Studies of Idiotope Expression in B-Cell Chronic Lymphocytic Leukaemia

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DECLARATION

I declare that the experiments described in this work were performed by myself and that this thesis was composed by myself.

ACKNOWLEDGMENTS

There are many people whose effort and support have been invaluable to me in completing this work.

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PUBLICATIONS

None of the experimental results described in this work have, as yet, been published. However, two papers, discussing the main findings and their relevance for anti-idiotypic immunotherapy, are in preparation and are shortly to be submitted for publication.

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ABBREVIATIONS

Ab	Antibody
ADCC	Antibody Dependent Cellular Cytotoxicity
Anti-Id	Anti-idiotypic antibody
CLL	Chronic Lymphocytic Leukaemia
DDW	Deionised, Distilled Water
EDTA	Ethylenediamine Tetra-Acetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
HEPES	4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid
Id	Idiotype
Ig	Immunoglobulin
McAb	Monoclonal Antibody
NHL	Non-Hodgkin's Lymphoma
NP-40	Nonidet P-40
NRS	Normal Rabbit Serum
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PBMNC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PC	Phosphorylcholine
PLL	Pro-Lymphocytic Leukaemia
RID	Radial Immunodiffusion
SAPU	Scottish Antibody Production Unit
SDS	Sodium Dodecylsulphate
sIg	Surface Immunoglobulin
SRBC	Sheep Red Blood Cells

TBS	Tris Buffered Saline
TNP	Trinitrophenyl
TPA	12-O-Tetradecanoylphorbol 13-Acetate
V	Variable

ABSTRACT

Over the past few years there has been considerable interest in the use of anti-idiotypic (anti-Id) antibodies for immunotherapy of B-lymphocyte tumours. In spite of some promising results clinical trials have been restricted by the need to produce unique antibodies for each patient to be treated. This study has attempted to define recurring idiotopes in a group of 42 cases of chronic lymphocytic leukaemia (CLL) with the ultimate aim of using pre-existing anti-Ids to treat the disease. The reactivity of a panel of 14 monclonal anti-Ids with normal serum immunoglobulin (Ig) was determined using an enzyme-linked immunosorbent assay (ELISA) technique. The anti-Ids were divided into three groups; seven antibodies reacting with determinants unique to the immunising paraprotein, four antibodies reacting with public idiotopes strongly expressed by serum Ig, and three antibodies reacting with restricted public idiotopes, ie with low levels of expression by serum Ig. Expression of the various idiotopes by the CLLs was tested by indirect immunoperoxidase. To confirm the specificity of cellular reactions, cells from 18 patients were stimulated by the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) to secrete Ig in vitro. The presence of polyclonal Ig in

the culture supernatants was found to be related to the presence of autologous T-cells. Secreted tumour Ig was tested for anti-Id specificity by ELISA. The majority of cellular reactions were confirmed as Ig specific, but a number of cross-reactions also occurred both in unstimulated cells and after TPA stimulation. The results confirmed that private idiotypic determinants are not generally expressed by CLLs. However, the public idiotopes are expressed by between 7% and 29% of cases and the restricted public idiotopes also show variable expression ranging from 5% to 14% of cases. The potential value of the last group of antibodies as therapeutic agents in B-cell malignancies is discussed.

CHAPTER 1

INTRODUCTION

For many years one goal of tumour immunologists has been to produce antibodies directed against tumour specific cell surface components for therapeutic use (Currie, 1972). Early attempts at such immunotherapy were frustrated by the heterogeneity of anti-sera and the side effects associated with the administration of large quantities of foreign proteins. Since the advent of monoclonal antibody technology (Köhler and Milstein, 1975.) with the ability to produce unlimited amounts of antibody against a single antigenic determinant, these problems have been largely solved, but have been replaced by those of defining suitable tumour cell antigens. The ideal target for immunotherapy would be a stable epitope expressed by the entire neoplastic clone but not by non-malignant cells.

Unfortunately, the majority of tumour associated antigens described are not absolutely specific for the malignant cells as they are also expressed by normal cells at certain stages of differentiation. The closest approximation to a true tumour specific antigen is the immunoglobulin (Ig) idiotype (Id) of those leukaemias and

lymphomas of the B-lymphocyte series which express surface immunoglobulin (sIg). In the future it is possible that T-cell receptor idiotypes expressed by T-lymphocyte malignancies may also be exploited as tumour specific antigens.

1.1. The Immunoglobulin Idiotype

It is estimated that the immune system is capable of generating up to 10^{8} different antibody specificities (Roitt et al, 1981,) partly by the control and expression of the germline Ig variable (V) region heavy and light chain genes, and partly by a process of somatic mutation (Tonegawa, 1983). As a result each antibody clone expresses unique V region associated antigenic determinants collectively known as the idiotype; a term derived from the Greek for "individual" and "form" and first used by Oudin (1966).

Even before the structure of Ig was elucidated Slater et al (1955,) showed that rabbit anti-sera against human myeloma proteins would react with the original paraprotein even after extensive adsorption with normal Ig and other myeloma proteins; the first description of an anti-idiotypic (anti-Id,) anti-serum. Similar properties for normal Ig were described by Kunkel et al

(1963,) who showed that human antibodies produced in response to immunisation with dextran, levan and blood group antigens expressed unique antigenic determinants. These were localised to the Fab segment by Grey et al (1965,) and eventually to the Fv portion of the immunoglobulin molecule (Wells et al, 1973).

Most idiotypic determinants require the interaction of heavy and light chains for their expression (Carson & Weigert, 1973,) and are therefore the products of 2 unrelated genes. There are, however, idiotypic determinants which are localised on heavy (Yarmush et al, 1977; Zeldis et al, 1979,) or light (Chen et al, 1984,) chains and may be phenotypic markers of single variable (V) region genes.

Williams et al (1968,) had shown that IgM cold agglutinins with similar antigenic specificity also shared idiotypic specificity which was partly blocked when the antibodies were bound to their antigens. Similar findings for human IgM rheumatoid factors (Kunkel et al, 1973,) led to the generally held belief that the antigenic and idiotypic specificities of an Ig molecule were both dependent on the hypervariable regions of the V genes and that the antibody combining site contained the major idiotypic determinants (Capra, 1977).

However, V region structures which are physically distant from the antigen binding site can also be defined serologically (Mudgett et al, 1978). These are generally framework determinants common to V region gene families or cross-isotypic epitopes (Rivat-Peran et al, 1980). In addition it is possible that V region allotypes such as have been described for the V_H genes of rabbits (Kindt, 1975,) may also exist in humans. Such determinants are not clone specific and are therefore not strictly idiotypic but the term is frequently used to describe any V region associated antigenic determinant.

Thus a picture has gradually emerged of the idiotype as a complex structure expressing a number of different antigenic determinants or idiotopes which are structurally and functionally distinct (Rajewsky and Takemori, 1983). Each idiotope can be defined by a monoclonal anti-idiotypic antibody and the sum total of all the individual idiotopes defines the idiotype.

1.2. Anti-Idiotypic Immunotherapy of Human B-Cell Tumours

Any B-cell tumour which expresses sIg can be considered a candidate for anti-Id immunotherapy providing tumour Id secretion is not excessive as high serum Id levels would neutralise the therapeutic antibody. Thus the most suitable tumours for this form of therapy are likely to be chronic lymphocytic leukaemia (CLL), pro-lymphocytic leukaemia (PLL), and the majority of the low and intermediate grade non-Hodgkin's lymphomas (Ersböll et al, 1985).

Practical experience has confirmed that it is relatively easy to generate polyclonal (Stevenson & Stevenson, 1975,) and monoclonal (Thielemans, 1984,) anti-Ids against tumour associated Ig. Such antibodies are highly specific for the malignant clone to the extent that a unique antibody has to be produced for each patient to be treated.

There are reports of 25 patients with B-cell tumours being treated with anti-Ids since 1980 (table 1.1). Although the overall results of treatment have been disappointing there is clear evidence that such therapy can have a significant anti-tumour effect. It must be remembered when assessing these results that the majority of the patients involved have had end-stage disease,

often refractory to conventional forms of therapy.

Two groups have pioneered the use of anti-Ids as therapeutic agents in human B-cell tumours. Stevenson's group at the Tenovus Research Laboratory in Southampton have reported treating a total of 7 patients with sheep polyclonal anti-Id preparations and 1 patient with a derivative of a monoclonal antibody (table 1.1).

Three patients with NHL did not show clinically significant effects after polyclonal anti-Id therapy (Macbeth et al, 1983,) but in 3 of 4 patients with CLL or PLL treatment with polyclonal anti-Id was associated with a marked reduction in circulating tumour cells and evidence of cell death (Hamblin et al, 1980; Gordon et al, 1984a). However, there was no reduction in palpable disease and the blood counts returned to pre-treatment levels within 3 days. No sustained beneficial effects were seen in any of the patients.

Levy's group at Stanford University reported the first apparent cure due to anti-Id therapy using a monoclonal antibody raised against a private idiotypic determinant from tumour specific Ig (Miller et al, 1982). The patient had a non-Hodgkin's lymphoma which had undergone transformation from a follicular to a more aggressive, diffuse histology and was refractory to conventional

cytotoxic therapy. Ten days after starting intravenous infusions of a monoclonal IgG2b anti-Id the tumour showed evidence of regression and ultimately the patient achieved a sustained, unmaintained complete remission which has currently lasted over 4 years. Biopsy of the regressing lesion demonstrated large numbers of macrophages and activated T-lymphocytes by immunophenotyping (Levy and Miller, 1983).

This group have since reported their experience of using monoclonal anti-Ids to treat 13 patients (12 with NHL and 1 with PLL). In 6 cases there was significant tumour regression lasting up to 6 months but only the original patient achieved a durable, complete remission (Meeker et al, 1985, and Miller et al, 1987).

Two patients with NHL (Rankin et al, 1985,) and two patients with CLL (Bertoli et al, 1984; Capel et al, 1985,) have also been treated with monoclonal anti-Ids and have had transient anti-tumour effects but no sustained response.

TABLE 1.1

Clinical Trials of Anti-Id Therapy in Humans

Disease	No. Pts.	Anti-Id	Reference
PLL	1	Poly}	Hamblin et al, 1980
CLL	3	Poly}	Gordon et al, 1984a
NHL	3	Poly	Macbeth et al, 1983
NHL	12	Mono}	Miller et al, 1982
PLL	1	Mono}	Meeker et al, 1985
		}	Miller et al, 1987
CLL	1	Mono	Bertoli et al, 1984
NHL	2	Mono	Rankin et al, 1985
CLL	1	Mono	Capel et al, 1985
CLL	1	Mono*	Hamblin et al, 1987

PLL = Prolymphocytic Leukaemia, CLL = Chronic Lymphocytic Leukaemia, NHL = Non-Hodgkin's Lymphoma, Poly = polyclonal, Mono = Monoclonal.
No. Pts. = Number of patients treated with anti-idiotype

*Univalent chimeric derivative of monoclonal antibody

1.3. Mechanism of Action of Anti-Idiotypic Therapy

It has been suggested that the anti-tumour effects of anti-Id could be mediated by two entirely separate actions (Stevenson & Stevenson, 1983,); i) direct cytotoxicity and ii) immune regulation.

1.3.1 Direct Cytotoxicity

Tumour cells coated with therapeutically administered anti-Id could be lysed by host immune effector mechanisms such as complement or antibody dependent cellular cytotoxicity (ADCC). Macrophages (Shin et al, 1975, Langlois et al, 1981,) natural killer cells (Ojo and Wigzell, 1978,) and killer lymphocytes (Cerrotini et al, 1974,) have all been suggested as the cell responsible for destruction of antibody coated tumour cells.

There is evidence from animal models of tumour immunotherapy that complement and ADCC can both mediate tumour cell destruction (Bernstein et al, 1980,) but that ADCC is the more important mechanism in vivo (Kirch and Hammerling, 1981; Levy and Miller, 1983).

1.3.2 Immune Regulation

As a target for immunotherapy the idiotype is unique, not only because of its specificity for the malignant clone, but also because it is a physiological receptor through which clonal regulation is normally mediated.

There is circumstantial evidence that malignant B-cells are capable of responding to such regulatory signals (Jaffe, 1983,) and it is possible that therapeutic anti-Id would induce immunoregulatory circuits which ultimately control the malignant clone.

There is some evidence from the treatment of human patients to support the participation of both these mechanisms in the anti-tumour effect.

The first patient treated with polyclonal anti-Id by Stevenson's group (Hamblin et al, 1980,) had a marked reduction in serum complement levels associated with therapy. Cells from 4 leukaemic patients treated by this group were lysed in vitro in the presence of polyclonal anti-Id when rabbit serum was used as a source of complement although ABO compatible human serum was less effective (Gordon et al, 1984a).

Results with monoclonal anti-Ids, however, have been more variable. A post-therapy lymph node biopsy from a patient with NHL revealed large numbers of lysed tumour cells suggesting a cytotoxic effect (Rankin et al, 1985,) and a patient with CLL treated with a derivative of a monoclonal anti-Id showed some evidence of complement activation in vivo although tumour cells were resistant to complement and ADCC in vitro (Hamblin et al, 1987).

However, the patient achieving a complete remission in the Stanford series (Meeker et al, 1985,) was treated with an anti-Id which was not cytotoxic to the tumour cells in vitro and had no effect on mitogen induced proliferation in vitro (Lowder et al, 1987). In this study the only pre-therapy parameter which did correlate with the clinical response to anti-Id was the number of T-lymphocytes in the tumour biopsy.

It is possible that more aggressive tumours are less likely to respond to anti-Id therapy and also tend to contain fewer normal cells. However, another explanation could be that the infiltrating T-cells are part of an ineffectual host immune response against the tumour which is augmented by anti-Id therapy.

Animal models have been used in an attempt to determine which factors and by what mechanisms tumour response to anti-Id therapy is mediated.

1.4 Animal Models

Only two models, the L_2C guinea pig leukaemia and the murine myelomas will be described in detail as they provide evidence in favour of each of the two mechanisms by which anti-Id therapy is thought to work.

1.4.1. L_2C Guinea Pig Leukaemia

This spontaneously arising IgMλ expressing leukaemia is a useful model because the cells express high levels of sIg but do not secrete Ig.

In vitro studies showed that only the IgM and IgG1 fractions of polyclonal sheep anti-Id antisera were capable of lysing tumour cells in the presence of complement and that the IgG2 fraction could mediate ADCC using sheep or human leucocytes (Stevenson & Elliot, 1978). Further studies with guinea pig and murine peritoneal macrophages as the effector cells showed that these cells were incapable of phagocytosing anti-Id

coated tumour cells, were not directly cytotoxic but that they did inhibit tumour cell proliferation in vitro (Lawson & Stevenson, 1983). The in vivo significance of these findings is uncertain and it may be that tumour cell destruction is dependent on a variety of effector functions.

Guinea pigs challenged with 2 x 10^5 tumour cells in vivo invariably die within 20 days. Survival times were slightly improved (by a median of three days,) by a single dose of polyclonal sheep anti-idiotypic IgG administered six hours after the tumour inoculum (Stevenson et al, 1977).

The group then looked at the effects of active immunisation with purified tumour Id prior to challenge with tumour cells (Stevenson & Gordon, 1983,) and found that all immunised animals developed anti-Id antibodies capable of lysing L_2C tumour cells in vitro in the presence of complement. The immunised animals showed clearly improved survival times (all the control animals died around day 12, whereas all the immunised animals died between days 19 and 26). Estimating the doubling time of the tumour in vivo the authors concluded that a high proportion of the tumour cells were destroyed but there were no long term survivors.

1.4.2. Murine Myelomas

Multiple myeloma in man is not a likely candidate for anti-Id therapy as the tumour cells express little or no Ig to serve as a target and also the large amounts of serum idiotype would neutralise infused anti-Id. The series of murine myelomas are different from the human disease. They are morphologically heterogeneous including small round sIg positive lymphocytes as well as classical plasmacytic forms. When an animal is challenged with tumour the more plasmacytic cells die off rapidly and serum Id levels remain low for a number of days until the sIg positive precursor cells differentiate into Ig secreting forms (Lynch et al, 1979). During this period idiotype specific manipulation of the tumour is possible.

The MOPC 315 myeloma is a tumour of BALB/c mice and purified MOPC 315Id is capable of inducing a syngeneic anti-Id response (Sirisinha & Eisen, 1971). Lynch et al (1972,) were the first to show that immunising BALB/c mice with purified MOPC 315Id provided 90% long term protection from lethal challenge with the tumour and that the protection was idiotype specific. The protective effect was not associated with complement mediated lysis nor with the levels of anti-Id in the serum (Frikke et al, 1977).

Passive immunisation with large amounts of syngeneic immune serum was capable of suppressing tumour growth (Bridges, 1978). This group also reported that tumour immunity could be mediated by adoptive transfer of spleen cells from immunised animals to irradiated animals but only when the recipient animals received a small dose of tumour Id in addition.

Evidence that the protective effect involved more than a simple antibody response was obtained when it was shown that post-immunisation thymectomy abolished the anti-tumour activity even though the serum anti-Id levels were not significantly changed (Daley et al, 1978).

Further studies found a population of Id specific suppressor T-cells capable of inhibiting Id secretion in vivo (Rohrer, 1979,) and of inhibiting tumour cell proliferation and Ig secretion in vitro without being directly cytotoxic (Flood et al, 1980; Milburn et al, 1982). Lynch & Milburn (1983,) showed that reduced Ig secretion induced by suppressor T-cells in vitro was due to reduced light chain transcription and production while heavy chain production was reduced but mRNA transcription was unaltered.

Monoclonal anti-Ids were also shown to be capable of suppressing myeloma cell growth and Ig secretion in vitro (Kodama et al, 1986,) and of protecting animals from tumour cell challenge in vivo (Bridges et al, 1984).

1.4.3. BCL$_1$ Murine Leukaemia

This spontaneously occurring IgMλ leukaemia has morphological and phenotypic characteristics similar to the pro-lymphocytic variant of CLL in man (Krolick et al, 1979). Administration of polyclonal anti-Id before and after challenge with a lethal dose of tumour cells resulted in complete suppression of the malignant clone. In vitro studies also showed reduced tumour cell proliferation in the presence of anti-Id.

However, Ciavarra et al (1986,) showed that some strains of mice were naturally resistant to tumour challenge and that this was mediated by T-cells. They further showed that a response against both the BCL$_1$ idiotype and a histocompatability antigen expressed by the tumour was required for successful spontaneous immunity to the tumour.

1.4.4 CH Murine Lymphomas

This series of lymphomas were induced in response to intense immunisation with sheep red blood cells (SRBC) and were found to be transplantable (Lanier et al, 1982). Original work on the CH1 lymphoma had shown that polyclonal anti-Id could completely protect animals from tumour challenge (Haughton et al, 1978). Increasing the dose of anti-Id resulted in reduced animal survival and the authors concluded that the effects were probably mediated by immune regulation rather than direct cytotoxicity. Further work showed that the protective effect of anti-Id was only mediated by the intact Ig molecule and that it did not depend on either an intact thymus or on complement (Lanier et al, 1980).

Polyclonal anti-Ids raised against 7 of the 27 B-cell tumours characterised in the CH series showed significant cross-reactivity with 21 of the tumours (Pennell et al, 1985). Not all of the cross-reacting tumours reacted with SRBC and the authors postulated that the expression of the recurring idiotypes could be related to a common immunoregulatory defect making certain B-cell clones more likely to undergo malignant transformation.

1.4.5 Murine Lymphoma 141

Sugai et al (1974,) described this tumour which arose spontaneously in stock NZB/NZW F1 mice which also have a high incidence of auto-immune disorders. Mice immunised with purified tumour Id and control animals both develop tumour nodules 15 days after tumour challenge. In 3 out of 5 immunised mice, however, the tumours regressed after 35 plus days. In vitro tests showed evidence of weak humeral immunity but no evidence of ADCC. Spleen cells on the other hand were cytotoxic for the tumour cells suggesting that a cellular immune response was responsible for the regression.

1.4.6 Murine Lymphoma 38C13

This carcinogen induced, transplantable, IgM expressing lymphoma was produced in a T-cell depleted C3H/eB mouse (Bergman & Haimovich, 1977 and Bergman, Haimovich & Melchers, 1977). Perek et al (1983,) showed that mice could be protected from lethal tumour challenge not only by monoclonal anti-Id but also by the $F(ab)_2$ portion of the parent molecule suggesting that the protective effect was not dependent on complement or ADCC.

Using the same model, however, Kaminski et al (1986,) showed that monoclonal anti-Id was capable of mediating ADCC in vitro. Using class-switch variants of the same anti-Id clone they further demonstrated that an IgG2a variant was more effective than other IgG isotypes both at protecting mice from tumour challenge in vivo and at mediating ADCC of tumour cells in vitro.

The immunisation of animals with tumour idiotype or anti-Id prior to challenge with a relatively small dose of tumour cells is far removed from the treatment of an established lymphoma or leukaemia in man. There are, however, a number of useful observations arising from such models.

Different experiments using the same animal models often provide conflicting evidence about the likely mechanism of action. This suggests that both direct cytotoxicity and immune regulation can be induced depending on factors such as the isotype, specificity and dose of anti-Id.

There is clear evidence that malignant B-cells are capable of responding to immunoregulatory signals both in vivo and in vitro with long lasting effects. Such

regulation is idiotype specific since the anti-tumour effect of immunising mice with one tumour idiotype does not extend to similar tumours expressing different idiotypes. In addition idiotype specific T-cells capable of inhibiting tumour cell growth and differentiation in vitro have been identified.

If normal immunoregulatory signals are to be exploited for the therapy of human B-cell tumours it is necessary to understand the role of idiotype in the regulation of the normal immune system and this is discussed in the following section.

1.5 Idiotypic Regulation in the Normal Immune System

1.5.1 The Idiotypic Network Theory

Soon after their discovery it was clear that idiotypic determinants were associated with Ig diversity and antigenic specificity (Williams et al, 1968 and, Kunkel et al, 1973). The concept that the idiotype might play a central role in immune regulation was first put forward by Jerne (1974). His theory assumed that the total Ig repertoire was very large and that the variable region of each Ig molecule consisted of a paratope or antigen binding site and a set of idiotopes or antigenic determinants which were immunogenic. He proposed that the immune system consisted of a series of interacting clones of lymphocytes which were capable of stimulating and suppressing each other through idiotypic interactions and suggested the predominant outcome in a steady state must be suppressive.

He also postulated that T-cells would be involved in idiotypic interactions thus converting a series of unrelated lymphocyte clones into an integrated system capable of self-regulation.

Jerne suggested a model of network regulation in which the clonal expansion of B-cells stimulated by antigen resulted in an increase in the concentration of their idiotypic determinants to immunogenic levels. As a consequence anti-idiotypic clones would be stimulated and expanded and could further stimulate or suppress the original clones depending on other factors. The idiotopes expressed by the anti-idiotypic clones would be capable of stimulating a third set of antibodies. The ultimate outcome of these various interactions would be to return the immune system to a state of equilibrium once the antigen was removed.

Experimental support for Jerne's hypothesis has mostly come from studies of inbred animals. For instance, the antibody response in BALB/c mice to the bacterial antigen phosphorylcholine (PC) is dominated by B-cell clones expressing the T15 Id. After repeated immunisations with PC, anti-idiotypic antibodies against the T15 Id are produced (Kluskens and Köhler, 1974). Kelsoe and Cerny (1979,) showed that the numbers of T15 positive cells peaked on day 5 and day 12 after immunisation while the number of anti-T15 positive clones peaked on days 10/11 and after 13 days. The successive peaks of Id positive and anti-Id positive clones is consistent with Jerne's original model.

Similar results have been obtained with other systems including the immune responses to trinitrophenyl (TNP) in BALB/c mice (Schrater et al, 1979, and Bona et al, 1979,) and to Micrococcus lysodeikticus in rabbits (Brown and Rodkey, 1979).

The absence of strain specific immunodominant idiotypes in outbred populations make it difficult to study similar systems in man. However, individuals hyperimmunised with antigens such as tetanus toxoid can be shown to develop auto-anti-idiotypic antibodies (Geha, 1982,) and circulating anti-Id positive lymphocytes (Geha, 1983).

Another investigative approach has been to influence the immune response by idiotype specific manipulation. For instance, antibodies (Ab1) produced in response to Micrococcus lysodeikticus in outbred rabbits only share idiotypic specificity once in 60 animals. Ab1 was used to immunise a second, allotype matched, rabbit to produce an anti-Id (Ab2) response. A third rabbit was immunised with Ab2 to produce an anti-anti-Id (Ab3) response. If Ab3 was used to immunise another animal which was then challenged with the original antigen the resulting antibody response was dominated by antibodies sharing idiotypic specificities with Ab1 (Urbain et al, 1977).

The authors suggest that Ab1 clones are normally suppressed by Id specific Ab2 clones. Pre-treatment with Ab3 temporarily suppresses the Ab2 clones allowing the preferential expansion of Ab1 clones in response to antigen.

Similar idiotypic manipulations have been described in BALB/c mice immunised with TNP. An idiotype expressed by the TNP binding myeloma MOPC 460 is normally a minor component of the anti-TNP response (Bona and Paul, 1979). Immunisation with 460Id induces an anti-Id response which results in a reduction of 460Id positive cells in response to TNP (Bernabé et al, 1981b). This suppression of 460Id positive clones is transferred to the progeny of female mice immunised with 460Id (Bernabé et al, 1981a).

Conversely, immunisation with anti-460Id antibody induces an anti-anti-460Id response which results in the inhibition of naturally occurring 460Id specific suppressor T-cells (Bona & Paul, 1979,) ultimately resulting in an increase in 460Id positive cells (Bernabé et al, 1981b).

Idiotype specific T-cells have also been shown to be generated during the course of an immune response. Thus Owen et al (1977,) demonstrated Id specific T-suppressor cells produced in response to anti-idiotypic antibodies

in A/J mice while Id specific helper T-cells are generated in the immune response of B10.A mice to hen egg white lysozyme (Adorini et al, 1979.) and probably augment the T15 Id response to PC in BALB/c mice (Bottomly et al, 1979).

These results show that idiotypic interactions occur in the normal immune response and can be used to manipulate that response. The demonstration of Id specific T-cells which can stimulate or suppress B-cells in the absence of antigen may shed some light on the nature of the idiotypic interactions which are involved in network regulation although no single model of the idiotypic network enjoys universal acceptance.

It is conceivable that disturbances in the normal idiotypic regulation of B-cells could result in uncontrolled clonal proliferation even though the critical defect lies outwith the malignant population. If such tumours do exist they would be ideal candidates for idiotype specific immunotherapy.

There is also the possibility that any B-cell tumour expressing sIg would be susceptible to network regulation if the immune system is intact and can be stimulated with the appropriate anti-Id.

1.5.2 Shared Idiotopes

Within a few years of the discovery of idiotypy evidence began to emerge that some idiotypic determinants were not specific for a particular antibody clone but were shared by many different clones (Kunkel et al, 1973).

Shared or cross-reacting idiotopes in the normal immune system must either be derived from genetically unrelated B-cell clones which have been selected by virtue of affinity for the same antigen or by related clones using the same or similar variable region genes.

In the former case the shared idiotopes are likely to be closely related to the antigen combining site and to depend on intact immunoglobulin for their expression. In the latter case the idiotopes concerned may be the phenotypic markers of the expression of specific variable region genes and may therefore be located on isolated heavy or light chains.

Shared idiotopes have been well described in the immune responses of inbred mice to a number of simple haptens (Claflin & Davie, 1975; Mäkelä & Karjanainen, 1977; Ju et al, 1978). In such cases the shared idiotope can usually

be shown to be the product of a specific germ line variable region gene, usually from the heavy chain. The cross-reacting idiotope from the anti-GAT response of BALB/c mice has been localised to a specific amino acid sequence in the second hypervariable region of the heavy chain although its expression seems to depend on combination with a specific light chain (Clevinger et al, 1980).

Even in an outbred population such as man it is possible to identify shared idiotopes as in antibodies against Rhesus antigens (Forre et al, 1977,) and hepatitis B surface antigen (Kennedy & Dreesman, 1983). The majority of shared idiotopes that have been described in man are associated with auto-immune disorders for instance in anti-thyroglobulin antibodies in Grave's Disease (Matsuyama et al, 1983,) anti-acetylcholine receptor antibodies in myasthenia gravis (Lefvert, 1982,) and in non-organ specific auto-antibodies such as anti-DNA antibodies (Diamond & Soloman, 1983,) and Rheumatoid factors (Kunkel et al, 1974).

Immunoglobulins expressing shared idiotopes associated with cold agglutinins (Feizi et al, 1977,) and Rheumatoid factors (Goñi et al, 1985; Fong et al, 1986,) have been shown to utilise a restricted number of V region genes. In such instances the shared idiotopes are phenotypic

markers of these genes.

Thus there is evidence in health and in disease that shared idiotopes are the products of specific V region heavy or light chain genes. The incidence of shared idiotopes in auto-immune disorders is most interesting and suggests that specific clones or V region genes are particularly likely to be involved in disordered immunoregulation.

The conservation of specific V region genes and their idiotypic determinants by the normal immune system would strongly suggest that there are selective pressures favouring their retention and expression. One possible explanation would be that such shared idiotopes have specific roles in the normal regulatory processes of the idiotypic network.

1.5.3 Regulatory Idiotopes

In the early 1980s Bona and his colleagues put forward a theory that the immune system preferentially used a specific set of idiotopes for internal regulation (Bona et al, 1981, Paul & Bona, 1982, and reviewed by Bona, 1987). They attempted to prove this using mouse myelomas as models.

Idiotypic Ig from the ABPC48 and UPC10 myelomas of BALB/c mice share antigenic specificity for the β2-6 polyfructosan linkages found in bacterial and grass levans. In addition they share a number of cross-reacting idiotopes some of which are not inhibited by antigen and are therefore distant from the antigen combining site (Bona et al, 1984). Genetic analysis demonstrates that both myeloma idiotypes use the same V_H subgroup derived from a single germline V_H gene (p441-4) (Ollo et al, 1981).

BALB/c mice immunised with A48Id (Ab1) produced an anti-Id (Ab2) response. Ab2 antibodies were then used to generate Ab3 which were used to generate Ab4 antibodies in other mice. If each antibody expresses a set of idiotopes all of which are immunogenic then each anti-Id response should result in the generation of a new and unrelated set of antibodies.

However, when Bona looked at the binding properties of these antibodies he found that Ab1 and Ab3 antibodies shared A48 idiotopes and were both capable of binding Ab2 and Ab4.

In order to explain these results he postulated that Ab1 expressed a special set of "regulatory idiotopes" which

were preferentially used by the immune system, so that immunisation with Ab2 results in the activation of clones bearing the A48 regulatory idiotopes.

An alternative explanation is that since the Ab2 response is polyclonal it will consist of different clones of B-cells linked only by a common affinity for antigenic determinants on Ab1. It is therefore possible that the expression of immunogenic idiotopes by these heterogeneous clones is not sufficient to stimulate a true Ab3 response whereas the common specificity they share for Ab1 gives rise to the preferential stimulation of clones expressing similar idiotypic determinants to Ab1. If this were so then the use of a monoclonal Ab2 with a single, uniform set of idiotopes would induce a true Ab3 response which would consist of antibody clones idiotypically and genetically unrelated to Ab1.

However, the immunisation of BALB/c mice with different monoclonal anti-A48Ids (Ab2) still resulted in the stimulation of clones bearing A48 idiotopes including both levan binding and non-binding molecules (Legrain and Buttin, 1983).

The A48 Id system can also be used to investigate normal immune responses, since non-immunised BALB/c mice have naturally high titres of anti-levan antibodies, only a minor population of which express A48 idiotopes. Immunisation with levan does not stimulate A48 Id positive cells possibly due to naturally occurring A48 Id specific T-suppressor cells (Liebermann et al, 1979).

However, Bona discovered that A48 Id positive clones could be preferentially stimulated by administration to neonatal animals of either a minute amount of anti-A48 Id or A48 Id (Bona et al, 1984). Giving A48 Id also resulted in the production of idiotype specific T-helper cells which, presumably, had a positive regulatory effect.

Bona interpreted this as further evidence that A48 cross-reacting idiotopes have a special role in clonal regulation and performed further experiments with the related myeloma protein UPC10 which was used to immunise pregnant mice. When their progeny were subsequently challenged with bacterial levan the antibody response was dominated by UPC10 positive clones and idiotype specific T-helper cells were also detected (Bona et al, 1984).

As a result of these experiments Bona has suggested that regulatory idiotopes can be defined by the following

criteria:

i. They are autoimmunogens.

ii. They are recognised by regulatory T-cells and have the potential to become dominantly expressed as a result of such interactions.

iii. They are the products of conserved V region germ line genes and therefore recurring within species.

iv. They are shared by antibodies with varying antigenic specificities.

The concept of regulatory idiotopes has enormous implications for anti-idiotypic therapy of B-cell tumours. Activating T-cell clones with specificity for a regulatory idiotope expressed by surface Ig may be the key to controlling the malignant cells. Successful anti-Id therapy would then depend on the identification of the most appropriate idiotypic targets.

Using the ABPC48 mouse myeloma model Bona's group have obtained experimental evidence that the target idiotope may be crucial to the outcome of idiotype based

immunotherapy. BALB/c mice were immunised with 1 of 14 different monoclonal Ids all derived from the same germ line V_H genes and all sharing idiotopes with A48Id. All antibodies induced a similar anti-Id response but only 5 of them protected immunised mice from challenge with ABPC48 myeloma cells (Rubinstein & Bona, 1986).

The detection of specific regulatory idiotopes amongst the set of idiotypic determinants expressed by each tumour is, however, a far more complex problem in man than it is in an inbred laboratory animal.

1.6 <u>Anti-Id Production</u>

The ideal immunogen is purified tumour specific idiotype, a problem in tumours likely to respond to anti-Id therapy since these, largely, do not secrete Ig. However, it is possible to produce sufficient amounts of Id from such tumours in vitro by forming heterohybrids between tumour cells and mouse myelomas cells and selecting variants which secrete human Ig (Brown et al, 1980).

Chooosing between polyclonal and monoclonal anti-Id as a therapeutic agent is important as they have different characteristics:-

1.6.1 Polyclonal Anti-Ids

Polyclonal anti-Ids can be fairly easily raised by immunising an animal such as a sheep with tumour Id and adsorbing the antibodies produced with normal human Ig to remove activity against Ig isotypes and allotypes. This will, however, result in the loss of reactivity against idiotopes commonly expressed by normal Ig and these may include regulatory idiotopes.

The resulting anti-Id preparation will contain antibodies from a variety of different B-cell clones reacting with a variety of idiotypic determinants on the tumour Ig and may therefore show high avidity in binding tumour Id.

Large amounts of purified Id would be required for anti-Id production which would also have the disadvantage of batch variation. Nevertheless, polyclonal anti-Id can be quickly produced and high avidity would be advantageous for cell lysis via ADCC or complement.

1.6.2 Monoclonal Anti-Ids

Monoclonal antibodies have the advantages that they can can be produced in unlimited quantities without batch variation. They can also be generated using much smaller amounts of the immunogen although the process is complicated, time consuming and expensive.

Antibodies against private idiotypic determinants can be produced fairly easily by selecting and cloning hybrids which react with the tumour idiotype but not with other, unrelated paraproteins or with pooled normal immunoglobulin (Giardina et al, 1985). The production of antibodies against shared idiotopes would be much more complicated as large numbers of hybrids showing restricted reactivity with a small panel of paraproteins may have to be cloned and further characterised (Walker et al, 1987).

Since a monoclonal antibody reacts with a single epitope avidity is unlikely to to be as high as in a polyclonal preparation but the specificity of monoclonal anti-Ids would make them most appropriate for therapy dependent on effects on regulatory idiotopes.

1.7 Selection of Monoclonal Anti-Ids for Tumour Immunotherapy

Conventional strategy relies on producing anti-Ids against private idiotypic determinants with the expectation that such reagents offer the advantage of absolute specificity for the malignant clone. However, the production of a unique anti-Id for each patient to be treated may be impractical on a large scale. In the Stanford trial it took an average of 1 year between selecting a patient as a candidate for therapy and having a monoclonal anti-Id available for therapeutic use (Thielemans et al, 1984).

Private determinants are also characteristic of somatic mutations and may therefore be prone to further mutations associated with changes in idiotype expression in time (Rajewsky & Takemori, 1983).

Furthermore, if successful anti-Id therapy depended on selecting antibodies against specific regulatory idiotopes then the random selection of antibodies against private determinants will not be optimal and this may explain the variable results of clinical trials.

1.8 Cross-Reacting Idiotopes in B-Cell Tumours

An alternative approach would be to identify idiotopes which recurred at significant frequencies within a group of tumours and to produce a panel of antibodies against such determinants.

The Stanford group looked for evidence of shared idiotopes amongst their patients but found no cross-reactions in 100 different lymphomas with a panel of 15 monoclonal anti-Ids (Thielemans et al, 1984). However, as the authors themselves point out, the screening methods used to select anti-Ids in their studies favoured the selection of antibodies against private determinants.

Using a different screening protocol Stevenson et al (1986,) demonstrated that it is possible to generate monoclonal anti-Ids against public determinants of tumour idiotypes but also pointed out that the expression of such idiotopes by normal serum Ig could prevent infused anti-Id from reaching the tumour cells. Rankin and Hekman (1984,) have also reported that 2 monoclonal anti-Ids produced against individual NHL idiotypes cross-reacted with 1 out of 45 other NHLs.

Mayumi et al (1982,) found that an anti-Id against Ig expressed by a case of acute lymphoblastic leukaemia reacted with 1-3% of normal B-lymphocytes and plasma cells. Kiyotaki et al (1987,) used 39 anti-Ids to screen 28 different paraproteins and found that 5 of the antibodies reacted with one or more paraprotein. Walker et al (1987,) produced a panel of 14 anti-Ids against a single paraprotein and extensively characterised them against a panel of 159 paraproteins and 50 normal sera. Seven of the antibodies reacted, in varying degrees, with paraproteins other than the immunogen.

There are a number of possible explanations for the recurrent expression of idiotopes by different tumour populations thought to arise randomly from the total B-cell pool.

i. They could simply be phenotypic markers of a common V region gene and the high expression in the tumour population a reflection of their expression in the normal B-cell pool.

ii. They could be phenotypic markers of a particular V gene rearrangement which is associated with the process of malignant transformation.

iii. A common idiotope may be expressed by genetically unrelated clones if they are all selected by a common antigen. Thus a network defect involving idiotype specific T-cell regulation could result in the oligoclonal expansion of B-cell expressing the relevant Id.

iv. Finally, the shared idiotope could be a regulatory idiotope expressed by the malignant B-cells either because it is commonly expressed by normal B-cells or because a specific regulatory defect results in clones expressing that idiotope being more susceptible to malignant transformation.

The last two possibilites are particularly relevant to anti-Id therapy since tumours associated with a defect in immunoregulation might be particularly susceptible to immunotherapy.

This work is concerned with the identification of shared idiotopes in normal Ig and within tumour populations. The aim will be to define a group of idiotopes which are recurrently expressed by B-cell tumours but which are detectable only at very low levels in normal Ig.

If such determinants can be defined they could be valuable tools for further investigating the biology of the malignant process as well as making ideal targets for immunotherapy.

CHAPTER 2

MATERIALS AND METHODS

2.1 PATIENTS

The 45 patients involved in this study had chronic lymphocytic leukaemia (CLL) as defined by conventional morphological and immunocytochemical criteria (Gale, 1985). All the patients attended the Haematology Department at the Western General Hospital, Edinburgh and were from a group of about 60 patients who have been followed up for a number of years.

Diagnostic investigations included peripheral blood and bone marrow morphology, phenotyping by indirect immunofluorescence, indirect immunoperoxidase and rosetting with mouse erythrocytes before and after treatment of the lymphocytes with neuraminidase. All patients were fully phenotyped before inclusion in this study.

Patient characteristics are shown in tables 2.1, 2.2 and, 2.3. Sixteen patients had stage A disease 15 had stage B and, 14 had stage C (Binet et al, 1981). During the study 1 patient changed from stage A to B and 3 changed from

stage B to C. Twenty eight patients had never received cytotoxic therapy and only 9 out of 17 patients who had been treated were on therapy at the time they were sampled. There were 17 IgMκ, 18 IgMλ, 5 IgGκ, 2 IgGλ and, 1 IgAκ and 2 IgAλ expressing tumours.

2.2 THE JS MONOCLONAL ANTIBODY PANEL

This panel of 14 monoclonal antibodies (McAbs), generated against a single IgGλ paraprotein from a patient with lymphoplasmacytoid lymphoma, was a gift from Dr. J.A. Habeshaw (ICRF Oncology Unit, St. Bartholomew's Hospital, London). Each antibody was characterised by determining its specificity for isolated heavy and light chains of the immunogen and its reactivity with 50 normal sera and 159 paraproteins (table 2.4,)(Walker et al, 1987).

The V region specificity of the 14 McAbs is suggested by their reactivity with the Fab fragment of the immunogen and by the patterns of inhibition of binding to the immunogen by other monoclonals to variable and constant region epitopes. The idiotypic specificity is suggested by the normal distribution of reactivity with the normal sera; a pattern which would not be seen with allotypic

determinants (Walker et al, 1987). It remains possible, however, that antibodies which reacted strongly with the normal sera and frequently with the paraprotein panel (44.3C6, 44.3C1, 44.4B4 and, 47.5A2,) may detect variant constant region epitopes expressed by Fab or variable region framework determinants which may not be strictly idiotypic.

Seven of the JS McAbs (2G5, DPF11, 1B2, 49.8B4, 47.1B2, 47.1D5, and 47.2B3,) react with private determinants which are almost unique to the JS paraprotein. These antibodies showed weak binding to normal serum and, apart from 2G5, DPF11, and 1B2 which all reacted with the same IgM paraprotein, did not react with any of 159 paraproteins (Walker et al, 1987).

The remaining 7 antibodies react with V region associated epitopes which are expressed to varying degrees by normal serum and the paraprotein panel (Walker et al, 1987.)

Antibodies 44.3C6, 44.3C1, 44.4B4, and 47.7B3 react with lambda specific determinants while 44.2A1, 2H3.D6, and 45.7A2 react with heavy chain specific epitopes expressed by paraproteins with differing isotypes. A further antibody (49.3C1,) was found to detect the IgG allotype Glm(f) (Walker et al, 1987,) and was included in the panel.

All the antibodies are IgG1 subclass and were supplied as ascites preparations which were aliquoted and stored at -70°C.

2.3 METHODS

2.3.1 Cell Collection and Preparation

Venous blood samples (15mls) were taken from patients and mixed with 2mls of 2% (weight/volume) disodium ethylenediamine tetra-acetic acid (EDTA) in a universal container. Peripheral blood mononuclear cells (PBMNC) were then separated by layering the whole blood onto lymphopaque (Nycomed Diagnostics,) (density - 1,086g/ml,) and centrifuging for 20 minutes at 500g. The layer of cells at the interface of plasma and lymphopaque was removed and washed three times in RPMI-1640 (Gibco).

2.3.2 Cell Counting

The cell suspension was counted in an improved Neubauer chamber. Viability was determined by trypan blue exclusion.

2.3.3 T-Cell Depletion

Three mls of sheep blood was washed 3 times in phosphate buffered saline, pH 7.3 (PBS) and 1ml of packed red blood cells was mixed with 1ml of 0.1 unit/ml of neuraminidase (Sigma N 2876,) and incubated at 37°C for 30 minutes. The treated cells were washed a further 3 times in RPMI, resuspended in RPMI and counted. The treated sheep cells were added, at a ratio of 40:1, to PBMNC in 10mls RPMI-1640 with 10% fetal calf serum (FCS) and incubated at 37°C for 10 minutes. The cells were then centrifuged for 3 minutes at 60g and incubated for a further 45 minutes at 0°C. The cells were gently resuspended using a pipette and layered onto lymphopaque and centrifuged for 20 minutes at 500g. The cells at the interface of RPMI and lymphopaque were then collected using a pipette and washed three times in RPMI. Such cells were referred to as "E-depleted cells."

2.3.4 Preparation of Cell Lysate

100 x 10^6 cells were washed 3 times in RPMI and resuspended in 2 mls of PBS. Two mls of 1% Nonidet P-40 (NP-40,) in PBS containing 2 mM phenylmethyl sulphonyl fluoride (Sigma) was added, thoroughly mixed and incubated at 0°C for 30 minutes. After this time the samples were centrifuged at 2000g for 15 minutes and the supernatants kept at 4°C.

2.3.5 Cell Culture

The culture medium consisted of RPMI-1640 with 25mM 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid (HEPES) (Gibco 041-2402) containing 2mM L-Glutamine (Gibco 043-5030), 1mM sodium pyruvate (Gibco 043-1360), 50 units/ml Penicillin and Streptomycin (Gibco 043-5140) and 10% FCS (Northumbria Biologicals). Two batches of FCS were used during this study which were selected for their ability to support normal and CLL PBMNC proliferative responses to stimulation with the mitogens phytohaemagglutinin (Difco Labs,) and the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) (Scientific Marketing Associates).

Cells were cultured at 37°C in humidified air with 5% CO_2. 10 x 10^6 cells were added to 70ml tissue culture flasks (Costar 3055.) containing 10mls of medium. Immunoglobulin secretion was induced by 100ng/ml of TPA and the culture supernatants were collected after 6 days (Polliack et al, 1986). Supernatants were stored at 4°C with a final concentration of 0.1% (weight/volume) sodium azide in deionised distilled water (DDW).

2.3.6 Cryopreservation of Cells

Cells were suspended in FCS at 10 x 10^6/ml and kept at 4°C for 10 minutes. Dimethyl sulphoxide was added to a final concentration of 10% (volume/volume) and the cells quickly transferred to 2 ml cryopreservation vials (Costar 2028). The vials were placed in a polystyrene box, put into a -70°C deep freeze overnight and transferred to liquid nitrogen the following day.

Cells were recovered by taking a vial from liquid nitrogen, warming it in a 37°C waterbath and transferring the cells into 10mls of pre-warmed culture medium containing 10% FCS before they had completely thawed. The cells were then centrifuged at 150g for 8 minutes, the supernatant poured off and replaced with another 10 mls of pre-warmed culture medium. The cells were then counted

and viability assessed as previously.

2.3.7 Purification of IgM

Serum containing a monoclonal IgMκ paraprotein band of 22g/l was obtained from a patient with Waldenström's Macroglobulinaemia.

2.3.7.1 Boric Acid Precipitation

10ml of serum was added to 190ml of 2% (weight/volume) boric acid ($B(OH)_3$) in DDW for 30 minutes, then centrifuged for 30 minutes at 1200g and the supernatant discarded. The precipitate was washed twice in 2% boric acid and redissolved in 10ml tris buffered saline (TBS) (0.1M tris, 0.5M NaCl, pH 8.1 with 1M HCl,) according to Mauch et al (1980).

2.3.7.2 Gel Filtration

A 100cm column (Pharmacia C 26/100,) was packed with Sepharose 6B, attached to a peristaltic pump (LKB Varioperex,) and washed with TBS. The sample was centrifuged at 1200g for 10 minutes and any precipitate

discarded. The supernatant was layered onto the column and run at 3mls per minute. 6ml fractions were collected by an automated fraction collector (Gibson Microcol TDC 80). The protein content of samples was estimated by measuring UV absorbance at 280nm using a spectrophotometer (Pye Unicam SP6-550 UV/VIS,) and samples from a single peak were pooled.

2.3.7.3 Protein Concentration

Protein in the eluate from the column was concentrated by positive pressure ultrafiltration using a filter with a 10kD cut-off (Diaflo ultrafilter PM10,) and a pressure of 55 psi. The sample was concentrated to approximately 1mg/ml as judged by absorbance at 280nm (Johnstone and Thorpe, 1985).

2.3.7.4 Protein assay

This assay was adapted from the method of Bradford (1976). 20µl of each sample was placed in duplicate wells of a micro-ELISA plate (Dynatech M24 U well,) and 20µl of PBS added to separate wells as negative controls. Standards consisted of doubling dilutions of bovine albumin (Sigma A-4503,) from 1mg/ml. Protein assay

solution (Bio-Rad 500-0006, Gaston Way, Watford,) was diluted 1:5 in DDW and filtered. 200µl of this solution was added to each well and left for 15 minutes. Optical density at 620nm was measured in a plate reader (Titertek Multiscan MC 340,) which was zeroed with 200µl of the diluted Bio-rad solution. The protein concentration of the unknown samples were then estimated from the standard curve (figure 2.1).

2.3.7.5 Polyacrylamide Gel Electrophoresis (PAGE)

PAGE was run using the Pharmacia Phastsystem according to the manufacturers recommendations. The samples were run on a pre-formed 0.45mm 10-15% gradient gel under reducing conditions. The gel buffer was 0.112M acetate and 0.112M tris, pH 6.4 and the elctrode buffers were 0.20M tricine, 0.2M tris and 0.55% sodium dodecylsulphate (SDS) pH 7.5 in 2% Agarose strips. 100µl of the samples were added to 30µl of a mixture of 40mM tris, 4mM EDTA, 10% SDS and, 20% dithiothreitol, pH 8.0 with 1M HCl, and boiled for 2 minutes immediately prior to running the gel.

2.3.7.6 Radial Immunodiffusion

1% Agarose (weight/volume) was dissolved in PBS and cooled to 60°C. 1ml of anti-Ig anti-sera (Scottish Antibody Production Unit,) (SAPU,) was added to 25mls of agarose, poured into a plastic petri dish (diameter 9cm,) and left to set on a levelling table. 2mm circular holes were made using a gel punch and the agar plugs removed. 10µl of each test sample, undiluted and 1/10 in PBS, was added to the appropriate wells, the lids replaced and the petri dishes left at 4°C overnight. The plates were examined for precipitation lines the following morning.

2.3.8 Immunocytochemistry

2.3.8.1 Cytospin Preparation

100µl of a cell suspension at 1 x 10^6/ml in RPMI was added to the carrier of a Shandon Cytospin II. The addition of 5% FCS was beneficial in cases with consistently poor morphology. The cells were centrifuged at 500rpm for 4 minutes, and the slides were allowed to dry in air for 2 minutes before being fixed in fresh acetone for 4 minutes. The slides were again dried in air, wrapped in polythene bags and either stored at 4°C or at -20°C if they were not to be used within two weeks.

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2.3.8.2 May-Grünwald Giemsa's Stain

Slides were fixed in absolute methanol for 10 minutes and transferred directly to May-Grünwald's stain for 10 minutes and then to Giemsa's stain diluted 1:20 in Sörensen' Buffer, pH 6.8, for a further 10 minutes. The slides were then washed three times in Sörensen's buffer and left to stand for 2 minutes for differentiation to take place. The slides were then air dried and mounted in Histomount.

2.3.8.3 Indirect Immunoperoxidase

The method was adapted from Salter et al, (1985.) Acetone fixed slides were rehydrated with with 50µl of TBS (0.5M tris, 1M HCl, pH 7.6,) containing 20% normal rabbit serum (NRS.) After 10 minutes excess fluid was tipped off and 50µl of the primary antibody diluted in TBS/NRS was added and incubated in a wet box for 30 minutes. The slides were then washed in TBS for five minutes twice, the area around the cells was wiped with a tissue and 50µl of rabbit anti-mouse Ig conjugated to peroxidase (Dako P-260,) diluted 1:20 in TBS/NRS added. After 30 minutes the slides were washed twice in TBS for

five minutes. The areas around the cells was wiped dry and the slide flooded with freshly prepared substrate (1mg/ml diaminobenzidine in 0.02M tris-HCl with 0.01M imidazole, pH 7.6). 100µl of 1% H_2O_2 was added to the substrate immediately before use and the reaction was stopped after 5 minutes by immersing the slides in water for 2 minutes. The slides were then counterstained in haematoxylin, washed in water and then briefly dipped in lithium carbonate. After washing in water again the slides were dehydrated through alcohol to xylene and mounted in Histomount.

2.3.9 Enzyme Linked Immunosorbent Assays (ELISA)

2.3.9.1 Materials

96 well Micro-ELISA plates (Dynatech M24 U well)

Capture antibodies:-

Polyclonal goat anti-human Ig (Sigma I-9631)
Polyclonal goat anti-mouse Ig adsorbed against human serum proteins (Sigma M-8645)

Goat Anti-Human Ig Alkaline Phosphatase Conjugates:-

Anti-γ	(Sigma A-0287)
Anti-μ	(Sigma A-3275)
Anti-α	(Sigma A-3400)
Anti-κ	(Sigma A-2779)
Anti-λ	(Sigma A-2904)
Anti-Ig (polyvalent)	(Sigma A-5034)

Buffers:-

Washing and Blocking Buffer

0.05 M Na_2HPO_4 containing 0.5M NaCl, 2.5g/l gelatin, 1% Tween-20, adjusted to pH 7.7

Substrate Buffer

0.1M glycine containing 0.01M $MgCl_2$ and 0.01M $ZnCl_2$ adjusted to pH 10.4 with 5M NaOH

Plate reader:- Titertek Multiscan MC 340 (Flow Labs)

2.3.9.2 Three-Stage Immunoglobulin Assay

The Ig content of culture supernatants was quantitated using an ELISA assay modified from the method of Lems-Van Kan (1983.) The micro-ELISA plates were coated with 100µl of polyclonal goat anti-human Ig antibody at 100µg/ml in PBS and incubated at room temperature for 60 minutes. The plates were washed 4 times in washing buffer and left for 4 minutes during the final wash to block unbound sites. 100µl of each culture supernatant was added to duplicate wells and incubated for a further 90 minutes. After a further 4 washes in blocking buffer 100µl of goat anti-human Ig alkaline phosphatase conjugate diluted in blocking buffer was added and incubated for 90 minutes. The optimal dilution of each conjugate varied between batches but a protein concentration of 2µg/ml was generally satisfactory. After 4 washes in blocking buffer 200µl of p-nitrophenyl phosphate disodium (Sigma 104,) freshly diluted in substrate buffer was added. The plates were read at 405 nm after 40-45 minutes at room temperature.

2.3.9.3 Quantitation of Ig

Ig levels in culture supernatants were quantitated by comparison with dilutions of calibrated normal serum (Protein Reference Unit, Royal Hallamshire Hospital, Sheffield). A single batch of serum, containing 9.97mg/ml IgG, 1.10mg/ml IgM and, 1.69mg/ml IgA, was used throughout the study. Standard curves for each of the Ig assays are shown in figures 2.2, 2.3 and, 2.4.

It was possible that competition by the different isotypes in the calibrated serum for the anti-human Ig capture antibody might affect the results. This would particularly apply to the IgM and IgA assays where the high levels of IgG could block the available binding sites. In order to assess the effects of such competition IgM was purified from the serum of a patient with Waldenstrom's Macroglobulinaemia as described previously. Two protein peaks were separated by gel filtration (figure 2.5.) and radial immunodiffusion using anti-heavy chain specific anti-sera (SAPU) demonstrated IgM only in samples from the first peak and mixture of IgG and IgA in the second peak (table 2.5). Samples 36-40 from the first peak and 52-54 from the second peak (figure 2.5.) were pooled and concentrated. The absence of contaminating proteins was confirmed by SDS-PAGE (plate 2.1.) and the protein concentration in the IgM

containing sample determined using the protein assay described previously. Standard curves for IgM using the calibrated normal serum and purified IgM at the same concentrations of IgM show reduced binding with the serum, probably as a result of inhibition by IgG and IgA (figure 2.6). Thus accurate quantitation of Ig using polyvalent anti-Ig capture antibody with diluted normal serum for the standard curves is not possible. Since absolute quantitation of Ig was not essential for this work the calibrated serum was used for all assays.

2.3.9.4 Four-stage Idiotope Specific Assay

Micro-ELISA plates were coated with 100µl of goat anti-mouse IgG antibody at 2µg/ml in PBS and incubated at room temperature for 60 minutes. They were then washed 4 times in washing buffer and left to stand for 4 minutes during the final wash to block unbound sites. All the JS McAbs were used at 1:90 in the blocking buffer and anti-κ and anti-λ (SAPU) were used at 1:40 and 1:90 respectively. The plates were incubated for a further 90 minutes at room temperature, washed 4 times in blocking buffer and 100µl of human Ig preparation (culture supernatant or serum,) was then added and incubated at room temperature for 90 minutes. After a further 4 washes in blocking buffer 100µl of the appropriate goat anti-human Ig alkaline phosphatase conjugate diluted in blocking buffer was added and incubated for 90 minutes. The plates were developed and read as before.

TABLE 2.1

Characteristics of Clinical Stage A Patients

Pt	Sex	Age at Dx	Year of Dx	Ig Iso	Date of 1st sample	WCC	Lymph	Rx
JB	M	72	1985	IgMλ	10/85	10.8	42	N
HB	F	71	1986	IgMλ	1/86	17.3	52	N
PC	M	73	1985	IgMκ	6/86	15.6	56	N
AD	F	72	1982	IgGλ	6/87	16.2	68	N
WG	M	69	1978	IgMκ	3/86	11.6	71	N
EG	F	65	1983	IgMλ	8/87	16.8	76	N
MG	F	60	1975	IgMκ	6/87	6.2	35	Y
AG	F	72	1984	IgMκ	11/85	13.1	75	N
AI	M	55	1985	IgMκ	2/86	21.0	86	N
JJ	F	65	1979	IgMκ	8/85	15.7	64	N
AN	F	58	1983	IgMλ	10/85	8.4	55	N
JR	M	71	1987	IgMλ	9/87	47.4	94	N
AR	M	51	1970	IgMκ	9/87	32.6	98	N
JS	F	55	1983	IgGλ	10/85	14.4	72	N
MSm	F	68	1983	IgAκ	10/85	19.6	64	N
AS	F	82	1983	IgMκ	1/86	33.1	88	N

M = male
F = female
Pt = Patient
Dx = Diagnosis
Ig Iso = Ig isotype
WCC = total white cell count x 10^9/l of 1st sample
Lymph = lymphocyte count (% of WCC)
Rx = Cytotoxic therapy at time of sampling
(Y = yes, N = never treated,
O = off treatment)

TABLE 2.2

Characteristics of Clinical Stage B Patients

Pt	Sex	Age at Dx	Year of Dx	Ig Iso	Date of 1st sample	WCC	Lymph	Rx
AB	F	56	1979	IgAλ	6/87	25.1	74	O
MC	F	88	1986	IgMλ	2/86	40.0	89	N
WC	M	62	1984	IgGκ	10/87	14.6	72	N
LC	F	75	1984	IgMλ	9/87	88.0	96	N
MGr	F	75	1982	IgMκ	9/87	41.7	96	N
LG	F	75	1983	IgMλ	6/87	53.2	94	O
RH	F	61	1983	IgMλ	10/85	28.0	81	O
PK	M	73	1986	IgMλ	3/86	180	92	N
AM	M	62	1978	IgMλ	11/85	67.7	95	Y
IM	M	64	1979	IgMκ	11/85	36.3	82	O
DN	F	54	1977	IgAλ	10/85	18.8	73	N
RPa	M	71	1982	IgMκ	3/86	142	99	N
MS	F	81	1985	IgGκ	6/87	127	95	N
ASh	M	36	1982	IgMκ	1/86	11.2	87	Y
ES	F	60	1985	IgMλ	11/85	6.4	64	N

M = male
F = female
Pt = Patient
Dx = Diagnosis
Ig Iso = Ig isotype
WCC = total white cell count x 10^9/l of 1st sample
Lymph = lymphocyte count (% WCC)
Rx = Cytotoxic therapy at time of sampling
(Y = yes, N = never treated,
O = off treatment)

TABLE 2.3

Characteristics of Clinical Stage C Patients

Pt	Sex	Age at Dx	Year of Dx	Ig Iso	Date of 1st sample	WCC	Lymph	Rx
LA	M	48	1981	IgGκ	12/86	28.0	92	Y
CC	F	64	1982	IgMκ	11/85	5.8	45	Y
CD	F	82	1984	IgMλ	10/87	68.0	94	N
TE	M	66	1985	IgMκ	6/87	17.7	91	O
PM	M	51	1982	IgMκ	9/87	64.7	97	Y
WM	M	77	1985	IgMλ	10/85	18.0	81	N
RP	M	40	1974	IgMκ	11/85	157	97	Y
CP	M	70	1980	IgMλ	1/86	31.7	99	Y
JSk	M	53	1979	IgGκ	8/85	17.3	95	O
JSm	M	63	1984	IgMλ	8/87	28.1	95	N
GS	M	60	1980	IgMλ	8/87	37.1	90	O
MT	F	65	1973	IgGκ	8/87	155	97	N
JT	M	56	1984	IgMλ	7/87	16.6	85	Y
WT	M	47	1980	IgMκ	3/86	293	98	O

M = male
F = female
Pt = Patient
Dx = Diagnosis
Ig Iso = Ig isotype
WCC = total white cell count x 10^9/l of 1st sample
Lymph = lymphocyte count (% WCC)
Rx = Cytotoxic therapy at time of sampling
(Y = yes, N = never treated,
O = off treatment)

TABLE 2.4

The JS Monoclonal Antibody Panel Characterised by Radioimmunoassay

Antibody	Frequency of Reactivity with 159 Paraproteins (number +ve)	Binding to Normal Sera (cpm)
44.3C6	15	1488.6 +/- 266.4
44.3C1	8	931.8 +/- 244.6
44.4B4	6	331.9 +/- 87.3
47.7B3	2	144.7 +/- 31.0
44.2A1	3	471.1 +/- 82.0
2H3.D6	3	206.9 +/- 36.3
47.5A2	8	480.3 +/- 167.3
2G5	1	166.8 +/- 34.7
DPF11	1	167.8 +/- 55.4
1B2	1	296.2 +/- 87.2
49.8B4	0	237.8 +/- 185.9
47.1B2	0	193.0 +/- 42.0
47.1D5	0	201.1 +/- 48.9
47.2B3	0	295.1 +/- 92.1

McAbs were positive for paraproteins when binding was significantly greater than background.

Normal serum results are means of 50 individual sera +/- standard deviation.
cpm = counts per minute

Results reproduced by kind permission of Dr. J.A. Habeshaw.

TABLE 2.5

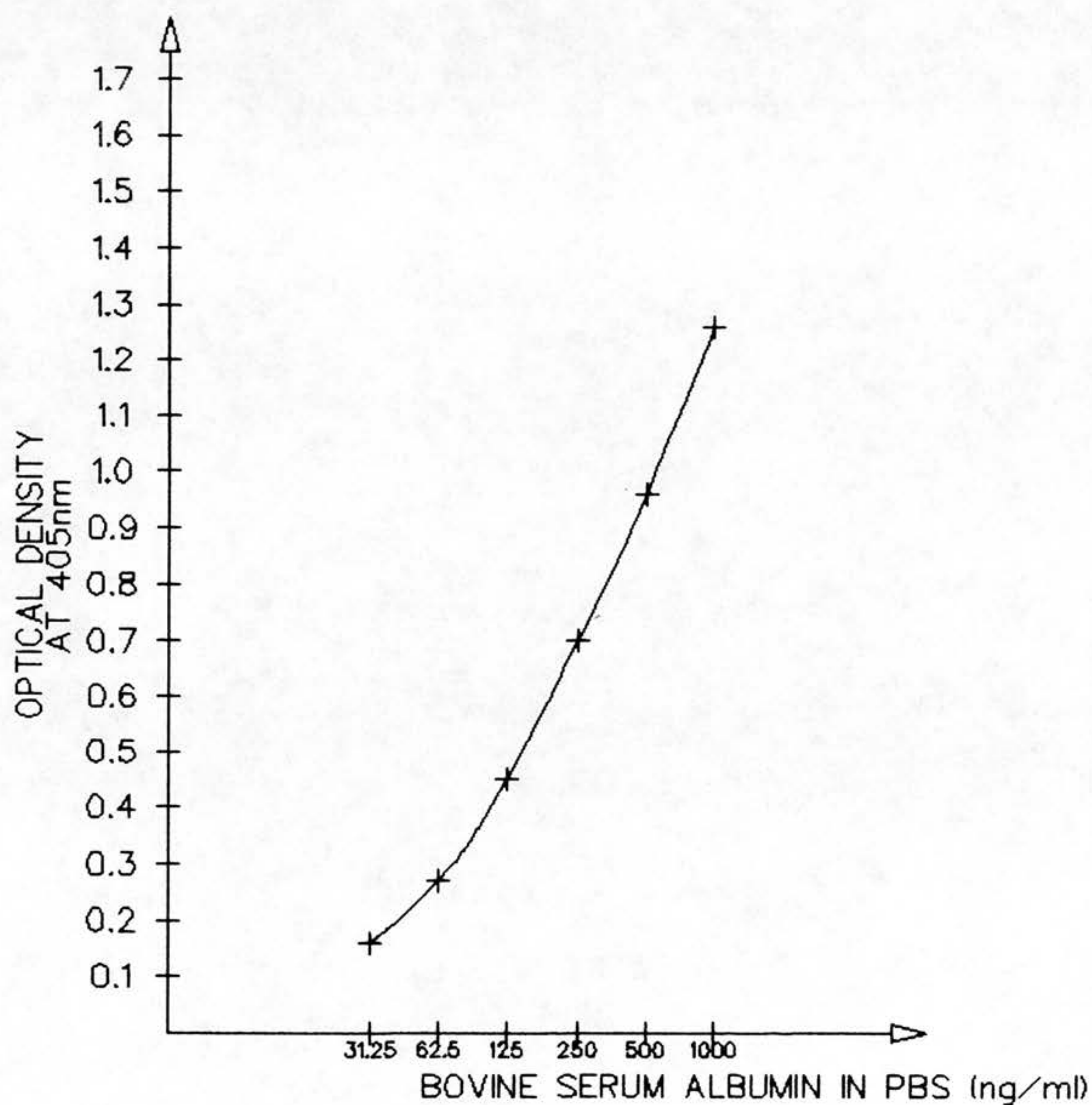
Ig Isotypes in Gel Filtration Eluate Measured by Radial Immunodiffusion

	IgM mm	IgG mm	IgA mm
Sample 38 1:10	3	0	0
Sample 38 neat	4	0	0
Sample 53 1:10	0	0	0
Sample 53 neat	0	5	6

All values refer to diameter of precipitation rings in millimetres at 24 hours.

FIGURE 2.1

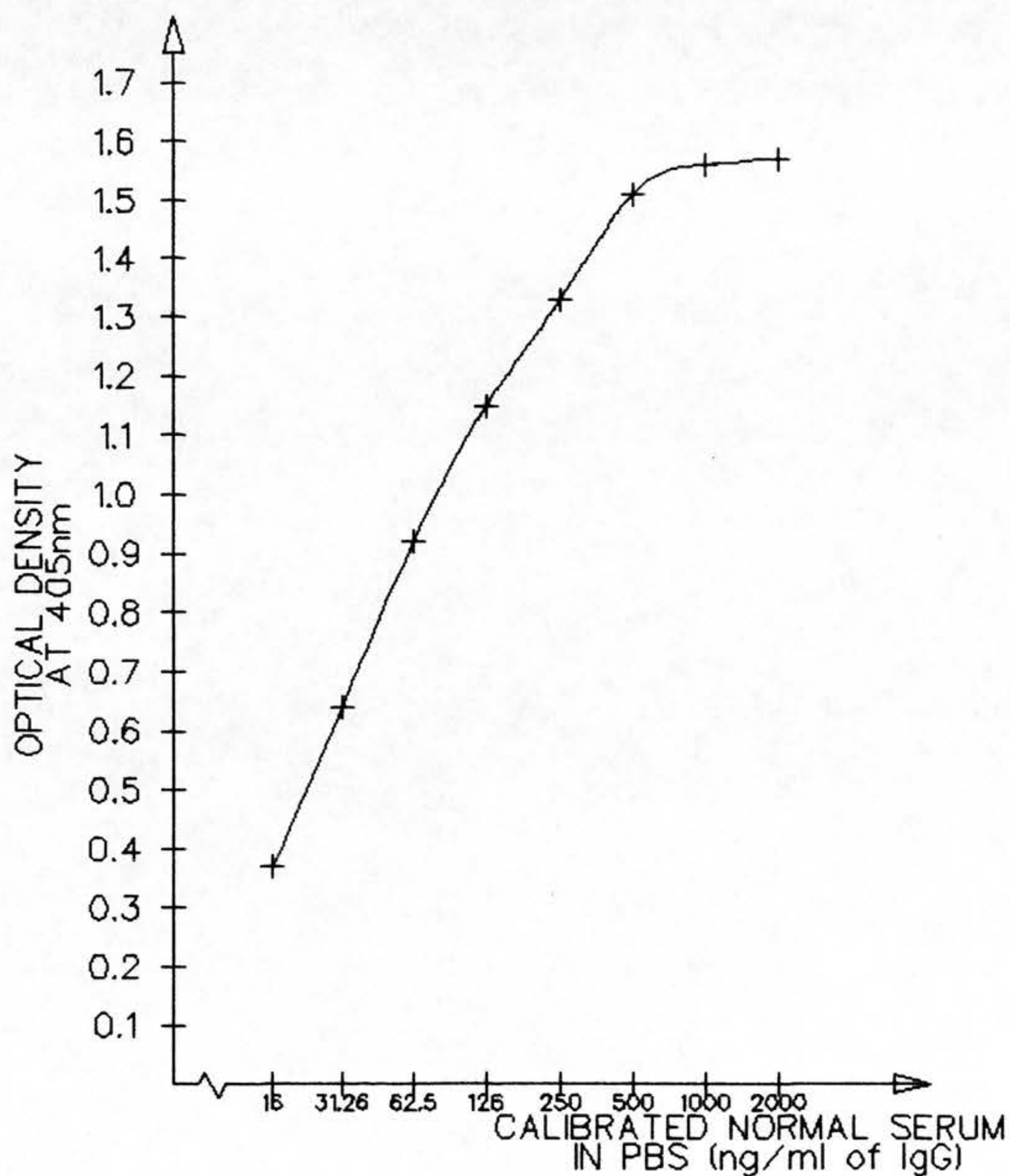
STANDARD CURVE FROM PROTEIN ASSAY



ALL RESULTS ARE MEANS OF DUPLICATE WELLS.
BACKGROUND OPTICAL DENSITY (PBS ONLY) = 0

FIGURE 2.2

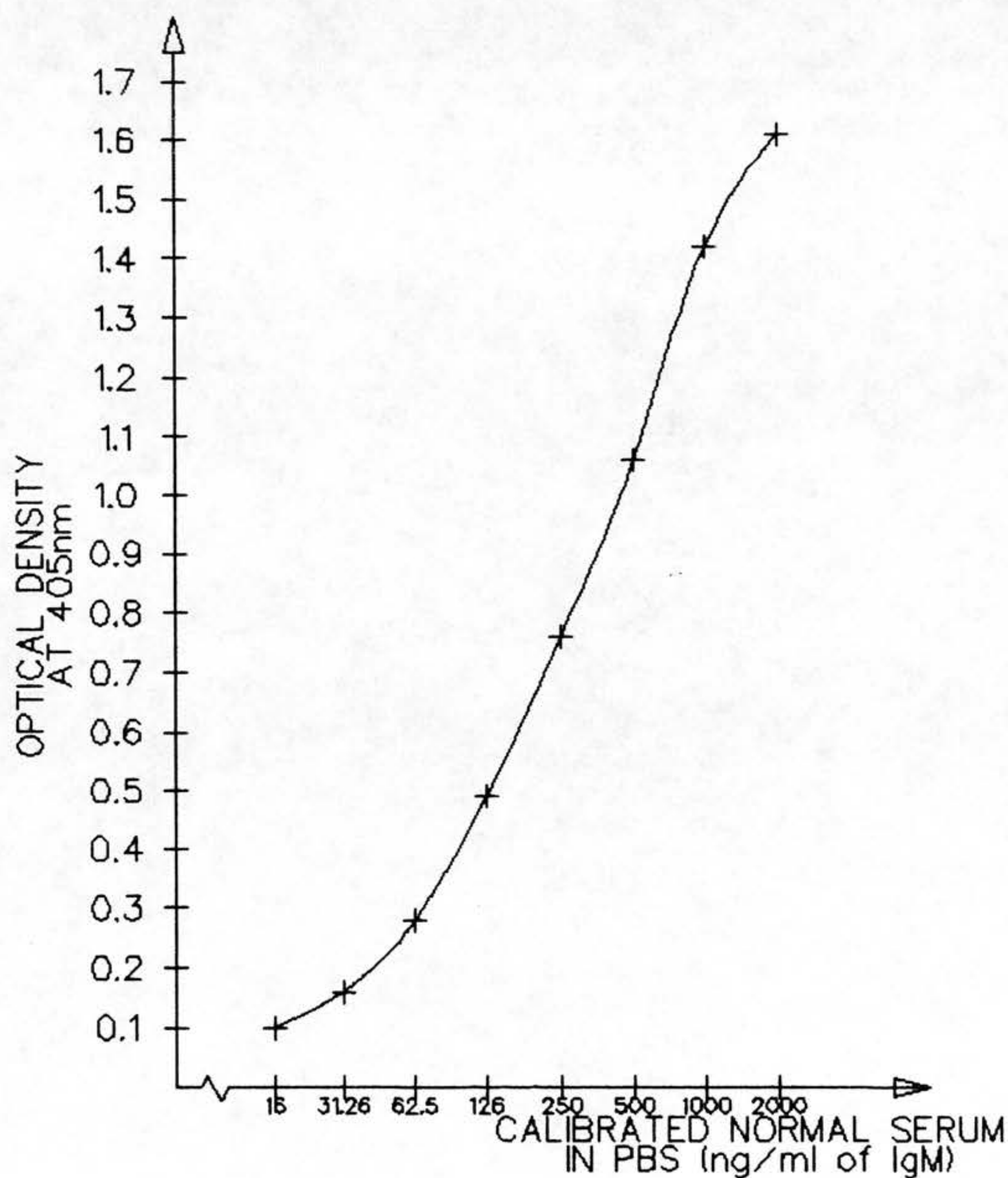
STANDARD CURVE FOR IgG SPECIFIC ELISA ASSAY



ALL RESULTS ARE MEANS OF DUPLICATE WELLS.
BACKGROUND OPTICAL DENSITY (CULTURE MEDIUM ONLY)= 0

FIGURE 2.3

STANDARD CURVE FOR IgM SPECIFIC ELISA ASSAY

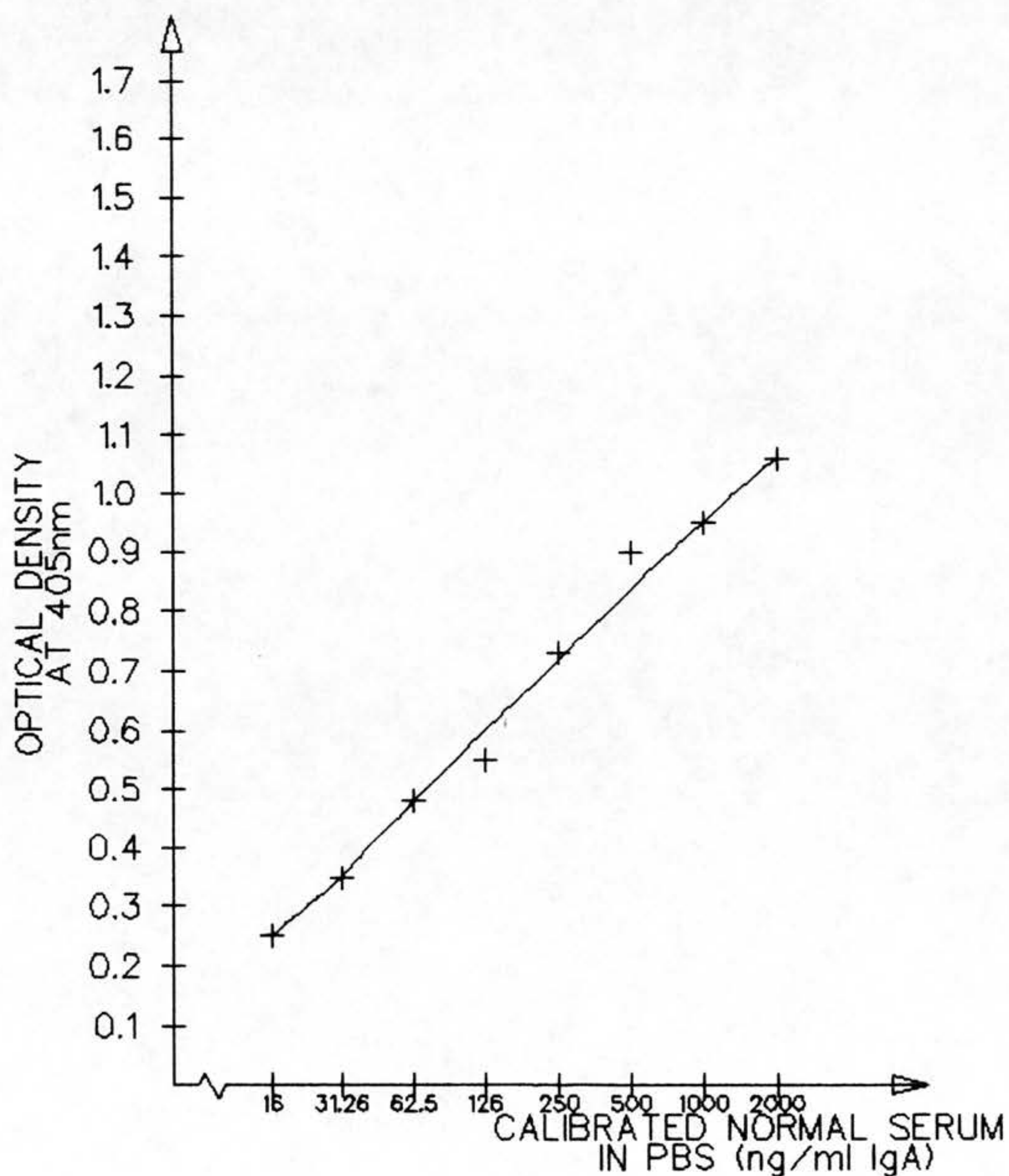


ALL RESULTS ARE MEANS OF DUPLICATE WELLS.

BACKGROUND OPTICAL DENSITY (CULTURE MEDIUM ONLY)= 0.02

FIGURE 2.4

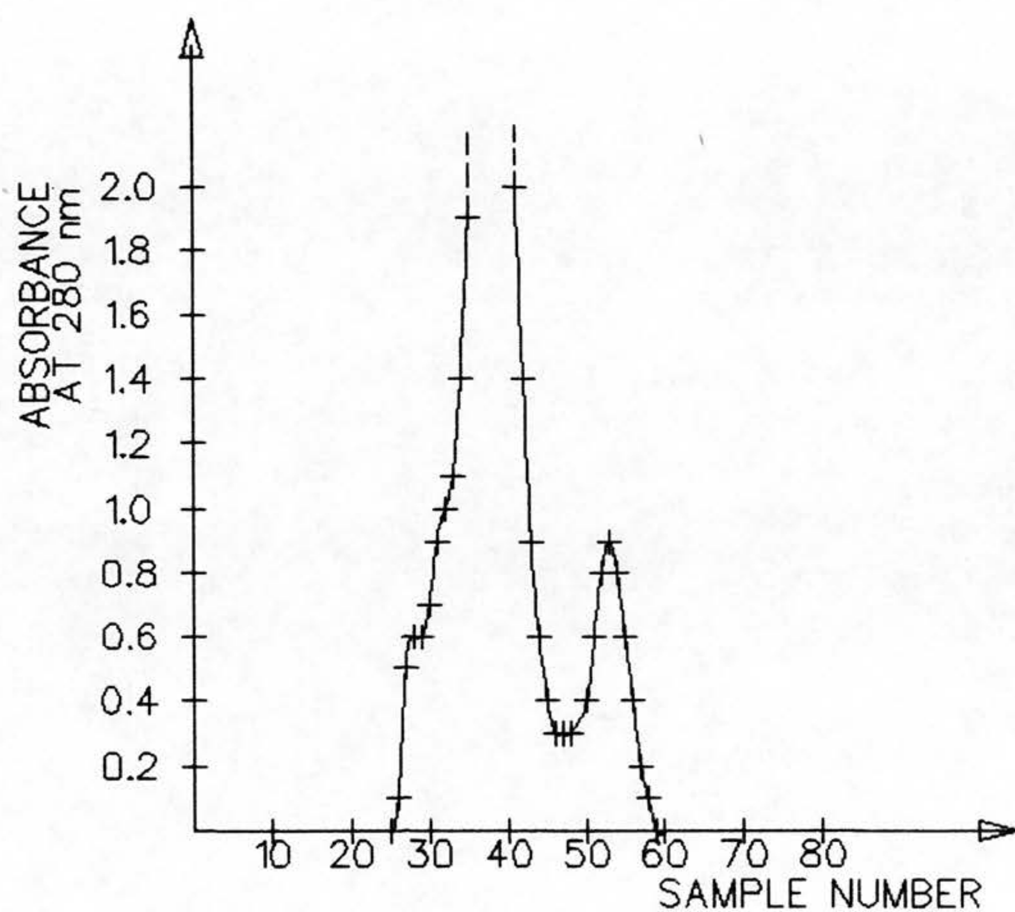
STANDARD CURVE FOR IgA SPECIFIC ELISA ASSAY



ALL RESULTS ARE MEANS OF DUPLICATE WELLS.

BACKGROUND OPTICAL DENSITY (CULTURE MEDIUM ONLY)= 0.05

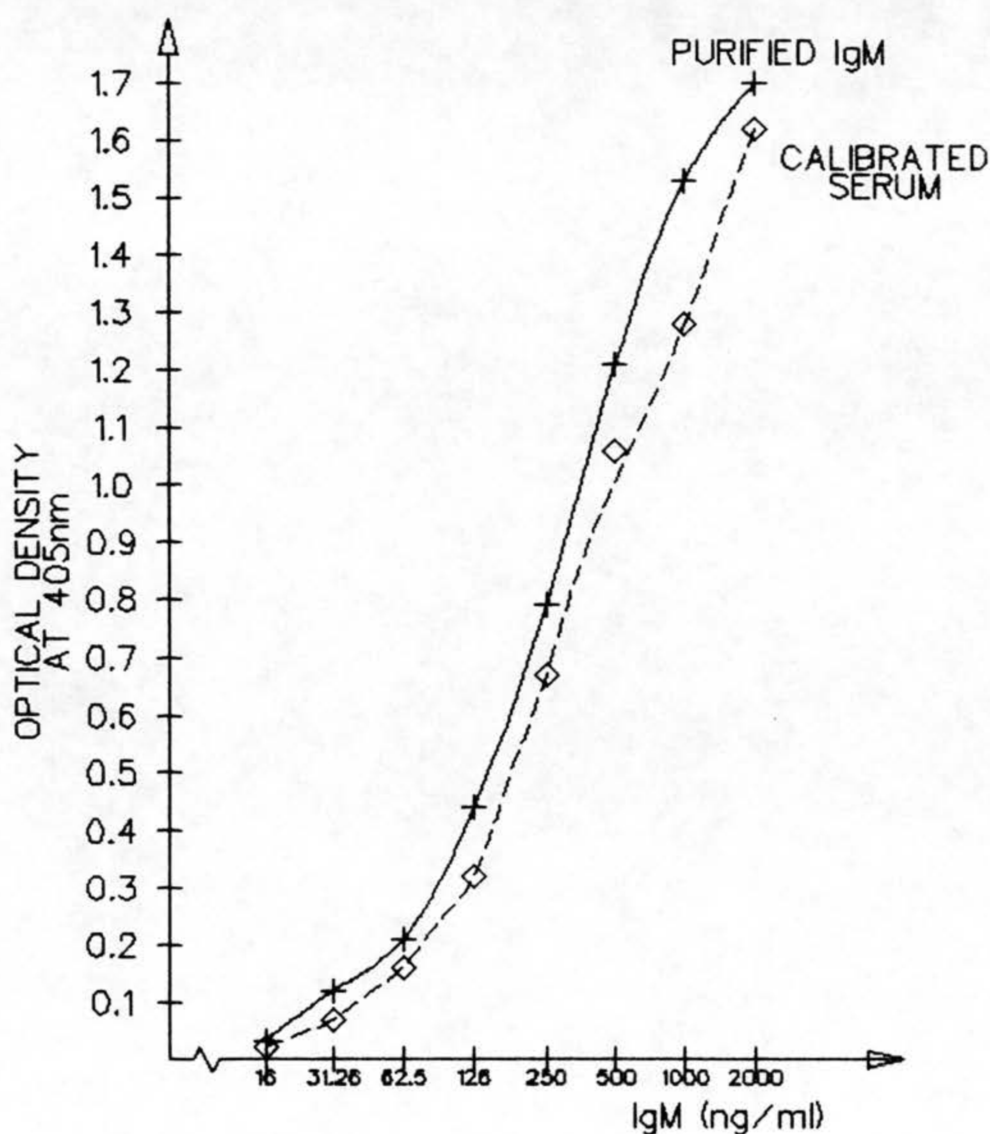
FIGURE 2.5



6ML SAMPLES OF ELUATE COLLECTED AND PROTEIN CONTENT ESTIMATED BY MEASURING UV ABSORBANCE AT 280nm

FIGURE 2.6

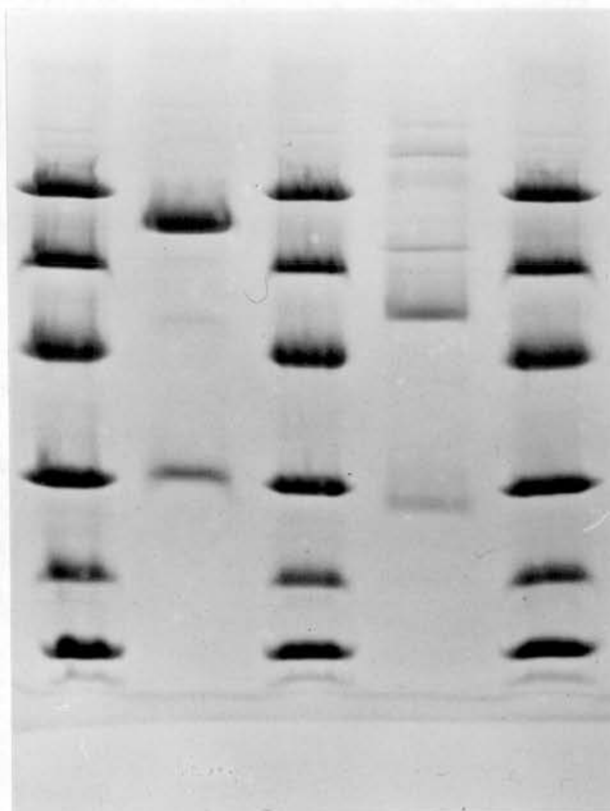
COMPARISON OF STANDARD CURVES FROM IgM ELISA ASSAY USING PURIFIED IgM AND CALIBRATED NORMAL SERUM



ALL RESULTS ARE MEANS OF DUPLICATE WELLS.
BACKGROUND OPTICAL DENSITY (PBS only)= 0

PLATE 2.1

SDS-PAGE of Samples from Gel Filtration Eluate



Tracks 1, 3, and 5:

Low Molecular Weight Markers (Pharmacia)
From top to bottom:

	MW
Phosphorylase B	94000
Albumin	67000
Ovalbumin	43000
Carbonic Anhydrase	30000
Trypsin Inhibitor	20100
α-Lactalbumin	14400

Track 2: Pooled samples 36-40 (1mg/ml)

Track 4: Pooled samples 52-54 (1mg/ml)

Track 2 shows single bands corresponding to IgM heavy and light chains. Very faint band at 50000 may represent IgG or IgA heavy chains.

Track 4 shows single band at 30000, corresponding to Ig light chain, and several bands >50000 which would correspond to IgG and IgA heavy chain.

CHAPTER 3

REACTIVITY OF JS MONOCLONAL ANTIBODIES WITH NORMAL IMMUNOGLOBULIN

3.1 MATERIALS

Pooled Human Serum	Protein Reference Unit, Royal Hallamshire Hospital, Sheffield.
Pooled Human IgG	Scottish National Blood Transfusion Service.
Normal Sera	Regional Virus Laboratory, City Hospital, Edinburgh.

3.2 METHODS

4-stage Idiotope specific ELISA assay
(as described in chapter 2.)

3.3 INTRODUCTION

In their original characterisation the reactivity of the JS McAbs against 50 normal sera and 159 paraproteins was determined by a solid phase radioimmunoassay (Walker et al, 1987). Antibodies 44.3C6, 44.3C1 and, 47.5A2 which each reacted with 8 or more paraproteins, also showed the highest binding to the normal sera (see table 2.4). For the remaining antibodies, however, the correlation between the frequency of reactivity with the paraprotein

panel and the reactivity with the normal sera was less clear. For instance, antibody 47.2B3, which does not react with any of the paraproteins, had a higher binding to normal serum than antibody 2H3.D6 which reacts with 3 of the paraproteins (see table 2.4).

These findings could be due to the different expression of specific V region associated epitopes by the B-cell clones represented in normal serum Ig and the paraprotein panel. However, an alternative explanation is that the radioimmunoassay assay was not sensitive enough at low levels to discriminate between antibodies with low reactivity for normal serum and those with no significant reactivity.

It was important, for the purposes of this investigation, to compare the expression, in normal serum, of the different epitopes recognised by the JS McAb panel. In order to do so, a highly sensitive ELISA assay, capable of detecting as little as 10ng/ml of a specific idiotope, was developed. The assay was adapted from a 3-stage Ig specific ELISA assay (Lems Van-Kan et al, 1983,) and was first standardised using a purified preparation of the JS paraprotein. The reactivity of the JS McAbs with pooled human IgG, pooled human serum and 14 individual sera was then tested using this assay.

3.4 IDIOTOPE SPECIFIC ELISA ASSAY

The assay is shown diagramatically in figure 3.1. Initial attempts to bind the JS McAbs directly to the wells were unsatisfactory. This was overcome by the use of goat anti-mouse Ig (adsorbed against human serum proteins,) as a capture antibody. This increased the chances of inter-species cross-reactions between different Ig molecules so negative controls (PBS in place of the human Ig at the third stage,) were included for each JS McAb in each assay. The background optical density (OD) rarely exceeded 0.1 units except for antibodies 44.3C6 and 44.4B4 where backgrounds up to 0.40 were seen particularly if the blocking buffer was more than 48 hours old.

Controls without mouse Ig but with the human Ig preparation were also included. Some preparations of human Ig did show significant binding to the goat anti-mouse Ig capture antibody. In such cases the binding in the absence of mouse monoclonal Ig was greater than that seen with non-reacting antibodies in the panel. This suggests that the binding sites available on the goat anti-mouse Ig antibody are effectively saturated by the mouse Ig thus preventing binding by human Ig.

Results were taken as positive when the difference

between the observed result and the background for that particular JS McAb exceeded 0.15 units of optical density.

3.5 RESULTS

3.5.1 Binding of JS McAbs to JS Paraprotein

The assay was standardised using purified JS paraprotein (gifted by Dr. J.A. Habeshaw). Figure 3.2 shows that the assay was sensitive enough to detect binding in 10 out of 14 JS McAbs at 10ng/ml of JS paraprotein and 13 out of 14 at 100ng/ml using the anti-γ antibody conjugate. All the antibodies were strongly positive (ODs > 1.4,) with 1000ng/ml of paraprotein.

The 4 antibodies which were negative at 10ng/ml were known to have relatively low affinity for the immunogen (Walker et al, 1987). The remaining antibodies all had similar affinities for the JS paraprotein so their binding patterns to normal Ig could be compared.

3.5.2 Binding of JS McAbs to Pooled Normal IgG

Figure 3.3 shows the binding pattern using pooled normal IgG diluted to 10μg/ml in PBS and the anti-γ antibody conjugate. Higher concentrations of IgG gave markedly increased background results. The strong binding to monoclonal anti-κ, anti-λ and, antibody 49.3C1 confirm the presence of polyclonal IgG. There is insignificant binding of IgG to 7 of the JS McAbs (1B2, 2G5, DPF11, 49.8B4, 47.1B2, 47.1D5 and, 47.2B3,) with all ODs <0.10. The remaining 7 JS McAbs are split into 2 groups; 4 antibodies (44.3C6, 44.3C1, 44.4B4 and, 47.5A2,) show strong binding to IgG (ODs >1.0,) while an intermediate group of 3 antibodies (47.7B3, 44.2A1 and, 2H3.D6,) show much weaker binding (ODs 0.34 - 0.45).

3.5.3 Binding of JS McAbs to Pooled Normal Serum

The pooled, calibrated serum contained 12.76 mg/ml of Ig and was diluted in PBS to a final Ig concentration of 10μg/ml. The assay was performed using a polyvalent detection antibody recognising all Ig isotypes and the results are shown in figure 3.4. There are minor changes in the relative affinities of different antibodies compared to the results using pooled IgG but the overall pattern of reactivity is similar with the same antibodies

in the strong (ODs >0.48,) intermediate (ODs 0.15 - 0.21,) and, weak (ODs <0.07,) groups.

3.5.4 Binding of Selected JS McAbs to Individual Normal Sera

The weak binding of pooled normal Ig to the intermediate group of JS McAbs could have been a consequence of strong expression of the relevant V region associated epitopes by small numbers of individuals or to weak expression by many individuals. To investigate this 14 individual sera were obtained from samples which had been sent for a routine virological screen and were all negative for antibodies to common viruses. Each serum was diluted 1:1000 and tested in duplicate for binding to the intermediate group of JS McAbs (47.7B3, 44.2A1 and 2H3.D6,) as well as 1 antibody (44.3C6,) which reacted strongly with pooled Ig and 1 (47.1B2,) which did not bind significantly.

The results are shown in table 3.1. None of the sera bind significantly to 47.1B2 (ODs <0.09,) whereas all the sera show strong binding to 44.3C6 with mean ODs from 0.49 - 0.73. Most of the sera do react with the intermediate group of JS McAbs although serum 11 did not react significantly with any of them in spite of reactivity

with 44.3C6 and serum 3 did not react significantly with 2H3.D6.

The mean ODs of the binding to the different antibodies were compared by Student's t test. There is no significant difference between the means for antibodies 2H3.D6 and 47.7B3 although the mean OD for 44.2A1 is higher than 2H3.D6 ($p < 0.01$,) and 47.7B3 ($p < 0.05$). The means of all these intermediate group antibodies are, however, very significantly lower than the mean of antibody 44.3C6 ($p < 0.001$,) and higher than the mean of 47.1B2 ($p < 0.001$).

3.6 DISCUSSION

These results extend the original characterisation of the JS McAbs (Walker et al, 1987,) by clearly sub-dividing the public idiotopes into two groups which may be of functional and practical significance. Thus the epitopes recognised by the panel antibodies can be divided into three distinct groups expressed by normal serum immunoglobulin (table 3.2):-

3.6.1 Private Determinants Recognised by JS McAbs

The 7 antibodies (2G5, DPF11, 1B2, 49.8B4, 47.1B2, 47.1D5, and 47.2B3,) which do not react significantly with normal Ig by ELISA or with the paraprotein panel by radio-binding assay (Walker et al, 1987,) recognise private determinants on the JS paraprotein. None of these antibodies reacted with isolated JS heavy or light chains suggesting that they recognise conformational determinants which are dependent on 2 unrelated genes (V_H and V_L,) for their expression.

These antibodies are typical of anti-idiotypic antibodies recognising private, antigen binding site related determinants which characteristically arise by somatic mutation (Rajewsky & Takemori, 1983).

3.6.2 Public Determinants Recognised by JS McAbs

The 4 antibodies reacting with this group each reacted with 6 or more paraproteins and include 3 antibodies specific for lambda chain (44.3C6, 44.3C1, and 44.4B4,) and 1 (47.5A2,) specific for heavy chain (Walker et al, 1987). They each react strongly with the pooled normal Ig preparations and antibody 44.3C6 reacts strongly with all the individual sera.

These determinants, which are obviously common to many different B-cell clones, are likely to be framework determinants characteristic of a major V gene sub-group or variant constant region determinants. Analysis of the primary structures of the JS and other paraproteins expressing these determinants may be required to characterise the antibodies further.

3.6.3 Restricted Public Determinants Recognised by JS McAbs

The 3 antibodies reacting with these determinants each react with 2 or 3 paraproteins and include 1 specific for lambda chain (47.7B3,) and 2 which are specific for heavy chain (44.2A1 and 2H3.D6.) Their expression in normal Ig is much reduced compared to the public determinants.

These determinants are likely to be shared idiotopes conserved by the immune system and expressed by small numbers of B-cell clones. Their specificity for heavy or light chain suggests that they may be products of single V region genes. These features are characteristic of regulatory idiotopes (Bona et al, 1987).

TABLE 3.1

Binding of Individual Normal Sera to Selected JS McAbs

Serum	JS McAb				
	44.3C6	47.7B3	44.2A1	2H3.D6	47.1B2
1	0.73	0.29	0.40	0.35	0.05
2	0.66	0.23	0.42	0.18	0.02
3	0.74	0.17	0.24	0.11	0.03
4	0.70	0.47	0.37	0.27	0.05
5	0.67	0.30	0.33	0.24	0.01
6	0.62	0.17	0.48	0.23	0.03
7	0.62	0.23	0.30	0.19	0.06
8	0.60	0.25	0.33	0.21	0.05
9	0.63	0.30	0.48	0.33	0.09
10	0.52	0.18	0.29	0.20	0
11	0.52	0.14	0.13	0.08	0
12	0.60	0.19	0.17	0.16	0.02
13	0.49	0.16	0.23	0.18	0.04
14	0.52	0.24	0.26	0.19	0.03
Mean	0.616	0.237	0.316	0.209	0.034
SD	0.081	0.096	0.107	0.074	0.025

All results are the means of duplicate wells and are expressed in units of optical density. All background ODs < 0.03

SD = standard deviation

TABLE 3.2

VARIABLE REGION ASSOCIATED EPITOPES DEFINED BY JS MONOCLONAL ANTIBODY PANEL:

Correlation of specificity for JS paraprotein with expression in normal serum

JS McAb	Specificity for immunising paraprotein	Expression in normal serum by ELISA
44.3C6	Lambda	Public
44.3C6	Lambda	Public
44.4B4	Lambda	Public
47.7B3	Lambda	Restricted
44.2A1	Heavy Chain	Restricted
2H3.D6	Heavy Chain	Restricted
47.5A2	Heavy Chain	Public
2G5	Intact Ig	Private
DPF11	Intact Ig	Private
1B2	Intact Ig	Private
49.8B4	Intact Ig	Private
47.1B2	Intact Ig	Private
47.1D5	Intact Ig	Private
47.2B3	Intact Ig	Private

Public = optical density > 0.5 by ELISA
Restricted = optical density <0.3 >0.14 by ELISA
Private = optical density <0.08 by ELISA

FIGURE 3.1

FOUR STAGE ELISA ASSAY

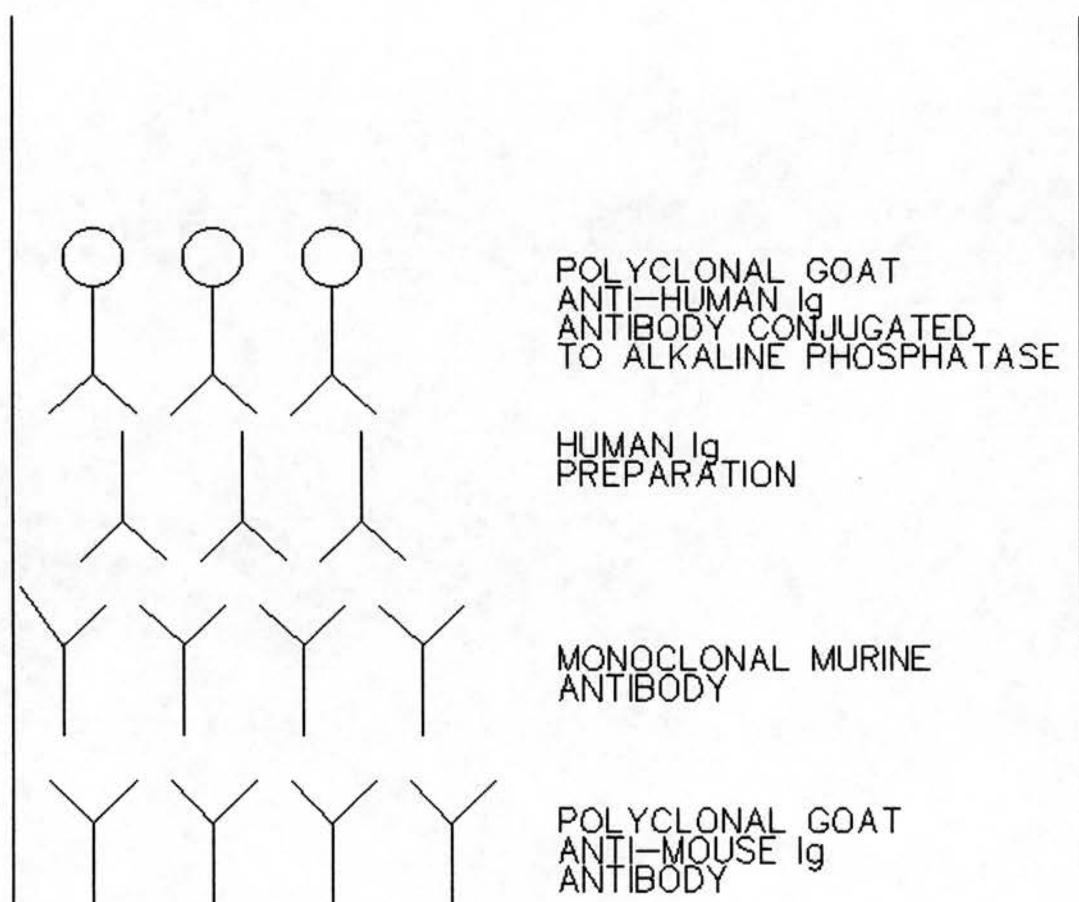
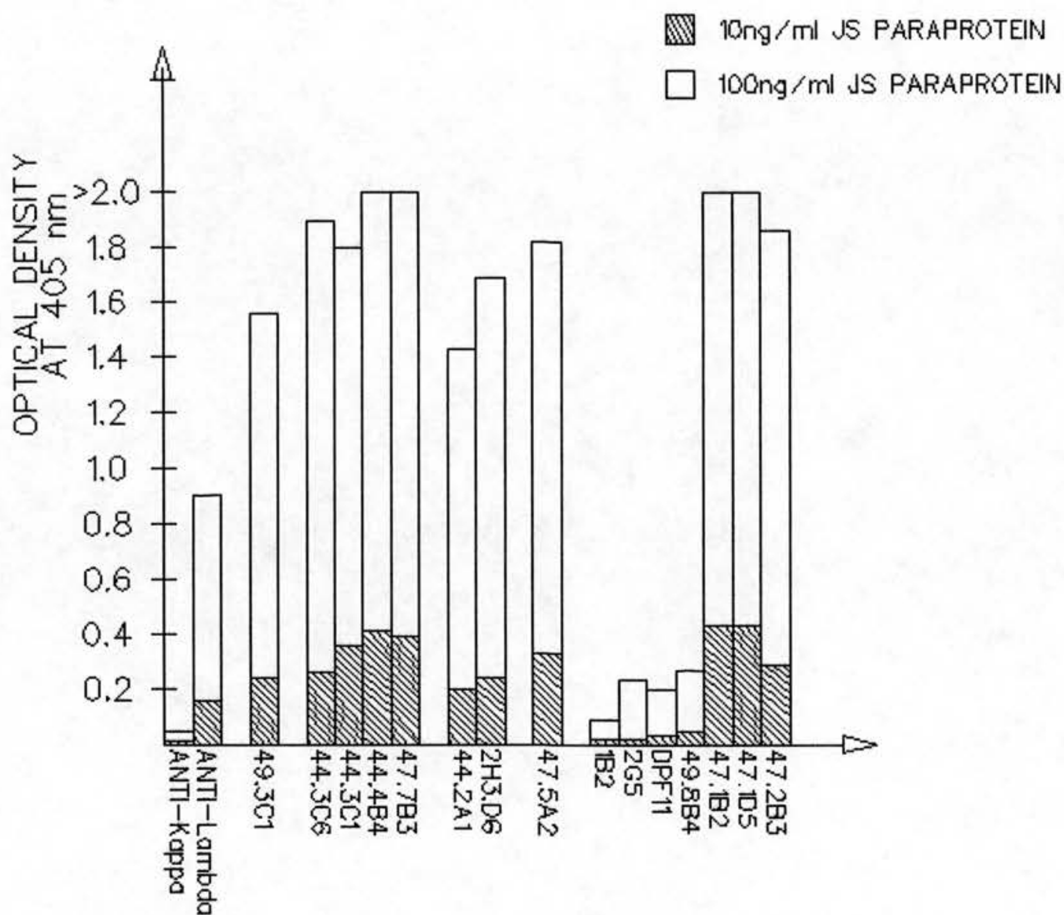


FIGURE 3.2

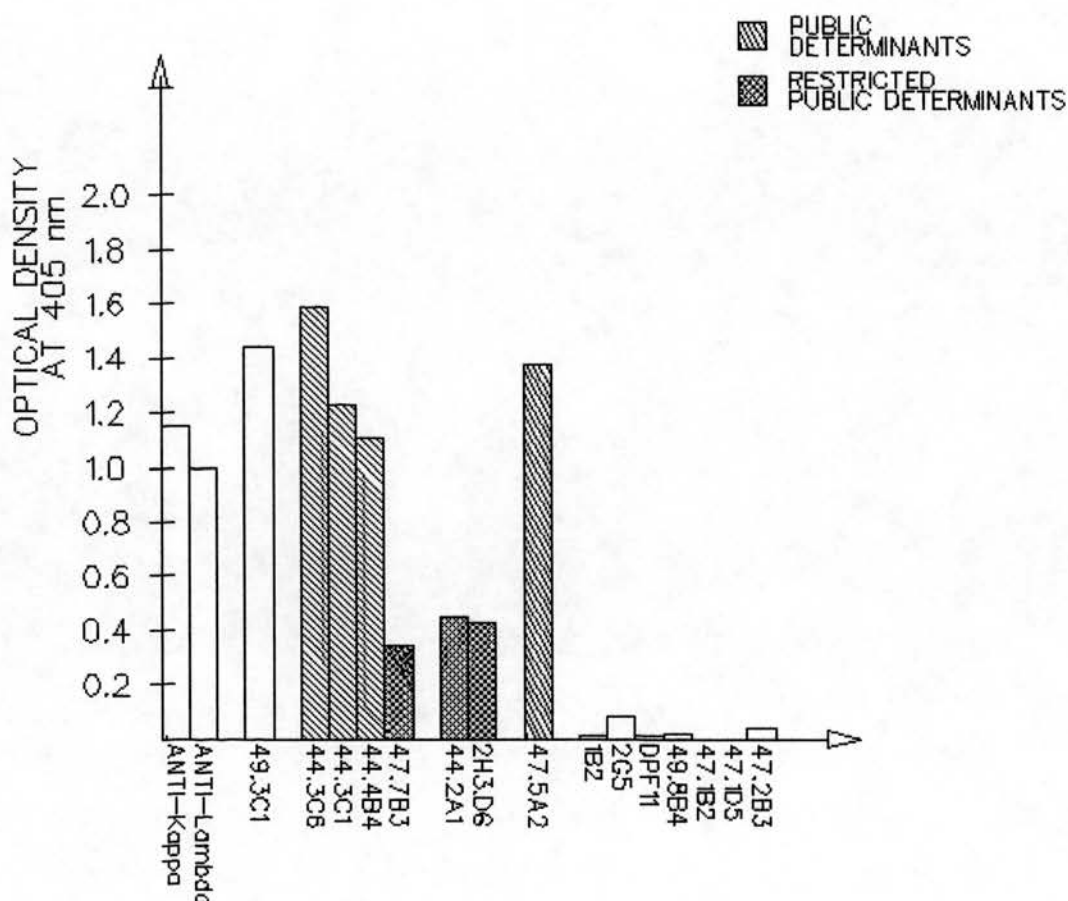
BINDING OF JS PARAPROTEIN TO JS MONOCLONAL ANTIBODY PANEL BY ELISA



BINDING DETECTED USING ANTI-IgG ALKALINE PHOSPHATASE CONJUGATE
ALL RESULTS ARE MEANS OF DUPLICATE WELLS
ALL BACKGROUND RESULTS < 0.06

FIGURE 3.3

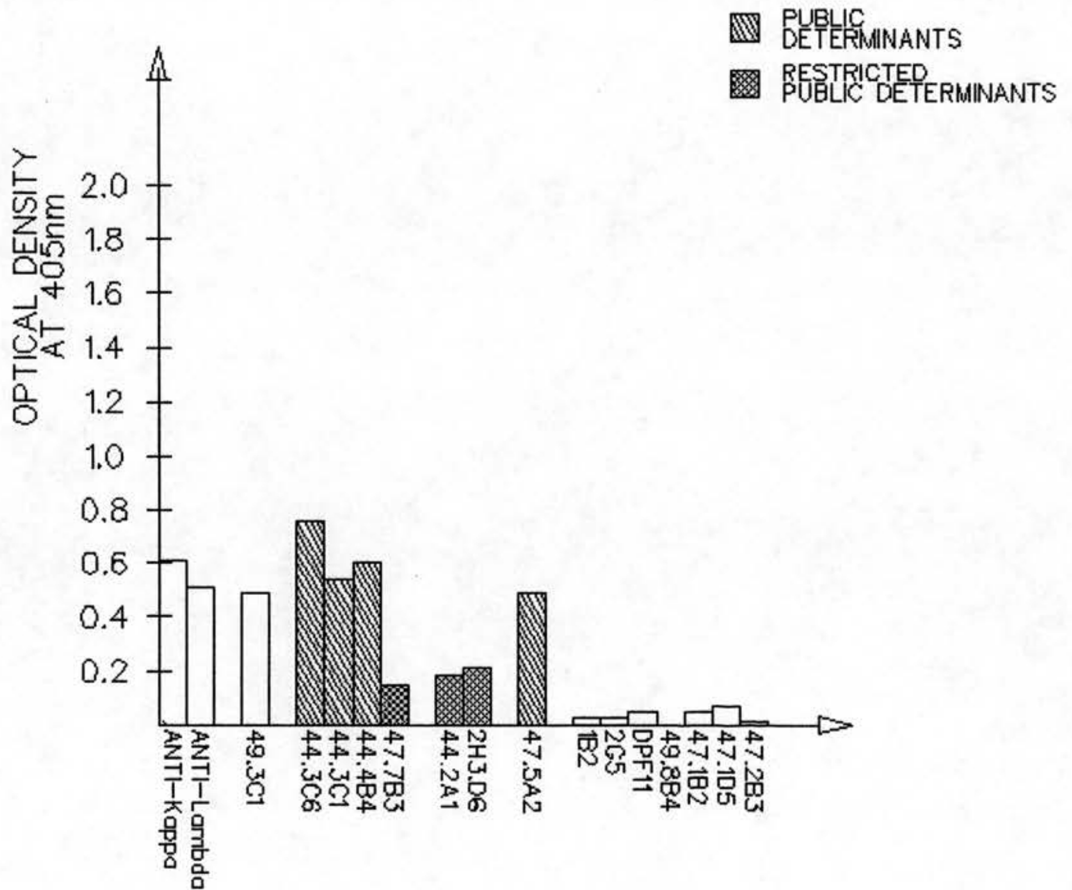
BINDING OF POOLED NORMAL IgG TO JS MONOCLONAL ANTIBODY PANEL BY ELISA



BINDING DETECTED USING ANTI-IgG ALKALINE PHOSPHATASE CONJUGATE
ALL RESULTS ARE MEANS OF DUPLICATE WELLS
ALL BACKGROUND RESULTS < 0.02

FIGURE 3.4

BINDING OF NORMAL SERUM Ig TO JS MONOCLONAL ANTIBODY PANEL BY ELISA



BINDING DETECTED USING ANTI-POLYVALENT Ig ALKALINE PHOSPHATE CONJUGATE
ALL RESULTS ARE MEANS OF DUPLICATE WELLS
ALL BACKGROUND RESULTS < 0.04

CHAPTER 4

REACTIVITY OF JS MONOCLONAL ANTIBODY PANEL WITH CHRONIC LYMPHOCYTIC LEUKAEMIA CELLS

4.1 MATERIALS

Peripheral Blood Mononuclear Cells from CLL Patients

4.2 METHODS

Indirect Immunoperoxidase
Preparation of Cell Lysate
3-Stage Human Ig ELISA Assay
4-Stage Idiotope Specific ELISA Assay

4.3 INTRODUCTION

The reactivity of each JS McAb with cells from 42 patients with CLL was determined by indirect immunoperoxidase on acetone fixed cytospin preparations of PBMNC.

In most cases the staining pattern seen with a positive JS McAb was identical to that seen with the tumour specific anti-light chain antibody suggesting that the JS McAbs were binding tumour specific Ig. There were, however, a number of instances when the staining pattern seen with JS McAbs was either very weak or was qualitatively different from that seen with the

anti-light chain antibody.

The JS antibody panel has not been extensively tested against the complex range of proteins expressed by cells although antibody 1B2 has been shown to cross-react with an epitope expressed by human epithelial cells (J.A. Habeshaw, personal communication). The existance of such cross-reactions would not be surprising in view of idiotypic diversity and, as a consequence, it was necessary to demonstrate that the reactivity of JS McAbs with CLL cells was specific for tumour Ig.

Characteristically CLL cells express low levels of surface Ig but in vitro stimulation with phorbol esters has been shown to increase Ig production and secretion in most cases (Tötterman et al, 1980 and, Gordon et al, 1984b). Thus reactivity of JS McAbs with tumour specific Ig will be associated with increased cytoplasmic staining of TPA stimulated cells by immunoperoxidase and, the specificity of the reaction for tumour specific Ig can be confirmed using the 4-stage ELISA assay already described.

4.4 RESULTS

4.4.1 JS McAb Binding to CLL Cells

In each immunoperoxidase run a single patient's cells were tested against the complete JS McAb panel as well as anti-κ and anti-λ antibodies (SAPU) and a negative control (no primary antibody). The light chain specific antibodies were a positive control for the presence of monoclonal Ig and also a control for the non-specific binding of mouse Ig. In addition, the 14 JS McAbs, which were all ascites preparations raised in the same strain of mice and, were all IgG1 sub-class, acted as their own negative controls for each other.

The number of cells reacting with each antibody was assessed semi-quantitatively and patients were only considered positive when the same population of cells reacted with a JS McAb and the tumour specific anti-light chain antibody. Sixteen patients reacted with at least 1 of the JS McAbs and 10 reacted with 2 or more but these included a number of cases where the staining was weak or of questionable specificity (table 4.1). Examples of the various staining patterns seen are shown in plates 4.1 to 4.10.

4.4.1.1 Typical Staining Patterns

In plate 4.1 almost all the cells from patient CD are positive with anti-λ used as the primary antibody. The granular pattern was typical of Ig staining in the majority of CLLs and an identical pattern was seen with 4 of the JS McAbs including 44.3C6 (plate 4.2.) suggesting that the JS McAbs are also reacting with tumour specific Ig.

In a minority of CLLs cytoplasmic Ig forms crystalline inclusion bodies (Guglielmi et al, 1982). For instance, the majority of cells from patient LA contain needle-like cytoplasmic inclusions reacting with anti-κ. Plate 4.3 shows the same pattern of reactivity with the JS McAb 2H3.D6. Cells from another patient (HB) show strong staining with anti-λ with the occasional cell containing a positively staining cytoplasmic block. The same pattern is seen with the JS McAbs 44.3C6 and 44.4B4 (plate 4.4). In such cases there can be little doubt about the specificity of the JS McAbs for tumour specific Ig.

4.4.1.2 Weak Staining Patterns

In a number of cases the staining patterns seen with JS McAbs (particularly 44.2A1,) were similar to those seen with tumour specific anti-light chain antibodies but were very weak. For instance, cells from patient TE show strong staining with anti-κ (plate 4.5,) but much weaker staining with antibody 44.2A1 although the granular pattern is still visible (plate 4.6). Repeating the immunoperoxidase with a more concentrated solution of 44.2A1 as the primary antibody was unhelpful because of significant background staining. This was probably due to non-specific binding of murine proteins in the ascites preparation.

4.4.1.3 Questionable Staining Patterns

In a number of cases the staining patterns seen with JS McAbs were qualitatively different from the tumour specific anti-light chain antibodies. For instance cells from patient MT show typical granular staining with anti-κ (plate 4.7,) but with 49.8B4 the staining pattern is different with the majority of cells showing discrete blocks of cytoplasmic staining (plate 4.8).

Plate 4.9 shows typical granular staining of cells from patient LG with anti-λ as the primary antibody. Plate 4.10, however, shows a weak diffuse deposition of the reaction product with antibody 47.5A2. This pattern is suggestive of non-specific binding but was seen in 5 cases with antibody 47.5A2.

4.4.2 <u>JS McAb Binding to TPA Stimulated Cells</u>

Fresh or frozen cells were available for TPA stimulation from 14 of the 16 patients whose cells reacted with 1 or more of the JS McAbs by immunoperoxidase. Cells from 5 patients who were negative with all the antibodies were also tested. Those not available for further study were patient RK whose cells were strongly positive with all but one of the JS McAbs and, patient AS whose cells were weakly positive with antibody 49.8B4.

The cells were cultured in the presence of 100ng/ml of TPA for 6 days after which the supernatants were collected and stored. The TPA stimulated cells were washed 3 times, resuspended at 2 x 10^6/ml in RPMI and cytospin preparations made as before. In 5 of the patients the cells were E-depleted prior to culture.

An MGG stain of unstimulated cells from patient PC (plate 4.11.) shows the majority of cells to be small round lymphocytes with scanty cytoplasm and clumped nuclear chromatin. Occasional cells have more prominent cytoplasm and azurophilic granules. After TPA stimulation, cultures contained cells of varying morphology (plate 4.12.) ranging from small lymphocytes to large blast cells with some plasmacytic features; eccentrically placed nuclei, plentiful basophilic cytoplasm, a prominent Golgi area and some cytoplasmic vacuoles.

The binding of the JS McAb panel to the TPA stimulated cells was tested by indirect immunoperoxidase as previously. All cases showed an increase in cytoplasmic staining with the tumour specific anti-light chain antibody (plates 4.13 and 4.14). Three patterns of results with the JS McAbs were identified other than those which were negative before and after stimulation:-

i) Positive unstimulated and stimulated cells
ii) Positive unstimulated but negative stimulated cells
iii) Negative unstimulated but positive stimulated cells

4.4.2.1 Positive Unstimulated and Stimulated Cells

All available cases which were positive with the lambda specific "public" antibodies, the "restricted public" antibodies and, 2 out of 7 cases reacting with the heavy chain specific "public" antibody (47.5A2) showed an increase in cytoplasmic reactivity with the JS McAb after TPA stimulation (table 4.2).

In contrast, out of 7 cases of "private" JS McAbs reacting with unstimulated cells only 1 case (patient MT with antibody 47.2B3,) showed staining of TPA stimulated cells.

4.4.2.2 Positive Unstimulated but Negative Stimulated Cells

All 5 of the patients whose cells showed the diffuse staining pattern with antibody 47.5A2 were studied and in none of them did the TPA stimulated cells show significant reactivity with this antibody. Similarly 5 of the 7 patients whose cells had reacted with antibody 49.8B4 and cells from patient LG which reacted with 47.1B2 were studied and none of the TPA stimulated cells

reacted with these antibodies.

In these cases it is likely that the reactivity of JS McAbs with unstimulated cells is due to non-specific binding or cross-reactivity with an epitope expressed by a molecule other than Ig. However, the possibility that TPA stimulation resulted in altered idiotype expression by the tumour cells cannot be excluded.

4.4.2.3 Negative Unstimulated but Positive Stimulated Cells.

In a number of instances TPA stimulated cells were positive with antibodies when the unstimulated cells had been negative (table 4.3). For instance, unstimulated cells from patient AB were positive with 3 JS McAbs (47.7B3, 44.2A1 and, 47.5A2). The TPA stimulated cells only reacted with 2 of these (47.7B3 and, 44.2A1,) but, additionally, a further 6 antibodies (table 4.3). The pattern of staining with these antibodies was indistinguishable from that seen with anti-λ (plates 4.15 and 4.16).

Similarly unstimulated cells from patient PM were negative for all the antibodies but a proportion of the TPA stimulated cells reacted strongly with 5 of the JS

McAbs. In 3 other instances TPA stimulated cells were positive with antibodies when the unstimulated cells were negative (patient LG with 44.3C1, MS with 44.2A1 and, CD with 47.7B3.)

4.4.3 Ig Secretion by CLL Cells

The Ig levels in the supernatants of TPA stimulated cells were assayed using the 3-stage ELISA. All the samples were done in duplicate and IgG, IgM, and IgA were quantitated from standard curves as described in chapter 2. Kappa and lambda results were expressed as units of optical density. Only 1 supernatant (patient LA,) did not contain significant amounts of Ig. The results from the other 18 culture supernatants are shown in table 4.4.

Out of 14 PBMNC cultures only 2 supernatants (HB and PM) contained monotypic Ig with a single isotype and a single light chain. A further 3 (MS, MT, and RP) show a single isotype and a marked predominance of the light chain expressed by the tumour cells. The remaining 8 supernatants contained polyclonal Ig.

The 5 supernatants from E-depleted cultured cells include 4 which contain monotypic Ig and one (RPa) with a single isotype and both light chains.

4.4.4 Binding of Secreted Ig to JS McAb Panel

The reactivity of Ig in culture supernatants with the JS McAbs and murine anti-κ and anti-λ was assessed using the 4-stage ELISA assay as described previously. Results were considered positive when the difference between the result and the background for that antibody was greater than 0.15 units of optical density.

In supernatants containing monotypic Ig the assay was only performed using the relevant anti-isotypic antibody conjugates and all samples were done in duplicate.

In supernatants containing polyclonal Ig binding of tumour specific Ig to a JS McAb could be demonstrated in 2 ways:-

i. Binding of secreted Ig to the anti-κ and anti-λ murine monoclonal capture antibodies demonstrated that the isotype corresponding to the tumour cell phenotype had the same light chain restriction as the tumour cells (figure 4.1a).

ii. Secreted Ig binding a JS McAb was shown to have the same isotype and light chain restriction as

the tumour cells using the polyclonal goat anti-isotype and anti-light chain antibody conjugates (figure 4.1b).

In assays where antibody conjugates specific for both light chains and all 3 isotypes were required the numbers involved made it impractical to include duplicate samples although inconclusive results were repeated in duplicate. Furthermore, the detection of tumour specific Id reacting with a JS McAb in such assays depended on positive results with the relevant anti-isotypic and anti-light chain antibodies so 2 separate results were still required before a sample was considered positive.

In 7 out of 18 supernatants there was no significant binding to any of the JS McAbs. The results of the 11 remaining cases are shown in tables 4.5 to 4.15 and can be divided into 5 groups:-

i) Positive Supernatants, Stimulated and Unstimulated Cells

ii) Negative Supernatants but Positive Stimulated and Unstimulated Cells

iii) Positive Supernatants but Negative Stimulated and Unstimulated Cells

iv) Negative Supernatants and Stimulated Cells but Positive Unstimulated Cells

v) Negative Supernatants and Unstimulated Cells but Positive Stimulated Cells

4.4.4.1 Positive Supernatants, Stimulated and Unstimulated Cells

In 18 instances the specificity of the cellular reactions of JS McAbs with the unstimulated and stimulated cells was confirmed by binding of secreted Id to the JS McAbs in the ELISA (see tables 4.5 to 4.13). Eight of these involved the antibodies 44.2A1, 2H3.D6, or 47.5A2 and in all these cases the reactivity was due to monotypic Ig with the same isotype and light chain as the tumour (tables 4.8 to 4.13).

There were a further 10 instances when supernatants from lambda expressing tumours showed significant reactivity with the lambda specific McAbs (tables 4.5 to 4.9). In 9 of these the reactivity could be confirmed as tumour Ig specific on the basis of isotype matching and/or the identification of monotypic Ig in the supernatant. In patient LG the binding of 44.4B4 to IgM in the supernatant could not be confirmed due to a cross-reaction between 44.4B4 and one batch of goat anti-human IgM conjugate.

4.4.4.2 Negative Supernatants but Positive Stimulated and Unstimulated Cells

In 3 instances JS McAbs which reacted with stimulated and unstimulated cells did not react with secreted Ig despite the demonstration of monotypic Ig in the culture supernatants. These involved patient AB whose cells reacted with 47.7B3 and 44.2A1 (table 4.14.) and patient MT whose cells reacted with 47.2B3.

4.4.4.3 Positive Supernatant but Negative Stimulated and Unstimulated Cells

Significant binding of secreted Ig to the JS McAbs in the absence of cellular reactivity only occurred in culture supernatants containing polyclonal Ig and mainly with the public determinants recognised by 44.3C6, 44.3C1, 44.4B4, and 47.5A2. The first 3 are lambda specific antibodies but reacted with polyclonal IgG in supernatants from kappa and lambda expressing tumours (tables 4.10, 4.11, 4.13, 4.14 and 4.15). Similarly antibody 47.5A2 reacted with polyclonal IgG in 5 supernatants from IgM and IgA expressing tumours in the absence of cellular reactivity. The binding of secreted Ig to 47.5A2 detected by the anti-light chain antibodies was much weaker than with the

anti-γ. Light chain restriction was only apparent in 1 of these cases (table 5.15.) but this may be a reflection of the normal 2:1 kappa:lambda ratio in humans.

In two patients with IgG expressing tumours there was weak binding of secreted IgG to the "restricted public" antibody 44.2A1 (tables 4.12 and 4.13.) but, in both cases the light chain specific antibodies were negative.

4.4.4.4 Negative Supernatants and Stimulated Cells but, Positive Unstimulated Cells

In all 11 instances in which JS McAb reactivity with unstimulated cells was lost after TPA stimulation (table 4.2.) secreted Ig was unreactive with the antibodies by ELISA.

Antibody 49.8B4 did not react with secreted Ig in culture supernatants from 5 patients whose cells reacted with the antibody even though all of them contained monotypic Ig. Similarly, secreted tumour specific Ig from LG cells did not react with antibody 47.1B2 by ELISA (table 4.6).

Secreted tumour specific Ig did not react with 47.5A2 in any of the 5 cases where unstimulated cells had shown

diffuse reactivity with the antibody (plates 4.9 and 4.10). In 3 of these cases, however, secreted polyclonal IgG did bind to 47.5A2.

4.4.4.5 Negative Supernatants and Unstimulated Cells but Positive Stimulated Cells

There were 15 instances in which TPA stimulated cells had reacted with JS McAbs when the unstimulated cells had been negative (table 4.3,) but in none of them did the culture supernatants react with the antibodies involved.

These results could be explained either by antigenic differences between secretory and cytoplasmic Ig or by reactivity of the JS McAbs with epitopes expressed by molecules other than Ig.

In order to investigate this further cell lysates were prepared from TPA stimulated cells and the extract tested for reactivity with the JS McAbs using the ELISA system.

4.4.5 Binding of Cytoplasmic Ig to the JS McAb Panel

Fresh or frozen cells were obtained from the 5 patients in whom JS McAbs had reacted with TPA stimulated cells but not with unstimulated cells or with secreted Ig (table 4.3). In addition cells from patient MT were studied as cellular reactivity with antibody 47.2B3 before and after TPA stimulation could not be confirmed as specific by the ELISA.

Bulk cultures (100 $\times 10^6$ cells in 100mls culture medium,) were stimulated with TPA at 100ng/ml. After 6 days the cells were harvested, washed x3 in RPMI and the lysates prepared as described in the methods. The Ig content of each preparation was measured by the 3-stage ELISA using serial dilutions of calibrated serum in PBS containing 1% NP-40 as the standards. Monotypic Ig was obtained from all 6 cases (table 4.16).

The lysates were then tested for reactivity with the JS McAbs using the 4-stage ELISA with the anti-isotypic antibody conjugate appropriate for each sample. Cell lysates from patients AB and PM, whose TPA stimulated cells had reacted with multiple JS McAbs, were tested against the entire JS McAb panel except for antibody 47.5A2. The sample from patient PM showed weak binding to a number of antibodies including anti-λ and the lambda

specific JS McAbs (table 4.17). This is unlikely to represent specific binding as the sample contained monotypic IgMκ by the 3-stage ELISA (table 4.16).

The remaining samples were only tested against the antibody in question plus a JS McAb known to react with tumour specific Ig or anti-light chain antibodies as positive controls (table 4.17). The results show that none of the cases in which JS McAbs react with TPA stimulated cells but not unstimulated cells or secreted Ig is due to reactivity with cytoplasmic Ig. The positive staining by immunoperoxidase in these cases must be due to cross-reactivity with an epitope expressed by another molecule.

4.5 DISCUSSION

Indirect immunoperoxidase is a highly sensitive technique for demonstrating antibody/antigen complexes in cells and there was no reason to doubt the specificity of the results obtained with the JS McAbs on cytospin preparations of CLL cells in the majority of cases.

There were, however, a number of cases in which the staining patterns seen with a JS McAb were qualitatively different from those seen with the tumour specific anti-light chain antibody. It was therefore necessary to demonstrate that the JS McAbs were reacting with tumour specific Ig.

A simple method of doing this would be to demonstrate loss of JS McAb binding in the presence of high concentrations of the JS paraprotein but this approach was rejected for 2 reasons.

First, the potential blocking ability of the JS paraprotein is dependent on the paratope of a JS McAb recognising an epitope shared by the CLL and JS idiotypes. However, the JS McAbs were selected by virtue

of affinity for the JS paraprotein V region and it is just as likely that the JS paratope recognises an idiotope expressed by a JS McAb. In that case it would be possible for the paratope of the JS McAb to continue to bind to an idiotope expressed by the CLL Id in spite of the presence of excess JS paraprotein.

Secondly, monoclonal antibodies recognise epitopes rather than antigens so the ability of the JS paraprotein to block a JS McAb binding does not demonstrate specificity for tumour Ig as the epitope recognised may be expressed by other molecules.

A more direct method of demonstrating specificity would be to stimulate Ig production by CLL cells in vitro using a culture medium deficient in an essential amino acid (for instance methionine,) and to provide that amino acid labelled with a radio-active isotope such as ^{35}Sulphur. Ig molecules synthesised by the cells would incorporate the label and could be used as a probe to detect binding to the JS McAbs in a solid phase radioimmunoassay or by Western blotting.

Preliminary studies demonstrated that TPA was highly effective at inducing Ig secretion. However, the presence of polyclonal Ig in many of the culture supernatants could result in false positive results because of

radio-labelled polyclonal Ig. This problem could be circumvented using the 4-stage ELISA assay because it was possible to demonstrate that the Ig molecules reacting with a particular JS McAb were restricted to the same isotype and light chain as the tumour Id even when the culture supernatant contained polyclonal Ig.

JS McAbs reacted with unstimulated CLL cells in 47 instances. In 32 of these the reactivity of the JS McAbs for secreted Ig was tested by ELISA and in 18 cases the cellular reactions were confirmed as specific for Ig.

The number of cellular reactions which were shown not to be Ig specific (table 4.18) demonstrate the importance of using such a method to prove specificity.

4.5.1 <u>False Positive Results</u>

There were 14 instances when unstimulated CLL cells reacted with JS McAbs by indirect immunoperoxidase but secreted tumour specific Ig did not bind to the same antibodies in the ELISA.

Five of these involved antibody 47.5A2 which reacts with a public idiotope commonly expressed by IgG (see chapters

2 and 3). In 2 patients with IgG expressing tumours 47.5A2 reacted specifically with cells and secreted Ig (table 4.15 and 4.16,) but the weak, diffuse staining pattern seen with 5 other patients was not specific for tumour Ig. A similar staining pattern was seen with the Glm(f) allotype specific antibody 49.3C1 in 1 case (table 4.10,) and it is probable that the diffuse staining in these cases is due to McAb reactivity with polyclonal IgG bound to the cells by Fc receptors (Stevenson et al, 1981).

Most of the other false positive results involved antibody 49.8B4 which reacted with unstimulated cells from 7 patients but, in the 5 of these who could be studied further, 49.8B4 was unreactive with TPA stimulated cells or secreted Ig. In these cases and also that of antibody 47.1B2 with cells from patient LG the reactivity of the JS McAb with unstimulated cells was lost after TPA stimulation suggesting that the reactions were due to non-specific binding or the determinant involved had been lost from the cell surface and not re-synthesised during culture.

In 3 instances (cells from patient AB with antibodies 47.7B3 and 44.2A1 and cells from patient MT and antibody 47.2B3,) JS McAbs reacted with both stimulated and unstimulated cells but not with secreted Ig. Cytoplasmic

Ig was extracted from TPA stimulated cells from both these patients but this was also negative for the JS McAbs by ELISA demonstrating that the JS McAbs were not reacting with cellular Ig.

In addition to these false positive results there were 14 instances when TPA stimulated cells reacted with JS McAbs when the unstimulated cells had been negative. In all these cases cytoplasmic Ig from the positive cells was unreactive with the JS McAbs by ELISA suggesting that TPA had induced the synthesis of a molecule expressing a cross-reacting epitope.

In most cases which were subsequently shown to be false positives, the cellular reactions were quantitatively or qualitatively different from those seen with the tumour specific anti-light chain antibody.

A great deal of effort would be required to identify the cross-reacting determinants involved in false positive results and this was not considered necessary for the purposes of this investigation. Preliminary characterisation might be to run cell lysates in SDS-PAGE and agarose iso-electric focussing and to determine the molecular weight and iso-electric points of the molecules that the JS McAbs react with by Western blotting.

4.5.2 True Positive Results

The cellular reactions shown not to be specific for Ig have been excluded from tables 4.19 and 4.20 which show the reactivity of the JS McAbs with tumour specific Ig in lambda and kappa expressing CLLs. The only results which have not been confirmed as specific for secreted Ig by ELISA are those of patients RK and LA.

Patient RK's cells react with all but one of the JS McAbs with a staining pattern similar to that seen with anti-λ suggesting that the reaction is specific for Ig. Assuming this is so then either the tumour idiotype is very similar to that of the original immunogen or it has antigen binding specificity for an epitope expressed by mouse Ig. The latter possibility is reduced by the negative reactions with with anti-κ and antibody DPF11. Unfortunately, patient RK died shortly after diagnosis before any cells had been cryopreserved and could not be investigated further.

This finding could be of great interest as the chances of 2 randomly selected paraproteins having almost identical idiotypes is extremely small and it is therefore possible that the 2 tumours shared a common immunoregulatory defect associated with the expression of

certain idiotypic determinants. However, a question mark must remain over the results as the specificity of the reactions for tumour Ig have not been proven and the possibility of the tumour Id reacting with a murine Ig epitope cannot be excluded.

Patient LA's cells contain crystalline inclusion bodies reacting with anti-κ strongly suggesting that the reaction with antibody 2H3.D6 (plate 4.3) is also specific for tumour Ig.

In 2 patients (LA and CD,) positive for 2H3.D6 there are minor populations of cells (5-10%) reacting with antibody 44.2A1. In patient LA the 44.2A1 positive cells show the characteristic cytoplasmic staining and are thus part of the malignant clone. In patient CD secreted IgMλ reacts with 44.2A1 and it is therefore highly likely that the cells staining with the antibody are also part of the malignant clone.

Patient LA has been followed up over a 2 year period during which the populations of cells expressing these idiotopes have remained static.

In the remaining patients the population of cell staining with the JS McAbs is always comparable to those positive for the relevant anti-light chain antibody although any

minor changes in proportion cannot be assessed on cytospins.

TABLE 4.1

Reactivity of Cells from 42 Cases of CLL with JS McAb Panel by Indirect Immunoperoxidase

Antibody	Postive Cases	Weak Staining	Questionable Staining
44.3C6	6	0	0
44.3C1	3	0	0
44.4B4	4	0	0
47.7B3	2	1	0
44.2A1	4	3	0
2H3.D6	6	1	0
47.5A2	8	5	5
2G5	1	0	0
DPF11	0	0	0
1B2	1	0	0
49.8B4	7	4	6
47.1B2	2	1	0
47.1D5	1	0	0
47.2B3	2	0	0

Positive cases = Number of cases in which the same population of cells stained with tumour specific anti-light chain antibody and JS McAb.

Weak staining = Number of cases in which staining pattern seen with JS McAb much weaker than that of tumour specific anti-light chain antibody.

Questionable staining = Number of cases in which staining pattern seen with JS McAb was qualitatively different from that seen with tumour specific light chain antibody.

TABLE 4.2

Reactivity of JS McAbs with TPA Stimulated CLL Cells by Immunoperoxidase

Antibody	No. Cases	Positive	Negative
44.3C6	5	5	0
44.3C1	2	2	0
44.4B4	3	3	0
47.7B3	1	1	0
44.2A1	3	3	0
2H3.D6	4	4	0
47.5A2	7	2	5
49.8B4	5	0	5
47.1B2	1	0	1
47.2B3	1	1	0

No. cases = Number of cases in which PBMNC positive with 1 or more JS McAbs were available for TPA stimulation studies

Positive = Number of cases in which JS McAb reacted with unstimulated and TPA stimulated cells

Negative = Number of cases in which JS McAb reacted with unstimulated but not TPA stimulated cells

TABLE 4.3

Reactivity of JS McAbs with TPA Stimulated CLL Cells in the Absence of Reactivity with Unstimulated Cells by Immunoperoxidase

Antibody	Patient				
	AB	PM	LG	CD	MS
44.3C1	+		+		
47.7B3		+		+	
44.2A1		+			+
2H3.D6	+				
2G5	+	+			
47.1B2	+	+			
47.1D5	+				
49.8B4	+	+			

+ = JS McAb reacting with TPA stimulated cells but not with unstimulated cells from same patient.

TABLE 4.4

Ig Levels in TPA Stimulated Peripheral Blood Culture Supernatants from 18 Cases of CLL Assayed by 3-Stage ELISA

Patient	Isotype	IgM ng/ml	IgG ng/ml	IgA ng/ml	Kappa OD	Lambda OD
HB	IgMλ	700	<16	<16	0.04	1.68
PM	IgMκ	8000	<16	<16	>2	0.10
MS	IgGκ	<16	130	<16	1.67	0.21
MT	IgGκ	<16	40	<16	0.80	0.17
RP	IgMκ	92	<16	<16	1.03	0.26
CD	IgMλ	>2000	>2000	50	1.20	>2
PC	IgMλ	1150	310	110	0.74	>2
LG	IgMλ	84	18	<16	0.43	1.41
AD	IgGλ	<16	250	<16	0.68	1.01
MSm	IgAκ	<16	57	43	0.85	1.22
AB	IgAλ	<16	110	28	0.49	0.88
TE	IgMκ	120	135	76	1.15	1.02
MG	IgMκ	620	750	520	1.70	>2
MGr	IgMκ	84	<16	<16	0.48	0.08
JT	IgMλ	3800	<16	<16	0.02	>2
GS	IgMλ	1100	<16	<16	0.02	>2
AN	IgMλ	44	<16	<16	0.08	0.35
RPa	IgMκ	275	<16	<16	0.58	0.17

Isotype = Tumour specific Ig detected by immunocytochemistry
OD = Optical Density
All results are means of duplicate wells.
MGr, JT, GS, AN, RPa cultured after E-depletion.

TABLE 4.5

Reactivity of JS McAbs with Cells and Secreted Ig from Patient HB

McAb	CELLS		SUPERNATANT		
	Unstim	TPA Stim	Kappa	Lambda	IgM
Anti-κ	+	+			0.04
Anti-λ	+++	+++			≥2
49.3C1	-	-	0.03	0	0.06
44.3C6	+++	+++	0	≥2	1.46
44.3C1	-	-	0	0.06	0
44.4B4	+++	+++	0	≥2	ND
47.7B3	-	-	0	0.06	0.02
44.2A1	-	-	0.01	0.05	0.03
2H3.D6	-	-	0	0.09	0.03
47.5A2	-	ND	ND	ND	ND
2G5	-	-	0.02	0.10	0.03
DPF11	-	-	0	0.07	0
1B2	-	-	0	0.05	0.01
49.8B4	-	-	0	0.05	0.03
47.1B2	-	-	0.02	0.09	0
47.1D5	-	-	0	0.05	0
47.2B3	-	-	0.01	0.11	0.02

Tumour phenotype: IgMλ
Culture supernatant contained IgMλ (table 4.4)

CELLS = number of cells reacting with McAb on cytospin preparations by immunoperoxidase before and after TPA stimulation

- = negative
+/- = <1% positive
+ = 1-20% positive
++ = 20-80% positive
+++ = >80% positive

ND = not done
w = weak
D = diffuse staining pattern

SUPERNATANT = Reactivity (optical density minus background,) of secreted Ig from TPA stimulated cultures with McAb using isotype and light chain specific ELISA assays.
Positive results underlined.

TABLE 4.6

Reactivity of JS McAbs with Cells and Secreted Ig from Patient LG

McAb	CELLS		SUPERNATANT			
	Unstim	TPA Stim	Kappa	Lambda	IgM	IgG
Anti-κ	+/-	+/-			0.02	<u>0.72</u>
Anti-λ	++	++			<u>0.26</u>	<u>0.17</u>
49.3C1	+/-	+/-	<u>0.23</u>	<u>0.18</u>	0.09	<u>0.74</u>
44.3C6	++	+++	0	<u>≥2</u>	<u>0.36</u>	0
44.3C1	-	+++	0.01	0.03	0	0.02
44.4B4	++	+++	0	<u>≥2</u>	ND	0
47.7B3	-	-	0.01	0.01	0.03	0
44.2A1	-	-	0.01	0	0	0.01
2H3.D6	-	-	0.07	0	0.03	0
47.5A2	+++wD	-	0.05	0	0	0.04
2G5	-	-	0.01	0	ND	ND
DPF11	-	-	0.04	0	ND	ND
1B2	-	-	0	0	ND	ND
49.8B4	++	-	0.04	0	0	0.01
47.1B2	++	-	0.02	0	ND	ND
47.1D5	-	-	0.01	0	ND	ND
47.2B3	-	-	0	0	ND	ND

Tumour phenotype: IgMλ
Culture supernatant contained IgM and IgG (Table 4.4)

CELLS = number of cells reacting with McAb on cytospin preparations by immunoperoxidase before and after TPA stimulation

- = negative
+/- = <1% positive
+ = 1-20% positive
++ = 20-80% positive
+++ = >80% positive

ND = not done
w = weak
D = diffuse staining pattern

SUPERNATANT = Reactivity (optical density minus background,) of secreted Ig from TPA stimulated cultures with McAb using isotype and light chain specific ELISA assays.
Positive results underlined.

TABLE 4.7

Reactivity of JS McAbs with Cells and Secreted Ig from Patient AN

McAb	CELLS		SUPERNATANT
	Unstim	TPA Stim	IgM
Anti-κ	+/-	+/-	0.04
Anti-λ	++	++	<u>0.22</u>
49.3C1	-	-	0.01
44.3C6	++	++	<u>0.17</u>
44.3C1	-	-	0
44.4B4	-	-	0.03
47.7B3	-	-	0
44.2A1	-	-	0.02
2H3.D6	-	-	0.01
47.5A2	ND	ND	ND
2G5	-	-	0.01
DPF11	-	-	0.01
1B2	-	-	0.03
49.8B4	-	-	0.01
47.1B2	-	-	0.02
47.1D5	-	-	0
47.2B3	-	-	0.02

Tumour phenotype: IgMλ
Culture supernatant contained IgMλ (table 4.4)

CELLS = number of cells reacting with McAb on cytospin preparations by immunoperoxidase before and after TPA stimulation

- = negative
+/- = <1% positive
+ = 1-20% positive
++ = 20-80% positive
+++ = >80% positive

ND = not done
w = weak
D = diffuse staining pattern

SUPERNATANT = Reactivity (optical density minus background,) of secreted Ig from TPA stimulated cultures with McAb using isotype and light chain specific ELISA assays.
Positive results underlined.

TABLE 4.8

Reactivity of JS McAbs with Cells and Secreted Ig from Patient CD

McAb	CELLS		SUPERNATANT	
	Unstim	TPA Stim	IgM	IgG
Anti-κ	+/-	+/-	0.01	0.81
Anti-λ	+++	+++	0.97	0.45
49.3C1	+/-	+/-	0	1.44
44.3C6	+++	+++	1.24	0.93
44.3C1	+++	+++	1.85	0.12
44.4B4	+++	+++	≥2	0.13
47.7B3	-	++	0.05	0.05
44.2A1	+	+	0.53	0.12
2H3.D6	+++	+++	≥2	0.07
47.5A2	-	-	0	1.16
2G5	-	-	0	0.04
DPF11	-	-	0.02	0.03
1B2	-	-	0	0.01
49.8B4	-	-	0	0
47.1B2	-	-	0	0.02
47.1D5	-	-	0	0.01
47.2B3	-	-	0	0.05

Tumour phenotype: IgMλ
Culture supernatant contained IgM, IgG and IgA (table 4.4)

CELLS = number of cells reacting with McAb on cytospin preparations by immunoperoxidase before and after TPA stimulation

- = negative
+/- = <1% positive
+ = 1-20% positive
++ = 20-80% positive
+++ = >80% positive

ND = not done
w = weak
D = diffuse staining pattern

SUPERNATANT = Reactivity (optical density minus background,) of secreted Ig from TPA stimulated cultures with McAb using isotype and light chain specific ELISA assays.
Positive results underlined. Anti-α not tested

TABLE 4.9

Reactivity of JS McAbs with Cells and Secreted Ig from Patient PC

McAb	CELLS		SUPERNATANT	
	Unstim	TPA Stim	IgM	IgG
Anti-κ	+/-	+/-	0	0.71
Anti-λ	+++	+++	0.31	0.21
49.3C1	+/-	+/-	0	0.60
44.3C6	+++	+++	0.46	0
44.3C1	+++	+++	0.45	0
44.4B4	-	-	0.02	0.04
47.7B3	-	-	0	0
44.2A1	-	-	0	0
2H3.D6	+++	+++	0.91	0.01
47.5A2	++w	-	0	0.12
2G5	-	-	0.06	0
DPF11	-	-	0	0.01
1B2	-	-	0.07	0
49.8B4	-	-	0	0
47.1B2	-	-	0.08	0
47.1D5	-	-	0.08	0
47.2B3	-	-	0.08	0

Tumour phenotype: IgMλ
Culture supernatant contained IgM, IgG and IgA (table 4.4)

CELLS = number of cells reacting with McAb on cytospin preparations by immunoperoxidase before and after TPA stimulation

- = negative
+/- = <1% positive
+ = 1-20% positive
++ = 20-80% positive
+++ = >80% positive

ND = not done
w = weak
D = diffuse staining pattern

SUPERNATANT = Reactivity (optical density minus background,) of secreted Ig from TPA stimulated cultures with McAb using isotype and light chain specific ELISA assays.
Positive results underlined. Anti-α not tested

TABLE 4.10

Reactivity of JS McAbs with Cells and
Secreted Ig from Patient MG

McAb	CELLS		SUPERNATANT				
	Unstim	TPA Stim	Kappa	Lambda	IgM	IgG	IgA
Anti-κ	++	++			0.54	1.67	1.03
Anti-λ	+/-	+/-			0.11	1.32	0.38
49.3C1	+++wD	++wD	1.11	1.18	0	1.96	0
44.3C6	-	-	0.07	≥2	0.02	≥2	0.09
44.3C1	-	-	0.12	≥2	0.05	≥2	0.01
44.4B4	-	-	0.10	1.21	ND	1.37	0
47.7B3	-	-	0.08	0.09	0.06	0.07	0.02
44.2A1	++	++	1.87	0.05	1.85	0.02	0.01
2H3.D6	-	-	0.10	0.05	0.08	0.07	0
47.5A2	+++wD	+++wD	0.15	0.12	0.06	0.77	0.01
2G5	-	-	0	0.02	ND	ND	ND
DPF11	-	-	0	0	ND	ND	ND
1B2	-	-	0	0	ND	ND	ND
49.8B4	-	-	0.10	0.03	0.05	0	0.02
47.1B2	-	-	0.03	0.03	ND	ND	ND
47.1D5	-	-	0	0	ND	ND	ND
47.2B3	-	-	0.10	0.04	0.06	0.03	0

Tumour phenotype: IgMκ
Culture supernatant contained IgM, IgG and IgA (table 4.4)

CELLS = number of cells reacting with McAb on cytospin preparations by immunoperoxidase before and after TPA stimulation

- = negative
+/- = <1% positive
+ = 1-20% positive
++ = 20-80% positive
+++ = >80% positive

ND = not done
w = weak
D = diffuse staining pattern

SUPERNATANT = Reactivity (optical density minus background,) of secreted Ig from TPA stimulated cultures with McAb using isotype and light chain specific ELISA assays.
Positive results underlined.

TABLE 4.11

Reactivity of JS McAbs with Cells and
Secreted Ig from Patient TE

McAb	CELLS		SUPERNATANT				
	Unstim	TPA Stim	Kappa	Lambda	IgM	IgG	IgA
Anti-κ	++	++			0.27	1.32	0.30
Anti-λ	-	-			0.06	0.71	0.28
49.3C1	-	-	0.92	0.71	0.02	>2	0.10
44.3C6	-	-	0	0.61	0.03	0.60	0.13
44.3C1	-	-	0	0.29	0.01	0.32	0.11
44.4B4	-	-	0.02	0.24	ND	0.13	0.07
47.7B3	-	-	0.04	0.01	0.03	0.04	0.07
44.2A1	++w	++	0.31	0	0.21	0.04	0.07
2H3.D6	++w	+++	0.34	0	0.43	0.12	0.10
47.5A2	++wD	+++wD	0.03	0.05	0.01	0.60	ND
2G5	-	-	0.08	0.10	ND	ND	0
DPF11	-	-	0.06	0.08	ND	ND	0.01
1B2	-	-	0.04	0.10	ND	ND	0.01
49.8B4	++w	-	0	0	0.05	0.01	0.11
47.1B2	-	-	0	0	0.02	0	0
47.1D5	-	-	0.06	0.11	ND	ND	0
47.2B3	-	-	0.09	0.10	ND	ND	0

Tumour phenotype: IgMκ
Culture supernatant contained IgM, IgG and IgA (table 4.4)

CELLS = number of cells reacting with McAb on cytospin preparations by immunoperoxidase before and after TPA stimulation

- = negative
+/- = <1% positive
\+ = 1-20% positive
++ = 20-80% positive
+++ = >80% positive

ND = not done
w = weak
D = diffuse staining pattern

SUPERNATANT = Reactivity (optical density minus background.) of secreted Ig from TPA stimulated cultures with McAb using isotype and light chain specific ELISA assays.
Positive results underlined.

TABLE 4.12

Reactivity of JS McAbs with Cells and
Secreted Ig from Patient MS

McAb	CELLS		SUPERNATANT		
	Unstim	TPA Stim	Kappa	Lambda	IgG
Anti-κ	+++	+++			1.93
Anti-λ	-	-			0.25
49.3C1	-	-	0.05	0.06	0.43
44.3C6	-	-	0	0	0
44.3C1	-	-	0	0	0.03
44.4B4	-	-	0.03	0.02	0
47.7B3	-	-	0.01	0.10	0.06
44.2A1	-	++	0.10	0.06	0.19
2H3.D6	++w	++	0.83	0.05	0.96
47.5A2	++	+++	0.59	0.02	>2
2G5	-	-	0.03	0	ND
DPF11	-	-	0.01	0	ND
1B2	-	-	0	0	ND
49.8B4	-	-	0.02	0.04	0.03
47.1B2	-	-	0.01	0	ND
47.1D5	-	-	0.03	0	ND
47.2B3	-	-	0	0.02	0.03

Tumour phenotype: IgGκ
Culture supernatant contained IgGκ+λ (table 4.4)

CELLS = number of cells reacting with McAb on cytospin preparations by immunoperoxidase before and after TPA stimulation

- = negative
+/- = <1% positive
+ = 1-20% positive
++ = 20-80% positive
+++ = >80% positive

ND = not done
w = weak
D = diffuse staining pattern

SUPERNATANT = Reactivity (optical density minus background.) of secreted Ig from TPA stimulated cultures with McAb using isotype and light chain specific ELISA assays.
Positive results underlined.

TABLE 4.13

Reactivity of JS McAbs with Cells and
Secreted Ig from Patient AD

McAb	CELLS		SUPERNATANT		
	Unstim	TPA Stim	Kappa	Lambda	IgG
Anti-κ	+/-	+/-			>2
Anti-λ	++w	+++			>2
49.3C1	-	-	1.48	1.64	>2
44.3C6	-	-	0.03	0.82	1.47
44.3C1	-	-	0.05	0.41	0.76
44.4B4	-	-	0.10	0.27	0.53
47.7B3	-	-	0.05	0.05	0.13
44.2A1	-	-	0.05	0.02	0.15
2H3.D6	-	-	0	0	0.03
47.5A2	+++	+++	0.11	0.78	>2
2G5	-	-	0.02	0	0.10
DPF11	-	-	0.01	0	0.05
1B2	-	-	0	0	0.06
49.8B4	-	-	0.03	0	0.03
47.1B2	-	-	0.05	0.01	0.06
47.1D5	-	-	0	0	0
47.2B3	-	-	0.04	0	0.09

Tumour phenotype: IgGλ
Culture supernatant contained IgGλ+κ (table 4.4)

CELLS = number of cells reacting with McAb on cytospin preparations by immunoperoxidase before and after TPA stimulation

- = negative
+/- = <1% positive
+ = 1-20% positive
++ = 20-80% positive
+++ = >80% positive

ND = not done
w = weak
D = diffuse staining pattern

SUPERNATANT = Reactivity (optical density minus background,) of secreted Ig from TPA stimulated cultures with McAb using isotype and light chain specific ELISA assays.
Positive results underlined.

TABLE 4.14

Reactivity of JS McAbs with Cells and
Secreted Ig from Patient AB

McAb	CELLS		SUPERNATANT			
	Unstim	TPA Stim	Kappa	Lambda	IgG	IgA
Anti-κ	+/-	+/-			>2	0
Anti-λ	+++	+++			1.72	0.50
49.3C1	+/-	+/-	0.79	0.85	>2	0.03
44.3C6	-	-	0	0.45	0.88	0.03
44.3C1	-	+++w	0	0.09	0.36	0.02
44.4B4	-	-	0.04	0	0.05	0.04
47.7B3	++w	++	0	0	0.05	0.04
44.2A1	++w	+++w	0	0	0.08	0.03
2H3.D6	-	+++	0	0	0.13	0.02
47.5A2	++w	-	0	0.07	0.82	0.03
2G5	-	++w	0	0	0	0
DPF11	-	-	0	0	0	0
1B2	-	-	0	0	0.03	0
49.8B4	-	+++	0	0	0.05	0.05
47.1B2	-	++	0	0	0.03	0.05
47.1D5	-	++	0.01	0	0.07	0.02
47.2B3	-	-	0	0	0.02	0.01

Tumour phenotype: IgAλ
Culture supernatant contained IgG and IgA (table 4.4)

CELLS = number of cells reacting with McAb on cytospin preparations by immunoperoxidase before and after TPA stimulation

- = negative
+/- = <1% positive
+ = 1-20% positive
++ = 20-80% positive
+++ = >80% positive

ND = not done
w = weak
D = diffuse staining pattern

SUPERNATANT = Reactivity (optical density minus background,) of secreted Ig from TPA stimulated cultures with McAb using isotype and light chain specific ELISA assays.
Positive results underlined.

TABLE 4.15

Reactivity of JS McAbs with Cells and Secreted Ig from Patient MSm

McAb	CELLS		SUPERNATANT			
	Unstim	TPA Stim	Kappa	Lambda	IgA	IgG
Anti-κ	++	++			0.23	>2
Anti-λ	+/-	+/-			0.06	1.18
49.3C1	-	-	0.75	0.90	0	>2
44.3C6	-	-	0	0.84	0.09	1.08
44.3C1	-	-	0	0.40	0	0.47
44.4B4	-	-	0.01	0.19	0	0.25
47.7B3	-	-	0.01	0.08	0	0.09
44.2A1	-	-	0.02	0.07	0	0.08
2H3.D6	-	-	0.04	0.11	0	0.12
47.5A2	-	-	0.12	0.44	0	1.32
2G5	-	-	0	0.03	0.02	0.06
DPF11	-	-	0	0.04	0.08	0.02
1B2	-	-	0	0.04	0.02	0.03
49.8B4	+++w	-	0.01	0.03	0	0.08
47.1B2	-	-	0	0.03	0.01	0.03
47.1D5	-	-	0	0.04	0.01	0.02
47.2B3	-	-	0	0.04	0.02	0.01

Tumour phenotype: IgAκ
Culture supernatant contained IgG and IgA (table 4.4)

CELLS = number of cells reacting with McAb on cytospin preparations by immunoperoxidase before and after TPA stimulation

- = negative
+/- = <1% positive
+ = 1-20% positive
++ = 20-80% positive
+++ = >80% positive

ND = not done
w = weak
D = diffuse staining pattern

SUPERNATANT = Reactivity (optical density minus background,) of secreted Ig from TPA stimulated cultures with McAb using isotype and light chain specific ELISA assays.
Positive results underlined.

TABLE 4.16

Ig Levels in Lysates from TPA Stimulated CLL Cells
Assayed by 3-Stage ELISA

Patient	Isotype	IgM ng/ml	IgG ng/ml	IgA ng/ml	Kappa OD	Lambda OD
PM	IgMκ	440	<16	<16	0.45	0.04
AB	IgAλ	<16	<16	220	0.08	0.25
LG	IgMλ	200	<16	<16	0.06	0.53
MS	IgGκ	<16	30	<16	0.33	0.05
CD	IgMλ	84	<16	<16	0	0.93
MT	IgGκ	<16	42	<16	0.29	0.06

Isotype = Tumour specific Ig by immunocytochemistry
All results are means of duplicate wells
OD = optical density

TABLE 4.17

Reactivity of JS McAbs with Cytoplasmic Ig from TPA Stimulated CLL Cells by 4-Stage ELISA

Antibody	Patient					
	AB	PM	CD	LG	MS	MT
Anti-κ	0	1.87	0.05	ND	ND	0.31
Anti-λ	0.21	0.17	0.95	ND	ND	0
44.3C6	0	0.18	*1.78	*0.77		
44.3C1	0	0.14	*>2	0.01		
44.4B4	0	0.13	*>2			
47.7B3	0.01	0.13	0.12			
44.2A1	0	0.07			0.01	
2H3.D6	0	0.12			*0.16	
2G5	0.01	0.12				
DPF11	0.01	0.10				
1B2	0.02	0.09				
49.8B4	0	0.12				0
47.1B2	0.01	0.11				
47.1D5	0	0.09				
47.2B3	0.02	0.12				0

Results (optical density minus background,) are means of duplicate wells.

*signifies JS McAbs reacting with tumour specific Ig used as positive controls.

ND = not done

TABLE 4.18

Patterns of Reactivity of 14 JS McAbs with Cells and Secreted Ig from 18 Cases of CLL.

Unstimulated Cells	TPA Stim. Cells	Secreted Id	No.
+ve	+ve	+ve	18
+ve	+ve	-ve	3
+ve	-ve	-ve	11
-ve	+ve	-ve	14
-ve	-ve	-ve	200

Unstimulated Cells = peripheral blood cells from CLL patients
TPA Stim. Cells = TPA stimulated cells from same patients

+ve = reactivity with JS McAb by immunoperoxidase
-ve = no reactivity with JS McAb

Secreted Id = tumour specific Ig secreted by TPA stimulated cells in vitro

+ve = binding of secreted Id to JS McAb detected by ELISA
-ve = no binding by ELISA

No. = number of instances in which a JS McAb reacted with cells and secreted Ig from a single CLL in the pattern shown.
Six tests not performed.

TABLE 4.19

Reactivity of JS McAbs with Tumour Idiotype in 21 Cases of Lambda Expressing CLLs by Immunoperoxidase

JS McAb		Patient						
		RK	HB	CD	PC	LG	AN	AD
λ	44.3C6	+++	+++	+++	+++	+++	++	-
	44.3C1	+++	-	+++	+++	-	-	-
	44.4B4	+++	+++	+++	-	+++	-	-
	47.7B3	+++	-	-	-	-	-	-
HC	44.2A1	+++	-	+	-	-	-	-
	2H3.D6	+++	-	+++	+++w	-	-	-
	47.5A2	+++	-	-	-	-	-	++
Con	2G5	+++	-	-	-	-	-	-
	DPF11	-	-	-	-	-	-	-
	1B2	+++	-	-	-	-	-	-
	49.8B4	+++	-	-	-	-	-	-
	47.1B2	+++	-	-	-	-	-	-
	47.1D5	+++	-	-	-	-	-	-
	47.2B3	+++	-	-	-	-	-	-

14 patients negative with all antibodies.

λ = lambda specific determinants
HC = heavy chain specific determinants
Con = conformational determinants

- =	negative	++ =	20-80% cells positive
+/- =	<1% cells positive	+++ =	>80% cells positive
+ =	1-20% cells positive	w =	weak reaction

TABLE 4.20

Reactivity of JS McAbs with Tumour Idiotype in 21 Cases of Kappa Expressing CLLs

JS McAb		Patient			
		TE	MG	LA	MS
λ	44.3C6	-	-	-	-
	44.3C1	-	-	-	-
	47.4B4	-	-	-	-
	47.7B3	-	-	-	-
HC	44.2A1	++w	++w	-	+w
	2H3.D6	++w	-	++	+++
	47.5A2	-	-	++	-
Con	2G5	-	-	-	-
	DPF11	-	-	-	-
	1B2	-	-	-	-
	49.8B4	-	-	-	-
	47.1B2	-	-	-	-
	47.1D5	-	-	-	-
	47.2B3	-	-	-	-

17 patients negative with all antibodies.

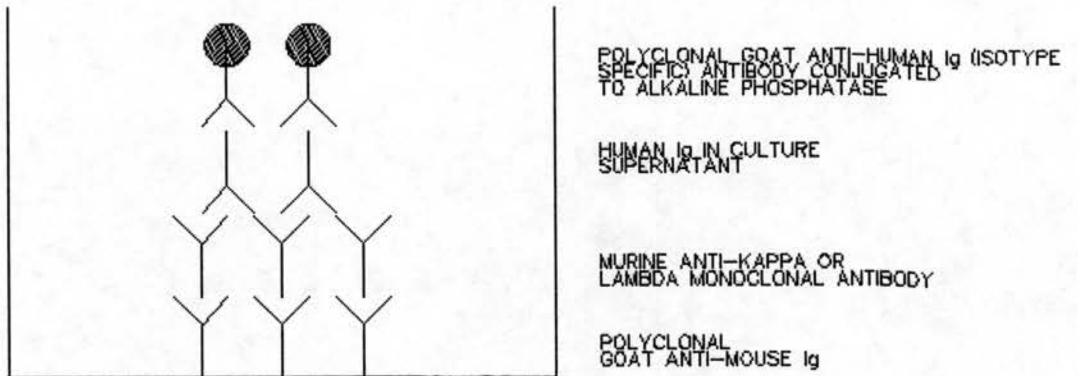
λ = lambda specific determinants
HC = heavy chain specific determinants
Con = conformational determinants

- =	negative	++ =	20-80% cells positive
+/- =	<1% cells positive	+++ =	>80% cells positive
+ =	1-20% cells positive	w =	weak reaction

FIGURE 4.1

METHODS OF PROVING MONOCLONALITY OF Ig IN CULTURE SUPERNATANTS USING 4 STAGE ELISA

A – USING MONOCLONAL ANTI-KAPPA AND ANTI-LAMBDA CAPTURE ANTIBODIES



B – USING POLYCLONAL ANTI-KAPPA AND ANTI-LAMBDA ANTIBODY CONJUGATES

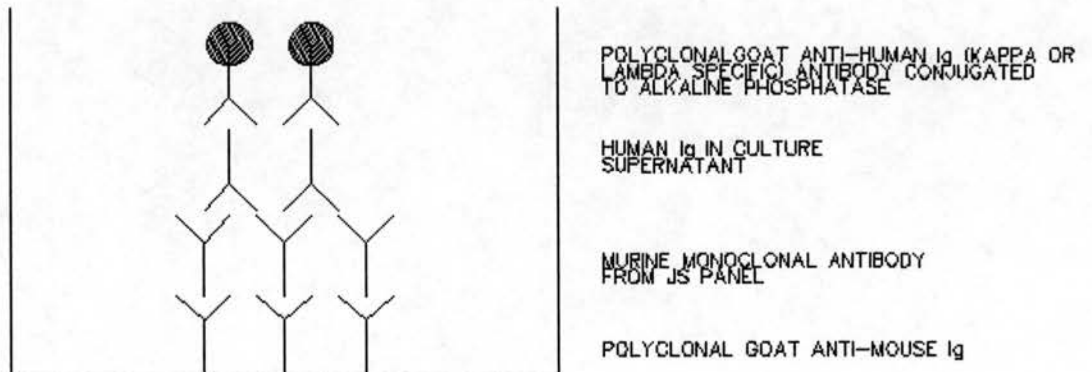
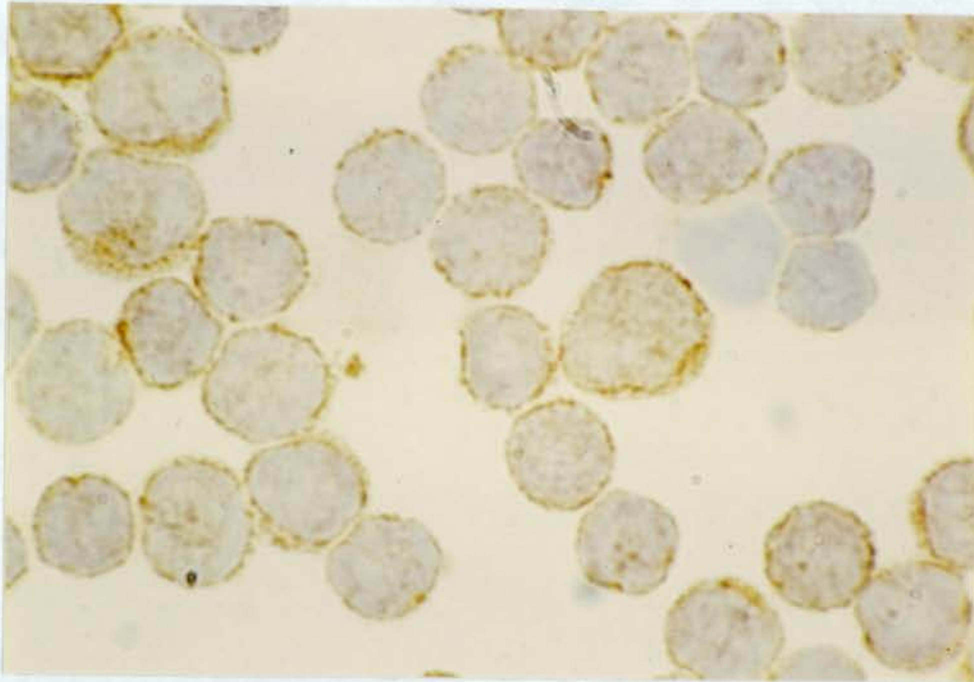
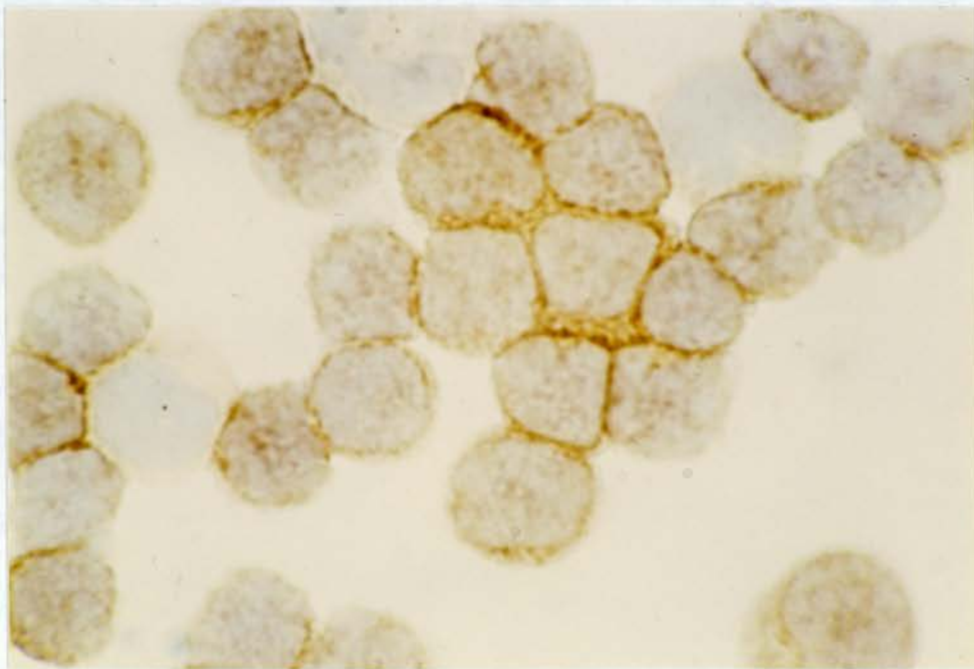


PLATE 4.1



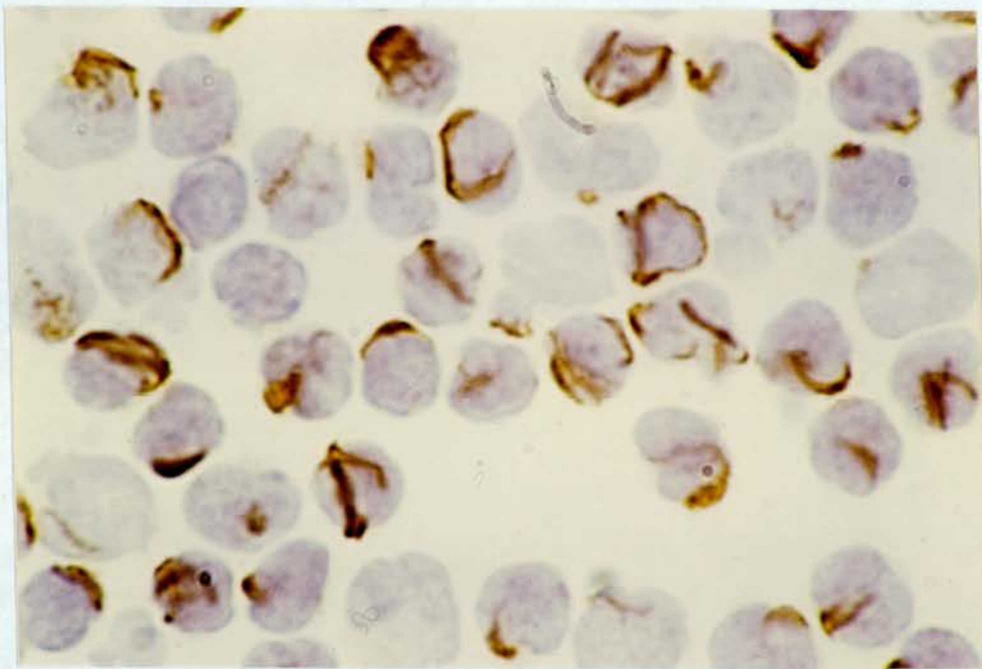
Cells from patient CD reacting with anti-λ by immunoperoxidase. Granular reactivity typical of immunoglobulin. (x1600)

PLATE 4.2



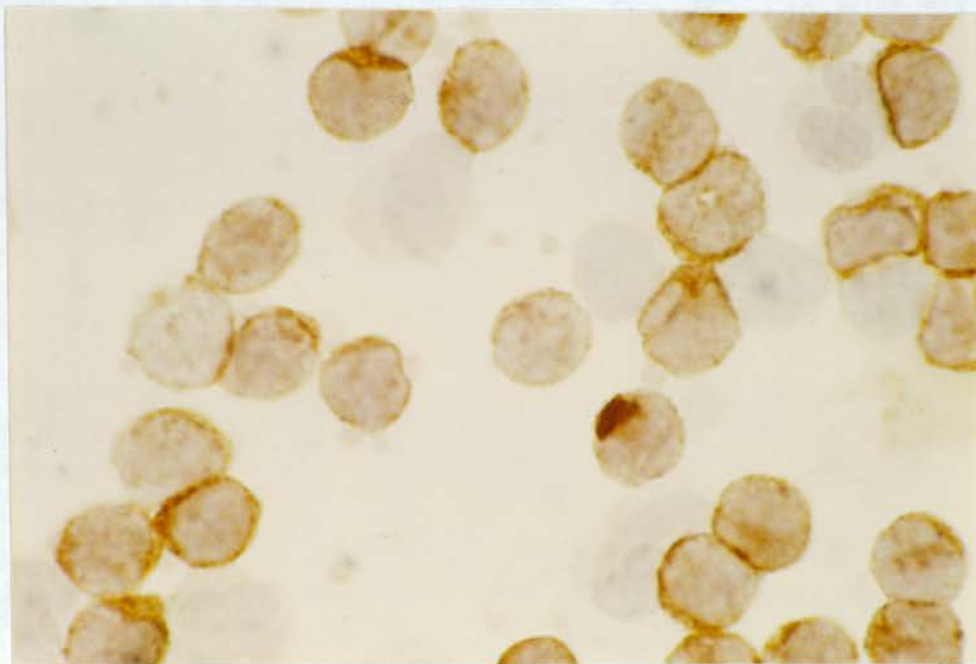
Cells from patient CD showing similar pattern of reactivity with JS McAb 44.3C6. (x1600)

PLATE 4.3



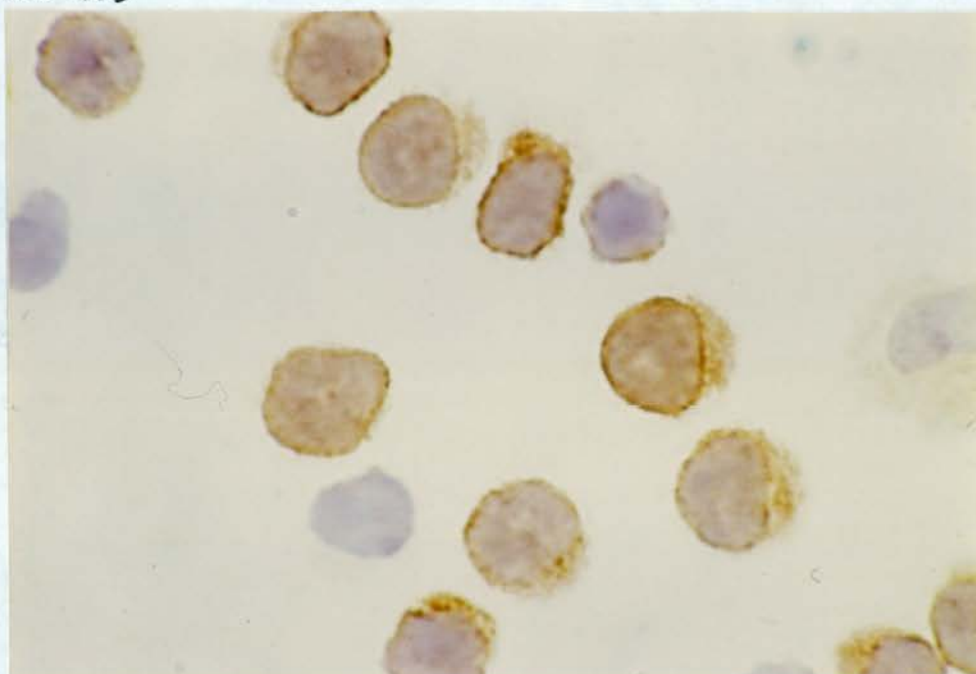
Immunoperoxidase of cells from patient LA with JS McAb 2H3.D6. Identical staining pattern seen with anti-κ (x1600)

PLATE 4.4



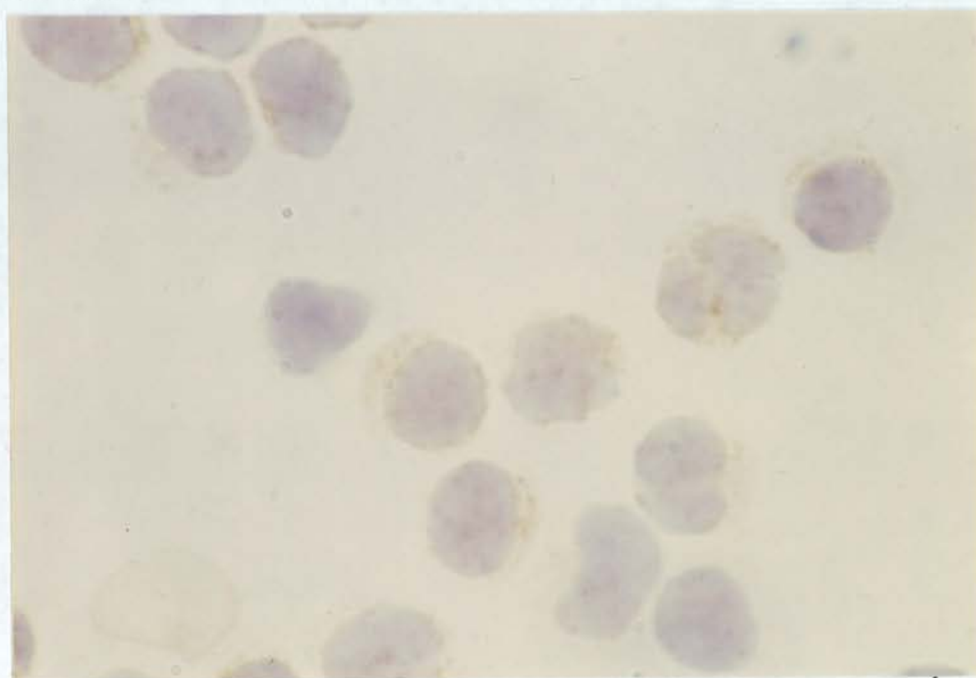
Immunoperoxidase of cells from patient HB with JS McAb 44.4B4. Occasional cell showing block positiviity as seen with anti-λ. (x1600)

PLATE 4.5



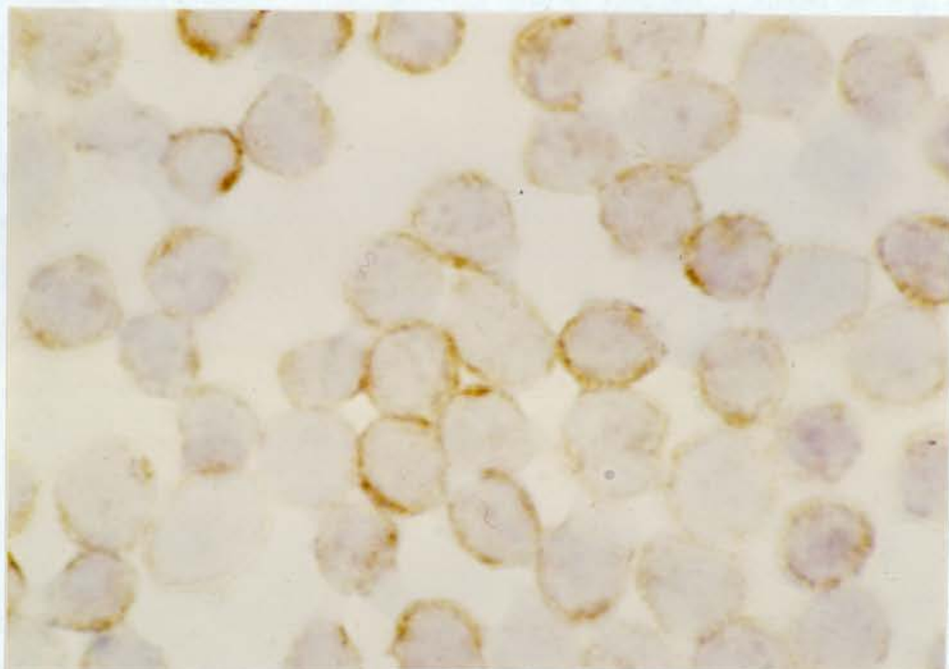
Immunoperoxidase of cells from patient TE with Anti-κ. (x1600)

PLATE 4.6



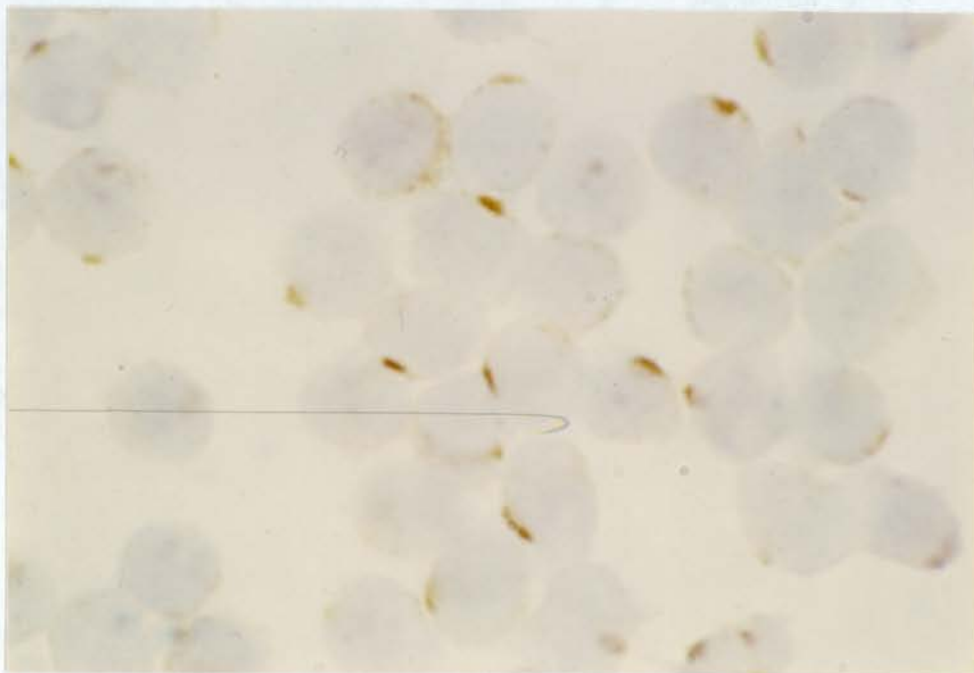
Immunoperoxidase of cells from patient TE with JS McAb 44.2A1. Much weaker staining than with anti-κ but similar granular pattern (x1600)

PLATE 4.7



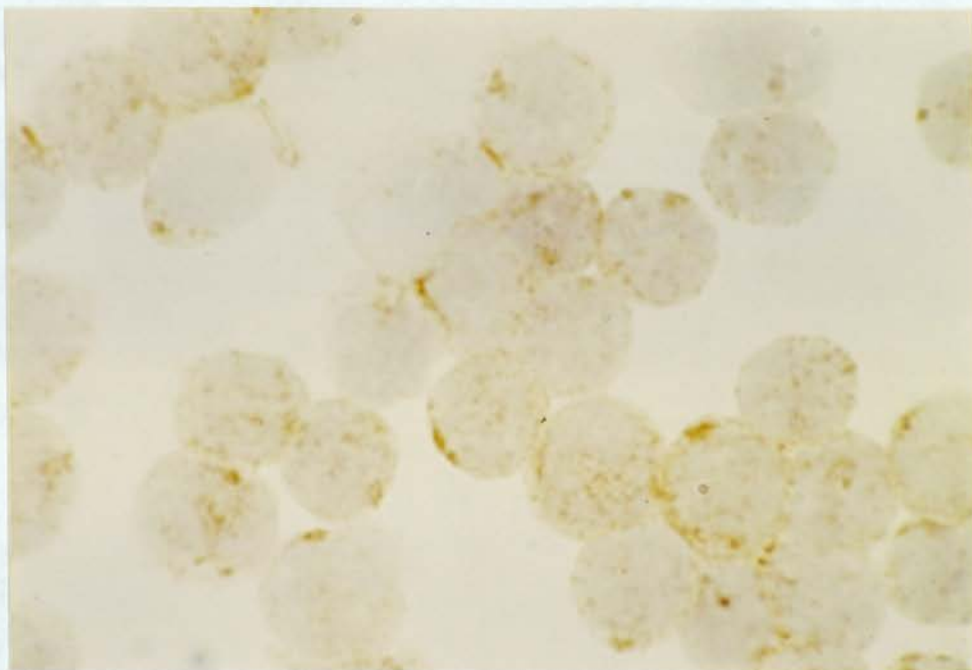
Immunoperoxidase of cells from patient MT with anti-κ. (x1600)

PLATE 4.8



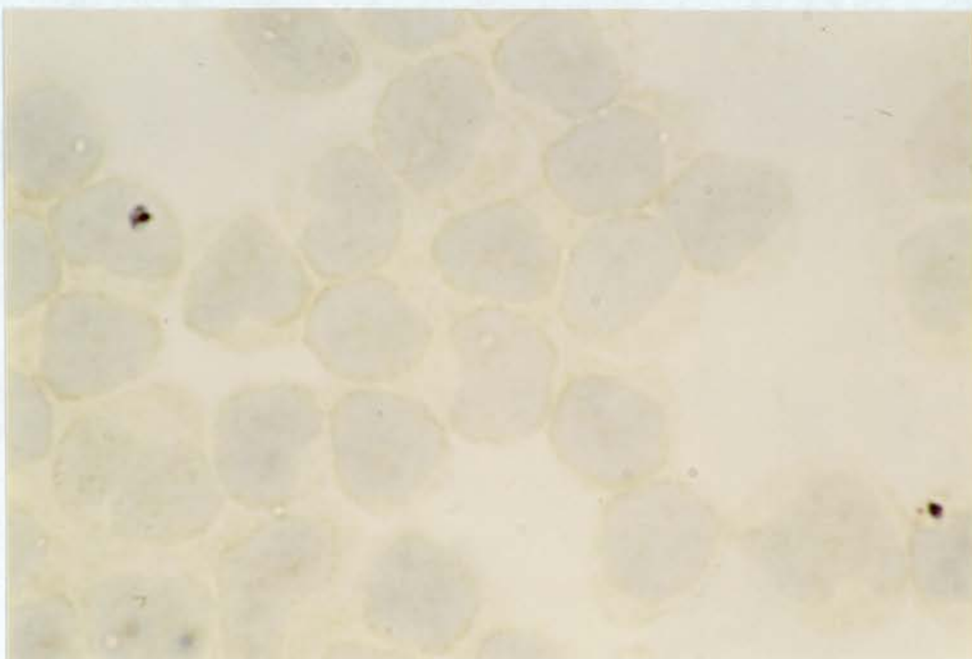
Immunoperoxidase of cells from patient MT with JS McAb 49.8B4 showing different pattern of reactivity from anti-κ. (x1600)

PLATE 4.9



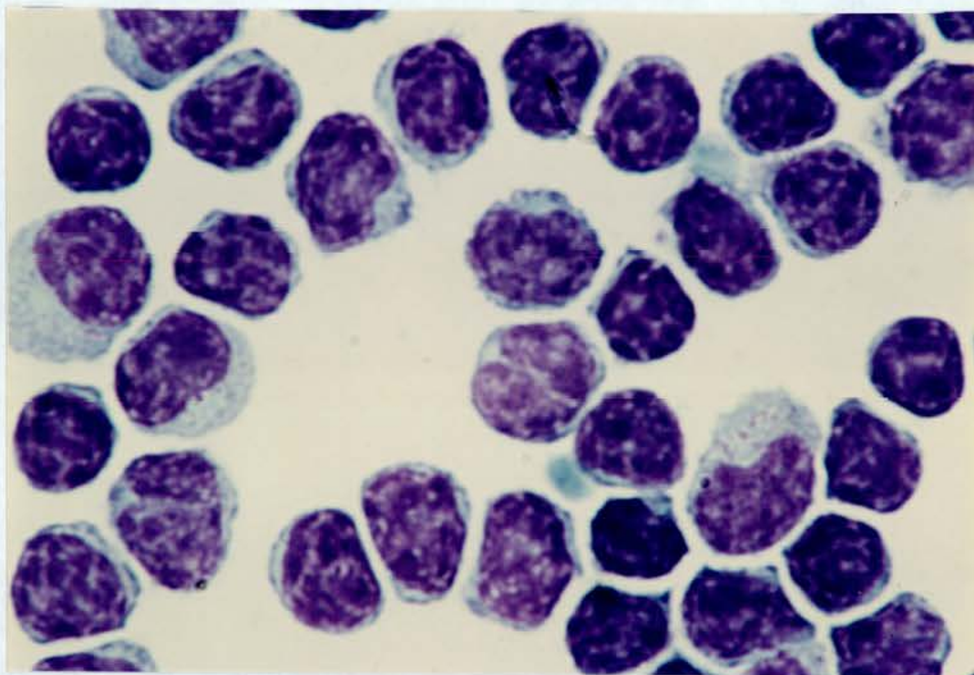
Immunoperoxidase of cells from patient LG with anti-λ. (x1600)

PLATE 4.10



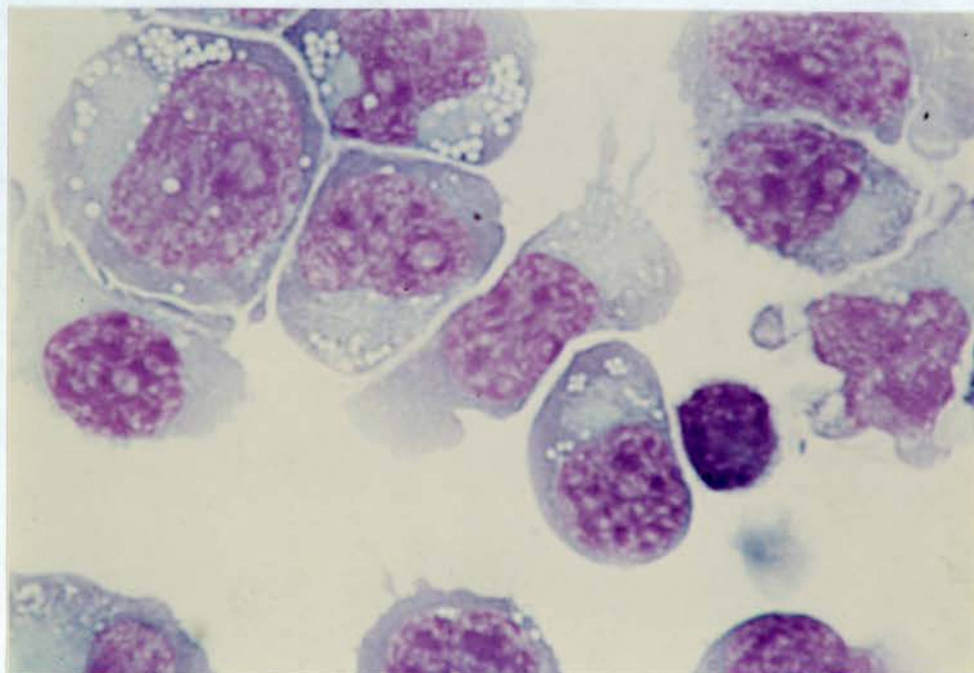
Immunoperoxidase of cells from patient LG with JS McAb 47.5A2 showing weak, diffuse staining reaction. (x1600)

PLATE 4.11



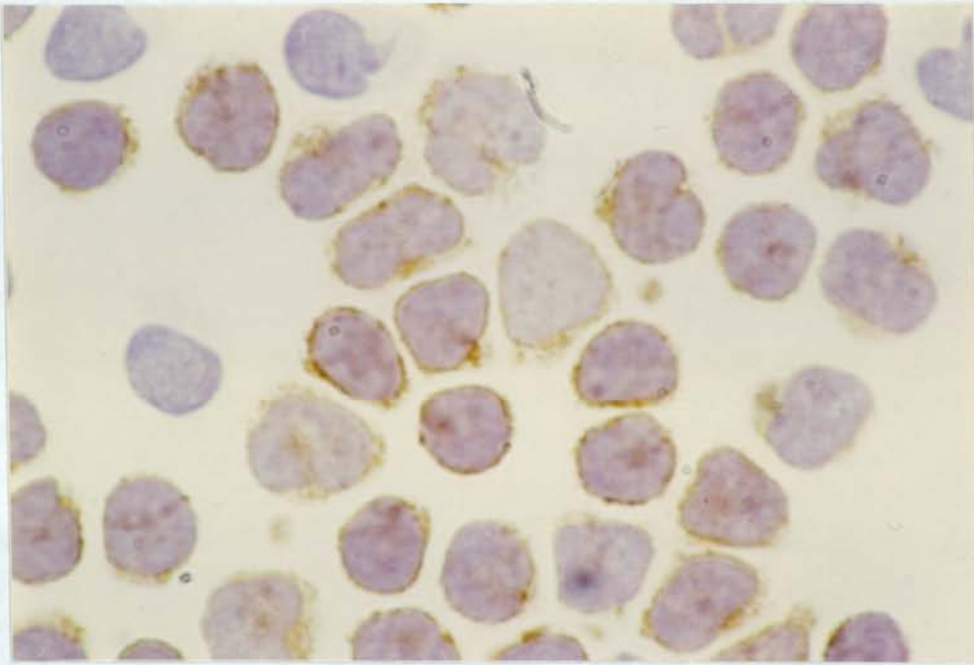
MGG stain of cells from patient PC. Mostly small round lymphocytes with scanty cytoplasm. (x1600)

PLATE 4.12



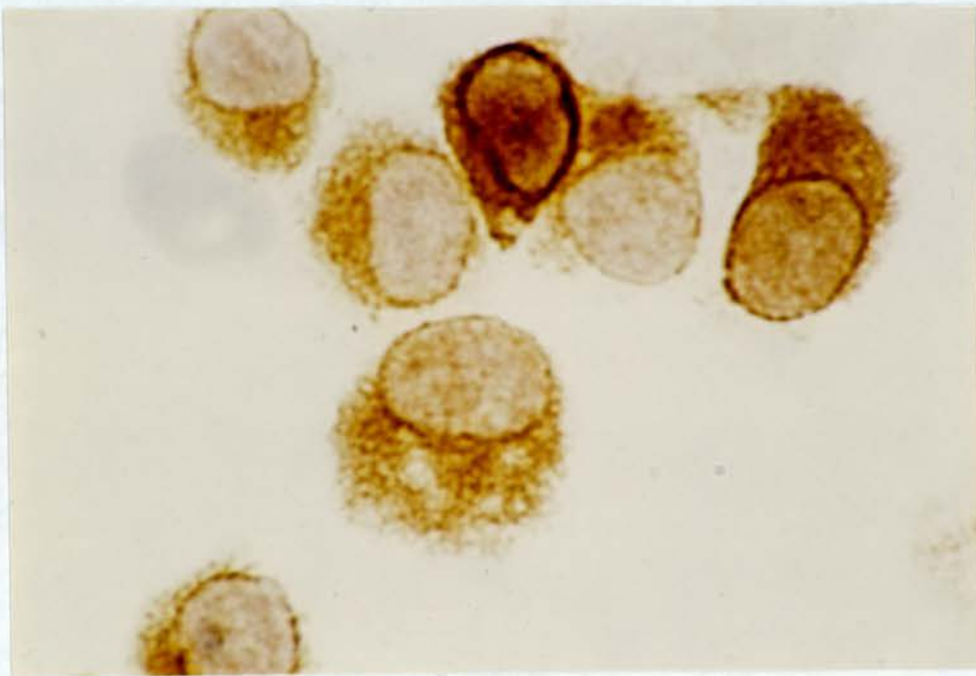
MGG stain of cells from patient PC after TPA stimulation. Majority of cells have undergone blast transformation and have plasmacytoid features. (x1600)

PLATE 4.13



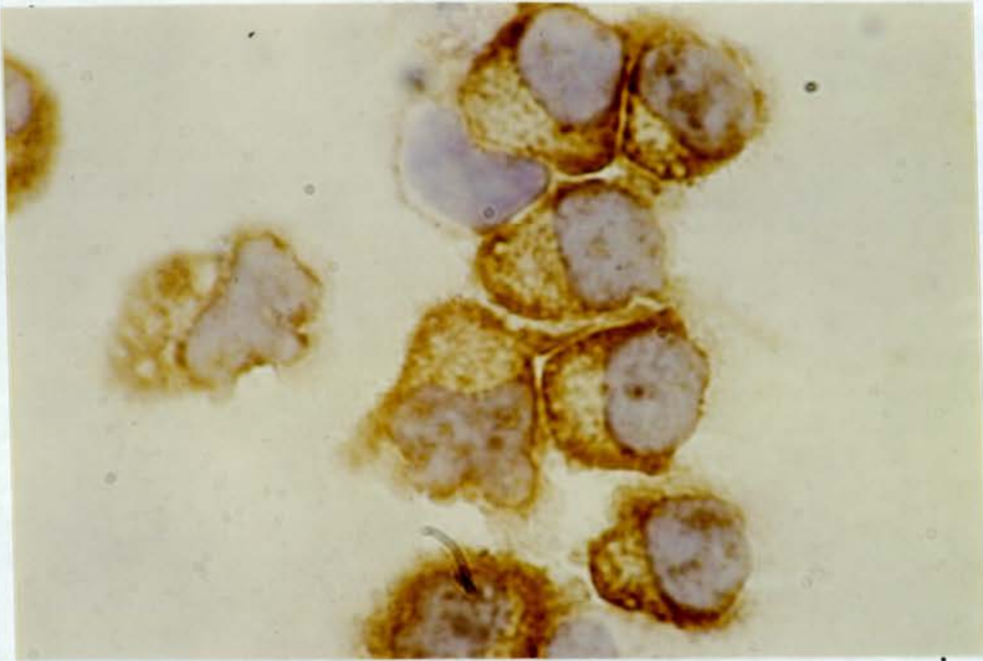
Immunoperoxidase of unstimulated cells from patient PC with anti-λ. (x1600)

PLATE 4.14



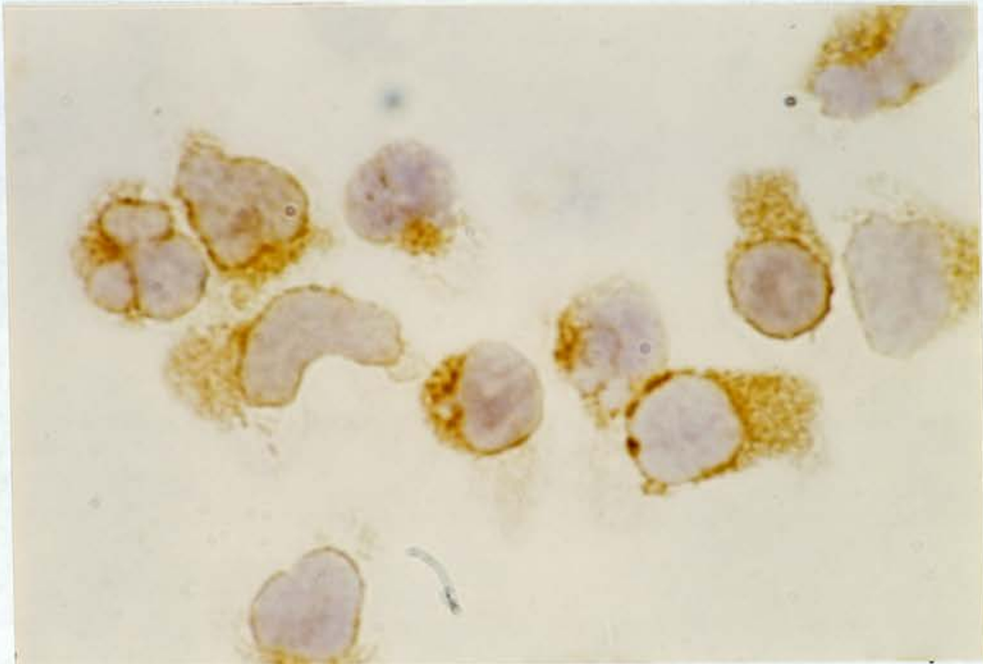
Immunoperoxidase of TPA stimulated cells from patient PC with anti-λ showing increased cytoplasmic Ig. (x1600)

PLATE 4.15



Immunoperoxidase of TPA stimulated cells from patient AB with anti-λ. (x1600)

PLATE 4.16



Immunoperoxidase of TPA stimulated cells from patient AB with JS McAb 49.8B4. Reactivity not specific for tumour Ig. (x1600)

CHAPTER 5

IMMUNOGLOBULIN SECRETION BY PERIPHERAL BLOOD CULTURES FROM CLL PATIENTS

5.1 MATERIALS

PBMNC and E-Depleted Cells from CLL Patients

5.2 METHODS

Indirect Immunoperoxidase
Cell Culture
3-Stage Ig ELISA Assay

5.3 INTRODUCTION

In the preceding chapter, the majority of TPA stimulated PBMNC cultures from CLL patients were found to contain polyclonal Ig even though most cultures contained less than 1% of cells expressing the opposite Ig light chain to the leukaemic clone (chapter 4). This was contrary to published experience (Gordon et al, 1984b,) in which only monotypic, tumour specific Ig was detected using a similar ELISA assay sensitive to 10ng/ml.

In vitro Ig secretion by normal B-cells in response to phorbol esters is dependent on helper T-cell factors (Ralph & Kishimoto, 1982 and, Sugawara, 1982). In order to investigate whether autologous T-cells contributed to the production of polyclonal Ig in CLL cultures 15 cases were studied before and after E-depletion.

5.4 RESULTS

The patients studied included all clinical stages (3 stage A, 6 stage B, and 6 stage C). Ten had IgM and 5 had IgG expressing tumours.

The number of T-cells before and after E-depletion was assessed by immunoperoxidase studies on cytospin preparations using monoclonal antibodies against the CD3, CD4, and CD8 antigens (SAPU). A minimum of five hundred cells were counted and the percentage positive with each antibody is shown in table 5.1.

Ig levels in culture supernatants were measured using the 3-stage ELISA assay described previously. The difference in mean ODs of samples analysed in the same assay was analysed using Student's t test.

The supernatants were divided into 4 groups depending on the Ig levels in PBMNC cultures. Only 4 samples, (group 1,) showed no significant increase in Ig levels after TPA stimulation. The remaining supernatants were divided into those containing polyclonal Ig with multiple isotypes (group 2,) those with polyclonal Ig but a single isotype (group 3,) and those containing monoclonal Ig (group 4).

5.4.1 Group 1: No Change in Ig Secretion

In 4 cases there was no significant increase in Ig levels in the TPA stimulated cultures compared to control cultures containing no mitogen (Table 5.2). This group included 1 IgM and 3 IgG expressing tumours and patients with all clinical stages.

Two supernatants (from patients AR and JSk,) contained a single isotype but both light chains while the other 2 (from JS and WC,) both IgG expressing tumours, contained all three isotypes including high levels of IgG.

In all 4 cases Ig levels in the E-depleted cultures (Table 5.3,) were reduced compared to PBMNC cultures.

Control and TPA stimulated E-depleted culture supernatants from AR were negative for IgG, IgM, and IgA but reacted weakly with anti-κ in keeping with the known ability of unstimulated CLL cells to secrete free light chains in vitro (Gordon et al, 1984b).

TPA stimulated, E-depleted cultures from patient JSk (whose tumour expressed IgGκ,) contained small amounts of IgG and IgM and were positive for kappa but not lambda. The mean OD for IgG in the TPA stimulated culture was significantly greater ($p < 0.01$,) than in the control

cultures whereas the IgM levels were not significantly different. These results suggest that TPA stimulation resulted in the secretion of small amount of tumour Id. A similar effect in PBMNC cultures would be masked by the presence of polyclonal IgG.

In supernatants from patients JS and WC, who had IgG expressing tumours, there was a marked reduction of polyclonal Ig production after E-depletion but there was no significant difference between control and TPA stimulated Ig levels. The supernatant from patient JS contained polyclonal IgG while patient WC's contained light chains only.

5.4.2 Group 2: Polyclonal Ig with More than One Isotype

In 4 supernatants from PBMNC cultures TPA stimulation resulted in increased Ig secretion over control cultures but more than one isotype was present (table 5.4). All 4 tumours expressed IgM and the patients were all stage A or B.

In 3 patients (JR, MGr, and LG,) there was a significant increase in IgM but not IgG or IgA in PBMNC cultures after TPA stimulation. This was almost certainly due to

increased tumour Id as only the tumour specific light chain was increased over control cultures. E-depleted TPA stimulated culture supernatants from these patients contained monotypic, tumour specific IgM (table 5.5). The IgM levels in TPA stimulated E-depleted cultures were significantly increased compared to TPA stimulated PBMNC cultures in patient JR and MGr but decreased in patient LG.

PBMNC cultures from the remaining patient (JSm,) were negative for IgM (the tumour isotype,) but did contain polyclonal IgG and IgA, which were increased after TPA stimulation. After E-depletion only lambda light chains were detected and were significantly increased after TPA stimulation ($p < 0.02$).

5.4.3 Group 3: Polyclonal Ig with a Single Isotype

The 4 patients in this group were all stage B or C. There were 2 IgM and 2 IgG expressing tumours. Supernatants from PBMNC cultures contained a single isotype, corresponding to the tumour, but both kappa and lambda light chains. All cultures showed increased Ig secretion after TPA stimulation (table 5.6).

E-depleted cells from 3 patients (RPa, WT, and MS,) secreted monotypic tumour specific Ig after TPA stimulation (table 5.7). In WT and MS the levels were significantly reduced compared to PBMNC cultures while in RPa they were unchanged.

No isotypes were detected in the control or TPA stimulated E-depleted culture supernatants from LA but tumour specific light chain was present and was increased after TPA stimulation.

5.4.4 Group 4: Monotypic Ig

PBMNC culture supernatants from 3 patients with IgM expressing tumours contained monotypic tumour Id after TPA stimulation (table 5.8). Control cultures from patient PM contained large amounts of tumour Id which was greatly enhanced by TPA stimulation.

There was no significant difference in Ig levels between TPA stimulated PBMNC and E-depleted cultures in patients LC and GS whereas IgM levels in the E-depleted culture from patient PM were significantly reduced compared to the PBMNC culture (table 5.9).

5.5 DISCUSSION

A previous study of TPA induced Ig secretion by PBMNC cultures from CLL patients demonstrated increased tumour specific Ig secretion in 18 out of 22 cases using an ELISA assay similar to the one used here (Gordon et al, 1984b). All the tumours expressed IgM and no cultures contained other isotypes or polyclonal Ig. Two other studies reported a similar frequency of Ig secretion by CLL cultures stimulated by phorbol esters but did not attempt to demonstrate isotype or light chain restriction in the secreted Ig (Cossman et al, 1982, and Polliack et al, 1986). Deegan and Meada (1984,) reported increased tumour specific Ig secretion in only 2 out of 9 CLLs but used a haemolytic plaque assay which may be less sensitive than the ELISA.

5.5.1 Polyclonal Ig Secretion by CLL PBMNC Cultures

The results in chapter 4 confirmed that the majority of PBMNC cultures from CLL patients secrete Ig in response to TPA stimulation. However, the finding of polyclonal Ig in most of the supernatants was unexpected. This problem had not been encountered in pilot studies but, for convenience, these had used cells from patients who attended the clinic regularly and who had high total

white cell counts and relatively low numbers of T-cells. As can be seen from the data presented this increases the probability of monotypic Ig secretion.

TPA induced Ig secretion by normal B-cells in vitro is enhanced by T-helper cells and monocytes (Sugawara, 1982,) and inhibited by T-suppressor cells (Ralph & Kishimoto, 1982). It is known that T-helper/suppressor ratios are abnormal in CLL, that absolute numbers of T-suppressor cells are increased in advanced disease (Kay et al, 1982,) and that T-helper/suppressor ratios in CLL correlate positively with IgG and IgA serum levels in vivo (Platsoucas et al, 1982). If the presence of polyclonal Ig in CLL culture supernatants is dependent on autologous T-cells then cultures from patients with early stage disease who have relatively high numbers of T-cells and high helper/suppressor ratios will be more likely to produce polyclonal Ig. Conversely, PBMNC from patients with advanced disease and high leukaemic cell counts will contain fewer normal B-cells and fewer T-cells with a lower helper/suppressor ratio and will therefore be less likely to produce polyclonal Ig in culture.

The estimation of T-cell numbers in cytospin preparations stained by immunoperoxidase may not be as accurate as suspension counts but did confirm the efficiency of

T-cell depletion with only 1 E-depleted sample containing more than 5% CD3 positive cells.

The results show that autologous T-cells can have a marked effect on Ig secretion by residual normal B-cells in PBMNC cultures from CLL patients. Cultures containing the highest numbers of T-cells (table 5.1.) were most likely to contain polyclonal Ig. However, the Ig levels in control cultures show that the secretion of polyclonal Ig is not due to TPA stimulation in these cases. Thus the absence of polyclonal Ig in the previous report (Gordon et al, 1984b.) must have been due to variation in culture conditions or in the selection of patients for study.

The culture medium used here differed from that used by Gordon et al (1984b.) in the addition of L-glutamine, sodium pyruvate and HEPES buffer. A major difference, however, could be batch variation in FCS which is used as a supply of trace elements and growth factors. The FCS used in our study had been selected for its efficiency at supporting cell growth and may have been responsible for inducing T-cell dependent Ig secretion by the small numbers of non-malignant B-cells in the cultures.

5.5.2 Light Chain Secretion by CLL Cultures

Supernatants in which no Ig isotype was detected but which were positive for light chain were assumed to contain free light chains. Intact Ig below the sensitivity of the isotype assays (16ng/ml,) would give similar results but was unlikely as the ODs for the light chains were similar to other samples containing whole Ig at levels above 16ng/ml.

In vitro production of free light chains was observed in 7 cases in which no isotype was detected and in three patients light chain production was increased after TPA stimulation. This contrasted with the experience of Gordon et al (1984,) who found the secretion of free light chains to be unaffected by TPA stimulation. However, this could be due to the lower dose of TPA (10ng/ml,) used in their study.

5.5.3 Tumour Idiotype Secretion by CLL Cultures

Eleven out of 15 E-depleted CLL cultures secreted tumour Id in response to TPA stimulation. Of the remaining cultures, 2 showed an increase in the tumour specific light chain after TPA stimulation while the other two did not secrete tumour specific Ig.

Interestingly, IgM and IgG expressing tumours had different patterns of Ig secretion. Cultures from 8 out of 10 IgM expressing CLLs secreted tumour Id in response to TPA and in 5 of these spontaneous tumour Id secretion was detected in the control cultures. In contrast none of the control cultures from IgG expressing tumours contained tumour Id and only 2 cases secreted Id after TPA stimulation.

This is consistent with the results of Gordon et al (1984b,) who found that CLLs with an early B-cell phenotype (cytoplasmic IgM in the absence of detectable light chain, or surface IgM and IgD,) were more likely to secrete tumour Id in response to TPA than cases with a more mature B-cell phenotype (diffuse cytoplasmic IgM).

E-depletion had no consistent effect on the level of tumour Id in comparison to PBMNC cultures. In 4 cases Id levels were reduced in E-depleted cultures, in 2 cases they were increased, and in 3 cases they were not significantly different.

The results show that whole Ig and free light chain secretion by CLL cultures in response to phorbol ester is heterogeneous and can also be independently affected by autologous T-cells.

TABLE 5.1

T-Cell Subsets in Pre-Culture Samples before and after E-Depletion

Pt.	PBMNC			E-Depleted Cells		
	CD3	CD4	CD8	CD3	CD4	CD8
AR	9	5	6	2	1	1
JSk	15	3	7	1	<1	1
JS	10	8	5	1	<1	<1
WC	16	10	3	4	ND	ND
JR	20	6	15	2	<1	2
JSm	8	5	2	<1	ND	ND
MGr	19	11	10	3	1	<1
LG	29	13	14	6	3	5
RPa	1	<1	3	<1	<1	<1
WT	<1	<1	<1	<1	<1	<1
MS	3	2	<1	<1	ND	ND
LA	1	2	1	<1	ND	ND
LC	8	4	3	<1	<1	<1
GS	1	<1	<1	<1	ND	ND
PM	1	<1	1	<1	ND	ND

All numbers are percentages of cells on cytospins reacting with McAb against CD3, CD4, or CD8 T-cell antigens by immunoperoxidase.
ND = Not Done
Pt = patient

TABLE 5.2

Ig Levels in PBMNC Cultures from
Group 1 Samples

Patient (Isotype)		IgG ng/ml	IgM ng/ml	IgA ng/ml	Kappa OD	Lambda OD
AR	Con	26	<16	<16	0.53	0.66
(IgMκ)	TPA	22	<16	<16	0.53	0.60
JSk	Con	36	<16	<16	0.48	0.76
(IgGκ)	TPA	37	<16	<16	0.62	0.65
JS	Con	1350	60	210	1.21	1.28
(IgGλ)	TPA	1500	72	230	1.28	1.23
WC	Con	620	50	135	1.03	1.36
(IgGκ)	TPA	490	45	100	1.04	1.36

TABLE 5.3

Ig Levels in E-Depleted Cultures from
Group 1 Samples

Patient (Isotype)		IgG ng/ml	IgM ng/ml	IgA ng/ml	Kappa OD	Lambda OD
AR	Con	<16	<16	<16	0.12	0.02
(IgMκ)	TPA	<16	<16	<16	0.15	0.02
JSk	Con	<16	<16	<16	0.42	0.07
(IgGκ)	TPA	21	17	<16	0.62	0.07
JS	Con	36	<16	<16	0.55	0.44
(IgGλ)	TPA	46	<16	<16	0.67	0.43
WC	Con	<16	<16	<16	0.17	0.19
(IgGκ)	TPA	<16	<16	<16	0.27	0.23

All results are means of duplicate wells. Isotypes quantitated from standard curves Light chains expressed in units of optical density

Con = control culture TPA = TPA stimulated culture

TABLE 5.4

Ig Levels in PBMNC Cultures from Group 2 Samples

Patient (Isotype)		IgG ng/ml	IgM ng/ml	IgA ng/ml	Kappa OD	Lambda OD
JR	Con	125	28	100	1.08	1.48
(IgMλ)	TPA	88	110	72	1.00	1.69
JSm	Con	43	<16	40	0.54	1.36
(IgMλ)	TPA	100	<16	90	0.96	1.80
MGr	Con	58	<16	<16	0.86	1.00
(IgMκ)	TPA	37	50	<16	0.97	0.89
LG	Con	17	<16	<16	0.42	0.77
(IgMλ)	TPA	18	84	<16	0.43	1.41

TABLE 5.5

Ig Levels in E-Depleted Cultures from Group 2 Samples

Patient (Isotype)		IgG ng/ml	IgM ng/ml	IgA ng/ml	Kappa OD	Lambda OD
JR	Con	<16	16	<16	0.12	0.36
(IgMλ)	TPA	<16	180	<16	0.13	1.00
JSm	Con	<16	<16	<16	0.02	0.28
(IgMλ)	TPA	<16	<16	<16	0.06	0.58
MGr	Con	<16	<16	<16	0.17	0.07
(IgMκ)	TPA	<16	84	<16	0.48	0.08
LG	Con	<16	<16	<16	0.05	0.15
(IgMλ)	TPA	<16	32	<16	0.05	0.44

All results are means of duplicate wells. Isotypes quantitated from standard curves Light chains expressed in units of optical density

Con = control culture TPA = TPA stimulated culture

TABLE 5.6

Ig Levels in PBMNC Cultures from
Group 3 Samples

Patient (Isotype)		IgG ng/ml	IgM ng/ml	IgA ng/ml	Kappa OD	Lambda OD
RP	Con	<16	16	<16	0.29	0.53
(IgMκ)	TPA	<16	275	<16	0.81	0.45
WT	Con	<16	19	<16	0.22	0.37
(IgMκ)	TPA	<16	240	<16	0.88	0.27
MS	Con	21	16	<16	0.61	0.34
(IgGκ)	TPA	100	<16	<16	1.97	0.27
LA	Con	26	<16	<16	0.38	0.42
(IgGκ)	TPA	45	<16	<16	1.10	0.57

TABLE 5.7

Ig Levels in E-Depleted Cultures from
Group 3 Samples

Patient (Isotype)		IgG ng/ml	IgM ng/ml	IgA ng/ml	Kappa OD	Lambda OD
RP	Con	<16	16	<16	0.07	0.15
(IgMκ)	TPA	<16	250	<16	0.58	0.17
WT	Con	<16	23	<16	0.13	0.01
(IgMκ)	TPA	<16	110	<16	0.53	0.03
MS	Con	<16	<16	<16	0.30	0.01
(IgGκ)	TPA	46	<16	<16	1.64	0.06
LA	Con	<16	<16	<16	0.34	0.11
(IgGκ)	TPA	<16	<16	<16	0.72	0.14

All results are means of duplicate wells. Isotypes quantitated from standard curves Light chains expressed in units of optical density

Con = control culture TPA = TPA stimulated culture

TABLE 5.8

Ig Levels in PBMNC Cultures from
Group 4 Samples

Patient		IgG ng/ml	IgM ng/ml	IgA ng/ml	Kappa OD	Lambda OD
LC	Con	<16	<16	<16	0.21	0.29
(IgMλ)	TPA	<16	56	<16	0.07	0.50
GS	Con	<16	23	<16	0.04	0.28
(IgMλ)	TPA	<16	1300	<16	0.08	>2
PM	Con	<16	700	<16	1.30	0.11
(IgMκ)	TPA	<16	8000	<16	>2	0.10

TABLE 5.9

Ig Levels in E-Depleted Cultures from
Group 4 Samples

Patient (Isotype)		IgG ng/ml	IgM ng/ml	IgA ng/ml	Kappa OD	Lambda OD
LC	Con	<16	<16	<16	0.08	0.23
(IgMλ)	TPA	<16	41	<16	0.05	0.38
GS	Con	<16	84	<16	0.02	0.57
(IgMλ)	TPA	<16	1100	<16	0.02	>2
PM	Con	<16	720	<16	1.17	0.04
(IgMκ)	TPA	<16	2900	<16	>2	0.03

All results are means of duplicate wells. Isotypes quantitated from standard curves and light chains expressed in units of optical density.

Con = control culture
TPA = TPA stimulated culture

CHAPTER 6

GENERAL DISCUSSION

This study has shown that it is possible to identify shared idiotopes in CLL using a panel of V region reactive monoclonal antibodies produced against a randomly selected paraprotein.

Immunocytochemical studies had to be interpreted with care because of cross-reactions between the JS McAbs and epitopes expressed by molecules other than Ig. Such cross-reactions are not surprising in view of idiotypic diversity but they do emphasise the importance of extensive characterisation of such antibodies.

Weak cross-reactions may not be of practical significance in vivo as strong cross-linking of surface receptor is generally required for the recruitment of cytotoxic effectors (Hamblin et al, 1987).

6.1 Expression of Shared Idiotopes in CLL

The division, by ELISA, of the V region associated epitopes defined by the JS McAb panel into 3 groups recognisable in normal Ig could be exploited to select potentially useful antibodies for immunotherapy of B-cell tumours.

Antibodies against private idiotopes, conventionally used for anti-Id therapy (Thielemans et al, 1984,) were shown not to react significantly with either serum Ig or CLL tumour Ids.

Antibodies against the public epitopes reacted with 14-29% of CLLs but are not attractive as therapeutic agents because of the blocking potential of normal serum. The expression of the public idiotopes by polyclonal Ig in supernatants from CLL cultures containing less than 1% normal B-cells confirmed that they were common to many different clones.

Antibodies against restricted public epitopes have far greater potential as therapeutic agents because the blocking effects of normal serum are much lower. For instance, the binding of normal IgG at 10µg/ml to antibodies 47.7B3, 44.2A1, and 2H3.D6 is less than the binding of JS paraprotein at 10ng/ml in the ELISA assay

(chapter 3). With a serum Ig concentration up to 20mg/ml the potential serum blocking activity would be no greater than 20µg/ml, well below the 30µg/ml suggested as a cut-off for choosing cross-reacting anti-Ids for therapy (Stevenson et al, 1986).

In spite of similar binding to normal serum Ig the 3 antibodies recognising restricted public idiotopes differ in their reactivities with the CLLs. The lambda specific antibody 47.7B3 reacts with 1 (5%) out of 21 lambda expressing tumours and the heavy chain specific antibodies 44.2A1 and 2H3.D6 react with 3 (7%) and 6 (14%) out of 42 cases respectively.

The level of expression of these restricted public idiotopes in CLL is sufficient to make clinical trials and, ultimately, routine therapy with pre-formed antibodies a practical proposition.

6.2 Preferential Expression of Idiotope defined by Antibody 2H3.D6 in CLL

In the original characterisation of the JS McAbs, 47.7B3 reacted with 2/72 (3%) lambda positive paraproteins while 44.2A1 and 2H3.D6 each reacted with 3/159 (2%) paraproteins (Walker et al, 1987). The difference between

McAb reactivity with the paraprotein panel and the CLL group was compared using the chi-squared test with the Yate's correction factor. The only antibody to show a significant difference was 2H3.D6 which reacted more frequently with the CLLs ($p < 0.01$).

This preferential expression of an idiotope in CLL compared to the B-cell tumours represented by the paraprotein panel (mostly multiple myeloma and Waldenstrõm's Macroglobulinaemia,) could be due to a number of reasons:-

Firstly, the high incidence of IgM positive CLLs (31/42 cases,) could affect idiotype expression as somatic mutation in B-cells appears to be associated with class switching (Allen et al, 1987,) and, consequently, IgM shows less idiotypic variation than other isotypes (Bothwell et al, 1981 and, Gearhart et al, 1981). However, the paraprotein panel used in the characterisation of the JS McAbs included 36 IgM paraproteins only one of which reacted with either 44.2A1 or 2H3.D6.

Secondly, CLL could be derived from a specific set of B-cells with a limited idiotypic repertoire. Infrequent cells in normal human lymph nodes and tonsils have a phenotype similar to B-CLL cells (Caligaris-Cappio et al,

1982). However, little is known about the relationship of such cells to normal B-cell development or to CLL.

Thirdly, the preferentially expressed idiotope could be the product of a specific V region gene rearrangement associated with CLL.

A new family of V_H genes (V_H(V)), located close to the D_H-J_H complex, have recently been described and were rearranged in 9 out of 33 cases of CLL and 6 out of 16 cases of acute lymphoblastic leukaemia but not in 38 IgM expressing Epstein-Barr Virus transformed human B-cell lines (Shen et al, 1987, and Humphries et al, 1988).

Evidence from murine studies suggests that the V_H genes nearest to the D_H-J_H complex are preferentially rearranged in pre-B cells (Rathbun et al, 1987). If the idiotope recognised by antibody 2H3.D6 is a product of this gene family, its expression in CLL may be due to the preferential use of the V_H(V) genes by B-cells frozen at a specific stage of differentiation. The expression of the idiotope in other classes of B-cell tumours as well as genetic studies of those tumours reacting with 2H3.D6 would help clarify this.

Finally, the preferential expression of this idiotope could be due to an immunoregulatory defect commonly associated with CLL. For instance, an abnormality of idiotypic network regulation or an abnormal regulatory T-cell clone may have driven B-cell clones expressing a specific regulatory idiotope to autonomous proliferation. Tumours such as myeloma, which do not express sIg, would not arise from such a mechanism and would therefore be less likely to express the idiotope.

The reason(s) for the preferential expression of the idiotope defined by 2H3.D6 in CLL compared to myeloma and Waldenström's Macroglobulinaemia must remain a matter for speculation at present.

In vitro studies comparing the effects of 2H3.D6 with other JS McAbs on CLL cells expressing the relevant idiotopes could demonstrate whether the preferentially expressed idiotope has a particular role in cellular regulation in this disease and would therefore be an ideal target for anti-Id therapy.

6.3 Factors Influencing the Outcome of Anti-Id Therapy of Human B-Cell Tumours

Experience has shown that it is possible to produce polyclonal and monoclonal anti-Ids which are highly specific for individual neoplastic B-lymphocyte clones and which are capable of mediating tumour cell destruction in vivo and in vitro. In spite of this the results of anti-Id therapy of human B-cell malignancies have been disappointing. The failure to produce a sustained, beneficial response in all but one of the patient's treated so far has been attributed to a number of mechanisms (table 6.1) which are discussed below:

TABLE 6.1

Factors Influencing the Outcome of Anti-Id Therapy

Factors Influencing the Outcome of Anti-Id Therapy
Dose of anti-Id
Extracellular idiotype
Inaccessability of malignant cells
Reduced surface Ig density
Lack of effector cells
Host anti-mouse Ig immune response
Antigenic Modulation
Idiotype variant tumour cell populations

6.3.1 Dose of Anti-Id

The purity and unlimited supply of monoclonal antibodies allow the administration of large quantities of therapeutic anti-Id with a minimum of side effects. In the Stanford series patients received total doses of 400 - 3,183mg of anti-Id over 7 - 57 days (Meeker et al, 1985). Although there was no relationship between anti-Id dose and a beneficial therapeutic response, no anti-tumour effects were seen unless free serum anti-Id could be detected.

Similar regimes have been used by others with up to 10g of monoclonal anti-Id given to one patient over 17 days (Capel et al, 1985, and Rankin et al, 1985).

Patients treated with polyclonal anti-Id have not received such prolonged courses but have received up to 2g of purified anti-Id in single intravenous infusions (Gordon et al, 1984a).

6.3.2 Extracellular Idiotype

Serum Ig reacting with infused anti-Id could prevent the therapeutic antibody from binding to the tumour cells. In addition, Id/anti-Id immune complexes might have toxic side effects and the clearance of such complexes could significantly reduce the number of phagocytic cells available for tumour cell destruction.

Binding to normal serum Ig is not a problem with anti-Ids against private tumour determinants such as have been used in therapeutic trials so far. However, many B-cell tumours which are not commonly associated with serum paraprotein bands by conventional electrophoresis have been shown to secrete significant amounts of tumour Id by more sensitive techniques such as ELISA assays (Meeker et al, 1985,) radio-immunoassays (Stevenson et al, 1980,) and iso-electric focussing (Sinclair et al, 1984).

In the Stanford series the serum Id levels prior to therapy ranged from 0 to 400µg/ml. Significant side effects were only seen in patients with serum Id levels > 1µg/ml.

The blocking effect of low levels of serum Id can probably be overcome by increasing the dose of anti-Id in the majority of patients. Higher serum Id levels can be

reduced by plasmapheresis prior to immunotherapy (Meeker et al, 1985,) or by reduction of tumour mass with conventional chemotherapy (Brown et al, 1980).

6.3.3 Inaccessability of Malignant Cells

Assuming successful anti-Id therapy requires antibody binding to all tumour cells the infused antibody must be able to penetrate all tissues containing malignant cells.

In the Stanford series 6 out of 8 patients who had biopsies of lymph nodes or marrow within 24 hours of anti-Id therapy showed murine Ig bound to tumour cells indicating successful penetration by anti-Id. One patient with a malignant pleural effusion also had detectable anti-Id levels in the pleural fluid within 24 hours (Meeker et al, 1985).

Rankin et al (1985,) demonstrated that a 10mg infusion of monoclonal anti-Id was sufficient to coat circulating lymphoma cells in 2 patients with NHL but an intravenous bolus of 150 - 300mg followed by a low dose infusion was required to saturate tumour cells in lymph nodes and marrow.

6.3.4 Surface Ig Density

CLL cells are unusual in having a much lower surface Ig density than normal lymphocytes (Ternynck et al, 1974,) and most other Ig expressing B-cell tumours (Mason et al, 1980). As a consequence CLL cells may be more resistant to complement mediated cytotoxicity for which the number of cell membrane receptor molecules is critical (Gordon et al, 1982). However, sIg density is not important for effective ADCC in vitro (Halloran and Stylianos, 1980,) so the consequences for anti-Id therapy of the reduced sIg in CLL may depend on the mechanism of action in vivo.

6.3.5 Lack of Effector Cells

Where anti-Id therapy is dependent on mechanisms such as complement or ADCC the number of tumour cells may exceed the capacity for cell lysis. Protection from tumour challenge in a number of animal models of immunotherapy was only seen when the tumour burden was low (reviewed by Ritz and Schlossman, 1982). As a consequence it has been

suggested that anti-Id therapy of humans should be preceded by conventional cytotoxic therapy to reduce the tumour mass. However, effective anti-Id therapy is possible even when disease is advanced and widespread (Miller et al, 1982).

6.3.6 Host Anti-Mouse Ig Response

In the Stanford series the repeated administration of murine anti-Id was associated with the production of host anti-mouse antibodies in 5 out of 11 patients (Meeker et al, 1985). These cases had an increased incidence of side effects and no further therapeutic benefit after developing the anti-mouse Ig response which was therefore considered a reason for terminating anti-Id therapy.

6.3.7 Antigenic Modulation

Originally described as cellular resistance to the cytotoxic action of complement plus specific antibody due to prior exposure to antibody (Boyse and Old, 1969,) this phenomenon is due to redistribution, often followed by endocytosis, of the surface receptor after binding by antibody (Gordon and Stevenson, 1981). The process is

rapid and reversible with affected cells re-expressing the surface receptor 24 hours after removal of the antibody.

Modulation was shown to be the mechanism of tumour escape in treating acute lymphoblastic leukaemia with antibodies against a differentiation antigen expressed by the malignant cells (Ritz et al, 1981). It has also been shown to occur in vivo after anti-Id therapy with polyclonal (Gordon et al, 1984a,) and monoclonal (Capel et al, 1985,) antibodies.

Since cross-linking of surface Ig by bivalent antibodies is necessary for modulation (Gordon & Stevenson, 1981,) the possibility of using univalent anti-Ids as therapeutic antibodies has been explored by Stevenson's group. Modulation of L_2C guinea pig leukaemia cell sIg in vitro did not occur after exposure to univalent derivatives of polyclonal anti-Id which still effectively mediated both complement and ADCC lysis (Glennie and Stevenson, 1982, and Stevenson et al, 1982).

Stevenson et al (1985,) described the preparation of a hybrid molecule in which an Fab' from polyclonal anti-Id was coupled via thioether bonds to normal IgG. This has the advantages of anti-idiotypic specificity and univalency, combined with a human Fc receptor which is

likely to be the most efficient at complement lysis or ADCC in therapy.

A similar molecule derived from monoclonal anti-Id and human IgG has been used to treat a human patient with CLL (Hamblin et al, 1987). There was no evidence of modulation and after 4 infusions the patient had achieved a partial remission.

6.3.8 Idiotype Variant Tumour Cell Populations

Using panels of monoclonal anti-Ids each produced against a specific tumour Carroll et al (1986,) examined biopsies from 2 patients with follicular lymphomas by indirect immunoperoxidase. They showed that the number of tumour cells reacting with individual anti-Ids varied both at different biopsy sites and over time.

The Stanford group have further investigated this phenonemon using hybridoma technology to produce a series of monoclonal tumour specific Ids each derived from a single tumour cell from individual patients. The amino acid sequence of individual tumour Id V regions can be determined and related to differences in idiotope expression (reviewed in Levy et al, 1987).

Their results show that spontaneous somatic mutation of Ig V region genes in B-cell tumours results in altered idiotype expression so that phenotypically homogeneous tumours may include a number of idiotypically distinct sub-clones. Immunoglobulin gene rearrangement studies in these and other cases (Raffeld et al, 1985,) confirmed that the idiotype variant cell populations were derived from the same original clone.

Biopsy from one patient whose tumour had become resistant to anti-Id therapy contained tumour cells which expressed surface Ig but were negative with the therapeutic anti-Id. The altered idiotype was found to be due to a single point mutation in the heavy chain second hypervariable region (Cleary et al, 1986). Altogether 4 patients in the Stanford series who had temporary partial responses to anti-Id therapy had a surface Ig positive, Id negative phenotype after treatment (Miller et al, 1987).

Similar studies in the one patient who achieved a complete remission demonstrated that somatic mutation occurred in the V_H genes as frequently as in the other tumours but the anti-Id used in therapy still reacted with all 6 tumour cell lines established suggesting that the idiotope recognised was conserved by selective forces

within the host (Kon et al, 1987).

Thus anti-Id therapy with an antibody which does not characterise the entire malignant clone may simply result in a selective advantage for sub-clones which do not express the idiotope concerned.

6.4 Future Progress

Clinical trials have provided useful information about the pharmacokinetics and potential toxicity of infused anti-Id and have highlighted some of the reasons for failure of therapy as well as the possible benefits. The existance of idiotype variant tumour cell populations both spontaneously and after therapy with anti-Id has emerged as an important mechanism by which tumours can escape the effects of monoclonal antibody therapy.

In addition, there are at least 2 fundamental problems which, in the opinion of this author, are crucial to improving the results of anti-Id therapy;

Firstly, the need to produce individual anti-Ids for each patient to be treated has limited the number of patients involved in trials and could also limit the practical application of this therapy.

Secondly, lack of understanding both about the biology of B-cell tumours and the mechanisms of effective anti-Id therapy means there is no logical basis for selecting the patients to be treated or the most appropriate therapeutic antibodies.

Evidence from animal models suggests that the induction of idiotype-specific regulatory T-cells is the likeliest mechanism by which idiotypic manipulation could result in the long term control of a malignant B-cell clone. It is not clear whether this also applies in humans nor whether the administration of antibodies reacting with tumour Id is the most effective method of inducing such a response.

In vitro studies using cells from human B-cell tumours or B-cell lines could be used to investigate the effects of anti-Ids on cellular proliferation and metabolism. For instance, it may be possible to define tumour associated idiotopes which are susceptible to T-cell regulation. However, a fundamental limitation of such studies is that they cannot mimic or predict the effects of anti-Id administered in vivo.

Human clinical trials have to be the ultimate test of the efficacy of anti-Id therapy. However, the treatment of small numbers of patients with randomly generated

anti-Ids against private determinants is unlikely to provide sufficient information to be able to identify either the patients most likely to respond to therapy or the most appropriate anti-Ids to use.

This study has demonstrated that it is possible to define idiotopes which are not strongly expressed in normal serum Ig but which recur at reasonable frequencies in CLL. In addition, there is evidence that idiotopes can be preferentially expressed by different classes of B-cell tumours. These results have important implications for anti-Id therapy:

i. Antibodies against shared idiotopes which are also regulatory idiotopes (Bona et al, 1987,) may be more effective than conventional therapeutic anti-Ids at inducing a host immunoregulatory response capable of long term control of the malignant B-cell clone.

ii. Idiotopes which are conserved by the immune system may be less prone to somatic mutation than private idiotopes and would, therfore, be more likely to characterise the entire malignant clone.

iii. Pre-formed antibodies against shared idiotopes expressed by B-cell tumours would make this

therapy more practical. Larger numbers of patients could be treated with standardised antibodies allowing a more effective analysis of the factors which determine the outcome of therapy.

The extension of this work to include other classes of B-cell tumours and the generation of other V region reactive antibody panels will allow larger clinical trials to be established. In addition, antibodies against idiotopes which are preferentially expressed by single classes of B-cell tumour may prove to be the ideal therapeutic agents and may be powerful tools for investigating the biology of these diseases in vitro.

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