the two heavy and two light chains. The antigen binding site is composed of one VL and one VH domain and contributes to what is termed the Fab region of the antibody molecule. Each basic unit of immunoglobulin (2 heavy and 2 light chains) contains two identical binding sites for antigen.

As a result of the systematic analysis of light chain sequences (Wu and Kabat, 1970) and of heavy chain sequences (Kehoe and Capra, 1971) the molecular basis of antibody specificity (complementation for antigen) was revealed. The variable domain was found to consist of three hypervariable regions, referred to as complementarity determining regions (CDR) bounded by four less variable framework regions (FR). The remaining domains of the antibody molecule were found to be relatively constant, and are thus referred to as constant domains. Within the basic IgG structure there are eight constant domains, each of these globular domains being stabilised by intrachain disulphide bonds.

These eight constant domains are made up of two constant domains on the light chains, one on each, referred to as the CL domain and three constant domains on each of the two heavy chains (CH domains). In IgG the CH1 and CH2 domains are separated by a vaguely defined region of the heavy chain, known as the hinge region. Flexibility within this hinge region permits variation in the distance between the two antigen binding sites, allowing them to operate independently. The remaining CH2 and CH3 constant domains are located beyond the hinge region, that is, toward the C-terminal end (carboxy terminus). These four constant domains (2 x CH2, 2 x CH3) form what is known as the Fc portion of the immunoglobulin molecule. It is to the CH2 domain that complement binds, whilst binding to Fc receptors on a variety of cell types takes place through the CH3 domain. As seen in Table 1, both IgM and IgE differ from this basic structure in that the heavy chains of both have another constant domain, referred to in the case of IgM as the Cu4 domain, and in IgE as the C 4 domain.

Although the carboxyterminal half of the heavy chain is essentially constant, variations may occur two types of variation are known, these are allotypic variations and isotypic variations. Allotypic variations are genetic variations within a species. For example the variant of IgG3 called G3m (bo), which is characterised by having phenylalanine instead of tyrosine at position 436 of its heavy chain. This variant of IgG3 is not found in all people and therefore is an allotype.

Isotypic variations are amino acid sequences which characterise $\gamma_1, \gamma_2, \gamma_3, \gamma_4$ and μ heavy chains and kappa or lambda light chains. All the genes for isotypic variations are present in all healthy members of a species.

A third type of variation, which results from differences in the amino acid sequence of both heavy and light chain hypervariable regions, or complementarity determining regions (CDR), is known as the idiotypic variation. Individual variants are termed idiotypes. Idiotypes are normally specific for a single antibody clone (private idiotypes) but are sometimes shared between different antibody clones (public idiotypes).

Table 1 illustrates the basic characteristcs of the five human immunoglobulin classes.

TABLE 1

	IgG	IgA	IgM	IgD	IgE
Heavy chain:	γ	α	μ	δ	ε
Subclasses:	γ1, γ2, γ3, γ4	α1, α2	μ	-	-
Light chain:	K or L	K or L	K or L	K or L	K or L
J-chain	-	+	+	-	-
Molecular weight:	146,000	150-350,000	970,000	184,000	188,000
Sedemention coefficient:	7S	7(9-15)S	19S	7S	8S
% carbohydrate:	3	7	12	12	12
T-1/2 in plasma (days):	21	6	5	3	2
Complement activation:	+	-	+	-	-
Placental Transfer:	+	-	-	-	-
Serum concentration: (mg/ml)	13	3.5	1.5	0.03	0.0001
Heavy chain domains:	4	4	5	4	5

Figure Legend

Figure 1 shows the basic four chain polypeptide immunoglobulin structure, with letters A to E referring to the following.

A Papain cuts here to give two univalent Fab fragments which bind antigen

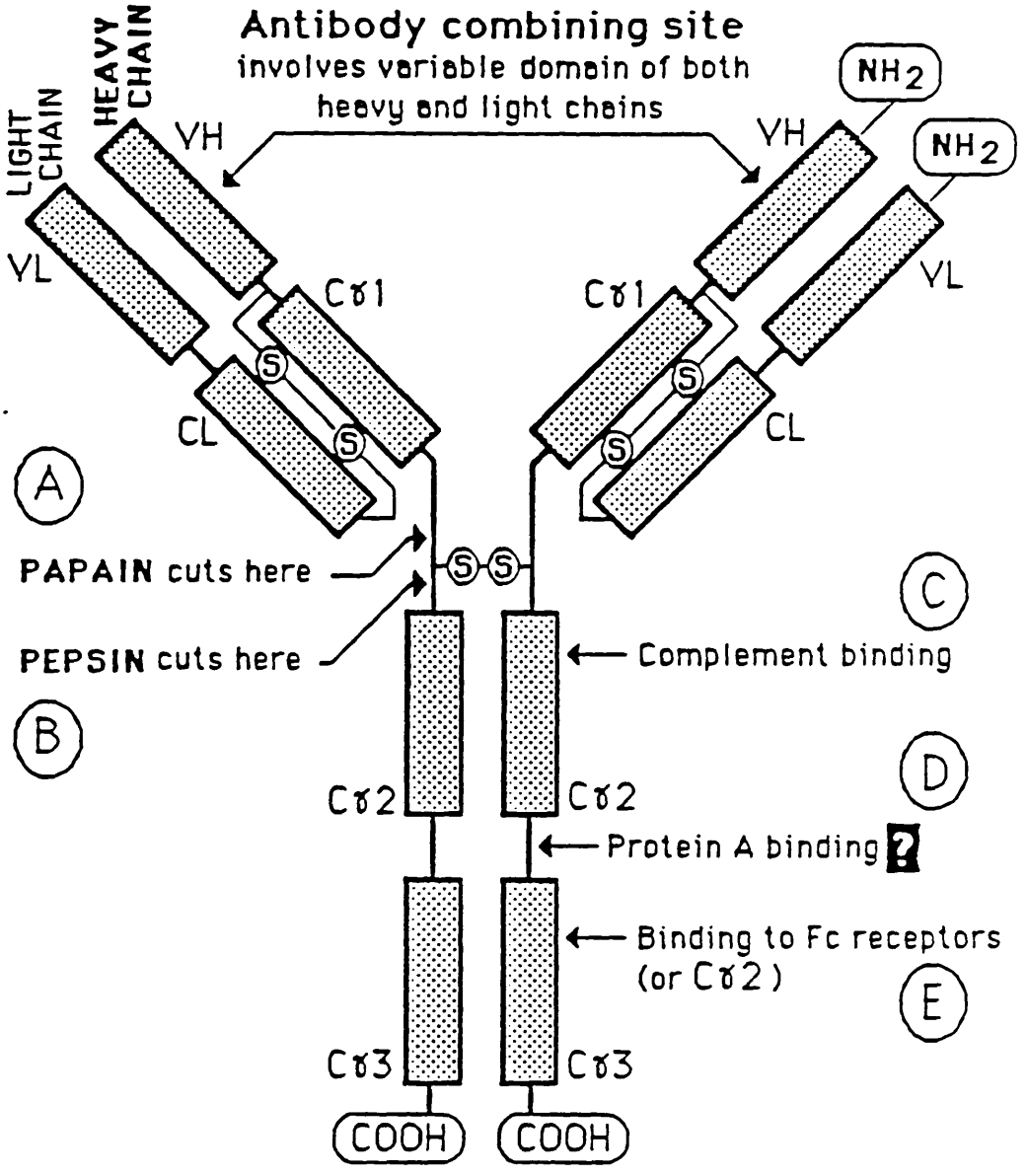
B Pepsin cuts here to give one divalent F(ab')2 fragment which retains the antigen binding properties of the original IgG.

C Complement binding (classical pathway) is initiated by binding C1q to CH2 domain: later, C4 binds to CH1 or V_H.

D Protein A binding probably occurs here.

E Binding to Fc receptors on cell surface is usually via the CH3 domain and CH2 domain is sometimes also involved.

The antibody molecule



1.3 ANTIBODY FUNCTION

Antibodies can be thought of as being bifunctional, in that they firstly bind to antigen and secondly via the Fc portion, activate a host of other immune system functions. The latter are known as antibody effector functions.

Antibody effector functions include antibody-dependent cell-mediated cytotoxicity, opsonization leading to phagocytosis and activation of the complement system a complex group of serum proteins which mediate immune adherence, cell lysis and inflammatory reactions. The primary role of antibody in antibody-dependent cell mediated cytotoxicity (ADCC) is to bind to target cells and then allow potential cytotoxic cells, such as killer cells (K-cells) to bind via their Fc receptors for IgG, and lyse the target cell. An example would be a tumour cell coated with tumour-specific antibody. The K-cell could bind to the antibody via its Fc receptor and then lyse the bound tumour cell.

Phagocytic cells of the reticuloendothelial system can often bind in a non-specific manner to infectious agents, via non-specific cell surface receptors. Problems arise when the phagocyte lacks a suitable receptor for the infectious agent or the infectious agent does not activate complement. In these circumstances antibody can bind to antigenic determinants on the infectious agent, i.e. opsonise it, and facilitate phagocytosis via binding of the phagocyte through its Fc receptor.

In man, IgG1, IgG3 and IgM are capable of fixing C1q the first component of the classical complement pathway In the case of IgM only a single molecule is required for C1q fixing, whereas with IgG two molecules are required.

Once C1q has bound to the immunoglobulin, a series of other complement components bind. The pathway components are numbered C1q-C9. When all the complement components have been fixed, disruption of the cell membrane occurs, resulting in the free exchange of solutes. It is this exchange of solutes which is primarily responsible for the eventual cell lysis. Partial activation of the complement cascade leads to other effector functions. For example, antigen-antibody complexes coated with C3b are very efficiently (via C3b receptors) bound and phagocytosed by macrophages and neutrophils, whilst the soluble complement products C3a and C5a are potent mediators of inflammation.

1.4 TUMOUR ASSOCIATED ANTIGENS

Later in this chapter, the role of antibodies in passive immunotherapy will be discussed. For serotherapy to be effective as an anti-cancer modality, either tumour-specific, or tumour - associated antigens must be present on the cell surface of the malignant cells in the tumour.

It is widely assumed that tumour "specific" antigens have been demonstrated in human cancer, although there is no basis for this

belief (Old 1981). Heteroimmune sera, initially claimed to identify tumour-specific antigens have, on further analysis, been found to be detecting quantitative differences between tumour and normal cell expression of differentiation antigens characterizing a step in the corresponding normal cell lineage.

Although not tumour-specific, a large number of antigens have been identified as being tumour "associated". A number of these tumour-associated antigens are fetal antigens. Alpha-fetoprotein is a globulin detected in the serum of patients with hepatoma and embryonal carcinoma. This antigen is normally only synthesised by parenchymal cells of fetal liver and is not found at detectable levels in the serum (Stillman, 1970).

Carcinoembryonic antigen (CEA), an antigen present in fetal gut, (Holyoke et al., 1972) is expressed by human colonic tumours (Mach et al 1981). Other fetal antigens have been described in association with human tumours; these include a sulphoglycoprotein found in the gastric juice of some patients with carcinoma of the stomach (De Boer et al., 1969), and placental alkaline phosphatase (PLAP), which is normally only synthesised by trophoblast tissue from the 12th week of pregnancy, but which has been found in elevated amounts in patients with ovarian (Fishman et al., 1979) and testicular germ cell tumours (Epenetos et al., 1984). Another tumour-associated antigen is human chorionic gonadotrophin (hCG) which is produced by malignant teratoma, carcinoma and hydatiform mole (Begent et al., 1980).

In recent years a large, complex and heterogeneous family of glycoproteins, known as mucins, has been identified as containing tumour-associated antigens. These mucins have been shown to be expressed by a wide variety of tumour types including those of the breast (Taylor-Papadimitriou et al., 1981), ovary (Canney et al., 1984), Pancreas (Metzger et al., 1984), gastrointestinal tract (Magnani et al., 1983), and lung (Rosen et al., 1984). Although mucins have been identified as being tumour-associated, they are expressed and secreted by healthy individuals, as the main role of these high molecular weight mucins is in biological lubrication. In this capacity they provide a barrier that protects normal cells against osmotic and pH gradients and physical trauma, and are secreted by the seroviscous tissue found in the mouth, lungs, cervix, and intestines (Rittenhouse et al., 1985).

In further studies of tumour-associated antigen expression a number of receptors have been shown to have elevated expression in tumour cells. Of these receptors, epidermal growth factor receptor (EGF-R) has been shown to be expressed at elevated levels on a variety of tumours including cervical, ovarian and vulval (Gullick et al., 1985), lung (Cerny et al., 1986) and brain tumours (Libermann et al., 1984).

The close similarity between the sequence of the v-erb-B oncogene of AEV-H (a strain of the avian erythroblastosis virus) and the nucleotide sequence encoding the cytoplasmic and transmembrane part of EGF-R (truncated EGF-R) (Downward et al., 1984) have

given rise to the hypothesis that inappropriate activation of the human erb-B gene resulting in over expression of EGF-R plays a role in the development of malignancy (Newmark, 1984). Although EGF-R is over expressed by some tumour types, it is, as with the other antigens discussed, by no means tumour- specific, in fact, its expression is found in a wide range of normal epithelial tissues (Gusterson et al., 1984).

As molecular biology techniques improve, it may well be that antigens whose expression is associated specifically with the transformed state and linked directly with the mechanisms responsible for transformation will be identified. However, such antigens have not yet been found. Nevertheless the large number of tumour-associated antigens, with their common feature of being expressed in greater amounts on tumour cells than on the normal cell counterpart have provided the foundation for the targetting of antibodies to tumour cells - passive immunotherapy.

1.5 TUMOUR-ASSOCIATED ANTIBODIES

1.5.1 Polyclonal

The "magic bullet" concept of antibody therapy was proposed by Paul Ehrlich in 1908. Attempts to use antibodies made against malignant tissues for cancer treatment had in fact already been attempted in 1895. Heterologous anti-tumour antisera had been raised in an ass and two dogs, against human osteogenic sarcoma, (Hericourt and Ricket, 1895). The investigators claimed that these sera were effective against a fibrosarcoma of the chest wall and a stomach cancer; subsequently, fifty cases of cancer were treated, and the results were reported to be excellent.

Further attempts at such passive immunotherapy using heteroantisera were made in 1927, when eleven patients with acute leukaemia were treated with X-ray therapy and infused with rabbit antisera to human leukaemia cells (Lindstrom, 1927). Of the eleven patients treated, four were reported to have obtained complete remissions. Unfortunately the results of this study were not reproducable (Hueper and Russel, 1932). In fact, in 1929, in a cancer review, it was written "although some tangible basis for immunity, something of the general nature of a specific antibody, has been sought for thirty years, none has yet been discovered, though the methods employed leave nothing to be desired in the way either of completeness or ingenuity;" (Woglom, 1929). However, in that same year a rabbit antiserum was raised against uterine carcinoma (Witebsky, 1929), this

antiserum had no demonstrable reaction with normal tissue.

More recently, patients with chronic lymphocytic leukaemia (CLL) have been treated with horse anti-lymphocyte serum. In one trial involving six patients (Tsirimbas et al., 1968) only one patient obtained transient lymphopenia. In another study on the use of passive immunotherapy for the treatment of chronic lymphocytic leukaemia (Lazlo et al., 1968) patients were treated with isologous plasma from donors who had been immunized with lymphocytes. Thirty minute infusion of the isoimmune plasma produced a decrease in circulating lymphocytes, and shrinkage of lymph nodes and spleens in some patients. Although side effects such as low-grade fever and chills were encountered, one patient received seven courses of such therapy, and was managed for a two year period.

One of the best reports of clinical success in patients with T-cell malignancies was a trial in which patients with Sezary syndrome who received horse anti-thymocyte globulin (ATG), showed a 75% reduction in lymphadenopathy, resolution of erythroderma, and a decrease in the number of circulating T cells (Fisher et al., 1978). In this trial ATG was given over a six hour period. Complications included rigors and hypertension. Retreatmentwith diphenhydramine and steroids prevented such toxicity during six subsequent daily treatments. In another trial where four patients with cutaneous T-cell lymphoma (CTCL) received intravenous administrations of ATG a reduction in the number of circulating T cells was also shown. As in the patients treated

by Fisher, toxicity was observed but overcome with diphenhydramine and corticosteroid therapy (Edelson et al., 1979). Tumour regression was observed in three patients, but was relatively transient in duration.

Although many attempts to use hetero or allo-antisera for the treatment of various malignancies have been made, the results in general are poor. Investigators have failed to produce consistent positive results; this may be due to a number of reasons. Firstly, it is unclear whether the few remissions seen using heteroantisera are due to an anti-tumour effect of the antibodies or to a non-specific adjuvant effect of infusing a foreign protein. It is also possible that the sera contained no antibodies which could react with any part of the tumour tissue, or that the antibodies were cross-reactive with other tissue and were therefore bound by these tissues, thus being unavailable to the tumour. Whatever the reason for the relatively poor results of such passive immunotherapy, it became clear that purer preparations of antisera of well defined specificity were required if the clinical investigation of serotherapy as an anti-cancer modality was to continue and advance.

1.5.2. Monoclonal Antibodies

Although antisera raised to tumour cells or tumour-associated antigens contained antibody capable of reacting with the antigen of interest, the concentration of the desired antibody was often low, the total amount produced was limited and there was

considerable batch-to-batch variability. However in 1975 a technique which would enable antibodies of predefined specificity to be produced in large quantities was developed (Kohler and Milstein, 1975).

Hybrid cells (hybridomas) which secreted the required antibody could be made, and immortalized by the fusion of a myeloma cell with a spleen cell from an immunised donor. The hybrid cells could be cloned-out and a single clone, producing antibody of desired specificity could be selected. The clone could then be expanded in tissue culture medium, and large quantities of antibody obtained. The antibodies produced by a single clone, monoclonal antibodies, would all be of a single isotype and binding specificity. The advantage of monoclonal antibodies over polyclonal antisera, in oncology, would be the unlimited availability of a specific reagent which was homogeneous and contained antibodies of unique specificity and affinity with no variability between lots.

The production of monoclonal antibodies and the obviation of several of the limitations of polyclonal antisera resulted in a rekindling of interest in the clinical investigation of immunotherapy. The first clinical trial reported, using a monoclonal antibody directed against a lymphoma-associated antigen, was carried out in a single patient (Nadler et al., 1980). The patient, a man with non-Hodgkins lymphoma, received three administrations of monoclonal antibody (25mg, 75mg and 150mg) over three days.

Although no clinical toxicity was noted, the patient complained of both lymph node and liver tenderness. The result of the monoclonal antibody therapy was a drop in white cell count from 388,000 to 240,000/cu mm on day two. By day four the white cell count had risen to its pre-therapy level of 392,000/cu mm. After one month the patient received an infusion of 1.5g of antibody over a six hour period, the only result noted was a transient drop in the white cell count to 200,000/cu mm. The investigators concluded that "the quantity of circulating antigen was too great to effectively deliver Ab89 to the patient's tumour cells".

In other trials employing unmodified monoclonal antibodies for the treatment of gastrointestinal tumours (Sears et al., 1982) and B-cell lymphoma, where anti-idiotypic antibodies were used (Meeker et al., 1985) the results have been disappointing. There are a number of possible reasons for this; these include high levels of circulating tumour antigen, target antigen modulation and failure of host effector mechanisms to eliminate antibody-coated tumour cells.

In an attempt to circumvent the problem of poor tumour cell clearance by host effector mechanisms, toxic molecules have been coupled to monoclonal antibodies. Monoclonal antibodies have now been used as specific vectors for a variety of anti-tumour agents, including chemotherapeutic drugs, toxins and radioisotopes.

1.6 MONOCLONAL ANTIBODIES AS VECTORS

As discussed in the previous sections, the results of immunotherapy using unmodified antibodies (where destruction is dependant upon host effector mechanisms) have been disappointing. An alternative to the use of unmodified antibodies in tumour therapy was proposed nearly eighty years ago by Paul Ehrlich when he discussed the potential use of antibodies as carriers of pharmacological agents.

In oncology three groups of such pharmacological agents have, and are still being, investigated as potential therapeutic agents for antibody guided therapy of human neoplasia. These three groups are: drugs which are already employed in the treatment of cancer; toxins, which when coupled to antibodies are called "immunotoxins", for example the plant toxin, ricin; and cytotoxic radionuclides such as Iodine-131 and Yttrium-90.

In the early seventies, an absorbed polyclonal antiserum carrying chlorambucil was used for the treatment of melanoma (Ghose et al 1972). The investigators reported that one patient had obtained clinical remission. Since the advent of monoclonal antibodies a number of *in vitro* studies, in which monoclonal antibodies have been used as vectors for cytotoxic drugs, have been reported (Garnett et al., 1983). In one study using a monoclonal antibody against antigens expressed on an osteogenic sarcoma cell line it has been shown that when the drug, Methotrexate, is attached to a carrier which in turn is coupled to the monoclonal antibody the

cytotoxicity is greater than that obtained using free Methotrexate (Garnett and Baldwin., 1986).

In another study (Dillman et al., 1986) it was observed that when the drug Doxorubicin, commonly used in the treatment of chronic lymphocytic leukaemia, was injected together with an anti-human T-cell monoclonal antibody, into athymic mice bearing subcutaneous tumours of the Molt-4 cell line, tumour regression was observed. When the antibody and drug were administered by separate routes, the degree of tumour regression was found to be less. The investigators concluded from these studies that there were "weak complexes via hydrophobic bonds" formed between doxorubicin and the anti-human T-cell monoclonal antibody T101. Such a weak bond between antibody and drug could cause problems in clinical trials. In the event of the drug conjugate being unstable, non-specific drug induced tissue damage would possibly occur. Nevertheless the use of monoclonal antibodies as vectors for cytotoxic drugs appears promising.

Immunotoxins, plant or bacterial toxins conjugated to antibodies, have been synthesised in several laboratories and their in vitro toxicity to tumour cells studied (Thorpe and Ross, 1982). Ricin, a plant toxin from the plant Ricinus communis, has been studied in depth. In its natural form ricin is composed of a toxic A chain coupled to the B chain which is responsible for binding to and entry into a target cell. The A chain of ricin has been conjugated to a number of monoclonal antibodies and shown to be highly toxic to cancer cells when bound to tumour associated

antigen via the antibody moiety. After binding the conjugate is internalised by the target cell and the A-chain inhibits protein synthesis by binding to the 60S subunit of ribosomes.

Recent studies comparing the toxicity of antibody-drug and antibody-toxin conjugates have shown that although the antibody-ricin A-chain conjugate is more toxic than a drug conjugated to the same antibody, the antibodyg -ricin A-chain was less discriminative between cells which weakly to moderately expressed the target antigen and those with high antigen expression (Embleton et al., 1986). In an animal study where rats received multiple dose administration of an anti-melanoma antibody-ricin A-chain immunotoxin a number of side effects were observed (Harkonen et al., 1987). In order to reduce the non-specific uptake of ricin by cells with mannose and fucose receptors, such as hepatic parenchymal and nonparenchymal cells, the carbohydrate moiety of the ricin A-chain has been removed (Blakey et al., 1987). As with the drug antibody conjugates, the role of immunotoxins as therapeutic agents in the treatment of cancer, and their toxicity to normal tissues will be evaluated in clinical trials.

One limitation to the use of toxin-antibody conjugates is that once at the site of a tumour, an immunotoxin can only kill the tumour cell to which it is attached. However, studies of human tumour biopsies have shown tumours to be antigenically heterogeneous (Edwards et al., 1985); with only a proportion of the cells bearing a given tumour-associated antigen. This

problem of antigenic heterogeneity on tumour cells will mean that a given tumour-associated monoclonal antibody can bind to only a proportion of the tumour cells and hence, with an immunotoxin a number of tumour cells will not be targetted. In contrast, efficacy of drug-antibody conjugates would not depend so greatly on each cell being targetted if the drug could be released into the tumour area after the antibody vector had bound to the tumour- associated antigen.

As with drug antibody conjugates, the advantage of targetted radiation over targetted toxins is that tumour cells without the appropriate antigenic determinants can be killed by radiation cross-fire from adjacent cells to which the radiolabelled antibody has bound.

Research on serotherapy using radiolabelled antibodies was first introduced in 1948 (Pressman and Keighley, 1948). Since then several workers have demonstrated in experimental models the potential clinical usefulness of radiolabelled tumour-associated polyclonal and monoclonal antibodies (Mach et al., 1974; Quinones et al., 1971; Pimm and Baldwin, 1985; Goldenberg et al., 1974; Primus et al., 1973), and of radiolabelled antibody fragments (Rogers et al., 1986). Based on the encouraging results from animal studies, clinical trials of radioimmunodiagnosis and therapy commenced (Mach et al., 1980; Berche et al., 1982; Farrands et al., 1982).

There have now been a number of reports indicating clinical response to antibody targetted radionuclides (Order et al., 1980; Carrasquillo et al., 1984).

A number of problems associated with the in vivo use of monoclonal antibodies have been recognised. The presence of circulating tumour antigen (Nadler et al., 1980; Hagen et al., 1983), poses a problem. Reduction in this circulating antigen can be achieved by plasmaphoresis, but the reduction is only short lived (Meeker et al., 1985). Persistance of circulating administered antibody can give high "background" effects. Clearance of this residual circulating antibody from the blood would lead to a reduction in background activity, and hence, would make imaging of tumour sites possible in a shorter period of time and would also reduce irradiation of sensitive normal tissues. Two ways in which this may be achieved have been proposed. The first involves the use of lyosomally entrapped second antibody (Begent et al., 1982) and the other of using a second antibody alone, to clear the first radiolablled antibody (Begent et al., 1984).

Another factor affecting the percentage of tumour uptake of the injected tumour-associated antibody is the route of administration. To maximise the percentage of injected dose targetting to the tumour, regional administration has been used (Epenetos et al., 1985; Pectasides et al., 1986; Ashorn et al., 1985). The results of regional administration have been encouraging.

Finally, although it has been proposed that human monoclonal antibodies could be used in tumour diagnosis and therapy (Sikora et al., 1985), very few such reagents are available - due to many technical problems encountered in their production. Hence, the majority of trials have employed xenogeneic monoclonal antibodies, in particular those raised in the mouse system. Thus, the question of sensitization to the administered diagnostic or therapeutic monoclonal antibody is therefore an important one. Where the recipient's immune response to the monoclonal antibody is high, any potential therapeutic efficacy could be lost, and where the route of administration is intravenous the possibility of immune commplex disease would be increased.

1.7 AIM OF THIS THESIS

Due to antisera impurity, a large number of groups studying the role of passive immunotherapy in the treatment of human neoplasia noted significant toxicity in patients receiving either heterologous or isologous antisera. In contrast, the use of monoclonal antibody infusions has not resulted in many reports of significant toxicity. A few cases of anaphylactic shock, hypotension and dysponea have been reported (Dillman et al., 1982).

Although toxicities were observed, they had all occurred in patients receiving intravenous monoclonal antibody therapy. Although there are theoretical concerns of serum sickness and of

any delayed complications of immune complex disease, for example glomerulonephritis, there are no clear reports in the literature on this matter. Moreover skin testing prior to treatment has not been a useful predictor of toxicity since reactions have been seen in the absence of a positive skin test.

The aim of this thesis is to study the generation, specificity and clinical significance of the humoral immune response that develops in patients receiving regionally administered radiolabelled murine monoclonal antibodies given for the therapy of neoplasia.

CHAPTER 2

MATERIALS AND METHODS

2.1 INTRODUCTION

Methods and materials are described in this chapter. Further details will be presented in each chapter as appropriate.

2.2 MONOCLONAL ANTIBODIES

HMFG1 is a mouse IgG1 monoclonal antibody raised against human milk fat globule membranes. The determinants recognised by this antibody are found in high molecular weight components (>400KD) of milk fat globule membranes (Burchell et al., 1983) and are strongly expressed in secretory epithelium of lactating breast and in a wide range of carcinomas, including those of ovary, colon and lung.

HMFG2 is an IgG1 monoclonal antibody derived from a mouse receiving an initial injection of human milk fat globule membranes followed by a boost with HumE cells (Taylor-Papadimitriou et al.,1981). This antibody reacts with high molecular weight components (>400KD) which are expressed weakly by normal non-lactating breast epithelium and strongly by lactating breast and a range of neoplasms of epithelial origin, particularly adenocarcinomas of breast, ovary and gastrointestinal tract.

AUA-1 is an IgG1 mouse monoclonal antibody which reacts with a 35KD epithelial cell surface antigen coded by the gene MIC 18 on chromosome 2. This antigen is expressed by nearly all normal

epithelial cells except those of stomach and upper gastrointestinal tract (Spurr et al., 1986). The antigen is also expressed by a wide range of adenocarcinomas including those of colon, breast and ovary.

HI7E2 is a mouse IgG1 monoclonal antibody directed against placental and placental-like alkaline phosphatase (Travers and Bodmer, 1984). This enzyme is expressed as a surface membrane antigen of many neoplasms including 60-85% of ovarian carcinomas.

11-4.1 is an IgG1 mouse monoclonal antibody which is directed against the mouse $H\text{-}2K^{k}$ antigen (murine MHC Class I). This antibody does not react with any tissues of human origin so far tested (Oi et al., 1979).

9A is a mouse IgG3 monoclonal antibody generated by immunising a mouse with cells of a human epidermoid carcinoma cell line, A431, and is directed against an oligosaccharide component of the epidermal growth factor receptor (Parker et al., 1985). This antibody reacts with a wide range of neoplastic tissues, including brain gliomas.

EGF-R1 is a mouse IgG2b monoclonal antibody raised to the human epidermal growth factor receptor (Waterfield et al., 1982).

AB44 is an IgG1 mouse monoclonal antibody which is specific for the ic3b complement receptor on human granulocytes and monocytes (Danpure et al., 1986).

2.3 MONOCLONAL ANTIBODY PRODUCTION

All of the monoclonal antibodies (section 2.2) were obtained from the Imperial Cancer Research Fund, Lincolns Inn Fields, London. None of the antibodies were obtained as mouse ascites, they were all obtained as tissue culture supernatants. The hybridomas were all grown in bicarbonate buffered RPMl medium supplemented with 10% foetal calf serum (FCS), penicillin (100 units/ml) and streptomycin (100 units/ml). Supernatants of HMFG1 and EGF-R1 were produced at the Imperial Cancer Research Fund. Hybridomas of HMFG2 were grown and the supernatant collected at the RPMS as part of the research work for this thesis. All monoclonal antibodies were purified as described below, prior to use.

2.4 MONOCLONAL ANTIBODY PURIFICATION

Prior to purification by column chromatography hybridoma supernatants were spun at 2000g for 15 minutes to remove cells. The pH of the cell free supernatant was adjusted to 8 using IM Tris [hydroxymethyl] aminomethane, and then purified by affinity chromatography at 4°C on a staphylococcal Protein-A-Sepharose CL 4B (Pharmacia) column, as described by Ey (1978).

After all the supernatant had been passed through the column the protein A-beads were washed with 0.1M phosphate buffer pH8, to remove any non-bound material. Sequential elution of the immunoglobulin using 0.1M citrate buffer of decreasing pH was carried out at a flow rate of 20ml per hour, with four fractions

collected per hour. After elution the monoclonal antibody containing fractions were pooled and the pH adjusted to 7 using 1M Tris [hydroxymethyl] aminomethane (Tris-base). Antibodies were then "Millipore" filtered (0.22μm pore size) and aliquotted into sterile ampoules and stored at 4°C.

The protein-A-Sepharose column was regenerated, for future use, by washing with 10 column volumes of 0.1M Tris buffer pH 8.5 containing 0.5M sodium chloride and 10 column volumes of 0.1M citrate buffer pH 4.5 containing 0.5M sodium chloride. The column was finally washed with 0.1M phosphate buffer pH8. All of the above purification steps were carried out at 4°C, and all buffers used were prepared and autoclaved just prior to use, in order, so far as possible, to maintain sterility.

2.5 DETERMINATION OF ANTIBODY PURITY

After purification of the monoclonal antibodies, the degree of purity was checked using 7.5% non-reduced polyacrylamide gel electrophoresis as described by Laemmli (1970) and isoelectric focussing as described by Awde et al (1968).

2.6 DETERMINATION OF ANTIBODY CLASS

Immunoglobulin class and subclass were determined by the double immunodiffusion technique (Ouchterlony and Nilsson, 1978) using commercially available rabbit anti-mouse immunoglobulin, (class and subclass specific antisera) obtained from Miles Laboratories

(U.K.).

2.7 STERILITY AND PYROGENICITY TESTING

After purification, those antibodies intended for patient use were "Millipore" filtered and aliquotted into sterile ampoules. Ampoules from the beginning, middle and end of the alliquoting procedure were dated, labelled and sent for independent sterility and pyrogenicity testing to Safepharm Laboratories Ltd., Derby, England, where sterility was tested by culturing and freedom from pyrogenic material tested according to the European pharmacopoeia, the temperature rise in rabbits was given as a summed temperature rise and as individual rabbit responses. Any antibodies failing independent testing were discarded.

2.8 PREPARATION OF ANTIGEN-COATED MICROTITER PLATES

Antigen was coated onto 96 well microtiter plates in a number of different ways depending on the nature of the antigen. For non-solubilised antigen such as delipidated milk fat globule the following method was used. Each of the 96 wells of the microtiter plate were filled with 0.25% glutaraldehyde (Sigma) in phosphate buffered saline (PBS) and left for five minutes, after which the glutaraldehyde was removed and the plates blot dried. To each of the 96 wells 10µl of a stock solution of 300µg per ml of milk fat globule (MFG) was added. The plates were stored overnight at 37°C to dry the antigen onto the wells, and then stored at 4°C for up to 6 weeks.

For antigen present on whole cells the following method was used. Cells were washed and diluted in PBS to 10^7/ml 50µl of PBS containing 5×10^5 cells was then added to each well of 96 well plates that had previously been treated with poly-l-lysine (0.1 mg/ml) for 1 hour at room temperature. Plates were then centrifuged and 0.025% of glutaraldehyde was gently added to fill each well and left for 30 minutes at room temperature. The glutaraldehyde was removed and the wells then filled with gelatin (0.2mg/ml) containing 0.02% azide after which they were sealed with Titertek covers and stored at 4°C for up to six weeks.

For soluble antigens, such as purified placental alkaline phosphatase or purified immunoglobulins, 96 well microtiter plates were incubated overnight at 37°C using pH 9.6 bicarbonate buffer as the antigen solvent. The plates were then washed in PBS containing 0.05% Tween-20 (Polyoxyethylene-Sorbitol Monolaurate) for 5 minutes at room temperature, and used immediately.

2.9 ANTIBODY IMMUNOREACTIVITY

2.9.1 ENZYME-LINKED IMMUNOSORBANT ASSAY (ELISA)

In order to determine the immunoreactivity of monoclonal antibodies after preparation (section 2.3) the enzyme-linked immunosorbant assay (ELISA) principle of Engvoll and Perlmann (1972), which employed antigen-coated plates and enzyme-labelled anti-globulin, was used.

To the first horizontal row of wells of the microtiter plate, coated with respective antigen, 200µl of PBS-Tween containing 10µg of monoclonal antibody was added. Using a 12-channel, digital multichannel pipette (Titertek), 20µl from the this row was added to a 180µl of PBS-Tween in wells of the second row, this 10-fold dilution sequence was repeated down the plate.

After titration, the microtiter plate was incubated in a 10% CO_2 gassed humidified incubator for 2 hours at 37°C. After incubation the plate was washed 3 times in PBS-Tween with two minutes per wash, after which 100µl of a 1:500 dilution of horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin antibody was added, using PBS-Tween or RPMI containing 10% FCS as diluent, and the plate incubated at 37°C for one hour. After incubation the plate was washed 3 times in PBS-Tween, as before, and 100µl of substrate added to each well, and left for 30 minutes in the dark room or in silver foil, at room temperature. The optical density of the contents of each well of the plate was then read at 405nm on a Multiskan MCC/340 (Titertek) microtiter plate reader and the results plotted as absorbance at 405nm (y-axis) against antibody concentration (x-axis).

2.9.2. Radioimmunoassay (RIA)

In order to determine the immunoreactivity of monoclonal antibodies, intended for either *in vitro* or *in vivo* use, after radioiodination, a direct, competitive binding radioimmunoassay,

based on the method of Williams (1977) was used.

In this assay, iodinated antibody was titrated out as previously described (section 2.9.1) alone, or with increasing amounts of cold, unlabelled antibody, which would compete for antigen on the microtiter-plate wells. After titration the plate was incubated at 37°C for 2 hours, washed 3 times in PBS-Tween and the wells cut out and counted for bound radioactivity in a gamma-counter and the results used to construct a plot of counts per minute (Y-axis) against antibody concentration (x-axis).

2.10 RADIOIODINATION OF ANTIBODIES

2.10.1 Iodogen Method

Prior to administration of monoclonal antibodies to patients, the antibodies were labelled with Iodine-131 - a radionuclide having a physical half-life of 8 days and principle radiations of beta-rays of 0.61 Mev and gamma-rays of 0.36 Mev.

The radionuclide was covalently linked to the antibody by oxidation, using either 1,3,4,6-tetrachloro-3a, 6a-diphenylglycoluril (Iodogen, Pierce Chemicals) or N-bromosuccinimide (NBS; Sigma U.K.) as follows.

Using the iodogen method (Salacinski et al., 1978) one milligram of Iodogen was dissolved in 25mls of dichloromethane, the solution mixed and 2.0ml aliquots aseptically dispensed into

sterile polypropylene test-tubes (Sterilin U.K.). The tubes were evaporated to dryness at room temperature, under sterile conditions, resulting in a film of iodogen on the walls of the polypropylene test-tubes. After drying, the tubes were capped, and stored at 4°C in a dessicator containing silica gel until required.

The required amount of antibody, between 2 and 30 milligrams concentrated to give a final volume of between 0.2 and 0.5ml, was added to the iodogen tube, after which between 10 and 150 MCi (370-5550 MBq) of Iodine-131 (Amersham) was added. The tube was then capped, vortexed and incubated at room temperature for between 5 and 10 minutes. After incubation the incorporation of iodine into immunoglobulin was assessed by ascending chromatography, in the following way. 5µl of the iodogen tube reactants were spotted into chromatography paper (1 x 10cm) and developed in an ascending manner in 10% trichloroacetic acid.

The distribution of radioactivity on the chromatography paper was determined by counting in an ionisation chamber (SIEL). The iodinated antibody was precipitated at the origin, while the free iodine was chromatographed with the solvent front.

In order to separate the free, unincorporated iodine from the radiolabelled immunoglobulin the contents of the iodogen tube were purified by gel-filtration, using a 20ml disposable syringe barrel, plugged with sterile dressing and containing 2 grammes of sterile swollen Sephadex G-50 (fine grade) in 30mls of water.

The column was eluted with PBS containing 1% human serum albumin (HSA) and 2ml fractions collected. The fractions containing the protein peak were pooled and millipore filtered using a 0.22μ membrane filter, prior to being assayed for radioactivity and administration to the patient.

2.10.2 N-bromosuccinimide Method

N-bromosuccinimide is a mild oxidant used for the iodination of proteins (Reay 1982). Using this oxidant, high iodination efficiences were obtained. When labelling with N-bromosuccinimide (NBS), reagents identical to those described for the iodogen procedure were used. For high activity iodinations, 20mg of NBS was dissolved in 20mls of water for injection BP. The solution was vortexed and left for 10 minutes prior to use. The volume of NBS required was dependent upon the total volume of reactants, that is, antibody and radionuclide. Normally the volume of oxidant used was between 20 and 150μl for activities of between 20 and 150 MCi of Iodine-131.

After five minutes of incubation the percentage of radionuclide incorporated into the immunoglobulin was determined by chromatography, as described previously. To stop the iodination reaction 4mls of 1% human serum albumin in PBS was added, and the mixture purified and filtered as previously described. To ensure sterility was maintained, all iodinations were carried out in a special area designated for iodinations only. The reagents were all tested for sterility and pyrogenicity prior to use. After

iodination the immunoreactivity of the labelled antibody was determined by radioimmunoassay and enzyme-linked immunosorbant assay, as described previously.

2.11 INDIUM-111 LABELLING OF ANTIBODIES

The main area of expansion in Indium-labelling technology at the present time is in the use of Indium for labelling monoclonal antibodies (Mather, 1987). Although this low energy isotope is not of use as a therapeutic agent, the nuclide Indium-111 is suitable for immunoscintigraphy, since its half-life of 67 hours permits imaging of patients at time points later than is possible with some other isotopes used in nuclear medicine, such as Tc-99 and I-123.

Unlike Iodine-131, Indium-111 cannot be directly coupled to antibody. In order to attach Indium to antibodies a number of chelating agents were studied (Hnatowich et al., 1982), and one of the most suitable agents found was diethylenetriaminepentacetic acid (DTPA).

In order to attach the chelating group to the antibody, the following procedure was used. Cyclic-DTPA-anhydride (Sigma) was dissolved in DMSO to a concentration of 2-10 mgs per ml. Sufficient cyclic-DTPA-anhydride (CDTPAA) solution is added, dropwise to the antibody to give an anhydride: antibody molar ratio of 2-4:1. The mixture is stirred and then purified to

remove the unbound DTPA component. Purification can be carried out using either an FPLC sepharose column CS-200 (Pharmacia) or alternatively a 30-60 cm Sephacryl column.

After coupling of the chelating agent CDTPAA to the antibody, a process known as derivatisation, the isotope can be attached in the following manner. To a vial of 111-Indium chloride, sufficient 5M acetate buffer pH 5.5 is added to produce a final concentration of 0.5M acetate. The CDTPAA-antibody conjugate is then added dropwise to the Indium acetate solution. The mixture is incubated for 10-30 minutes at room temperture after mixing, and the indium-DTPA labelled antibody purified on a Sephadex (G50) column, as described for the purification of iodinated antibodies.

2.12 PURIFICATION OF HUMAN ANTI-MURINE IMMUNOGLOBULIN ANTIBODIES

In order to separate the human anti-mouse immunoglobulin antibody fraction of patients' serum, after therapy, their serum was passed through a cyanogen bromide activated sepharose 4B column (Pharmacia) to which either an isotypically or idiotypically related mouse monoclonal antibody had been coupled.

The coupling of mouse monoclonal antibody to cyanogen bromide (CN-Br) activated sepharose 4B beads was carried out as follows. 1 gram of CNBr-activated sepharose 4B was washed on a sintered

having previously been dialysed overnight in 0.1M sodium-hydrogen-carbonate pH 8.3 containing 0.5M sodium chloride, was added to the gel and the mixture placed on a rotator overnight at 4°C in a sterile plastic tube.

After overnight mixing the antibody/gel mixture was transferred to a 1M ethanolamine buffer, pH 8, and again left overnight at 4°C. This step is required to block any uncombined binding sites on the gel. After blocking, the unabsorbed antibody and ethanolamine was washed away with 100mls of sodium hydrogen carbonate (0.1M pH 8.3) followed by 100mls of 0.1M sodium-acetate buffer pH 4 containing 0.5M soidum chloride, and finally 100mls of the sodium hydrogen carbonate buffer.

After all of the above steps had been completed the gel was ready for use. 1 gram of gel gave a final volume of swollen beads, of 3.5ml.

The antibody-coupled beads were then added to patient's serum and placed on a rotator for two hours at room temperature, after which the beads were packed into a suitable column and any unbound material removed using PBS. When the PBS eluted fractions had at absorbance of 280nm of zero the bound material was eluted with proprionic acid (1M) which was neutralised after passing through the column with 1M Tris. 0.5ml fractions were collected until the protein peak had been eluted. The pooled protein was dialysed overnight at 4°C in PBS pH 7.4, and analysed by SDS-PAGE under non-reducing conditions and then assayed by

ELISA for anti-mouse immunoglobulin immunoreactivity.

2.13 PEPSIN DIGESTION OF MONOCLONAL ANTIBODY HMFG1

In contrast to rabbit and human IgG, there have been few reports on the formation of $F(ab')_2$ from mouse monoclonal IgG antibodies by pepsin digestion (Parham, 1983). The optimal conditions of digestion vary with each antibody and it is essential to use optimal conditions in order to obtain satisfactory yields of $F(ab')_2$ fragments. These can then be analyzed by SDS-PAGE under non-reducing conditions.

In order to obtain $F(ab')_2$ fragments of HMFG1, the following procedure was used. Antibody was concentrated to 10mg/ml and dialysed in 0.1M sodium acetate buffer pH4 for 40 hours at 4°C, with several changes of buffer.

After dialysis the antibody was transferred to a 10ml plastic centrifuge tube (Sterilin U.K.) to which was added porcine pepsin (3200 units/mg, Sigma U.K.) to give pepsin concentrations of 3% by weight. The antibody/pepsin mixture, pH4, was then placed on a rotator in a warm room at 37°C. Using continual rotation, digestion of the antibody was monitored by taking 50µl aliquots of the reaction mixture every hour.

In order to stop the pepsin digestion of the 50µl aliquots, the pH was increased to 8 and the aliquots stored at 4°C until they

were required for analysis by 7.5% SDS-PAGE under non-reducing conditions, the results are discussed in chapter 4.

2.14 DEGLYCOSYLATION OF MONOCLONAL ANTIBODIES

In order to remove the carbohydrate moeity of two murine monoclonal antibodies, AUA1 and HMFG1, the deglycosylation method of Thorpe et al (1985) was used. Deglycosylation was achieved by concentrating the murine monoclonal antibodies to 2.5mg/ml followed by dialysis against 0.2M sodium acetate buffer pH 3.5 for 16 hours. Conducting the procedure at pH 3.5 minimises both the possibility of Schiff's base formation between aldehyde groups and amino groups in the protein and the possibility of non-specific oxidation of amino acids.

After dialysis antibodies were mixed with an equal volume of a solution containing 80mM sodium cyanoborohydrate and 40 mM sodium metaperiodate in acetate buffer pH 3.5. The mixture was left on ice at 4°C for 120 minutes in a fume cupboard and the reaction stopped by adding 100µl of 20% glycerol for every 2.8mls of total sample reaction mixture. This was then left overnight at 4°C, after which it was dialysed against 3 x 5 litres of 0.05M ammonium hydrogen carbonate and finally 1 x 5 litres of PBS, pH7.

The mixture of sodium metaperiodate and sodium cyanoborohydrate at pH 3.5 results in the oxidative cleavage of the carbohydrates and reduction of the aldehyde groups thus formed to primary alcohols.

2.15 IMMUNOPEROXIDASE STAINING OF FORMALIN FIXED AND PARAFFIN EMBEDDED TISSUE SECTIONS

Prior to immunoperoxidase staining of 4 u tissue sections on poly-L-lysine coated slides, the wax was removed with xylene and the section rehydrated by passage through 100% ethanol, 70% ethanol and water respectively. Endogenous peroxidase activity was removed by incubating the sections in a solution containing 1 part 3% hydrogen peroxide in water and 5 parts methanol.

In order to saturate the non-specific protein binding sites, slides were placed in a solution of 0.2% rabbit serum in PBS and incubated at room temperature for 20 minutes. After incubation, the rabbit serum was rinsed off and the sections incubated with antibody or antibody fragments (1-10μg/ml) for 30 minutes at room temperature. Sections were then given three 5 minute washes in PBS. A rabbit anti-mouse IgG peroxidase conjugated second antibody (DAKO Denmark) diluted 1 in 200 with PBS was then added and the sections incubated at room temperature for 30 minutes.

The second antibody was removed after incubation by washing the sections in PBS, three 5 minute washes, and the peroxidase substrate, diaminobenzidine (DAB), (25mg in 100ml of 0.03% hydrogen peroxide in PBS) added. The peroxidase reaction was allowed to continue for between 5 and 10 minutes after which sections were washed and counter-stained with haematoxylin, taken

through alcohols to xylene and mounted using Permount (BDH Chemicals Ltd U.K.).

2.16 ANTI-IMMUNOGLOBULIN RESPONSE ASSAYS

2.16.1 Hybridoma targeting

In order to assay patient's serum for the presence of human anti-mouse immunoglobulin antibodies, cytospins of the relevant hybridoma cells (i.e. those that secrete the monoclonal antibodies used clinically in the patient) were prepared using 3×10^4 cells per slide. Cytospins were fixed for 10 minutes in methanol, at room temperature, washed in PBS, and incubated for 30 minutes at room temperature with 50µl of a 1:100 dilution of patients serum, after which they were washed for 15 minutes at room temperature (3 x 5 minute washes) in PBS.

Cytospins were then incubated for 15 minutes at room temperature with a 1:40 dilution of a fluorescein conjugated sheep anti-human immunoglobulin reagent (Wellcome, Beckenham, Kent). Slides were then washed as before, mounted in Hydromount (National Diagnostics, Somerville, N.J.) and screened using a Leitz U.V. microscope equipped with epi-illumination optics.

The assay controls consisted of PBS, serum from healthy controls and serum from patients with neoplastic conditions identical to those of the patients under study, but receiving no monoclonal antibody therapy.

2.16.2 Enzyme linked Immunosorbant Assay (ELISA)

Monoclonal antibodies were diluted to 5µg/ml in bicarbonate buffer, pH 9.6 (coating buffer), and used to coat 96-well microtiter plates (Sterilin, U.K.) 100µl/well (500ng/well). The plates were incubated overnight at 37°C and then washed in PBS pH 7.4, containing 0.05% Tween-20.

Serial dilutions of each patient's serum together with control sera were made and the plates incubated for 2 hours at 37°C, washed three times in PBS Tween-20 (0.05%) (3 x 2 min washes), and then incubated for 1 hour at 37°C with 50µl of a 1:1000 dilution of peroxidase conjugated goat anti-human immunoglobulin species specific reagent (Amersham International U.K.).

Alternatively, for IgM determinations, a peroxidase conjugated rabbit-anti-human µ-chain specific reagent (Dako, Denmark) was used at the second layer stage. Plates were then washed three times in PBS Tween-20 (0.05%) and incubated at room temperature with 100µl of substrate (2,2'-azino-di(30ethyl-benzthiazolinesulfonate) (Amersham International U.K.), and the absorbance determined in a Titertek Multiscan plate reader at 405nm.

CHAPTER 3

PRE-EXISTING HUMAN ANTI-MURINE Ig REACTIVITY

3.1 INTRODUCTION

Over the last few years several workers have reported interference in immunoassay procedures from circulating antibodies in human serum samples. These antibodies have been shown to be binding specifically to the immunoglobulins being used in the assay, usually sheep or rabbit antiserum (Hunter and Budd, 1980).

With the advent of monoclonal antibodies and the increasing use of these reagents in labelled antibody assays, the occurrence of circulating antibodies directed against murine immunoglobulin in particular becomes of practical importance, because any agent capable of cross-linking the labelled antibody with antibody on a solid phase could generate a false positive result in the absence of antigen.

Circulating antibodies directed against murine immunoglobulin could also result in a number of problems in patients receiving monoclonal antibodies by intravenous administration for tumour localisation. In one study the pre-existing antibody has been shown to bind to murine-immunoglobulin *in vivo*, following the administration of 131-Iodine labelled murine monoclonal antibody for radioimmunolocalisation (Davies et al., 1986).

Although the existence of such antibodies has been known for some time, little has been done to determine their specificity or possible aetiology. In this chapter the role of rheumatoid

factors which have specificity for antigenic determinants on the Fc fragment of IgG (Talal, 1977) in the pre-existing human anti-murine immunoglobulin response is examined.

3.2 SPECIFIC VERSUS NON-SPECIFIC BINDING

In order to maximise the sensitivity of the enzyme-linked immunosorbant assay to be used in studying the anti-immunoglobulin response a number of commercially available 96-well microtiter plates were screened for non-specific binding of immunoglobulins present in human serum.

Figure 2 shows the results of an assay carried out to determine the component of non-specific binding to the plastic of three commercially available microtiter plates. In this assay all the wells in rows 1-6 were coated with murine monoclonal antibody (500ng/well in 100µl of bicarbonate coating buffer). The wells in rows 7-12 were left uncoated with antigen but were incubated overnight at 37°C with 100µl of coating-buffer. The use of one plate with half the wells with, and half without, antigen enabled specific, or total binding to be compared with non-specific binding under identical experimental conditions.

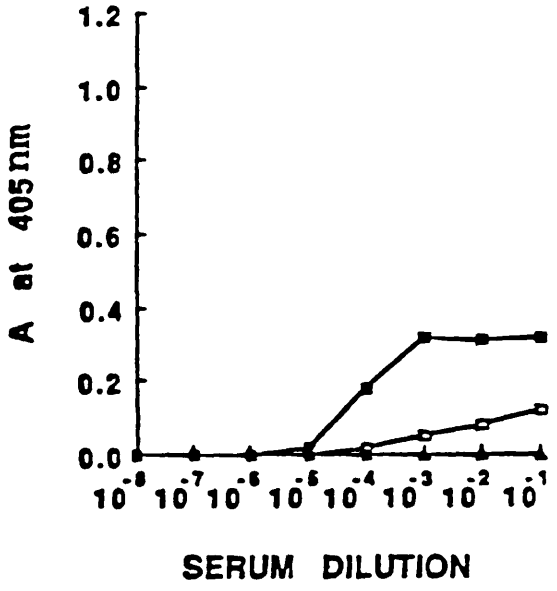
The assay was carried out as described in Materials and Methods (2.16.2) and the results expressed as absorbance at 405nM against serum dilution. In this experiment, immunoglobulin from patient's serum bound non-specifically to two of the three plates. In the microtiter plate where no non-specific binding

occurred, the best results with respect to titration of anti-immunoglobulin antibody could also be seen. This assay was repeated three times with the sera from five patients, and the same relative data obtained. Sterilin plates were therefore used in all subsequent experiments.

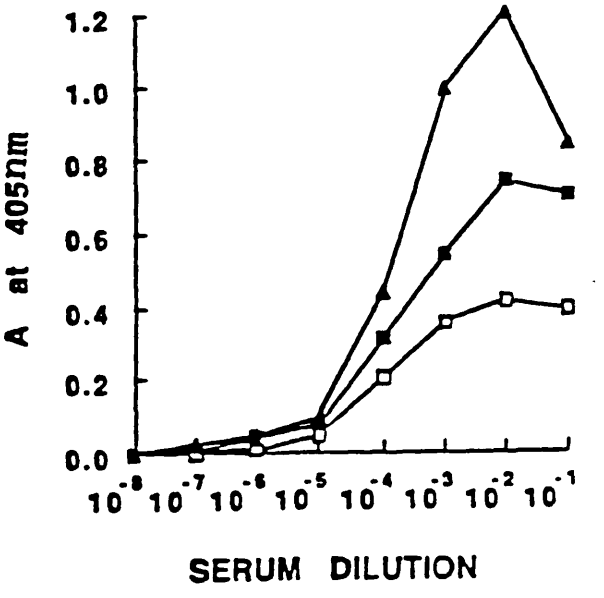
Figure Legend

Figure 2. Data from an ELISA showing non-specific and total binding of human immunoglobulin to uncoated wells (NON-SPECIFIC BINDING) and to antigen (murine monoclonal antibody) coated (TOTAL BINDING) wells of three commercially available microtiter plates. These are Dynatech (□—□), Falcon (■—■) and Sterilin (▲—▲).

NON-SPECIFIC BINDING



TOTAL BINDING



3.3 LOCATION OF ANTIGENIC DETERMINANTS

In a series of experiments Franklin et al. (1957) identified in the sera of some *rheumatoid* patients, by ultracentrifugation, large protein complexes (22S) which proved to be dissociable into 19S IgM with rheumatoid factor activity, and native 7S IgG. The antigenic determinants (or epitopes) to which the rheumatoid factors bound were located to heavy chain constant regions on IgG, in particular the CH2 and CH3 homology regions.

In order to analyse the pre-existing response and to determine the location of antigenic determinants recognised by the human anti-murine immunoglobulin antibodies the following experiment was carried out. The wells in rows 1-6 of a 96 well microtitre plate were coated with intact murine monoclonal antibody and wells in rows 7-12 with $F(ab')_2$ fragments of the same antibody. Binding of antibodies from 13 patients' and 50 blood donors' sera, to the intact IgG and its $F(ab')_2$ fragments was then compared under identical experimental conditions.

After incubation with a rabbit anti-human μ-chain specific, peroxidase linked second layer reagent, and substrate development, binding could only be detected in rows 1-6, that is, in those rows with intact murine IgG. No binding was detectable in wells of rows 7-12 where the $F(ab')_2$ fragments were used as antigen. This experiment was repeated and the results found to be consistent with those obtained in the first experiment.

These experiments indicated that the IgM anti-murine immunoglobulin antibodies present in the human sera tested, recognised determinants other than those on the variable domain and CH1 constant domain located on the $F(ab')_2$ fragments. Thus the reactivity of these IgM antibodies showing pre-existing, anti-murine immunoglobulin activity must be directed towards heavy-chain CH2 and/or CH3 constant-region determinants on the Fc portion of the murine IgG. These results were later substantiated in experiments designed to determine the location of antigenic determinants of anti-murine immunoglobulin antibodies generated by patients receiving murine monoclonal antibody therapy (Chapter 5).

3.4 ANTI-MURINE AND ANTI-HUMAN IgG REACTIVITY

As described above, the experiments located the antigenic determinants recognised by these IgM anti-murine IgG antibodies in human sera, to the Fc portion of the molecule. The question that was prompted by these findings was, did the sera with demonstrable human IgM anti-murine Ig antibodies also contain anti-human Ig antibodies?

In order to obtain an answer to the above question the following experimental system was used. Wells in rows 1-6 of a 96 well microtitre plate were coated with a murine monoclonal antibody, HMFG1 (500ng/well) and wells in rows 7-12 with an equal amount of purified human IgG (Sigma, U.K.). Using the anti-μ-chain specific reagent, IgM anti-human IgG antibodies were

demonstrable. The results shown in Figure 3 were typical of those obtained when assaying sera for these anti-Ig antibodies, although the level of pre-existing anti-Ig antibody was variable. In total 50 blood donors' and 13 patients' sera were analysed and the data are summarized in Table 3.

3.5 POLYCLONAL RHEUMATOID FACTOR

Polyclonal rheumatoid factor was obtained from a patient with active rheumatoid disease. The patient presented with renal failure and was plasmapheresed. The serum of this patient was found to contain 13 grammes per litre, of rheumatoid factor. This was purified by staff from the Department of Renal Medicine.

If the conclusions drawn from the previous experiments were correct, that is, that pre-existing human anti-murine Ig reactivity is due to IgM rheumatoid factors, then elevated activity to both murine and human IgG should be seen when purified polyclonal rheumatoid factor is assayed. The assay was carried out as described above, except that purified polyclonal rheumatoid factor (IgM) was used in place of sera.

As shown in Figure 4, the purified polyclonal rheumatoid factor bound to both murine and human IgG, indicating shared antigenic determinants.

Figure Legend

Figure 3 ELISA to compare the binding of pre-existing IgM antibodies, found in the serum of one of the 50 blood donors sera tested, to a) murine IgG and b) human IgG. The assay was carried out in a single plate to ensure comparability of data.

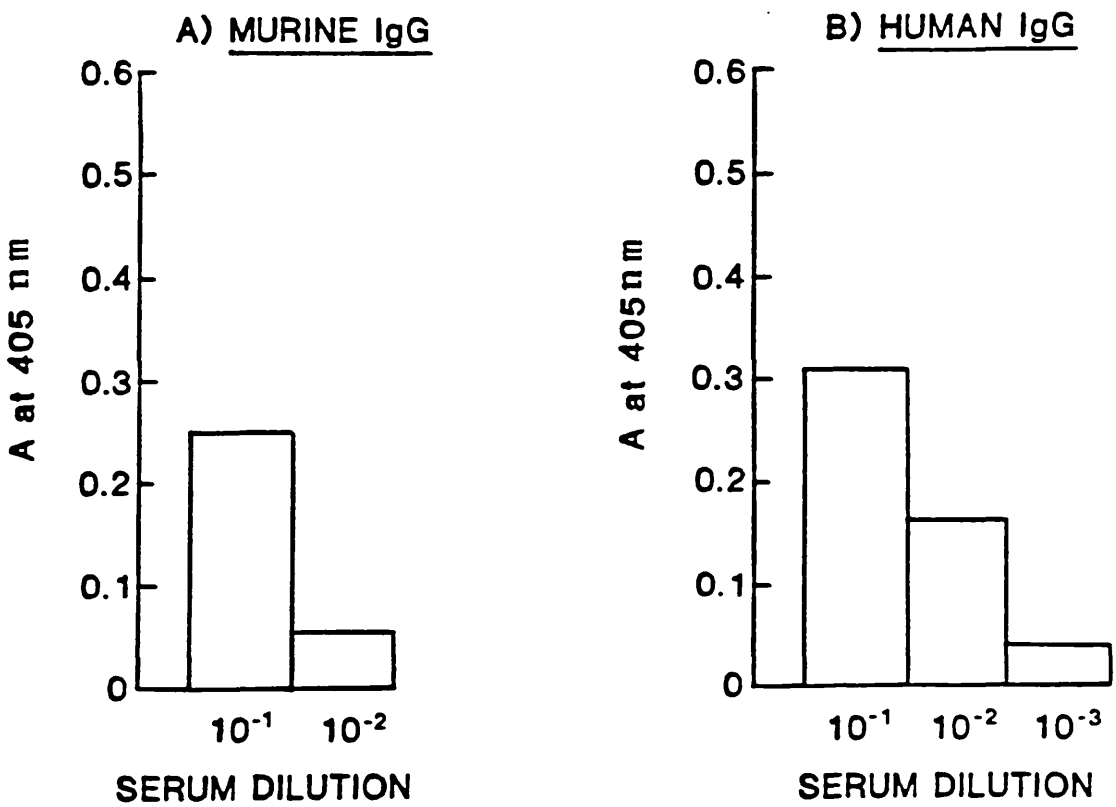
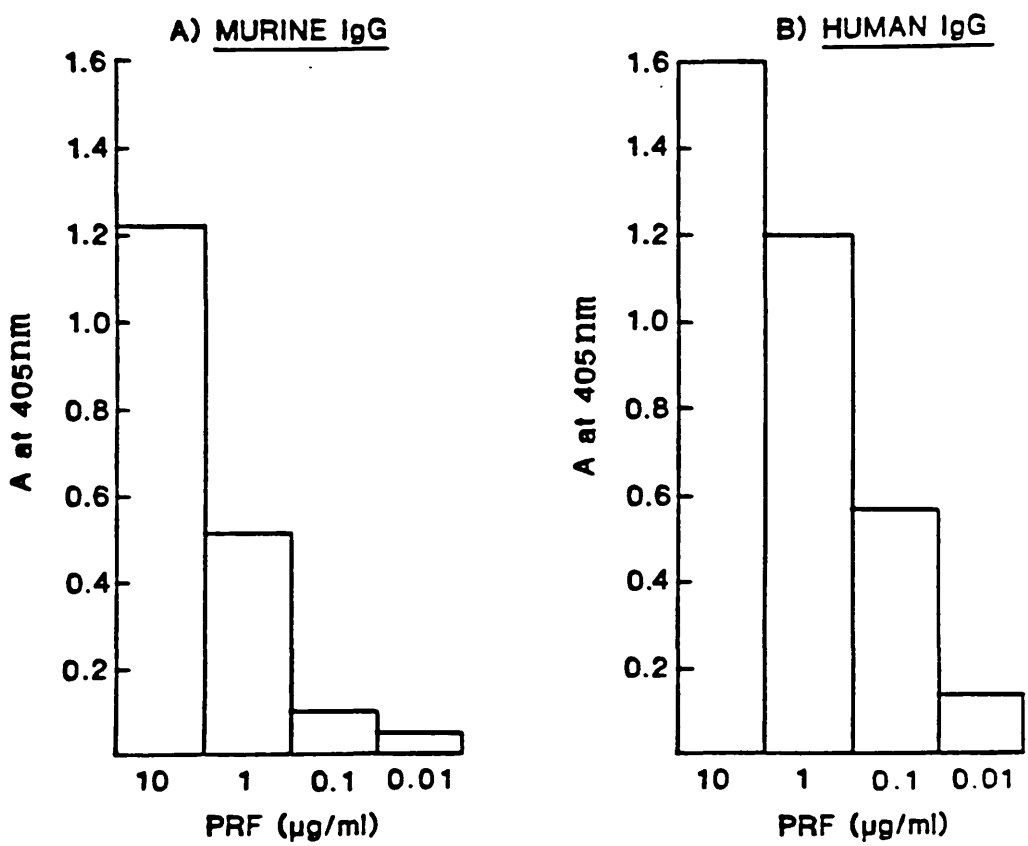

A) MURINE IgG
A at 405 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
10^{-1}
10^{-2}
SERUM DILUTION
B) HUMAN IgG
A at 405nm
0.6
0.5
0.4
0.3
0.2
0.1
0
10^{-1}
10^{-2}
10^{-3}
SERUM DILUTION

Figure Legend

Figure 4 shows the results obtained when comparing the binding of purified IgM polyclonal rheumatoid factor (PRF, from a patient with rheumatoid arthritis) to a) murine IgG and b) human IgG. The assay was carried out in a single plate to ensure comparability of data.


A) MURINE IgG
B) HUMAN IgG
A at 405nm
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
10
1
0.1
0.01
PRF (µg/ml)

3.6 MONOCLONAL RHEUMATOID FACTOR

In order to determine whether or not all the antigenic determinants on the Fc portions of murine and human IgG are shared, monoclonal rheumatoid factor binding to the two IgG species was compared.

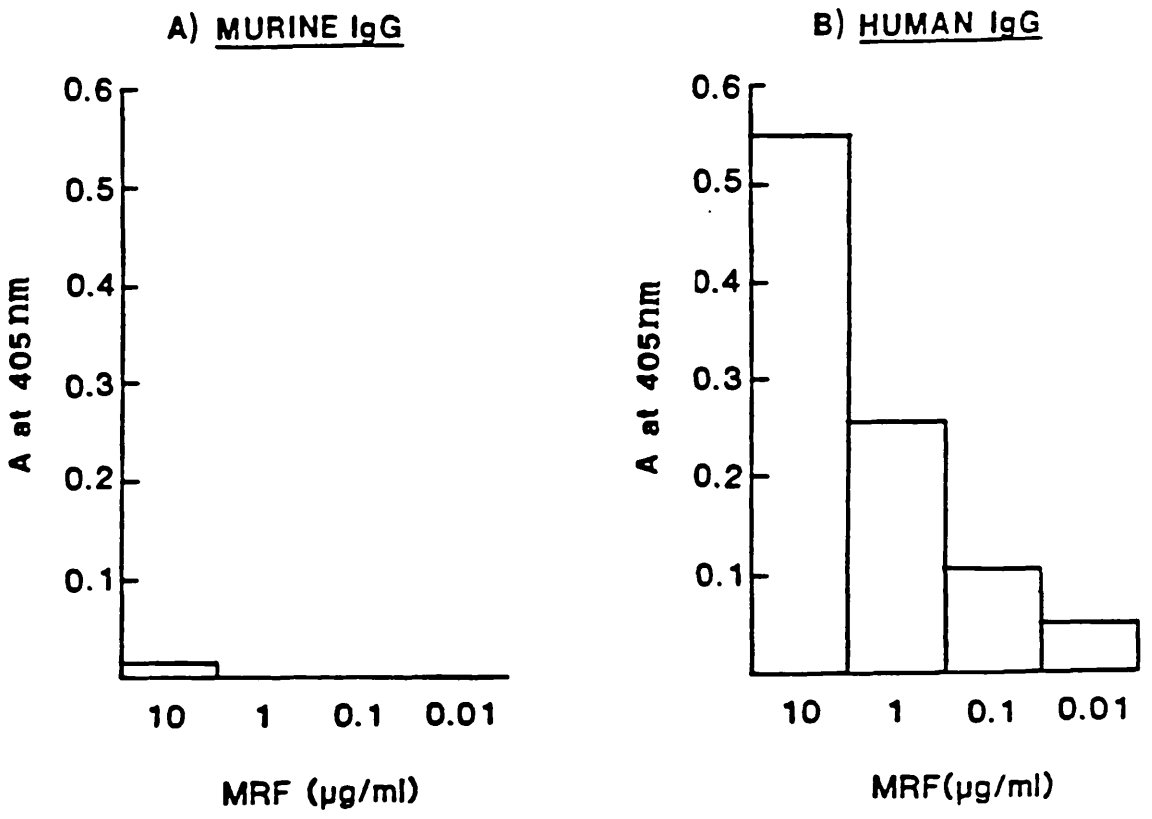
Unlike polyclonal rheumatoid factors, monoclonal rheumatoid factors see a single antigenic determinant, or epitope. Such rheumatoid factors are consistently found to be present in the serum of patients with mixed essential cyroglobulinaemia.

Four monoclonal rheumatoid factors were obtained (generous gift of Dr.C.Winearls).These factors had been affinity purified, by use of a Sepharose 6B column, from the serum of 4 patients with mixed essential cryoglobulinaemia (Ono et al., 1987). All four of these monoclonal rheumatoid factors were IgM Kappa proteins.

Microtiter plate wells were coated with murine (rows 1-6) and human (rows 7-12) IgG (500ng/well) and the binding of the purified monoclonal rheumatoid factor to each, determined. As can be seen in Figure 5 the monoclonal rheumatoid factor only bound to human IgG. All four monoclonal rheumatoid factors showed this restricted binding specificity.

Figure Legend

Figure 5 shows a comparison of binding of a purified, IgM (Kappa), monoclonal rheumatoid factor (MRF, from a patient with mixed essential cryoglobulinaemia) to a) murine IgG and b) human IgG. Again, the assay was carried out in a single plate to ensure comparability of data.


A) MURINE IgG
B) HUMAN IgG
A at 405nm
0.6
0.5
0.4
0.3
0.2
0.1
10
1
0.1
0.01
MRF (µg/ml)
MRF(µg/ml)

3.7 Fc-Fc INTERACTION

In order to discount the possibility that results obtained with the enzyme-linked immunosorbant assay used to determine the binding of rheumatoid factor to purified human and murine IgG antibodies were due to non-specific Fc-Fc interaction, the following experiment was carried out.

Monoclonal rheumatoid factor, used in the previous experiment (3.6), was incubated with a 10-fold molar excess of a monoclonal IgG1 antibody, directed against the antigen binding site of the rheumatoid factor. After pre-incubation with this anti-idiotypic antibody the ability of the monoclonal rheumatoid factor to bind to its target IgG antigen was tested as before (3.6). In this experiment, as can be seen in Figure 6A, the anti- idiotypic antibody, C8E3, inhibited the monoclonal rheumatoid factor binding to human IgG, indicating that the binding detectable in this assay system is due to specific antibody-antigen interaction, and not, non-specific Fc-Fc interaction.

In separate assays it was seen that the anti-idiotypic antibody C8E3 was unable to block the binding of three other, different purified monoclonal rheumatoid factors, Figure 6B. Thus, the ELISA system is detecting genuine antigen-antibody binding, and is not giving false positive results due to Fc-Fc interactions.

Figure Legend

Figure 6A shows the binding of monoclonal rheumatoid factor MRF1 alone, and after preincubation with an IgG1 anti-idiotype antibody, C8E3, to its target antigen, human IgG. All the data shown were obtained using a single microtitre plate.

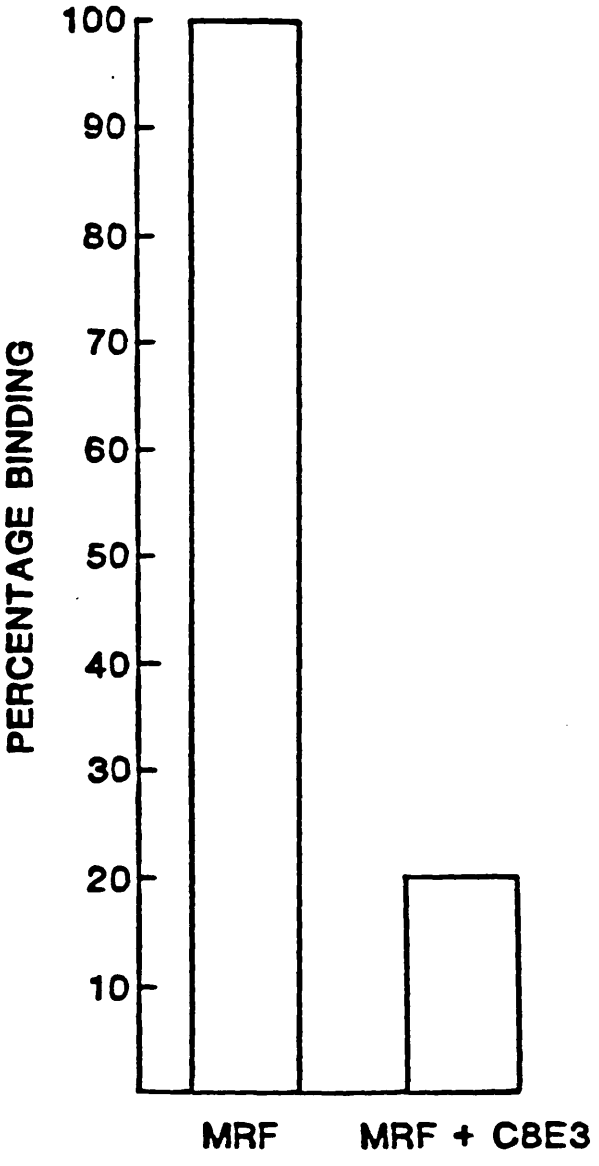
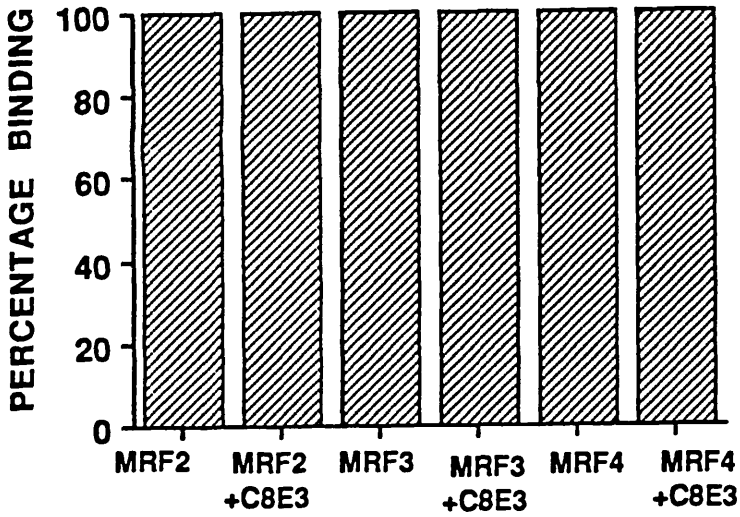

PERCENTAGE BINDING
100
90
80
70
60
50
40
30
20
10
MRF
MRF + C8E3

Figure Legend

Figure 6B shows that the binding of three unrelated monoclonal rheumatoid factors (MRF2, MRF3, MRF4) to their human IgG target antigen is unaffected by preincubation with the monoclonal anti-idiotypic antibody C8E3 (specific for the idiotype of MRF1, but not MRF2, MRF3 or MRF4) see figure 6A. All the data shown were obtained using a single microtitre plate.


PERCENTAGE BINDING
100
80
60
40
20
0
MRF2
MRF2 +C8E3
MRF3
MRF3 +C8E3
MRF4
MRF4 +C8E3

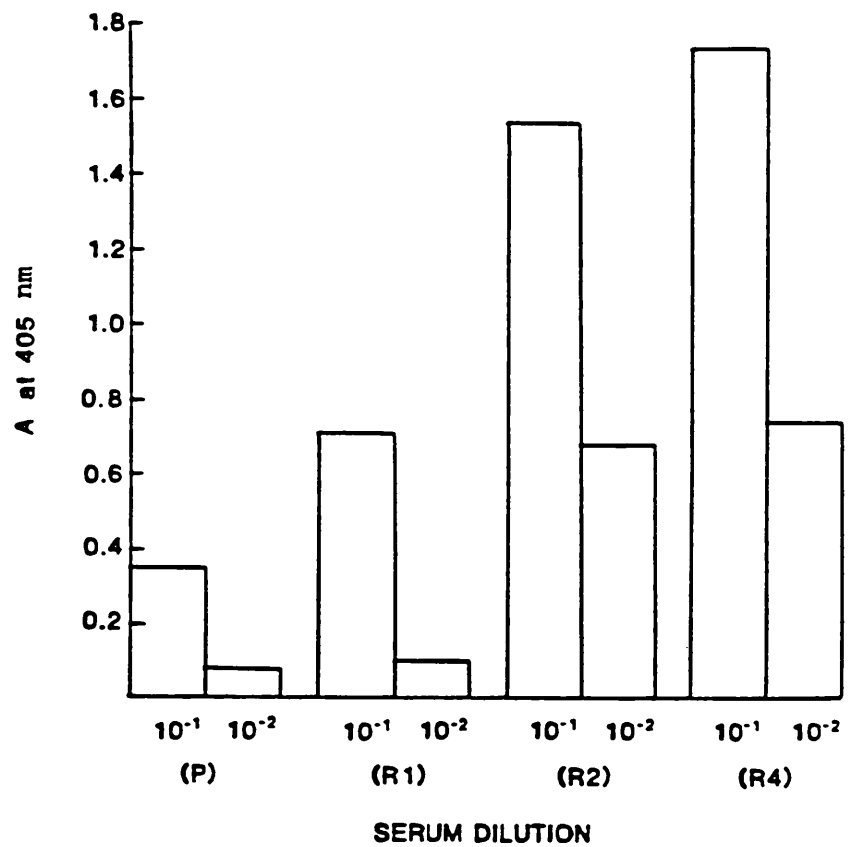
3.8 RESPONSE OF PATIENTS WITH RHEUMATOID ARTHRITIS TO MURINE AND HUMAN IgG

In order to further demonstrate the role of polyclonal IgM rheumatoid factors in the anti-murine immunoglobulin response, sera from patients with active rheumatoid arthritis were assayed for their anti-murine Ig activity. In the pre-existing anti-murine immunoglobulin response, unpurified sera were used in the enzyme-linked immunosorbant-assay being employed. At this point in the experiments it was felt important to demonstrate not only that the purified polyclonal rheumatoid factor bound to antigenic determinants on murine immunoglobulin, but that IgM antibodies present in the sera of patients with active rheumatoid disease also had anti-murine immunoglobulin activity. This would test the generality of the findings.

As shown in Figure 7, IgM antibodies present in the sera of 3 patients with varying degrees of rheumatoid arthritis and a healthy control, bind to murine IgG. A consistant feature of this experiment was that sera from patients with rheumatoid disease showed higher levels of IgM anti-murine IgG activity than that found in healthy control (blood donors, or patients prior to the antibody therapy).

Figure Legend

Figure 7 shows the elevated human IgM anti-murine IgG response from three patients with rheumatoid arthritis (samples R1, R2, R4) compared to the pre-exisiting IgM anti-murine IgG response of a healthy control (sample P). All data shown were obtained from samples analysed on a single ELISA plate under identical experimental conditions.


A at 405 nm
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
10⁻¹ 10⁻²
(P)
10⁻¹ 10⁻²
(R1)
10⁻¹ 10⁻²
(R2)
10⁻¹ 10⁻²
(R4)
SERUM DILUTION

3.9 NATURE OF ANTIGENIC DETERMINANTS

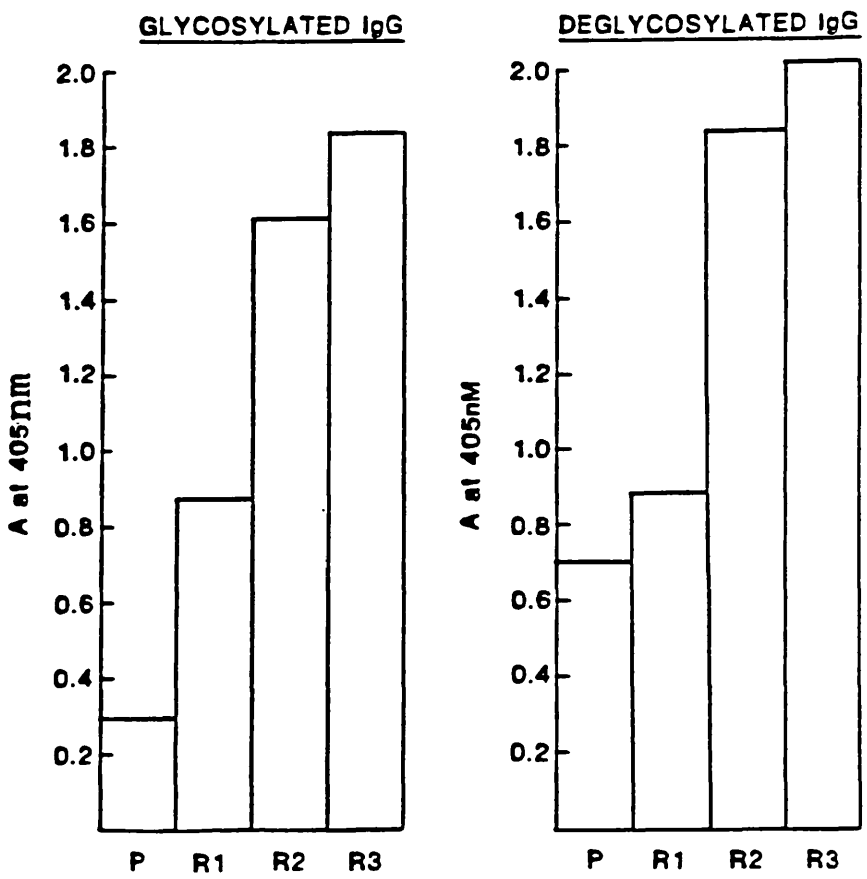
Research on the association of rheumatoid factors with changes in the glycosylation pattern of total human serum IgG content of patients with rheumatoid arthritis and primary osteoarthritis (Parekh et al., 1985) has indicated that IgM rheumatoid factors are directed against antigenic determinants which are protein rather than carbohydrate in nature.

The contribution made by carbohydrate to the antigenic determinants on murine Ig to which IgM rheumatoid factors and pre-existing antibodies bind, was determined by deglycosylation experiments. The IgG1 monoclonal antibody HMFG1 was deglycosylated using the method of Thorpe et al, (1985). This was then coated onto microtiter plates and the ability of sera from 1 healthy control and 3 rheumatoid arthritis patients to bind to this was tested by ELISA.

Results, shown in Figure 8, demonstrate no difference in the binding to deglycosylated as compared to glycosylated HMFG1, indicating that the nature of antigenic determinants recognised by the IgM rheumatoid factors is protein rather than carbohydrate. If the determinant being recognised was a sugar, then one would expect reduced binding to the deglycosylated murine monoclonal antibody, HMFG1. This was not seen.

Figure Legend

Figure 8 compares the binding of IgM serum antibodies to glycosylated (native) and deglycosylated murine IgG, using an ELISA system. Serum samples are as follows: P - healthy individual, (pre-existing response) R1, R2, R3 - patients with active rheumatoid arthritis (polyclonal rheumatoid factor). All data shown were obtained from samples analysed on a single ELISA plate under identical assay conditions.


GLYCOSYLATED IgG
DEGLYCOSYLATED IgG
A at 405nm
A at 405nM
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
P
R1
R2
R3

3.10 CONSERVED, SHARED ANTIGENIC DETERMINANTS

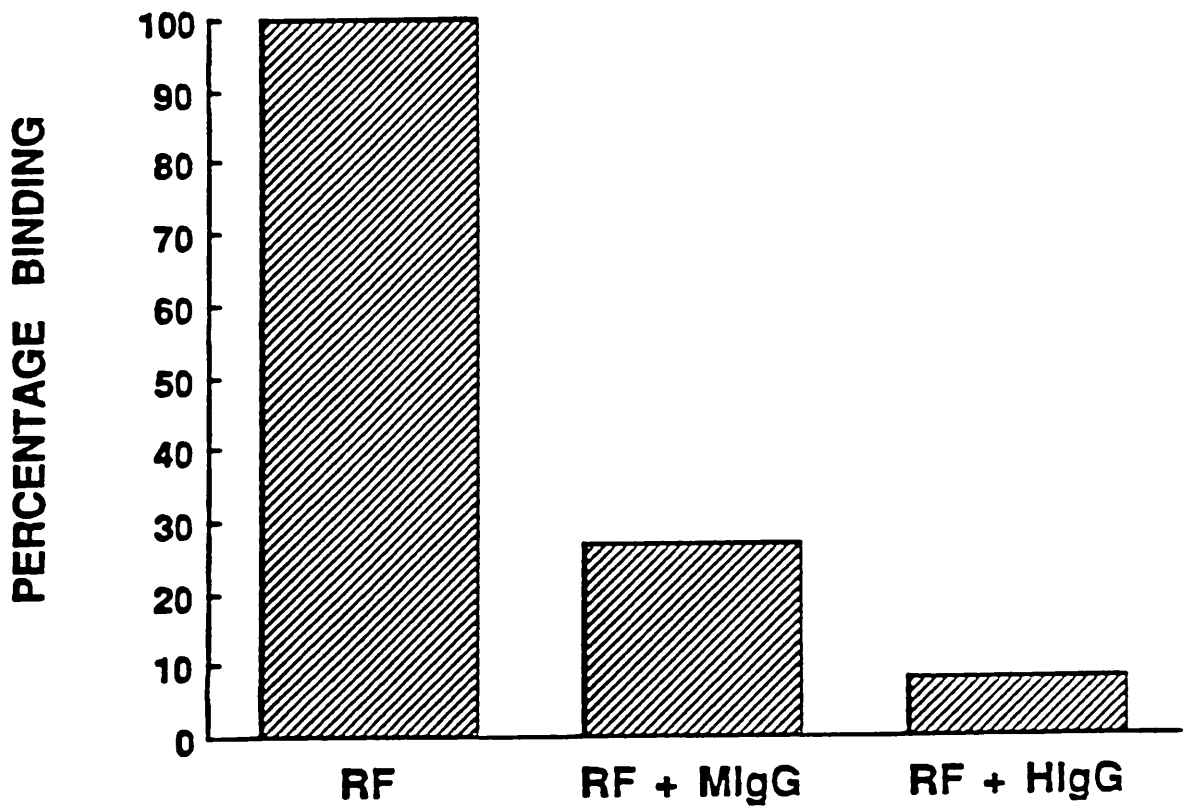
Experiments described within this chapter have been initiated and carried out with the intent of showing that IgM antibodies present in human serum, and purified IgM rheumatoid factors, bind to antigenic determinants (epitopes) present on both human and murine IgG. What has not been shown is whether these determinants are shared.

In an attempt to demonstrate this point, the IgM polyclonal rheumatoid factor was pre-incubated with 10-fold concentrations of either purified human or murine IgG. After incubation at 4°C overnight the polyclonal rheumatoid factor binding was assayed against human IgG (500ng/well), which had been coated onto the wells of a 96 well microtiter plate.

As can be seen in Figure 9, pre-incubation with human IgG absorbs out nearly all the human IgM polyclonal rheumatoid factor activity, as would be expected. Murine IgG was capable of absorbing approximately 70% of the human IgM polyclonal rheumatoid factor activity. From this experiment it can be seen that a high percentage of antigenic determinants recognised by the purified human IgM polyclonal rheumatoid factors are shared, and possibly conserved, between human and murine IgG.

Figure Legend

Figure 9 shows the results of pre-incubating purified human IgM polyclonal rheumatoid factor with either murine IgG (MIgG) or human IgG (HIgG). Absobed sera were then assayed for rheumatoid activity against human IgG used to coat the wells of a 96-well microtiter plate. All samples were assayed on a single ELISA plate to ensure comarability of data.


PERCENTAGE BINDING
100
90
80
70
60
50
40
30
20
10
0
RF
RF + MIgG
RF + HIgG

3.11 ELEVATION OF PRE-EXISTING REACTIVITY

Results presented in this chapter have shown that IgM antibodies present in human serum bind to antigenic determinants present on both murine and human IgG, and that a proportion of these determinants are shared. An important question to be answered therefore was, do patients receiving a single therapeutic dose of radiolabelled murine IgG monoclonal antibodies generate an elevated IgM anti-murine and anti-human IgG response?

In order to address this question, serum was obtained from the blood of 12 patients 15 days post administration of radiolabelled murine monoclonal IgG antibodies, used in the attempted therapy of ovarian cancer. As can be seen in Table 2 and Figure 10 both the IgM anti-murine IgG and the anti- human IgG response were elevated. Although the response to both species of IgG increased, that to the murine IgG was consistantly higher than that to the human IgG as shown in Table 2, indicating a murine-specific component of the response. The location of antigenic determinants recognised by human anti-murine immunoglobulin antibodies after therapeutic administration of radiolabelled murine monoclonal antibodies is discussed in Chapter 5.

Figure Legend

Figure 10 shows the binding of both pre-existing (PRE) and post-therapeutic (POST) of IgM serum antibodies, to a) murine and b) human IgG. These samples were from patient 11 (see table 2) who received a single therapeutic dose of radiolabelled murine monoclonal antibodies for the attempted therapy of ovarian cancer.

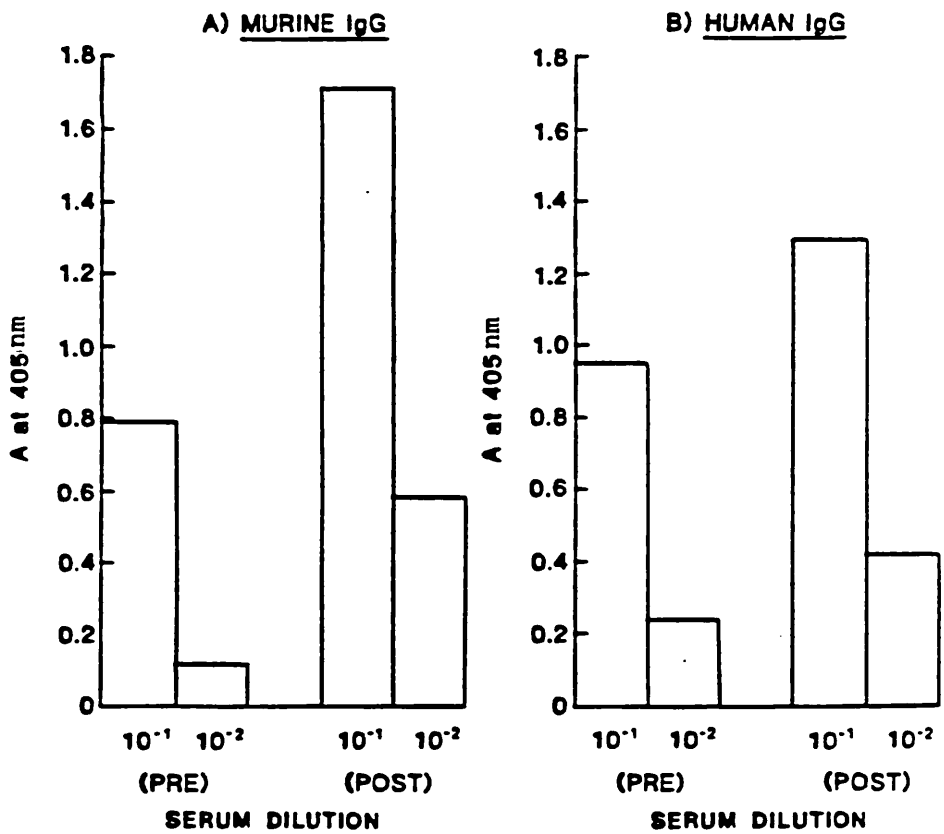

A) MURINE IgG
B) HUMAN IgG
A at 405 nm
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
10^{-1} 10^{-2} 10^{-1} 10^{-2}
(PRE) (POST)
SERUM DILUTION

TABLE 2

Table 2 shows the results (Absorbance at 405nm) for the response of ovarian cancer patients to murine IgG and human IgG, both prior to (pre) and after (post) receiving radiolabelled murine monoclonal antibody therapy.

Patient	Binding to Murine IgG		Binding to Human IgG	
	Pre	Post	Pre	Post
1	0.544	1.655	0.674	1.021
2	1.315	1.713	1.432	1.614
3	0.248	1.480	0.310	0.540
4	0.367	1.472	0.708	0.958
5	0.364	1.721	0.900	1.230
6	0.114	0.618	0.238	0.470
7	0.354	1.810	0.392	0.542
8	0.708	1.974	0.914	1.121
9	1.215	1.987	1.690	1.846
10	0.266	1.264	0.375	0.512
11	0.794	1.721	0.948	1.321*
12	0.083	0.571	0.800	1.204

*Data for this patient is also presented graphically (Figure 10).

Data shown are for all sera diluted 10^{-1}. This was the maximum absorbance, since no prozone effects were seen in these experiments

Table 3

Human IgM anti-IgG Response

Disease	Number of patients Studied	Binding to murine IgG	Binding to Human IgG
B.D.	50	+	+
O.C.	13	+	+
M.E.C.	4	-	+
R.A.	12	+	+
L.C.	4	+	+
P.R.F.	1	+	+

Sera from healthy blood donors and from patients with a variety of diseases were tested for IgM anti-murine and anti-human IgG antibodies, using an ELISA system.

(a) B.D. = Blood donor

O.C. = Ovarian cancer

M.E.C. = Mixed essential cryoglobulinaemia

R.A. = Rheumatoid arthritis

L.C. = Lung cancer

P.R.F. = Purified polyclonal rheumatoid factor

(b) + = positive (> 2 x background)

- = negative

3.12 Discussion

Results presented in this chapter show that IgM antibodies present in human serum have specificity for evolutionarily conserved antigenic determinants that are shared by murine and human IgG. These IgM rheumatoid factors are detectable in the serum of healthy individuals and patients with various disease, as shown in table 3.

From these data it is seen that these rheumatoid factors bind to murine IgG in solid phase assays and when incubated in solution. The ability of these factors to bind to murine IgG in solution poses several problems; these include, the blocking of tumour targetting when radiolabelled murine monoclonal antibodies are used for in vivo tumour localisation or therapy, and the possible formation of immune complexes, which could result in immune complex mediated disease.

Having identified the role of rheumatoid factors in the anti-murine immunoglobulin response, several lines of action can be taken to avert possible adverse or inhibitory effects they may cause. Firstly, as the serum IgM anti-murine IgG response is elevated after a single therapeutic administration, one could try to avoid these antibodies by administering the radiolabelled antibodies directly into a body cavity, wherever possible. For example, antibodies for tumour imaging have been administered intraperitoneally for patients with ovarian cancer. In addition, for tumour diagnosis F(ab')2 fragments could be used.

CHAPTER 4

FRAGMENTATION OF MONOCLONAL ANTIBODY HMFG1

4.1 INTRODUCTION

Radiolabelled murine monoclonal antibodies are increasingly being used for *in vivo* tumour localization studies and more recently for tumour therapy. During the course of these studies it has been shown that although intact antibody is cleared relatively rapidly from the bloodstream, significant background radioactivity remains for several days after injection (Wahl et al., 1983).

In order to reduce the background radioactivity and hence obtain earlier images of tumour localization, the use of antibody fragments, such as $F(ab')_2$ fragments has been employed. Use of $F(ab')_2$ fragments removes the problem of Ig binding to Fc receptors in the liver, which produces a rapid accumulation in that organ (Buraggi et al., 1985). Antibody fragments devoid of the Fc would also overcome any possible interaction of murine IgG with human monocyte Fc receptors (Lubeck et al., 1985).

In this chapter the results obtained from studies on the optimization of enzyme (Pepsin) digestion, purification, stability, immunoreactivity and radiolabelling of $F(ab')_2$ fragments of HMFG1 are discussed. This antibody was selected as it has been shown to be clinically useful due to its specificity for tumour-associated antigen and is currently in use in clinical trials at the Hammersmith Hospital (Epenetos et al

4.2 OPTIMAL DIGEST CONDITIONS

In order to obtain the highest yield of immunoreactive $F(ab')_2$ fragments from a given weight of intact HMFG1, four variables were studied. These variables, each capable of determining the success of pepsin digestion, were pH, period of digestion, monoclonal antibody concentration and pepsin concentration.

Previous studies had indicated that the optimal pH at which IgG1 murine monoclonal antibodies were digested with pepsin was between 3.5 and 4.0 (Parham, 1983). Therefore using a digest pH of 4, and an antibody concentration of 10mg/ml the optimal period of digestion was studied using a pepsin concentration of 2% by weight of total IgG. The products were then analysed by SDS PAGE. The results obtained showed the period of digestion resulting in the highest percentage of cleavage of HMFG1 by pepsin to be 16 hours.

In order to increase the rate at which HMFG1 was being cleaved, the pepsin concentration was increased to 3% of the weight of antibody. Using a pH of 4, obtained by dialysing the antibody in 0.1M sodium acetate buffer, as described in Materials and Methods, the optimal time of digestion was reduced from 16 to 8 hours. The optimal period of digestion was found by stopping the digestion at a number of time points and analysing, by non-reduced SDS-PAGE, the digest progression.

Using a pH of 4, a pepsin concentration of 3%, by weight, an antibody concentration of 10mg/ml and an 8 hour period of digestion at 37°C, optimal yields of HMFG1 fragments were obtained. After 8 hours of digestion only a small percentage of the intact antibody remained (between 2% and 5%), as shown in Figure 11A.

In Figure 11A it can be seen that at time 0, no band is present at the 100KD $F(ab')_2$ point. As incubation at 37°C with pepsin proceeds the IgG (150kD) band diminishes as the intensity of the $F(ab')_2$ band increases. After 8 hours only a small IgG band is seen. If the digest were allowed to continue further, the primary substrate, IgG, would be totally used, and the $F(ab')_2$ fragments would then be digested, acting as secondary substrate for pepsin, giving rise to the formation of an unidentified 60KD fragment (possibly of monomeric Fab fragments), as seen at the 24 hour time-point.

Figure Legend

Figure 11A, shows the results obtained by 7.5% SDS-PAGE (non-reduced) of a pepsin digest of HMFG1, stopped at various time points. The gel was stained with Coomassie blue.

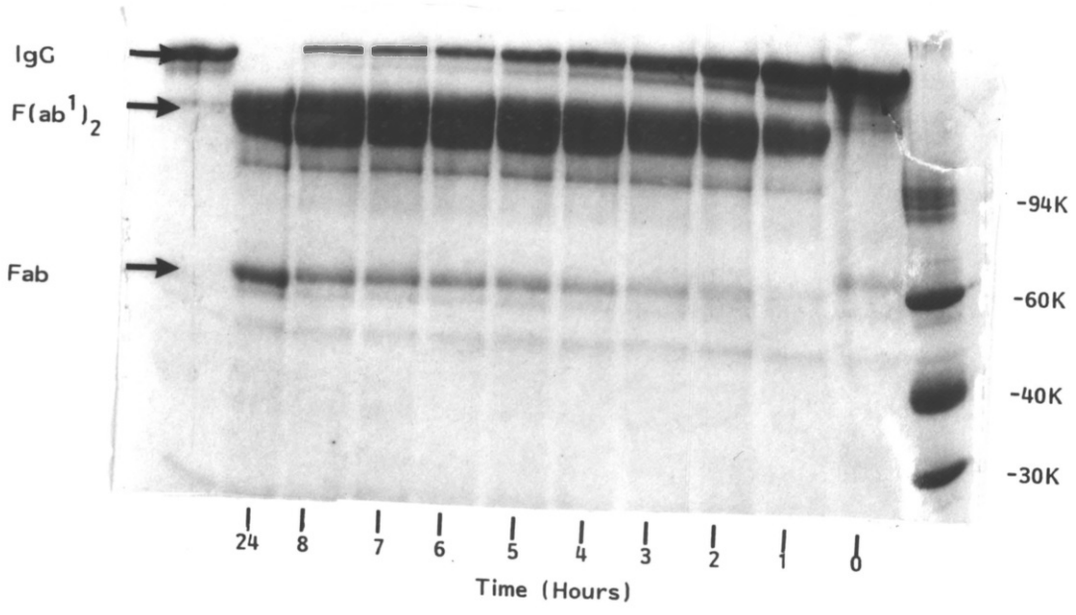
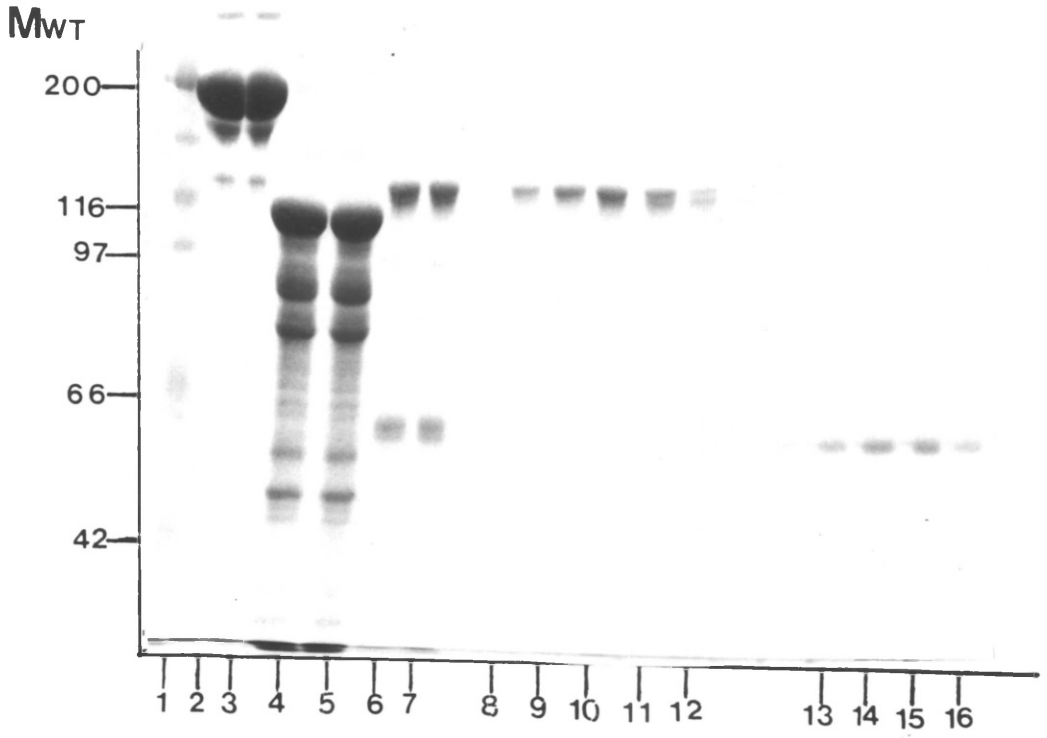

IgG
F(ab¹)₂
Fab
-94K
-60K
-40K
-30K
24
8
7
6
5
4
3
2
1
0
Time (Hours)

Figure Legend

Figure 11B, SDS-PAGE (7.5%) analysis of the purity of the $F(ab')_2$ fragments of the monoclonal antibody HMFG1 (tracks 8-12), after purification, using a Sephadex G-100 column. Also shown, is the intact monoclonal antibody, HMFG1 (tracks 2-3), a commercial $F(ab')_2$ preparation (tracks 4-5), the HMFG1 digest prior to purification (tracks 6-7) and the lower molecular weight contaminants (tracks 13-16). Track 1: molecular weight markers. Mwt = molecular weight X 10^3


MWT
200
116
97
66
42
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

4.3 PURIFICATION OF F(ab')$_2$ FRAGMENTS

Having optimized the conditions required for the pepsin digestion of HMFG1, the methods of purification were examined. In purifying the F(ab')$_2$ fragments the goals are to remove any intact, undigested antibody, and remove any other products of digestion, such as Fc portions and breakdown products of F(ab')$_2$ fragments. In order to remove the intact antibody, the digest pH was adjusted to 8 using 1M TRIS buffer: the digest was then passed through a protein-A column.

After protein-A purification, the partially purified F(ab')$_2$ preparation was further purified on a Sephadex G-100 column. The F(ab')$_2$ preparation was then assayed for contaminants by loading samples onto a non-reduced 7.5% polyacrylamide gel (SDS-PAGE) as described, along with the purification methods in Chapter 2. Results obtained by SDS-PAGE showed the F(ab')$_2$ fragments to be free of any contaminants, Figure 11B.

After purification, the fractions containing F(ab')$_2$ fragments were pooled, the protein concentrations determined and the efficiency of the digest calculated. The efficiency was calculated as the weight of F(ab')$_2$ fragments obtained, divided by the weight of intact antibody minus 33%, assuming the Fc to be 1/3 of the immunoglobulin weight, this figure was then multiplied by 100 to give the percentage efficiency. For three separate preparations the percentage efficiencies, using the optimal condition described above, were 84%, 86% and 79%. In each

preparation the weight of intact HMFG1 used to prepare $F(ab')_2$ fragments was 50mg.

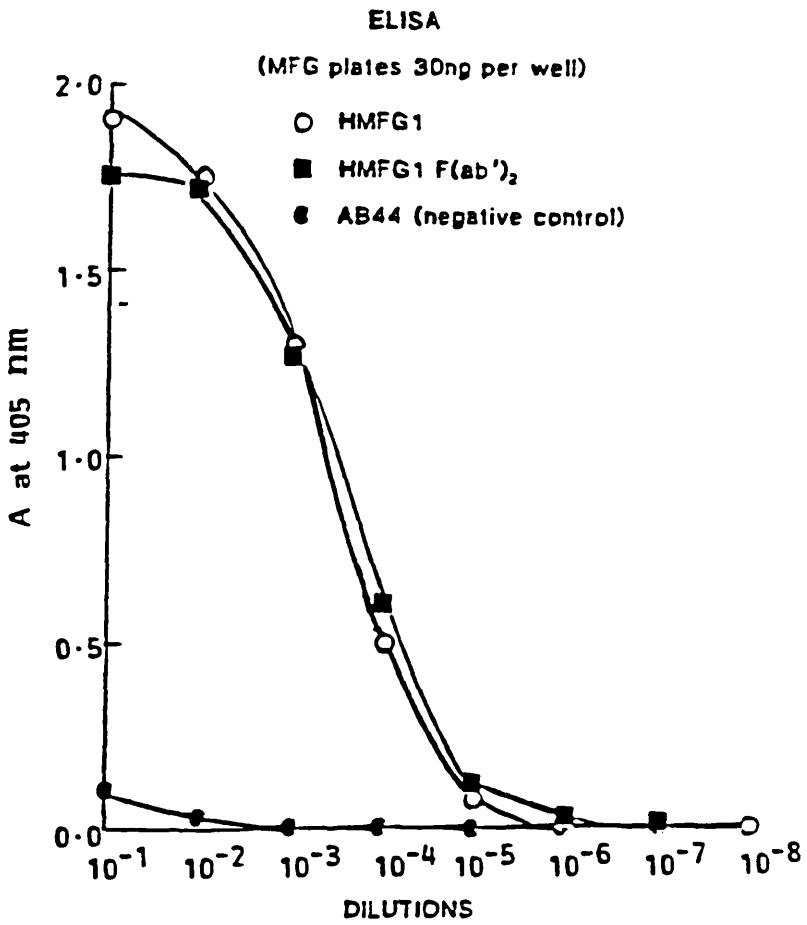
4.4 IMMUNOREACTIVITY OF $F(ab')_2$ FRAGMENTS

After pepsin digestion and purification of HMFG1 $F(ab')_2$ fragments, their immunoreactivity was determined by ELISA. 96-well microtiter plates were coated with antigen, 30ng/well of milk fat globule (MFG), and 10-fold dilutions of intact HMFG-1 and AB44 (negative control) and of $F(ab')_2$ fragments of HMFG1 were made in PBS, using an initial concentration of 2µg/ml of each antibody.

As can be seen in Figure 12 neither the conditions used to pepsin digest HMFG1, nor those used to purify the $F(ab')_2$ fragments resulted in any significant loss of immunoreactivity. The negative control antibody AB44, an IgG1 murine monoclonal antibody raised to the C3b receptor of human granulocytes, showed no significant binding, hence, the binding of HMFG1 and its $F(ab')_2$ fragments was specific for the MFG antigen and not due to an artefact of the conditions used.

Figure Legend

Figure 12 shows a comparison of the binding of intact and $F(ab')_2$ fragments of HMFG1 antibody to target MFG antigen . Intact AB44 was used as a negative control antibody, being of the same isotype as HMFG1 (IgG1). The initial concentration of each antibody was 2μg/ml, from which ten-fold dilutions were made. All assays were performed on a single ELISA plate.


ELISA
(MFG plates 30ng per well)
HMFG1
HMFG1 F(ab')2
AB44 (negative control)
A at 405 nm
2·0
1·5
1·0
0·5
0·0
10-1
10-2
10-3
10-4
10-5
10-6
10-7
10-8
DILUTIONS

4.5 RADIOIODINATION OF F(ab')$_2$ FRAGMENTS

In order to carry out localization studies in either patients or tumour xenografted nude mice, the F(ab')$_2$ fragments would need to retain their immunoreactivity after iodination, using either the iodogen or N-bromosuccinimide methods to covalently attach the isotope, as described in Chapter 2.

To test the stability of the fragments after radioiodination, they were labelled with Iodine-125 to a specific activity of 5MCi/mg. After labelling, the free iodine was removed by gel-filtration (Sephadex G50) and the fractions containing labelled antibody fragments pooled, as shown in Figure 13. In order to test the immunoreactivity of the Iodine-125 labelled fragments a direct competitive radioimmunoassay was carried out using a starting concentration of 2μg/ml and 10-fold dilutions down the length of the plate. A fixed amount of cold antibody (2μg/ml) was added to each of the wells in which the labelled antibody had been diluted.

In order to determine the specificity of labelled antibody binding in the radioimmunoassay, non-specific antibody F(ab')$_2$ fragments were labelled to the same specific activity as the HMFG1 F(ab')$_2$ fragments. The non-specific antibody fragments were of the IgG1 monoclonal antibody AUA-1 (generous gift of H.Durbin, ICRF). After incubation, the wells of the microtiter plate were cut-out and counted. The results were plotted as

counts per minute (cpm) against dilution of antibody $F(ab')_2$ fragments, as shown in Figure 14. From the results, the binding of the radiolabelled $F(ab')_2$ fragments of HMFG1 can be seen to be specific in two ways. Firstly, the binding of the labelled fragments can be competed out with increasing concentrations of cold antibody fragments and secondly, $F(ab')_2$ fragments of another antibody show no significant binding to the milk fat globule antigen recognised by HMFG1 and its $F(ab')_2$ fragments.

Figure Legend

Figure 13 shows two peaks obtained in the elution of Iodine-125 labelled $F(ab')_2$ fragments from a G50-Sephadex column. The first peak contained the labelled fragments and the second contained the free radioisotope.

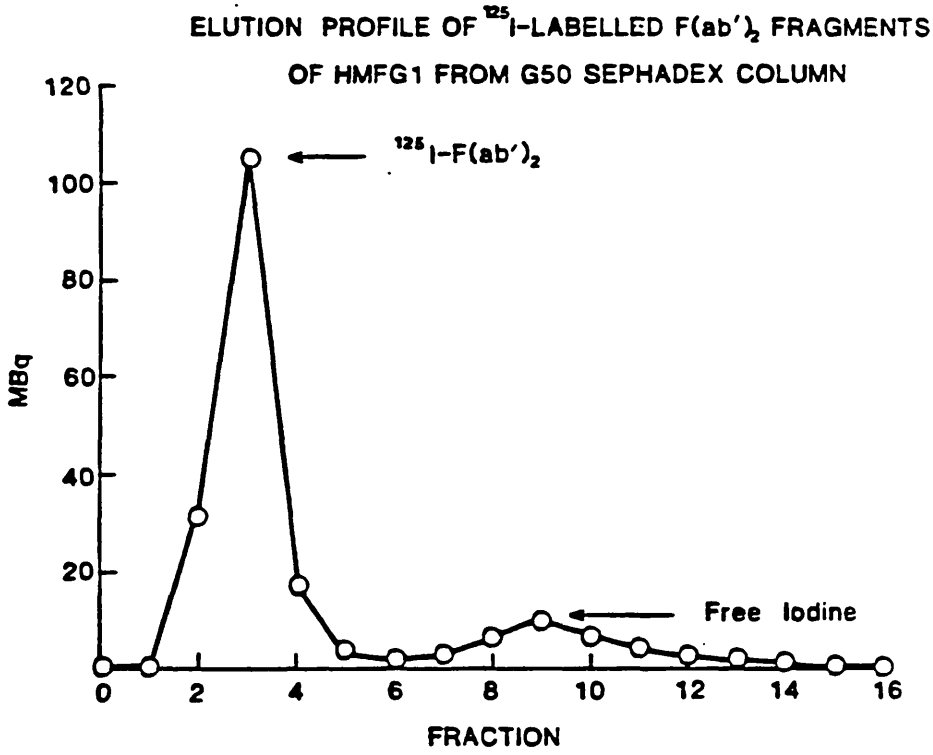
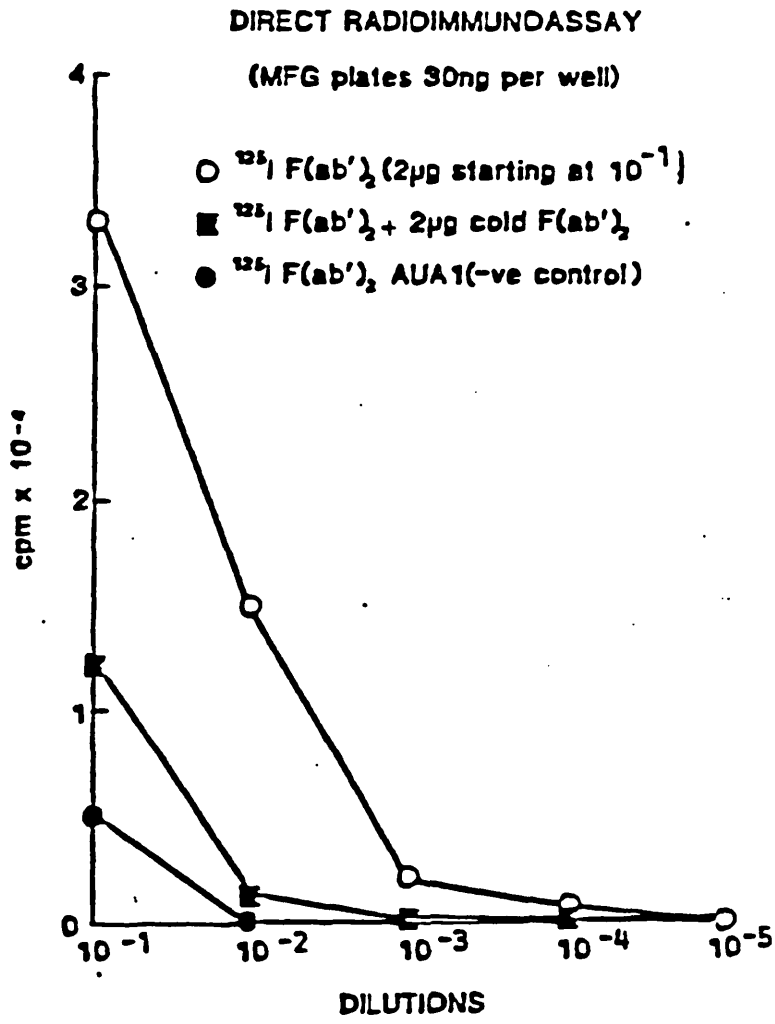

ELUTION PROFILE OF ^{125}I-LABELLED F(ab')$_2$ FRAGMENTS
OF HMFG1 FROM G50 SEPHADEX COLUMN
^{125}I-F(ab')$_2$
Free Iodine
MBq
120
100
80
60
40
20
0
2
4
6
8
10
12
14
16
FRACTION

Figure Legend

Figure 14 shows the results obtained in a direct radioimmunoassay carried out to determine the immunoreactivity and specificity of Iodine-125 labelled $F(ab')_2$ fragments of HMFG1. Cold (unlabelled) fragments were used to compete with the labelled fragments for the MFG antigen on the plate. Iodine-125 labelled $F(ab)_2$ fragments of AUA-1 were used as a negative control. Stock concentration of each antibody was 20 ug/ml. Cold competitive antibody was added at 2ug/well. All assays were performed on a single microtitre plate.


DIRECT RADIOIMMUNOASSAY
(MFG plates 30ng per well)
^{125}I F(ab')$_2$ (2µg starting at 10^{-1})
^{125}I F(ab')$_2$ + 2µg cold F(ab')$_2$
^{125}I F(ab')$_2$ AUA1(-ve control)
cpm x 10^{-4}
4
3
2
1
0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
DILUTIONS

4.6 INDIUM-111 LABELLING OF F(ab')$_2$ FRAGMENTS

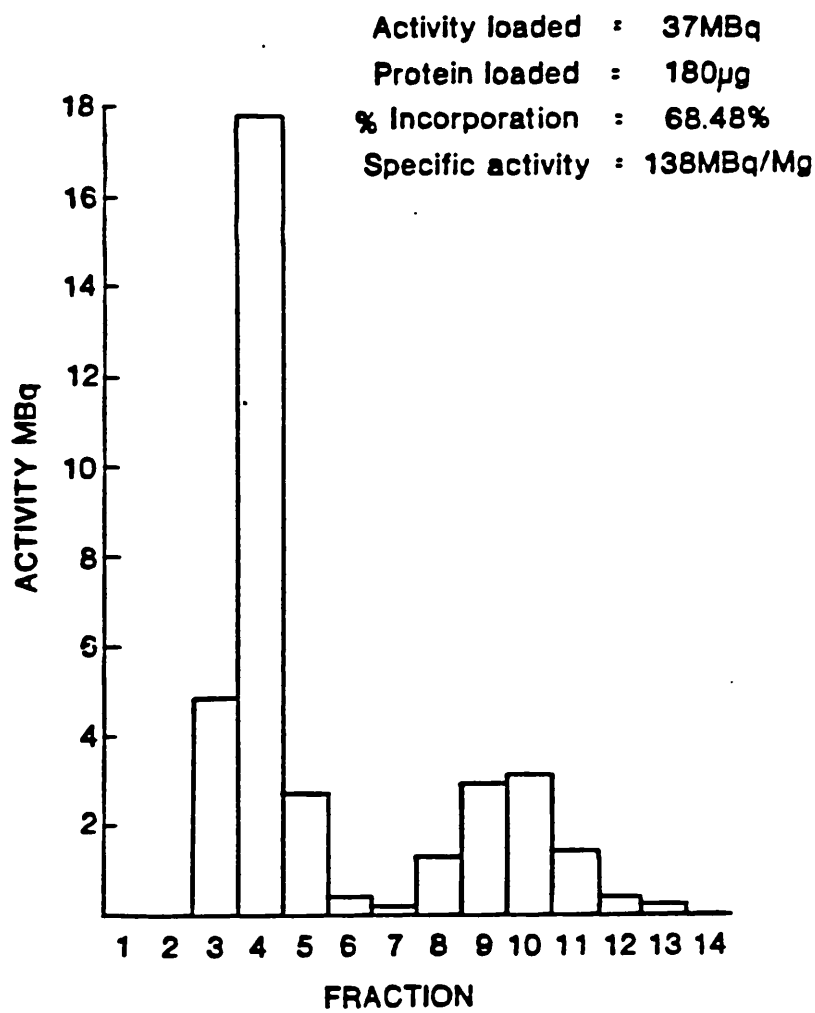
Indium-111 has become one of the most used isotopes for labelling monoclonal antibodies being administered to patients for *in vivo* tumour localization. Prior to labelling, the antibody has first to be attached to a chelating agent, such as DTPA, a bi-cyclic anhydride which utilises one of its carboxyl groups to form an amide bond with the protein while leaving the others free to bind with Indium-111.

F(ab')$_2$ antibody fragments labelled with Indium-111 have been successfully used in immunoscintigraphy studies of patients with melanoma (Siccardi et al., 1986) and various other neoplasms. In order to exploit the potential of this useful imaging agent the F(ab')$_2$ fragment of HMFG1 were first coupled to DTPA and then labelled with Indium-111. To remove any free indium-111 from the preparation, the preparation was passed through a G50-Sephadex column, which resulted in two distinguishable peaks, the first containing labelled fragments and the second, free indium, as shown in Figure 15. As with the Iodine-125 labelled F(ab')$_2$ fragments, no significant loss of immunoreactivity was observed when specific activities of between 1 - 5 MCi/mg (37 and 185 MBq) were used.

Figure Legend

Figure 15 shows the elution profile of Indium-111 labelled $F(ab)_2$ fragments of HMFG1 purified on a G50-Sephadex column. The first peak (fractions 3-6) contained 68.48 per cent of the total activity used (36 MBq) which was coupled to the HMFG1 $F(ab')_2$ fragments. The remaining free Indium-111 was eluted later and represented the second peak (fractions 8-11).

^{111}In-LABELLED F(ab')$_2$ FRAGMENTS OF HMFG1



4.7 DISCUSSION

The results presented in this chapter show that with careful evaluation of the variables associated with the enzymatic (pepsin) digestion of a murine monoclonal antibody (HMFG1), acceptable yields of immunoreactive $F(ab')_2$ fragments can be obtained and that this immunoreactivity is not significantly impaired by radio-labelling, thus making them useful as imaging agents in immunoscintigraphy studies.

Although neither isotope (Iodine-125 or Indium-111) when used to obtain specific activities of between 1 and 5 MCi/mg of $F(ab')_2$ fragments, resulted in significant loss of immunoreactivity, the coupling of DTPA to antibody fragments did. When high molar ratios of DTPA to $F(ab')_2$ fragments were used the immunoreactivity was reduced. Experiments showed that as the molar ratio of DTPA: $F(ab')_2$ fragments was reduced, the immunoreactivity increased, with a molar ratio of between 2 and 10 DTPA molecules per antibody fragment molecule being optimal.

Animal studies using the HMFG1 $F(ab')_2$ fragments have produced results which indicate that these fragments may have some clinical potential. The clinical usefulness of the HMFG1 $F(ab')_2$ fragment is at present being investigated in the localization of tumour lesions in patients with non-small cell lung cancer.

CHAPTER 5

HUMAN ANTI-MURINE IMMUNOGLOBULIN RESPONSE

5.1 INTRODUCTION

Monoclonal antibodies are used therapeutically in man in a number of clinical situations, essentially as immunosuppressive (Chatenoud et al., 1986) or anti-tumour agents (Schlom, 1986). To date, nearly all the monoclonal antibodies used in clinical situations have been derived from mice, and therefore, sensitization to xenogeneic protein has been viewed as a potential major complication to their use.

Development of anti-murine Ig antibodies by patients could result in a number of problems, such as hypersensitivity reactions, for example, allergic (TYPE-I), or immune-complex mediated (TYPE-III serum sickness) reactions. Although such problems could result from the use of murine antibodies, few have been reported.

However, apart from hypersensitivity reactions, the production of human anti-murine Ig antibodies could lead to the rapid clearance of potentially therapeutic antibodies and hence abrogate any therapeutic effect.

Production of human anti-murine Ig antibodies was observed in 3 of 4 patients receiving murine antibodies for the treatment of gastrointestinal tumours (Sears et al., 1982). In another study, their presence in patients' sera was observed 7 days after administration, and they were found to be present for at least a further 10 months (Pimm et al., 1985).

In the majority of studies the potentially therapeutic murine monoclonal antibodies have been administered intravenously. In an attempt to overcome the problems of immune-complex formation, and hence rapid clearance and possible immune complex mediated disease, the administration of labelled antibodies into body regions by intrapericardial, intrapleural and intraarterial routes has been used (Epenetos et al., 1984). Although this regional administration may avoid the formation and consequences of immune complexes, and result in a higher percentage of the potential therapeutic radiolabelled mouse monoclonal antibody reaching its target, it was not known whether it would in any way alter the humoral immune response to the xenogeneic monoclonal antibodies being used.

In this chapter the development, specificity and kinetics of immune responses made by patients with stage III or IV ovarian carcinoma receiving intraperitoneally administered radiolabelled murine monoclonal antibodies is discussed.

5.2 MEASUREMENT OF THE HUMAN ANTI-MURINE IMMUNOGLOBULIN RESPONSE

In order to measure the development of human anti-murine Ig antibody responses made by patients, two assay systems were developed. The first assay, referred to as "hybridoma targeting", was a non-quantitative assay. The binding of patients' serum antibodies to antigenic determinants located on the mouse monoclonal antibody was assessed by

immunocytochemistry using cytospin preparations of the relevant hybridoma cells.

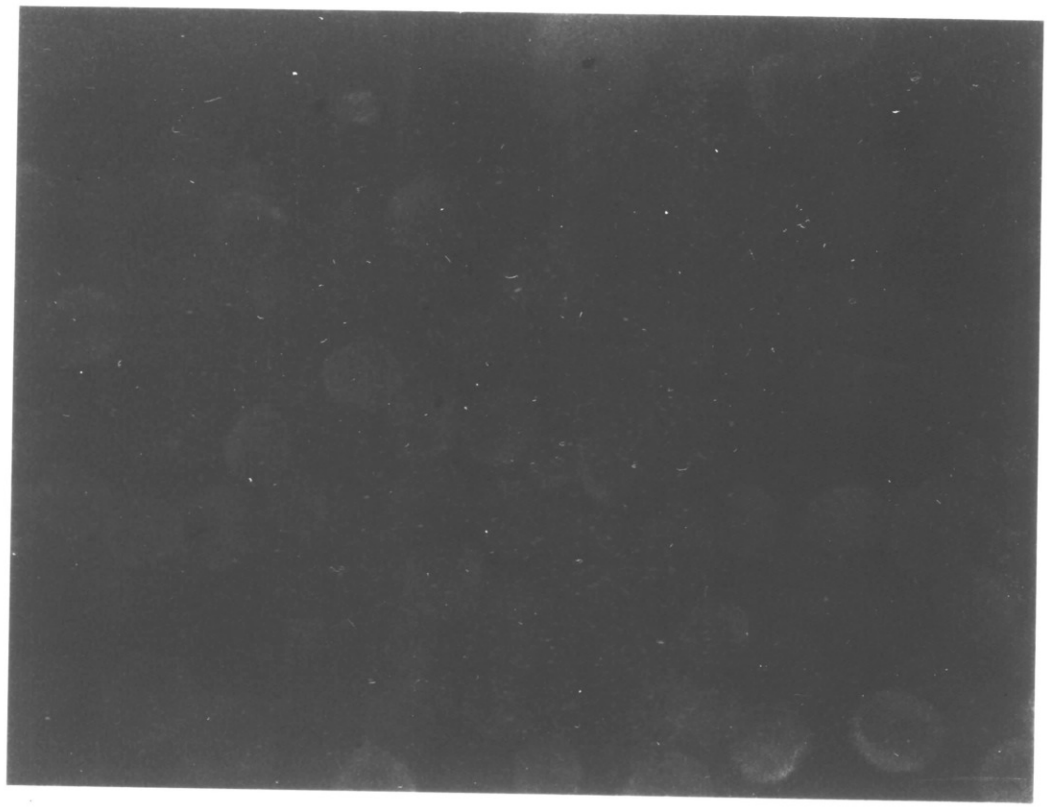
In the hybridoma targetting assay the use of a second layer fluorescein conjugated sheep anti-human Ig reagent enabled a clear distinction between the detection of anti-murine Ig antibodies present in patient's serum prior to, and after administration of radiolabelled murine monoclonal antibodies to be made, as shown in Figure 16. This assay, although reproducable and relatively easy, was not sufficiently sensitive to permit detection of pre-existing anti-murine Ig antibodies.

In order to semi-quantitate the anti-murine Ig response, an ELISA was developed. A number of problems were associated with the development of this assay. These included cross-reactivity of the peroxidase conjugated second layer antibody with the antigen (murine Ig), non-specific binding of serum antibodies to the plastic of some microtiter plates (Chapter 3) and the constituents of the diluent media for the sera.

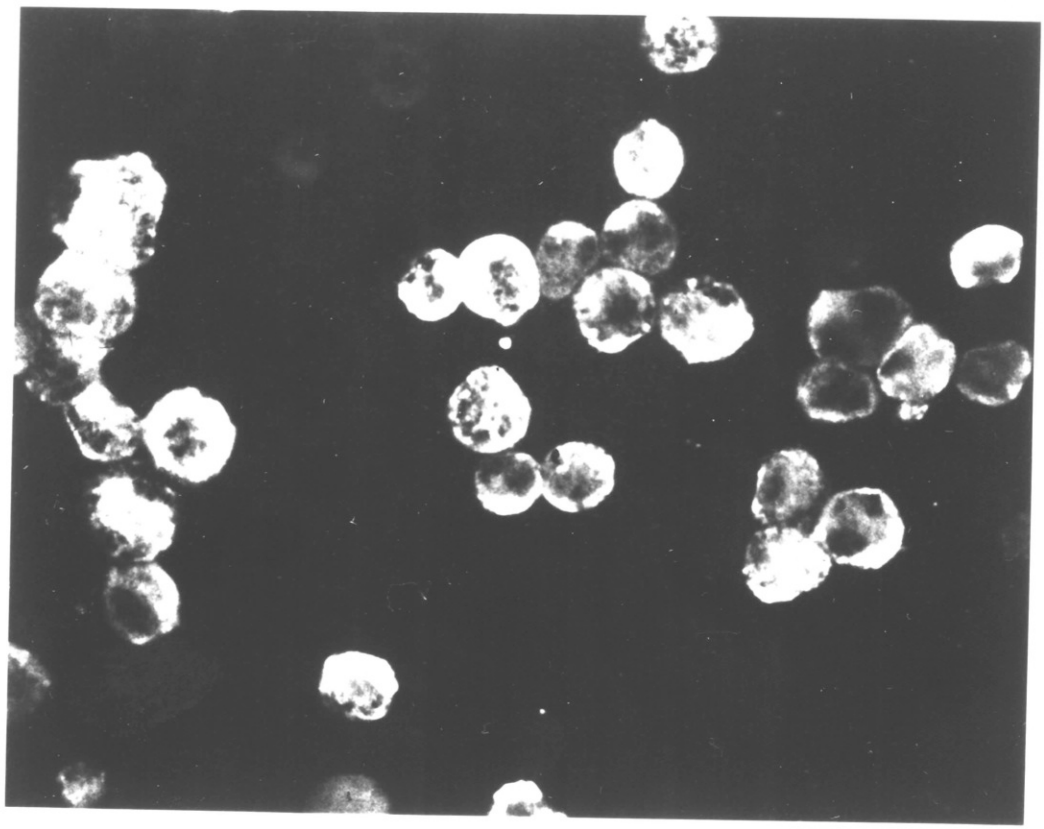
Figure Legend

Figure 16 illustrates the results of hybridoma targeting using a patient's pre (A) and post (B) therapy serum. Cytospin preparations of the HMFG2 secreting hybridoma were incubated with the patient's serum. Binding of human antibodies to the murine HMFG2 antibody in the target cells was visualized by incubation with a FITC-conjugated sheep anti-human Ig reagent.

A



B

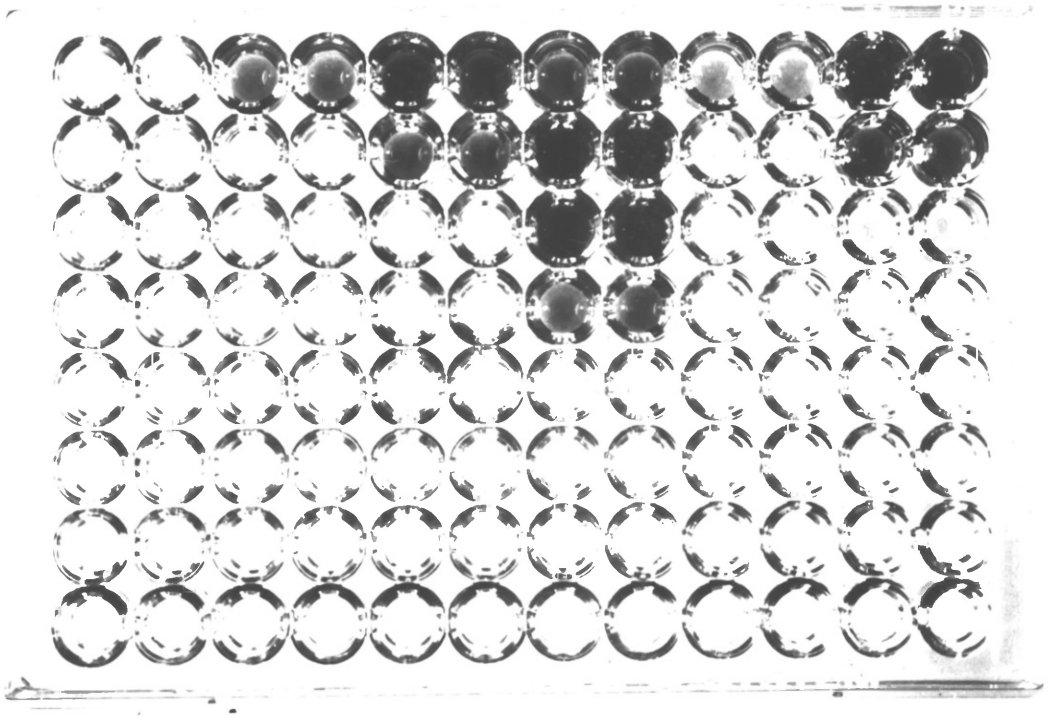


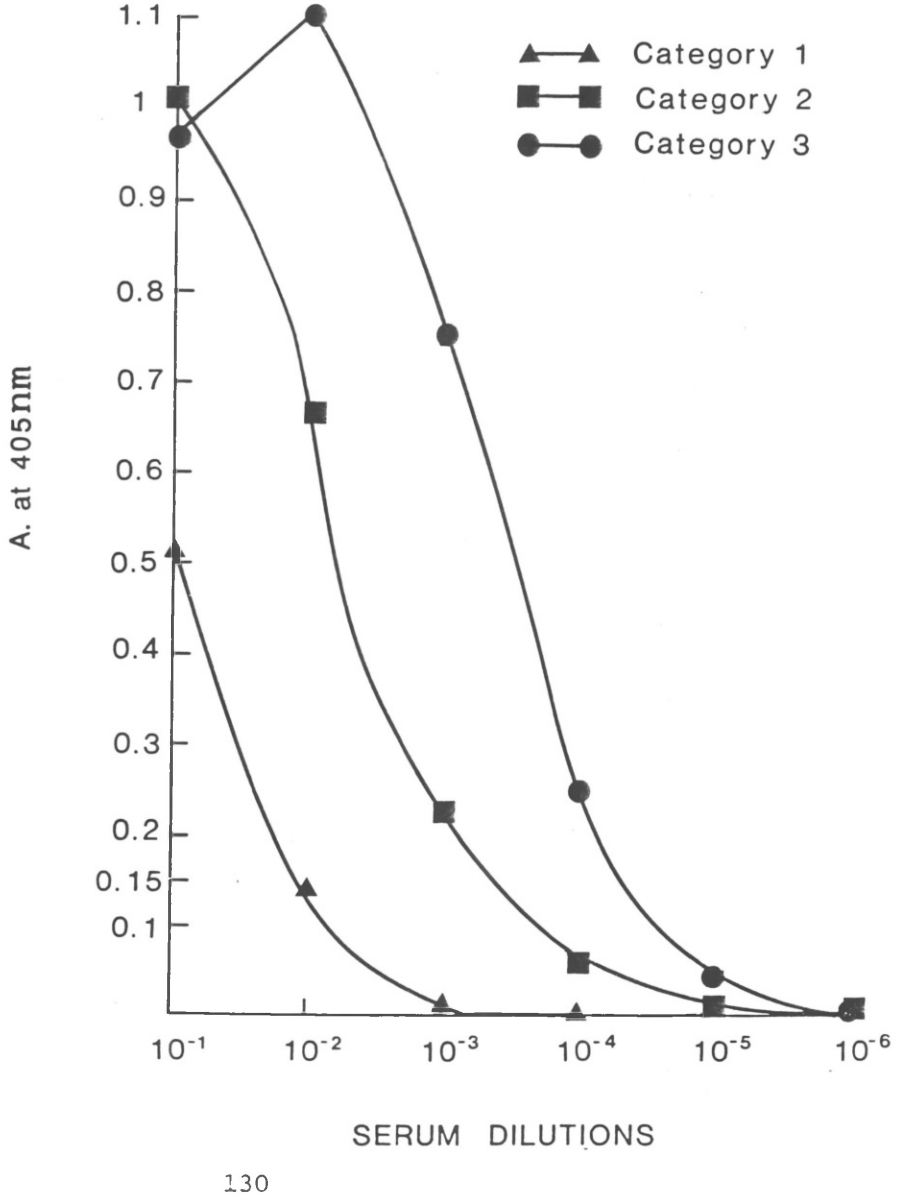
Having optimized the assay conditions, the ELISA system proved to be not only more sensitive than hybridoma targeting, but also more reproducible with respect to measuring elevated titres of anti-Ig antibodies present in human serum. For this assay, serum was obtained from peripheral blood of patients prior to antibody therapy and at a number of time intervals after therapy. It was then aliquotted, frozen and stored at -20° until assayed.

Using the ELISA system, patients' responses could be divided into three main categories. Those patients that did not respond above their pre-existing level were referred to as category 1 responders, those who had elevated post therapeutic levels of anti-murine Ig antibodies were referred to as category 2 responders and those with elevated levels high enough to produce a prozone effect (Chapter 6) were referred to as category 3 responders.

Figure Legend

Figure 17 shows the results of an ELISA, used to detect human anti-murine Ig antibodies in the serum of patient 14 at 3 seperate stages: pre therapy; post 1st therapy and post second therapy. Going from left to right across the microtiter plate, three categories of response can be seen. These are also shown graphically below the ELISA plate. In rows 3 and 4 the pre-existing category 1 response can be seen. In rows 5 and 6 an elevated anti-murine Ig response is seen after one administration of therapeutic radiolabelled murine monoclonal antibodies, this is a category 2 response. In rows 7 and 8, the same patient's response after a second therapeutic administration is shown. In this category 3 response a clear prozone effect is observable at a serum dilution of 1 in 10. All samples were assayed on a single microtitre plate.




Category 1
Category 2
Category 3
A. at 405nm
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.15
0.1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
SERUM DILUTIONS

5.3 SPECIFICITY OF HUMAN ANTI-MURINE IMMUNOGLOBULIN ANTIBODIES

In the pre-existing human anti-murine Ig response, discussed in Chapter 3, the antigenic determinants were located on the Fc portion of the murine monoclonal antibody. In order to determine the location of antigenic determinants recognised by anti-Ig antibodies present in patients with post therapeutic elevated levels, the following assay was carried out. Half of a 96-well microtiter plate (rows 1-6) were coated with intact murine Ig and the other half (rows 7-12) with the $F(ab')_2$ fragments of the IgG used in rows 1-6. The concentration of both intact IgG and $F(ab')_2$ used, was 500ng/well. Using a single plate coated with both intact IgG and $F(ab')_2$ fragments enabled the binding of patients' serum antibodies to these two antigen preparations to be compared under indentical experimental conditions.

Employing the above assay, patients who showed a moderate, (category 2) response after receiving a single therapeutic dose of between 2 and 10mg of radiolabelled monoclonal antibody, produced anti-Ig antibodies which were directed to antigenic determinants located on the Fc portion of the murine Ig. This anti-Fc activity was predominantly due to an IgM response (Table 1, Chapter 3). In these patients, no significant binding of serum antibodies to the $F(ab')_2$ portion of the murine monoclonal antibody was seen, as shown in Figure 18.

Results obtained for those patients receiving a 2 to 10mg dose of monoclonal antibody suggested that the elevated human anti-murine

Ig response was due, at least in part, to a boosting of the pre-existing response. The majority of the post first therapy anti-murine Ig antibodies were of the IgM class, although significant levels of IgG anti-Ig antibodies were also detectable.

Using serum from patients who had received a second therapeutic administration of radiolabelled murine monoclonal antibodies, the assays were repeated. In these patients, the responses found were nearly always elevated from a moderate category 2, to a pronounced category 3 type (Fig. 17). Not only had the category of response changed, but also the number of antigenic determinants being recognised on the murine monoclonal antibody had increased. Some of the determinants detected by the anti-murine Ig antibodies were now located on the $F(ab')_2$ region of the therapeutic monoclonal antibody (Figure 18).

As shown in Figure 18, the post second therapy response contained a signficant anti-$F(ab')_2$ component. This switch from anti-Fc alone after 1 therapeutic dose, to anti-Fc and anti-$F(ab')_2$, after a second dose, was consistently found. Together with this increase in the number of determinants being recognised, there was also an increase in the IgG components of the anti-murine Ig response, as shown in Figure 19.

Figure Legend

Figure 18 ELISE showing the binding of human anti-murine Ig antibodies to the intact Ig (murine) and $F(ab')_2$ fragments of the same antibody after the first (○—○) and second (●—●) therapeutic administrations of radiolabelled murine monoclonal antibodies.All samples were assayed together on a single micrtitre plate.

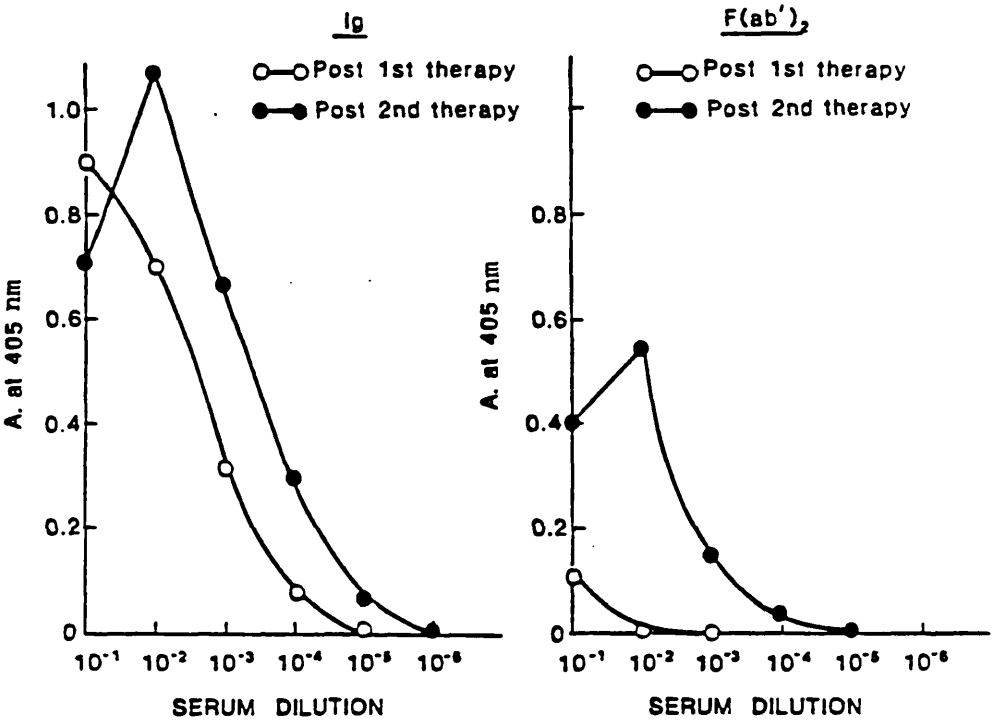
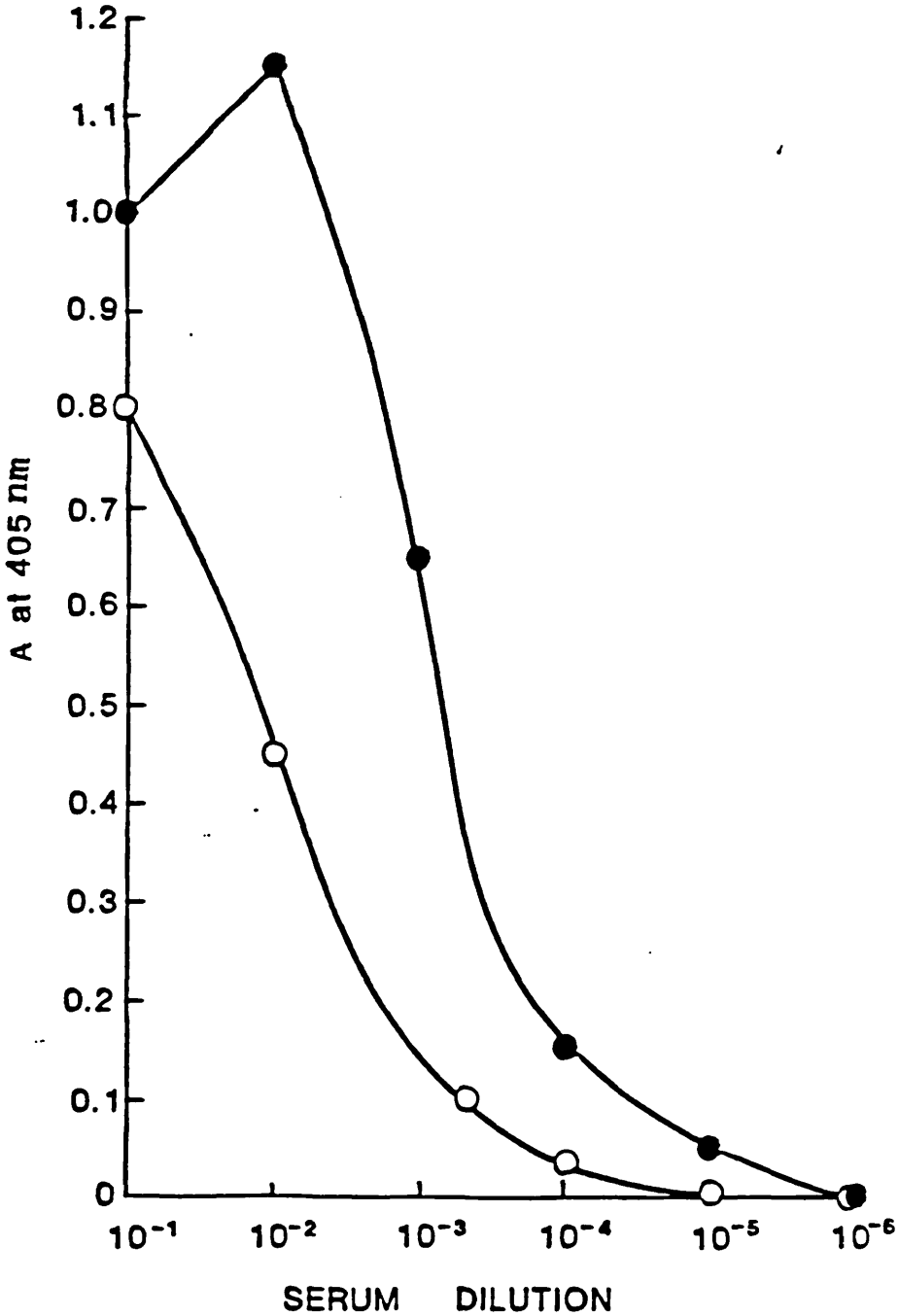

Ig
Post 1st therapy
Post 2nd therapy
A. at 405 nm
1.0
0.8
0.6
0.4
0.2
0
10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6}
SERUM DILUTION
F(ab')2
Post 1st therapy
Post 2nd therapy
A. at 405 nm
0.8
0.6
0.4
0.2
0
10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6}
SERUM DILUTION

Figure Legend

Figure 19 shows the IgM (○—○) component of the human anti-murine Ig response (●—●) made by a patient after receiving two intraperitoneal administrations of radiolabelled murine monoclonal antibodies for the treatment of advanced ovarian cancer.

IgM anti-murine Ig antibodies were detected using a u-chain specific secondary reagent. Total anti-murine Ig antibodies were detected using a secondary reagent that binds to both IgM and IgG.Allsamples were assayed together on a single microtitre plate.


1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
A at 405 nm
10⁻¹
10⁻²
10⁻³
10⁻⁴
10⁻⁵
10⁻⁶
SERUM DILUTION

5.4 QUANTITATION OF THE HUMAN ANTI-MURINE IMMUNOGLOBULIN RESPONSE

As shown in Figure 18 the serum from patients receiving a single therapeutic dose of radiolabelled murine monoclonal antibody, when assayed 15 days post therapy, contained very little antibody directed to antigenic determinants on the $F(ab')_2$ portion of the murine Ig molecule. In contrast, serum taken from the same patient 15 days after a second administration, contained very significant levels of antibody binding to the $F(ab')_2$ portion.

In an attempt to correlate the absorbance results with µg/ml, i.e. to quantitate these responses, the human anti-murine Ig antibody was separated from one patient's post second therapy serum by affinity chromatography, using mouse monoclonal antibody bound to cyanogen-bromide activated sepharose beads. After purification, the anti-mIg antibody concentration was determined and a standard curve constructed by coating the wells in 2 rows of a microtiter plate with known amounts of anti-murine Ig antibody, while half of the remaining wells were coated with the intact murine IgG and the other half with the $F(ab')_2$ fragments of the same antibody.

Using this microtiter plate, containing the standard curve, the patient's post first and second therapy serum was reassayed. From this experiment a significant correlation ($r=0.985$, $P<0.001$) between absorbance at 405nm and concentrations over a range of 0.1-10µg/ml of human anti-murine Ig was found, as shown in Figure 20A. The purity of the anti-murine Ig fraction of this patient's

serum was tested by using SDS-PAGE, as described in Materials and Methods. The purified fraction was run beside two dilutions of the patient's post first and second therapy serum samples, also shown in Figure 20A.

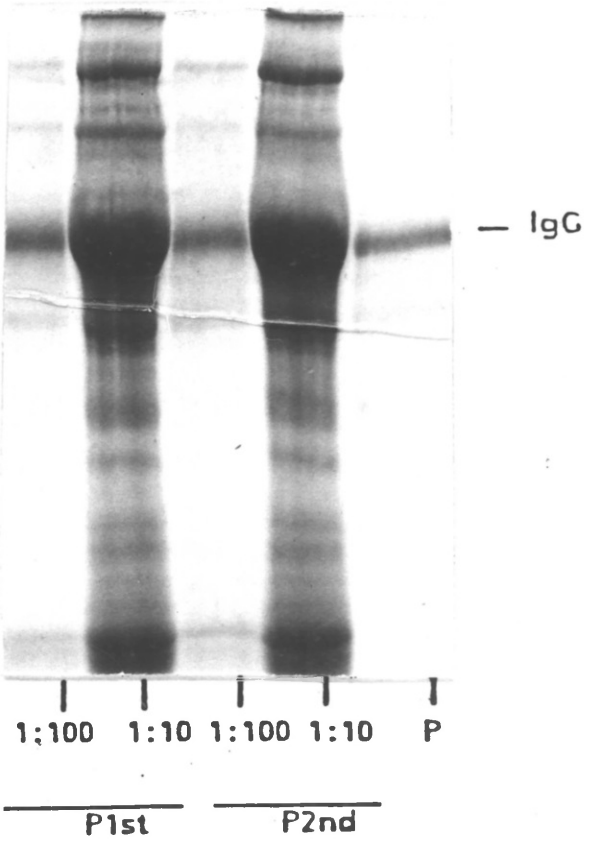
From the equation for a straight line (Y=C+MX), the concentrations of anti-IgG and anti-F(ab')$_2$ antibodies present in the patient's post first and second therapy serum were calculated. It was found that the post first therapy serum contained 600ug/ml of anti-murine IgG antibody and 12µg/ml of anti-F(ab')$_2$ antibody. The post second therapy serum contained 5.67mg/ml of anti-murine IgG antibody and 524µg/ml of anti-F(ab')$_2$ antibody. When the experiment was repeated no significant difference in these values was obtained.

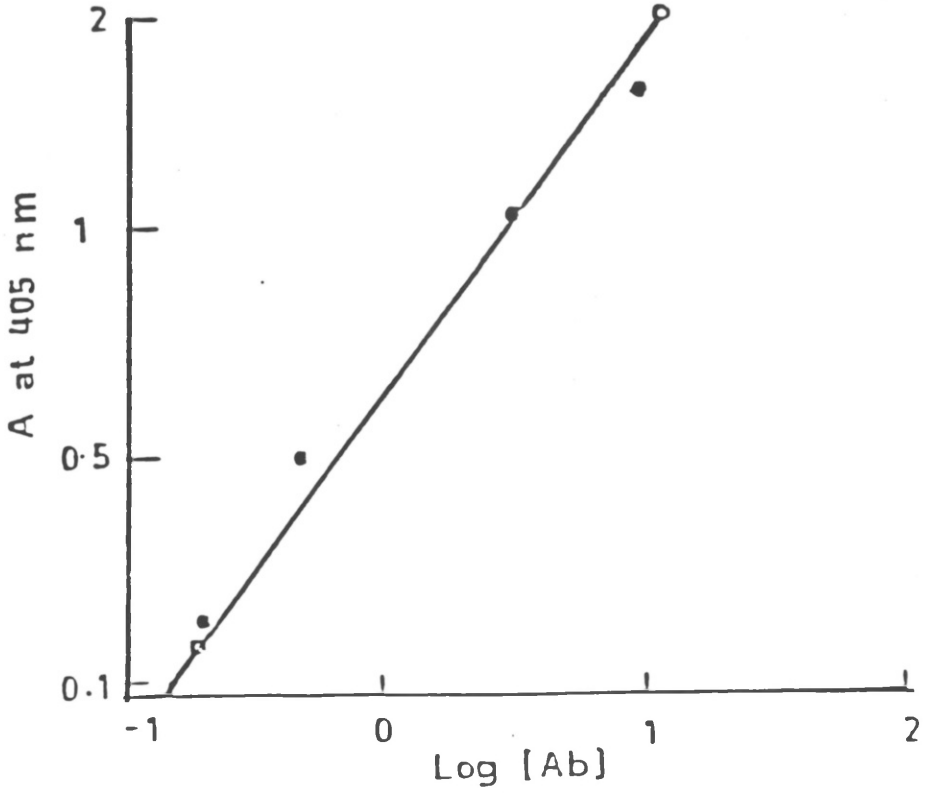
From this quantitative assay the conclusion drawn from the experiments shown in Figure 18 were confirmed. That is, there are only low levels of human anti-murine Ig antibodies with specificity for F(ab')$_2$ fragment of the murine antibody in the serum of patients after the first therapy, even though, significant levels of anti-Ig directed to determinants on the Fc portion of the murine immunoglobulin are present. This patient's pre-therapy serum was found to contain 52µg/ml of anti-murine IgG antibody. None of this anti-murine IgG antibody was found to react with the F(ab')$_2$ fragments.

Figure Legend

Figure 20A shows the bands obtained by 7.5% SDS-PAGE for a patient's post first therapy (P1st) and post second therapy (P2nd) serum at 1 in 100 and 1 in 10 dilutions. In the last track a single band which represents the affinity purified human anti-murine Ig antibody is seen (P).

Below, is a standard curve constructed by plotting the various concentrations of the purified human anti-murine Ig antibody used, Log [Ab], against absorbance at 405nm.


— IgG
1:100
1:10
1:100
1:10
P
P1st
P2nd


A at 405 nm
2
1
0·5
0·1
-1
0
1
2
Log [Ab]

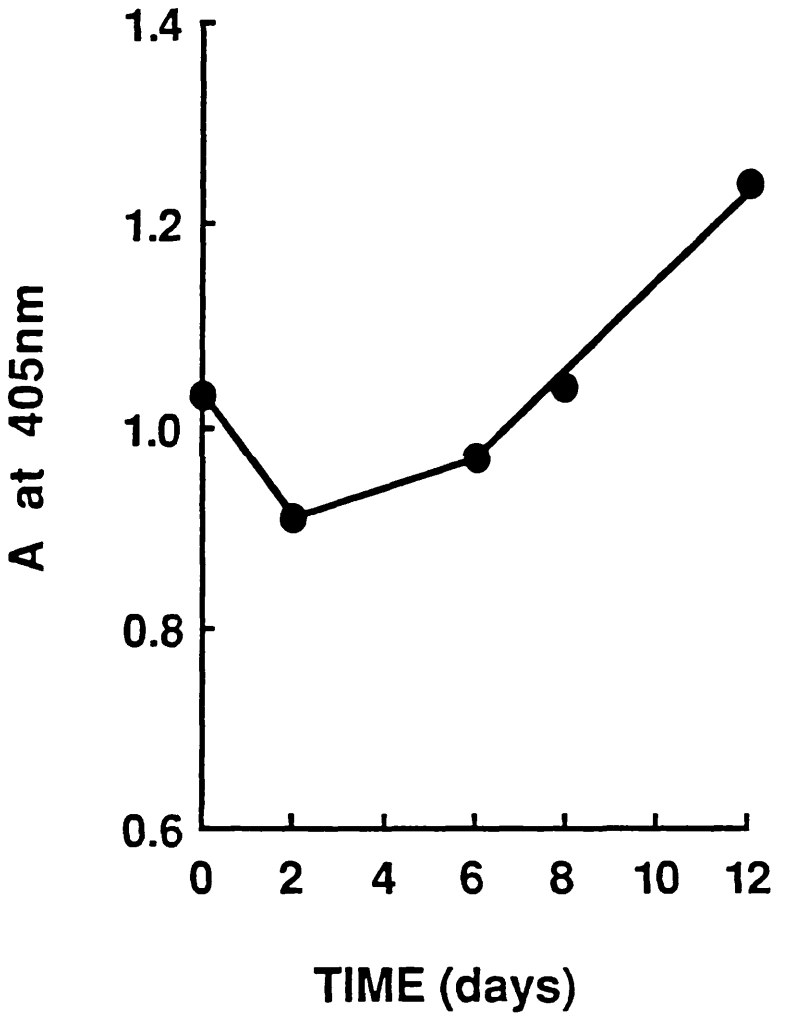
5.5 KINETICS OF THE ANTI-MURINE IMMUNOGLOBULIN RESPONSE

In Chapter 3 the role of rheumatoid factors in the pre-existing human anti-murine Ig response was discussed. In this chapter the elevation of the IgM anti-murine IgG response after a single therapeutic administration of radiolabelled murine monoclonal antibodies, has been discussed. Together, these two observations suggest that rather than obtaining a primary response to the first dose of murine monoclonal antibodies, a boost in the pre-existing IgM anti-murine IgG response is obtained.

Patients receiving a single therapeutic dose of murine monoclonals of between 2 and 10mg, consistantly showed the category 2 response at seven to fifteen days after administration. The response in patients receiving a second administration was rapid, elevated levels of anti-Ig antibodies being seen at 4 days post administration, and the levels of human IgG anti-murine IgG increased by day 10. This rapid IgM/IgG response is consistent with a secondary immune response. What was also noticable in those patients receiving a second administration was an initial drop in circulating human anti-murine Ig antibodies, in the first 24 to 48 hours, presumably due to complexing with the murine monoclonal antibody, Figure 20B.

Figure Legend

Figure 20B shows the ELISA data obtained for a patient's serum obtained after a second administration of radiolabelled monoclonal antibody. This figure shows that within the first two days post injection there is an initial drop in the amount of assayable anti-murine antibody ,suggesting the formation of immune complexes between the murine monoclonal antibody and the patients anti-murine Ig antibodies.


1.4
1.2
1.0
0.8
0.6
A at 405nm
0
2
4
6
8
10
12
TIME (days)

5.6 DISCUSSION

During the course of studying the humoral immune responses of patients receiving radiolabelled murine monoclonal antibodies, the anti-mIg response resulted in only 1 of 25 patients developing a hypersensitivity reaction. In all the 25 patients, pre-existing antibodies have been found, and after therapy, the level of anti-murine Ig antibodies was elevated in 21 of the 25 patients.

Other studies have shown that nearly all patients who possess pre-existing human anti-murine Ig antibodies develop an elevated post-therapeutic response. In contrast, in patients with chronic lymphocytic leukaemia (CLL), pre-existing anti-murine Ig antibodies have not been detected, and these patients fail to develop anti-Ig responses following therapy (Schroff et al., 1985). However CLL patients are hypogammaglobulinaemic and incapable of mounting normal humoral responses.

Consistently, a specificity for antigenic determinants on the Fc portion of the murine Ig was observed after the first therapy. This result suggests a boosting of the pre-existing response. The elevated levels of IgM anti-mIg after the first therapy could also, in part, be due to a primary response to unshared (mouse specific rather than human-mouse shared) antigenic determinants on the murine antibody.

Elevated levels of human IgG anti-murine Ig were found in all those patients receiving a second administration of murine antibodies. This result indicates class-switching and, as discussed in Chapter 1, cellular and humoral immune responses.

Although many groups have observed the development of anti-murine Ig responses, their reports on the effects of such responses on the efficacy of therapy are conflicting. In one report, 4 out of 7 patients with T-cell lymphoma were treated with anti-Leu-1 monoclonal antibody; in 3 of these 4 patients the development of anti-murine Ig antibodies was said to contribute to tumour escape from therapy (Miller et al.,1983). Similarly, patients with T-cell lymphoma who were treated with T101 monoclonal antibody and who developed anti-Ig antibodies were said to show a lack of response to therapy (Dillman et al., 1983).

In other studies the development of anti-Ig responses in patients receiving murine monoclonal antibodies has not been thought to reduce the efficacy of therapy (Foon et al., 1984). In those patients observed in this study one could not say with any degree of certainty whether the anti-murine Ig response resulted in any loss of therapeutic effect, although it would be less likely to as the antibodies were administered intraperitoneally rather than intravenously where there would be a higher risk of complexing and hence reduced tumour targeting.

In renal allograft recipients receiving monoclonal antibody OKT3, the effect of sensitization has been clearly seen. In these patients their anti-murine Ig response was seen to completely neutralise the therapeutic effect of the monoclonal antibody. Interestingly, only the anti-Ig antibodies of the IgG class were seen to exert a clear neutralizing effect on the immunosuppresive capacity of OKT3, possibly because of the low affinity of IgM antibodies (Chatenoud et al., 1986).

CHAPTER 6

THE PROZONE EFFECT

6.1 INTRODUCTION

From the results presented in Chapter 5 it can be seen that characteristic of the pronounced category 3 type response curve, is a decrease in antibody binding at low serum dilutions (1 in 10) in the ELISA system. This phenomenon is known as the prozone effect, and has been observed by a number of groups studying the humoral response of patients to various antigens.

In a study of the parameters affecting solid phase radioimmunoassay quantitation of antibodies to meningococcal antigens, investigators found that the binding curves obtained with most test sera exhibited pronounced prozones (Zollinger et al., 1976). After a number of further studies, this group's explanation for this prozone phenomenon was that there were an excess of antigenic sites present on the surface of the antigen coated tubes used in their assays.

Other studies have given rise to alternative explanations for such a phenomenon. In one study on the parameters affecting the assaying of serum antibodies to a mutant of Salmonella minnesota, the investigators concluded that the "easiest explanation of this prozone effect is that the early IgG antibodies are not able to resist the washing procedure when crowded and not ideally aligned on the antigen site as happens at low serum dilutions, although they are able to resist washing when they are not crowded and can properly align themselves on the antigenic site" (Bruins et al., 1978).

The non-alignment of antibody with antigen theory was supported by work undertaken to investigate the role of the IgM component of patients sera (DeSavigney and Voller, 1980). From this study it was shown that removal of the IgM component of patients' sera by affinity chromatography resulted in the removal of the prozone effect even when serum was assayed at low dilutions. In assays carried out to determine the isotypes of antibodies produced by patients receiving radiolabelled murine monoclonal antibodies (Chapter 5) a large component of the anti-murine Ig antibody pool was found to be of the IgM isotype. In this chapter the kinetics, effect of serum IgM depletion and significance of the prozone effect observed when assaying patients' post-therapy serum by the ELISA method are discussed.

6.2 KINETICS OF PROZONE DEVELOPMENT

Using the enzyme-linked immunosorbant assay, the development of a prozone effect was observed in two groups of patients receiving radiolabelled monoclonal antibody therapy. The first group contained those who had received two therapeutic administrations, of between 2 and 10 milligrams for each of the two therapies. The second group were patients who had received only a single therapeutic dose of radiolabelled monoclonal antibody; however, these patients all received at least double the amount of murine monoclonal, between 25 and 30 milligrams, as compared to those patients in the first group. The kinetics of prozone development for the two groups were quite different.

In assays using serum from patients in the first group (those receiving two administrations) a prozone developed within 4 to 10 days of receiving the second therapeutic administration. For patients in the second group (i.e. those receiving a single large therapeutic dose) the prozone effect was only seen in assays carried out using sera taken between 12 and 18 days. In Figure 21 these two distinctly different kinetics can be seen; the results for a patient from group one shows the prozone developed by six days (Figure 21A), whereas those for a patient from group 2 shows it developing between days 16 and 17 (Figure 21B).In order to determine the exact serum dilution at which the prozone effect was lost, two patients' sera were assayed using both ten-fold and doubling dilutions (Figure 21C). As seen in Figure 21C, using doubling dilutions, the serum dilution at which the prozone effect is lost is between 1 in 80 and 1 in 100 for patient 1 and between 1 in 60 and 1 in 80 for patient 2. Using ten fold dilutions the prozone effect is seen to be lost at 1 in 100 dilution of both patients' sera.

This difference in the kinetics of prozone development was observed for eight patients in group one and six patients in group two. The mean time for prozone development for group one patients was 7 days, after receiving the second therapeutic administration of radiolabelled murine monoclonal antibodies and for patients in group two the mean time for prozone development was 15 days, after a single therapeutic administration of between 25 and 30 milligrams of radiolabelled murine monoclonal antibody. Other variables such as the route of antibody administration

(intraperitoneal), period of administration (30-60 minutes), volume of saline used to infuse the radiolabelled antibodies (500-1000ml), and the class of murine immunoglobulin (IgG1) were identical for all patients in groups one and two.

Figure Legend

Figure 21 shows the difference in time taken for the development of a prozone effect between patients receiving two therapeutic administrations of radiolabelled antibody each of between 2 and 10 milligrams (Figure 21A), and patients receiving a single therapeutic dose of between 25 and 30 mg (Figure 21B).

FIGURE 21A

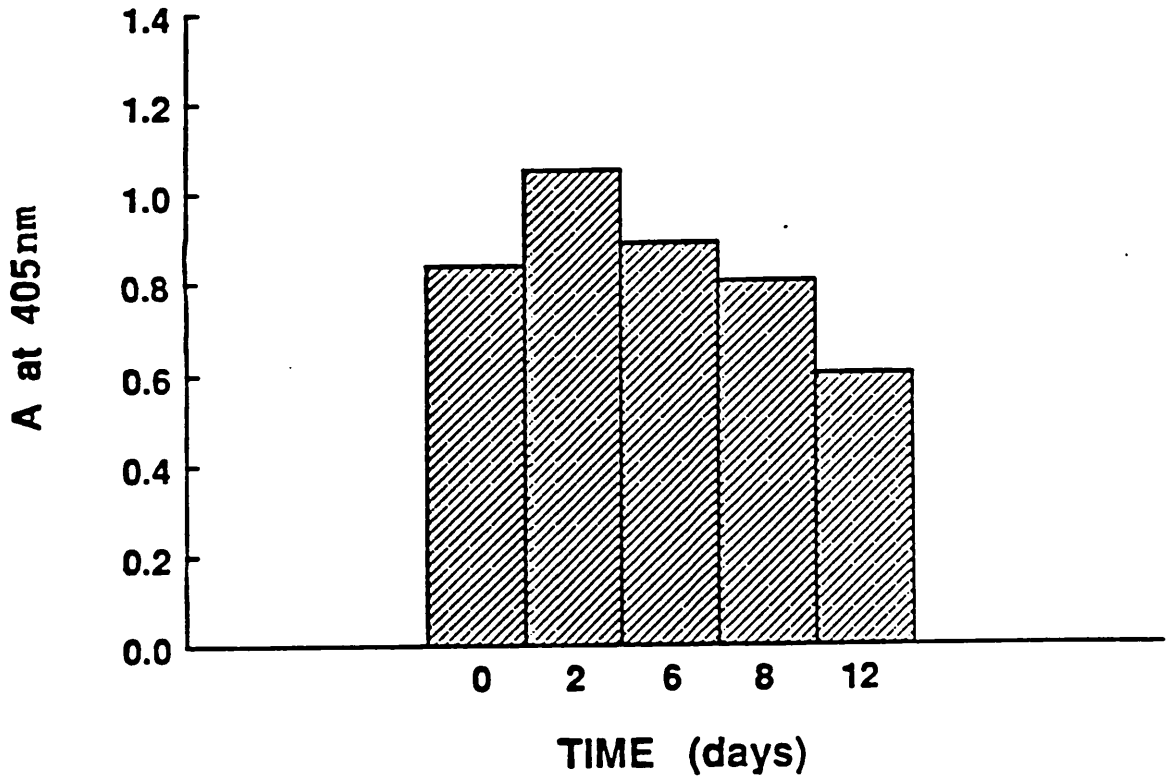


FIGURE 21B

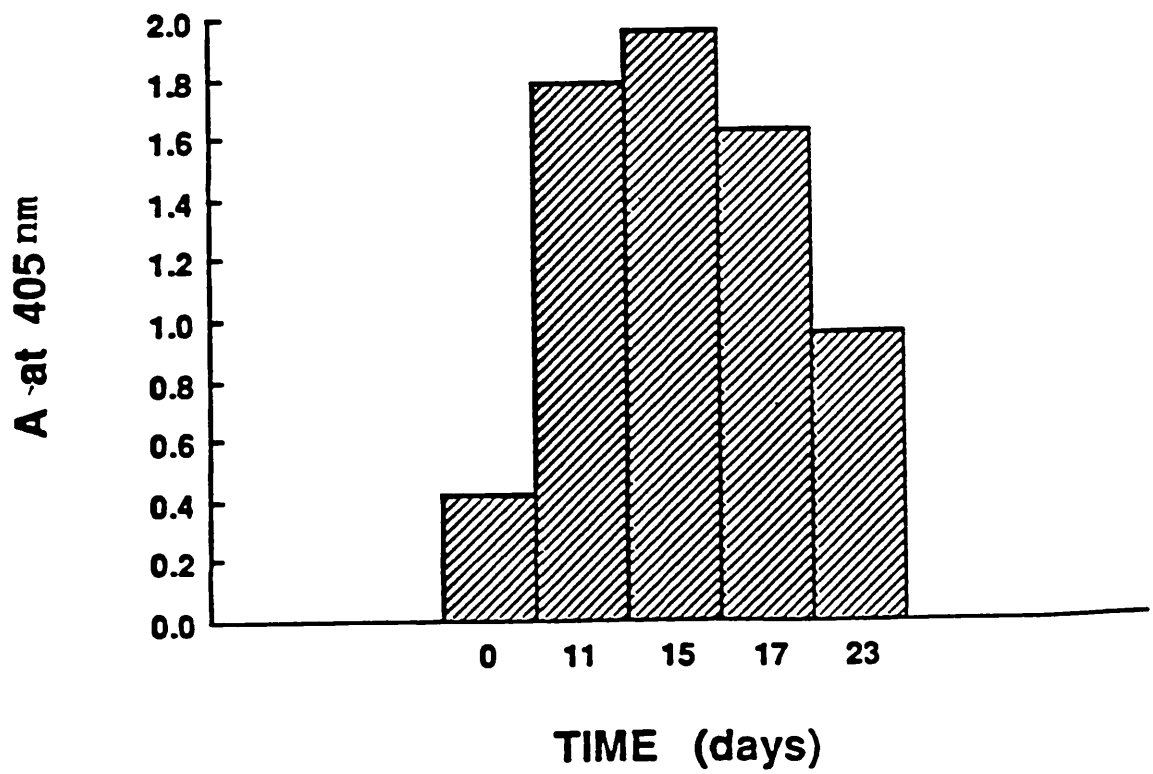
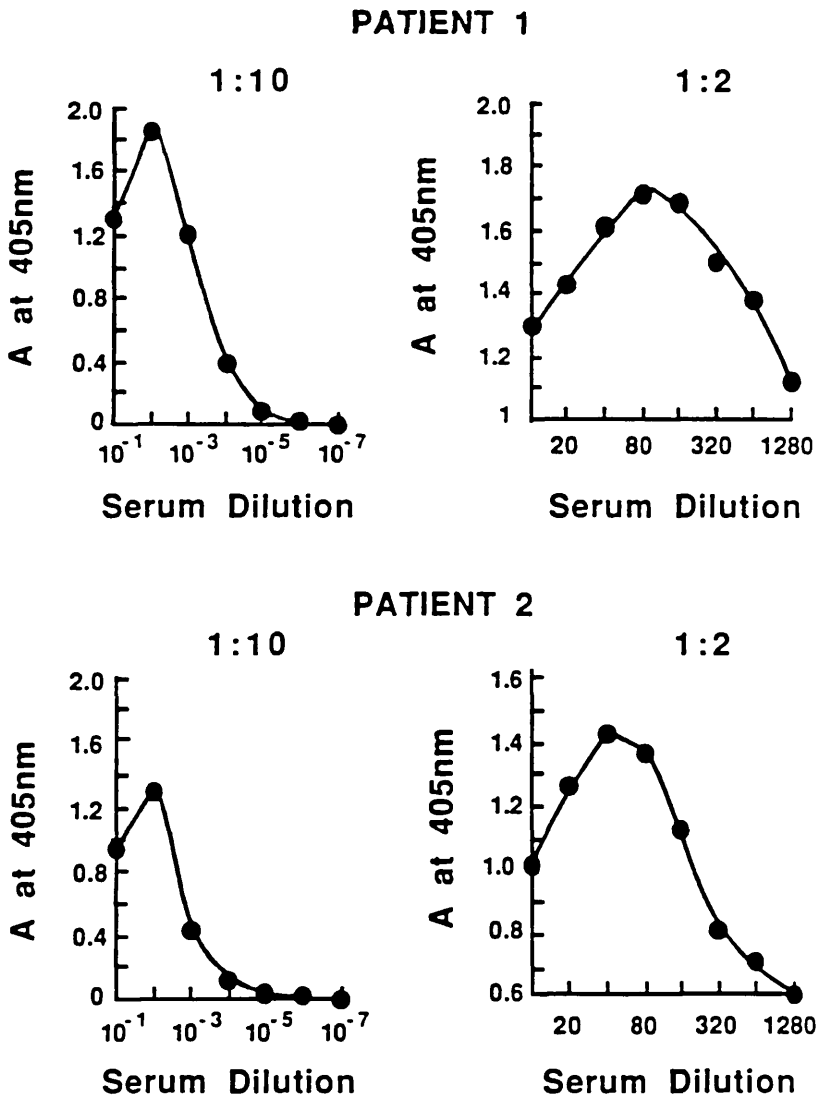


Figure Legend

Figure 21C shows that when 10-fold dilutions of patients' sera are used in the ELISA assay the prozone effect is seen at between 1:10 and 1:100 dilution. The exact point at which the prozone effect is lost is illustrated more accurately by using doubling dilutions of patients' sera.


PATIENT 1
1:10
A at 405nm
2.0
1.6
1.2
0.8
0.4
0
10^{-1} 10^{-3} 10^{-5} 10^{-7}
Serum Dilution
1:2
A at 405nm
2.0
1.8
1.6
1.4
1.2
1
20 80 320 1280
Serum Dilution
PATIENT 2
1:10
A at 405nm
2.0
1.6
1.2
0.8
0.4
0
10^{-1} 10^{-3} 10^{-5} 10^{-7}
Serum Dilution
1:2
A at 405nm
1.6
1.4
1.2
1.0
0.8
0.6
20 80 320 1280
Serum Dilution

6.3 EFFECT OF SERUM IgM DEPLETION

In a series of experiments conducted by DeSavigney and Voller (1980) it was found that depletion of the IgM component of human serum resulted in a loss of the prozone effect obtained with whole serum at low dilutions. In order to study the role of this isotype in the prozone effect observed when screening patients' post therapy sera by ELISA for anti-murine Ig responses, the IgM component was separated from the IgG using an affinity column.

Serum (5ml) taken from a patient ten days after a second administration of radiolabelled murine monoclonal antibody and showing a pronounced prozone effect by ELISA, was affinity purified on an activated Bio-gel column to which mouse Ig had been coupled. 5ml of serum was loaded and the column was equilibrated with phosphate buffered saline, pH 7.4. Under these conditions the human IgG anti-murine Ig will bind while the lower affinity IgM anti-murine Ig will not (DeSavigney and Voller, 1980).

The bound IgG human anti-murine Ig was eluted using 3 molar magnesium chloride, and the protein containing fractions pooled and dialyzed. The dialyzed material was then concentrated, Millipore filtered and the protein concentration determined. To determine the purity of the affinity isolated human IgG anti-murine Ig it was analysed by SDS-PAGE using a 3-15% gradient gel. In this purification assay a major single band of approximately 150KD molecular weight, corresponding to IgG was

found. No IgM was detectable even though a gradient gel was used (Figure 22A).

After purification the human IgG anti-murine Ig was assayed by ELISA in parallel with a sample of the intact serum. From this experiment it was clear that the purified IgG fraction did not give a prozone effect when assayed against either intact murine Ig or $F(ab')_2$ fragments, as shown in Figure 22B. By contrast the pre column serum showed a marked prozone effect against both the intact and $F(ab')_2$ fragments of murine IgG.

Figure Legend

Figure 22A shows, in tracks 1-3 , the 150KD molecular weight bands obtained after affinity purification of a patients' serum using an activated murine Ig coupled Bio-gel column. In tracks 4-6 the bands of a purified monoclonal antibody, used as a molecular weight marker, are seen.

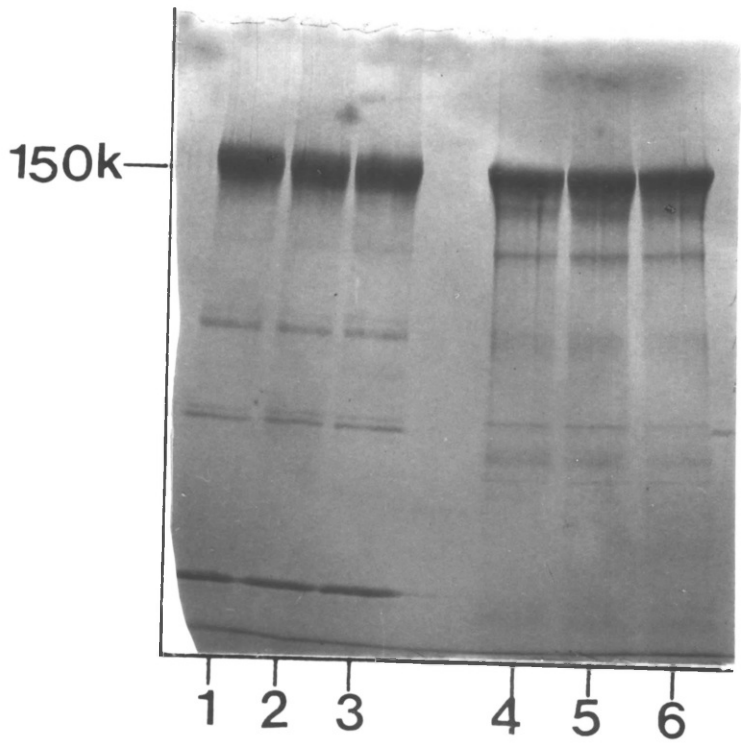
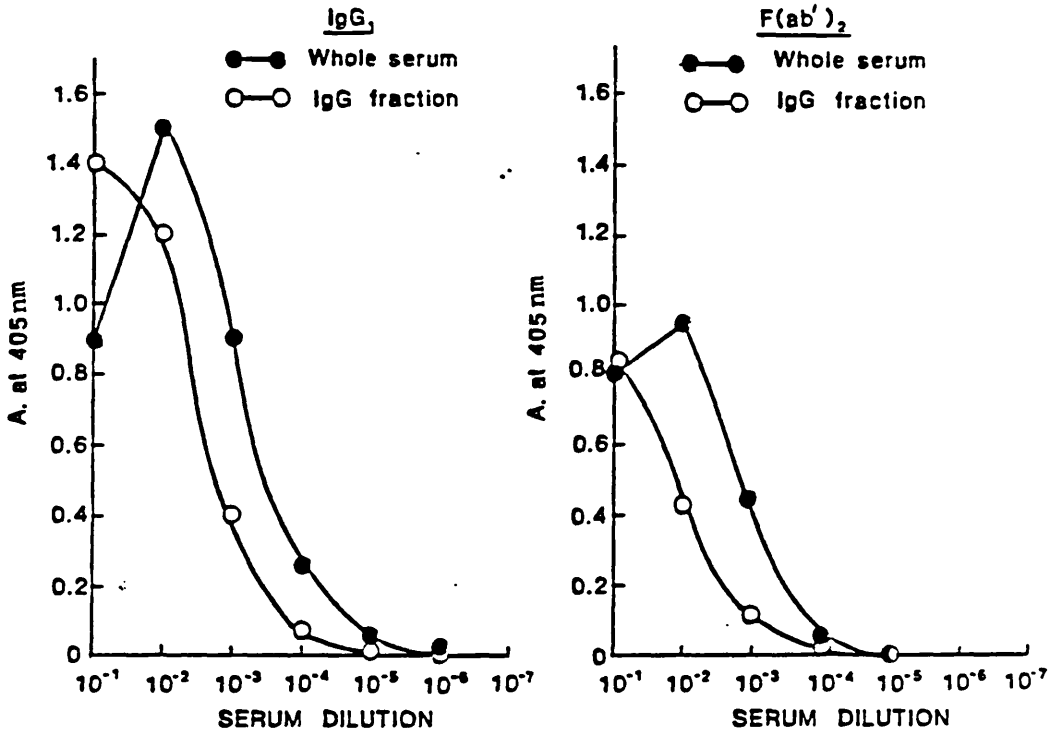

150k
1
2
3
4
5
6

Figure Legend

Figure 22B shows that the affinity purification of the IgG component from a patient's 10 day post second therapy serum sample (and hence removal of the IgM componant), results in a loss of prozone effect when assayed against either whole murine monoclonal IgG or the $F(ab')_2$ fragments of that monoclonal. All samples were assayed on a single microtitre plate.


IgG1
Whole serum
IgG fraction
A. at 405nm
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
10-1 10-2 10-3 10-4 10-5 10-6 10-7
SERUM DILUTION
F(ab')2
Whole serum
IgG fraction
A. at 405nm
SERUM DILUTION

6.4 SIGNIFICANCE OF THE PROZONE EFFECT

As shown in Figure 22B, when whole serum containing both IgG and IgM human anti-murine Ig is assayed against murine IgG a pronounced prozone effect is seen. This prozone effect is present only at low dilutions of a patient's serum where the antibody concentration is high.

In order to determine the relevance of the prozone effect on assaying different dilutions of a patient's serum, taken at various time intervals before and after therapy, the following experiments were conducted. Serum was obtained from a patient just prior to receiving a third therapeutic administration of radiolabelled murine monoclonal antibodies. This patient already had a significant anti-murine Ig response. After administration of the third therapeutic dose, serum samples were obtained at various time points, as shown in Figure 23.

The pre-third therapy together with the post-third therapy serum samples were assayed for anti-Ig activity at two serum dilutions, 1 in 10 and 1 in 1000. The results obtained from the 1 in 1000 dilution showed that the level of anti-murine Ig antibody dropped slightly, just after administration, and then, after 48 hours increased. The results obtained for the same serum samples assayed at a 1 in 10 dilution (the dilution at which the prozone is seen), showed a mirror image of these results, as shown in Figure 23.

In addition to testing the pre and post-third therapy serum samples for anti-Ig activity they were also assayed for their total human IgM and IgG content. As shown in Table 4, neither the IgM nor IgG content of the five serum samples collected showed any significant change in concentration, irrespective of their anti-Ig activity. This result shows that during the human anti-murine Ig response, the concentration of the two isotypes, IgM and IgG present in the total serum Ig pool does not noticeably increase: only the percentage of these two isotypes reacting with antigenic determinants located on the murine Ig increases.

Figure Legend

Figure 23. Serum was obtained from a patient just before (time 0) and at 2 day intervals after the administration of a third therapeutic dose of monoclonal antibody, HMFG1. Serum samples diluted to 1:1000 (●—●) show the true response, unlike the sample used at 1:10 dilution which are lower due to the prozone effect (○—○).

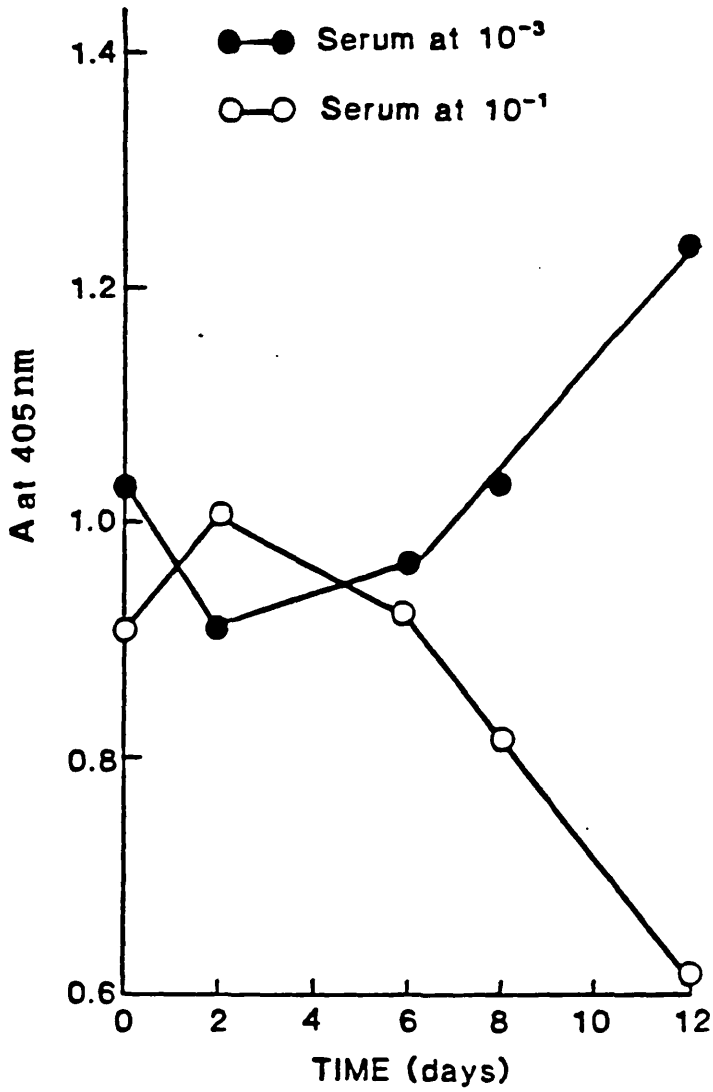

Serum at 10^{-3}
Serum at 10^{-1}
A at 405 nm
1.4
1.2
1.0
0.8
0.6
0
2
4
6
8
10
12
TIME (days)

TABLE 4

Table 4a shows sequential analysis of a patients serum IgG and IgM levels after radiolabelled antibody therapy with HMFG1.

Table 4a

TIME (DAYS)	TOTAL SERUM IgG (g/L)	TOTAL SERUM IgM (g/L)
0	8.5	0.95
2	8.0	0.95
6	8.0	0.90
8	8.0	0.95
12	8.5	0.85

Table 4b shows the binding (absorbance at 405nm) to murine Ig of a 1:10 and 1:1000 dilution of patient's serum, with time after administration of radiolabelled HMFG1 . All samples were assayed by ELISA on a single plate.

Table 4b

Absorbance at 405nm

TIME (DAYS)	SERUM DILUTION 1:10	SERUM DILUTION 1:1000
0	0.914	1.030
2	1.080	0.910
6	0.926	0.966
8	0.818	1.034
12	0.616	1.238

6.5 DISCUSSION

A prozone effect was characteristic of all enzyme-linked immunosorbant assay tests on serum from patients with high levels of anti-murine Ig antibodies.

This probably results from an increase in monovalent (only one binding site per Ig molecule being occupied by antigen) binding of antibody to antigen, imposed by the high antibody concentration present in the microtiter plate wells, at low serum dilutions.

This effect would be more extreme for the IgM component since IgM antibodies are generally of lower affinity (strength of monovalent binding to antigen) than IgG antibodies, and the difference in overall affinity of pentavalent versus monovalent binding to antigen is considerable (in the region of 10^6), whereas for IgG the difference in overall affinity between divalent versus monovalent is much less (approximately 10^3). The consequence of very low binding affinities is that such antibodies will be easily washed off the antigen coated plate during the assay hence giving spuriously low results at high antibody concentrations, i.e. the prozone effect.

This is supported by the observation that removal of a patient's serum IgM anti-murine Ig antibodies also removed the prozone effect. However, IgM depletion would also obviously reduce the total anti-murine Ig concentration, which in itself would

contribute to loss of the prozone. Whatever the exact reason for the prozone, the practical importance of this phenomenon is clear. In order to evaluate a patient's true humoral response to radiolabelled (xenogeneic) monoclonal antibody therapy full titrations of patient's serum must be assayed in order to avoid the possibility of falsely low values of anti-murine Ig activity at the higher serum concentrations. Finally, the fact that a prozone occurs suggests that the ELISA test is probably affected by antibody affinity, and therefore any results obtained would be an underestimation of low affinity antibodies, mainly IgM, which means that any attempt to correlate ELISA absorbance values at a set serum dilution with a set amount of antibody protein is hazardous at best. It could only be done at high serum dilutions where a drop in the absorbance value from one dilution to the next correlated with the actual decrease in the number of antibody molecules available for binding with antigen. Hence a titration curve is necessary.

Some workers have suggested that numerical values given for ELISA results are not a gravimetric measure of antibody in μg or mg, and hence data obtained with an ELISA should be expressed as ELISA units rather than μg/ml (Butler et al., 1977). Whilst it is true for human sera which contain a wide range of antibodies with different affinities for a given antigen, it is not the case for monoclonal antibodies which are of a single affinity.

CHAPTER 7

THE ANTI-IDIOTYPIC RESPONSE

7.1 INTRODUCTION

Lymphocytes recognise antigens by receptors on their surface membranes. In the case of B cells, these are immunoglobulin molecules, and for a single B cell the immunoglobulin surface receptors are all of a single specificity, with identical variable domains (Chapter 1).

The structures (resulting from amino acid sequence and conformation) on antigen molecules which lymphocytes recognise are called antigenic determinants, or epitopes. The antibody binding site which recognizes a given epitope is termed a paratope; this together with determinants outside the binding site displays epitopes which may be recognized by the paratopes of other antibody molecules. The variable domain epitopes of a given immunoglobulin molecule are termed idiotopes, and the collection of idiotopes specific to the variable regions of that immunoglobulin molecule comprise its idiotype. It was proposed by Jerne (1974) that through idiotopes, receptor specific regulation of the immune system may be achieved - the "network theory" of the immune system.

Jerne then proposed that "within the B cell repertoire of variable domains we distinguish a paratopic and an idiotypic repertoire. The paratopic repertoire is complete, degenerate and redundant: any epitope in the universe can be recognised by many different paratopes, and a given paratope can recognise many different similar epitopes. Since paratopes and idiotopes of one

variable domain are determined by the same pair of variable regions of an antibody light chain and heavy chain, it seems plausible that the idiotopic and paratopic repertoires are of comparable size. Hence, within the same immune system, every idiotope may be recognised by several different antibody molecules and every antibody molecule may recognise several different idiotopes. The immune system thus consitutes a network of paratope-idiotope interactions".

Since the network theory was first postulated by Jerne, a number of investigators have provided experimental evidence for it. Suppression by anti-idiotypic suppressor T cells was documented by Bona and Paul (1979), who demonstrated the natural presence of T-suppressor cells specific for the MOPC-460 idiotype by increasing the 460 idiotope in the anti-trinitrophenyl plaque-forming-cell response through removal of 460-binding cells on 460-coated dishes. Anti-idiotypic T help has been observed in the response to streptoccocal group A by Eichmann et al, (1978). The crosslinkage by anti-idiotypic antibodies of T helper cells and B cells which both had the same idiotype was used by Eichmann (1978) to explain the observation that the A5A idiotype could be stimulated in vitro. However, the relevance of this finding might be questioned by the fact that attempts to induce stimulation in vivo were not successful.

In Chapter 5 the immune response of patients to antigenic determinants present on not only the Fc, but also the $F(ab')_2$ portion of murine Ig was discussed. The response by patients to

the $F(ab')_2$ portion of the murine Ig raised the possibility of an anti-idiotypic response since each injection of monoclonal antibody would contain a relatively high immunogenic dose of idiotypic determinants.

7.2 RESPONSES TO IDIOTYPICALLY UNRELATED MONOCLONAL ANTIBODIES

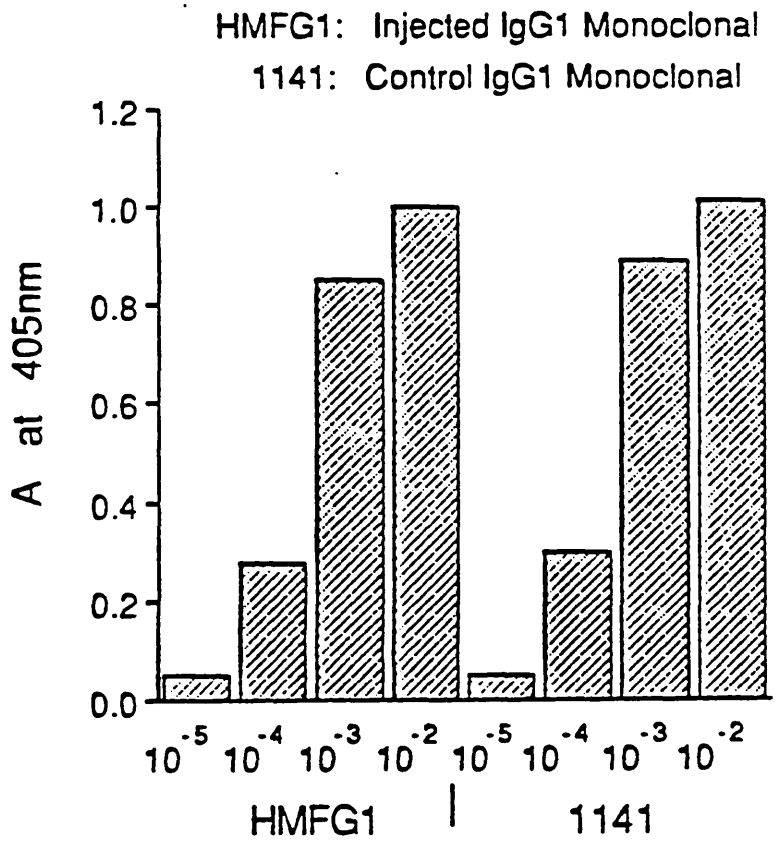
In order to determine the presence of serum antibodies having specificity for variable region determinants (idiotopes) present on the murine monoclonal antibody administered to patients for therapeutic purposes, the following experiment was carried out. Wells in rows 1-6 of a 96 well microtiter plate were coated with 500ng of the relevant (administered) HMFG1 murine monoclonal antibody, the wells in rows 7-12 were coated with a equal amount of an antibody which was isotypically related, but idiotypically unrelated. This enabled a comparison of anti-murine Ig antibody binding to the two immunoglobulins to be compared under identical experimental conditions.

Serum from patients receiving HMFG1 therapy was titrated into the murine Ig coated plates, and the binding of any serum antibodies to the relevant (same antibody as was administered) and idiotypically unrelated monoclonal antibodies compared.

In the first series of experiments, patients' pre-therapy and post-first therapy sera were compared, using the assay described above. Out of twenty-seven patients' sera tested, not one showed elevated binding to the administered monoclonal antibody, over that to the idiotypically unrelated monoclonal antibody (Figure 24). No increased binding was seen, irrespective of the amount of murine monoclonal antibody administered or of the level of pre-existing anti-murine Ig found. From these experiments it was apparent that, either antibodies to idiotypic determinants on the variable domains of the murine monoclonal antibodies were not being generated, or if they were, they were of low affinity and therefore unable to withstand the assay washing procedures. The inability to detect anti-idiotypic antibodies was consistent with the finding that very low amounts, if any, of antibodies to the $F(ab')_2$ portion of the murine monoclonal antibodies administered could be detected after a single therapeutic dose (Chapter 5).

Figure Legend

Figure 24 shows the binding of serum anti-murine Ig antibodies from a patient who had received a single therapeutic administration of radiolabelled HMFG1 to the relevant monoclonal antibody (HMFG1) and to an idiotypically unrelated monoclonal antibody (1141) of the same IgG1 isotype. Both assays were performed on a single plate.


HMFG1: Injected IgG1 Monoclonal
1141: Control IgG1 Monoclonal
A at 405nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10-5 10-4 10-3 10-2 10-5 10-4 10-3 10-2
HMFG1
1141

Employing the same assay system used previously to screen patients pre and post-first therapy sera, a second series of experiments was carried out to assay the serum of 5 patients (Table 5) who had received multiple administrations (two or more) of radiolabelled murine monoclonal antibodies. From this second series of experiments it was found that after two or more administrations, an increase in binding to the administered antibody could be seen, over that to the isotypically identical but idiotypically unrelated monoclonal antibody (Figure 25).

However, although the two monoclonal antibodies used as antigen on the microtiter plate were of the same isotype, the question of allotypic variation still existed. As allotypes occur mostly as variants of heavy chain constant regions, the second phase experiments were repeated using $F(ab')_2$ fragments of the two monoclonal antibodies. Not only would the use of $F(ab')_2$ fragments of the administered and idiotypically unrelated monoclonal antibodies reduce the number of allotypic variants, but it would reduce also the number of constant region determinants, and should thus provide a clearer "background" against which idiotypic variants might be seen.

The use of $F(ab')_2$ fragments did not abolish the difference in response to the administered and idiotypically unrelated antibodies indicating that the difference was due to idiotypic, rather than allotypic variations (Figure 26).

Figure Legend

Figure 25 shows the binding of human anti-murine Ig antibodies present in a patient's serum, after receiving two therapeutic administrations of radiolabelled HMFG1 to the relevant monoclonal antibody, (HMFG1) and to an idiotypically unrelated monoclonal antibody, 1141, which was of the same IgG1 isotype. The relative difference in binding, shown as absorbance at 405nm, is presented as the anti-idiotypic response. Non-specific binding is shown as the binding to antigen-free wells, incubated with ELISA coating buffer (E.C.B) alone. The assays were performed on a single ELISA plate to ensure comparability.

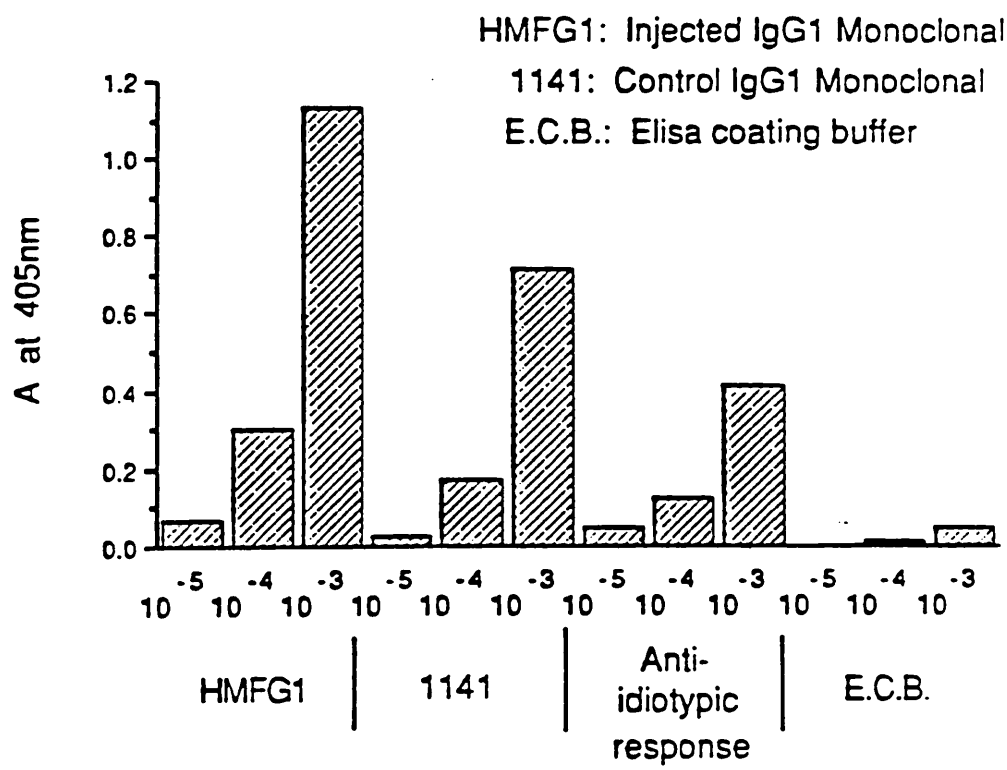
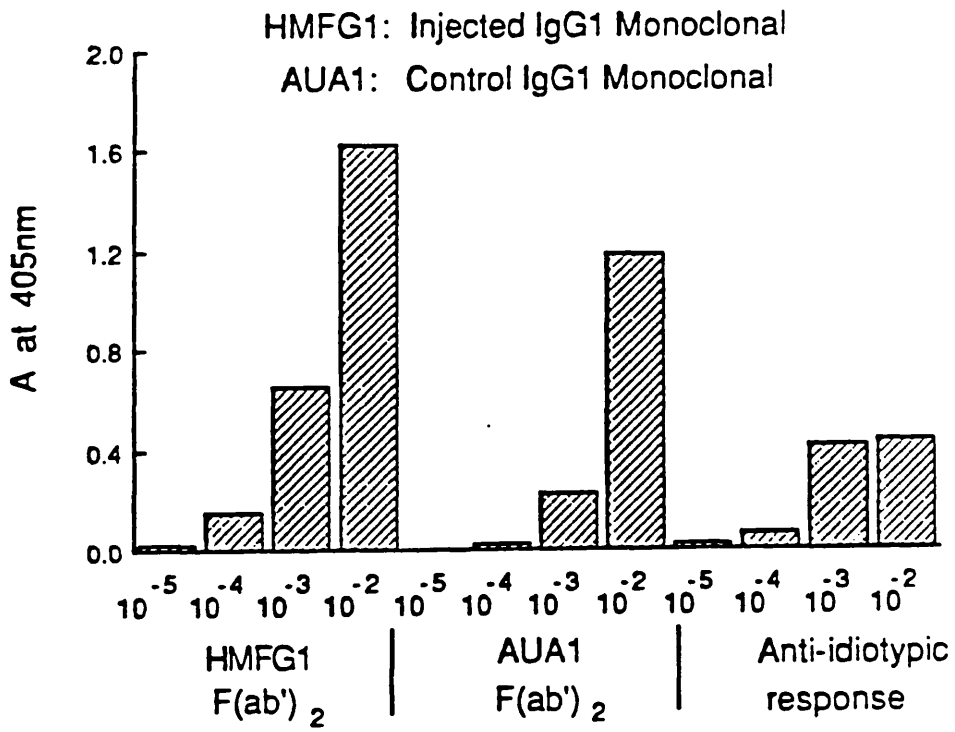

HMFG1: Injected IgG1 Monoclonal
1141: Control IgG1 Monoclonal
E.C.B.: Elisa coating buffer
A at 405nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10 -5 10 -4 10 -3 10 -5 10 -4 10 -3 10 -5 10 -4 10 -3 10 -5 10 -4 10 -3
HMFG1
1141
Anti-idiotypic response
E.C.B.

Figure Legend

Figure 26 shows the binding of a patient's serum anti-murine Ig antibodies to antigenic determinants located on the $F(ab')_2$ fragments of the relevant murine monoclonal antibody HMFG1 (administered antibody), and to those on the $F(ab')_2$ fragments of an idiotypically unrelated but isotypically related IgG1 monoclonal antibody, AUA1. The anti-idiotypic response is obtained by subtraction. The assays were performed on a single ELISA plate to ensure comparability.


HMFG1: Injected IgG1 Monoclonal
AUA1: Control IgG1 Monoclonal
A at 405nm
2.0
1.6
1.2
0.8
0.4
0.0
10-5 10-4 10-3 10-2 10-5 10-4 10-3 10-2 10-5 10-4 10-3 10-2
HMFG1 F(ab')2
AUA1 F(ab')2
Anti-idiotypic response

7.3 INHIBITION OF MURINE ANTIBODY BINDING

From the above experiments it appeared that patients who had received two or more therapeutic administrations of the same monoclonal antibody, developed an anti-idiotypic response. If some of these anti-idiotypic antibodies are directed towards the combining site of the murine monoclonal antibody, they should be able to inhibit the binding of the murine monoclonal antibody to its antigen. In order to test this theory, patients' sera containing anti-idiotypic antibodies were pre-incubated overnight at 4°C with the administered (HMFG1) murine monoclonal antibody (10µg/ml).

The ability of patients' sera to inhibit the binding of the administered murine monoclonal antibody to its target antigen was assayed by ELISA using purified milk fat globule antigen as target (3µg/ml - 30ng per well). Two positive controls were used: firstly, the administered murine monoclonal antibody was incubated for the same period of time in PBS in order to determine any non-specific background inhibition; secondly, the patient's pre-therapy serum diluted 1 in 10 was preincubated with the monoclonal antibody solution (10µg/ml). As a negative control, the patient's pre-therapy serum alone was used.

Pre-therapy serum from each patient tested gave very little inhibition of binding (8%-25%) compared to the serum from a patient who had received three administrations of the same murine monoclonal antibody (Figure 27). In order to determine the

percentage of binding inhibition due to steric hindrance, (binding to non-paratopic epitopes on either the varable or constant domain) this patient's post third therapy serum was diluted 1 in 10 with a 10μg/ml dilution of HI7E2, a control IgG1 monoclonal antibody against placental alkaline phosphatase (PLAP). As shown in Figure 27, this patient's post third therapy serum was able to block HI7E2 binding by approximately 36% even though she had never received this antibody for therapy. This experiment showed, as would be expected, that not all the inhibition of binding of HMFG1 (administered antibody) to its target antigen was due to anti-paratopic serum antibodies. This blocking experiment was repeated with several other "irrelevant" IgG1,IgG2b and IgG3 monoclonal antibodies (Figure 27). In each case, the patient's post third therapy serum gave between 35 and 47 percent inhibition of binding. These data suggest that , since each of the four irrelevant antibodies has a different binding specificity, the partial blocking effect must result from anti-constant region antibodies in the patient's serum, rather than from antibodies to non-paratotic idiotopes.

Figure Legend

Figure 27 *shows* the percentage inhibition of the binding of monoclonal antibodies to their target antigen by a patient's pre-therapy (pre) and post third therapy (P3) serum.

HMFG1 (therapeutic monoclonal antibody received by this patient,IgG1).

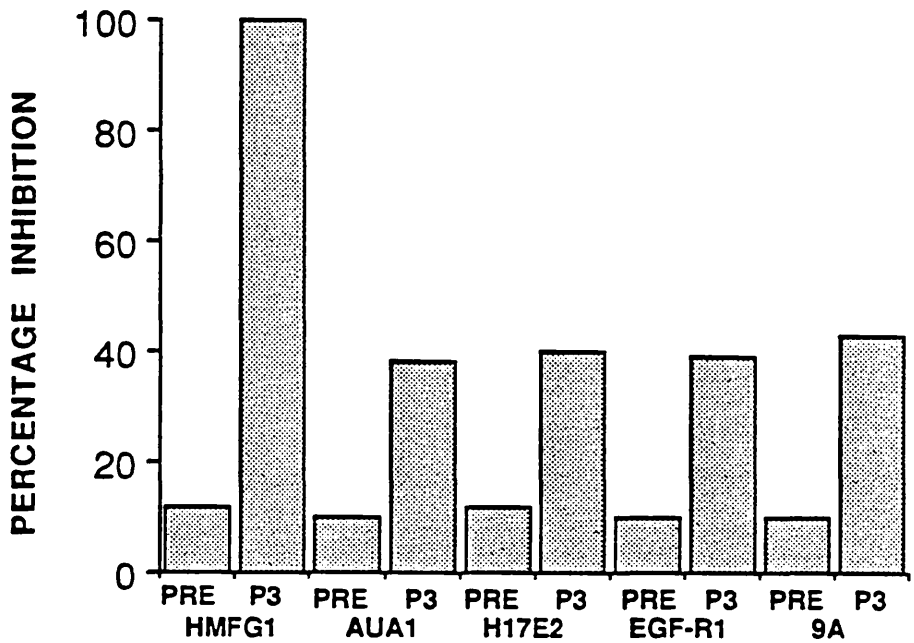
AUA1 (IgG1), H17E2 (IgG1), EGF-R1 (IgG2b) and 9A (IgG3) are "control" monoclonal antibodies in that they are frequently used for therapy, but were not administered to this patient. Their target antigens are:

AUA1: Antigen on Lovo cell line.

H17E2: Antigen is placental alkaline phosphatase.

EGF-R1: Antigen is the epidermal growth factor receptor on A431 cell line.

9A: Antigen is the epidermal growth factor receptor on A431 cell line.


PERCENTAGE INHIBITION
100
80
60
40
20
0
PRE
P3
HMFG1
PRE
P3
AUA1
PRE
P3
H17E2
PRE
P3
EGF-R1
PRE
P3
9A

In order to test the generality of this finding, that patients develop an anti-idiotypic response, fifteen patients' pre- and post-therapy serum samples were screened by the inhibition assay. From these experiments it was found that: none of the fifteen patients produced anti-idiotypic antibodies after a single antibody therapy, Table 5; three patients (patients 11, 12 and 15) produced anti-idiotypic antibodies after two administrations; and two (patients 13 and 14) produced anti-idiotypic antibodies only after three therapeutic administrations.

Patient 15 received four therapeutic administrations of HMFG1 murine monoclonal antibody (Table 5). To evaluate the effect of such therapy on the level of anti-idiotypic antibodies, each of this patient's sequential serum samples was preincubated with HMFG1 (10μg/ml) and assayed as described above. The level of inhibition increased with the number of administrations, until a point where the murine monoclonal antibody was totally inhibited by the post third therapy serum sample (Figure 28).

TABLE 5

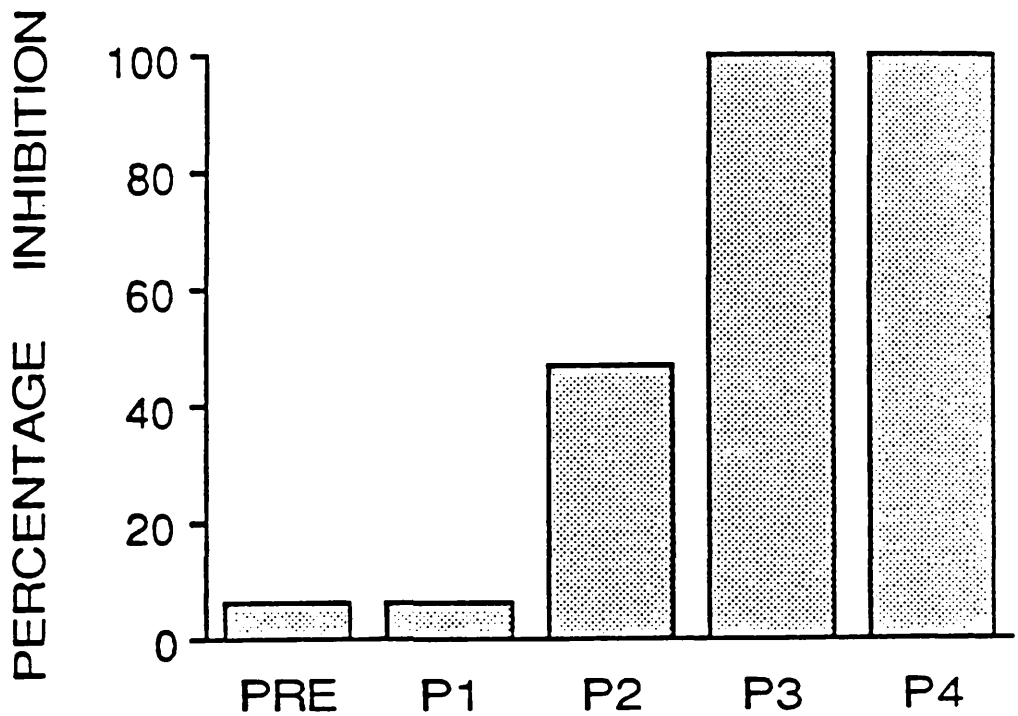
Development of anti-idiotypic responses by patients receiving single or multiple intraperitoneal administrations of radiolabelled murine monoclonal antibodies.

Patient	Administered Monoclonal Antibody	Dose Administered	Presence of anti-idiotype antibody in serum
1	HMFG2	10mg	-
2	HMFG2	10mg	-
3	HMFG2/H17E2	10mg	-
4	HMFG2	10mg	-
5	H17E2	10mg	-
6	H17E2	10mg	-
7	HMFG2	17mg	-
8	HMFG1	20mg	-
9	AUA1	20mg	-
10	HMFG1	10mg	-
	HMFG2	10mg	-
11	HMFG2	7mg	-
	HMFG2	6mg	+
12	HMFG1	12mg	-
	HMFG1/H17E2	10mg	+
13	HMFG1	2mg	-
	HMFG1	10mg	-
	HMFG1	12mg	+
14	HMFG2	2mg	-
	HMFG2	10mg	-
	HMFG2	10mg	+
15	HMFG1	10mg	-
	HMFG1	15mg	+
	HMFG1	10mg	+
	HMFG1	8mg	+

Serum samples were assayed by ELISA for their ability to inhibit binding of the therapeutic antibody, to their target antigen.

Figure Legend

Figure 28 shows the percentage inhibition of HMFG1 binding to its target antigen (MFG) by patient 15's post therapeutic sequential serum samples, P1, P2, P3 and P4. Inhibitory activity present prior to therapy is controlled for by using the patient's pre-therapy serum (PRE). Allassays were performed on a single ELISA plate.


PERCENTAGE INHIBITION
100
80
60
40
20
0
PRE
P1
P2
P3
P4

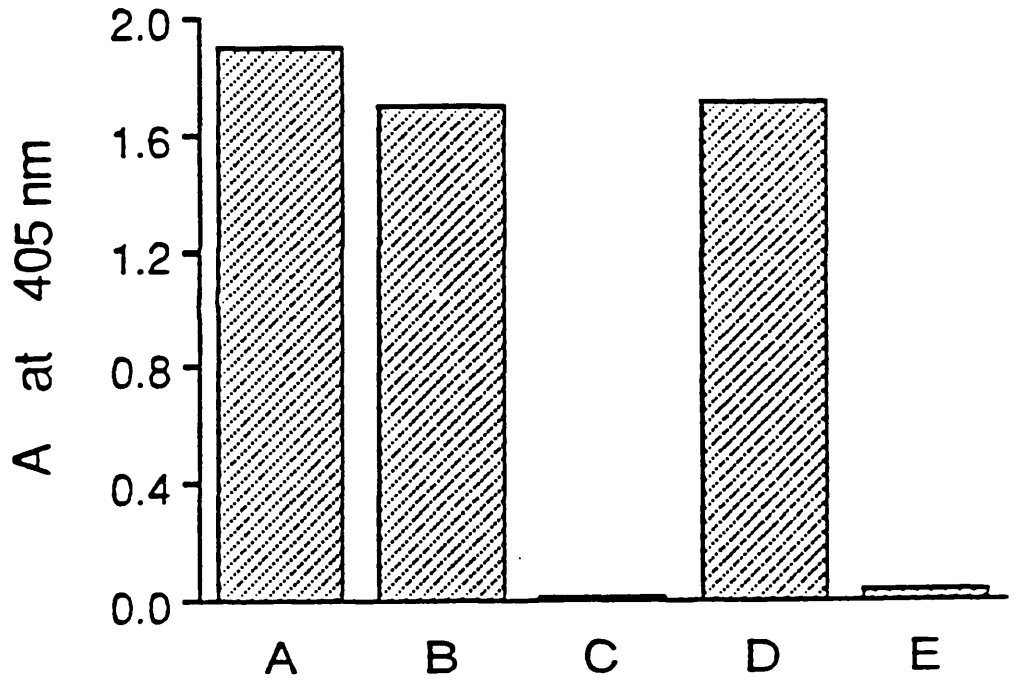
7.4 NATURE OF SERUM INHIBITION FACTOR

Although these results indicate the presence of an antigen-specific inhibitory factor in the patient's serum, the exact nature of this factor is unclear. One possible explanation of the data is that the serum contains anti-idiotypic antibodies with specificity for the paratope of the administered monoclonal antibody. Such anti-idiotypic antibodies would mimic, and hence form an internal image of the MFG antigen. Alternatively, the serum could actually contain MFG antigen itself, shed from tumour cells that were lysed during the radiolabelled monoclonal antibody therapy.

In order to determine the nature of the inhibition factor, the post fourth therapy serum from patient 15 (Table 5) was fractionated by protein A affinity chromatography. This fractionation enabled the inhibition studies to be repeated using Ig devoid serum and purified serum Ig. From this experiment it was found that only the purified serum immunoglobulin fraction inhibited the binding of the administered murine monoclonal antibody to its target antigen (Figure 29). The Ig devoid serum, which would contain any circulating antigen, gave very little inhibition, comparable with that seen with the pre-therapy serum (Figure 29).

Figure Legend

Figure 29 shows the binding of HMFG1 (10μg/ml) (A) to its target antigen (MFG). The level of inhibition by patient 15's pre (B) and post 4th therapy serum (C) is also shown. After fractionation of the post 4th therapy serum by protein A affinity chromatography, the degree of inhibition produced by the Ig devoid serum (D) and the purified serum Ig (E) fractions was assayed. All assays were performed on a single ELISA plate to ensure comparability of data.


A at 405 nm
2.0
1.6
1.2
0.8
0.4
0.0
A
B
C
D
E

7.5 DISCUSSION

Results presented in this chapter show that the serum from patients receiving two or more infusions of radiolabelled murine monoclonal antibodies, contains antibodies directed against variable domain determinants (idiotopes) of the murine immunoglobulin molecule. Inhibition experiments show that some of these anti-idiotypic antibodies can mimic the original antigen (to which the murine monoclonal antibody was raised) and hence are anti-paratopic.

This anti-idiotypic response has been observed by a number of other investigators studying the immune response of patients receiving therapeutic amounts of murine Igs. In one study where renal allograft recipients received multiple infusions of an anti-T-cell antibody, OKT3, a rapid and strong sensitization that completely neutralized the therapeutic effectiveness of the monoclonal antibody was observed. The anti-OKT3 (IgG2a) sensitization was manifested by accelerated OKT3 clearance and abrupt reappearance of circulating OKT3-positive cells. When the fine specificity of the antibodies produced was studied, using patients' whole sera and various mouse IgG2a affinity chromatography-purified serum fractions, the anti-OKT3 response was found to be remarkably restricted. Two main categories of antibodies were detected: anti-idiotypic antibodies that inhibited OKT3 binding to T cells and abrogated its therapeutic activity; and anti-isotypic antibodies that did not neutralize OKT3 immunosuppressive activity (Chatenoud et al., 1986).

In another study of patients with cutaneous T cell lymphoma (CTCL) receiving multiple injections of the murine monoclonal antibody T101, the titer of anti-murine immunoglobulin antibody was found to increase with the number of infusions. The anti-idiotypic portion of this anti-murine Ig antibody response steadily increased with each infusion until eventually, in one patient, well over fifty per cent of the serum anti-murine Ig antibodies recognized only T101 and not four other murine antibodies of the same isotype (Shawler et al., 1985).

Studies carried out at the Wistar Institute on patients receiving the monoclonal anti-colon carcinoma antibody 17-1A showed that of the 41 patients who raised an immune response against the murine monoclonal antibody, 35 produced anti-idiotypic antibodies, which persisted in the blood for up to 475 days following administrations. These anti-idiotypic antibodies were found to be against both combining site and non-combining site idiotopes (Herlyn et al., 1985).

Results presented in this chapter show that patients receiving intraperitoneal administrations of radiolabelled murine monoclonal antibodies develop anti-idiotypic antibodies which are directed towards idiotopes outside (and/or constant region epitopes), as well as inside the paratope of the monoclonal antibody. The significance of these findings to therapeutic efficacy in both the short and long term are discussed in Chapter 9.

CHAPTER 8

INDUCTION OF *IN VIVO* ANTI-TUMOUR AND AUTOANTIBODY RESPONSES

8.1 INTRODUCTION

Jerne has postulated a network of interacting antibody molecules and lymphocytes in the immune system in which idiotypes of antibody molecules are recognised by anti-idiotypic antibodies (Jerne, 1980) as discussed in Chapter seven. Accessibility computations have revealed the existance of about 40 distinct determinants including idiotopes, in both the hypervariable and framework regions of a single immunoglobulin molecule. If as postulated by Jerne, antibody epitopes are potential targets for a self-regulatory stable network of antibody-antibody interactions, then each molecule may be recognised and its concentration regulated by some 40 complementary anti-Ig antibodies (Novotiny et al., 1987).

Within the set of complementary anti-Ig antibodies one would expect there to be those that were directly complementary to the paratope of the antibody inducing their production. This has led to the concept that such anti-paratopic antibodies are internal images of the original/target antigen. A number of studies, including that of Dunn et al, (1980), have shown that anti-idiotypic antibodies raised to monoclonal antibodies, can themselves be used to raise second generation antibodies that have binding specificities similar to that of the original monoclonal antibody (an anti-paratopic response to the first generation of anti-paratopic antibodies).

In a study of the monoclonal anti-idiotypic antibody 87.92.6 (directed against the 9B.G5 antibody specific for the virus neutralising epitope on the mammalian reovirus type 3 haemagglutinin) it was shown to express an internal image of the receptor-binding epitope of reovirus type 3. Amino acid sequence comparision between the viral haemagglutinin and the 87.92.6 monoclonal antibody light chain "internal image" revealed an area of significant homology (Bruck et al., 1986) indicating that antigen mimicry by antibodies may be achieved by sharing primary structure.

With respect to monoclonal antibodies administered to patients with various malignancies, an idiotypic network could be generated by the following sequence of events. Murine monoclonal antibody (antigen) will induce a first generation anti-idiotypic response ($anti\text{-}id^{1}$). Such $anti\text{-}id^{1}$ antibodies could then themselves act as an antigen, and hence induce the formation of a second generation of anti-idiotypic antibodies ($anti\text{-}id^{2}$). The anti-paratopic components of the $anti\text{-}id^{1}$ antibodies would mimic the original tumour antigen, whilst the anti-paratopic components of the $anti\text{-}id^{2}$ generation would mimic the binding site of the murine anti-tumour monoclonal antibody. In the latter case, an <u>in vivo</u> human anti-tumour antibody would be produced.

Thus, from the above possible sequence of events which may occur within the idiotypic network, modulation of the immune system in cancer patients receiving murine monoclonal antibodies may be beneficial, with respect to the induction of anti-tumour

antibodies. However, due to the inter-relationship between antigen, idiotype and anti-idiotype the dynamic equilibrium within the network may be perturbed. This pertubation could then give rise to auto-reactive idiotypes.

Evidence exists to suggest that anti-self reactivity is based on a delicate dynamic equilibrium between idiotype and anti-idiotype which can be upset by appropriate manipulation of the idiotypic network (Zanetti et al., 1986). It has been shown that immunological B cell self-tolerance can be circumvented by anti-idiotypic antibodies alone (Brown et al., 1979; Zanetti and Bigazzi, 1981). Anti-idiotypic antibodies have also been demonstrated to be important in the recovery of patients from anti-VIII: C (anti-haemophilic factor) autoimmune disease (Sultan et al., 1987). In another study it has been shown that anti-idiotypic antibodies to a human monoclonal IgM autoantibody resulted in the production of multiple organ-reactive IgG antibodies (Essani et al., 1986). It has also been proposed (Plotz , 1983) that autoantibodies are anti-idiotypic antibodies generated to idiotopes of anti-viral antibodies.

In this chapter the results of anti-idiotypic antibody induced modulation of the immune network, with respect to the <u>in vivo</u> generation of anti-tumour and autoantibodies in patients receiving intraperitoneally administered radiolabelled tumour associated murine monoclonal antibodies are discussed.

8.2 IN VIVO GENERATION OF HUMAN ANTI-TUMOUR ANTIBODIES

In order to determine the presence of pre-therapy human antibodies with binding specificity similar to that of the anti-tumour monoclonal antibody, with which the patients were to be treated, serum was assayed on microtiter plates coated with the tumour-associated antigen. Results from these assays showed that prior to therapy, nearly all patients had serum antibodies which bound to tumour-associated antigen. However, these serum antibodies showed a broad spectrum of cross-reactivity when tested on soluble or membrane bound antigen of tumour derived cell lines.

In contrast to the pre-therapy levels of cross-reactive serum antibodies, a number of patients after receiving multiple infusions (two or more) developed serum antibodies which demonstrated binding specificity similar to that of the murine monoclonal antibody administered (Table 6 and 7). More specifically, only those patients with serum antibodies which were able to compete with antigens for the combining site of the administered murine monoclonal antibody (i.e. those with anti-id^1 antibodies), had elevated levels of anti-tumour (anti-id^2) antibodies. Serum from those patients who had received a single therapeutic dose of radiolabelled murine monoclonal antibodies showed no increase in antibody binding to the tumour associated antigen.

This elevation in post therapy serum antibody binding to the tumour associated antigen is shown in Tables 6 and 7, and Figure 30 (patient 15). The results for other patients whose serum contained elevated levels of antibodies with anti-tumour activity are shown in Table 7. From these results it can be seen that the response was at least twice as high for the post therapy serum, compared to the pre-therapy serum. The other 10 patients (Table 6) showed no such post therapy elevation.

The results obtained from the anti-tumour response experiments show that patients receiving multiple infusions of murine monoclonal antibodies generate human antibodies which have binding specificity similar to that of the administered murine monoclonal antibody. However, what is not clear from the anti-tumour assay is the origin of such anti-tumour antibodies. One possibility is that they are generated as a result of an idiotypic network, and are therefore genuine anti-id ^{2}'antibodies. Alternatively, they could be antibodies that are generated in the anti-murine Ig response which cross react with shared antigenic determinants located on the tumour associated-antigen. Finally, they could have been raised in direct response to shed tumour antigen.

In order to resolve the question of specificity of these anti-tumour antibodies, the following experiments were carried out. Firstly, in order to remove patients' serum antibodies which were directed against antigenic determinants located on the adminis - tered murine monoclonal antibody an affinity column was prepared

by coupling murine monoclonal antibody to the cyanogen bromide activated beads, Patient's serum, after being dialysed overnight at 4°C in 0.1M sodium carbonate buffer, pH 8.3, was then added to the column.

After depletion of the anti-mouse Ig component by column chromatography, the patient's serum was tested for both anti-murine Ig reactivity and anti-tumour associated antigen reactivity. These assays showed that depletion of anti-murine Ig had no significant effect on the anti-tumour response assay (Figure 31). Therefore, antibodies reacting with the tumour associated antigen are not cross-reactive antibodies which have primary specificity for either constant domain determinants or variable domain determinants (idiotopes) of the administered murine monoclonal antibody.

TABLE 6

Table 6 shows the development of both anti-tumour and autoantibodies in patients receiving monoclonal antibody therapy.

Patient	Administered Monoclonal Antibody	Dose Given	Presence of anti-id1 antibodies in serum	Presence of anti-id2 antibodies in serum	Presence Auto-anti-bodies in serum
1	HMFG2	10mg	-	-	-
2	HMFG2	10mg	-	-	-
3	HMFG2/H17E2	10mg	-	-	-
4	HMFG2	10mg	-	-	-
5	H17E2	10mg	-	-	-
6	H17E2	10mg	-	-	-
7	HMFG2	17mg	-	-	-
8	HMFG1	20mg	-	-	-
9	AUA1	20mg	-	-	-
10	HMFG1	10mg	-	-	-
	HMFG2	10mg	-	-	-
11	HMFG2	7mg	-	-	-
	HMFG2	6mg	+	+	+
12	HMFG1	12mg	-	-	-
	HMFG1/H17E2	10mg	+	+	-
13	HMFG1	2mg	-	-	-
	HMFG1	10mg	-	-	-
	HMFG1	12mg	+	+	+
14	HMFG2	2mg	-	-	-
	HMFG2	10mg	-	-	-
	HMFG2	10mg	+	+	+
15	HMFG1	10mg	-	-	-
	HMFG1	15mg	+	+	-
	HMFG1	10mg	+	+	+
	HMFG1	8mg	+	+	-

Figure Legend

Figure 30 shows the pre-therapy (●—●) and post third therapy (○—○) response of patient 15's serum antibodies to the tumour associated antigen (MFG), which was the same antigen as that which was used to raise the monoclonal antibody (HMFG1) with which this patient was treated. All samples were assayed on a single ELISA plate.

ANTI-TUMOUR RESPONSE

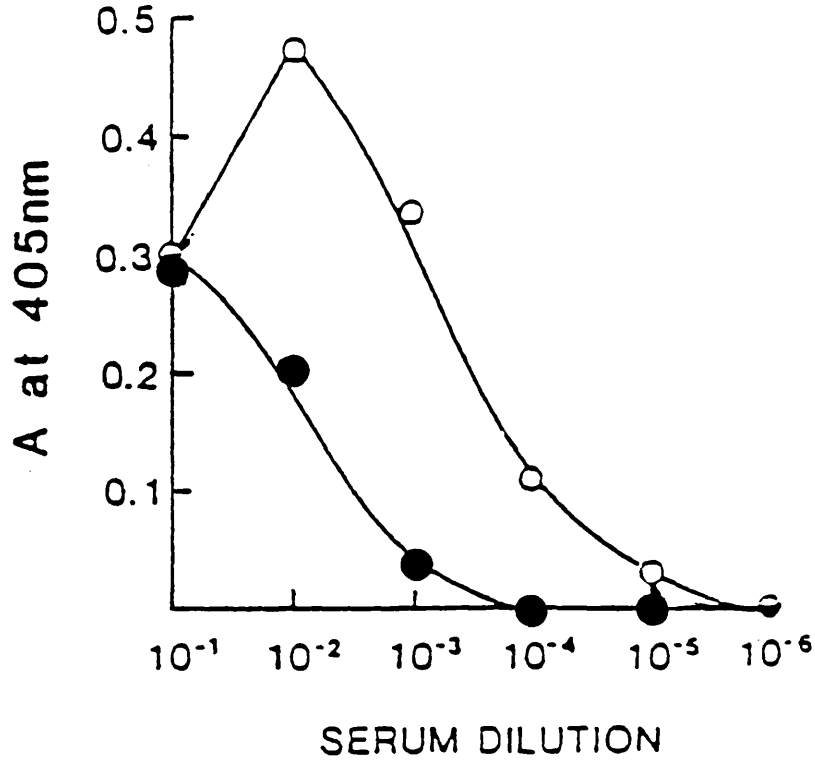


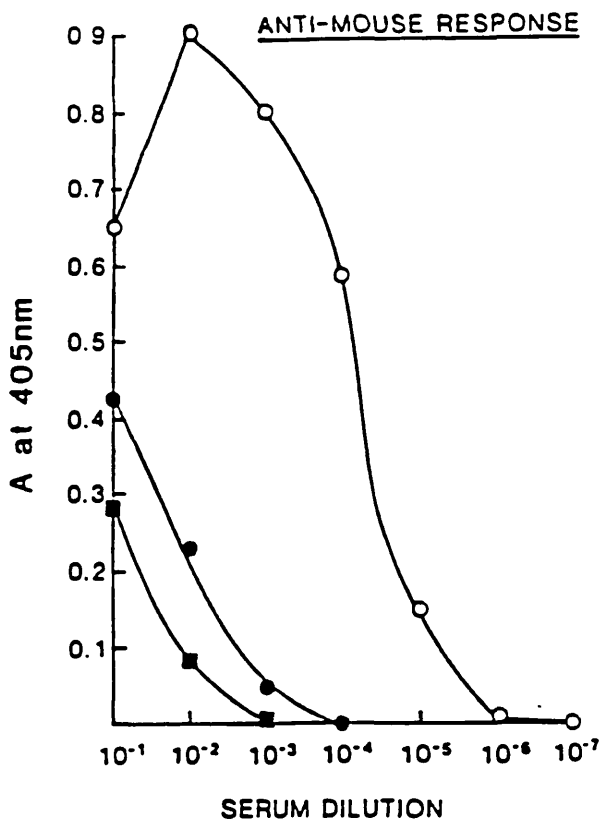
TABLE 7

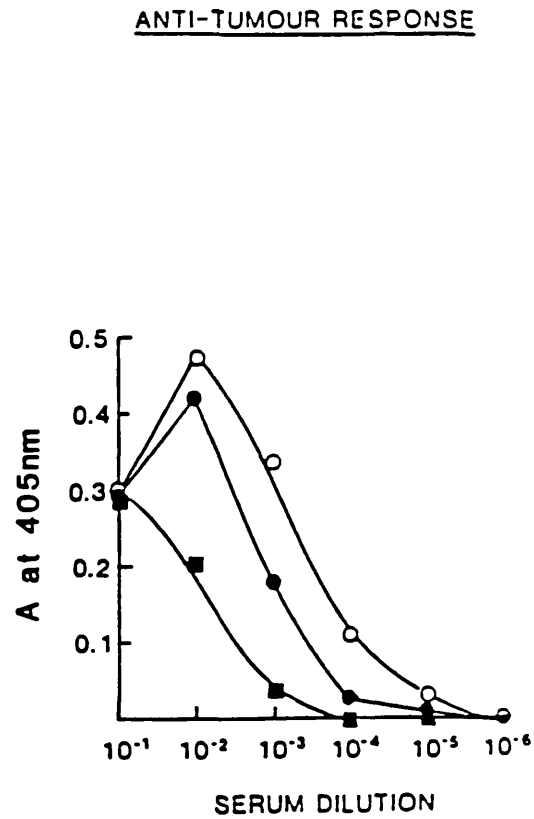
Presence of anti-tumour antibodies in patients' sera after monoclonal antibody therapy.

Patient	Absorbance at 405nm for serum samples diluted 1 in 100		number of therapies
	pre-therapy	post-therapy	
11	0.13	0.34	2
12	0.14	0.38	2
13	0.23	0.47	3
14	0.18	0.43	3
15	0.20	0.48	3

Figure Legend

Figure 31 shows the binding of patient 15's pre-therapy (■—■) and post therapy (○—○) serum antibodies to HMFG1, the administered murine monoclonal antibody (anti-mouse Ig response), and to the tumour-associated antigen MFG (anti-tumour response). The effect of removing serum antibodies reacting with antigenic determinants of the murine Ig on the post therapy anti-mouse Ig and anti-tumour response (●—●) is also shown. All samples were assayed on a single ELISA plate.


ANTI-MOUSE RESPONSE
A at 405nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7}
SERUM DILUTION


ANTI-TUMOUR RESPONSE
A at 405nm
0.5
0.4
0.3
0.2
0.1
10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6}
SERUM DILUTION

8.3 POST-THERAPY RESPONSE TO AUTOANTIGENS

Perturbation of the autoimmune network is reported to occur when the dynamic equilibrium between idiotype and anti-idiotype is upset by manipulation of the idiotypic network. In order to test this hypothesis, the serum samples of patients prior to and after each therapeutic administration of murine monoclonal antibodies were screened by an indirect immunofluorescence assay for the presence of autoantibodies.

In this assay fresh frozen tissue sections of rat liver, kidney, diaphragm and stomach were used. Sections were air-dried and stored at -20°C. Just prior to use, sections were thawed, washed with phosphate buffered saline, pH 7.4 and incubated for one hour at room temperature with 50µl of patient's serum at dilutions of 1:40, 1:80, 1:160, 1:320. Following this, sections were washed for 30 minutes in phosphate buffer, incubated for 15 minutes with a 1:40 dilution of a fluorescein conjugated sheep anti-human IgG antibody, then washed as before and mounted in hydromount. The sections were then screened using a Leitz UV microscope equipped with epi-illumination optics.

For controls, patient's serum was replaced by PBS, serum from healthy controls, serum from patients with neoplastic conditions identical to those patients under study but receiving no monoclonal antibody therapy, and lastly, pre-therapy serum from patients participating in the monoclonal antibody therapy study. When sections were incubated with fluorescein labelled rabbit

anti-rat Ig antibody alone, the results were negative, thus discounting the possibility of false positives due to anti-mouse Ig in the patient's serum binding to endogenous rat Ig in the tissue sections.

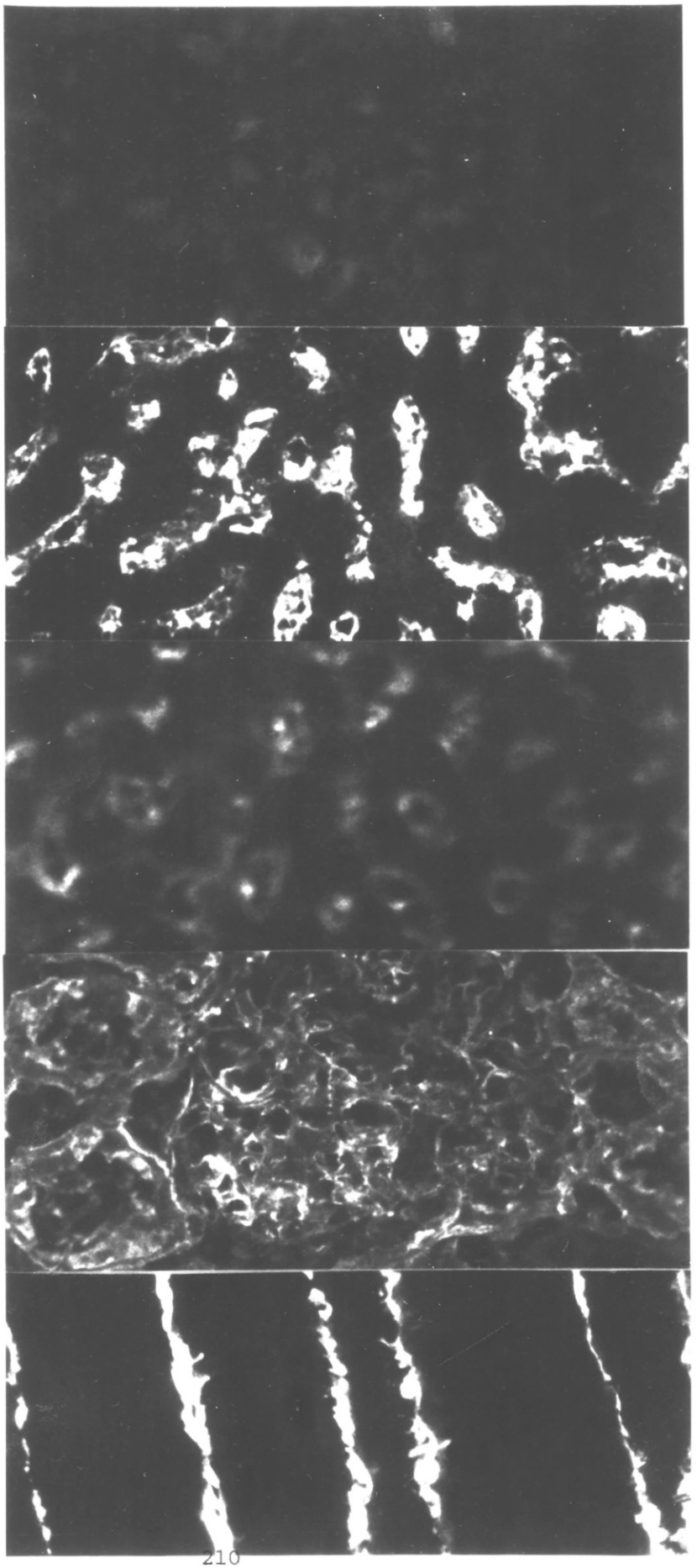
Patient's pre-therapy serum and serum from healthy controls, were all negative in the auto-antibody assays (Figure 32A), as were sera from patients who had received a single therapeutic dose of radiolabelled murine monoclonal antibody, irrespective of the dose administered. Next, the serum of patients receiving multiple infusions of murine monoclonal antibody was screened. From this group of patients, four of the five sera that were positive for anti-id^{2} antibodies (Table 7), also showed binding to connective tissue components of liver (Figure 32B) kidney glomerulus (Figure 32D) and diaphragm (Figure 32E).

When patient's sequential serum samples were assayed the results for patient 15 (Table 7) were found to be unique. Patient 15's pre-therapy (Figure 32A), post-first and post-second therapy samples were all negative. The post-third therapy serum gave intense staining, Figure 32B D and E. However, this patient's post fourth therapy serum was virtually negative again (Figure 32C). Thus, the development of auto-antibodies in patient 15 was transient. No other patient received further therapy, after the development of auto-antibodies.

Figure Legend

Figure 32 shows the results obtained for autoantibody screening of patient 15's serum prior to monoclonal antibody therapy (A), post third therapy (B) and fourth therapy (C) using cryostat sections of rat liver. The positive, post third therapy result is also shown on cryostat sections of rat kidney (D) and diaphragm (E). Frozen sections were stained using an indirect immunofluorescence technique. Magnification 1200X

FIGURE



8.4 DISCUSSION

Whether or not the "complete" idiotype network is an "absurd" immune system (Langman and Cohn, 1986) evidence for idiotype anti-idiotype interactions exists. The development of an anti-idiotypic response in patients receiving administrations of murine monoclonal antibodies has been reported by a number of groups (Shawler et al., 1985; Schroff et al, 1985; Herlyn et al., 1986 and Chatenoud et al., 1986). The serum of some patients has been found to contain human antibodies which have specificity for the paratope of the murine monoclonal antibody, that is anti-id^1 antibodies (Chapter 7).

On theoretical grounds it might be expected that the two structures which interact with the binding site of the murine monoclonal antibody, that is, antigen and anti-paratopic antibodies have a similar molecular shape. The anti-paratopic antibody could elicit an antibody (anti-id^2) which also recognises antigen. The anti-paratopic antibodies of the anti-id^1 response can therefore act as "surrogate antigen" (Stevenson, 1986).

Data presented in this chapter demonstrate that multiple therapeutic adminstrations with murine monoclonal antibodies do lead to the <u>in vivo</u> generation of human anti-tumour antibodies. It is not possible to determine whether these are genuine anti-id^2 antibodies or whether they have arisen in direct response to circulating shed tumour antigen. However, the fact that the

appearance of anti-tumour antibodies is always preceded by the appearance of serum anti-id[1] antibodies lends support to their generation via an idiotypic network.

Similar antibodies have been described by others. In patients treated with monoclonal antibodies directed against antigens of gastrointestinal tumours, some developed anti-idiotypic antibodies which were hapten-inhibited suggesting that an internal image of the antigen was produced by the anti-idiotype response. Patients who developed the anti-idiotype antibody were said to improve clinically and obtain long term remission from their disease (Koprowski et al., 1984). Later, (Herlyn et al., 1985) peripheral blood lymphocytes from the monoclonal antibody treated patients were found to respond to stimulation with purified anti-id by producing human anti-colorectal carcinoma antibody which had binding specificities similar to the administered monoclonal antibody .

In studies using ricin A chain conjugated to monoclonal antibodies with specificity for melanoma antigens, patients were reported to obtain tumour regression over periods of 2 to 13 months (Spitler et al.,1987). Since this is a time far in excess of the half life of the injected monoclonal antibody, the authors concluded that a variety of biological and immunological factors may be responsible for this, although they did not look for anti-idiotypic antibodies. The patients participating in this study who developed antibodies with binding specificities similar to that of the administered murine monoclonal antibody did not show

any significant difference in tumour regression, over those patients not developing anti-idiotypic antibodies. This however is not surprising as many of the patients had large tumours which one would not expect to respond. The results do however show that patients receiving multiple intraperitoneal infusions of radiolabelled murine monoclonal antibodies develop antibodies with specificity for the tumour-associated antigen. This raises the interesting possibility for the future that, with more knowledge of idiotypic network regulation, the production of such anti-tumour antibodies could be enhanced and form an effective component of the patient's anti-cancer therapy.

Finally, using the traditional immunofluorescence method for the demonstration of auto-antibodies (Gripenberg and Kurki, 1986), 4 out of the 5 patients whose serum contained anti-idiotypic antibodies also contained antibodies which showed reactivity against self antigens located on connective tissue. These sera were negative in assays for anti-nuclear, anti-smooth muscle, anti-skeletal muscle, anti-gastric paretial cell, anti-reticulin and anti-mitochondrial antibodies.

Of the four patients developing auto-antibodies, one, patient 15, received further therapy. After this fourth and final therapy the serum no longer showed significant binding to autoantigens, suggesting that the emergence of the auto-antibodies is transient rather than maintained. This regulation may therefore be due to anti-idiotypic control, as has been observed in other autoimmune conditions (Sultan et al., 1987). It is difficult to draw any firm conclusions from such observations given that only four

patients developed auto-antibodies. However, the data do support the suggestion that perturbation of the idiotypic network can lead to the generation of auto-antibodies. The transient nature of these antibodies in patient 15 suggests that they may not present a serious clinical problem in cancer patients undergoing repeated monoclonal antibody therapy.

CHAPTER 9

GENERAL DISCUSSION

Ovarian carcinoma is the most common fatal gynaecological malignancy. Approximately 60 to 80 percent of patients have disease that has spread through the peritoneal cavity (stage III) at the time of diagnosis (Zimm et al., 1987). Cisplatin-based, combination chemotherapeutic regimens produce pathologically documented complete remissions in 20 to 30 per cent of patients with advanced disease (Ozols and Young, 1984), but the vast majority of patients will not be cured. Ovarian carcinoma tends to remain confined to the peritoneal cavity throughout most of its natural history even in its most advanced stages.

A recent pharmacological approach to the treatment of malignancies that are principally confined to the peritoneal cavity has been the direct administration of chemotherapeutic agents in large fluid volumes into the peritoneal cavity. Several anti-neoplastic agents administered intraperitoneally have been investigated in phase I pharmacokinetic studies. These include Cisplatin (Howell et al 1982), methotroxate (Jones et al., 1981; Howell et al., 1981). Cytarbine (King et al., 1984) and fluorouracil (Speyer et al., 1980). In a recent study where Cisplatin was administered intraperitoneally to ovarian cancer patients with small volume (tumour nodules less than 2cm in diameter) disease, clinical activity was demonstrated with 30% of patients achieving a surgically documented complete remission (McVie et al., 1985).

These responses are almost certainly due to the high levels of drug delivery that are obtainable by regional (intraperitoneal)

administration. However, a major problem associated with chemotherapy is that of multiple drug resistance (Riordan et al., 1985). The gene responsible for multidrug resistance, termed Mdr, encodes a membrane glycoprotein (P-glycoprotein) that acts as a pump to transport cytotoxic drugs out of the cell (Fitzgerald et al., 1987). This resistance to chemotherapy agents therefore necessitates the use of alternative cytotoxic agents, such as radionuclides.

Monoclonal antibodies directed against tumour associated antigens provide a means for delivering preferentially cytotoxic radionuclides, such as Iodine-131, to the cells of primary and secondary tumours. The factors influencing the efficacy of the radiation in the tumour compared to its effect on the radiosensitive normal tissues, include the specificity of the monoclonal antibody, the distribution of targeted energy within the tumour and the host's immune response to the injected xenogeneic antibody.

Development of host antibodies against passively administered murine immunoglobulin, with possible subsequent neutralisation and abrogation of therapeutic efficacy, together with the risk of immune complex mediated complications such as serum sickness, has been viewed as a potential major complication to the use of murine monoclonal antibodies in vivo. This thesis describes the generation, specificity, immunoregulation and clinical significance of the humoral immune responses that are generated in patients receiving regionally administered radiolabelled

murine monoclonal antibodies for the therapy of neoplasia.

Having developed a reliable assay to detect serum antibodies which bound to murine immunoglobulin, experiments showed that the majority of both patients and healthy controls possess pre-existing anti-murine immunoglobulin antibodies mainly of the IgM class. The antigenic determinants to which these antibodies bound were located on the Fc portion of the murine immunoglobulin. Further studies showed that these IgM pre-existing human anti-murine Ig antibodies and polyclonal rheumatoid factors bound to both human and murine immunoglobulin.

Since the major part of the binding of rheumatoid factors to human IgG and of the pre-existing antibodies to mouse IgG could be blocked by pre-absorption with mouse and human IgG respectively, it can be concluded that the majority of antibody molecules in these sera have specificity for evolutionary conserved antigenic determinants that are shared by the IgG (Fc) immunoglobulins of man and mouse. Only a small component appears to be against species specific determinants.

In rheumatoid arthritis, immune complexes consisting exclusively of immunoglobulin are found. Autoantigenic reactivity has been localised to the constant region (CH2) domain of IgG. In studies comparing in detail the N-glycosylation pattern of serum IgG isolated from normal individuals and from patients with either primary osteoarthritis or rheumatoid arthritis, changes in the glycosylation pattern were observed (Parekh et al., 1985). The

investigators concluded that both osteo and rheumatoid arthritis may be glycosylation diseases, reflecting changes in the intracellular processing or post secretory degradation of N-linked oligosaccharides.

Secretion of IgG with an altered level of glycosylation could expose previously masked protein determinants or create new protein-oligosaccharide determinants that may be immunogenic, resulting in the generation of autoreactive antibodies. In order to determine the nature of the antigenic determinants on the murine immunoglobulin that are recognised by both the pre-existing human IgM anti-murine IgG and the polyclonal rheumatoid factors murine, target monoclonal antibodies (antigen) were deglycosylated.

Oxidative cleavage of carbohydrate residues (mannose and fucose) on the murine monoclonal antibody was achieved by employing the deglycosylation method of Thorpe et al, (1985). When the binding of pre-existing human IgM anti-murine IgG antibodies and polyclonal rheumatoid factos to glyclosylated and deglycosylated murine IgG was compared, no difference was observed. One could therefore conclude from these experiments that essentially all the antibody activity of pre-existing anti-murine immunoglobulin antibodies and of polyclonal rheumatoid factors is directed against protein rather than sugar determinants. These antibodies therefore cannot be classified as typical heterophile antibodies (IgM antibodies that bind to carbohydrate determinants and hence show broad cross-reactiviry on many glycosylated molecules).

However, the exact degree of deglycosylation must first be determined before detailed conclusions can be drawn.

Although the studies on pre-existing human anti-murine immunoglobulin antibodies (chapter 3) looked at antibody binding *in vitro* using an ELISA detection system, where the antibody binding to antigen fixed on a solid surface is probably in the order of two to three logs greater than binding to soluble antigen, the clinical implications of these findings are two-fold. Firstly, as rheumatoid factors which bind to antigenic determinants located on the Fc region of the murine monoclonal antibody are present in a high percentage of the population at subclinical levels, the finding would favour the use of $F(ab')_2$ fragments rather than intact IgG for *in vivo* tumour localisation.

Secondly, patients with active rheumatoid disease were shown to have elevated serum levels of IgM anti-murine immunoglobulin antibodies. This, combined with the elevation in anti-human IgG antibodies in patients treated with radiolabelled murine monoclonal antibodies, leads one to conclude that patients with elevated serum rheumatoid factors could be at higher risk of developing immune complex disease in response to antibody therapy.

Although significant levels of IgM anti-murine IgG antibodies remained detectable in patients receiving therapy, and gave rise to prozones in the ELISA assay, class switching did occur. That is, the serum of patients treated with murine monoclonal

antibodies contained both elevated levels of IgM and significant levels of IgG anti-murine IgG antibodies. This class switching implies that the murine Ig is a T cell dependent rather than a T cell independent antigen, as antibody isotype production during an immune response is controlled by antibody class induction signals from regulatory T cells (Teale and Abraham, 1987).

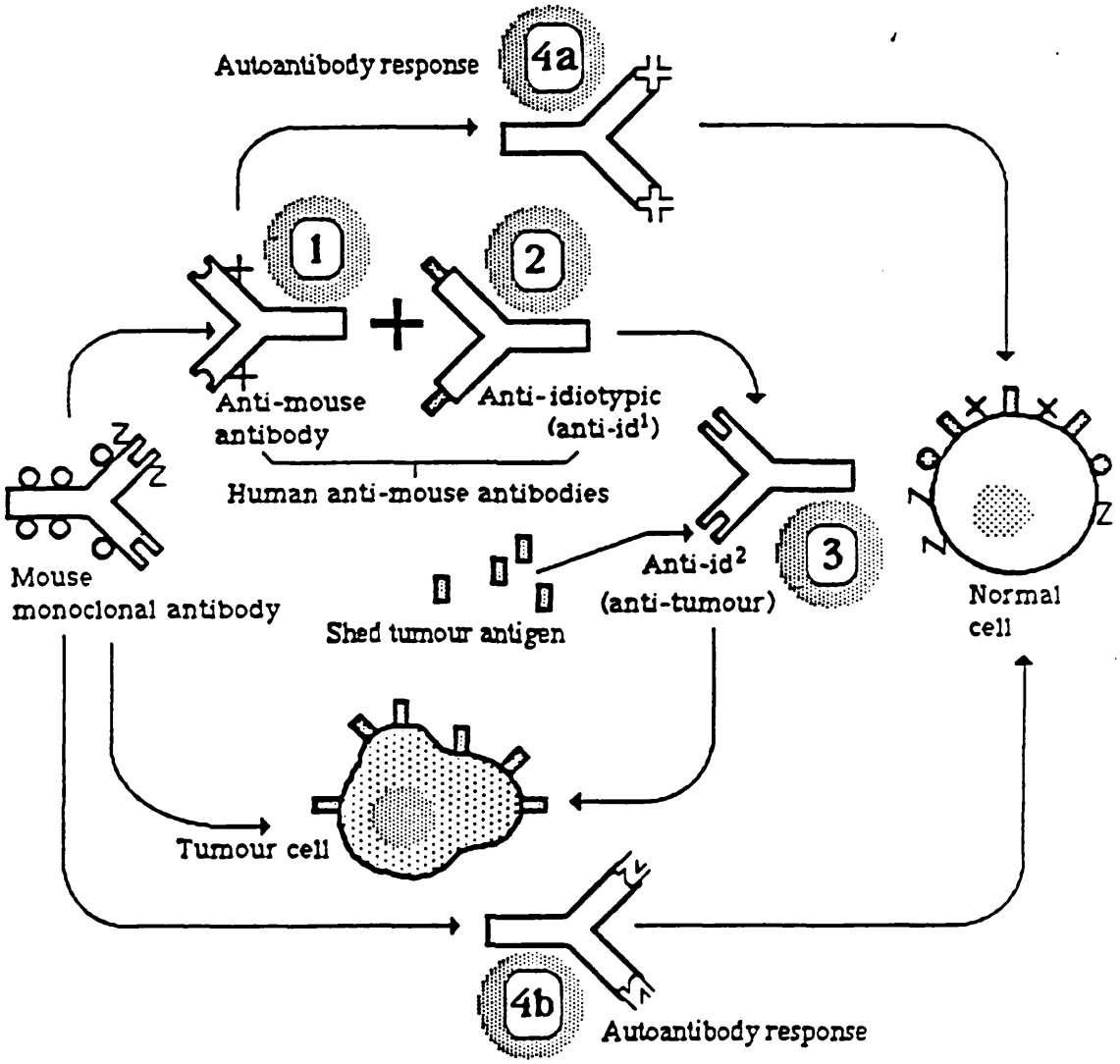
In addition to class switching, the preceding chapters show that as the number of radiolabelled murine monoclonal antibody administrations received by patients increases, the number of antigenic determinants recognised by anti-murine Ig antibodies also increases. Progressing from Fc only, to Fc and $F(ab')_2$, to Fc, $F(ab')_2$ and anti-id$_1$ and finally to include anti-id$_2$ and autoantibodies. In Figure 33 these separate observations are brought together and represented diagramatically in the context of an idiotypic network.

In this final chapter the arguments for, and against, these observations coming together to support the network theory (Jerne, 1975) will be discussed. As shown in Figure 33 the antibodies generated against the therapeutic monoclonal antibody, after a single administration, recognise constant region antigenic determinants, restricted to the Fc portion of the murine immunoglobulin. This restriction to determinants that reside in the Fc region has also been observed in studies on the therapy of primates using a murine IgG2a monoclonal antibody (Stratte et al., 1982) and in cutaneous T cell lymphoma patients

treated with murine monoclonal antibodies (Schroff et al., 1985).

Figure Legend

Figure 33 shows diagrammatic representation of the human anti-mouse *Ig* response in patients receiving radiolabelled murine monoclonal antibodies. It shows the generation of anti-constant region antibodies (1), and anti-idiotypic antibodies which reflect an internal image of the tumour associated antigen (2). It then shows the generation of a second anti-idiotypic antibody (3) which has a binding specificity similar to that of the original mouse monoclonal antibody. This figure also shows that antibodies generated to either epitopes on the antibodies generated in this response (4a) , or against the mouse monoclonal antibody (4b) could crossreact with antigens found on normal cells, giving rise to the generation of autoantibodies. These individual responses are intergrated to form an "idiotypic network".


Autoantibody response
4a
1
2
Anti-mouse antibody
Anti-idiotypic (anti-id¹)
Human anti-mouse antibodies
Mouse monoclonal antibody
Shed tumour antigen
Anti-id²
(anti-tumour)
3
Normal cell
Tumour cell
4b
Autoantibody response

A consistent feature of the anti-immunoglobulin response in patients after a single administration, was the increase in detectable levels of human IgM anti-murine IgG antibodies, chapter 3. This increase could therefore be due to a boosting of the pre-existing response, brought about by an expansion of B-cell clones producing anti-IgG (Fc) antibodies. This argument is supported in part by an observation that, of three groups of patients with melanoma, cutaneous T cell lymphoma or chronic lymphocytic leukaemia, only the chronic lymphocytic leukaemia patients who had no detectable pre-existing anti-murine immunoglobulin response, failed to develop a response following monoclonal antibody therapy (Schroff et al .,1985.)

Subsequent administrations of radiolabelled murine monoclonal antibodies to the patients studied in this thesis resulted in the generation of antibodies directed to both constant and variable domain determinants. A component of this anti-IgG response, represented by the number 2 in Figure 33, was anti-idiotypic. These anti-idiotypic antibodies were against idiotopes both inside (paratope) and outside the combining site. In vitro inhibition assays showed that the ability of patients' serum antibodies to inhibit the binding of the administered monoclonal antibody to its target antigen increased with the number of administrations (Chapter 7), hence indicating an increase in the anti-paratopic reponse.

The emergence of anti-paratopic antibodies to the therapeutic murine monoclonal antibody, could, and has, as discussed in chapter 7, lead to a loss of therapeutic efficacy. They could also play a beneficial role in the modulation of the patient's response to their own tumour. However, an anti-tumour response would only occur if the anti-id^{1} antibodies gave rise to second generation, anti-id^{2} anti-paratopic antibodies. According to Jerne this would occur as a result of part of immune regulation. Recognition of the tumour antigen by the anti-id^{2} anti-paratopic antibodies would result as a consequence of structural homology between the anti-id^{1} antibody combining site and the tumour antigen. It is due to this structural homology between anti-id^{1} and antigen that the term "internal image" has been used to describe the anti-id^{1}. In addition to the potential immunogenicity of this anti-id^{1} antibody <u>in vivo</u> in the generation of an anti-tumour response, its production could be exploited for the large scale manufacture of idiotypic vaccine. The potential use of idiotypic vaccines will be discussed later.

Fractionation of a patient's serum into immunoglobulin and immunoglobulin devoid fractions, demonstrated that inhibiton of the binding of the murine monoclonal antibody to its target antigen was due to antibody rather than to shed circulating antigen. However, what neither this, nor any other experiment can show is what acted as antigen for the anti-tumour response described in chapter 8. From the results presented in this thesis, one could argue that the human serum antibodies with binding specificities similar to those of the administered murine

monoclonal antibody were generated as a result of immune regulation via the idiotypic network (anti-id[1] antibodies acted as antigen). Alternatively, one could argue that it was shed tumour antigen, resulting from tumour cell lysis, that was responsible for the induction of *in vivo* anti-tumour antibodies (Figure 31).

Circulating tumour-associated antigens have been used as markers to predict and to monitor the presence of tumours and their response to therapy. In ovarian cancer patients, at least three antigens have been used for both diagnosis and prognosis; these are the HMFG1 and HMFG2 antigens (Dhokia et al., 1986) and the CA125 antigen (Bast et al., 1983; Caney et al., 1984). The level of all three antigens has been shown to vary with treatment. Chemotherapy normally results in a transient elevation of tumour markers. The level of circulating antigen then drops, and remains normal in those patients obtaining complete remissions, while disease progression is accompanied by a marked increase in serum antigen levels. How, then, in the presence of tumour associated antigen, is immunological escape of tumour destruction possible? Firstly, antibodies may exist but are complexed with the shed tumour-associated antigen and therefore escape detection in *in vitro* assays. Alternatively, the antigen could be recognised as being self (and therefore not immunogenic) because it is tumour-associated and not tumour-specific.

With respect to the patients participating in this study, who developed antibodies with binding specificities similar to those of the administered murine monoclonal antibodies, one could argue that the idiotypic network is the most likely origin of these anti-tumour antibodies. Prior to radiolabelled murine monoclonal antibody therapy, all patients studied had serum levels of tumour associated antigen above those found for healthy controls. This probably reflects the fact that these patients all had disease progression. However, the only patients who developed elevated levels of anti-tumour antibodies with binding specificity similar to that of the administered murine monoclonal antibody were those who had detectable anti-id^{1} antibodies.

In the generation of an idiotypic network not all antibodies will be anti-paratopic. Those that are not, may cross react with self antigens in the host (Figure 32). As shown in chapter 8, such autoreactivity was found in four patients receiving two or more doses of radiolabelled murine monoclonal antibodies. The autoreactive antibodies produced showed reactivity against self antigens located on connective tissues.

Of the four patients who developed auto-antibodies, one received further therapy, that is, a fourth administration. After this final therapy, the serum no longer showed any significant binding to auto-antigens suggesting that the emergence of these auto-antibodies is transient rather than maintained. This regulation may be due to anti-idiotypic control as has been observed in other autoimmune conditions (Sultan et al., 1987). The data

presented in this thesis therefore suggest that such autoimmunity will not present serious clinical problems.

However, given that only four patients developed auto-antibodies, firm conclusions cannot be drawn from these studies. However, the observations are consistent with a perturbation of the immune network due to the generation of anti-idiotypic antibodies. Clearly in depth studies are required to determine the nature of the antigens to which these auto-antibodies bind, to identify the patients most likely to develop auto-antibodies, to elucidate the the clinical significance of these auto-antibodies and to identify the controlling mechanisms that are responsible for their suppression.

The aim of the project presented in this thesis, was to study the humoral immune response of patients to therapeutically administered radiolabelled murine monoclonal antibodies. The results show the following. Firstly, both patients and healthy controls (e.g. blood donors) have rheumatoid factors which bind to both human and murine IgG, therefore giving rise to a pre-existing anti-murine IgG response. Administration of a single therapeutic dose leads to a boosting of the pre-existing response. Subsequent doses may lead to anti-id[1], anti-tumour and, in some cases, auto-antibodies. These immune responses are detrimental in that anti-murine Ig antibodies increase the rate of clearance (and hence efficacy) of the administered monoclonal antibody and lead to a risk of an immune-complex mediated hypersensitivity reaction (Type III, serum sickness). The use of

a routine mouse Ig skin test was found to be of no predictive value. This skin test, however, primarily detects mast cell bound IgE-mediated type I hypersensity (anaphylaxis) and so may be inappropriate for monitoring a disease that results from circulating IgM and IgG immune complexes. It is hoped that in the future an increased understanding of idiotypic regulation may make it possible to turn these immune responses to clinical advantage.

FUTURE PROSPECTS

Results from this and other studies have shown the the present use of murine monoclonal antibodies for *in vivo* diagnosis and therapy of human diseases to be limited. However, elucidation of the problems has given rise to new approaches. These include suppression of cellular and humoral immune responses, identification and generation of monoclonal antibodies to more tumour specific antigens, reduction in the xenogeneic content of the therapeutic antibodies and manipulation of the idiotypic network by peptides or anti-idiotypic vaccines.

Two factors which play an important role in the success of monoclonal antibody therapy are a) the specificity of the antibody for its antigen and b) the specificity of the antigen for tumour cells. Specificity of the monoclonal antibody can to a large extent be ensured by careful screening of the cell-fusion derived hybridomas. Specificity of the target antigen for tumour cells is much more difficult to ensure. The majority of tumour-associated antigens have, at best, quantitative differences in their distribution. It could be argued that it may be possible to reduce the number of antibody administrations required if increased targeting efficiency can be achieved by the use of antibodies to more highly specific tumour antigens.

The idiotypic determinants on the surface immunoglobulin of B cell lymphomas are a good example of tumour specific markers. There are many reports which show therapeutic effectiveness of

anti-idiotype antibodies on B cell cancers, such as lymphomas and leukaemias (Stevenson and Stevenson, 1983). Because of their high degree of specificity for tumour cells the anti-idiotype antibodies could be thought of as the ultimate in the potential of monoclonal antibodies. Why then, can such malignancies as lymphomas and leukaemias not be erradicated? Studies have shown that functional differences between monoclonal anti-idiotype antibodies exist. These differences affect the fate of targeted cells (Kodoma et al., 1986). The actual mechanism of the anti-tumour effect may vary. Some antibodies after binding may result in cell destruction by host effector functions whilst others may not. Another possibility is that antigen-negative mutant tumour clones may arise which are resistent to serotherapy.

A further obstacle to treatment has been the immune response to the murine antibodies, which in one study developed in 5 of the 11 patients treated (Meeker et al., 1985). Once the 5 patients had developed an anti-murine Ig response, further infusions of antibody did not reach the tumour or induce tumour regression. In fact, further therapy was associated with toxicity. Of the 11 patients treated for B lymphocytic malignancy five were said to have had clinically significant remissions.

For an antigen to be tumour specific its expression should be a result of the malignancy. Apart from the idiotypic determinants on surface Ig of B cell tumours only, one other antigen has emerged as being tumour specific. The antigen, a 170kD efflux pump called p-glycoprotein, has to date only been found on tumour

cells showing multiple drug resistance. P-glycoprotein is expressed as a result of chemotherapy. As the cells expressing p-glycoprotein are drug resistant, new means for their destruction must be found. Recently, two monoclonal antibodies have been raised to epitopes of p-glycoprotein present on the cell surface (Hamada and Tsuruo., 1986). Having raised these monoclonal antibodies it will be interesting to see whether the p-glycoprotein is in fact tumour specific or whether its expression is only quantitatively increased on multiple drug resistant cells, as compared to that of normal tissue cells. In the event of the p-glycoprotein being found to be tumour specific the problem of the xenogeneic nature of the monoclonal antibodies will still exist. The efficacy of monoclonal antibody therapy as an anti-cancer modality in the future could be improved in two ways. Firstly, by preventing the host response to the therapeutic antibodies by immunosuppression. Secondly, by reducing the xenogeneic content of the therapeutic monoclonal antibody reagent.

A number of attempts at immunosuppressing recipients of murine monoclonal antibodies have been made. These include giving large initial doses of monoclonal antibodies (Sears et al., 1984) in order to achieve high zone tolerance; this was only successful in one patient. Immunosuppressive drugs such as prednisone and azathioprine (Cosimi et al., 1981, Ortho study group 1985) have been shown to be of limited use. In addition to drugs, monoclonal antibodies raised against T cell surface activation molecules (CD3, CD4 and CD8) have been used in the attempt to

immunosuppress patients (McMichael et al.,1987). Even the concurrent use of corticosteroids, azathrioprine and the anti-T cell monoclonal antibody, OKT3 could not abolish the anti-murine Ig response in renal allograft recipients (Chatenoud et al., 1986), although both the incidence and intensity of the immune response were delayed and reduced.

A recent study of the anti-murine immunoglobulin response in cynomologus monkeys showed that the use of total lymphoid irradiation prior to the administration of the anti-T cell antibodies anti-Leu-2b (CD8), Leu-3a (CD4) and Leu-5 (CD2) reduced it to less than 1% of that of the control group (Lowder et al., 1987).

Cyclophosphamide, a potent immunosuppresive agent, has also been reported to have failed in preventing anti-murine immunoglobulin responses in a patient receiving anti-Leu-1 (CD5) antibody (Miller et al., 1983). This drug has however been shown to be immunosuppressive when administered to cancer patients in low doses (Berd and Mastrangelo, 1987). The major determinant as to whether cyclophosphomide potentiates or suppresses immunity appears to be related to the time of administration in relationship to the presentation of antigen (Turk and Parker., 1982).

Immunosuppression of the response to xenogeneic monoclonal antibodies has been difficult. It could be argued that a reduction of the xenogeneic content of the antibody would obviate

the need for immunosuppression, or at least reduce it. This reduction in xenogeneic antibody content could be achieved in two ways: partially, by substitution of the constant domains, framework regions and variable domain of the murine monoclonal antibody with the equivalent human domains and regions; and totally, by the use of human monoclonal antibodies.

Recombinant DNA technology has made it possible to construct human/murine chimaeric antibodies (Neuberger et al., 1984). This method consists of cloning the genomic DNA fragments encoding the heavy and light chain variable regions (Brown et al., 1987) or those encoding just the complementarity determining regions (Jones et al., 1986) of a murine monoclonal antibody and inserting them into a mammalian expression vector containing genomic DNA segments encoding the human constant regions. These expression vectors can then be transfered into mouse myeloma cells (Sun et al., 1987) resulting in the production of functional mouse-human chimaeric immunoglobulin. When chimaeric antibodies are eventually used in clinical trials one would expect them to have at least two advantages over existing murine monoclonal antibodies. Firstly, they should prove to be less immunogeneic, and secondly, the human constant domains should interact more efficiently with human effector cells which could enhance tumour destruction. The recent publication of Riechmann et al., (1988) demonstrates the feasibility and functional effectiveness of this approach.

The alternative to chimaeric monoclonal antibodies is to make human monoclonals. In the last few years several methods have been developed to generate cell lines producing human monoclonal antibodies. Somatic cell hybridization between specific B lymphocytes and mouse myeloma cells have been hampered by stability problems due to selective loss of human chromosomes (Brom et al., 1985). Epstein-Barr virus (EBV) immortalization of sensitized B-lymphocytes to give B lymphoblastoid cell lines has been successfully applied to the production of human monoclonals (Irie et al., 1982). However, lymphobastoid cell lines usually secrete IgM antibodies which are generally of lower affinity than IgG antibodies and may pose problems in penetrating tumour tissue due to their large molecular weight. The first true human-human hybridoma secreting human monoclonal antibody of predefined specificy was produced by Kaplan and Olsson (1980). The level of antibody production however was too low for practical applications. Anti-tumour binding activity of antibodies from hybridoma cells produced by the fusion of lymphocytes from patients with a variety of malignancies and human myeloma-derived cells has been observed (Sikora et al., 1983). In this particular study, 12 antibodies with anti-tumour binding activity were isolated, all however, showed a low level of specificity and affinity.

It is hoped that both current and future research will result in stable human hybridomas secreting both IgM and IgG monoclonal antibodies with predefined specificity for tumour cell surface antigens. Whether the use of either chimaeric or human

monoclonal antibodies will result in development of anti-idiotypic antibodies which abrogate their therapeutic efficacy remains unclear. Some investigators doubt that the use of human or chimaeric monoclonal antibodies will be sufficient to eliminate the anti-immunoglobulin response. These doubts are based on the finding that full tolerance is difficult to achieve. Thus, even in the presence of tolerance to constant region determinants, monoclonal antibodies to cell surface determinants still elicit strong anti-idiotypic responses (Benjamin et al

Recently, there has been considerable interest in the possibility of using anti-idiotypic antibodies for the modulation of human immune responses, with particular interest being in the use of such antibodies for the treatment of cancer. The concept of idiotype-vaccines is based on antigen mimicry. Anti-idiotypes that mimic antigen have been generated in several systems, and their administration in vivo has been shown to either enhance or suppress a relevant immune response (Sharp et al., 1984; Stein and Soderstrom, 1984; Uytdehaag and Osterhaus, 1985; Raychaudhuri et al ., 1986).

In a recent study, rats immunized with a monoclonal anti-idiotypic antibody raised against a tumour-reactive monoclonal antibody showed reduced tumour take, following an intravenous challenge with tumour cells. The serum of these rats was also shown to contain significant levels of antibody which was indistinguishable in antigen specificity from that of the

original tumour-reactive monoclonal antibody (Dunn et al., 1987). Other studies have shown that mice vaccinated with anti-idiotypic antibody in order to induce immunity to SV-40-transformed cells, demonstrated prolonged survival after tumour transfer (Kennedy et al., 1985).

It is hoped that these antigen mimicry properties of anti-idiotypic antibodies will be further exploited for making clinically useful idiotype vaccines against tumours, viruses, bacteria and parasites.

In addition to idiotype vaccines, a great deal of interest has been shown in the use of synthetic peptide vaccines. In a recent study, thirteen volunteers were given orally a synthetic peptide vaccine for enterotoxigenic E.coli. The vaccine consisted of 26 amino acids of the heat-labile toxin B subunit joined to an 18 amino acid sequence of the heat-stable toxin. The volunteers received a total of 60mg over 4-6 weeks. Anti-toxin titres in both serum and jejunal aspirates were 4-7 times greater than in control subjects, and the jejunal aspirates neutralised the activity of both toxins (Klipstein et al., 1986). The results of challenge studies in immunized subjects are obviously being awaited with intense interest.

Although this study was the first to use a synthetic peptide immunogen in man, new findings by other groups should lead to other studies being undertaken. Recently, the mucin molecule carrying tumour associated epitopes recognised by tumour -

associated monoclonal antibodies HMFG1 and HMFG2 has been deglycosylated. This revealed a dominant polypeptide of 68KD molecular weight, which corresponded to that of the mucin core protein. Using the core protein as immunogen, a number of monoclonal antibodies which show pronounced tumour specificity on immunohistology have been raised (Burchell et al., 1987).

Utilizing these antibodies to the core protein, an expression DNA library made from the human mammary adenocarcinoma cell line MCF-7 has been screened. This screening has resulted in the identification and characterization of seven positive clones, encoding the antigenic portion of the core protein (Gendler et al., 1987). The study has also revealed that the human tumour - associated epithelial mucins are coded for by a single highly polymorphic autosomal gene locus, PUM, (Swallow et al., 1987). It is almost certain that new synthetic peptides, suitable for synthetic peptide vaccines will emerge from this in depth study.

Whichever of the possible strategies discussed above are employed in the passive immunotherapy of human disease including cancer, our understanding of the advantages and disadvantages from studies such as those discussed in this thesis should improve the prospects of future trials.

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