Characterisation of antibody populations in renal dialysis patients: their role in renal transplant outcome

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Characterisation of Antibody Populations in Renal Dialysis Patients: Their role in renal transplant outcome

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Dedication

This thesis is dedicated to my wife, Caroline Taylor, without whose support and encouragement it would not have been possible.

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Abbreviations used

Ab	antibody
AHG	anti-human-globulin
ASHI	American Society of Histocompatibility and Immunogenetics
ATG	anti-thymocyte Globulin
ATN	acute tubular necrosis
Btf	blood transfusion
BSA	bovine serum albumin
CDC	complement dependant cytotoxicity
cd	cluster of differentiation
CLL	chronic lymphatic leukaemia
CMV	cytomegalovirus
CPM	counts per minute
CTS	collaborative transplant study
CyA	cyclosporine A
DMSO	dimethyl-sulfoxide
DST	donor specific transfusions
DTT	dithiothreitol
EBV	Epstein Barr virus
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
FITC	fluorescein-isothiocyanate
GLB	Gidion Golstein lysis buffer
HAT	hypoxanthine aminopterin thymidine
HLA	Human Leucocyte Antigens
HSA	human serum albumin
IAA	iodo-acetamide
iv	intravenous
kD	kilo Daltons
LCL	lymphoblastoid cell line
lps	lipopolysaccharide
LRD	live related donor
MAb	monoclonal antibody
MCF	mean channel fluorescence
MES	(2[N-Morpholino]ethane-sulphonic acid
MHC	major histocompatibility complex

MLR	mixed lymphocyte reaction
NIMA	non-inherited maternal antigen
OKT3	mouse anti-human T cell monoclonal antibody
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PHA	phytohaemagglutinin
PMSF	phenylmethylsulphonyl fluoride
PRA	panel reactive antibodies
ptc	phosphatidylcholine
RA	rheumatoid arthritis
RBC	red blood cells
SDS	sodiumdodecyl sulphate
SEM	standard error of the mean
SLE	systemic lupus erythematosus
UCLA	University of California Los Angeles
UKTS	United Kingdom Transplant Service

Characterisation of Antibody Populations in Renal Dialysis Patients: Their role in renal transplant outcome

C.J.Taylor

The relevance of a positive crossmatch in renal transplantation is controversial. Factors which may influence the relationship between recipient antibody sensitization and transplant outcome include; the antibody class and specificity, the time interval between the positive crossmatch and transplantation and whether the transplant is a first graft or a regraft.

In highly sensitized patients the clinical relevance of a positive crossmatch against a particular donor is difficult to determine because it may be due to damaging (presumed anti-HLA) and/or non-damaging (presumed non-HLA) antibodies. An in-vitro assay has been developed which can reliably define the specificity of donor reactive antibodies even in complex mixtures. This technique was applied to define the immunoglobulin class and specificity of antibodies present in a series of positive crossmatch transplants. The clinical relevance of sensitization to HLA class I, DR, DQ and non-HLA was examined and a correlation sought with graft survival. The results showed good primary and regraft survival in the presence of peak positive and current positive crossmatches caused by IgM non-HLA antibodies. There was acceptable primary and regraft survival with peak positive-current negative crossmatches due to IgM HLA class I, but not with IgG antibodies. A positive B cell crossmatch caused by HLA antibodies was associated with good primary but poor regraft survival.

These findings may be applied prospectively to select kidney donor and recipient pairs who, despite a positive crossmatch, can be transplanted with a high probability of success.

The target molecule of the commonest non-HLA antibody has remained a mystery for over 15 years. This question was investigated by the production and characterisation of human monoclonal antibodies from a renal dialysis patient by the generation of a mouse/human heterohybridoma. The resulting antibodies are of the IgM class with reaction profiles identical to those found in renal dialysis patients. Screening against panels of cells demonstrated that identical reactivity patterns could be generated at a different dilution for each MAb. This implies that the apparently different specificities are explained by differential target cell sensitivity. Reactivity profiles in fluorescence binding assays showed that this target cell sensitivity is dictated not by antigen density alone but also by antibody/antigen affinity. The results from enzyme treatment of target cells and from lectin inhibition studies show that the antibodies are polyreactive and capable of binding sialic acid dependent epitopes and other negatively charged cell surface molecules.

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Renal transplantation is a well established method for the treatment of end stage renal failure. However the most common cause of graft failure remains immunological rejection, particularly within the first three months after transplantation. The immune mechanisms involved in renal allograft rejection are both humoral and cellular.

1.1 The crossmatch test

In the mid 1960's, donor reactive antibodies in kidney transplant recipients were associated with a high chance of early graft failure. This so called 'positive crossmatch' was defined by the ability of recipient antibodies to agglutinate donor leucocytes. Later, the complement mediated cytotoxicity test was introduced, in which donor specific antibody was identified through lysis of donor lymphocytes by recipient serum in the presence of rabbit complement. Preformed antibody, present at the time of transplantation was found to mediate 'hyperacute rejection' which may occur within the first 24 hours after revascularization of the graft.

The first report of hyperacute rejection associated with preformed recipient antibodies reactive with donor lymphocytes came from Terasaki in 1965 (Terasaki, Marchioro and Starzl, 1965). They noted that a kidney transplanted from a brother to a sister suffered immediate failure. Cytotoxic antibodies were demonstrated against donor lymphocytes and these were thought to be elicited as a result of pre-transplant blood transfusions. They suggested the role of a crossmatch test, in which serum from the prospective recipient is tested with the donor cells before transplantation, may be important in preventing some early rejections.

In 1966, Kissmeyer-Nielson and his co-workers (Kissmeyer-Nielsen et al,

1966) also reported the existence of humoral factors associated with rapid rejection. In a series of 21 consecutive human kidney allografts they reported two instances of hyperacute rejection associated with pre-existing humoral antibodies. Both recipients had received multiple blood transfusions (21 and 17 respectively) and were multigravida (5 and 3 pregnancies respectively). The kidneys were rejected during the operation, with urine flow for the first 10 minutes but complete cessation after one hour. The transplants were described as loosing tone, becoming soft and flabby with a mottled appearance. The transplanted kidneys were removed on days 14 and 3 and both patients died shortly after.

Histological studies revealed total cortical necrosis and thrombi in glomeruli and arterioles and the renal artery. There was an absence of interstitial cells infiltrating the graft, a characteristic of cellular rejection, however substantial numbers of granulocytes were found in the outer cortex.

The possibility of a reaction with donor blood carried in the kidney was excluded as the kidneys were perfused before transplantation. Both patients were shown to have pregraft leucocyte antibodies which agglutinated donor cells to titres of 1/512. In addition IgG and IgM were eluted from kidney extracts following separation using diethylaminoethyl-cellulose chromatography. They compared the mechanism of graft destruction to the Schwartzmann reaction in which antibody/antigen complexes are deposited in the vessels, causing complement activation and polymorphonuclear leucocyte infiltration leading to clotting and haemorrhage.

In 1968, Terasaki reported seven cases of hyperacute rejection in a series

of 218 kidney transplants (Terasaki, Thrasher and Hauber, 1968). Four of these occurred using living related donors. Serum samples taken before transplantation revealed that all seven recipients were sensitized when tested against a panel of 10-20 random blood donors and all five patients tested had a positive lymphocytotoxic crossmatch against their kidney donor. Six patients were female with previous pregnancies and were shown to have cytotoxic antibodies against their husbands lymphocytes. Using the limited tissue typing available at that time, several of the husbands HLA incompatibilities were also shared with their kidney donors. The seventh recipient was a multiply transfused male.

Cytotoxicity screening of the 218 recipients against a panel of cells showed that 23% of males and 46% of females had lymphocytotoxic antibodies. They identified blood transfusions as the priming agent for allostimulation, and the increased risk of sensitization in females was associated with previous pregnancies. They proposed that the cells contained in the blood transfusion, or that leaked across the placental barrier into the maternal blood stream during pregnancy, shared antigens expressed on the kidney.

A further report by Williams and colleagues (Williams et al, 1968) presented a study of 132 renal transplants in which biopsies had been taken within one hour after revascularization. Histological evidence of hyperacute rejection was found in seven patients, of which only one functioned beyond one week. This included one out of 111 first grafts and five of 20 regrafts. One patient was transplanted across a blood group incompatibility (blood group B into group O).

The histological features of hyperacute rejection were characterized by a marked degree of polymorphonuclear leucocytes accumulating in capillaries of glomeruli and peritubular areas. Day three biopsies showed focal vascular thrombosis and tubular cell necrosis with extensive fibrin deposition and cortical necrosis. Immunohistology of cryostat sections revealed IgG deposited on capillaries of the glomeruli and tubules.

Laboratory analysis of these leucocyte antibodies demonstrated that all the patients were sensitized to a panel of random blood donors and that two of four patients tested by cytotoxicity against their transplant donors were positive. Agglutination tests, performed against a panel of selected cell lines were positive and antibody binding to graft tissue was also demonstrated in all cases. In two cases the antibody was shown to disappear 24 hours after surgery, but high titres were found 5 days post nephrectomy, implying that the antibodies were binding to the graft in-situ.

The histological features of early acute rejection were characterised by Kincaid-Smith (Kincaid-Smith et al, 1969) in a study of 180 biopsies taken from 58 cadaveric transplants. They confirmed the involvement of antibody and complement components deposited within the graft, followed by a massive influx of polymorphonuclear leucocytes and platelet activation. This results in interstitial haemorrhage, vascular changes and thrombosis. In addition, the appearance of more than two polymorphs per glomeruli was associated with repeated rejection episodes indicating the involvement of antibody in acute rejection. They postulated that if the presence of polymorphs reflected an immediate antigen-antibody reaction and subsequent endothelial cell damage, they may be failing to demonstrate donor reactive antibodies in some cases.

Finally, the interpretation of a positive crossmatch as an absolute contra-indication to transplantation was established following a paper by Patel and Terasaki (Patel and Terasaki, 1969). Their concluding remarks were that "the ethics of transplanting kidneys without the prior knowledge of the crossmatch test, or across a known positive crossmatch result can reasonably be expected to be questioned in the face of this evidence". This statement clearly established a mandate to perform a prospective crossmatch. In a study of pretransplant sera taken from 226 recipients, 43% of sensitized patients (defined by cytotoxicity testing against a panel of lymphocytes derived from 10-40 blood donors) failed immediately. This compared to only 2% of patients without pregraft antibodies. Furthermore, 24 of 30 (80%) grafts with a direct positive crossmatch with donor lymphocytes failed immediately. They ascribed the 20% of successful positive crossmatch transplants to be due to false positive reactions. Technical reasons such as poor cell viability and the presence of granulocytes in the cytotoxicity test were thought to be responsible.

On analyzing serum samples from 681 potential recipients they identified a number of risk factors associated with the formation of allogenic antibodies and a subsequent predisposition to early graft failure. Positive crossmatches were three times greater with unrelated donors than related donors. Immediate failure was found with 23% of first grafts performed in women with previous pregnancies, compared to only 6% of nulliparous females and 6% in males. Moreover, 40% of regrafts failed immediately compared to only 6% of first transplants.

As a consequence of these findings the dogma arose that all pretransplant

donor reactive lymphocytotoxic antibodies were a barrier, precluding kidney transplantation. The policy of ensuring a negative lymphocytotoxic crossmatch became a prerequisite of transplantation.

1.2 HLA system

The main antigenic stimulus for kidney allograft rejection and antibody production is the MHC (Major Histocompatibility Complex). In humans this is known as the HLA (Human Leucocyte Antigen) system. Evidence that HLA is the human MHC came from early transplants preformed using related donors. Graft survival was shown to correlate with the number of HLA haplotypes shared between the kidney donor and recipient. Two haplotype matched transplants had a 90% one year graft survival compared to 70% and 60% for one and zero haplotype matched transplants respectively. The 10% of transplant failures between HLA identical siblings, despite immunosuppression, is thought to be due to multiple minor histocompatibility antigen differences. In addition, antibodies specific for antigens of the HLA system have been eluted from rejected kidneys after nephrectomy.

The HLA system consists of ten loci coded for on the short arm of chromosome six. These loci are divided into two classes of antigens based on biochemical and functional differences (Figure 1.2.1).

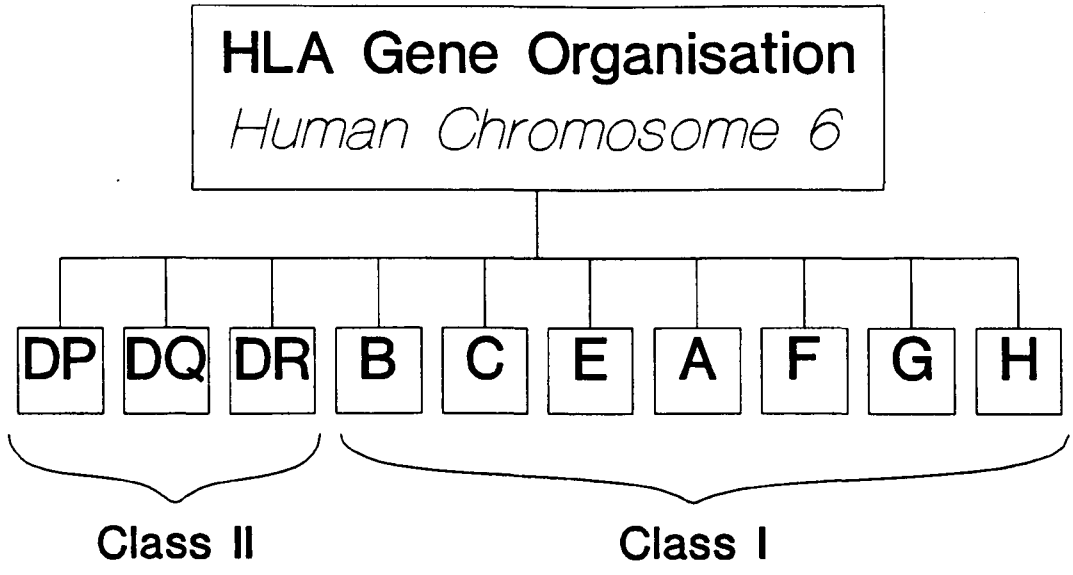

Figure 1.2.1

'Classical' HLA class I antigens, (HLA-A,-B,and -C) are expressed on all nucleated cells of the body, platelets and sperm (Halim, Wong and Mittal, 1982). They are made up of a highly polymorphic heavy chain (approximately 44 kD molecular weight) consisting of a glycoprotein with three extra-cellular immunoglobulin like domains, stabilised by disulphide bonds, a hydrophobic transmembrane region and an intra-cellular domain. This is non-covalently associated with the non-polymorphic molecule, beta-2-microglobulin (12 kD) coded for on chromosome 15. Beta-2-microglobulin is required for stable expression of HLA class I on the cell surface (Zijstra et al, 1990). HLA class I molecules are recognised by CD8 positive T cells and are associated with presentation of endogenous peptides to cytotoxic T cells (Guagliardi et al, 1990; Nuchtern, Beddison and Klausner, 1990). The 'non-classical' loci (HLA-E,-F,-G and -H) have been recently defined and there is little known about their expression and physiological role.

The major HLA class II loci comprise of HLA-DR,-DQ and -DP. The organisation of the genes encoding the HLA class II loci are shown in Figure 1.2.2.

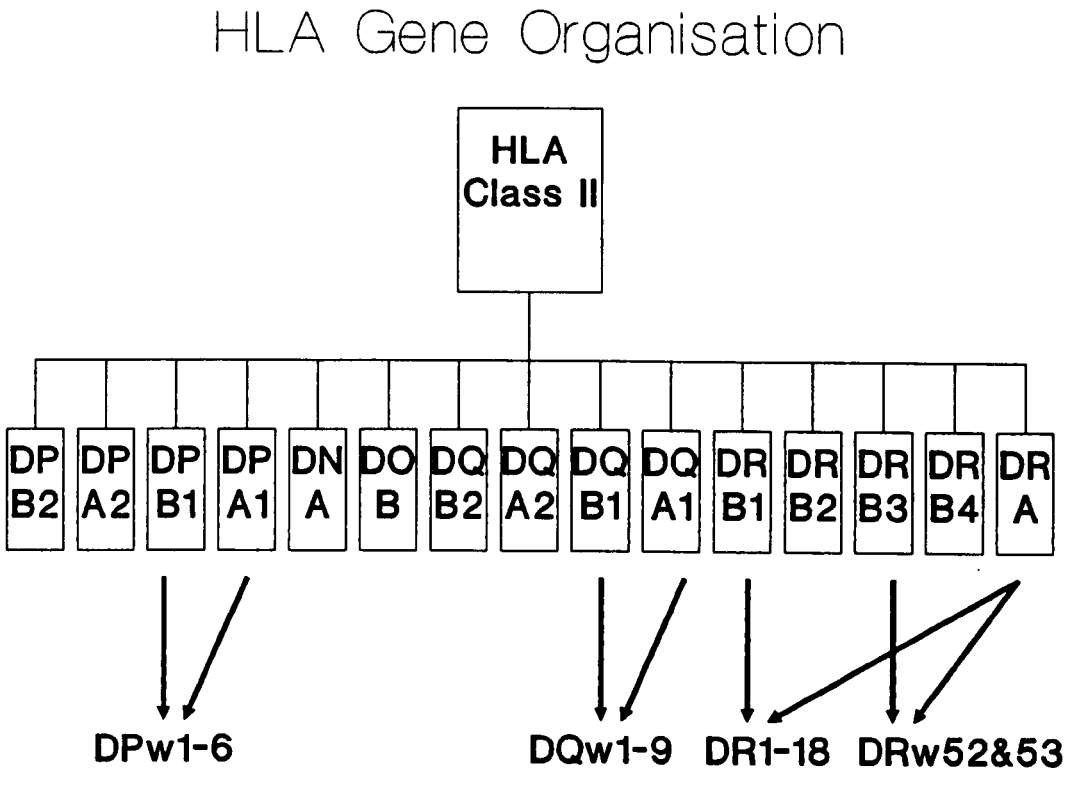

Figure 1.2.2

They are non-covalently linked heterodimers consisting of an alpha chain (34 kD) and a beta chain (28 kD). Both chains have two extracellular domains, a transmembrane region and a cytoplasmic tail. HLA class II molecules are expressed primarily on cells of the immune system such as B lymphocytes, monocytes, macrophages, dendritic cells and activated T cells. However expression is induced on most cell types in the presence of cytokines such as gamma interferon and interleukin 2.

The HLA-DR alpha chain (encoded by HLA-DRA) is non-polymorphic and can associate with either the beta chain (HLA-DRB1) encoding HLA-DR1 to 18, or with HLA-DRB3 encoding HLA-DRw52, or HLA-DRB4 encoding HLA-DRw53. HLA-DRB2 is a non-coding pseudogene. More recently an additional HLA-DRB5 gene has been identified on the HLA-DR2 haplotype (not shown). For the HLA-DQ locus, both the alpha and beta chains (encoded by HLA-DQA1 and HLA-DQB1 respectively) are polymorphic. These code for the alleles HLA-DQ1 to 9. As both genes are polymorphic the possibility of cis and trans associations exist, therefore generating new hybrid molecules in heterozygous individuals. HLA-DQA2, DQB2, DOB and DNA are thought to be non-coding genes which are not known to be expressed at the cell surface. HLA-DPA1, encoding the DP alpha chain has limited polymorphism. Its product associates with the DP beta chain, encoded by HLA-DPB1, which is a glycoprotein carrying the HLA-DP alleles DPw1 to 26. HLA-DPA2 and B2 are pseudogenes which are not transcribed and translated.

HLA class II molecules are recognised by CD4 positive T cells and are thought to be involved in presenting exogenous peptides to helper T cells (Mellins et al, 1990; Nuchtern, Beddison and Klausner, 1990).

1.3 Blood transfusion effect

Immunological sensitization of patients awaiting transplantation may be caused through three main routes: previous pregnancies; blood transfusion; and most commonly, rejection of a previous transplant (Opelz et al, 1981). In the early 1970's, studies on the degree of sensitization, defined by the frequency of antibodies reactive to lymphocytes derived from a random cell panel, correlated with poor graft survival. Also there was a lower chance of finding a suitable negative crossmatch donor for sensitized recipients.

As a consequence of this, to avoid unnecessary sensitization, the policy of avoiding blood transfusion except when clinically essential was adopted. However, during this period, despite improved surgical techniques (shorter warm ischaemia times etc) and improved immunosuppressive regimens, most transplant centres noted a decline in graft survival. Surprisingly, superior graft survival was seen in previously transfused patients.

The beneficial effect of pregraft blood transfusion on graft survival was first reported by Opelz (Opelz et al, 1973), and subsequently confirmed by many others (Chapman, 1990; Opelz et al, 1979). This almost universal finding, of about a 20% increase in graft survival in transfused patients (Opelz, 1988b) led to the policy of ensuring that all potential recipients were transfused prior to transplantation. The benefits of this outweighed the deleterious effects of sensitization and the associated dangers of disease transmission such as hepatitis and human immunodeficiency virus.

The UCLA transplant registry and Collaborative Transplant Study (CTS) data indicated that up to five units of blood was associated with a 20% improvement in one year graft outcome. Greater than five units did not

consistently produce additional benefit and greater than 20 transfusions were associated with poorer graft survival. Enhanced graft survival was only demonstrable when using fresh whole blood or buffy coats. Frozen and stored blood failed to produce the desired effect. The requirement for viable leucocytes seems to be an important factor for improved graft survival (Chapman, 1990).

Animal models showed that platelets, which only express class I molecules, may also produce enhanced graft survival without the potential deleterious effect of sensitization (Borleffs et al, 1983). However, human trials (Chapman et al, 1986b) showed that very low levels of leucocyte contamination ($15x10^6$) were associated with humoral sensitization (42% panel reactive antibodies) and that transfusion with 'pure' platelets (containing $<5x10^6$ leucocytes) failed to improve graft outcome, with only a 44% one year graft survival. These results indicated that the use of platelet transfusions has not fulfilled its original promise.

In contrast, Betuel (Betuel et al, 1985) studied 19 male patients transfused with only pure platelets, of whom 10 were subsequently transplanted. None of the patients were sensitized following transfusion, and one year graft survival of 79% was equivalent to control patients receiving blood transfusions (77% at one year). However the authors noted a higher incidence of rejection episodes in the platelet transfusion group which may become relevant with a longer follow up. Therefore the efficacy of platelet transfusion to improve kidney allograft survival is still open to debate.

More recently, the immunosuppressive agent 'cyclosporine A' (CyA) has been

introduced. The use of CyA, either in conjunction with, or in place of conventional immunosuppressive therapy (azathioprine and prednisolone) is associated with improved graft survival (about 12-15% at one year) when compared to azathioprine and prednisolone alone (Opelz, For the Collaborative Transplant Study, 1985; Morris, 1988). The introduction of CyA has led some centres to question the beneficial value of pregraft blood transfusions on transplant survival.

Several studies reported that they no longer found a transfusion effect (Klintmalm et al, 1985) and consequently, fewer centres adhere to a deliberate blood transfusion protocol. The UCLA registry has reported a steady increase of primary kidney transplants in non-transfused recipients, from 10% in 1984 to 25% in 1989 (Iwaki, Cecka and Terasaki, 1990).

In 1987, Opelz reported the partial disappearance of enhanced graft survival associated with pregraft blood transfusions (Opelz, 1987a). However, this was independent of the use of CyA and is due to increased graft survival in the non-transfused group since 1984. Absence of a transfusion effect was also reported by Groth following the Scandinavian Multi Centre Cyclosporine trial (Groth, 1987). Whilst the CTS data is no longer able to demonstrate a beneficial transfusion effect (Opelz, 1989), Cats (Cats et al, 1984) still reports a 16% improvement for transfused patients treated with CyA. There is now a debate whether the marginal benefits of transfusion outweighs their deleterious effects.

A recent analysis of the North American data by Iwaki (Iwaki, Cecka and Terasaki, 1990) and Cicciarelli (Cicciarelli and Terasaki, 1988) still shows enhanced graft survival following blood transfusion. Examination of

the UCLA transplant registry showed that this is true when the data is stratified for year of transplant, demonstrating the effect during the cyclosporin era (Iwaki, Cecka and Terasaki, 1990). The effect was strongest in the American black population (8-10% enhancement), although it was also present in Caucasoids (4-5% enhancement). Because of the large numbers of transplants recorded on the register, these relatively modest improvements were highly significant. However, they identified a subgroup patients, those transplants where the donor and recipient were HLA-DR matched, who gained no additional benefit from pregraft blood transfusions. In one and two HLA-DR antigen mismatched transplants they reported an 8% and 10% increase in graft survival respectively.

Borleffs (Borleffs et al, 1982) demonstrated bimodal graft survival in rhesus monkeys given a single third party transfusion. This indicated that some monkeys benefited from a pregraft blood transfusion whereas others did not. Following this observation, J.J.van Roods' group in Leiden investigated which factors were important to gain enhanced graft survival (Van Rood and Claas, 1990). They studied almost 200 renal allograft recipients who had received a single blood transfusion. Enhanced graft survival was only found in those patients where the blood donor and recipient shared a single HLA-DR antigen. Patients whose blood donor shared no HLA-DR antigens had a graft survival similar to the non-transfused patients.

These data are further supported by the use of donor specific transfusions (DST) in single haplotype matched live related donor transplants. Such transplants have a graft survival equal to HLA identical sibling donor transplants (Eklund et al, 1990; Salvatierra et al, 1985; Sanfilippo,

Thacker and Vaughn, 1990). These studies all show approximately 90% one year graft survival in two haplotype matched siblings and with one and zero haplotype matched related donor transplants receiving DST.

Subsequent studies by Lazda and colleagues (Lazda et al, 1990) indicated the requirement for the blood donor and recipient to share a single HLA-DR haplotype for improved transplant outcome following DST. In this study, single haplotype matched related donors and recipients who were HLA-DR compatible suffered more rejection episodes compared to those who shared only one HLA-DR allele which their donor.

It is therefore possible that a subgroup of patients, those who share an HLA-DR allele with their blood donor, benefit from pregraft blood transfusions. This effect may be abrogated if the recipient receives an HLA-DR matched donor.

1.4 Transplantation of highly sensitized patients

The policy of giving deliberate pregraft blood transfusions and also the inevitable increase in the numbers of patients sensitised through pregnancies and previous graft rejection, has led to a rise in the number of sensitised patients on most transplant waiting lists. The increase of 'highly sensitised patients' (those with antibody levels reactive with greater than 85% of a random cell panel) has led to the relevance of donor reactive antibodies as an 'absolute contra-indication to transplantation' to be examined in more detail. The definition of 'highly sensitized' varies with the transplant centre ranging from >50% to >90%. However, persistent reactivity of >85% is the criteria accepted by Eurotransplant and United Kingdom Transplant Service (UKTS). A number of strategies have been developed in order improve the chances of finding a negatively crossmatched donor, or identify circumstances when a positive crossmatch can be safely ignored.

1.4.1 Lymphocytotoxic autoantibodies

Following the publication of Patel and Terasaki in 1969, most transplants centres considered that all positive crossmatches would lead to acute graft failure. However in their report, 20% of transplants performed with a positive crossmatch were successful. At the time, they attributed these as being false positives due to technical difficulties in the laboratory.

In 1976, Cross (Cross, Greiner and Whittier, 1976) reported a series of nine successful transplants performed in the presence of a positive crossmatch. The antibodies were found to react with the patients own lymphocytes in addition to donor lymphocytes, and these lymphocytotoxic autoantibodies were not associated with graft failure. Similarly, Stastney

and Austin (Stastny and Austin, 1976) also reported successful transplantation in a HLA identical sibling pair. This patient had a positive crossmatch with the donor and also autologous lymphocytes. The cytotoxicity was shown to be complement dependent and reactivity was absorbed by autologous and donor lymphocytes, but not by platelets. These reports demonstrated that not all donor reactive antibodies are damaging to the graft, and that under some circumstances transplantation can be successful despite a positive crossmatch.

This observation was soon confirmed by Reekers (Reekers et al, 1977), who described nine out of 132 patients with autoantibodies. Seven of the nine patient sera reacted with B cells only, and two were reactive with both T and B cells. Two patients were subsequently transplanted with a positive donor crossmatch, both of which were successful.

In addition, seven such transplants were performed in highly sensitized patients in Oxford (Ting and Morris, 1977), of which six (86%) were successful at three months. In this study, the autoantibodies were identified not only by reactivity with a panel of B lymphocytes and autologous B cells, but also through lack of reactivity with a panel of B cells derived from patients with chronic lymphatic leukaemia (CLL). Of 150 patients on the Oxford transplant waiting list, 23 were shown to have lymphocytotoxic autoantibodies (Ting and Morris, 1978). Two different types were described; those with reactivity against B cells alone, or against both T and B cells. The autoantibodies were weak or negative with CLL cells which has also been confirmed by others (Ozturk and Terasaki, 1980). As CLL cells were known to express the normal complement of HLA-A,B,C and DR antigens on their cell surface, screening against panels of CLL cells

allowed discrimination between autoantibodies and alloantibodies.

The predominance of autoantibody reactivity with B cells was confirmed by Ting and Morris (Ting and Morris, 1979) where they had performed 11 B cell and three T and B cell (two HLA identical) positive crossmatch transplants with 91% and 100% three month graft survival respectively. This compared to 90% for sensitized patients transplanted with a negative crossmatch and 72% in non-sensitized patients. Moreover, in 15 cases the crossmatches were current positive, using serum taken at the time of transplantation. Of these, 73% were successful at three months, with no failures associated with hyperacute rejection.

Additional studies (Ting and Morris, 1983) confirmed that transplants could be successfully carried out with positive crossmatches caused by T and B cell autoantibodies. Of 16 such transplants, 10 were successful, including five of seven with a current positive crossmatch. However a low immediate function rate of 25% was noted. In this group of highly sensitized patients, those with mixtures of auto and alloantibodies were transplanted with an HLA matched donor, therefore excluding the possibility of sensitization to alloantigens.

Ettenger (Ettenger, Jordan and Fine, 1983) suggested an autoregulatory effect of B cell autoantibodies. They found enhanced graft survival of 90% at six months in ten patients with B cell autoantibodies compared to 48% with no antibodies.

1.4.2 Differentiation of autoantibodies from HLA antibodies

It is now generally accepted that transplantation can be performed safely

in the presence of a positive crossmatch due to lymphocytotoxic autoantibodies, even if they are present at the time of transplantation. However, in patients with a mixture of antibody populations (ie non-HLA and HLA antibodies), the specificity of a positive crossmatch against a particular donor is difficult to determine. The positive crossmatch may be due to damaging (presumed anti-HLA) or non-damaging (presumed non-HLA) antibodies and so the transplant is not usually performed. Several in-vitro methods have been employed in an attempt to differentiate between autoantibodies and HLA antibodies in highly sensitized patients.

i) Optimal temperature of reactivity

These include varying the pre-complement incubation temperature. Park (Park, Terasaki and Bernoco, 1977) demonstrated that B cell autoantibodies react more strongly at low temperatures (5°C) than at 20°C and described them as cold lymphocyte cytotoxins. Iwaki and his co-workers (Iwaki et al, 1979) showed that cold B cell lymphocytotoxic autoantibodies (reactive at 5°C), were associated with good graft survival whereas the putatively damaging HLA antibodies, which react more strongly at warm temperatures (37°C) had poor graft survival. In a series of 203 transplants they reported an 82% one year graft survival with cold B cell positive crossmatches compared to 36% with warm B cell or warm T and B cell positive crossmatches. This finding has also been confirmed by others (Ayoub et al, 1980; Posner et al, 1984), although in Posner's study the deleterious effect was only found with regrafts. However, other reports found no such effect (Coxe-Gilliland and Cross, 1981; Etheredge, Sicard and Anderson, 1979).

In a subsequent publication (Iwaki et al, 1979), Iwaki confirmed his

original finding in a series of 909 transplants using single haplotype matched related donors, first cadaver grafts and cadaver regrafts. The cold reactive B cell antibodies were found to be enhancing, a view also indicated by others (Fauchet et al, 1980). Graft survival in this group (68% at one year) was better than those transplants performed in patients with no antibodies (52% at one year). Corroborative evidence supporting this finding came from Jeannet's group (Jeannet, Vassali and Hufschmid, 1980) who reported 10 out of 10 six month graft survival with cold B cell antibodies, compared to 61% with no antibodies, and 62% and 50% with warm B and warm T antibodies respectively.

Exceptions to this have been noted. Deirehoi at al (Deierhoi, Ting and Morris, 1983) reported successful transplants in the presence of warm B cell crossmatches. Contrary to Parks' finding, lymphocytotoxic autoantibodies were shown to be reactive at 4°C, 22°C and 37°C. Of 25 transplants performed with warm B cell crossmatches, 21 were successful. Reactivity of lymphocytotoxic autoantibodies at multiple temperatures was also reported by Ettenger (Ettenger and Robertson, 1987). Therefore this method was not considered reliable to distinguish damaging from non-damaging antibodies.

ii) Characterisation of lymphocytotoxic autoantibodies

Lobo and colleagues (Lobo, 1981) proposed an immunoregulatory role for B cell autoantibodies. Using immunofluoresence techniques, autoantibodies were shown to be IgM whereas alloantibodies were IgG. They could be absorbed by autologous cells but not by red blood cells and their appearance was not associated with pregnancy or blood transfusion. They demonstrated a correlation between the presence of autoantibodies and an

absolute quantity of IgG in the serum, indicating that autoantibodies were modulating B cell function. However, the degree of alloimmunization following graft rejection was not effected.

Lobo (Lobo, Sturgill and Bolton, 1984) reported an increased incidence of primary non-function associated with cold reactive antibodies. Biopsies taken one hour following revascularization were found to contain IgM binding to kidney vascular endothelium in capillaries and arterioles. Warming of the kidney prior to revascularization did not reduce the incidence of initial non-function but did reduce its severity and duration.

The priming stimuli for, and molecular specificity of the autolymphocytotoxic antibodies are largely unknown. Their appearance has been associated with viral infection (Jeannet, Benzonana and Arni, 1981; Cross, Greiner and Whittier, 1976; Lobo, 1981; Jeannet, Vassali and Hufschmid, 1980), particularly with post-transplant Cytomegalovirus (CMV) infections (Macleod et al, 1987). Baldwin (Baldwin III et al, 1985) correlated their appearance with development of rheumatoid factor following CMV infection, although autoantibodies do not agglutinate IgG coated latex. Revillard proposed that they may result from immunization with cross-reacting antigens following microbial, parasitic or viral infection (Revillard, Rivera and Robert, 1980).

Ozturk and Terasaki (Ozturk and Terasaki, 1979) found lymphocytotoxic autoantibodies in patients with various diseases, particularly systemic lupus erythematosus (SLE, 72%) and rheumatoid arthritis (RA, 68%). These were mainly reactive with B cells although T cell reactivity was also present in 54% of SLE patients. However, lymphocytotoxic autoantibodies in

patients with SLE are often IgG. Their presence has also been correlated with lymphopenia. This may indicate that they constitute a different antibody population, or the same antibody having undergone immunoglobulin class switching. In addition, cold B cytotoxins were found in 7% of normal males. They were negative with monocytes, polymorphs and CLL cells. Park (Park, Terasaki and Bernoco, 1977) found B cell autoantibodies present in 20% of the normal population. Their presence in normal individuals has been associated with increasing age but they do not appear to have clinical significance (Cicciarelli et al, 1980).

The nature of the target molecule of the lymphocytotoxic autoantibodies is unknown. Ozturk (Ozturk and Terasaki, 1979) proposed that they are specific for cell surface IgM, accounting for the restricted B cell reactivity and also their apparent immunoregulatory role associated with enhanced graft survival. Following this Ozturk and Terasaki (Ozturk and Terasaki, 1980) demonstrated that human IgM caused inhibition of cold cytotoxins to B cells. This was not found using IgG or human serum albumin. In control studies, purified IgM did not block cytotoxicity of HLA antibodies. The cytotoxicity was proportional to the amount of immunoglobulin on the cell surface of different cell types, with B cells being strong targets whereas T cells, null cells (non-T non-B large lymphocytes), CLL cells and polymorphs were negative.

This data was supported by Takashashi (Takahashi et al, 1980) who found that B lymphocytes stripped of surface IgM using heterologous antisera (rabbit IgG anti-human-IgM) at 37°C were no longer susceptible to lysis by cold cytotoxins, but reactivity to HLA antibodies remained unaltered. IgM specificity was shown as pre-incubation of B cells with anti-human-IgG

failed to block autoantibody binding. In addition no effect was found using anti-human-IgG or -IgM at 4°C, demonstrating the requirement for the removal of IgM from the cell surface.

Furthermore, using affinity chromatography, Cicciarelli (Cicciarelli et al, 1980), demonstrated that cold B cytotoxins could be bound and eluted from IgM coated sepharose beads. Binding to IgG coated sepharose was weak and binding to HSA was completely negative. Competitive inhibition was only possible using purified human IgM and not with IgG nor HSA.

Taken together, these experiments indicate IgM on the B cell surface as the target molecule of cold cytotoxins. The molecular specificity of autoantibodies reactive with T cells has not been addressed.

iii) Autologous cell absorption

Autoantibodies are not absorbed by platelets (Stastny and Austin, 1976). Autologous cell absorptions using peripheral blood lymphocytes have been used to remove autoantibody reactivity. Sera, pre and post absorbtion have been used in the crossmatch test to determine the presence of auto and alloreactive antibodies against a particular donor. The difficulty encountered with this approach is the high numbers of autologous cells required for complete absorption of autoantibodies. Ettenger (Ettenger and Robertson, 1987a and b) described a protocol using a triple absorption technique which required a total of $9\text{-}15\text{x}10^6$ autologous PBL's to absorb 50μl of serum. Of 38 sera studied from highly sensitized patients, 21 (55%) had autoantibodies, including 11 of 13 patients with SLE. Autologous cell absorptions were successful in 10 of 11 patients. Transplantation using related donors was performed in three patients who had a negative

crossmatch following absorption and all were successful.

The use of autologous Epstein Barr virus (EBV) transformed lymphoblastoid cells lines (LCL's) has been applied to overcome the problem of high cell numbers required for autologous absorption. This has the advantage of continuous culture of large numbers of autologous B cells in-vitro, enabling absorption of patients autologous sera. Deirehoi (Deierhoi, Ting and Morris, 1984; Deierhoi, Ting and Morris, 1985) demonstrated the use of autologous EBV-LCL absorptions to distinguish auto and alloantibodies in highly sensitized patients. Similarly, Nicholls and Russ (Nicholls and Russ, 1989) also demonstrated specific absorption of autoantibodies using autologous LCL's in highly sensitized patients without depletion of alloantibodies. These studies used $8x10^7$ cells per 50μl of serum. They showed the usefulness of the technique for the detection of antibody specificities in complex sera.

Autoantibodies are reactive with the cell line K562 (Deierhoi, Ting and Morris, 1985; Deierhoi, Ting and Morris, 1984). This cell line, characterised as a primitive erythromyeloid leukaemia cell, does not express sufficient cell surface HLA to be detected by conventional serology. Of 940 HLA typing reagents, 10 were positive with K562 in extended cytotoxicity assays. These reactions had no definable HLA pattern. However, of 40 patients with lymphocytotoxic autoantibodies, 38 (95%) were positive. In highly sensitized patients without autoantibodies, 15% were positive. The extra reactivity may be directed towards granulocyte markers which are also expressed on K562. Autoantibody positive sera absorbed with autologous LCL's also removes K562 reactivity, confirming the autoantibody specificity.

1.5 The B cell crossmatch

The clinical relevance of a B cell positive crossmatch not caused by autoantibodies is also controversial. One reason is that in most studies the precise specificity of the B cell antibody has not been defined. Initial reports indicated that B cell alloantibodies were not associated with poor graft outcome. Ettenger (Ettenger et al, 1977) reported 12 of 15 transplants successful at three months, with no difference between serum creatine levels in the crossmatch positive and negative groups. Similarly, Morris (Morris et al, 1977) reported 10 of 13 successful transplants and Myeburgh (Myburgh et al, 1977) found 13 of 14 were successful at between 3 months and 3·5 years. However these early studies all assumed that the antibodies were alloreactive with no supporting evidence. Myeburgh (Myburgh and Smit, 1978) and Morris postulated that they were the same as rat anti-Ia (class II) antibodies which were known to cause active enhancement.

Ting and Morris (Ting and Morris, 1977) defined alloreactive B cell antibodies on the basis of CLL reactivity. They reported 8 of 11 successful transplants in the presence of alloantibodies assumed to be directed at donor HLA antigens. Further to this, in 1979 (Ting and Morris, 1979) they reported 9 of 13 (69%) successful at 3 months, compared to 91% with B cell autoantibodies and 72% with a negative crossmatch.

In a retrospective study, Jeannet (Jeannet, Benzonana and Arni, 1981) found good graft survival associated with both weak HLA-class I (11 of 13 grafts successful) and HLA-DR antibodies (14 of 16 grafts successful). In this study they utilised platelet absorption, autologous crossmatch and extended incubation times to differentiate auto from alloreactive antibodies. They concluded that weak HLA-class I and HLA-class II antibodies were not

associated with poor graft outcome.

The same techniques were used by d'Apice and Tait (d'Apice and Tait, 1979a and b), who reported improved function and enhanced graft survival with a positive crossmatch due to B cell alloantibodies (28 of 30 grafts successful compared to 15 of 48 successful with a negative crossmatch). They did note that the patients with positive crossmatches had received more blood transfusions (mean 12 units) compared to the negative group (mean 4 units). They hypothesized that only B cell antibodies to Ia (HLA-DR) led to passive enhancement and were beneficial.

However platelet absorption does not distinguish between B cell autoantibodies and HLA-class II antibodies and the autologous crossmatch on uraemic renal dialysis patients is technically difficult and unreliable. Therefore the results should be interpreted with caution.

Other investigators who supported the view that B cell alloantibodies were not damaging included Mohanakumar (Mohanakumar et al, 1979) with 22 of 26 transplants successful at one year, and Coxe-Gilliland and Cross (Coxe-Gilliland and Cross, 1981) who reported no deleterious effect of a warm B cell crossmatch. However Mohanakumar found a strong correlation with the development of post graft B cell antibodies and irreversible rejection. Fauchet (Fauchet et al, 1980) confirmed this, with only 38% (N=15) of patients who developed post graft B cell antibodies successful compared to 83% with no antibodies. Similar findings were later reported by Martin (Martin et al, 1985), who found only 35% of transplants were successful when post graft HLA class I or class II antibodies developed. These findings appeared to contradict the reports of their non-damaging role

before transplantation.

These early publications indicated that a positive B cell crossmatch was not an important indicator of acute graft failure and hence many transplant centres do not perform the test. Other studies have drawn different conclusions and believe that pre-graft B cell alloantibodies are damaging to renal allografts.

Dejelo and Williams (Dejelo and Williams, 1977) reported hyperacute rejection of a HLA-A,-B identical transplant with a positive B cell crossmatch thought to be directed at HLA-DR. The patient was highly sensitised following 51 blood transfusions and a previously failed transplant. Sirchia (Sirchia et al, 1979) examined the association of pregraft HLA-A,-B,-C and -DR antibodies (following platelet absorption) and graft survival. They reported 80% success at three years in patients with no pregraft antibodies compared to only 40% with B cell alloantibodies. Ayoub and colleagues (Ayoub et al, 1980) drew similar conclusions and found a 27% one year graft survival with pregraft HLA-DR antibodies (defined by reactivity at 37°C and not absorbed by platelets). They concluded that IgM-anti-IgM (auto) antibodies were enhancing whereas sensitization to HLA-DR (with titres of >1/8) were detrimental to graft survival.

Following their report of enhanced graft survival, d'Apice and Tait (d'Apice and Tait, 1980) examined the specificity of B cell antibodies in their series of 34 positive B cell crossmatch transplants. They correlated T and B cell panel reactivity with HLA-DR alleles, looked at antibody profiles pre- and post-platelet absorption, optimal thermal reactivity and the segregation of reactivity with HLA haplotypes within families. They

found twenty patients had non-HLA antibodies and 14 had HLA-DR antibodies (reactive at 37°C following platelet absorption). Of the 34 positive crossmatch transplants previously reported, only four were attributable to donor reactive HLA-DR antibodies. They concluded that most positive B cell crossmatches were not caused by HLA-DR antibodies.

Further to their initial findings of no overall deleterious effect at three months, Ting and Morris (Ting and Morris, 1981) reported 91% one year graft survival of 11 first grafts with B cell autoantibodies compared to only 30% in 10 transplants without autoantibodies. In most of the failures with positive crossmatches, the transplants never functioned. Regrafts also had poor success rates. They suggested that not all B cell crossmatches could be ignored and that B cell alloantibodies, particularly those produced after a previous graft failure, were damaging to the transplant.

Berg (Berg and Moller, 1981) reported the hyperacute rejection of a HLA-A,-B compatible regraft in the presence of a donor specific HLA-DR antibody. However, in this very short communication the antibody specificity was not clearly proven. Also Mohanakumar (Mohanakumar et al, 1981) reported the rapid failure of two regrafts performed with donor specific sensitization. Both recipients received kidneys sharing a repeat HLA-DR mismatch with their previous donors and were shown by CLL reactivity profiles and platelet absorption studies to have immediate pregraft antibodies to donor HLA-DR alleles. The antibody titres in one patient rose form 1/6 to 1/512 twenty-one days post-transplant and this was associated with graft loss. Furthermore Ahern (Ahern et al, 1982) reported two cases of hyperacute rejection with a positive B cell crossmatch caused by HLA-DR antibodies. Both patients were highly sensitized with 100% panel

reactive antibodies and both received kidneys from HLA-A,-B and -C identical donors having a negative T cell crossmatch. Platelet absorptions and absorptions using homozygous typing cells provided convincing evidence of the HLA-DR specificity. The titres were 1/64 and 1/32 immediately pre-transplant and in one case the titre decreased to 1/1 twenty-four hours post-transplant indicating absorption onto the renal vasculature. Using a skin biopsy crossmatch the antibody was shown to react with donor endothelium in one case. Both transplants suffered immediate histologically proven hyperacute rejection. These authors suggested that high titre donor specific HLA-DR antibodies may mediate graft destruction.

In subsequent years many other investigators have reported poor graft survival associated with B cell alloantibodies. Noreen (Noreen et al, 1983) found a significantly worse prognosis in transplants with a positive B cell crossmatch regardless of the temperature of reactivity. In 48 positive crossmatch transplants, 67% were successful at two years compared to 79% with a negative crossmatch. There was only a 43% graft survival in patients where autoantibodies could not be demonstrated. Posner (Posner et al, 1984), in a retrospective study of 84 transplants found 28 had a current positive B cell crossmatch. No hyperacute rejections were seen and there was no difference in overall graft survival rates. However, regrafts with a positive crossmatch fared slightly worse, although the difference was small.

In addition, Marrow (Morrow et al, 1984), in a series of 56 positive B cell crossmatch transplants reported a lower two year graft survival associated with both primary and regrafts (64% and 88% successful with a positive and negative crossmatch respectively). This was true for cadaver donor and

single haplotype matched live related donor transplants, and was also found regardless of optimal thermal reactivity. Moreover, patient survival was significantly worse in the positive crossmatch group (80% versus 95% at two years). In common with most other reports, although graft outcome was significantly poorer, 60% were still successful with no incidence of hyperacute rejection.

Rhodes (Rhodes et al, 1984) reported that patients with HLA-DR reactivity (defined as CLL positive) had poor graft outcome. They confirmed the HLA-DR specificity following immunoprecipitation of HLA-class II like molecules (29kD and 34kD) with patient sera. Blank and colleagues (Blank et al, 1982) found that a warm B cell crossmatch precedes the development of a positive T cell crossmatch following DST, which precludes transplantation with a living related donor. Vaidya (Vaidya et al, 1984) reported that in single haplotype matched LRD transplants who had received DST, 63% (N=16) with a positive B cell crossmatch had reversible acute rejection episodes compared to 37% with a negative crossmatch. There was however no difference in ultimate graft outcome.

Nunez and co-workers (Nunez, McPhaul and Stastny, 1983) utilized the technique of cytotoxicity inhibition to define the specificity of antibodies causing a positive B cell crossmatch. Using a non-cytotoxic monoclonal antibody (L227) reactive with a common framework determinant on HLA-DR molecules and a polyreactive chicken anti-human Ia (reactive with HLA-DR related supertypic class II specificities), they investigated the panel reactivity of patient sera pre- and post-transplant. They found that transplants with pregraft HLA-DR antibodies rejected more often (67%) than those without (44%). HLA-DR antibodies were present in 94% of patients who

lost their transplant due to rejection and were eluted from 85% of rejected kidneys. These results implied a role for HLA-DR antibodies in the pathogenesis of graft destruction although 55% of transplants had good function.

Fauchet and colleagues (Fauchet et al, 1985) prospectively examined the role of B warm and cold antibodies in allosensitized recipients in response to blood transfusions. In 90 transplants, 16% had B cold and 17% had B warm positive crossmatches. They found no difference in the incidence of acute rejection episodes or with graft outcome. However, presensitization to HLA-DR was considered a contra-indication and such transplants were prospectively avoided.

Salvatierra (Salvatierra et al, 1985) published their experience of 239 live related transplants following DST. Zero and one haplotype matched transfused recipients had an equal four year graft survival to HLA identical patients. However, despite administration of imuran or cyclosporin during DST to prevent sensitization, 62 patients developed B warm positive crossmatches which were considered a contra-indication to transplantation. The crossmatches were repeated using flow cytometry against T cells in order to identify and exclude weak (subliminal) HLA class I reactivity. Of the 62 patients, 45 (73%) were negative and subsequently transplanted. Forty-four (all with titres of <1/4) were successful. They noted that the single failure had a titre of 1/8 with donor B cells.

Reekers and Fluit (Reekers and Fluit, 1985) found no influence of a B cell crossmatch due to alloantibodies (defined as autoantibody negative) in

primary transplants (80% successful at one year, N=32) but reported poor regraft survival with 6/14 successful at one year compared to 70% with a negative crossmatch. Russ and colleagues (Russ et al, 1987) reported 32 positive B cell crossmatch transplants with 48% one year graft survival compared to 75% with a negative crossmatch. There was a higher proportion of regrafts and sensitized patients (>50% PRA) in the positive crossmatch group although increased rejection was found with both primary and regrafts. Further to this (Russ et al, 1987) they reported 47% of the positive B cell group had rejection within 7 days compared to only 15% without antibodies. Platelet absorption and screening with HLA typed panels were used to confirm the specificity which showed HLA-D and weak HLA-A,B,C antibodies were associated with an increased incidence of rejection. Biopsies showed evidence of antibody mediated damage with polymorph and monocyte infiltration and early acute glomerular rejection. Noreen (Noreen et al, 1987) found similar results, with 62% one year success in 81 primary grafts in the B cell positive group compared to 80% with a negative crossmatch.

Lazda and co-workers (Lazda et al, 1987) examined the influence of a current B cell positive crossmatch in the pre- and post-cyclosporin era. From 1978-1983 (pre cyclosporin) 56% and 40% were successful with a negative and positive B cell crossmatch respectively. This detrimental effect was only found with regrafts. In the negative crossmatch transplants, poor regraft survival was associated with sensitization, with 44% one year graft survival in patients with >10% PRA compared to 67% with <10% PRA. Graft survival with a positive crossmatch directed at HLA class II (not platelet absorbed) was no different to those with no antibodies. However, of six transplants with a current positive flow cytometry

crossmatch due to HLA-class I and II antibodies, only one (17%) was successful. With cyclosporin (post 1984) no difference in graft survival was seen although the positive B cell group had higher rejection rates (55% versus 75%) which again correlated with levels of T cell PRA. They proposed that the B cell crossmatch and flow cytometry technique detected 'subliminal' sensitization to HLA class I.

Alarif (Alarif et al, 1987) reported no overall difference in graft outcome but they also found a strong correlation with previous levels of sensitization. Van der Berg-Loonan (Van den Berg-Loonen et al, 1987) reported that after blood transfusion, 53% had B cell antibodies of which only half were against HLA-DR/DQ. Positive crossmatch transplants due to HLA antibodies were avoided and no difference in graft outcome was seen.

Tzakis (Tzakis et al, 1988) investigated transplants performed with a current weak positive crossmatch in 39 sensitized patients. Primary and regraft survival was 80% and 63% respectively, indicating the existence of a 'therapeutic window' through which patients can be successfully transplanted. These patients received intensive immunosuppression (OKT3) and an increased incidence of ATN was noted. Noreen (Noreen et al, 1989) examined the results of 49 B cell positive crossmatch transplants in patients treated with CyA. They found 88% one year success with a negative crossmatch compared to 71% with a positive crossmatch. The difference was most apparent in diabetic patients (89% vs 64%) and they concluded that a positive B cell crossmatch was predictive of poor graft outcome.

A study by Phelan (Phelan et al, 1989b) showed that ten of 17 positive B cell crossmatch transplants were due to HLA class I antibodies (confirmed

by flow cytometry). Poor graft survival (60%) despite extensive immunosuppressive treatment was found indicating that weak HLA class I sensitization was detrimental to graft outcome.

In summary, a positive B cell (but negative T cell) crossmatch may be caused by heterogeneous antibody populations directed towards non-HLA, weak HLA class I, or HLA class II (HLA-DR, DQ and DP). The significance of such antibodies in renal transplantation remains controversial, with some reports suggesting immediate graft failure or poor graft survival, whereas others suggest that they have no influence or are associated with improved graft outcome. Most studies in which the specificity has been defined and was shown to be towards HLA-DR reported poor graft survival. A positive B cell crossmatch in regrafts and high titre B cell antibodies may be additional risk factors associated with poor graft survival. It is therefore important to distinguish between B cell antibody specificities to determine which, if any, are prognostic of graft failure. Because the early studies showed that graft survival in B cell positive crossmatch transplants was similar to, or even better than grafts with a negative crossmatch, many transplant units do not perform B cell crossmatches. On the other hand some centres avoid altogether transplanting any B cell positive crossmatch donors.

1.6 Peak positive - current negative crossmatch transplants

Traditionally, both historical and current sera have been used for crossmatching. This was based on the rational that due to immunological memory, an anamnestic response would result if a transplant was performed using a donor to which the recipient was previously sensitized. This would cause uncontrolled acute or accelerated rejection.

Due to the accumulation of highly sensitized patients awaiting a negative crossmatch donor and following the introduction of more powerful immunosuppressive agents, pioneering work by Cardella and Falk (Cardella et al, 1982) questioned the role of non-current donor specific HLA antibodies. They performed 15 transplants in patients who had lost their antibodies during the course of time. These patients had a positive T and B cell crossmatch using sera taken in the past (ranging from 2 months to 3 years) but were negative on the day of the transplant. All were autoantibody negative. Of the 15 transplants, 9 were successful at one year which was not significantly different to the negative crossmatch transplants performed during the same period (75% successful at one year). They concluded that only the current antibody status is relevant when selecting a suitable donor and that immunological memory is not a precluding factor with the use of modern day immunosuppression. However, only six of the fifteen patients in this group were highly sensitized (>90% PRA). Graft rejection was treated with intensive immunosuppressive regimes of plasma exchange and rabbit anti-thymocyte sera which was associated with notable infectious morbidity.

Following this lead, many investigators drew similar conclusions. Laupacis (Laupacis et al, 1983) reported 3 of 4 transplants successful (3/3 first

and 0/1 regrafts). Matas (Matas et al, 1984) found 3 of 5 successful (2/2 first and 1/3 regrafts). All patients in this study were previously highly sensitized but had a current negative crossmatch for at least 18 months prior to the operation. Fuller and colleagues (Fuller, Forbes and Delmonico, 1985) reported 4 of 8 transplants negative for greater than 4 months were successful and Rosenthal (Rosenthal et al, 1985) found 10 of 12 transplants successful at one year. In a study by Norman and co-workers (Norman, Barry and Wetzsteon, 1985) all six transplants reported were successful. In this study, all were first grafts in patients sensitized through blood transfusion and in most cases the antibodies had a low titre.

These and other authors drew the conclusion that the memory B cell response was short lived and secondary responses could be controlled by the use of cyclosporin. Subsequently, the policy of crossmatching only current sera was widely adopted enabling transplantation of many highly sensitized patients. However despite these advances many transplants still underwent acute irreversible rejection.

In subsequent studies several risk factors which could identify those patients' at higher risk of loosing their graft were reported. These were i) whether the transplant was a first or a regraft, ii) unresponsiveness to donor alloantigens induced by anti-idiotypic antibodies and iii) the immunoglobulin class and specificity of the T cell crossmatch.

1.6.1 Primary and regrafts: Several reports indicated that graft survival in the presence of a peak positive but current negative crossmatch was acceptable in primary transplants but was associated with poor regraft survival. Kerman (Kerman et al, 1985) reported 12 of 14 (86%) first grafts

successful but none of 4 regrafts successful at one year. This compared to 81% and 74% successful for first and regrafts respectively, performed with a negative crossmatch. Cardella (Cardella et al, 1985) also found a poor regraft survival but this was no different to their regrafts carried out with a negative crossmatch. In a series of 61 T and B cell positive crossmatch transplants they found high risk patients (defined as rejection of a previous transplant in less than one year) had 41% success with a positive crossmatch compared to 46% with a negative crossmatch.

Goeken (Goeken and And the Clinical Affairs Committee, 1985) reported on the ASHI survey conducted by the clinical affairs committee. Of 216 historical positive crossmatch transplants from 27 reporting centres, 69% of first grafts (N=109) and 53% of second or subsequent transplants (N=107) were successful at one year. No other factor such as the time period between the positive crossmatch and transplantation, the level of peak PRA or whether the antibody was reactive with donor T and B cells or B cells only were significant. Hodge (Hodge et al, 1987) also found good primary (87%) but poor regraft survival (56%). The mean panel reactivity of this group was 66% (N=26).

Johnson (Johnson et al, 1987), examined 162 transplants with a negative T cell crossmatch using serum taken up to six months prior to transplantation. Using the more sensitive antiglobulin-augmented crossmatch test they found 17 were positive. This was associated with an increased incidence of accelerated rejection ($p < 0.01$) and increased loss of regrafts ($p < 0.02$) but not first grafts. Their data indicated that when crossmatching current serum only, it may be important to use the extra-sensitivity of the antiglobulin-augmented test. This detects sublytic

levels of donor reactive antibodies which are not seen in the conventional crossmatch.

Falk (Falk et al, 1987) re-examined the Canadian data and confirmed their previous finding that the timing of the first graft loss was the only factor which influenced regraft outcome. One year graft survival was 50% (N=113) in patients who had rejected a previous transplant in less than one year compared to 80% (N=93) in patients whose transplants failed beyond one year. No correlation was found with a positive crossmatch using non-current sera.

In contrast, Turka (Turka et al, 1989) found an adverse effect of a historical positive crossmatch in both first and regrafts. Primary graft survival at one year was 68% (N=41) with a positive crossmatch compared to 86% with a negative crossmatch. For second and subsequent transplants, 51% (N=29) of positive and 79% of negative crossmatch transplants were successful. Although a historical positive crossmatch was not an absolute contra-indication these authors considered it a 'relative increased risk' when selecting suitable donor and recipient pairs.

1.6.2 Anti-idiotypic antibodies: In 1980, Miyajima (Miyajima et al, 1980) described the presence of serum factors which caused MLR inhibition in a recipient of a successful transplant from a HLA mismatched cadaver donor on minimal immunosuppression. The donor specific inhibition was due to IgG antibodies reactive with responder cells and was postulated to block the antigen binding site of the T cell receptor on autologous cells. They proposed a mechanism of anti-idiotypic antibodies playing a central role in the immunoregulatory system, sustaining graft protection. Takeuchi

(Takeuchi et al, 1985) reported similar findings in patients following DST. They described MLR suppression of autologous responder cells with IgG purified from sera following blood transfusion. This correlated with a lower incidence of acute rejection. They advanced the idea that anti-idiotypic antibodies performed an important role in the prolongation of graft survival. Similar findings were also reported by others (Suzuki et al, 1985). However Daniel (Daniel, Wendler and Opelz, 1987) found non-specific inhibition of the primary MLR, in-vitro stimulated donor specific T cell blasts and PHA blasts using both patient and negative control sera.

Toma and colleagues (Toma et al, 1985) used the Lymphocytotoxic Inhibition Test (LIT) for the detection of anti-idiotypic antibodies. This involved pre-incubation of current and non-current serum (1:1) overnight at 4°C, followed by a cytotoxicity test. Inhibition of cytotoxicity (compared to an RPMI treated control) was interpreted to contain anti-idiotypic antibodies. The anti-anti-HLA specificity (Ab2) was confirmed by blocking reactivity of specific HLA antisera (Ab1). Thirteen patients sensitized through DST, who had subsequently become negative were transplanted and 11 (85%) were successful. They investigated one such successful transplant and correlated the appearance of Ab2 with MLR suppression and specificity to a mismatched donor antigen (HLA-Bw59). However in a follow-up study (Toma et al, 1987), no overall correlation with graft survival was found.

Using a similar technique, Phelan (Phelan et al, 1985) found cytotoxic inhibition of control HLA antisera using both whole serum, purified IgG and $F(ab')_2$. They interpreted >50% lysis inhibition in diluted sera as being Ab2 positive. Burlingham (Burlingham et al, 1988) also found $F(ab')_2$ of IgG contained inhibitory activity specific for donor antigens following DST

although only 2 of 12 patients studied were positive.

Barkley and co-workers (Barkley et al, 1987) developed a solid phase enzyme immunoassay to define blocking antibodies. They examined the ability of post transfusion sera to block both polymorphic and monomorphic monoclonal anti-HLA antibody binding to trays coated with gluteraldehyde fixed platelets. Binding was detected using horseradish peroxidase conjugated anti-human or mouse IgG in an ELISA technique. They found 8 of 16 post transfusion patients showed anti-idiotypic activity which resided in the $F(ab')_2$ of IgG. Furthermore, binding was directed at anti-HLA IgG (Ab1) $F(ab')_2$. Cytotoxicity inhibition of control HLA antibodies using $F(ab')_2$ fragments of IgG was also reported by MacLeod (MacLeod et al, 1989). They proposed that this was a possible mechanism of allograft enhancement following blood transfusion.

Reed (Reed et al, 1987) tested 20 patients transplanted with a positive crossmatch using non-current sera using the cytotoxicity inhibition test. Donor specific anti-HLA sensitization in these patients was demonstrated by panel screening with a correlation coefficient of r=>0.7 with mismatched donor antigens. Only sera from the last six months were prospectively crossmatched, without regard to previous sensitization history. The presence of Ab2 in current sera was found in 9 of the 10 successful transplants. None of the 10 failed transplant patients had Ab2 and 9 had potentiating antibodies (Ab3). The potentiating antibodies were postulated to be anti-anti-anti-HLA antibodies. They concluded that a historical positive crossmatch could only be ignored if the current serum contained anti-idiotypic HLA antibodies.

A paper from Michelle Jeannet's group in Geneva (Hann et al, 1988) used the same technique to identify inhibitory factors which blocked HLA class II antisera. They monitored 44 patients for anti-idiotypic antibodies following blood transfusion and their correlation with transplant outcome. Mean cytotoxic inhibition of HLA class II sera was significantly higher in patients with successful transplants compared to those that failed. Lack of inhibition was associated with irreversible rejection in patients treated with conventional immunosuppressive therapy (azathioprine and prednisolone) and with cyclosporin. However due to the inherent variability of the test, standard deviations were high. They noted variability with negative control sera, some of which also caused inhibition. There was no relationship of their appearance with pregraft blood transfusions, nor previous pregnancies. The lack of a correlation to HLA sensitization and therefore the production of HLA antibodies (Ab1) must bring into doubt the anti-idiotypic nature of these factors. In addition, cytotoxic inhibition was associated with the presence of immune complexes (determined by ^{125}I C1q binding) found in patients with systemic disease. One explanation was that these represent Ab1/Ab2 complexes which block Fc receptors causing suppression. Inhibitory factors which block the formation of EA rosettes with Fc receptors on donor B cells following blood transfusion are also associated with good renal allograft outcome (Macleod et al, 1982). One year graft survival of 85% with EA rosette inhibiting IgG antibodies was reported, compared to only 30% when absent. These findings however have not been confirmed by others (Soulillou, Peyrat and Guenal, 1978; Soulillou and Peyrat, 1979; Suthanthiran et al, 1978; Suthanthiran et al, 1977).

Phelan (Phelan, Rodey and Anderson, 1989a) investigated the occurrence of Ab2 in 16 patients following DST under the cover of azathioprine. Post

transfusion sera inhibited HLA-A,B,C,DR and DQ sera with no predilection for any particular locus. They appeared immediately after the first transfusion although some were found prior to transfusion (thought to be a consequence of previous transfusions). All 16 patients were successfully transplanted (13 LRD and 3 cadaver).

Similar results were reported by Al-Muzairai (Al-Muzairai et al, 1989). DST was administered with or without cyclosporin to 50 patients. Whilst the degree of sensitization was similar in both groups, those who received CyA quickly lost their antibodies. This was associated with the development of inhibiting Ab2 which was increased in the cyclosporin group (10/14 with CyA compared to 4/17 without CyA). All these patients were successfully transplanted. They concluded that persistent sensitization was prevented and Ab2 production was augmented by cyclosporin. They also found six patients who developed potentiating antibodies which were negative by the anti-human-globulin complement dependent cytotoxicity (AHG-CDC) test and cellular ELISA (for non-complement fixing antibodies). Unlike the experience of Reed, five of the six were transplanted, all of which were successful.

Garovoy's group (Pohanka et al, 1989) examined the disappearance of sensitization following DST in 9 transiently sensitized recipients. Four of 12 sera studied showed inhibition whereas six were enhancing (Ab3). However of the four patients with Ab2, three had been previously transfused and the fourth was unknown. None of the four previously non-transfused patients developed Ab2. Flow cytometry demonstrated subliminal Ab1 binding in the six sera with enhancing antibodies. This was not detected by the anti-globulin augmented cytotoxicity test. They correlated the appearance

of Ab2 with the duration of Ab1 (mean 2.5 months) whereas when enhancement occurred, Ab1 lasted significantly longer (mean 7.5 months). These results indicate that Ab3 is not an anti-anti-anti-HLA antibody as suggested by Reed, but they represent residual sublytic levels of Ab1.

Further reports of HLA anti-idiotypic antibodies correlating with graft protection came from Braun (Braun et al, 1988) following cardiac transplantation (specificity was not proven) and Rodey (Rodey and Phelan, 1989). The later paper documented a patient who had suffered hyperacute rejection of a previous transplant subsequently lost the antibody with the emergence of a transient specific Ab2. The patient then received a second successful transplant carrying the same repeat mismatched antigen.

Using the EBV technique, Singal (Singal et al, 1988; Singal et al, 1989a) produced and characterised an antiidiotypic antibody in vitro. Culture supernatants contained IgM which specifically blocked HLA-B8,B5 and DR3 alloantisera. This coincided with the mismatched antigen (HLA-B8) of a successful transplant performed 10 years earlier. In addition, supernatants caused MLR inhibition of responder/stimulator combinations sharing HLA-B and DR antigens with the donor and recipient. Absorption studies indicated that cytotoxicity inhibition and MLR suppression were independent, caused by distinct antibodies.

Following this, Singal and colleagues (Singal et al, 1989b) produced and characterised two further human monoclonal antibodies (one IgM and one IgG) which suppressed specific MLR responses. As previously described, these same antibodies did not block HLA alloantisera, confirming that distinct antibody populations were responsible for MLR suppression and cytotoxicity

inhibition. Immunochemical analysis, following precipitation of ^{35}S methionine labelled cell lysate derived from activated responder T cells, revealed 43kD and 51kD bands. No bands were found using activated T cells from irrelevant responder/stimulator combinations. The function of these idiotypic receptors was not defined but an immunoregulatory role in alloantigen recognition was clearly implicated.

To avoid the problems of multiple antibodies in HLA alloantisera, Pistillo (Pistillo et al, 1989) looked for putative anti-idiotypic inhibition using human HLA MAbs. Sera from patients previously sensitized to HLA-B7 following blood transfusion (N=9), a failed transplant (N=2), or mismatched for B7 with no antibody production, were used to block cytotoxicity of a HLA-B7 MAb and two irrelevant HLA MAbs. Four patients had Ab2 activity; two following graft rejection (8 to 10 years previously), one following rejection with no detectable HLA B7 antibody production and one following DST.

In conclusion anti-idiotypic antibodies have been shown to be present following third party and donor specific transfusions, and also after both failed and successful transplants. Their activity may be one mechanism of the transfusion effect associated with enhanced graft survival. They may also play a regulatory role leading to transplantation tolerance. They can block alloreactive T cell recognition or antibody binding, or act as a marker for other regulatory events. However, definitive confirmation of their existence is still lacking.

1.6.3 Antibody class and specificity: Ayoub, Terasaki and Tonai (Ayoub, Terasaki and Tonai, 1983), on examination of the UCLA international

registry, demonstrated that patients who once had high antibody levels but whose sensitization decreased before transplantation had higher failure rates. This was particularly apparent in regrafts. However the effect was not seen in their own data. They postulated that the reason for the difference was their policy of crossmatching both the highest and most recent serum samples, thus avoiding an anamnestic response due to immunological memory. The crossmatches were repeated using all available sera and stored donor cells and 19 were found to be positive with donor T cells. These had a 40% success rate compared to 51% (N=61) with a negative crossmatch (not significant). The crossmatches were retested in the presence of 2-mercaptoethanol which preferentially destroys the cytotoxicity of IgM but not IgG. Of 18 positive crossmatch transplants tested, six were reduced indicating an IgM antibody, all of which were successful. However twelve were not reduced indicating an IgG antibody and only two were successful. Whilst this data supported the Toronto experience that historical positive crossmatch transplants can be successful, many 'false positives' could be distinguished using this technique.

Support for this hypothesis came from our own data (Chapman et al, 1986a) in which the reducing agent dithiothreitol (DTT) was used to distinguish IgM and IgG antibodies causing a positive crossmatch. Cytotoxicity inhibition using a non-cytotoxic mouse monoclonal anti-HLA class I was used in conjunction with DTT to define the antibody class and specificity of a series of positive T cell crossmatch transplants. IgM non-HLA (mainly autoreactive) antibodies were found in 26 cases with 72% successful at three months. However none of the seven transplants caused by IgG HLA-class I, and 4 of 7 due to IgM HLA class I antibodies were successful.

Taken together, these preliminary studies indicated that not all peak reactive sera can be safely ignored and that there is a high probability of failure if the historical crossmatch is due to an IgG antibody. Following these observations and also some of the work presented in this thesis, the benign coarse of peak reactive IgM antibodies defined by the DTT technique has been established (Barger et al, 1989; Roy et al, 1990). Some apparently highly sensitized patients have high levels of IgM antibodies (Rudy and Opelz, 1987; Vaidya and Ruth, 1989) and thus can be successfully transplanted. However there is little information confirming the detrimental effect of peak reactive IgG HLA antibodies, nor concerning the class and specificity of antibodies causing a positive B cell crossmatch. The significance of the non current crossmatch is still an open question awaiting further clarification.

1.7 The objectives of this thesis

The aims were two-fold. Firstly to characterise the nature and molecular specificity of human lymphocytotoxic autoantibodies. This was attempted by the production human monoclonal lymphocytotoxic autoantibodies from a renal dialysis patient using the mouse/human heterohybridoma technique. These have the advantage of unlimited supply, monoclonal, and clones with a higher affinity can be selected. The resulting antibodies were then applied to determine if the multiple reactivity profiles seen with patient sera represented one, or many different autoantibody populations. In addition immunochemical analysis was performed to identify their molecular target. These goals have not previously been possible as only patient sera have been available, with limited supply usually having low antibody titre and affinity.

Secondly, discrepancies in the literature concerning the clinical relevance of the positive crossmatch to graft outcome may be due to the poor definition of the antibody specificities. In the majority of studies the precise specificity of antibodies causing a positive crossmatch has been poorly defined. The aim of this project was to develop an in vitro assay which could reliably define the specificity of donor reactive antibodies in highly sensitized patients. The technique of cytotoxicity inhibition of the T cell crossmatch using non-cytotoxic monomorphic anti-HLA class I MAb was extended to define the specificity of the B cell crossmatch. This involved the identification of suitable monomorphic anti-HLA class I, DR and DQ locus specific MAbs and the development of a blocking assay which could distinguish multiple antibody populations within the same serum. In combination with dithiothreitol (to reduce IgM) the techniques were applied to define the immunoglobulin class and antibody specificity of a series of positive crossmatch transplants. A correlation with graft outcome was then sought.

Chapter 2. Materials and Methods

2.1 Microlymphocytotoxicity assays

2.1.1 Reagents and buffers

a) Physiological saline
b) Phosphate buffered saline (PBS-DAB)
c) Neuraminidase treated sheep red blood cells
d) Polybrene
e) Gidion Goldstein lysis buffer
f) Rabbit complement
g) Eosin staining solution
h) Formal saline fixative
i) Dithiothreitol
j) Cystine
k) Sodium hydroxide

2.1.2 Cell separation methods

a) Preparation of peripheral blood lymphocytes
b) Preparation of lymph node and spleen lymphocytes
c) Separation of T and B lymphocytes
d) Removal of monocytes from B cells

2.1.3 Cytotoxicity assays

a) Cytotoxicity inhibition assay to define the antibody specificity
b) Microlymphocytotoxicity assay
c) Immunoglobulin class determination (DTT reduction)

2.2 Tissue culture techniques

2.2.1 Strategy for monoclonal lymphocytotoxic autoantibody production

2.2.2 Reagents

a) Growth medium
b) Selection medium
c) Fusion medium
d) Mouse spleen feeder cells

2.2.3 Methods

a) Generation of B-LCL
b) Production of heterohybridoma
c) Cloning. i. Clone picking, ii. Limiting dilution
d) Preparation of cell lines for cytotoxicity assays
e) Assessment of heterohybridoma growth characteristics
f) DNA content analysis of heterohybridoma
g) Determination of monoclonal antibody class
h) Mixed lymphocyte reaction inhibition

2.3 **Binding assays**

a) Cytotoxicity binding assay to select higher affinity clones
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e) Biotin/avidin binding assay
f) Competitive inhibition binding assay;
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g) Lectin inhibition of autoantibody binding;
    i) Using fluorescence binding assay
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h) Sialic acid inhibition of autoantibody binding;
    i) Cytofluorograph analysis
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I) Effect of pH on autoantibody binding

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2.4.1 Strategy for batch purification of IgM from tissue culture supernatant

2.4.2 Reagents

a) MES buffer, pH 5·5
b) Sodium acetate buffer, pH 6·8
c) Ammonium sulphate (200mM)
d) MES buffer, pH<4·0
e) Sodium azide (10%)

2.4.3 Methods

a) Preconditioning of ion exchange resin
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    i) Column preparation
    ii) Column calibration
    iii) Sample application

2.5 **Determination of IgM purity by SDS-PAGE**

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a) Acrylamide solution
b) Separation gel buffer
c) Stacking gel buffer
d) Sodium-dodecyl sulphate (10%)
e) Ammonium persulphate
f) Running buffer
g) Sample buffer
h) Running gel
i) Stacking gel
j) Bromo-phenol blue
k) Coomassie blue stain
l) Destain

m) Sample preparation reagents;
    i) Iodo-acetamide (40mM)
    ii) Dithiothreitol (40mM)
    iii) Iodo-acetamide (0·665M)

Table 2.5.1 Running gel concentrations

2.5.2 Method
a) Non-reduced sample preparation
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2.6 Biotinylation of purified IgM

2.6.1 Reagents
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2.6.2 Method

2.7 Immunoprecipitation

2.7.1 Lactoperoxidase catalysed cell surface iodination
a) Reagents
b) Method

2.7.2 Preparation of solubilized antigen
a) Reagents
b) Method

2.7.3 Immunoprecipitation
a) Reagents
b) Method

2.7.4 Autoradiography

2.1 Microlymphocytotoxicity assays

2.1.1 Reagents and buffers

a) Physiological saline:

9·0g of sodium chloride (NaCl, Sigma Chemical Company Ltd, Poole, Dorset) was dissolved in 1 litre of deionised water (dH_2O).

b) Dulbecco A and B solution (PBS-DAB):

10 phosphate buffered saline (PBS) tablets (Dulbecco A, Oxoid Ltd, Basingstoke) were dissolved in 1 litre of dH_2O followed by the addition of one vial (5ml) of mineral salts (Dulbecco B, Oxoid). The solution was passed through a grade 1 filter paper (Whatman Labsales Ltd, Maidstone, Kent) before use.

c) Neuraminidase treated sheep red blood cells (N-SRBC):

10ml of sheep blood in Alsever's solution, (Becton Dickinson UK Ltd, Oxford) was washed 3 times in PBS-DAB by centrifugation for 5 minutes at 700G. On each occasion the white blood cell buffy coat was removed from the red cell surface. 0·6ml of packed sheep red blood cells were resuspended in 10ml of PBS-DAB, with the addition of 300μl of neuraminidase (Type V, Sigma), at a concentration of 1 unit/ml in PBS-DAB, (stored in aliquots at -80°C). Red cells were incubated at 37°C for 30 minutes, washed x3 in PBS-DAB and finally resuspended in 50ml of PBS-DAB. Neuraminidase treated sheep red blood cells were stored at 4°C for up to one week before use.

d) Polybrene 5% w/v:

A stock solution of 5% polybrene (Sigma) was prepared by dissolving 5·0g in 100ml of dH_2O. This was dispensed into 20ml universal containers (Sterilin Ltd, Hounslow, Middlesex) and stored at 4°C. For use, the stock

solution of polybrene was diluted 1/30 (100μl in 3ml) with PBS-DAB and used immediately.

e) Gidion Goldstein lysis buffer (GLB):

8·29g of ammonium chloride (NH_4Cl, Sigma), 37mg of EDTA (ethylenediaminetetraacetic acid, disodium salt, Sigma) and 1g of potassium hydrogen carbonate ($KHCO_3$, Sigma) were dissolved in 1 litre of dH_2O. The pH of the solution was adjusted to 7·3 with the addition of 1M NaOH added drop-wise with continuous mixing. The pH of the lysis buffer is not stable when open to air, therefore 20ml volumes were dispensed into universal tubes (Sterilin) and the lids sealed.

f) Rabbit complement:

Fresh rabbit serum was used as a source of complement for cytotoxicity tests. This was prepared as a routine procedure in the Tissue Typing Laboratory. Ten litres of fresh rabbit blood (obtained from a local farm) was allowed to clot at 4°C overnight. The serum was poured from the clotted blood and residual cells were removed following centrifugation in 1 litre volumes at 2000G for 10 minutes (Beckman 6L, Beckman Instruments Ltd) at 4°C. The rabbit serum was stored on ice and dispensed in 2·5ml and 5ml volumes. The aliquots were frozen and stored at -80°C. For use, aliquots were thawed and used immediately.

g) Eosin staining solution:

A 5·0% w/v solution of eosin was prepared by dissolving 25·0g of eosin Y (Sigma) in a final volume of 500ml dH_2O. The solution was then filtered through a grade 1 filter paper (Whatman).

h) Formal-saline fixative:

200ml of 40% w/v formaldehyde (BDH, Atherstone) was mixed with 300ml of saline. A few drops of 5% phenol red (Sigma) in dH_2O was added as a pH indicator.

i) Dithiothreitol (DTT) 0·01M:

15·4mg of DTT (molecular weight 154·2, Sigma) was dissolved in 10ml of PBS-DAB. The stock solution of DTT was dispensed in 300μl aliquots and stored at -80°C and used immediately after thawing.

j) Cystine 20mM:

A stock solution (x10 concentrated) of L-cystine (molecular weight 240·8, Sigma) was prepared by dissolving 48mg in 10ml of dH_2O and stored at 4°C until use. The cystine is not completely soluble at this concentration and should therefore be mixed before use.

k) Sodium hydroxide 1·0M:

NaOH (Sigma), Molecular weight 40·0.

4·0g of NaOH was dissolved in 100ml dH_2O.

2.1.2 Cell Separation Methods

a) Preparation of peripheral blood lymphocytes:

Peripheral blood lymphocytes (PBL) were separated from anticoagulated (heparin or trisodium citrate) blood by density gradient centrifugation. Blood was diluted with an equal volume of PBS-DAB and 7ml volumes were underlayed with 3ml of lymphocyte separation medium (Nycomed UK Ltd, Birmingham) in 10ml tubes (16x100mm, L.I.P. Ltd, Shipley, West Yorkshire). The samples were centrifuged at 700G for 20 minutes, the mononuclear cells

at the interface were aspirated using a Pasteur pipette and transferred to a 10ml tube. The lymphocyte suspensions were diluted with an equal volume of PBS-DAB and centrifuged for seven minutes at 500G. Platelets were removed by differential centrifugation with three washes in PBS-DAB for five minutes at 350G. PBL's were stored at a concentration of $2\text{-}3x10^6$ in Terasaki Park medium (Gibco, Paisley) at 4°C.

b) Preparation of lymph node and spleen lymphocytes:

Lymphocytes from cadaver organ donor lymph node and spleen were purified by repeatedly perfusing the tissue with 20ml volumes of Terasaki Park medium (Gibco) using a 20ml syringe and 19 gauge needle (Monoject). Polymorphonuclear neutrophils were removed from spleen cells by the addition of a microspatular of carbonyl iron (Sigma) and incubating for 20 minutes at 37°C with periodic mixing. Phagocytes which had ingested the iron and any remaining free iron particles were then separated by placing a magnet on the side of the tube and pouring the free cell suspension into a new tube. Red cells were removed by density gradient centrifugation as above.

c) Separation of T and B lymphocytes:

T and B cells were separated using N-SRBC rosetting. Lymphocytes were suspended at $2\text{-}6x10^6$ cells/ml and 5ml volumes were transferred to 50ml round bottom tubes (Philip Harris Scientific, London). An equal volume of N-SRBC's and 100μl of freshly diluted polybrene was added. The tubes were centrifuged at 100G for 5 minutes, and the cells resuspended by gently rotating the tubes at an angle, allowing the meniscus to pass though the cell pellet. The suspensions were gently transferred to 10ml tubes and underlayed with lymphocyte separation medium and centrifuged at 500G for

15 minutes. B cells were aspirated from the interface and transferred to 4ml tubes (LP4, Luckham Ltd, Burgess Hill), diluted with an equal volume of Terasaki Park medium and centrifuged. The T cell/N-SRBC rosette pellets and the T cell depleted (B cell enriched) fractions were resuspended in 2ml of GLB for 5 minutes, to lyse the red cells by hypotonic shock. Equal volumes of medium were added and the tubes centrifuged. The cell pellets were resuspended and counted under a haemocytometer using a phase contrast microscope (Leitz, Luton). The lymphocyte concentrations were adjusted to $2\text{-}3x10^6$/ml in Terasaki Park medium.

d) Removal of monocytes from B cells:

Where necessary monocytes were removed from B cells by adherence of monocytes to plastic. The B cell enriched suspensions, in 2ml volumes, were transferred to 60mm petri dishes (Falcon Plastics, Becton Dickinson UK Ltd, Oxford) and incubated at 37°C for 30 minutes. Non-adherent B lymphocytes were removed from the plastic by gently washing the surface with two volumes of 2ml of medium using a Pasteur pipette, and transferred into 4ml tubes. B cells were then examined under a haemocytometer and suspended at a concentration of $2\text{-}3x10^6$/ml.

Routinely T and B cell purity was >90% as assessed by an anti-HLA-class II MAb in a cytotoxicity assay. Lymphocytes from patients with CLL were selected to have >90% B cell purity without the need to purify the lymphocytes further.

2.1.3 Cytotoxicity assays

a) Cytotoxic inhibition assay to define the antibody specificity:

The monoclonal antibodies selected for cytotoxicity inhibition studies were PA2.6 (mouse monomorphic IgG HLA class I; Prof A.McMichael, I.M.M., Oxford), NDS-22 (mouse monomorphic IgG HLA-DR; Fuggle, N.D.S., Oxford) and LEU-10 (mouse IgG HLA-DQ; Becton Dickinson). Donor T and B cells were suspended at a concentration of $3x10^6$/ml in Terasaki Park medium (Gibco) and divided into three and seven aliquots respectively. One aliquot of T and B cells remained untreated to act as a control, whilst the remainder were centrifuged, the supernatants removed and cells resuspended in equivalent volumes of appropriate monoclonal antibody at saturating concentration. Two aliquots of T and B cells were resuspended with PA2.6, two aliquots of B cells with NDS-22 and two with LEU-10. Following incubation at 4°C for one hour, one aliquot of each pair was washed in medium and resuspended with a non-cytotoxic affinity purified $F(ab')_2$ fragment of goat anti-mouse IgG light and heavy chain (Northeast Biomedical Laboratories, Uxbridge) at a predetermined dilution (batch dependant variation of 1/100 to 1/200). After a final incubation at 4°C for one hour, these aliquots were washed and the cells resuspended in an equivalent volume of cold medium.

Treated and untreated donor cells were then crossmatched against the recipient sera in the microcytotoxicity assay. To each serum dilution, 1ul volumes of untreated cells, cells incubated with the appropriate MAb alone or cells treated with both the MAb and the $F(ab')_2$ goat anti-mouse antibody were added. All tests were carried out in duplicate. In addition each aliquot of cells was tested with an appropriate anti-HLA-ABC, DR or DQ serum, to confirm locus specific inhibition of cytotoxicity. With one

exception locus specific inhibition was demonstrated for all the serologically defined HLA-ABC, DR and DQ antigens tested. The exception was that LEU-10 does not bind to HLA-DQw2 and does not inhibit alloantisera with specificity for this antigen. A reduction of cytotoxicity by at least one doubling dilution was interpreted as a positive result.

b) Microlymphocytotoxicity assay:

The cytotoxicity tests were carried out using the standard NIH microlymphocytotoxicity test with an extended two hour post complement incubation (Terasaki PI, 1980). One microlitre volumes of tissue culture supernatant or sera were dispensed in duplicate, at doubling dilutions into a 60 well microtest tray (HLA S plate, Gibco), under liquid paraffin oil (BDH, Atherstone). To these, 1μl volumes of cell suspension at $2x10^6$/ml were added. The trays were incubated at 22°C for 30 minutes followed by the addition of 5μl of rabbit complement and a further 2 hour incubation at 22°C. Viability was assessed after staining and fixation, with the addition of 3μl of eosin followed by 3μl of formalin. Wells were covered with a 3x2" coverslip (A.R.Horwell, London) and examined using an inverted phase contrast microscope (Diavert, Leitz, Luton). A positive result was interpreted as at least 20% cell lysis above background in duplicate wells.

c) Immunoglobulin class determination (DTT reduction):

The antibody class was determined using DTT reduction of IgM in the cytotoxicity assay. 1μl volumes of tissue culture supernatant or test serum were dispensed across six wells of a microtest tray under liquid paraffin oil. Two wells remained untreated as normal controls, two wells were inoculated with 1μl of 10mM DTT in PBS-DAB, and two wells received 1μl of PBS-DAB as diluent controls. The trays were incubated at 37°C for 30

minutes. Cells were tested in an extended microcytotoxicity test, with the addition of 100μl/ml of 20mM cystine to the rabbit complement, giving a final concentration of 2.0mM cystine. A negative cytotoxicity reaction with neat serum or supernatant after DTT reduction was considered to indicate IgM antibody only, whereas a positive reaction despite DTT treatment indicated an IgG antibody. A cytotoxic IgG monoclonal antibody and an IgM control was included for each test to confirm specific DTT reduction of IgM antibodies.

2.2 Tissue Culture Techniques

2.2.1 Strategy:

The strategy for the production of a human monoclonal lymphocytotoxic autoantibodies is shown in Figures 2.2.1 and 2.2.2. Initially, renal dialysis patients with lymphocytotoxic autoantibodies were identified. Lymphocytes from these patients were immortalised with Epstein Barr virus (EBV), and lymphocytotoxic autoantibody producing B-LCL's were identified by cytotoxicity screening of tissue culture supernatants. These were fused with the mouse fusion partner X63-Ag8.653 (a non-secreting HAT sensitive mouse myeloma cell line) to produce a mouse/human heterohybridoma (Fig 2.2.2). Lymphocytotoxic autoantibody producing clones were isolated and tissue culture supernatants were harvested for autoantibody and/or antigen characterization.

Fig 2.2.1 In Vitro Production of a Monoclonal Human Lymphocytotoxic Autoantibody

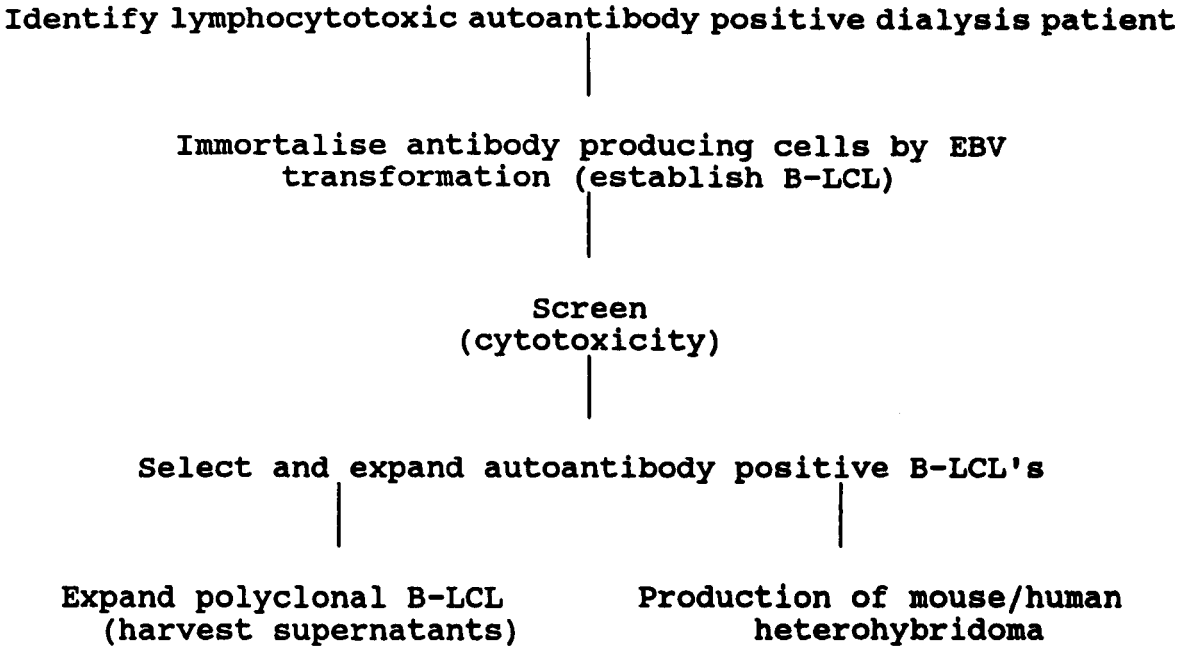
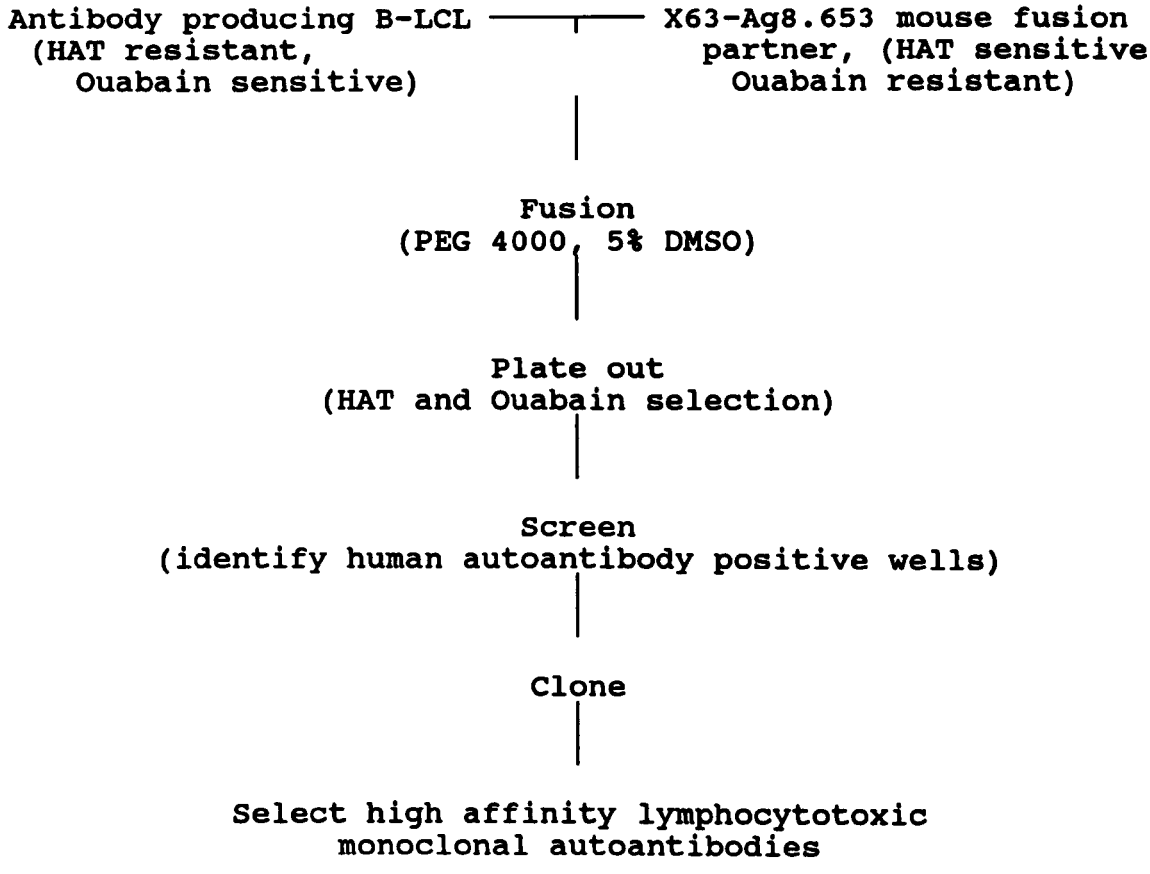


Fig 2.2.2 **Production of mouse/human heterohybridoma**



All the following procedures were carried out using aseptic technique, performed in a class II tissue culture cabinet (MHD Microflow Pathfinder).

2.2.2 **Reagents**

a) Growth medium:

RPMI 1640 supplemented with 10% heat inactivated foetal calf serum (HI-FCS, treated at 56°C for 20 minutes), 2mM L-Glutamine and 100 units/ml of Penicillin and Streptomycin (Gibco).

b) 2x Concentrated selection medium:

2ml of x100 concentrated HAT medium (0·08g hypoxanthine, 0·012g thymidine, 0·3g glycine, 0·002g aminopterin in 100ml of PBS-DAB) was added to 100ml of growth medium supplemented with 2ml of 0·1mM ouabain (Sigma, 7·29mg in 100ml PBS-DAB), giving 2x concentrated HAT and ouabain.

c) Fusion medium:

5ml of polyethylene glycol (PEG 4000, autoclaved for 15 minutes at 15 PSI, Merck) was mixed with 5ml of 2x concentrated RPMI 1640 supplemented with 10% Dimethyl Sulfoxide (DMSO, Sigma), 4·0mM L-Glutamine and 200 units/ml Penicillin and Streptomycin.

d) Mouse spleen feeder cells:

Freshly killed Balb-C mice, between 8 and 13 weeks old, were immersed in 70% alcohol for 1 minute, transferred to sterile 10cm petri dishes (Sterilin), and excess alcohol removed with sterile gauze swabs. To a second petri dish, 10ml of growth medium was added, ready to receive the spleen. An incision was made along the mid line of the abdomen, through the skin without penetrating the muscle layer. The skin was then parted and a second incision made through the muscle layer entering the abdomen, cutting up to the chest. The abdomen was opened and the spleen located (under rib cage on right side), removed (taking care not to rupture the gut wall), and transferred to the second petri dish.

The spleen was transversely dissected, each half was held at the tail, and using curved forceps the spleen cells were teased out by gently stroking towards the open end. Clumps of cells were dispersed by passage through a fine needle (21 gauge) and transferred to 15ml conical tubes (Falcon

plastics, Becton Dickinson) and washed twice in medium. The cell pellets were resuspended in 10ml of growth medium and the white cell population counted. The cells were irradiated with 2000 rads (500 rads/minute for 4 minutes, Gammacell 1000, Atomic Energy of Canada Ltd), centrifuged and resuspended at a concentration of $3x10^6$ white cells/ml in growth medium. Mouse spleen feeder cells were kept at 37°C for up to one week before use. The white cell yields were between $0{\cdot}6x10^8$ and $1{\cdot}0x10^8$ per spleen.

2.2.3 **Methods**

a) Generation of EBV transformed lymphoblastoid cell lines:

Peripheral blood lymphocytes were prepared from 20ml of heparinized blood by density gradient centrifugation as above.

PBL's were counted and then centrifuged in a 15ml conical tube. The lymphocyte pellet was resuspended in 1ml of tissue culture supernatant containing EBV (derived from EBV secreting marmoset cell line B95-8), and incubated for one hour at 37°C. The volume was made up to give a final concentration of $1x10^6$ cells/ml using growth medium supplemented with phytohaemagglutinin (PHA, HA-15, Wellcome Diagnostics, Dartford, reconstituted with 5ml dH_2O) at a dilution of 1/200. 1ml volumes of cell suspension were dispensed into the wells of a 24 well tissue culture tray (Nunc, Gibco). The trays were placed in a 37°C incubator with a humidified, 5% CO_2 atmosphere. The B-LCL's were fed after five days, then as necessary thereafter, replacing half the volume with fresh growth medium. When the cells reached >75% confluence they were transferred to 50ml and subsequently 250ml flasks and maintained at a concentration of $5x10^5$ to $1x10^6$/ml.

b) Production of heterohybridoma:

Fusion of the B-LCL with the mouse myeloma cell line X63-Ag8.653 (a kind gift from K.M.Thompson, MRC Unit, Cambridge) was carried out according to the method of Thompson (Thompson et al, 1986). Heterohybridoma selection was carried out in Hypoxanthine, Aminopterin, Thymidine (HAT) and Ouabain.

Both cell populations were subcultured for two consecutive days prior to fusion to obtain logarithmic growth. Equal numbers ($1x10^7$) of X63-Ag8.653 and B-LCL were pooled and centrifuged in a 50ml tube (Falcon). The cell pellet was loosened by gentle flicking and 1ml of fusion medium at 37°C was added drop-wise over a 60 second period with continuous agitation. The cells were placed in a 37°C waterbath for 90 seconds followed by the addition of 10ml of RPMI 1640 containing 25mM HEPES (Gibco) at 37°C, also added drop-wise over a 90 second period with continuous agitation. The suspension was then centrifuged at 340G for 5 minutes at 22°C and the cell pellet resuspended in 62ml of growth medium containing $1x10^6$/ml irradiated mouse spleen feeder cells, to give a final concentration of $1{\cdot}5x10^5$ of fused cells/ml. 1ml volumes were dispensed into the wells of 24 well trays (Nunc) which were incubated as above. After 24 hours, 1ml of 2x concentrated selection medium was added to each well. Cells were maintained in selection medium for two weeks post fusion, after which growth medium was used.

c) Cloning of heterohybridoma:

i) Clone picking: Macroscopically visible cell clusters were identified by viewing the trays next to a bright light source and their positions indicated by marking the underside of the trays with a marker pen. Individual clusters were carefully drawn up using a long form Pasteur pipette and transferred to individual wells of a 96 well tray. 50μl of 1x concentrated selection medium was added and after a 24 hour incubation 100μl of growth medium containing $3x10^5$ irradiated mouse spleen feeder cells was added. Picked clones were expanded into 24 well trays and subsequently into 50ml flasks.

ii) Limiting dilution: Hybridoma cells were cloned at a concentration of one cell per well in 100μl volumes of growth medium into flat bottomed 96 well tissue culture trays (Nunc), containing $3x10^5$ irradiated mouse spleen feeder cells. The trays were incubated as above for seven days, after which cells were fed initially with growth medium containing $3x10^6$/ml irradiated mouse spleen feeder cells, and subsequently with growth medium alone. When the hybridoma cells reached >75% confluence they were transferred into 24 well trays (Nunc) and subsequently into 50ml and 250ml tissue culture flasks (Nunc). Hybridoma cells were maintained at a concentration of between $1x10^5$ and $1x10^6$/ml.

d) Preparation of cell lines for cytotoxicity assays:

Cell lines were suspended at $5x10^5$/ml in 250ml tissue culture flasks (Falcon, Becton Dickinson, Oxford) in growth medium and incubated as above, being fed for two consecutive days before use, to obtain logarithmic growth phase. Cells were centrifuged, resuspended in 10ml of Terasaki Park medium and layered onto 4ml of lymphocyte separation medium in 15ml conical tubes

(Falcon) and centrifuged for 15 minutes at 500G. B-LCL's were aspirated from the interface and washed in Terasaki Park medium, then resuspended at a concentration of 1-2x10^6/ml for cytotoxicity screening.

e) Assessment of growth characteristics of heterohybridoma clones

Hybridoma cell lines (in growth phase) were counted, centrifuged and resuspended in fresh growth medium at a concentration of 5x10^4/ml. Each cell line was then dispensed in triplicate, in 1ml volumes, into 24 well culture trays (Nunc). A small aliquot (20μl) of each cell population was removed daily for five consecutive days and the number of viable cells was assessed by counting (in duplicate) on a haemocytometer, using a phase contrast microscope. A growth curve was then plotted for each cell line. To assess the antibody production relative to cell growth, supernatants from the triplicate samples were pooled and screened in duplicate, at doubling dilutions in the cytotoxicity assay.

f) DNA content analysis of heterohybridomas:

To confirm that the cell lines generated represented stable heterohybridomas and not out-growth of surviving parent cell lines, the DNA content of the parent cell lines (X63-Ag8.653 and B-LCL) and heterohybridomas were measured.

Reagents

i) Alcohol fixative:

70mls of ethanol (BDH) was mixed with 30ml dH_2O.

ii) RNAse enzyme solution:

A 10% RNAse (Sigma) stock solution was prepared by dissolving 100mg in 1ml of Terasaki Park medium and stored at -80°C. This was diluted 1/10 (0·5ml + 4·5ml) with medium before use.

iii) Staining solution:

2·5mg of propidium iodide (Sigma) was dissolved in 10ml of Terasaki Park medium.

Method

Cell lines were prepared as for the cytotoxicity method and resuspended at $2x10^6$/ml in Terasaki Park medium. Duplicate 250μl aliquots of each cell population were centrifuged, the pellets resuspended in ice cold alcohol fixative, and incubated for 15 minutes. The samples were then washed three times in 4ml volumes of Terasaki Park medium, by centrifuging at 400G for 5 minutes and resuspended in 200μl of medium. To this, 200μl of 1% RNAse solution and 100μl of staining solution was added, and the cells incubated at 37°C for 20 minutes. DNA content was then analyzed on an Ortho Cytofluorograph.

g) Determination of monoclonal antibody class:

Single radial immunodiffusion was used to define the immunoglobulin class and light chain isotype of the monoclonal antibodies. Wells of 'low level' anti-human IgG, IgM, kappa and lambda light chain single radial immunodiffusion plates (LC-Partigen, Hoechst UK Ltd, Hounslow, Middlesex) were inoculated with 20μl (for IgG and IgM) and 5μl (for kappa and lambda) of tissue culture supernatant or control sera at the appropriate dilutions. For quantitative evaluation of IgM levels, dilutions of protein standard

serum (LC-A, human) in PBS-DAB was used. For the measurement of IgG levels, protein standard serum (LC-V, human) was used, and for kappa and lambda light chain determination standard human serum (Hoechst) was used. The dilutions of protein standards used were batch dependant and are given in the results. Trays were placed in a moist chamber at 22°C for 72 hours. Precipitation bands were intensified by the addition of 10ml of 0·1M DL-DOPA (Sigma) in PBS-DAB for 24 hours and differentiated with multiple washes of dH_2O, after which the ring diameters were measured with an accuracy of 0·01mm using a micrometer. Quantitative immunoglobulin levels were determined from a standard curve, after plotting the square of the ring diameter (mm^2, Y axis) against the standard protein concentration ($\mu g/ml$, X axis). For the kappa and lambda light chain determination it was necessary to concentrate the supernatants x5 in a B15 Minicon ultra filtration concentrator (Amicon Ltd, Stonehouse, Gloucestershire).

h) Mixed lymphocyte reaction (MLR) inhibition:

PBL's were prepared from defibrinated blood as above with the omission of platelet washes. Lymphocytes were suspended in 10ml of RPMI with 25mM HEPES (Gibco) in conical 15ml tubes (Falcon) and counted on a haemocytometer. Each sample was then divided into two 5ml aliquots and lymphocytes counted a second time. The tubes were centrifuged at 340G for 5 minutes and the lymphocytes resuspended at $1x10^6/ml$ (calculated from the mean of the two cell counts) in growth medium supplemented with 15% heat inactivated human serum (pooled from 5 blood group AB untransfused males) in place of the 10% HI-FCS. One tube of each pair was irradiated with 2000 rads (500 rads per minute for 4 minutes on a Gammacell 1000) to use as stimulators, whilst the second tube of each pair remained untreated, as a responder cell population. Cells were dispensed in 50μl volumes, in quadruplicates, into

U bottomed 96 well trays (Sterilin), using a format testing all responder and stimulator combinations three times. To the first set of trays 10μl PBS-DAB was added, to the second and third set of trays, 10μl of purified IgM at 1·0mg/ml from two autoantibody clones (F15-DD5-26 and F36-CB10-18) in PBS-DAB was added.

The trays were incubated for five days at 37°C in a humidified 5% CO_2 atmosphere, then each well was inoculated with 1μl of tritiated thymidine (methyle ^3H, 1mCi/ml, NEN, Du-Pont, Luton, Bedfordshire). The trays were incubated for a further 18 hours, after which DNA was harvested onto glass fibre filter disks (Whatman), following cell lysis with dH_2O, using a cell harvester (Skatron, Norway). The disks were air dried and transferred into 5ml scintillation vials (Sterilin), and 2ml of scintillation fluid (Econofluor-2, NEN) was added. Counts per minute (CPM) were measured on an LKB Rackbeta 1280 ß counter (Pharmacia-LKB, Milton Keynes). Mean and median values of the quadruplicate CPM were calculated, and responses of each group with MAb treatment were compared to that of the controls.

2.3 <u>Binding Assays</u>

a) <u>Cytotoxicity binding assay to select higher affinity clones</u>:

To identify strong binding (higher affinity) antibodies, a single and double wash step was introduced in the cytotoxicity test, between incubation with MAb and the addition of complement. Purified T and B lymphocytes at a concentration of $2\text{-}3x10^6$/ml were divided into 250μl aliquots. Tubes were centrifuged and the cell pellets resuspended in an equivalent volume of the appropriate MAb at dilutions of 1/2 or 1/10. Tubes were incubated at 22°C for 30 minutes and a small aliquot (50μl) was removed from each sample and stored at 22°C (no washes). The remaining

cells were diluted with 4ml of Terasaki Park medium and centrifuged. The cell pellets were resuspended in 200μl of medium and a further 50μl aliquot was removed (one wash). Cells were washed a second time as above, and the cell pellets resuspended in 150μl of medium (two washes).

The above cell aliquots (having undergone no washes, one wash and two washes after antibody treatment) were then tested in a cytotoxicity assay. Duplicate volumes (1μl) of each cell suspension were added to the wells of a microtest tray (Nunc), under liquid paraffin oil (BDH), and 5μl of rabbit complement was added. The trays were incubated for two hours at 22°C followed by staining with eosin and fixation with formalin. Viability was assessed as for the cytotoxicity test.

b) Fluorescence binding assay:

Reagents

Fixation buffer:

A solution of 0·5% bovine serum albumin (BSA) and 1·5% formalin in PBS-DAB was prepared by dissolving 0·5g BSA (Sigma) in 10ml of PBS-DAB. This was mixed with 88·5ml of PBS-DAB and 1·5ml of 40% w/v formaldehyde (BDH).

Method

Separated T and B lymphocytes were suspended at $2x10^6$/ml in Terasaki Park medium and divided into 100μl duplicate aliquots in LP4 tubes (Luckham Ltd, Burgess Hill). One pair of each cell population remained as an untreated control, whilst the other aliquots were centrifuged, the supernatants removed and cells resuspended in 100μl of MAb supernatant at the appropriate dilution in Terasaki Park medium. All aliquots were incubated on ice for 1 hour, washed once in 4ml volumes of cold PBS-DAB and

subsequently resuspended in 100μl of FITC conjugated second layer antibody. FITC-goat-anti-human IgM (Incstar Ltd, Winnersh, Berkshire), μ chain specific, at a predetermined dilution (1/50 in Terasaki Park medium) was used for human IgM MAbs. FITC-goat-anti-mouse IgG (Sigma, diluted 1/20 with Terasaki Park medium supplemented with 25% heat inactivated pooled human blood group AB serum), was used for mouse IgG MAbs. Tubes were incubated on ice for a second 1 hour period, washed once in cold PBS-DAB, and the cell pellets resuspended in 100μl of fixation buffer. Fixed samples were stored in the dark at 4°C for up to one month before fluorescence profiles were assessed on a FAScan (logarithmic profiles) or an Ortho Cytofluorograph (linear profiles) (Becton Dickinson).

c) Enzyme cleavage of lymphocyte cell surface molecules:

Purified T and B lymphocytes were treated with a series of enzymes to remove antigens from their cell surface, in order to identify the biochemical nature of the molecule(s) to which the autoantibodies bind.

i) Enzyme preparation:

1. Neuraminidase (Sigma), type V from Clostridium perfringens.

Ten units of neuraminidase were dissolved in 10ml of PBS-DAB to give a concentration of 1 unit/ml and stored at -30°C.

2. Papain (P4762, Sigma).

Papain was dissolved in PBS-DAB to give a concentration of 4mg/ml (saturating concentration, not completely soluble). To this an equal volume of L-cysteine (free base, Sigma) at 4mg/ml in PBS-DAB was added, giving a final concentration of 2mg/ml of papain and cysteine. This was stored at -30°C until use.

3. Peptidase (Sigma).

Peptidase was dissolved in PBS-DAB to give a concentration of 2mg/ml and stored in at -30°C until use.

4. Trypsin (Sigma), type XIII from bovine pancreas.

A stock solution of trypsin was prepared by dissolving 100mg in 5ml of PBS-DAB to give a concentration of 20mg/ml. This stock solution was stored at -80°C. For use the stock solution was diluted 1/8 in PBS-DAB to give a final concentration of 2·5mg/ml (30,000 units/ml).

5. Lipase (Sigma), type I from wheat germ.

Lipase was dissolved at a concentration of 2mg/ml in PBS-DAB (18·5 units/ml) and stored at -30°C.

6. α-Amylase (Sigma), type I-A from porcine pancreas.

25mg of amylase was made up to 4ml in PBS-DAB to give a concentration of 6·25mg/ml and stored at -30°C. For use, this was diluted 1/3·1 to give a final concentration of 2mg/ml.

Method

Aliquots (1·5ml) of separated T and B lymphocytes were suspended at a concentration of $2x10^6$/ml in Terasaki Park medium. Cells were centrifuged and cell pellets resuspended in an equivalent volume of the appropriate enzyme, or PBS-DAB as a negative control, and incubated for 1 hour at 37°C. Enzyme treated cells were washed twice in PBS-DAB at 4°C, the pellets resuspended in cold FCS and incubated for 20 minutes on ice. The cells were washed in PBS-DAB at 4°C, resuspended in the original volume of cold

Terasaki Park medium and kept on ice. Cells were dispensed in 100μl aliquots and analyzed for antibody binding properties in a fluorescence binding assay.

d) Autoantibody binding inhibition assays:

Lymphocytes, at a concentration of $2x10^6$/ml were divided into 1·5ml aliquots in LP4 tubes (Luckham Ltd). Cells were centrifuged and the pellets resuspended in 1·5ml volumes of blocking agent. The inhibition agents used were; PBS-DAB (negative control); human IgG (Sigma) 0·5mg/ml in PBS-DAB; human IgM (Dako) 0·5mg/ml in PBS-DAB; lipid A (Sigma) 0·2mg/ml in PBS-DAB; or phosphatidylcholine (PtC, Sigma) prepared by warming PtC to 37°C and adding 1·0g to 10ml of pre-warmed PBS-DAB and vortexing to an emulsion.

Cells, suspended in the appropriate inhibition test reagents were dispensed in 100μl volumes in LP4 tubes. Autoantibody binding inhibition was analyzed in a fluorescence binding assay as above.

e) Biotin/FITC-avidin binding assay:

Biotin-avidin binding assays were performed as above using directly biotinylated primary MAbs, with the addition of 50μl of FITC-avidin (Sigma) at a predetermined dilution (1/20 in Terasaki Park medium), as the second layer reagent.

To confirm successful biotinylation, without the loss of antibody binding activity, biotinylated antibodies also were tested by conventional cytotoxicity, and by binding assay using FITC-goat-anti-human IgM second layer antibody, as previously described.

f) Competitive inhibition binding assay:

i) Inhibition by autoreactive MAbs:

PBL at a concentration of $2x10^6$ml were dispensed in 20 duplicate 100μl aliquots in LP4 tubes (Luckham Ltd). Tubes were centrifuged, and the cell pellets resuspended in 100μl volumes of the appropriate blocking agent according to the protocol (Table 2.3.1). Five pairs were suspended in PBS-DAB; five pairs in F36-CB10-18 purified IgM (0·05mg/ml in PBS-DAB); five in F15-DD5-26 purified IgM (0·05mg/ml in PBS-DAB) and five in FOM-A (human IgM anti-RhD) neat supernatant (0·05mg/ml). Cells were incubated for one hour on ice followed by the addition of 100μl of second antibody (F36-CB10-18 IgM biotinylated at 0·1mg/ml and 0·05mg/ml, or F15-DD5-26 IgM biotinylated at 0·1mg/ml and 0·05mg/ml) (Table 2·3.1). Negative control samples remained in PBS-DAB. Following a further one hour incubation on ice, cells were centrifuged, washed twice in 4ml volumes of cold PBS-DAB and cell pellets resuspended in 50μl of FITC-avidin (Sigma), diluted 1/20 with Terasaki Park medium. Cells were then washed twice, and cell pellets fixed in 100μl volumes of fixation buffer.

Inhibition results were expressed as percent binding of the test result (biotinylated MAb in the presence of inhibiting IgM) compared to the untreated control (PBS-DAB alone).

$$\% \text{ binding} = \frac{\text{MCF of test - MCF background}}{\text{MCF of control (PBS-DAB) - MCF background}} \times 100$$

MCF =mean channel fluorescence

Table 2.3.1

Protocol for cross inhibition of biotinylated MAbs

Tube No.	Inhibiting antibody	2nd incubation
1 & 2	PBS-DAB	-
3 & 4	PBS-DAB	CB10-18 IgM biotin 0.1mg/ml
5 & 6	PBS-DAB	CB10-18 IgM biotin 0.05mg/ml
7 & 8	PBS-DAB	DD5-26 IgM biotin 0.1mg/ml
9 & 10	PBS-DAB	DD5-26 IgM biotin 0.05mg/ml
11 & 12	CB10-18 IgM 0.05mg/ml	-
13 & 14	CB10-18 IgM 0.05mg/ml	CB10-18 IgM biotin 0.1mg/ml
15 & 16	CB10-18 IgM 0.05mg/ml	CB10-18 IgM biotin 0.05mg/ml
17 & 18	CB10-18 IgM 0.05mg/ml	DD5-26 IgM biotin 0.1mg/ml
19 & 20	CB10-18 IgM 0.05mg/ml	DD5-26 IgM biotin 0.05mg/ml
21 & 22	DD5-26 IgM 0.05mg/ml	-
23 & 24	DD5-26 IgM 0.05mg/ml	CB10-18 IgM biotin 0.1mg/ml
25 & 26	DD5-26 IgM 0.05mg/ml	CB10-18 IgM biotin 0.05mg/ml
27 & 28	DD5-26 IgM 0.05mg/ml	DD5-26 IgM biotin 0.1mg/ml
29 & 30	DD5-26 IgM 0.05mg/ml	DD5-26 IgM biotin 0.05mg/ml
31 & 32	FOM-A spnt neat	-
33 & 34	FOM-A spnt neat	CB10-18 IgM biotin 0.1mg/ml
35 & 36	FOM-A spnt neat	CB10-18 IgM biotin 0.05mg/ml
37 & 38	FOM-A spnt neat	DD5-26 IgM biotin 0.1mg/ml
39 & 40	FOM-A spnt neat	DD5-26 IgM biotin 0.05mg/ml

ii) Inhibition of autoreactive monoclonal antibodies by patient serum and serum proteins:

The ability of autoantibodies from renal dialysis patient serum and other serum proteins, to inhibit monoclonal autoantibody binding was assessed using binding inhibition of biotinylated autoreactive MAbs, tested against T and B cells according to the protocol in Tables 2.3.2 and 2.3.3.

Table 2.3.2

Protocol for inhibition of biotinylated MAbs by patients' sera

Tube No.	Inhibiting antibody	2nd incubation
1 & 2	PBS-DAB	-
3 & 4	PBS-DAB	CB10-18 IgM biotin 0.1mg/ml
5 & 6	PBS-DAB	CB10-18 IgM biotin 0.05mg/ml
7 & 8	PBS-DAB	DD5-26 IgM biotin 0.1mg/ml
9 & 10	PBS-DAB	DD5-26 IgM biotin 0.05mg/ml
11 & 12	AB serum	-
13 & 14	AB serum	CB10-18 IgM biotin 0.1mg/ml
15 & 16	AB serum	CB10-18 IgM biotin 0.05mg/ml
17 & 18	AB serum	DD5-26 IgM biotin 0.1mg/ml
19 & 20	AB serum	DD5-26 IgM biotin 0.05mg/ml
21 & 22	Patient serum (DV)	-
23 & 24	Patient serum (DV)	CB10-18 IgM biotin 0.1mg/ml
25 & 26	Patient serum (DV)	CB10-18 IgM biotin 0.05mg/ml
27 & 28	Patient serum (DV)	DD5-26 IgM biotin 0.1mg/ml
29 & 30	Patient serum (DV)	DD5-26 IgM biotin 0.05mg/ml
31 & 32	Patient serum (EW)	-
33 & 34	Patient serum (EW)	CB10-18 IgM biotin 0.1mg/ml
35 & 36	Patient serum (EW)	CB10-18 IgM biotin 0.05mg/ml
37 & 38	Patient serum (EW)	DD5-26 IgM biotin 0.1mg/ml
39 & 40	Patient serum (EW)	DD5-26 IgM biotin 0.05mg/ml

Table 2.3.3

Protocol for inhibition of biotinylated MAbs by human serum proteins

Tube No.	Inhibiting antibody	2nd incubation
1 & 2	PBS-DAB	-
3 & 4	PBS-DAB	CB10-18 IgM biotin 0.1mg/ml
5 & 6	PBS-DAB	CB10-18 IgM biotin 0.05mg/ml
7 & 8	PBS-DAB	DD5-26 IgM biotin 0.1mg/ml
9 & 10	PBS-DAB	DD5-26 IgM biotin 0.05mg/ml
11 & 12	Albumin 60mg/ml	-
13 & 14	Albumin 60mg/ml	CB10-18 IgM biotin 0.1mg/ml
15 & 16	Albumin 60mg/ml	CB10-18 IgM biotin 0.05mg/ml
17 & 18	Albumin 60mg/ml	DD5-26 IgM biotin 0.1mg/ml
19 & 20	Albumin 60mg/ml	DD5-26 IgM biotin 0.05mg/ml
21 & 22	Albumin 40mg/ml	-
23 & 24	Albumin 40mg/ml	CB10-18 IgM biotin 0.1mg/ml
25 & 26	Albumin 40mg/ml	CB10-18 IgM biotin 0.05mg/ml
27 & 28	Albumin 40mg/ml	DD5-26 IgM biotin 0.1mg/ml
29 & 30	Albumin 40mg/ml	DD5-26 IgM biotin 0.05mg/ml
31 & 32	Human IgM 1.0mg/ml	-
33 & 34	Human IgM 1.0mg/ml	CB10-18 IgM biotin 0.1mg/ml
35 & 36	Human IgM 1.0mg/ml	CB10-18 IgM biotin 0.05mg/ml
37 & 38	Human IgM 1.0mg/ml	DD5-26 IgM biotin 0.1mg/ml
39 & 40	Human IgM 1.0mg/ml	DD5-26 IgM biotin 0.05mg/ml
41 & 42	Human IgG 13mg/ml	-
43 & 44	Human IgG 13mg/ml	CB10-18 IgM biotin 0.1mg/ml
45 & 46	Human IgG 13mg/ml	CB10-18 IgM biotin 0.05mg/ml
47 & 48	Human IgG 13mg/ml	DD5-26 IgM biotin 0.1mg/ml
49 & 50	Human IgG 13mg/ml	DD5-26 IgM biotin 0.05mg/ml

g) Lectin inhibition of autoantibody binding:

i) Using fluorescence binding assay: Purified T and B lymphocytes at a concentration of $2x10^6$/ml were dispensed in 1·5ml aliquots into LP4 tubes (Luckham Ltd). Tubes were centrifuged and the cell pellets resuspended in the appropriate lectin (0·2mg/ml in PBS-DAB), and one aliquot in PBS-DAB as an untreated control. Cells were incubated at 22°C for one hour and washed twice in 4ml volumes of PBS-DAB. Cell pellets were resuspended in 1·5ml of Terasaki Park medium (clumps were dispersed on a vortex) and dispensed in 100μl aliquots into LP4 tubes. Duplicate samples were tested for inhibition of autoantibody binding in a fluorescence binding assay as

above. The lectins used in this study are shown in Table 2.3.4.

Table 2.3.4 Lectin Blocking

Lectin	Specificity	Blood Group	Mitogen
Triticum vulgaris	Neu NAc, NAc-D-Glc	A	+
Mycoplasma gallisepticum	Glycophorin	O	
Ptilota plumosa	α-D-gal	B	
Sophora japonica	ß-D-gal NAc	A,B	
Vicia faba	D-man, D-glc	O	+
Ulex europaeus	α-L-fuc	O	+
Euronymus europaeus	α-D-gal(1-3)-D-gal	H,B	+
Concanavalin A	α-D-man, α-D-glc	A	+
Lens culinaris	α-D-man	A	+
Phaseolus vulgaris	Oligosaccharides	A	+
Dolichos biflorus	α-D-gal NAc	A1	
Arachis hypogaea	ß-D-gal(1-3)-D-gal NAc	O	+
Helix pomatia	D-gal NAc	A	

ii) Red blood cell agglutination assay: 5ml volumes of heparinized blood were dilute with equal volumes of PBS-DAB and centrifuged at 600G for 5 minutes. The plasma and buffy coats were removed and the red cells washed twice, as above. A 2% RBC suspension was prepared by diluting 1ml of packed RBC's with 50ml of PBS-DAB.

Serial dilutions of the MAbs (50μl volumes diluted in PBS-DAB) were prepared in duplicate along the 12 rows of U bottomed 96 well trays (Nunc, Gibco), from neat to 1/2048 dilution. 50μl volumes of PBS-DAB were used as a negative control. Equal volumes of 2% RBC's were added and the cells incubated at 22°C for 1 hour, after which each well was assessed for RBC agglutination. Agglutination (positive) was seen as an even layer of RBC's covering the bottom of the well. No agglutination (negative) was seen as a central "spot" of RBC's.

iii) Inhibition of lectin agglutination of RBC by autoantibodies: Lectins were diluted to a concentration of 50μg/ml with PBS-DAB (1/4 from stock solution), and 50μl volumes were added to serial dilutions of MAbs prepared as above, in 96 well U bottomed trays. Trays were incubated for 1 hour at 22°C followed by the addition of 50μl of 2% RBC solution of the appropriate blood group. Trays were incubated for a further 1 hour at 22°C after which each well was examined for RBC agglutination as above.

iv) Detection of autoantibody binding to lectin coated RBC by cytotoxicity: 50μl volumes of 2% RBC in PBS-DAB, were dispensed into 96 well trays. Equal volumes of the appropriate lectins (0·05mg/ml in PBS-DAB) were added and the RBC's were allowed to agglutinate. Following an incubation of 1 hour at 22°C, 50μl volumes of MAbs at the appropriate dilution were added to duplicate wells. MAbs, titred at doubling dilution in PBS-DAB from neat to 1/2048 were used with PBS-DAB as a negative control. The trays were incubated at 22°C for one hour followed by the addition of 100μl of rabbit complement and a further incubation for 1 hour at 22°C. Positive cell lysis was assessed as no visible RBC's, whereas negative wells were seen as agglutinated RBC's. Additional controls with MAbs tested as above with the addition of 50μl of PBS-DAB in place of the lectin were included.

h) Sialic acid inhibition of autoantibody binding:

i) Cytofluorograph analysis; Sialic acid (N-Acetyl Neuramic acid (NANA), Sigma, type IV) was dissolved in Terasaki Park medium at concentration of 25·0 and 12·5mg/ml. The pH was measured and found to be 4·0. For comparison as an untreated control, the pH of Terasaki Park medium was adjusted to pH4·0 by the addition of 2μl of 2·5M HCl to 5ml of medium. The monoclonal antibodies at neat and 1/5 dilution (100μl volumes) were mixed with an equal volume of sialic acid at 25·0 and 12·5mg/ml, giving MAb dilutions 1/2 and 1/10. Duplicate samples of each combination were tested against T and B cells for inhibition of autoantibody binding in a fluorescence binding assay as above.

ii) Cytotoxicity; 1μl volumes of sialic acid at the appropriate concentrations were added to 1μl of MAbs at serial dilutions from neat to 1/128 in the wells of a microtest tray. Following incubation for one hour at 22°C, 1μl of T or B cells were added in a standard microlymphocytotoxicity test. As an untreated control, 1μl of Terasaki Park medium at pH4·0 (as above) and pH7·2 were added. Cytotoxicity titres were compared to the untreated controls.

I) Effect of pH on autoantibody binding:

Purified T lymphocytes at $2x10^5$ cells per tube were tested in a fluorescence binding assay as above, using 100μl volumes of F15-DD5-26 and F36-CB10-18 at doubling dilutions from 1/2 to 1/256, diluted in buffered saline at pH4·0 (18ml of 0·2M sodium acetate mixed with 82ml of 0·2M acetic acid), pH7·2 (PBS-DAB) and pH9·6 (45ml of 0·1M sodium carbonate mixed with 55ml of 0·1M sodium bicarbonate).

2.4 Immunochemical Techniques

2.4.1 Strategy for batch purification of IgM from tissue culture supernatant:

Low concentrations of IgM (50mg/l) were to be purified from large volumes of tissue culture supernatant. In the absence of preparative scale HPLC equipment, ion exchange chromatography using Bakerbond ABx 40μm ion exchange resin was selected. The manufactures claimed batch purification of IgM could be achieved using a slurry technique, giving 80 to 95% immunoglobulin yield and 97% purity. This method required no specialised equipment and as the ion exchange resin was reusable the technique was relatively cheap. However, application of the technique required adaptation of the manufacturers original specifications and the addition of a gel filtration step to obtain acceptable IgM purity.

2.4.2 Reagents:

a) 25mM (2[N-Morpholino]ethane-sulphonic acid (MES) pH5·5:

MES (Sigma, Poole), free acid, molecular weight 195·9.

A stock solution of 0·1M MES was made by dissolving 9·795g in 500ml of dH_2O.

25mM MES pH5·5 was prepared by mixing 250ml of 0·1M stock MES with 745ml of dH_2O. The solution was adjusted to pH5·5 by adding 1·0M NaOH drop-wise with continuous mixing (approximately 3ml) and the final volume was made up to 1 litre with dH_2O. The pH was checked and adjusted appropriately immediately prior to use.

b) 20mM sodium acetate in 500mM ammonium sulphate, pH6·8:

Sodium acetate (CH_3COO Na, BDH Analar) molecular weight 82·03. Ammonium sulphate ($[NH_4]_2SO_4$, BDH Analar) molecular weight 132·13.

66·06g of $(NH_4)_2SO_4$ was dissolved in 930ml dH_2O. To this, 1·64g of CH_3COO Na was added. The volume was adjusted to 1 litre with dH_2O. About 0·2ml of 1·0M NaOH was added drop-wise with continuous stirring to give pH 6·8.

c) 200mM Ammonium sulphate:

13·213g of $(NH_4)_2SO_4$ was dissolved in a final volume of 500ml dH_2O.

d) 25mM MES pH<4·0:

100ml of 0·1M stock MES was made up to 400ml with dH_2O (pH=3·6).

e) 10% Sodium azide:

Sodium azide (NaN_3, Sigma). 10g of sodium azide was dissolved in a final volume of 100ml of PBS-DAB. This was diluted 1/100 (0·1% final concentration) for use. (Caution; extremely toxic).

2.4.3 **Methods**

a) Preconditioning of Bakerbond ABx 40μm ion exchange resin:

The method was adapted for use from that described by the manufacturers. The volumes given for IgM purification are for 10ml of 10x concentrated tissue culture supernatant. Where different volumes of supernatant were used, the amount of resin and reagents were adjusted proportionately.

i) 4·0g of Bakerbond ABx40μm ion exchange resin (Technicol, Stockport) was equilibrated in 20ml of 25mM MES pH5·5 (solution A) in a 50ml tube (Falcon)

with continuous mixing, for five minutes.

ii) 2·0ml of heat inactivated foetal calf serum (HI-FCS, Gibco) was added and mixed for a further five minutes. The resin was allowed to settle and the supernatant decanted and discarded. The hydrated volume of ABx40μm resin was measured for future re-use (1·0g of equilibrated ABx40μm =2·8ml).

iii) The resin was washed in 40ml of 25mM MES pH5·5 (solution A) and mixed for two minutes. The resin was then allowed to settle and the supernatant discarded.

iv) 40ml of 20mM sodium acetate in 500mM ammonium sulphate pH6·8 (solution B) was added with continuous mixing for two minutes in a 250ml flask. 40ml of dH_2O was added, the resin mixed, allowed to settle, and the supernatant discarded.

v) 80ml of 25mM MES pH<4·0 (solution D) was added and mixed for two minutes. The resin was allowed to settle and the supernatant discarded.

vi) The resin was washed in 80ml of 25mM MES pH5·5 (solution A), mixed for two minutes, allowed to settle and the supernatant decanted. The pH of the supernatant was checked and found to be 5·5. The resin was stored in 25mM MES pH5·5 with 0·1% sodium azide until use.

b) Batch purification of IgM from tissue culture supernatant:

i) 10ml of 10x concentrated tissue culture supernatant was mixed with 30ml of 25mM MES pH<4·0 (solution D) in a 250ml flask, giving pH≈6·0. To this, 45ml of 25mM MES pH5·5 (solution A) was added, giving pH5·5.

ii) The diluted supernatant was mixed with 4·0g of equilibrated Bakerbond ABx40μm ion exchange resin. 15ml of 200mM ammonium sulphate (solution C) was added, giving a final concentration of 30mM ammonium sulphate. This was mixed continuously for 10 minutes and the resin allowed to settle for 10 minutes.

iii) The immunoglobulin depleted supernatant was decanted and the resin was washed, with the addition of 80ml of 25mM MES pH5·5 (solution A). The resin was allowed to settle and the supernatant discarded.

iv) 40ml of 20mM sodium acetate in 500mM ammonium sulphate pH6·8 (solution B) was added and mixed for 10 minutes. The resin was allowed to settle and the immunoglobulin containing fraction was decanted and stored. This step was repeated a second time to ensure complete elution of immunoglobulin from the resin.

v) The immunoglobulin containing fraction was then concentrated through a YM10 ultrafiltration membrane (Amicon) with positive pressure, to a volume of 4ml.

c) Purification of IgM by gel filtration chromatography:

i) Column preparation:

200ml of pre-swollen Sephacryl S300 (High resolution, Pharmacia-LKB, Milton Keynes) was washed in 400ml of buffer (PBS-DAB pH7·2) and allowed to settle (gel volume ≈150ml). The excess buffer was decanted and the gel equilibrated in PBS-DAB by washing twice more, as above. The gel was resuspended in 150ml of buffer and degassed under a vacuum in a Buchner flask for five minutes.

A 94x2cm chromatography column (Pharmacia-LKB) was mounted vertically and the lower bed support secured about 3cm from the base. The lower flow outlet was turned off and 15ml of buffer was poured into the column ensuring the release of air bubbles. The gel was gently mixed and using a glass rod the slurry was poured down the inner wall of the column, ensuring that no air was introduced. The gel was allowed to settle under gravity at room temperature, collecting excess buffer through the lower outlet. The gel was packed to about 4cm from the top of the column, ensuring the gel did not dry. The upper bed support was inserted, level with the gel bed, ensuring no air was trapped in the column.

The gel filtration column was run in an ascending direction, with the lower outlet tube placed in a reservoir containing buffer placed above the top of the column, and the upper outlet tube placed in an eluent reservoir positioned below the column. A syphoning action was started by gently drawing buffer through the upper outlet using a 10ml syringe to apply a vacuum. To equilibrate the column, buffer was allowed to flow through the column for 24 hours. The flow rate was measured at about 15ml per hour. The elution tube was then plugged until the column was ready for use.

ii) Column calibration:

High molecular weight protein standards thyroglobulin, ferritin, catalase and aldolase (Pharmacia-LKB, ART No. 17-0441-01) were dissolved in PBS-DAB at a concentration of 5mg/ml (with the exception of ferritin at 1mg/ml) and allowed to stand for 10 minutes. 3ml of thyroglobulin was applied to the column through the lower bed support tube (positioned above the column) ensuring that no air was introduced. The upper outlet tube was connected to a fraction collector (Redirac 2112, LKB-Pharmacia) preset at 80 drops per tube (≈4ml per fraction). When the protein solution had been applied, the lower bed support tube was placed in a PBS-DAB reservoir placed above the column. Elution of the protein standard was monitored by determining the absorbance of the each fraction at 280nm on a spectrophotometer (LKB Pharmacia Ltd). The protein elution profile was then calculated by plotting the absorbance of each fraction against the accumulative elution volume. Each protein standard was applied in turn, as above. Following use, the column was flushed with 200ml of PBS-DAB containing 0·1% sodium azide to prevent microbial growth. Before subsequent use the column was flushed over-night with PBS-DAB to remove residual sodium azide.

iii) Sample application:

4ml of the partially purified immunoglobulin fraction (following ion exchange extraction using the Bakerbond ABx 40μm) was applied to the column as above. The elution profile was monitored as for the protein standards.

2.5 Determination of IgM purity using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.5.1 Reagents

a) Solution A. Acrylamide solution:

30% acrylamide with 0·8% bis-acrylamide. Acrylamide (BDH, No.44299). NN'-Methylene-bis Acrylamide (Sigma, M-7256).

150g of acrylamide and 4·0g of bis-acrylamide were dissolved in 300ml of dH_2O and the volume made up to 500ml. The solution was filtered (Whatman, grade 1) and stored in the dark at 4°C.

b) Solution B. Separation gel buffer:

0·75M Tris buffer pH8·8. Trizma base, molecular weight 121·1 (Sigma, T-1503).

45·413g of Trizma base was dissolved in 400ml of dH_2O and then made up to a final volume of 500ml. The solution adjusted to pH8·8 with the addition of concentrated HCl added drop-wise with continuous stirring.

c) Solution C. Stacking gel buffer:

1·00M Tris pH6·8.

60.55g of Trizma base was dissolved in a final volume of 500ml of dH_2O. The pH was adjusted to 6·8 with the addition of concentrated HCl added drop-wise with continuous stirring.

d) Solution D. 10% Sodium-dodecyl sulphate:

Sodium-dodecyl Sulphate (SDS, Lauryl sulphate, sodium salt, Sigma, L-4509). 50g of SDS was dissolved in a final volume of 500ml of dH_2O.

e) Solution E. 1% Ammonium persulphate:

1g of ammonium persulphate (BDH, No44307) was dissolved in a final volume of 100ml of dH_2O. This reagent oxidises and was therefore prepared fresh, immediately before use.

f) Running buffer. Tris-glycine pH8·3 (X10 concentrated):

Glycine (Sigma, G-7126). Ten times concentrated running buffer was prepared by dissolving 60·6g of Trizma base and 288·0g of glycine in a final volume of two litres dH_2O giving pH 8·3. For use, 500ml of x10 concentrated running buffer was diluted with 4·5 litres of dH_2O, with the addition of 5·0g (0·1%) of SDS.

g) Sample buffer:

0·2M Tris, 8·0M Urea and 2% SDS pH8·0. Urea (Sigma, U-1250) molecular weight 60·06.

4·844g of Trizma base, 96·096g of Urea and 4·0g of SDS were dissolved in a final volume of 200ml of dH_2O with continuous stirring on a warm plate.

h) Running gel:

A 10% running gel was made up according to the volumes given in Table 2.5.1.

i) Stacking gel:

A 3% stacking gel was made up according to the volumes given in Table 2.5.1.

j) Bromo-phenol blue:

Bromophenol blue (BDH, No.44305). A microspatular of bromophenol blue was dissolved in 10ml of sample buffer.

k) Coomassie blue stain:

Brilliant blue R (Sigma, B-0630). Methanol (BDH, No.10158). Acetic acid (BDH, No.27013). (Detects ~1μg ml^{-1} protein). 1·0g of Brilliant blue R was dissolved in 364ml of dH_2O. To this, 73·6ml of acetic acid and 364ml of methanol was added and the solution passed through a filter (Whatman, grade 1, medium fast).

l) Destain:

5% methanol, 7·5% acetic acid. 150ml of acetic acid and 100ml of methanol was mixed with 1750ml of dH_2O. (The gel may be stored in 7% acetic acid).

m) Sample Preparation Reagents

i) 40mM iodo-acetamide (IAA):

IAA (Sigma, I-6125) molecular weight 185.

37mg of IAA was dissolved in 5ml of sample buffer.

ii) 40mM DL-dithiothreitol:

DTT (Sigma, D-0632) molecular weight 154·2.

31mg of DTT was dissolved in 5ml of sample buffer.

iii) 0·665M IAA:

123mg of IAA was dissolved in 1ml of dH_2O.

Table 2.5.1. Recipes for Running Gel Concentrations

Solution	Percentage Gel Concentration						
	5%	7·5%	10%	12·5%	15%	20%	3%
A	10.0*	15.0	20.0	25.0	30.0	40.0	1.8
B	30.0	30.0	30.0	30.0	26.4	9.0$	-
C	-	-	-	-	-	-	2.0
D	0.6	0.6	0.6	0.6	0.6	0.6	0.16
E	3.0	3.0	3.0	3.0	3.0	3.0	0.8
dH2O	16.4	11.4	6.4	1.4	-	10.0	11.0
TEMED	40.0	40.0	40.0	40.0	40.0	40.0	30.0

* *volumes are in millilitres except TEMED for which the volumes are in microlitres (μl). The volumes given are those required for each gel and should be mixed in order as above.*

TEMED; NNN'N' Tetramethylethylene diamine (Sigma).

$ *2·5M Tris pH8·8.*

For proteins of molecular weights 10,000 to 100,000 use a 10% or 12·5% gel. For peptides a 20% gel is recommended. The 3% gel is used as a stacking gel.

2.5.2 **Method**

a) Non-reduced sample preparation:

100μl of 40mM IAA was mixed with an equal volume of test sample in 1·5ml eppendorf tubes (Beckman). The tops were sealed and a small hole pierced through each lid. The tubes were placed in a boiling water bath for two minutes.

b) Reduced samples preparation:

100μl of test sample was mixed with an equal volume of 40mM DTT and boiled as above for two minutes. 10μl of 0·665M IAA was added and the tubes were placed in a boiling water bath for a further two minutes.

c) Calibration standard preparation:

Electrophoresis calibration kit, ART No.17-0446-01, Pharmacia-LKB.

Low molecular weight standards were dissolved in 100μl of 40mM DTT and boiled as above, for two minutes. 10μl of 0·665M IAA was added and the sample boiled for a further two minutes.

All samples were coloured by the addition of 1μl of bromophenol blue to each tube, to enable visualisation of the running front during electrophoresis.

d) Gel preparation:

The 14x16cm vertical slab gel electrophoresis apparatus (Biotech Instruments LTD, Luton, SE600) was assembled according to the manufactures instructions. Before use the apparatus was washed in 70% alcohol and rinsed in dH_2O. Gloves were worn at all stages.

i) The 10% SDS running gel (Table 2.5.1), prepared immediately prior to use, was poured evenly between the glass plates to a depth of 3cm below the height of the combs. Air bubbles were removed by running a 19G (1·5 inch) needle through the gel surface.

ii) Using a Pasteur pipette, dH_2O was gently layered onto the gel to a depth of 2mm to ensure gel had a flat surface. The gel was allowed to polymerise for about 15 minutes.

iii) After polymerisation, a 3% stacking gel was prepared immediately prior to use (Table 2.5.1). Water was blotted from the running gel using an absorbent towel, taking care not to disturb the gel surface. The stacking gel was poured to a depth of 3cm and air bubbles were released as above.

iv) The combs were inserted, ensuring no air bubbles were introduced and the gel was left to polymerise for about 15 minutes.

v) Five litres of running buffer containing 0·1% SDS was prepared from the x10 concentrated stock solution, and poured into the electrophoresis tank to three quarters full. The combs were removed from the stacking gel and air bubbles floated out using running buffer. The gel plates were located in the top gel reservoir and locked into position. The base supports were removed and the apparatus was inserted into the electrophoresis tank. The top reservoir was half filled with running buffer. Air bubbles were removed from the bottom of the plates by gently "swilling" with a 5ml pipette.

e) Sample application:

Between 10μl and 100μl of non-reduced or reduced sample (2-20μg of protein per band) was added to each alternate lane using a 100μl Hamilton syringe. When possible the two outer lanes (1 and 15) were left empty and 10μl of low molecular weight calibration markers were added to lanes 2 and 14.

The lid of the electrophoresis tank was secured and the electrodes connected. The gels were run at 35mA (constant current) per gel, with continuous water cooling, until the bromophenol blue dye front was 1-2cm from the end of the gel (about 4 hours).

The current was switched off and the electrodes and water cooling disconnected. The slab gel plates were dismantled and the gel floated off the glass plates into a large flat dish containing Coomassie blue staining solution. After 18 hours the gel was placed in destain, with three changes, until the protein bands were clearly differentiated. The gels were then photographed and dried.

f) Gel drying:

Gels were place onto chromatography card (Whatman) and covered with clear film (Saran Dow, Genetic Research Ltd, Felstead). They were placed onto a slab gel dryer (Bio-Rad Laboratories), the rubber mat placed over the gels and a vacuum applied. Gels were dried at 80°C for 2 hours.

2.6 Biotinylation of purified IgM:

The method for biotinylation of IgM antibodies was carried out as described by Gretch (Gretch, Suter and Stinski, 1987) for IgG antibodies. NHS-LC-Biotin was used to label IgM purified from autoreactive MAbs F15-DD5-26 and F36-CB10-18. NHS-LC-Biotin contains an extended spacer arm approximately 22Å in length. This spacer arm reduces steric hinderance caused by glycosylated residues and certain amino acids with subsequent improvement in the biotin/avidin association.

At alkaline pH the N-Hydroxysulfosuccinimide ester (NHS) reacts with primary amine groups (NH_2) such as lysine residues on the immunoglobulin molecule. This results in amide bond formation between the biotin ester and the immunoglobulin, with the release of N-Hydroxysulfosuccinimide.

2.6.1 Reagents

a) Sodium hydrogen carbonate buffer:

$NaHCO_3$, molecular weight 84·01 (Sigma). 4·2g of $NaHCO_3$ was dissolved in 500ml of dH_2O to give a 0·1M solution, pH8·3.

b) Biotin solution:

1·63mg of NHS-LC-Biotin (Pearce, Life Science Laboratories Ltd, Luton) was dissolved in 1ml of DMSO. Biotin is readily hydrolysed, therefore to avoid condensation the biotin bottle was allowed to reach room temperature before opening and the solution was made up immediately before use.

2.6.2 **Method**

The volume of purified IgM in PBS-DAB containing 1mg of protein (as determined by optical density, assuming that 1mg/ml of protein gives an absorbance of 1·4 at 280nm) at approximately 1mg/ml was dialysed against the $NaHCO_3$ pH8·3 buffer in a Centricon-30 microconcentrator (Amicon) according to the manufactures instructions. Microconcentrators containing the PBS-DAB/IgM solution were centrifuged at 6000 rpm at 4°C in an MSE High Spin centrifuge fitted with a 8x50ml fixed angle rotor. 1ml of $NaHCO_3$ buffer was added and the process repeated three times to ensure complete removal of all the PBS-DAB.

Following the final dialysis, 1ml of $NaHCO_3$ was added and the optical density measured as above. The volume of NHS-LC-Biotin ester required to give a concentration of 30μl per mg of protein was calculated (87·9mM) and added to the MAb and incubated at room temperature for 1 hour.

The samples were then dialysed x3 against PBS-DAB in a Centricon-30 microconcentrator as above, to remove all unbound biotin, and finally resuspended in PBS-DAB with 0·1% NaN_3, at 1mg/ml. The antibodies were stored at 4°C and tested for FITC-avidin binding on a FACScan (logarithmic profiles) or an Ortho Cytofluorograph (linear profiles).

2.7 Immunoprecipitation

2.7.1 Lactoperoxidase catalysed cell surface iodination

a) Reagents

1. Glucose 18mg/ml (Sigma); 1·8g of glucose was dissolved in 100ml of PBS-DAB.

2. Glucose oxidase (Sigma, type V from Aspergillus niger) 21 units/ml; 100μl of stock (1000 units/ml) was diluted with 4·7ml of PBS-DAB.

3. Lactoperoxidase (Sigma, from bovine milk) 0·2mg/ml; A stock solution was prepared by dissolving 5mg in 1ml of PBS-DAB. For use 100μl of stock solution was made up to 2·5ml in PBS-DAB.

b) Method; The autoantibody sensitive cell line K562 (logarithmic growth phase) was layered over lymphoprep and centrifuged as previously described. The cells were washed and $1x10^7$ cells suspended in 100μl of PBS-DAB in a 1·5ml microcentifuge tube. To this the following reagents were added; 10μl of I^{125} (1 mCi, danger extremely volatile), 30μl of glucose (18mg/ml), 30μl of glucose oxidase (21 units/ml) and 30μl of lactoperoxidase (0·2mg/ml). The prossess was performed in a fume hood designated for use with radio active isotopes. The tubes were incubated at room temperature for 15 minutes and then washed three times in cold PBS-DAB at 4°C, centrifuging for 1 minute at 13,000g.

2.7.2 Preparation of solubilized antigen

a) Reagents

Lysis Buffer; 1% NP-40 (Sigma, 1ml stock)

10mM Tris (Sigma, 0·2422g in 190ml H_2O)

150mM NaCl (Sigma 1·7532g in 200ml H_2O)

5mM EDTA (Sigma, 0·3722g in 100ml H_2O)

0·5% BSA (Sigma, 0·5g in 100ml)

Added fresh 1mM PMSF, 5mM IAA (x100 =0·0925/ml H_2O)

b) Method; The I^{125} labelled cells were centrifuged and the pellets resuspended in 1ml of lysis buffer and incubated on ice for 30 minutes. The nucleii were removed by centrifuging at 13,000g in a microcentrifuge for 5 minutes at 4°C. The supernatant was transfered into a fresh tube.

2.7.3 Immunoprecipitation

a) Reagents

i) Immune Precipitation Buffer

75ml of PBS-DAB pH7·5 was supplemented with the following:

0·1% SDS, (1ml from 10% stock solution in PBS),

1·0% NP-40, (BDH, 1ml stock),

0·5% DOC, (Sodium deoxycholate acetone purified, 0·5g),

0·02% NaN_3, (0·2ml of 10% stock solution in PBS),

100µg/ml PMSF, (Phenylmethylsulphonyl fluoride, 1ml of 100x concentrated stock solution, 10mg/ml freshly dissolved in isopropanol). The volume was made up to 100ml with PBS-DAB.

ii) Washing Buffer

100ml of PBS-DAB pH7·5 was supplemented with the following:

0·1% NP-40, (100µl stock),

100ug/ml PMSF, (1ml from 100x concentrated stock as above).

iii) Elution Buffer; 0·1M Glycine-HCl pH2·2.

A stock solution of 0·4M glycine (Sigma) was prepared by dissolving 3·0g in 100ml of H_2O. A 0·4M solution of HCl was prepared by diluting 2ml of

concentrated HCl (BDH) in 77·6ml of H_2O. To 25ml of 0·4M glycine, approximately 25ml of 0·4M HCl was added dropwise with continuous mixing to give pH2·2. The volume was then made up to 100ml with H_2O.

b) Method; Solubilized antigen was divided into two aliquots. To the first aliquot 20μl of biotinylated F15-DD5-26 (1mg/ml) was added and to the second aliquot 20μl of biotinylated F36-CB10-18 (1mg/ml) was added. Tubes were incubated overnight on ice followed by the addition of 200μl of Streptavidin-agarose. The tubes were mixed well for one hour at 4°C. The agarose bound immune complexes were washed three times using 1ml volumes of washing buffer, until no counts could be detected in the eluted washing buffer, followed by one wash with dH_2O.

0·5ml of elution buffer was added and tubes were rotated for 1 hour at 4°C. The agarose was then centrifuged and the supernatants removed. The eluted supernatant was neutralised with 50μl of 1·0M NaOH. Few counts were detectable in the supernatant, therefore the agarose was sequentially treated with 0·5ml of glycine HCL at 56°C for 15 minutes, and also boiled in DTT as previously described.

2.7.4 Autoradiography

Eluted fractions were run on an SDS-PAGE gel. The gels were stained for protein, destained and dried as previously described. Autoradiography was performed by exposing the dried gel to X-ray film (Sigma, XAR 5) placed in a cassette (Genetic Research Instrumentation Ltd, Bishops Stortford) at -80°C for seven days before developing.

Chapter 3

Generation of human monoclonal lymphocytotoxic autoantibodies

3.1 Generation of a lymphocytotoxic autoantibody producing B-LCL

3.1.1 Patient details:

Patient BN was placed on the renal transplant waiting list following end stage renal failure in 1984. He became sensitised, with the production of lymphocytotoxic autoantibodies and anti-HLA antibodies following multiple blood transfusions (15 units) and a failed renal allograft. An HLA-DR matched kidney became available in October 1986, which was found to have a current positive crossmatch with the donor B lymphocytes. The crossmatch was negative following DTT treatment, and was not blocked by monomorphic anti-HLA-ABC, DR, or DQ antibodies indicating an IgM non-HLA antibody. In addition the patients sera demonstrated cytotoxicity with autologous B lymphocytes. On the basis that this represented a non damaging lymphocytotoxic autoantibody the patient was transplanted. The post transplant course was uneventful and the patient has a serum creatinine of 164μM/l, 3 years post transplant.

In an attempt to produce a monoclonal lymphocytotoxic autoantibody this patients PBL's were EBV transformed to produce a B-lymphoblastoid cell line (B-LCL). Tissue culture supernatants were then tested for the presence of lymphocytotoxic autoantibody production.

3.1.2 Cytotoxicity screening of B-LCL (BN) supernatants:

EBV treated PBL's (BN) at a concentration of $1x10^6$/ml were initially dispensed into seven wells of a 24 well tissue culture tray. Screening of culture supernatants, harvested three weeks post transformation was performed using the microlymphocytotoxicity technique against a small panel of T cells, B cells and CLL cells. The results demonstrated typical lymphocytotoxic autoantibody profiles previously observed in renal dialysis

patient serum (Fig 3.1.1).

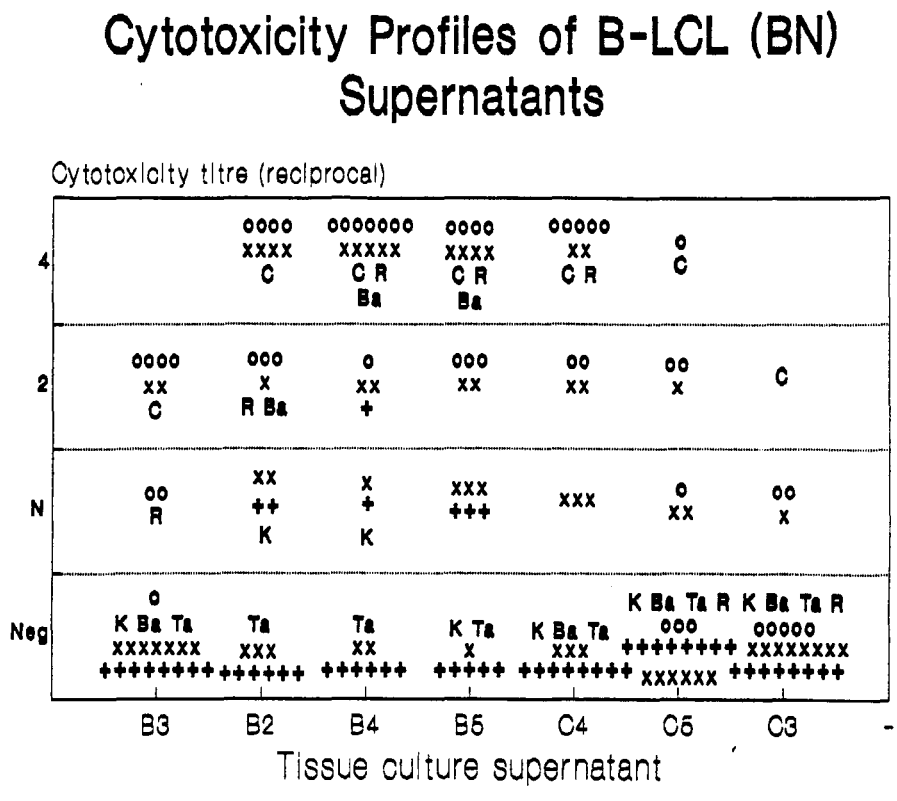


Figure 3.1.1 Cytotoxicity profiles of tissue culture supernatants derived from B-LCL (BN) harvested three weeks post transformation tested at neat, 1/2 and 1/4 dilutions against a panel of;

o = B cells, C and R =selected autoantibody sensitive CLL cells,

x = T cells, K =K562 autoantibody sensitive cell line,

+ = CLL cells Ta=autologous T cells,

Ba=autologous B-LCL.

Supernatants gave strongest cytotoxic reactivity when tested against B cells, with weaker reactivity against T cells and CLL cells. In addition, the supernatants reacted with two selected autoantibody sensitive CLL cells

(Cx and Rs), and B2 and B4 were weakly reactive with the cell line K562. Although screening was negative with the patients autologous T lymphocytes (Ta), B2, B4 and B5 had strong reactivity with the autologous B-LCL (BN). Of the seven wells, four supernatants (B2,B4,B5,C4) had lymphocytotoxic autoantibody reactivity with titres greater than 1/4 and were selected for expansion.

Antibody production was monitored by cytotoxicity, with positive flasks being subcultured twice weekly. Flasks were terminated following two consecutive negative screening results. Antibody production from wells B2 and C4 ceased after six weeks, B4 after two months, whereas B5 continued for three months. A total of 1·2 and 3·5 litres of supernatant were harvested from B4 and B5 respectively. Over the course of 3 months the initial broad panel reactivity was slowly lost. In each case, CLL and T cell reactivity was lost first, whereas B cell reactivity was maintained longest.

The cytotoxicity profiles were found to be dependant on antibody concentration. Supernatants pooled from B5 had only weak reactivity with B cells, Cx and Rs, whereas B4 was negative with all cell types. However on concentrating the supernatants 9 fold and 6 fold respectively (YM10 ultrafiltration membrane, Amicon) broad panel reactivity was obtained.

3.1.3 Determination of antibody class:

Tissue culture supernatants from wells B2, B4, B5 and C4, harvested at 4 and 6 weeks post transformation were tested by cytotoxicity against two autoantibody sensitive CLL cells (Cx and Rs), with the reducing agent DTT. Cytotoxic antibody reactivity was completely removed following DTT treatment, under conditions which reduce IgM. In control tests, reactivity of the MAb NDS-22 (IgG anti-HLA-DR, SV.Fuggle, NDS) at a dilution of 1/100 was not destroyed by DTT.

For quantitative determination of immunoglobulin levels, supernatants from exhausted cultures harvested at four weeks post transformation were tested using low level single radial immunodiffusion plates. For the determination of IgM levels, wells one, two and three were inoculated with 20μl of human standard protein (LC-A, IgM level 0·148g/l) at neat, and dilutions of 1/2 and 1/4 respectively (Fig 3.1.2). Duplicate wells (4 to 11) were inoculated with 20μl of neat tissue culture supernatant, and well 12 with fresh growth medium as a negative control. Immunoprecipitation ring diameters were measured, and the square of the ring diameters (mm^2) were plotted against the protein standard concentrations. Immunoglobulin levels of tissue culture supernatants were then determined from the calibration graph.

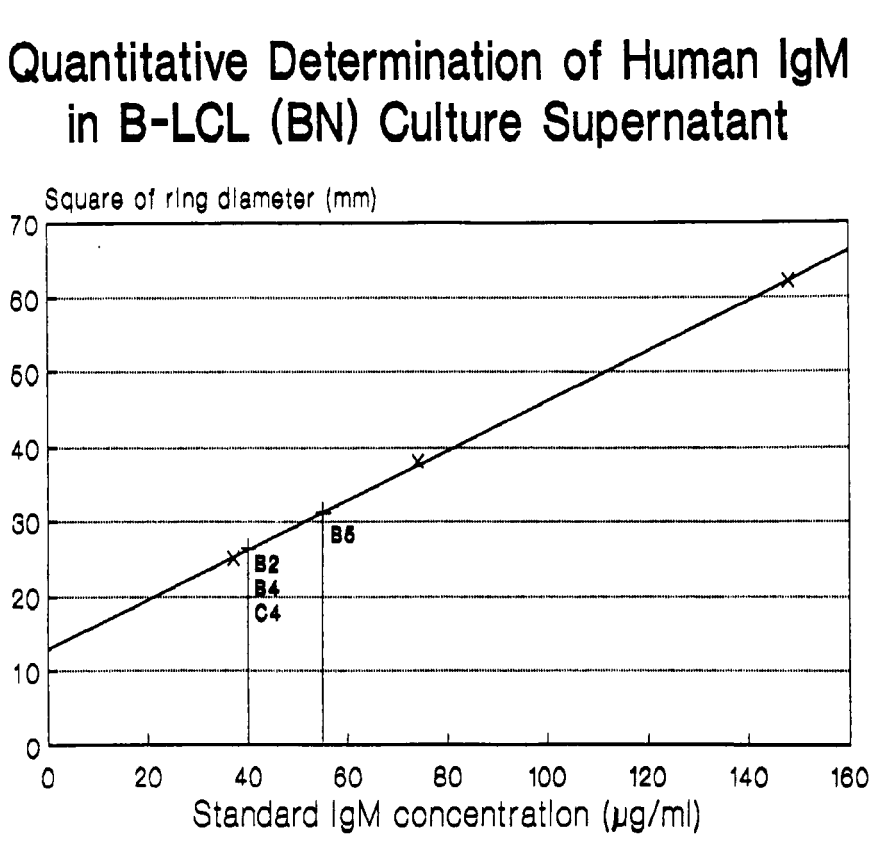


Figure 3.1.2 Quantitative determination of human IgM levels in B-LCL (BN) tissue culture supernatant harvested four weeks post transformation, measured by single radial immunodiffusion.

The results indicated total IgM levels of 40µg/ml for supernatants B2, B4, and C4 (ring diameters 5mm), and 55µg/ml (ring diameter 5·5mm) for B5. No precipitation rings were detectable in well 12 containing growth medium alone, nor in the test wells on the IgG plate, which was sensitive to levels of 8µg/ml.

3.2 Generation of lymphocytotoxic autoantibody producing heterohybridomas

3.2.1 Heterohybridoma fusion and DNA content analysis:

Antibody producing B-LCL (BN) cells pooled from wells B2,B4,B5 and C4 were fused four weeks post EBV transformation. The fused cells were dispensed into 63 wells. To establish that the cells derived represented hybridoma cells and not continued growth of parental cell lines, cells were analyzed for DNA content by flow cytometry. DNA histograms for each parental cell, fused cell lines and control cells were obtained.

Figure 3.2.1 demonstrates the DNA profile of the cell line K562. The first peak (left) represents fluorescence intensity of cells in G_0 and G_1 (resting) phase of the cell cycle. The second peak (right) indicates cells in G_2 and M (mitosis) phase and the central area are cells in S (synthesis) phase.

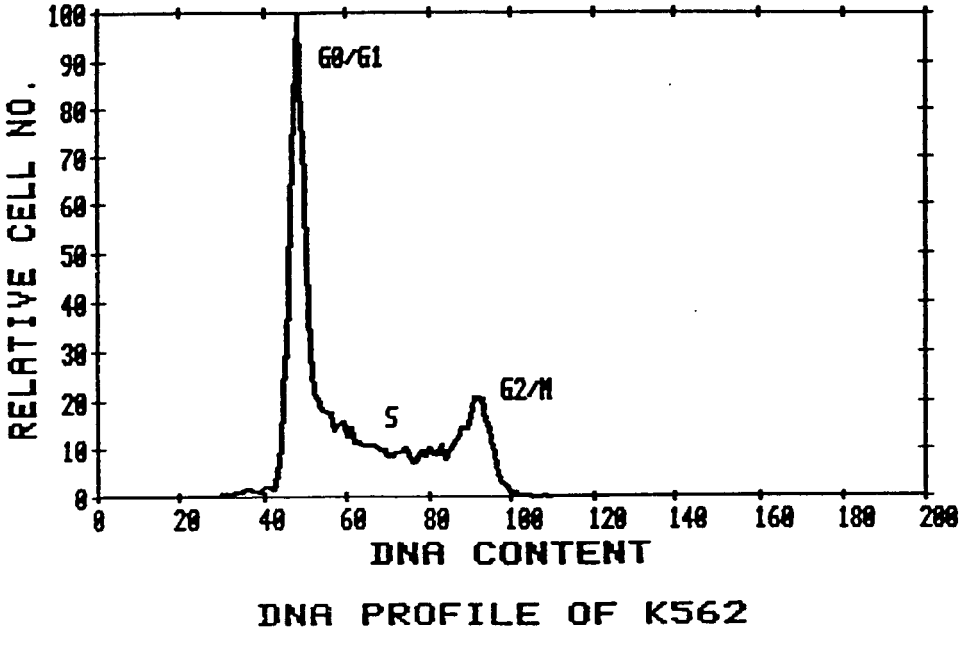
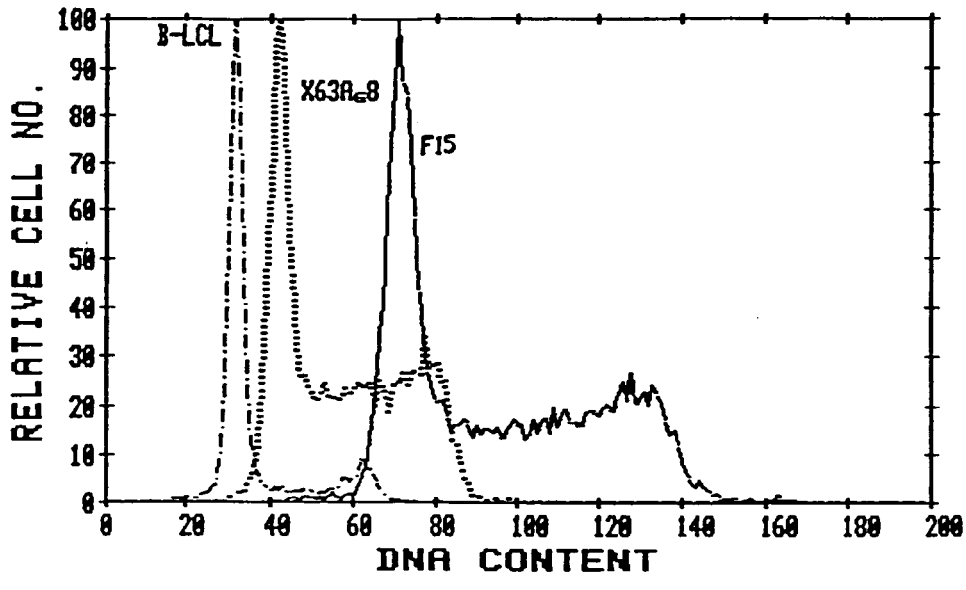


Figure 3.2.1 DNA content analysis of the cell line K562.



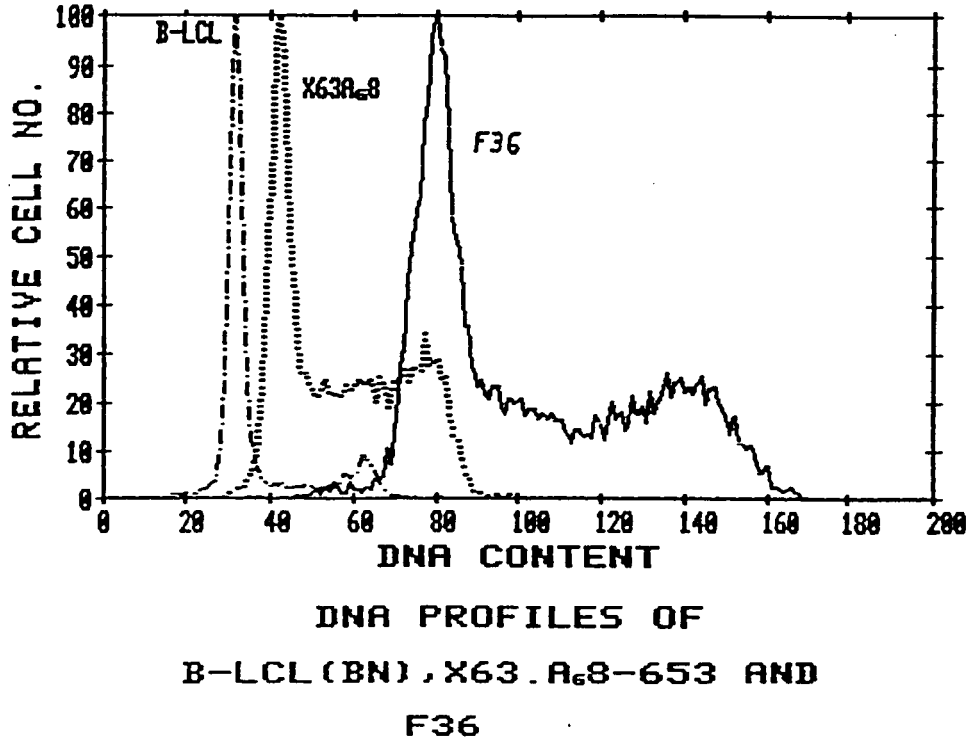


Figure 3.2.2 DNA content analysis of F15 (panel a) and F36 (panel b) compared to the parent cell lines X63.Ag8-653 and B-LCL BN.

Figure 3.2.2 shows the DNA profiles of the parental cell lines (X63.Ag8-653 and B-LCL BN) with two heterohybridoma cell lines, F15 (panel a) and F36 (panel b). Peak channel fluorescence of X63.Ag8-653 was 205 and B-LCL BN was 159, giving a total of 364. The peak channel fluorescence of F15 and F36 were 351 and 350 respectively. Therefore the heterohybridoma cells derived in HAT and Ouabain selection medium demonstrated approximately the sum DNA content of the two parental cell lines (peak channel fluorescence of 364 compared to 350). No DNA peaks representing either of the parental cells alone were seen following hybridoma selection. This indicates that parental cell lines X63.Ag8-653 and B-LCL (BN) had not survived heterohybridoma selection.

3.2.2 Heterohybridoma cloning:

Supernatants were screened 14 days post fusion when an average of 5 macroscopically visible clones could be seen in each well. Clone picking was performed three weeks post fusion. Fifty-five clones were transferred into 96 well trays, of which 35 were successfully expanded (Table 3.2.1).

Table 3.2.1 Heterohybridoma cloning and antibody screening results

Clone	No. of wells	Wells with growth	Wells expanded	No.antibody positive	Ab Class
Fusion (bulk)	63	5/well	all	15	IgM
Picked clones	55	35	35	11 (31%)	IgM
F15(1)	90	0	-	-	-
F15(2)	110	3	2	2 (100%)	IgM
F15-DD5	110	24	24	24 (100%)	IgM
F36(1)	90	8	6	5 (83%)	IgM
F36(2)	110	27	14	12 (86%)	IgM
F36-CB10	110	22	15	15 (100%)	IgM
F36-AF8	110	23	14	8 (57%)	IgM

On the basis of initial cytotoxicity profiles, two cell populations (F15 and F36) were selected for recloning. Initially cultures gave poor viability, despite attempts to maintain optimal growth conditions. F15 and F36 were cloned at one cell per well, each into 90 wells (Table 3.2.1). No clones were successfully derived from F15 and only 8 clones were derived from F36. Of the 8 F36 clones, six were successfully expanded, with two dying at an early stage. The poor cloning efficiency possibly reflects instability of the mouse/human heterohybridoma.

A second attempt at cloning into 110 wells produced 3 and 27 clones from F15 and F36 respectively (Table 3.2.1). However only 2/3 F15 clones and 14/27 F36 clones were successfully expanded.

F15-DD5, F36-CB10 and F36-AF8 were recloned into 110 wells giving 24, 22 and 23 subclones respectively (Table 3.2.1). Of these, all 24 F15-DD5 clones were successfully expanded and 15/22 and 14/23 clones were derived from F36-CB10 and F36-AF8 respectively.

The improved cloning efficiency of F15 probably reflects the derivation of stable cells following the first cloning step. Initial cloning of F15 produced only 2 stable clones but on recloning 24 subclones were derived, all being stable. However, initial cloning of F36(1) and F36(2) produced a total of 20 stable clones, and recloning generated 22 F36-CB10 and 23 F36-AF8 subclones, of which only 15 (68%) and 14 (61%) were stable respectively.

3.2.3 Cytotoxicity screening of heterohybridoma supernatants:

Following fusion, tissue culture supernatants were tested on day 14, at neat and 1/2 dilution. A small panel of two T and B cells, two CLL cells, two autoantibody sensitive CLL cells (Cx and Rs), the autologous B-LCL (BN) and autoantibody sensitive cell line K562 were used for screening. Of the 63 wells, 15 demonstrated strong positive cytotoxic antibody reactivity at both dilutions against B cells, B-LCL (BN), Cx and Rs and K562. All cytotoxicity reactivity was DTT reduced indicating IgM antibody (Table 3.2.1). Following clone picking, F36 was positive with both T and B cells and F15 was positive with B cells only. Both clones were negative with CLL cells.

After initial cloning, of the six stable clones derived from F36, 5 (83%) were antibody positive. Following the second cloning attempt 2/2 (100%) F15 clones, and 12/14 (86%) F36 clones were antibody positive (Table 3.2.1).

On recloning, 24/24 (100%) F15-DD5 subclones, 15/15 (100%) F36-CB10 subclones and 8/14 (57%) F36-AF8 subclones were antibody positive (Table 3.2.1). The low antibody producing frequency of F36-AF8 subclones may reflect derivation from an unstable clone or that it was not monoclonal.

3.2.4 Cytotoxicity profiles of heterohybridoma supernatants:

Several clones were lost at a late stage due to an incubator fault. Only seven clones which had been cryopreserved were available for testing. The cytotoxicity profiles of positive subclones were tested against a panel of T cells, B cells and CLL cells (Fig 3.2.3).

Cytotoxicity Profiles of Lymphocytotoxic Autoantibody Heterohybridoma Supernatant

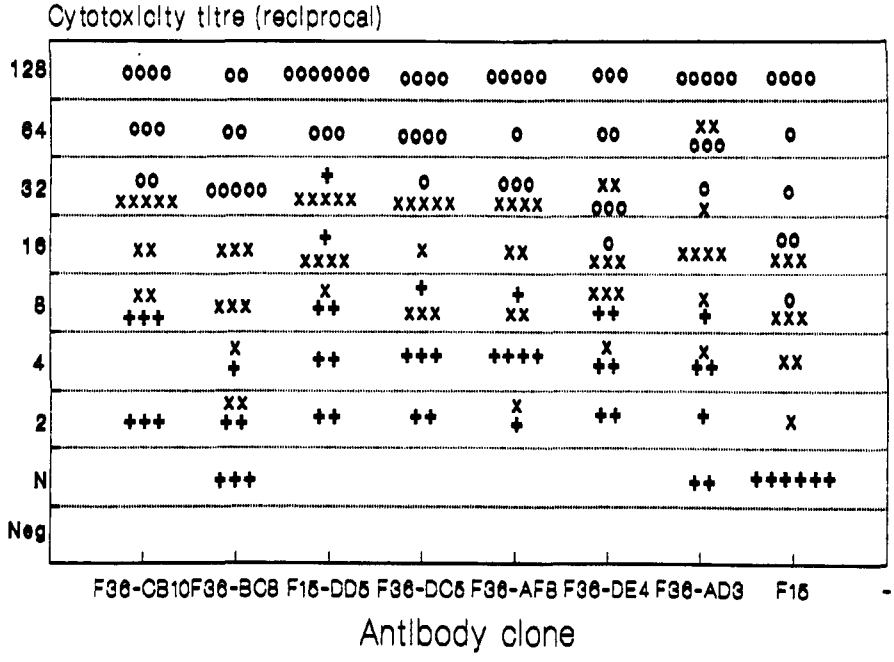


Figure 3.2.3 Cytotoxicity profiles of autoreactive heterohybridoma supernatants tested at doubling dilutions against a panel of B cells (o), T cells (x) and CLL cells (+).

The cytotoxicity profiles demonstrated similar panel reactive profiles to the B-LCL supernatants. However, cytotoxicity titres were higher with cloned heterohybridoma supernatants than those of the B-LCL supernatants. Neat supernatants gave broad panel reactive profiles being reactive with all T cells, B cells and CLL cells. On dilution, first CLL reactivity and then the T cell reactivity were lost and B cell reactivity was maintained at the highest dilutions. All clones were strongly positive with the autoantibody sensitive cell line K562 and the autologous B-LCL (data not shown). On the supposition that the antibodies are monoclonal, the three profiles described above probably represent one type of autoantibody with

differing target cell sensitivity. The initial profiles of T and B cell reactivity, or B cell reactivity only, but negative with CLL cells was dependant on antibody concentration. This data also confirms that CLL reactivity can be caused by autoreactive antibodies, although CLL cells are the least sensitive target.

3.3 Selection of clones for further study

Selection of clones for further study was assessed on the basis of; i) growth characteristics, ii) antibody binding profiles, and iii) immunoglobulin isotype.

3.3.1 Hybridoma growth characteristics:

Subclones derived from F15 and F36 demonstrated differing growth rates. In order to identify clones with good growth characteristics, cell growth was monitored over a five day period (Fig 3.3.1).

Clones F15-DD5, F36-AD3 and F36-DE4 demonstrated fastest growth characteristics. This was also evident during maintenance of bulk cultures. Clones F36-BC8, F36-CB10, F36-DC5 and F36-AF8 all demonstrated relatively slow growth characteristics. However these clones approximately doubled in cell number between days four and five. By design, cells initially in log phase were suspended at a low concentration ($5x10^4$/ml) in fresh growth medium. Growth of these cells may require a higher cell concentration and the presence of conditioned medium. Therefore optimal growth of these clones was only achieved by day four.

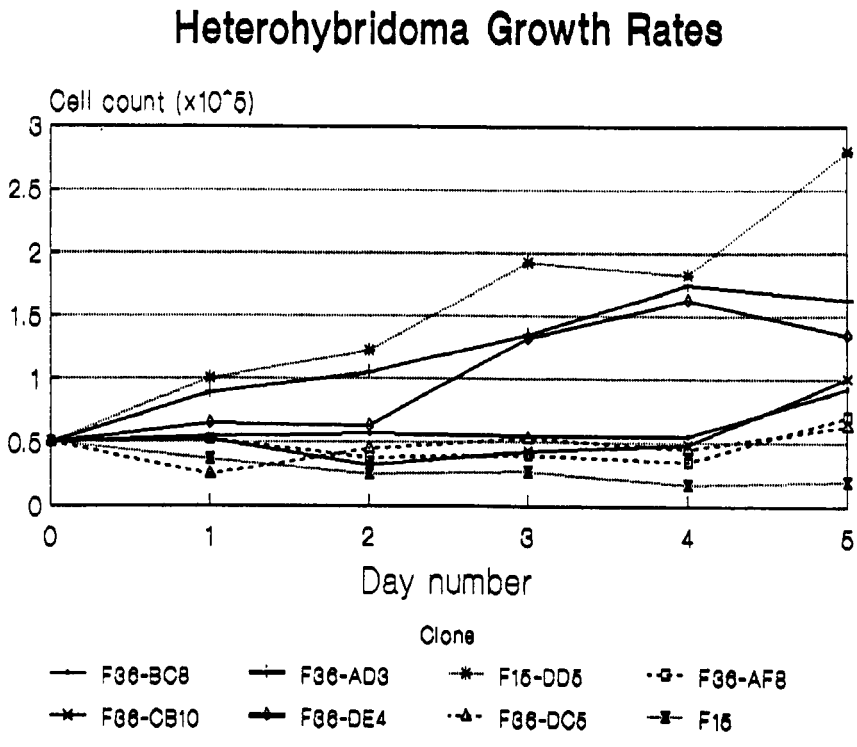


Figure 3.3.1 Analysis of heterohybridoma growth rate over a five day period to compare cell growth characteristics with immunoglobulin production and cytotoxicity profiles.

3.3.2 Antibody binding profiles:

It may be assumed that the fastest growing cells are not necessarily the best antibody producers. Therefore, following five consecutive days growth supernatants were tested by cytotoxicity against a panel of cells to assess the antibody binding profiles and titres.

Supernatants from the triplicate growth assay, harvested at five days, were individually screened against lymphocytes from two autoantibody sensitive CLL patients (Cx and Rs), two CLL patients and peripheral blood T and B

lymphocytes from three normal individuals. In addition, supernatants were also screened with K562 and the autologous B-LCL (BN). All tests were performed in duplicate. The mean of the end point titre (last dilution with positive cytotoxicity) for each cell type is shown in figure 3.3.2.

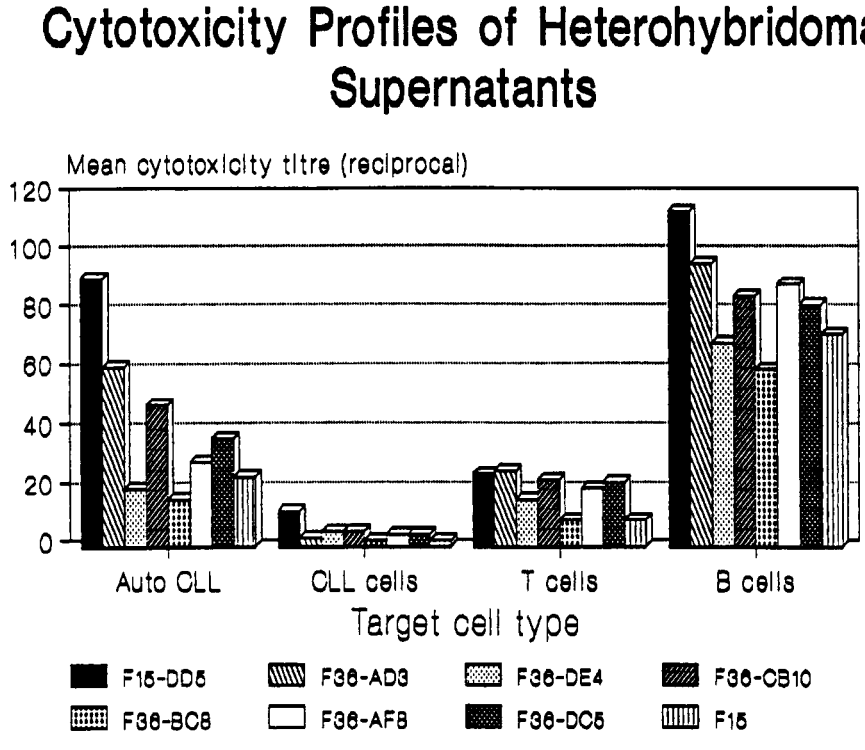


Figure 3.3.2 Cytotoxicity profiles of heterohybridoma supernatants derived from five day old cultures to compare cell growth characteristics with antibody production and cytotoxicity profiles. The clones are ranked in descending order of cell growth rate from left to right.

The cytotoxicity titres showed some correlation with cell growth rate when tested against B cells and the autoantibody sensitive CLL cells (Cx and Rs), with the exception of F36-DE4 and F36-BC8 which gave relatively low titres. No correlation of cell growth and antibody titre was observed for the CLL cells and the T cells. As was noted with the B-LCL (BN) supernatants, the cytotoxicity profiles were dependant on the antibody concentration. All cell types were positive with neat supernatant derived from all clones. However on dilution, initially CLL reactivity, then T cell reactivity was lost, with B lymphocytes being the most sensitive target.

3.3.3 Immunoglobulin class and light chain isotype:

Pooled supernatants from the above cultures were assayed for immunoglobulin class and light chain isotype by single radial immunodiffusion using low level human IgM, IgG, kappa and lambda trays (Fig 3.3.3).

All immunoglobulin production was of the IgM class. Immunoglobulin levels ranged from 76μg/ml (F15-DD5) to 12μg/ml (F36-DC5). F36-BC8 levels were only detectable in x5 concentrated supernatant and was 9·8μg/ml (data not shown). No IgG was detected using neat or x5 concentrated supernatant (sensitive to 9·0μg/ml).

Concentrated supernatants (x5) from antibody producing clones were analyzed for immunoglobulin light chain isotypes kappa and lambda (Table 3.3.1). The parent cell population F15, and its subclone F15-DD5 produced kappa light chains, whereas F36 and all F36 subclones produced lambda light chain.

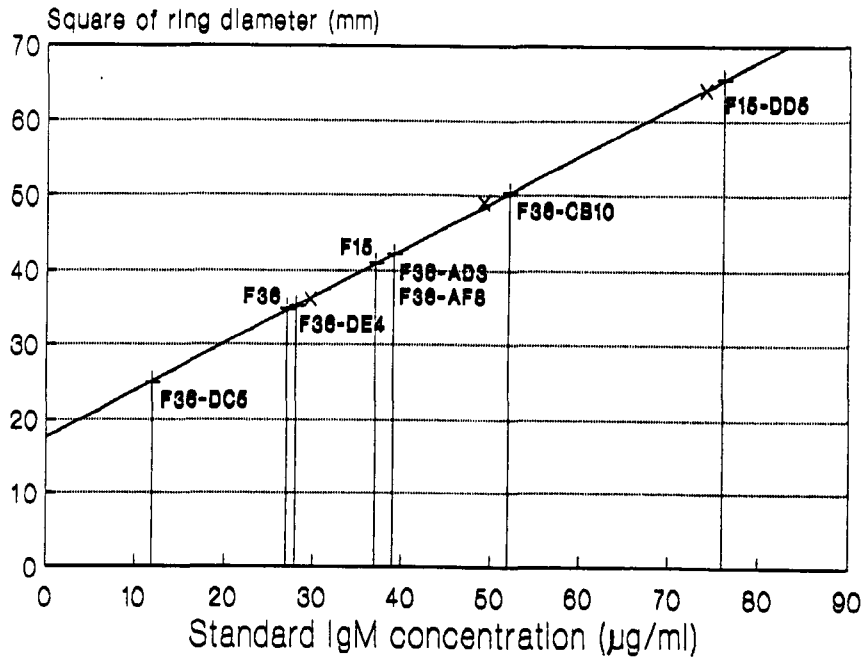


Figure 3.3.3 Quantitative determination of human IgM in heterohybridoma supernatants derived from five day old cultures to compare cell growth characteristics with immunoglobulin production and cytotoxicity profiles.

3.3.4 Selection of higher affinity clones:

We have previously found that lymphocytotoxic autoantibodies in renal patient serum react weakly or are negative in fluorescence binding assays. This is probably due to low antibody binding affinity. To identify higher affinity clones, a single and double wash step was introduced to the cytotoxicity test between incubation with MAb and the addition of complement. MAbs at dilutions of 1/2 and 1/10 were each tested against T cells and B cells from an individual donor (Table 3.3.1). Most clones were weak or negative with T cells following one and two washes with MAbs diluted 1/2 and 1/10, with the exception of F36-CB10 which was still

positive after two washes at 1/10 dilution. Binding to B cells was positive with all clones at both dilutions following one wash, and with F36-CB10, F36-AD3 and F36-AF8 following two washes at a dilution of 1/10. This indicates that B cells constitute a higher affinity target.

Table 3.3.1 Heterohybridoma immunoglobulin class, light chain isotype and antibody binding affinity results

Clone	IgM ug/ml	Isotype k	Isotype l	Growth rate x10-5	No. washes (affinity)@ 1 T cell Dilution (reciprocal) 2	1 T cell 10	2 T cell 2	2 T cell 10	1 B cell 2	1 B cell 10	2 B cell 2	2 B cell 10
F15	38	+	-	0.2	-	-	-	-	+	(+)	(+)	-
F15-DD5	76	+	-	2.8	(+)	-	-	-	+	+	+	-
F36*	26	-	+	NT	+	-	+	-	+	+	(+)	(+)
F36-CB10	52	-	+	1.0	+	(+)	(+)	(+)	+	+	+	+
F36-AD3	39	-	+	1.6	(+)	-	(+)	-	+	+	+	+
F36-AF8	39	-	+	0.7	(+)	-	(+)	-	+	+	+	+
F36-DE4	28	-	+	1.35	-	-	-	-	+	+	+	-
F36-DC5	12	-	+	0.63	(+)	-	-	-	+	+	+	-
F36-BC8	10	-	-	0.92	(+)	-	-	-	+	+	+	(+)

** F36 supernatant was obtained from an exhausted 5 day old culture.*

@ Number of washes between the addition of MAb (at dilutions of 1/2 and 1/10) and the addition of rabbit complement. + = strong positive (80% to 100% cell lysis), (+) = positive (21% to 79% cell lysis), - = negative (<20% cell lysis). NT Not tested.

3.4 Recloning of selected autoantibody producing heterohybridomas

3.4.1 Third cloning

From the above data, F15-DD5 demonstrated the highest growth rate and immunoglobulin production, whereas F36-CB10 and F36-AF8 had the best antibody binding affinities. In addition, F15-DD5 secreted IgM with the kappa light chain isotype whereas F36-CB10 and F36-AF8 produced lambda light chains. This indicates that the clones had originated from cells secreting immunoglobulin with different specificities. These cells were selected for further study and recloned (Table 3.2.1).

3.4.2 Selection of higher affinity clones

Following the third cloning, supernatants from 7 F36-AF8, 12 F36-CB10 and 5 F15-DD5 subclones were tested using the cytotoxicity binding assay, using two low affinity target cell types (peripheral blood T cells from one panel member and lymphocytes from one CLL patient). The results are shown in Table 3.4.1.

All of the F36-CB10 and F36-AF8 subclones tested demonstrated higher affinity binding to T cells as assessed by positive cytotoxicity at a dilution of 1/2 and 1/10 following two washes. Cytotoxicity with CLL cells at a MAb dilution of 1/2 following both one and two washes were positive. However most clones were either weak or negative with CLL cells at a dilution of 1/10 following only one wash. The results show that supernatants derived from F36-CB10 and F36-AF8 subclones bind to this CLL cell with lower affinity than T cells. All five of the F15-DD5 subclones tested had low affinity binding with both T cells and CLL cells, being weak or negative at both dilutions following one and two washes (Table 3.4.1).

Table 3.4.1 Analysis of the binding affinity of heterohybridoma subclones F15-DD5, F36-CB10 and F36-AF8 to T cells and CLL cells

MAb * Subclone	Dil	Target cell type: T cell — No. of washes 0	1	2	CLL cell — No. of washes 0	1	2
F36-AF8-1	1/2	+	+	+	+	+	+
	1/10	+	+	(+)	(+)	(+)	-
-3	1/2	+	(+)	(+)	+	+	+
	1/10	+	+	+	(+)	-	-
-4	1/2	+	+	+	+	+	+
	1/10	+	+	+	+	-	-
-7	1/2	+	+	+	+	+	+
	1/10	+	+	+	+	-	-
-11	1/2	+	+	+	+	+	+
	1/10	+	(+)	(+)	(+)	-	-
-12	1/2	+	(+)	+	+	+	+
	1/10	+	(+)	(+)	+	-	-
-15	1/2	+	+	+	+	+	+
	1/10	+	(+)	(+)	+	-	-
F36-CB10-2	1/2	+	+	+	+	+	+
	1/10	+	+	+	+	-	-
-3	1/2	+	+	+	+	+	+
	1/10	+	(+)	(+)	+	(+)	-
-4	1/2	+	+	+	+	+	+
	1/10	+	+	(+)	+	-	-
-5	1/2	+	+	+	+	+	+
	1/10	+	+	(+)	(+)	-	-
-10	1/2	+	(+)	(+)	+	+	(+)
	1/10	+	(+)	(+)	(+)	-	-
-11	1/2	+	+	+	+	+	(+)
	1/10	+	+	(+)	(+)	-	-
-12	1/2	+	+	+	+	+	+
	1/10	+	+	(+)	+	-	-
-13	1/2	+	+	(+)	+	+	+
	1/10	+	(+)	(+)	+	(+)	-
-14	1/2	+	+	+	+	+	+
	1/10	+	(+)	(+)	+	(+)	-
-15	1/2	+	+	+	+	+	+
	1/10	+	(+)	(+)	+	+	(+)
-16	1/2	+	+	(+)	+	+	+
	1/10	+	(+)	(+)	+	(+)	-
-18	1/2	+	+	+	+	+	+
	1/10	+	+	+	+	+	(+)

Table 3.4.1 continued

MAb Subclone	Dil	Target cell type: T cell, No. of washes 0	T cell, 1	T cell, 2	CLL cell, 0	CLL cell, 1	CLL cell, 2
F15-DD5-23	1/2	+	(+)	-	+	-	-
	1/10	+	-	-	(+)	-	-
-24	1/2	+	-	-	+	-	-
	1/10	+	-	-	-	-	-
-26	1/2	+	-	-	+	-	-
	1/10	+	-	-	(+)	-	-
-39	1/2	+	(+)	-	+	-	-
	1/10	+	-	-	(+)	-	-
-43	1/2	+	-	-	+	-	-
	1/10	+	-	-	+	-	-

** Supernatants from F15-DD5, F36-CB10 and F36-AF8 subclones were harvested from exhausted cultures.*

+ =strong positive cytotoxicity (>80% cell lysis),

(+) =positive cytotoxicity (20-79% cell lysis),

- =negative cytotoxicity (<20% cell lysis).

3.5 Characterisation of monoclonal lymphocytotoxic autoantibody binding by cytotoxicity

3.5.1 Comparison of target cell sensitivities:

The relative sensitivity of different target cell types was assessed using subclones derived from F15-DD5 (N=24), F36-CB10 (N=13) and F36-AF8 (N=8). Tissue culture supernatants from each subclone were individually tested at doubling dilutions by cytotoxicity against a panel of T and B cells derived from five cadaver donor spleen or lymph nodes and 12 random CLL patients. In addition the autoantibody sensitive cell line K562, and the autologous B-LCL (BN) were used. The mean of the end point of cytotoxicity was calculated for each family of subclones against each cell type (Fig 3.5.1). The results indicate that T cells, B-LCL and K562 have similar sensitivities, whereas CLL cells react at an average of one to two doubling dilutions below T cells. B cells constitute the strongest target, although the sensitivity above T cells was not assessed as most MAbs titred beyond 1/128. MAb cytotoxicity titres against CLL cell targets were variable, with some weakly reactive (positive at neat and 1/2 dilution) and some strongly reactive (positive at 1/128 dilution). Reactivity with T and B cells was less variable with MAb cytotoxicity titres between 1/4 and 1/64 for T cells and 1/32 to 1/128 for B cells.

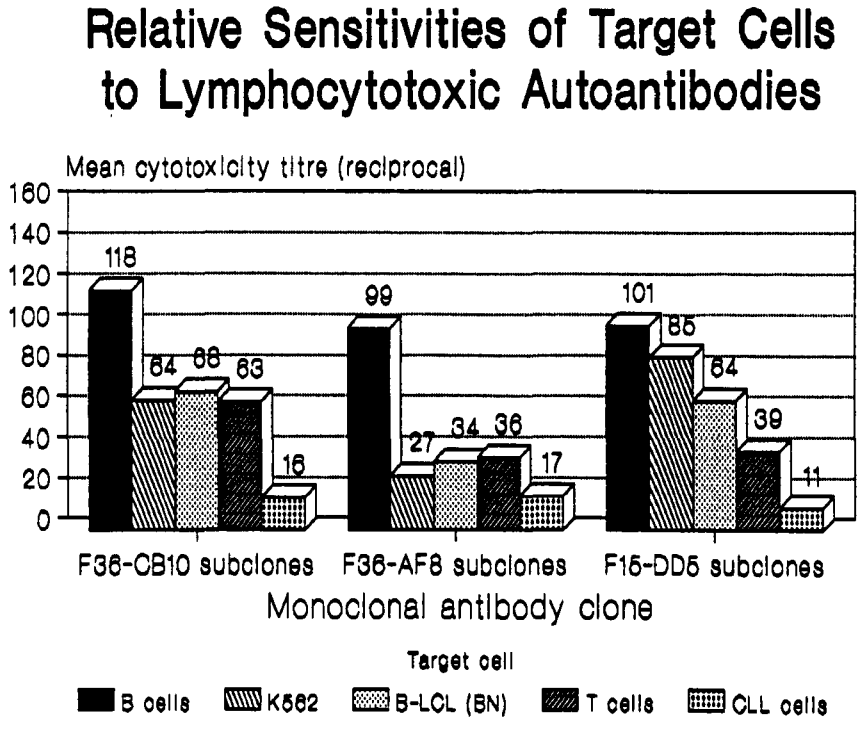


Figure 3.5.1 Comparison of the relative sensitivities of T cells, B cells, CLL cells, K562 and B-LCL (BN) to cytotoxicity by lymphocytotoxic autoantibodies.

3.5.2 Comparison of lymphocytotoxic autoantibody binding profiles with other human IgM monoclonal antibodies:

To determine if the cytotoxicity profiles were unique to lymphocytotoxic autoantibodies or a common feature of many human IgM antibodies, the cytotoxicity profiles of the monoclonal lymphocytotoxic autoantibodies were compared to five other human IgM MAbs, FOM-A, FOM-1 and MAD (all anti-RhD), BIO-4F11 (anti-tetanus toxoid) (all a kind gift from Dr Hugh-Jones group, Babraham, Cambridge) and RFAN (rheumatoid factor, a kind gift from Dr M.Steinitz, Hadassah Medical School, Jerusalem) (Fig 3.5.2).

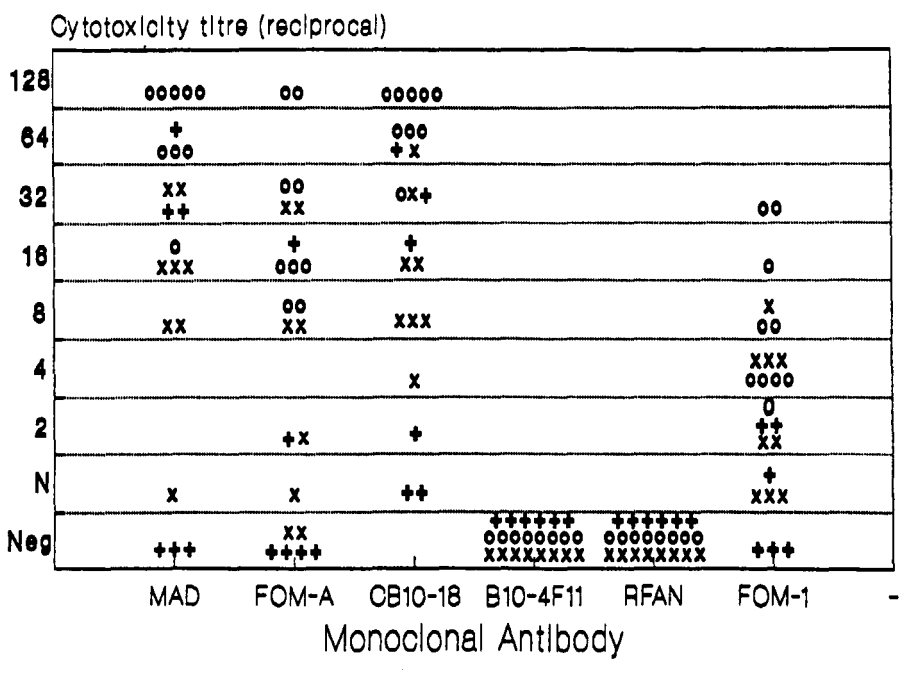


Figure 3.5.2 Comparison of cytotoxicity profiles of control human IgM heterohybridoma MAbs of defined specificities, with autoreactive monoclonal antibodies tested at doubling dilutions against a panel of B cells (o), T cells (x) and CLL cells (+).

The results showed similar cytotoxicity profiles between the anti-RhD MAbs (FOM-A, FOM-1 and MAD) and the autoantibodies. No difference was observed whether the target cells were derived from rhesus positive or negative donors. The anti-tetanus toxoid (B10-4F11) and anti-rheumatoid factor (RFAN) antibodies were negative against all target cells. These data demonstrated that well characterised RhD human IgM MAbs can give "non specific" cytotoxicity which mimic lymphocytotoxic autoantibody reactivity profiles.

3.5.3 Correlation of human IgM monoclonal antibody cytotoxicity profiles with immunoglobulin levels

To determine if the cytotoxicity profiles of the human IgM monoclonal antibodies were a reflection of the antibody specificity or merely dependant on IgM levels, the immunoglobulin concentration of each monoclonal antibody was measured (Fig 3.5.3).

The results show that cytotoxicity profiles were independent of immunoglobulin levels (the monoclonal antibodies in figure 3.5.2 are shown in descending order of IgM levels from left to right). The lowest IgM concentration of 18μg/ml was found in FOM-1 which was positive by cytotoxicity, whereas B10-4F11 and RFAN with levels of 34μg/ml and 22μg/ml respectively, were both negative. In addition, positive cytotoxicity had previously been found with F36-BC8 with IgM levels of <10μg/ml. Therefore cytotoxicity was a results of the antibody specificity and not solely the immunoglobulin concentration.

Quantitative Determination of Human IgM in Heterohybridoma Supernatants

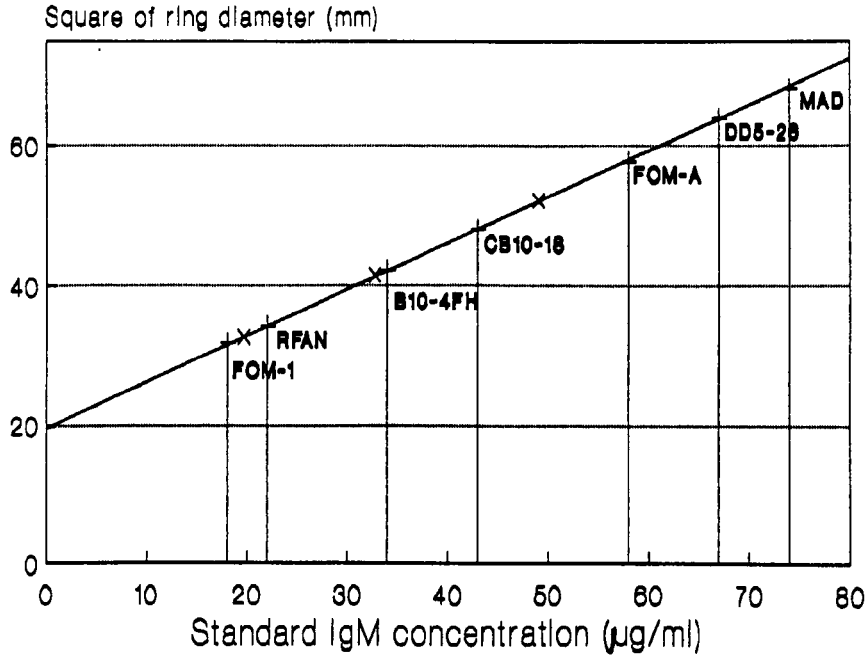


Figure 3.5.3 Immunoglobulin concentrations of human IgM monoclonal antibodies determined by single radial immunodiffusion. The calculated IgM levels for each monoclonal antibody are presented in descending order of antibody concentration given below.

MAD (anti-RhD) >70µg/ml; FOM-A (anti-RhD) 58µg/ml; F36-CB10-18 (autoreactive) 42µg/ml; BIO-4F11 (anti-Tetanus toxoid) 34µg/ml; RFAN (anti rheumatoid factor) 22µg/ml; FOM-1 (anti-RhD) 18µg/ml.

3.6 Discussion

Lymphocytotoxic autoantibodies in renal dialysis patients have been recognised for more than 14 years, but the nature of their specificity has not been fully defined. These antibodies have been shown to react with normal B cells alone (Ettenger et al, 1976), normal T and B cells (Ting and Morris, 1983) and with T, B and CLL cells (Ting and Morris, 1978). The different reactivity profiles suggest that the autoantibodies may represent multiple antibody populations or one population but with different sensitivities against different targets. The difficulty in reliably distinguishing autoreactive from alloreactive antibodies in the crossmatch test for kidney transplantation has lead to many patients being denied a kidney transplant, often due to a false positive crossmatch (Taylor et al, 1989).

In an attempt to resolve these questions we have generated human monoclonal lymphocytotoxic autoantibodies, to define their target cell specificity and develop a technique to distinguish autoreactive from alloreactive antibodies in highly sensitised patients.

The monoclonal antibodies generated in this study gave identical cytotoxicity profiles to autoantibodies found in the sera of renal dialysis patients. However the multiple profiles seen with sera from different renal patients could all be reproduced by single monoclonal antibodies at different dilutions. Cytotoxicity testing showed a progressive susceptibility of different cell types to lysis by the autoreactive monoclonal antibodies. B cells were strongly reactive, whereas T cells and CLL cells were less sensitive. This finding is similar to that of Ozturk

(Ozturk and Terasaki, 1979) who reported a marked difference in the susceptibility to lysis of B cells compared to T cells using cytotoxic sera from normal individuals and from patients with various diseases. It is therefore possible that these antibodies do not represent distinct specificities, but reflect differing target cell sensitivities, which is dependent on antibody concentration.

The finding of "autoantibody profiles" with well-characterised human IgM monoclonal antibodies to RhD suggests that binding is non-specific and that many IgM antibodies may result in apparent autoreactivity in-vitro. Red cell agglutination and fluorescence binding studies confirmed that these antibodies are reactive with RhD positive cells to dilutions of 1/1000, whereas the autoreactive monoclonal antibodies F15-DD5-26 and F36-CB10-18 do not bind red cells (see later). The finding is not related to IgM concentration as cytotoxicity was found in some monoclonal antibodies with IgM levels well below the two negative monoclonal antibodies (B10-4F11 and RFAN).

Polyreactive antibodies capable of binding multiple types of antigens have been described (Casali and Notkins, 1989). Polyreactive antibodies can recognise a variety of self and exogenous antigens, and bind with low intrinsic affinity (Nakamura et al, 1988). They are secreted by CD5 positive B cells (20% of PB-B cells in adults) and are thought to constitute the natural antibodies of serum giving a first line of defence. They use some conserved (germ line) V gene segments coding for sequences postulated to have large binding sites, capable of accommodating multiple antigens (Sanz et al, 1989; Pennell et al, 1988). Many polyreactive antibodies with low affinity for ssDNA, rheumatoid factor (IgG Fc),

thyroglobulin, insulin, Tetanus toxoid, bacterial lipopolysaccharides (lipid A), saccharides and phospholipids (phosphatidylcholine) have been characterised (Casali and Notkins, 1989; Van Rooijen, 1989). Their production is enhanced by exogenous antigen (Tetanus toxoid, bacterial lipopolysaccharides, viral, bacterial and parasitic infection), which is consistent with the association of their appearance with viral infection in renal transplant patients (Jeannet, Benzonana and Arni, 1981; Cross, Greiner and Whittier, 1976; Macleod et al, 1987). However, because of their low affinity their presence does not predispose to the pathogenesis of the autoimmune disease. This would be consistent with the lack of clinical evidence for lymphocyte related autoimmune diseases in renal dialysis patients.

Comparisons of the characteristics of polyreactive antibodies with lymphocytotoxic autoantibodies are consistent with the hypothesis that autoantibodies in renal dialysis patients are polyreactive and that they may represent a similar group of antibodies. The antibodies are low affinity (therefore of no clinical relevance), bind more strongly at 4°C, are easily washed off and react with self (autologous lymphocytes) in cytotoxicity assays.

The method employed for the production of the monoclonal autoantibodies in this study was through EBV transformation of B lymphocytes. It is now recognised that EBV activates CD5 positive B cells and stimulates polyclonal IgM production with polyreactivity (Casali and Notkins, 1989). This would explain the high frequency of autoantibody production from this and other B-LCLs (data not shown) and the correlation of such antibodies with lymphocytotoxic autoantibody profiles. Therefore, we would postulate

that there is no unique priming antigenic stimulus for the appearance of lymphocytotoxic autoantibodies and that they may result from polyclonal B cell activation by a variety of stimuli.

Chapter 4.

Characterisation of autoreactive monoclonal antibody binding profiles and specificity

4.1 Analysis of MAb binding profiles by fluorescence binding assays

4.1.1 FACScan analysis of autoreactive and anti-RhD MAb binding profiles
4.1.2 Cytofluorograph analysis of autoreactive and anti-RhD MAb binding profiles
4.1.3 Binding profiles of autoreactive and anti-RhD MAbs at serial dilutions
4.1.4 Fluorescence binding profiles of autoreactive and anti-RhD MAbs against a panel of T and B cells
4.1.5 Effect of temperature on autoantibody binding
4.1.6 Discussion

4.2 Autoantibody binding inhibition studies

4.2.1 Binding inhibition using lipid-A and phosphatidylcholine
4.2.2 Binding inhibition using human IgG and IgM
4.2.3 Discussion

4.1.1 FACScan analysis of autoreactive and anti-RhD MAb binding profiles: Fluorescence binding profiles with spleen T and B cells were analyzed using MAbs F15-DD5-26, F36-CB10-18 (autoreactive) and MAD (anti-RhD) at 1/2 and 1/10 dilutions on a FACScan (Fig 4.1.1). Similar T cell binding profiles were seen for each antibody, although MAD binding was weak. F36-CB10-18, which was selected for improved T cell binding affinity reacted the strongest, being positive with 82% of T cells at 1/2 dilution (Fig 4.1.1A, panel b). Lower mean channel fluorescence values were found at 1/10 dilution.

Binding profiles for B cells with FITC-goat-anti-human IgM alone (Fig 4.1.1B) demonstrated two peaks, the left peak representing surface IgM negative B cells, and the right peak indicating surface IgM positive B cells. Following the addition of the autoreactive (F15-DD5-26 and F36-CB10-18) and anti-RhD (MAD) MAbs, both cell populations were positive, as indicated by a shift of both peaks to the right. Due to the shape of the profiles it was not possible to determine the percentage of B cells positive as the weakly positive B cell peak overlapped with the IgM positive B cells in the negative control.

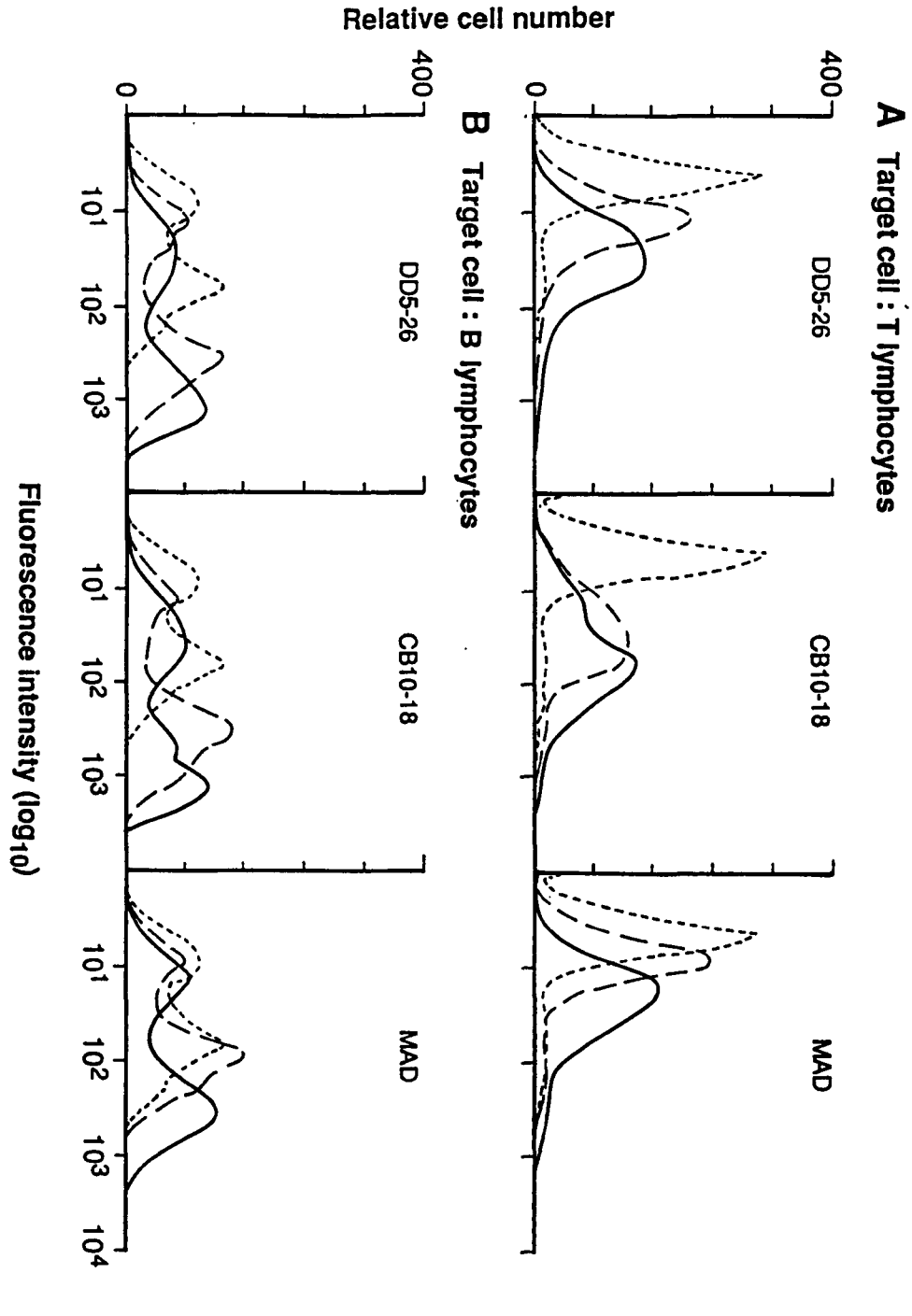


Figure 4.1.1 FACScan binding profiles of autoreactive (F15-DD5-26 and F36-CB10-18) and anti-RhD (MAD) human IgM MAbs with T cells (panel a) and B cells (panel b) at dilutions of 1/2 and 1/10.

----- =*FITC-goat-anti-human-IgM alone,*

——— =*MAb dilution 1/2,*

— — =*MAb dilution 1/10.*

4.1.2 Cytofluorograph analysis of autoreactive and anti-RhD MAb binding profiles

The use of logarithmic profiles by FACScan analysis was not routinely available, therefore an Ortho Cytofluorograph (linear profiles) was used. On each occasion the cytofluorograph was calibrated using fluorotrol standards (Becton Dickinson), to ensure consistent fluorescent values, allowing comparisons of results on a day to day basis. Test were performed in duplicate. The mean difference of 50 consecutive T cell duplicates was 4.6% and 50 B cell duplicates was 1.9% (data not shown).

Linear profiles derived from the autoreactive (F15-DD5-26 and F36-CB10-18) and anti-RhD (MAD) MAbs tested against spleen T and B cells are shown in Figures 4.1.2 and 4.1.3.

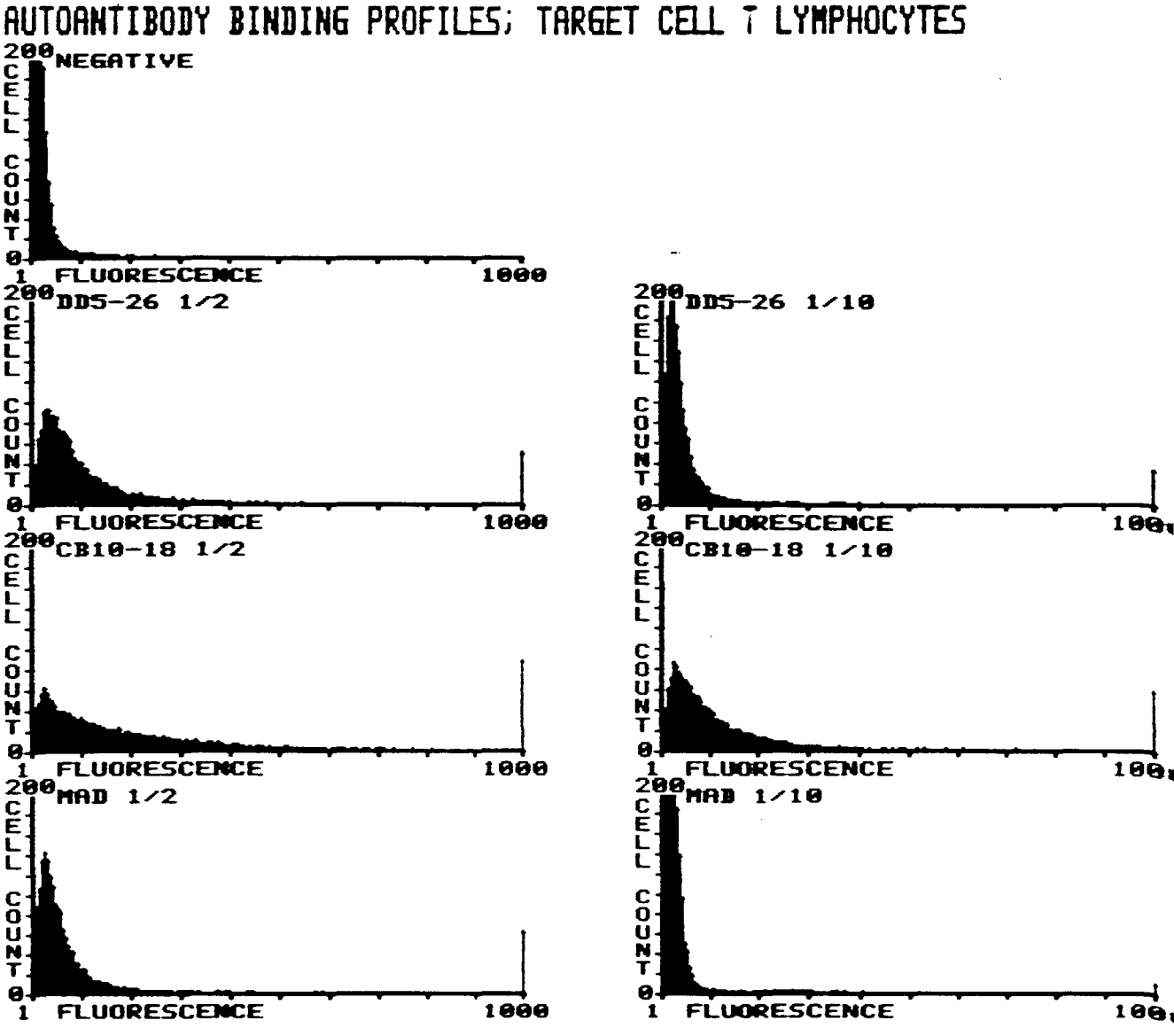


Figure 4.1.2 Cytofluorograph profiles of F15-DD5-26, F36-CB10-18 and MAD binding at 1/2 and 1/10 dilutions to T cells.

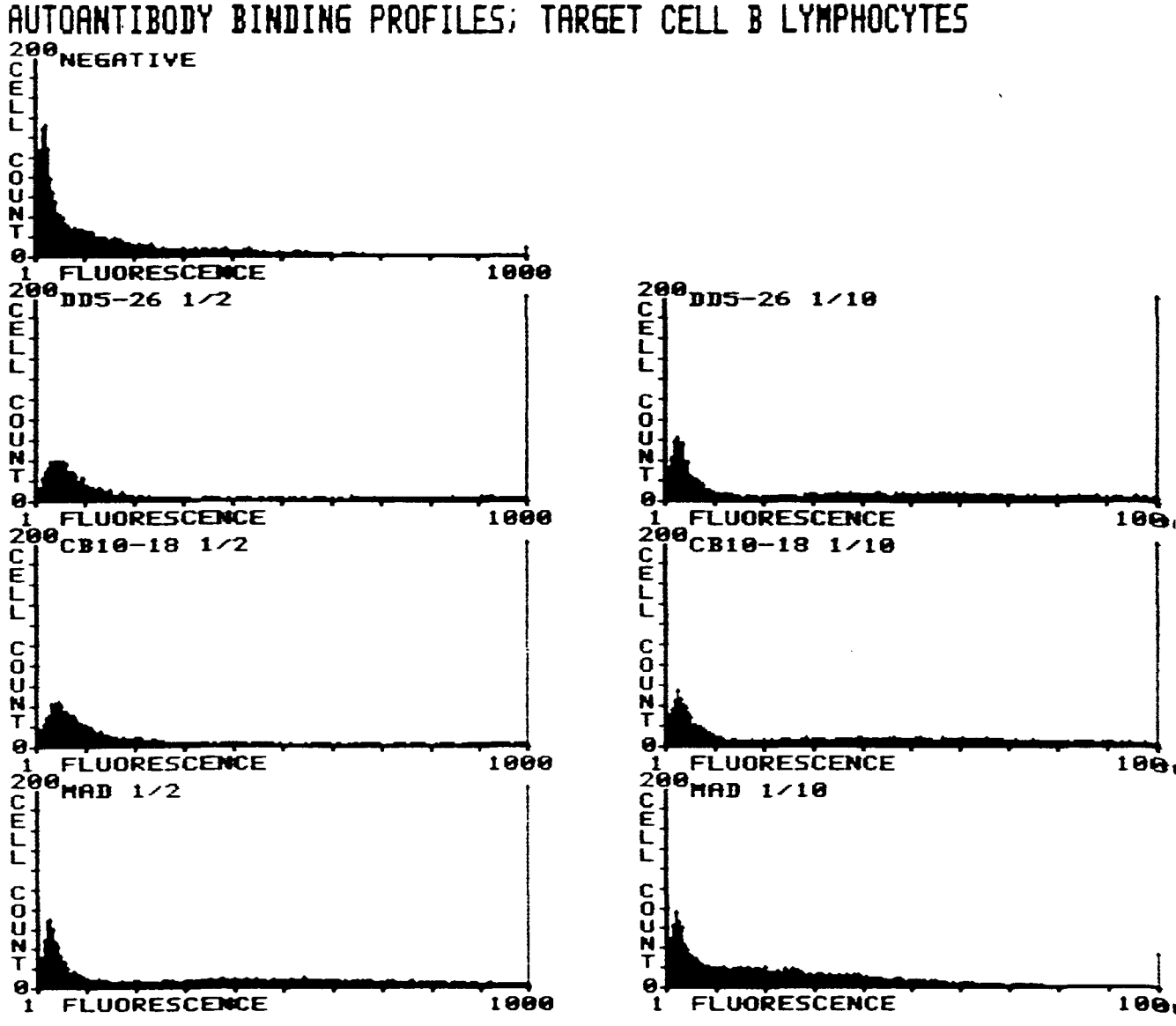


Figure 4.1.3 Cytofluorograph profiles of F15-DD5-26, F36-CB10-18 and MAD binding at 1/2 and 1/10 dilutions to B cells.

T cell binding was relatively weak for each antibody at 1/2 dilution and was further reduced at 1/10 dilution as indicated by movement of the peaks to the left (Fig 4.1.2). F36-CB10-18 gave highest mean channel fluorescence confirming improved T cell binding affinity. MAD (anti-RhD) gave weakest binding, having been selected for RhD binding by independent criteria.

By electronically subtracting the background fluorescence (FITC-anti-human-IgM alone) the percentage of T cells positive was determined (Fig 4.1.4). F15-DD5-26 was positive with 62% and 36% of T cells at 1/2 and 1/10 dilutions respectively. F36-CB10-18 was positive with 68% and 63% at dilutions 1/2 and 1/10 respectively. On comparison of F36-CB10-18 (IgM concentration 42μg/ml) and F15-DD5-26 (IgM concentration 68μg/ml), the higher binding reactivity of F36-CB10-18 at 1/10 dilution, despite lower IgM levels confirms improved binding affinity to T cells.

B cell binding with FITC anti-human-IgM alone (negative control) gave a negative peak and a positive 'tail', indicating cell surface IgM negative and positive B cells respectively. The two distinct B cell subpopulations could not be distinguished using linear profiles as was seen on the FACScan. Binding of the autoreactive MAbs showed no discrete peak of positive cells (Fig 4.1.3). The graphs demonstrated long flat profiles, ranging from weakly positive cells (left) to intensely positive B cells (right). Approximately 25% of B cells gave maximum fluorescence in channels 999-1000. This was true for both the autoreactive and anti-RhD MAbs.

On lowering the fluorescence intensity 'gain' (Fig 4.1.5), the intensely positive cells formed a broad peak, and the weakly positive cells entered channel 1 (negative). As with the logarithmic profiles on the FACScan, it

was not possible to determine the percentage of B cells positive, as the weakly positive cells overlapped with the cell surface IgM positive B cells in the negative control. Therefore in all subsequent fluorescence binding data, mean channel fluorescence values have been calculated following subtraction of the appropriate negative control (FITC-anti-human-IgM alone).

AUTOANTIBODY BINDING PROFILES; TARGET CELL T LYMPHOCYTES

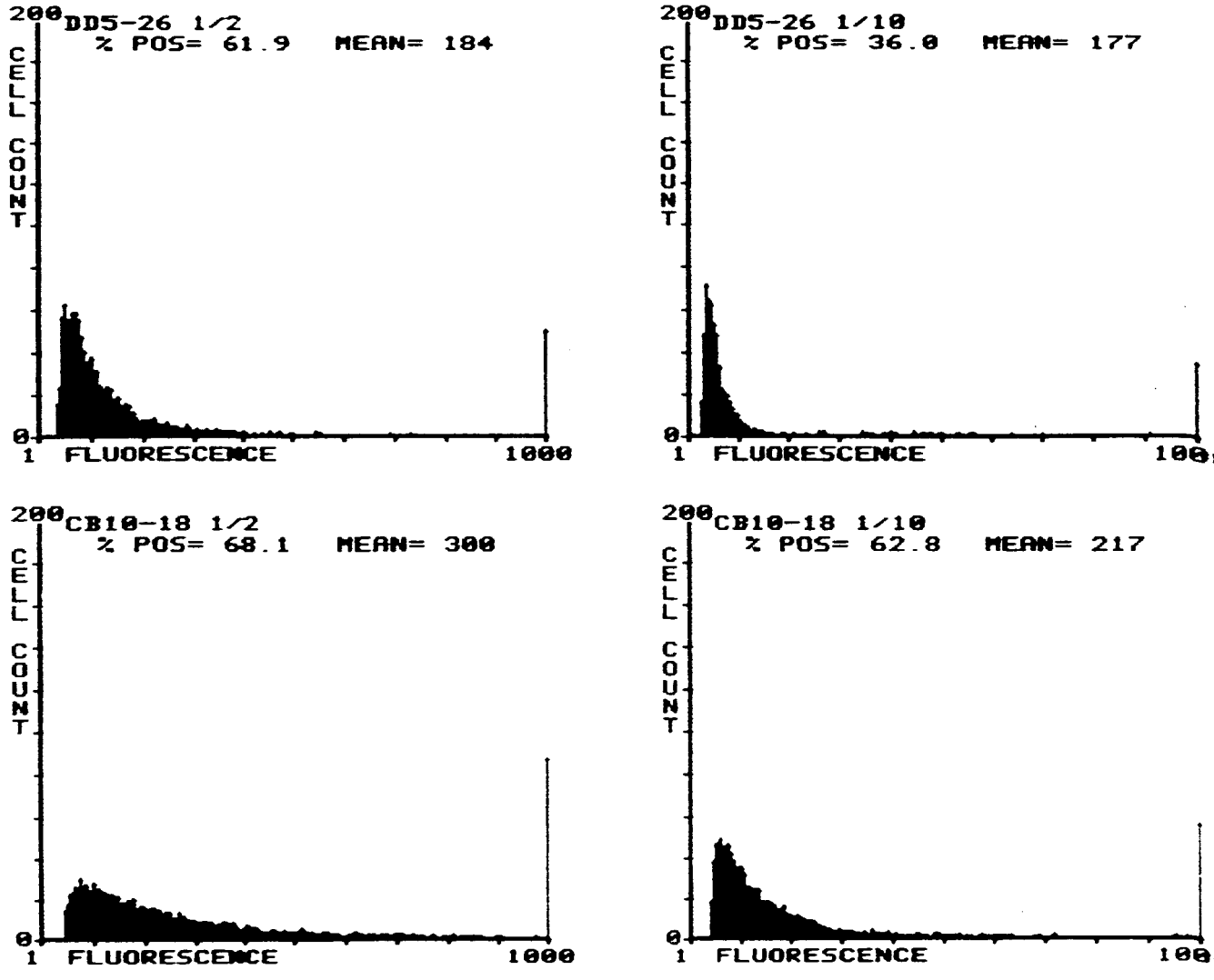


Figure 4.1.4 Cytofluorograph analysis of F15-DD5-26 and F36-CB10-18 binding at 1/2 and 1/10 dilutions to T cells. The negative control (FITC alone) has been electronically subtracted, allowing calculation of the percentage cell positive at each dilution.

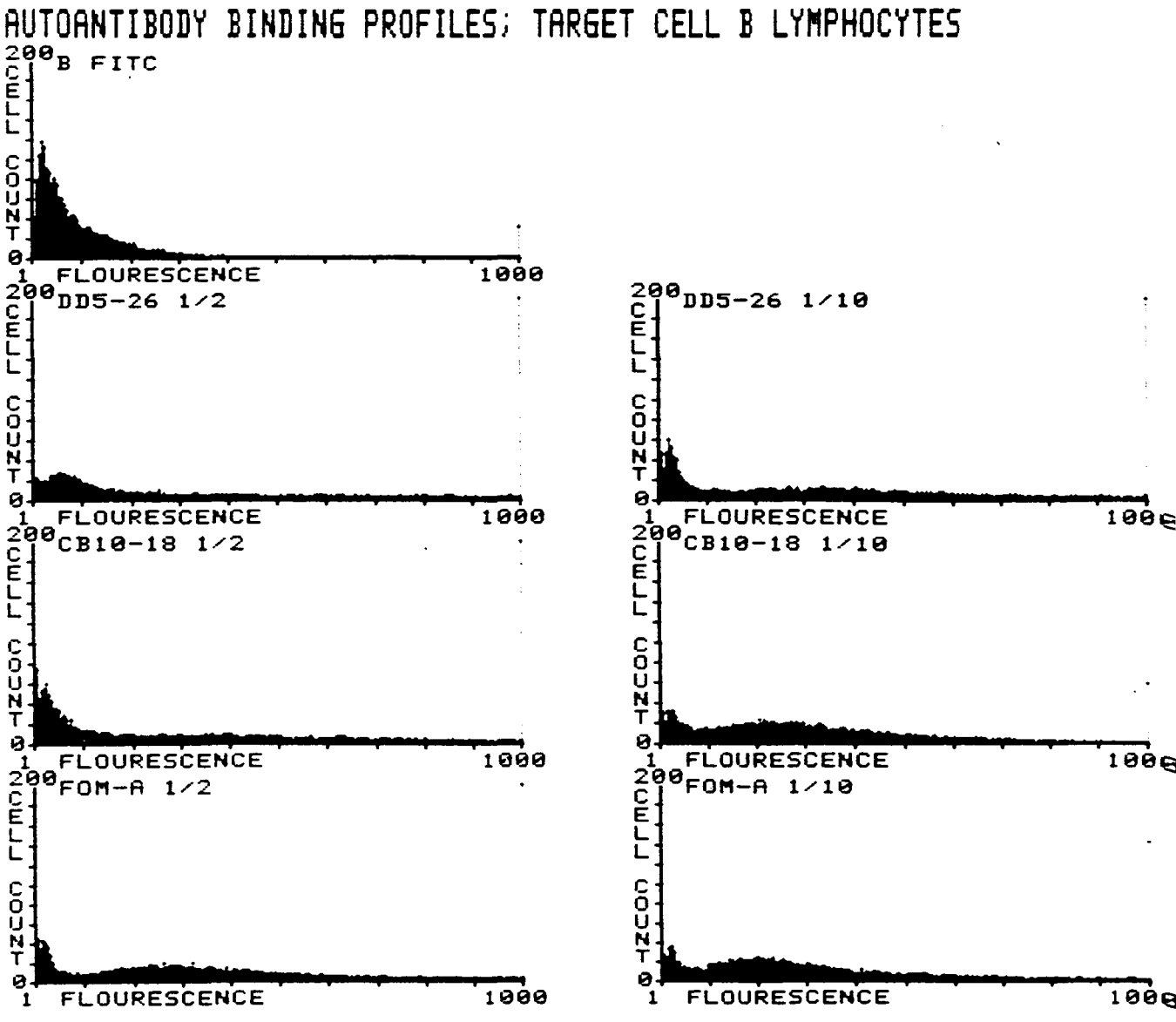


Figure 4.1.5 Cytofluorograph profiles of F15-DD5-26, F36-CB10-18 and MAD binding at 1/2 and 1/10 dilutions to B cells. The fluorescence 'gain' has been reduced to enable improved visualisation of the binding profile.

4.1.3 Binding profiles of autoreactive and anti-RhD MAbs at serial dilutions

MAbs F15-DD5-26 and F36-CB10-18 were tested at doubling dilutions against T and B cells on a cytofluorograph (Fig 4.1.6). The profiles demonstrated high mean channel fluorescence values with B cells at all dilutions. A mean of 21% and 28% of B cells were in channels 999-1000 for F15-DD5-26 and F36-CB10-18 respectively. This remained constant to a dilution of 1/64 and dropped to 9% at a dilution of 1/128. T cell profiles demonstrated relatively low mean channel fluorescence values which dropped on dilution. F36-CB10-18, which was selected for improved T cell binding affinity gave higher mean channel fluorescence at a dilution of 1/2. This fell sharply through dilutions 1/4, 1/8 and 1/16.

Fluorescence binding profiles of the lymphocytotoxic autoantibodies F15-DD5-26 and F36-CB10-18 were compared to FOM-A (anti-RhD), by testing at doubling dilutions against spleen T and B cells from a RhD negative donor (Fig 4.1.7a and b). Little difference in binding profiles was observed between the autoreactive MAbs and FOM-A (anti-RhD). This was true for RhD positive and RhD negative donors (data not shown). The results show relatively high binding to B cells to 1/32 dilution, whereas T cells gave lower mean channel fluorescence. The level of binding to T cells was still further reduced on dilution. These results, and those of Fig 4.1.6 indicate that the weaker binding profiles with T cells is not a result of antibody concentration, but that they constitute lower affinity targets.

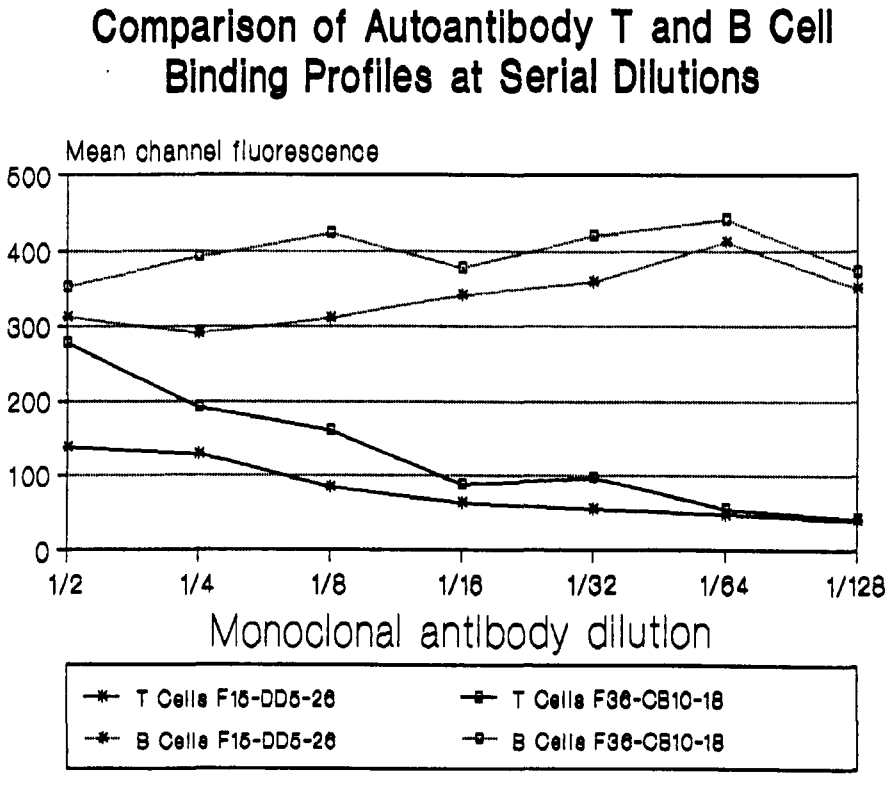


Figure 4.1.6 Cytofluorograph analysis of F15-DD5-26 and F36-CB10-18 binding to T and B cells at serial dilutions from 1/2 to 1/128. All mean channel fluorescence values in this and subsequent figures have had the appropriate negative control values (FITC-goat-anti-human IgM alone) subtracted.

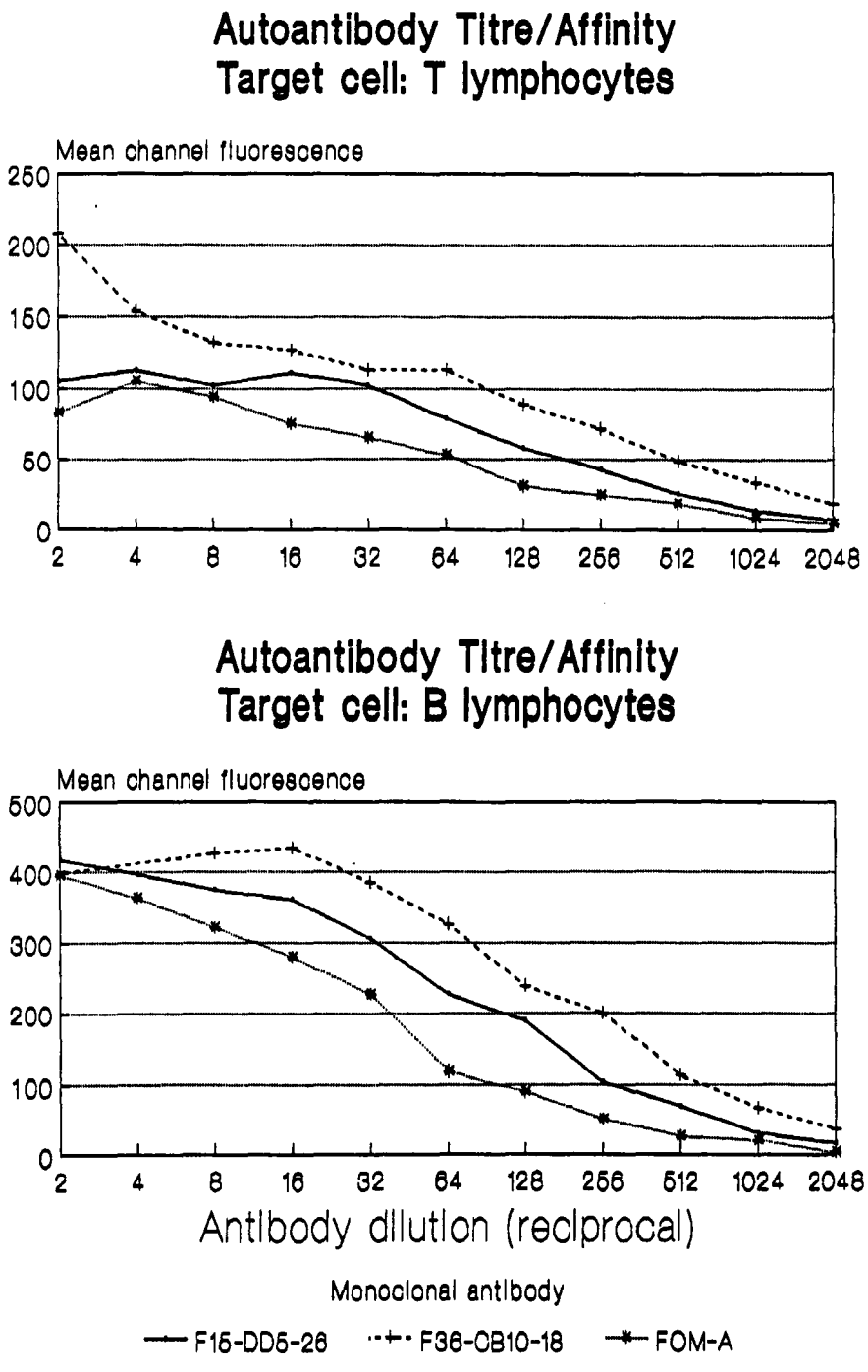


Figure 4.1.7 Comparison of cytofluorograph binding profiles of human IgM autoreactive (F15-DD5-26 and F36-CB10-18) and anti-RhD (FOM-A) MAbs tested at doubling dilutions using spleen T cells (panel a) and B cells (panel b) from a RhD negative donor.

4.1.4 Fluorescence binding profiles of autoreactive and anti-RhD MAbs against a panel of T and B cells

To confirm this observation, F15-DD5-26, F36-CB10-18 and FOM-A were tested at dilutions of 1/2 and 1/10 against a panel of donor lymph node and spleen T and B cells (Fig 4.1.8). B cell binding profiles revealed a relatively high mean channel fluorescence, which was not significantly changed between the dilutions 1/2 and 1/10 (366 ±26 and 330 ±23 respectively). A mean of 31% and 25% of B cells gave maximum fluorescence values at channels 999-1000 at dilutions 1/2 and 1/10 respectively. The binding profiles of T cells revealed a relatively low mean channel fluorescence at 1/2 dilution (172 ±11), which was further reduced at 1/10 (92 ±9). This data confirms that the lower sensitivity of T cells relative to B cell targets is not due to antibody concentration but is a result of low antigen density and/or low antibody binding affinity. The data also confirms higher T cell binding affinity by F36-CB10-18, and that high binding to B cells was consistent regardless of the selection of high affinity antibodies.

(Data comparing the mean channel fluorescence and standard errors are given numerically in Table 4.1.1)

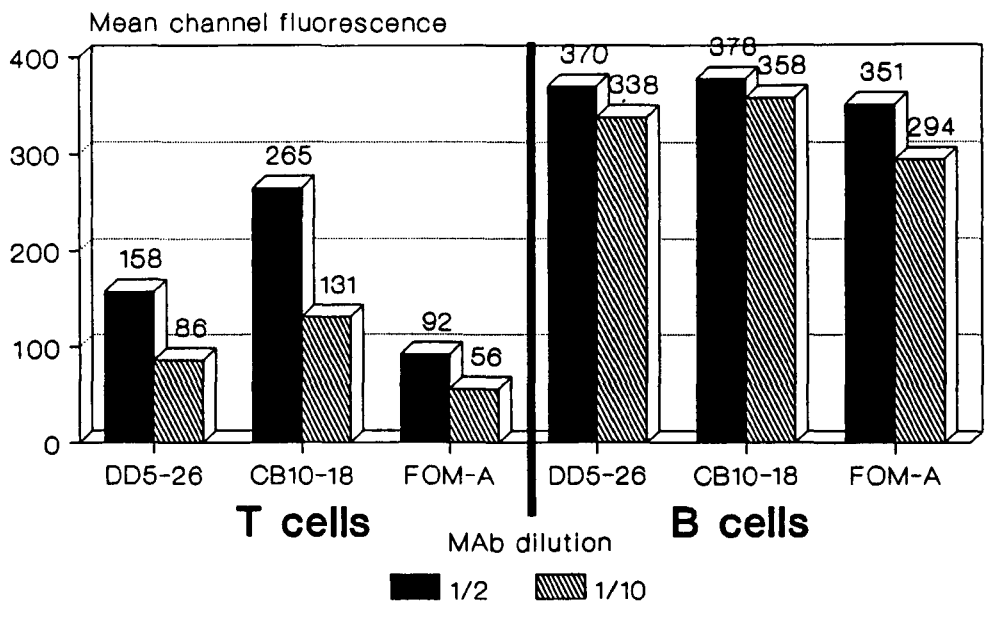


Figure 4.1.8 Cytofluorograph binding profiles of autoreactive (F15-DD5-26 and F36-CB10-18) and anti-RhD (FOM-A) human IgM MAbs tested at dilutions 1/2 and 1/10 against a panel of lymph node and spleen T and B cells. The number of panel cells used for each MAb are:

F15-DD5-26 =16 T cells and 12 B cells,

F36-CB10-18 =17 T cells and 13 B cells,

FOM-A =10 T cells and 11 B cells.

Table 4.1.1 Standard Error of the Mean (SEM)

Cell type	T cells		B cells	
Ab dilution	1/2	1/10	1/2	1/10
	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM
F15-DD5-26	158.3 ± 11.2	86.9 ± 7.7	369.8 ± 29.6	337.6 ± 24.3
F36-CB10-18	265.3 ± 13.7	131.5 ± 10.1	377.7 ± 27.4	358.0 ± 21.8
FOM-A	92.0 ± 8.1	56.4 ± 8.8	351.1 ± 20.6	294.1 ± 23.4
Mean	172 ± 11	92 ± 9	366 ± 26	330 ± 23

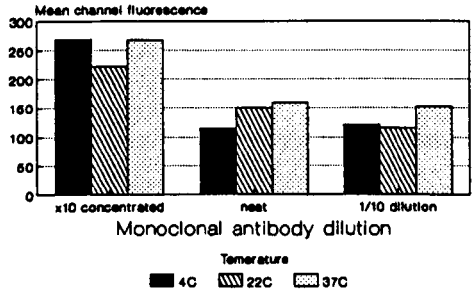
4.1.5 Effect of temperature on autoantibody binding

Varying the precomplement incubation temperature in cytotoxicity assays has been used to distinguish lymphocytotoxic autoantibodies from HLA antibodies. Autoantibodies have been reported to react more strongly at low temperatures (4°C) whereas anti-HLA antibodies react more strongly at warm temperatures (37°C). This test has been utilised in the crossmatch test to distinguish damaging and non-damaging antibodies in renal transplantation.

Binding profiles of the autoreactive MAbs F15-DD5-26 and F36-CB10-18 at x10 concentrated, neat and 1/10 dilution were assessed using low affinity T cell and CLL cell targets, tested at 4°C, 22°C and 37°C.

Binding of F15-DD5-26 to CLL cells and T cells showed no influence of temperature (Fig 4.1.9a and b). F36-CB10-18 demonstrated a weak trend towards higher affinity binding to T cells at 4°C compared to 37°C, but no influence was observed with CLL cell binding (Fig 4.1.9c and d). Therefore the results do not demonstrate improved binding affinity to T cells and CLL cells at low incubation temperatures.

a



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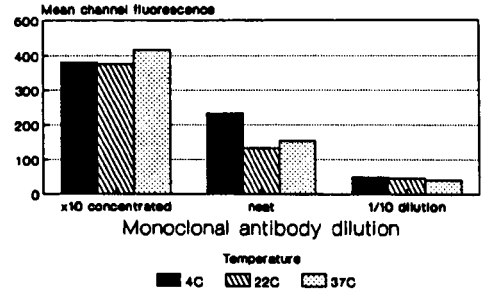

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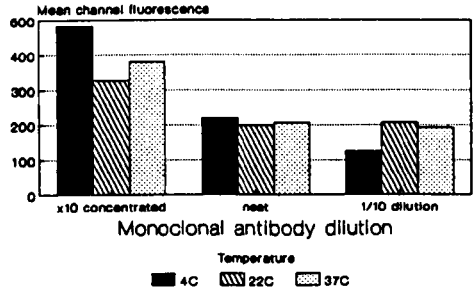

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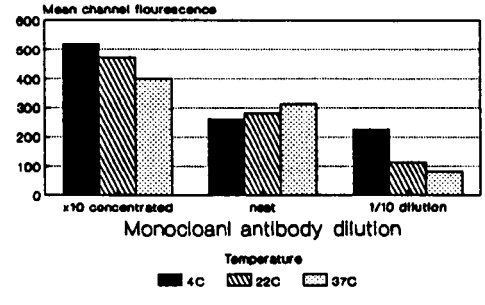


Figure 4.1.9 Cytofluorograph analysis of the effect of temperature on F15-DD5-26 and F36-CB10-18 binding (at x10 concentrated, neat and 1/10 dilution) to low affinity CLL cell and T cell targets.

4.1.6 Discussion

Fluorescent binding assays demonstrate that B cells constitute high affinity targets, whereas T cells and CLL cells are low affinity targets. Reliable absorption of B cell autoantibodies has only been achieved using autologous B-LCL's (Deierhoi, Ting and Morris, 1985; Nicholls and Russ, 1989) or extremely high concentrations of autologous PBL's (Ettenger and Robertson, 1987). Although these observations have indicated the existence of multiple autoantibody populations, the results are consistent with the view that they represent only one antibody population with differing target cell sensitivities. As B cells are the most sensitive target, autoantibody reactivity is only efficiently removed following absorption with high affinity targets such as B cell lines. T cell and CLL cell reactivity is more easily removed, as they represent low sensitivity targets.

FACScan binding profiles demonstrate that both surface IgM positive and IgM negative B cells are reactive with lymphocytotoxic autoantibodies. This suggests that these particular antibodies are not the IgM-anti-IgM type described by Cicciarelli and others (Ozturk and Terasaki, 1980; Takahashi et al, 1980; Cicciarelli et al, 1980; Ayoub et al, 1980).

Binding to low affinity targets such as T cells and CLL cells was not significantly improved at low incubation temperatures (4°C) compared to warm temperatures (37°C). Varying the pre-complement incubation temperature has been used to distinguish damaging (HLA antibodies) from non-damaging (non-HLA) antibodies in the crossmatch test (Revillard, Rivera and Robert, 1980; Lobo, Sturgill and Bolton, 1984; Iwaki et al, 1979). Poor graft survival has been reported in transplants performed with a warm reactive positive crossmatch compared to improved graft survival with a positive

crossmatch due to cold reactive antibodies. However this method of defining damaging and non-damaging antibodies is not reliable as many successful transplants have been performed in the presence of warm reactive positive crossmatches (Deierhoi, Ting and Morris, 1983).

The differential autoantibody binding to T and B cells indicates that the nature of the auto-antigen varies on cells of different lineage. This difference in cell surface expression may be due to post translational modification of the autoantigen, which may be different on T cells and B cells. Examples of such post translational modifications of one antigen present on cells of different lineage have been described (Barclay, Letarte-Muirhead and Williams, 1976; Standring et al, 1978). Alternatively, higher binding to B cells may be explained on the basis of differences in cell surface electrostatic charge. B cells adhere to tissue culture plastic and nylon wool with higher affinity than T cells. These properties have been used to separate T and B cells in vitro. In addition B cells have been shown to adsorb the blood group antigen 'Lewis' onto its cell surface, whereas T cells do not express Lewis (Park et al, 1979). An electrostatic interaction may enable stronger binding of polyreactive IgM antibodies to B cells relative to T cells. This may offer an explanation for the broad range of B cell binding, from weakly reactive to strongly reactive, seen on the linear cytofluorograph profiles.

Similar binding profiles were observed for T and B cells when comparing the autoreactive and the anti-RhD MAbs. This is consistent with the cytotoxicity profiles previously described (Chapter 3). Similarities between binding of the human IgM autoreactive antibodies and those of MAbs of defined specificities (anti-RhD) indicates that binding to T and B cells

may be non-specific. The results are in accordance with the proposal that lymphocytotoxic autoantibodies in renal dialysis patients share many of the characteristics described for polyreactive IgM antibodies. The data demonstrates that they represent a similar group of antibodies, which can bind multiple antigens which may vary on T cells, B cells and CLL cells.

4.2 Autoantibody binding inhibition studies

Reports on the specificities of lymphocytotoxic autoantibodies in renal dialysis patients have shown IgM and IgG Fc (rheumatoid factors) to be possible target molecules (Cicciarelli et al, 1980; Ozturk and Terasaki, 1980; Takahashi et al, 1980; Ayoub et al, 1980). Polyreactive antibodies may also react with IgM and IgG Fc and in addition with molecules such as lipid-A and phosphatidylcholine (PtC) (Schruster et al, 1979; Mercolino et al, 1988; Nakamura et al, 1988; Van Rooijen, 1989).

As possible candidates for the specificities of the autoreactive (polyreactive) antibodies generated in this study, binding inhibition studies were performed using IgM, IgG, lipid-A and phosphatidylcholine to inhibit binding of the monoclonal autoantibodies to T and B lymphocytes.

4.2.1 Binding inhibition using lipid-A and PtC:

Autoreactive MAbs (F15-DD5-26 and F36-CB10-18) and anti-RhD MAb (FOM-A) at dilutions 1/2 and 1/10, were incubated with T and B cells in the presence of lipid-A (0·2mg/ml) or phosphatidylcholine (100mg/ml), and mean channel fluorescence values were compared to untreated controls. PtC at this concentration produced an emulsion, most of which was washed away during the binding assay. The remaining PtC contaminants gave broad cytofluorograph scatter profiles, above which a distinct population of lymphocytes were gated.

No binding inhibition was found with lipid-A against T or B cell targets (Fig 4.2.1a and b) nor with PtC against T or B cell targets (Fig 4.2.2a and b).

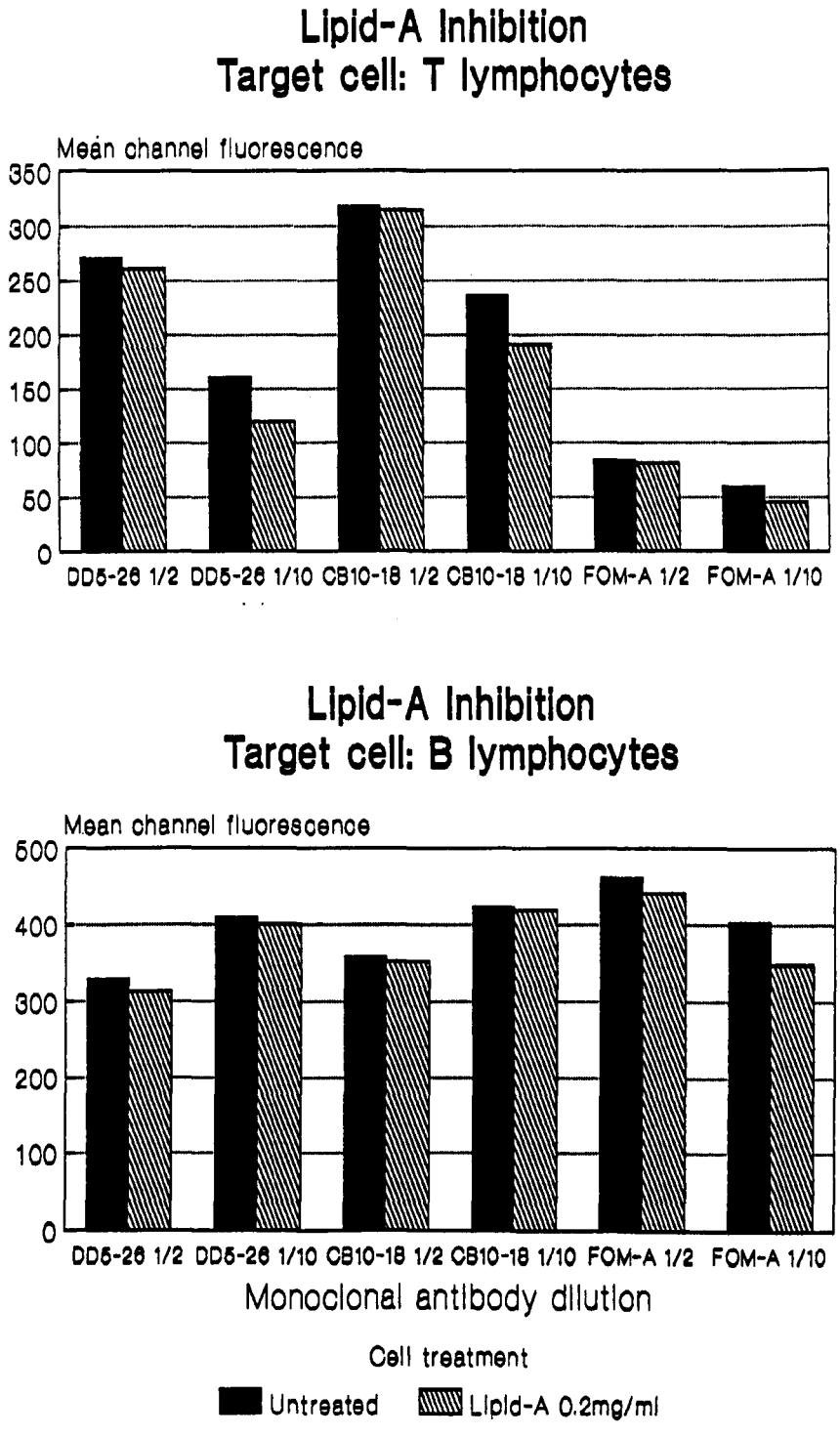


Figure 4.2.1 Binding inhibition of autoreactive (F15-DD5-26 and F36-CB10-18) and anti-RhD (FOM-A) human IgM MAbs tested at 1/2 and 1/10 dilutions against T cells (panel a) and B cells (panel b) by lipid-A.

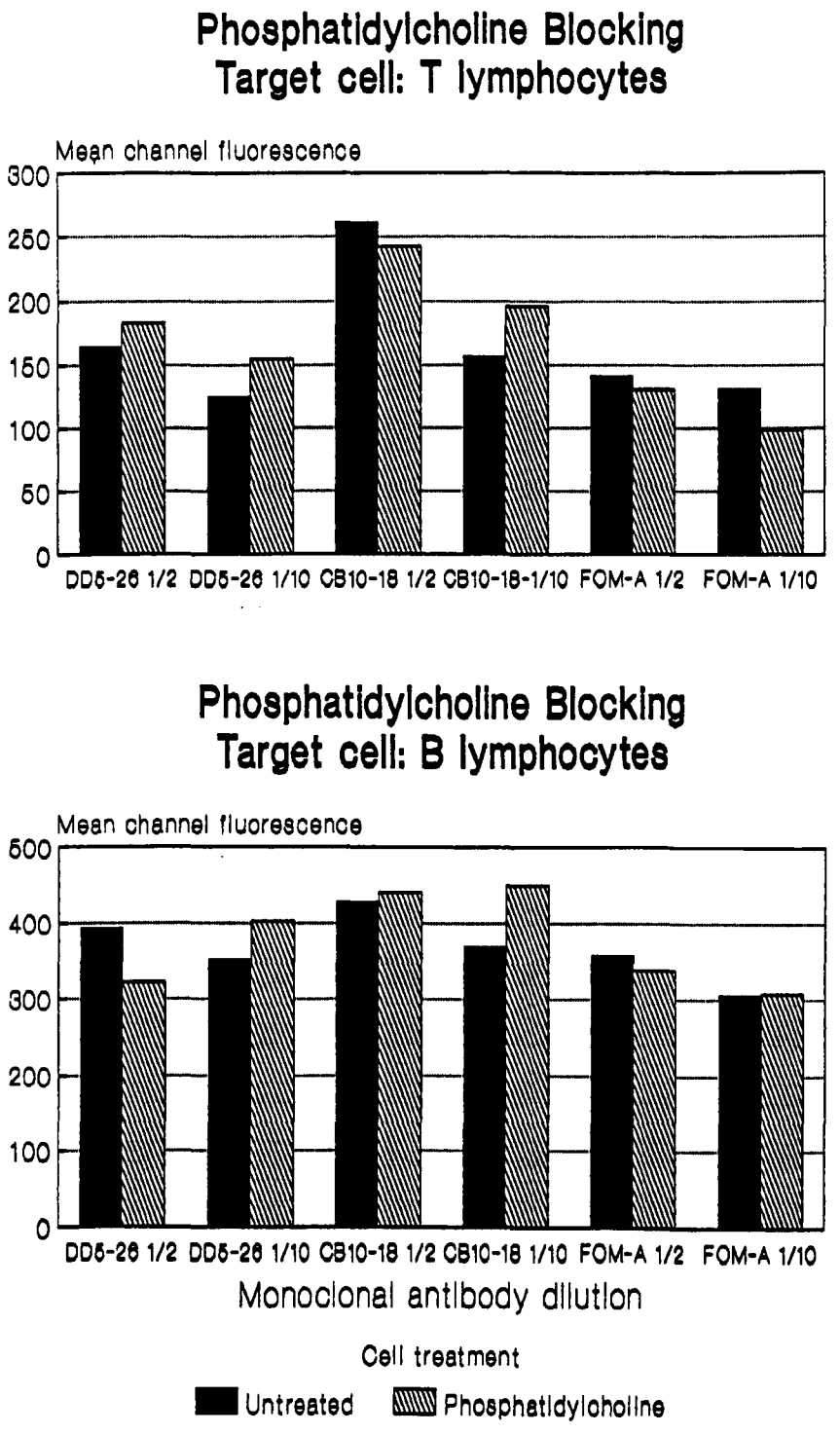


Figure 4.2.2 Binding inhibition of autoreactive (F15-DD5-26 and F36-CB10-18) and anti-RhD (FOM-A) human IgM MAbs tested at 1/2 and 1/10 dilutions against T cells (panel a) and B cells (panel b) by phosphatidylcholine.

4.2.2 Binding inhibition using human IgG and IgM:

Autoreactive and anti-RhD MAbs were tested as above for binding inhibition using human IgG and IgM. No binding inhibition was found for IgG against T or B cell targets (Fig 4.2.3a and b). The purified polyclonal IgM alone (negative control) gave strong background binding (Fig 4.2.4a and b) (no background values have been deducted from this data). The non-specific background binding was stronger with B cells than T cells, as has been noted for the autoreactive antibody binding. Despite the negative control binding, the addition of the autoreactive MAbs (F15-DD5-26 and F36-CB10-18) gave an increased mean channel fluorescence compared to the negative control (IgM alone). The increase in mean channel fluorescence shifts above background values was similar to those seen in normal binding assays using F15-DD5-26 and F36-CB10-18 alone. Therefore the results indicate normal binding profiles of the monoclonal autoantibodies to both T cells and B cells, in the presence of free and cell bound human IgM. In view of the relative concentration of IgM present (0·5mg/ml for polyclonal IgM, compared to between 6-30μg/ml for the autoreactive MAbs), there was no evidence of binding inhibition to T or B cells. The non-specific IgM binding in the 'negative controls' also supports the observation that many different human IgM antibodies may be polyreactive, capable of binding strongly to B cells and less strongly with T cells, resulting in apparent lymphocytotoxic autoantibody profiles.

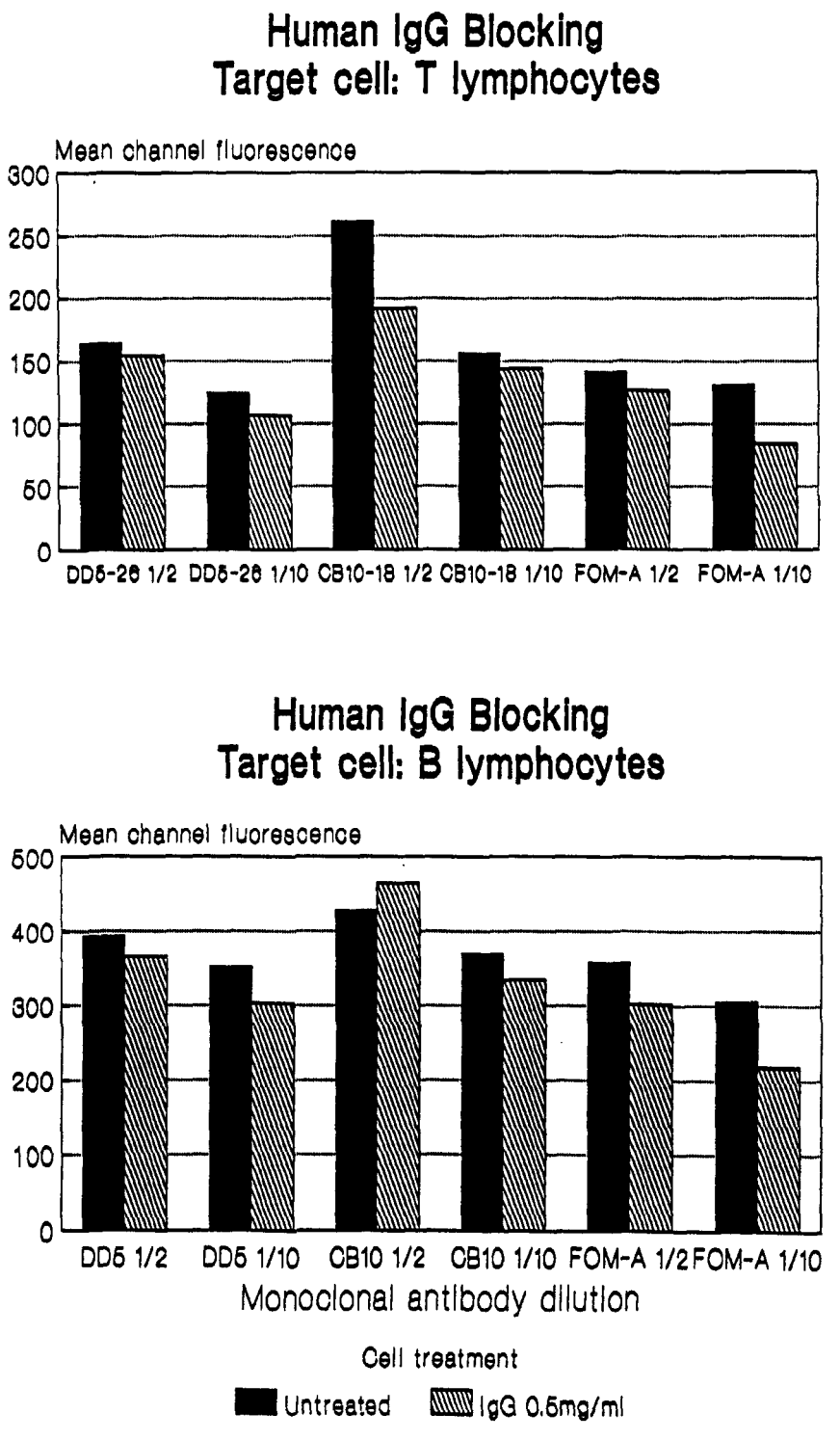
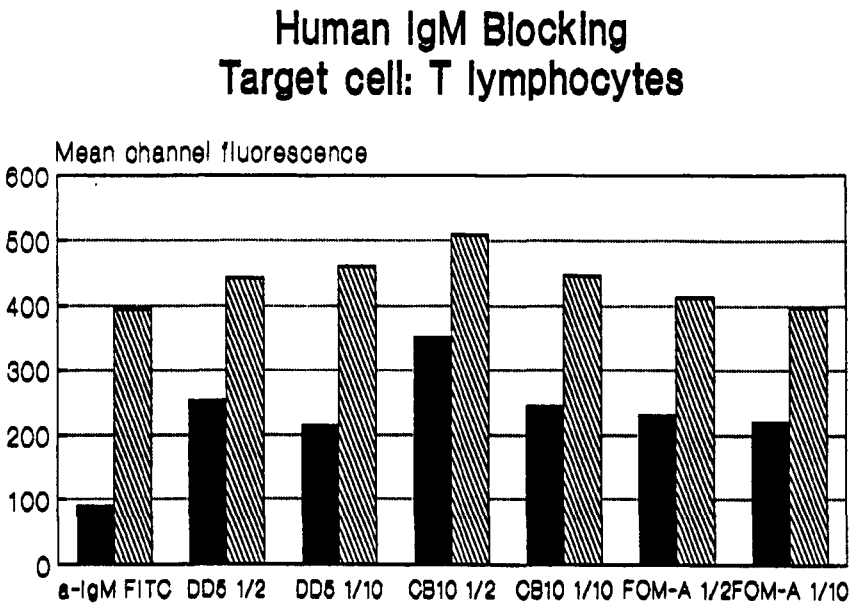


Figure 4.2.3 Binding inhibition of autoreactive (F15-DD5-26 and F36-CB10-18) and anti-RhD (FOM-A) human IgM MAbs tested at 1/2 and 1/10 dilutions against T cells (panel a) and B cells (panel b) by human IgG.



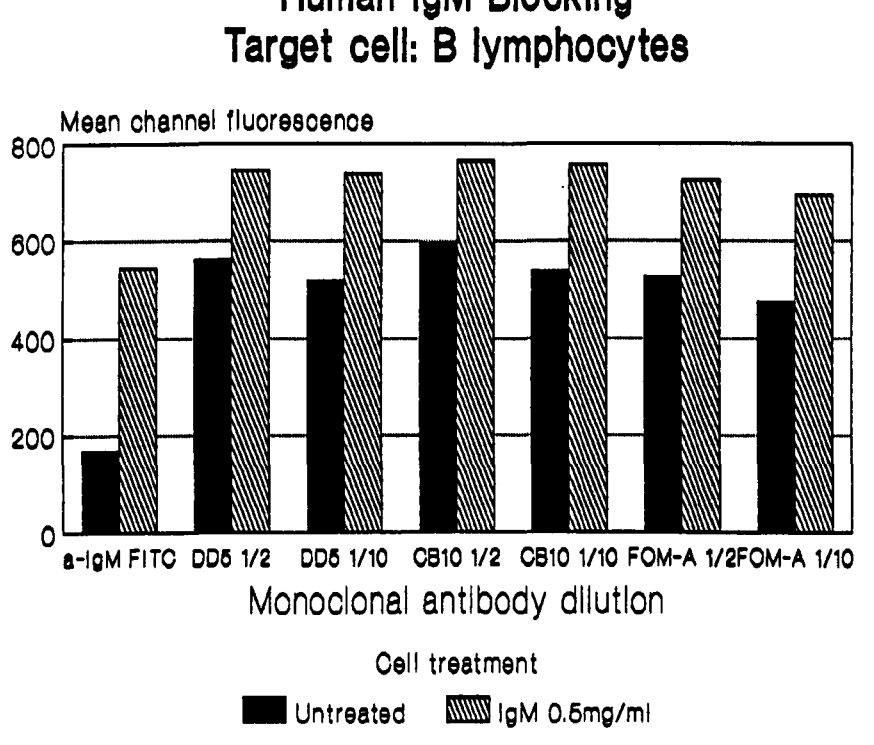


Figure 4.2.4 Binding inhibition of autoreactive (F15-DD5-26 and F36-CB10-18) and anti-RhD (FOM-A) human IgM MAbs tested at 1/2 and 1/10 dilutions against T cells (panel a) and B cells (panel b) by human IgM.

4.2.3 Discussion

Van Rooijen has described naturally occurring autoantibodies reactive with phospholipids in human and rabbit sera (Van Rooijen, 1989). These antibodies are postulated to arise from exposure to endotoxins (lipopolysaccharide) or their lipid containing fractions. Lipids derived from the endotoxins of Gram negative bacteria are absorbed onto the host cell membrane. This stimulates autoreactive IgM antibodies which bind to phospholipids on the cell surface. Antibody reactivity is thought to be directed at the polar heads of phospholipids found in the membrane of all cells (Mercolino et al, 1988). Phosphatidylcholine alone is non-immunogenic but lipid A (endotoxin fragment) insertion into the cell membrane can serve as a hapten/carrier, facilitating the generation of autoantibodies reactive with lipid A and with phosphatidylcholine (Schruster et al, 1979). Lipid A is an endotoxin from Gram negative bacteria and can absorb to the cell membrane of lymphocytes which may trigger autoreactive anti-phospholipid lymphocytotoxic antibodies. Endotoxins from Gram negative bacteria are widely found in the environment and are absorbed through the gut. They are also found in high concentrations in vaccines. Therefore normal individuals are exposed to endotoxins throughout their life. Lipopolysaccharide (LPS) and its lipid A fragments are thymus independent antigens which do not require T cell help. Therefore the antibodies produced are IgM, and there is no immunological memory to this response. Anti-phospholipid antibodies are produced by the CD5 positive subset of B cells which appear to have a limited repertoire of specificities and share common idiotypic determinants (Mercolino et al, 1988).

Casali and Notkins have described polyreactive IgM as natural antibodies of the serum, giving the first line of defence (Casali and Notkins, 1989).

The broad binding reactivity of these antibodies, although inefficient in the destruction of invading pathogens, may allow the vital time required for the induction of a more specific and effective secondary response. It is unclear whether the same CD5 positive B cells producing polyreactive IgM undergo antibody class switch and somatic mutation to produce specific high affinity IgG antibodies, or if these arise from a separate lineage of CD5 negative B cells.

CD5 positive B cells are increased in patients with rheumatoid arthritis (27-52%) compared to normal controls (20%). Polyreactive antibodies have also been identified in these patients. This correlates with the findings of Ozturke who reported a high incidence of lymphocytotoxic autoantibodies in patients with rheumatoid arthritis (Ozturk and Terasaki, 1979). Ozturke also found a marked difference in the susceptibility to lysis of B cells compared to T cells using cytotoxic sera from normal individuals and from patients with various diseases. In addition, Park (Park, Terasaki and Bernoco, 1977) reported lymphocytotoxic autoantibodies in 20% of normal healthy adults. The appearance of the autoantibodies has been correlated with infections and vaccinations (Jeannet, Benzonana and Arni, 1981; Cross, Greiner and Whittier, 1976; Macleod et al, 1987).

Lymphocytotoxic autoantibodies have also been reported in patients with Systemic Lupus Erythematosus (SLE). However unlike the autoantibodies previously described, these are often IgG and are equally reactive with T and B cells. Therefore it is possible that the autoantibodies in SLE patients represent a different type to those described in RA and renal dialysis patients. Alternatively, the same polyreactive autoantibody producing B cell in SLE patients clones may differentiate into CD5 negative

B cells, undergoing somatic mutation with the production of high affinity IgG which react equally with T cells and B cells.

However, despite observations correlating similarities between lymphocytotoxic autoantibodies and polyreactive IgM binding characteristics, inhibition data using lipid-A and PtC indicate that binding of the monoclonal autoantibodies, and the non-specific binding of anti-RhD MAb to T and B cells is not directed at a lipid component of the lymphocyte cell membrane. Although the non-specific binding of the anti-RhD MAbs to lymphocytes and the monoclonal autoantibody profiles indicate that binding may be due to polyreactivity, the target molecule in this case does not appear to be PtC as has been found for many naturally occurring and induced polyreactive antibodies in humans and rabbits (Van Rooijen, 1989).

Furthermore, these monoclonal autoantibodies were not inhibited by excess concentrations of human IgG or IgM. This indicates that the MAbs used in this study are not rheumatoid factors (IgM-anti-IgG) or reactive with IgM on the B cell surface (IgM-anti-IgM) as suggested by Cicciarelli. Two separate observations support this data. Firstly, cytotoxicity assays indicate that the monoclonal autoantibodies are reactive with both T and B cells, and that the different binding profiles are due to the target cell binding affinity. As T cells do not express cell surface immunoglobulin, and only a portion of adult spleen and lymph node B cells express IgM, the reactivity of lymphocytotoxic autoantibodies with all T and B cells indicate that cell surface IgM is an unlikely target. Furthermore, the FACScan profiles demonstrated autoantibody binding to both cell surface IgM positive and negative B cells. Secondly, the lack of reactivity of the

human IgM anti-rheumatoid factor MAb (RFAN) with T and B cells in cytotoxicity assays indicate that rheumatoid factors per se do not give rise to autoantibody profiles in cytotoxicity assays.

These data indicate that IgG, IgM, lipid-A and PtC are not the primary target molecules for lymphocytotoxic autoantibody reactivity.

Chapter 5

Characterisation of the biochemical nature of the autoantigen on lymphocytes

5.1 Enzyme digestion of lymphocyte cell surface antigens

5.1.1 Effect of Neuraminidase digestion on ALA MAb binding

a) Specificity controls
b) Cytotoxicity analysis of neuraminidase digestion on ALA MAb binding
c) Cytofluorograph analysis of neuraminidase digestion on ALA MAb binding to; i) CLL cells, ii) T and B cells

5.1.2 Effect of Papain digestion on ALA MAb binding

a) Control studies; i) Cytotoxicity, ii) Fluorescence binding assay
b) Cytofluorograph analysis of papain digestion on ALA MAb binding

5.1.3 Effect of Peptidase digestion on ALA MAb binding

5.1.4 Effect of Lipase digestion on ALA MAb binding

5.1.5 Effect of Amylase digestion on ALA MAb binding

5.1.6 Effect of Trypsin digestion on ALA MAb binding

5.1.7 Discussion

5.2 Lectin Inhibition Studies to Determine ALA Specificities

5.2.1 Cytofluorograph analysis of lectin inhibition of lymphocytotoxic autoantibody binding

Group 1. Lectins which gave no change in autoantibody binding
Group 2. Lectins which caused increased autoantibody binding
Group 3. Lectins which inhibited autoantibody binding

5.2.2 Lectin inhibition of autoantibody binding in cytotoxicity tests

5.2.3 Autoantibody binding to lectins

i) Lysis of lectin coated red blood cells by lymphocytotoxic autoantibodies and complement
ii) Inhibition of lectin agglutination of red blood cells by lymphocytotoxic autoantibodies
iii) Lectin inhibition of lymphocytotoxic autoantibody binding in the microlymphocytotoxicity test

5.1 Enzyme digestion of lymphocyte cell surface antigens

Enzyme cleavage of cell surface molecules from lymphocytes was used to determine the biochemical nature of the autoantigen. The effect of proteolytic, glycosidic and liposidic enzyme digestion of antigenic determinants on lymphocyte membranes was investigated. Subsequent binding of ALA's was analyzed to examine the protein, carbohydrate and lipid contributions to the antigenicity of the target molecule. The enzymes selected for this study were neuraminidase and amylase for carbohydrate analysis; papain, trypsin and peptidase for protein analysis and lipase for lipid and phospholipid analysis.

5.1.1 Effect of Neuraminidase digestion on ALA MAb binding:

Neuraminidase (sialidase) was used to cleave N-acetyl neuramic acid (NANA) determinants from the cell surface of lymphocytes.

a) Specificity controls

Positive and negative specificity controls were selected to demonstrate the specific cleavage of sialic acid residues from the lymphocyte cell surface by neuraminidase. The mouse IgG MAb, W6/32, which binds a protein defined monomorphic determinant on HLA class I molecules was used as a negative control. Reactivity of W6/32 with HLA class I is resistant to neuraminidase treatment. Cytofluorograph analysis showed binding of W6/32 at 1/10 dilution to T and B cells was increased following enzyme digestion (Fig 5.1.1). This suggests that sialic acid cleavage from HLA class I molecules allows increased binding of W6/32.

MAb DF-T1 (a kind gift from D.J. Flavell, Oxford [Stross et al, 1989a]) which binds a neuraminidase sensitive epitope on CD43 molecules present on

T cells (Stross et al, 1989b) was selected as a positive specificity control. Enzyme treated cells were tested for CD43 binding using DF-T1 at 1/2 and 1/10 dilutions (Fig 5.1.2). CD43 binding was completely removed at neuraminidase concentrations of 2·0 U/ml down to 0·25 U/ml, demonstrating specific activity of neuraminidase treatment.

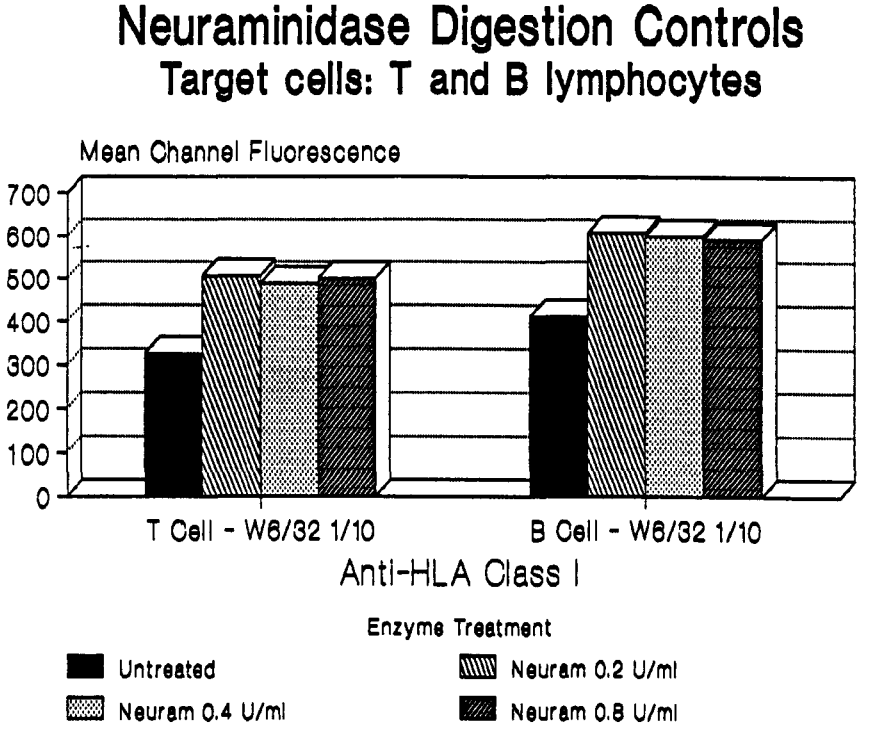


Figure 5.1.1 Cytofluorograph analysis of W6/32 (mouse IgG anti-HLA-class I) binding at 1/10 dilution to T and B cells following neuraminidase treatment using 0·8, 0·4 and 0·2 U/ml.

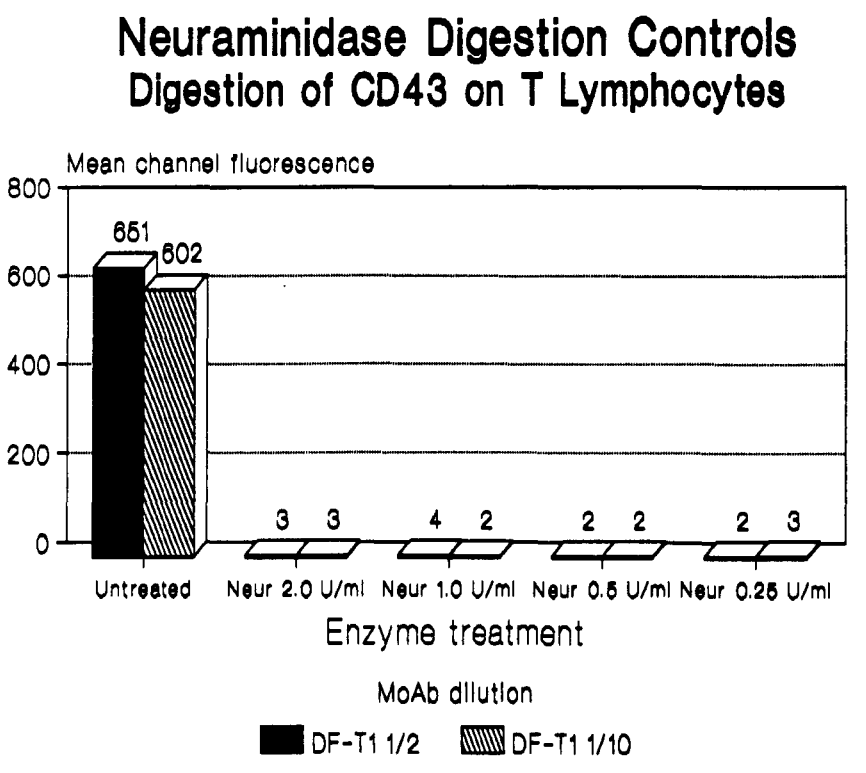


Figure 5.1.2 Cytofluorograph analysis of DF-T1 (mouse IgG anti-CD43) binding at 1/2 and 1/10 dilutions to T cells following neuraminidase treatment using 2·0, 1·0, 0·5, and 0·25 U/ml.

b) <u>Cytotoxicity analysis of neuraminidase digestion on ALA MAb binding</u>

Neuraminidase treated T and B lymphocytes were tested for ALA binding using F15-DD5-26 and F36-CB10-18 MAbs by cytotoxicity. Following enzyme treatment, the cells were >90% viable as assessed by eosin dye exclusion. However 100% background lysis was observed following cytotoxicity tests using enzyme concentrations down to 0·03 U/ml. Neuraminidase at 0·015 U/ml gave no background cell lysis, however there was no reduction in the titre of the autoantibodies using this enzyme concentration (data not shown).

The non-specific cell lysis was found to be due to complement toxicity as it did not occur in the absence of complement, or with heat inactivated complement. This finding is in accordance with reports that cleavage of sialic acid from the cell surface results in non-specific activation of the alternative complement pathway (Dr K.Wood, NDS, Oxford, personal communication). However, the test does demonstrate the cleavage of sialic acid residues from the cell surface using neuraminidase at concentrations of 0·03 U/ml.

c) Cytofluorograph analysis of neuraminidase digestion on ALA MAb binding: Neuraminidase treated CLL cells, T cells and B cells were tested for inhibition of monoclonal autoantibody binding in fluorescence binding assays. Cytofluorograph profiles of enzyme treated and untreated cells were compared.

i) CLL cells; CLL cells were tested for autoantibody binding with F15-DD5-26 and F36-CB10-18 at 1/2 and 1/10 dilutions following neuraminidase treatment using 0·1 U/ml and 0·05 U/ml (Figs 5.1.3a and 5.1.3b). The cytofluorograph profiles show that the autoantibody binding to CLL cells was markedly reduced following treatment at both enzyme concentrations.

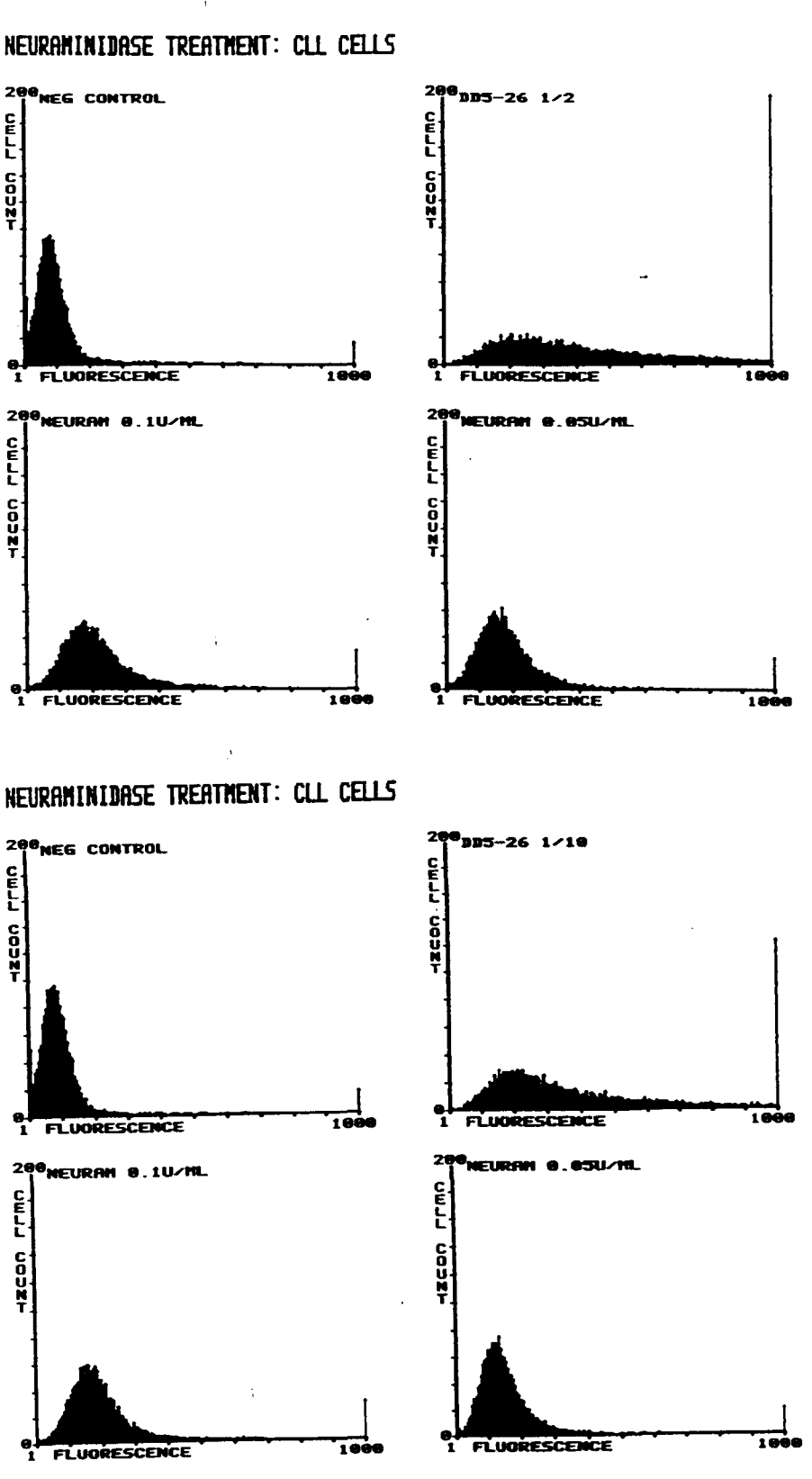


Figure 5.1.3a Cytofluorograph binding profiles of F15-DD5-26 at 1/2 and 1/10 dilutions to CLL cells following neuraminidase treatment at 0·1 and 0·05 U/ml.

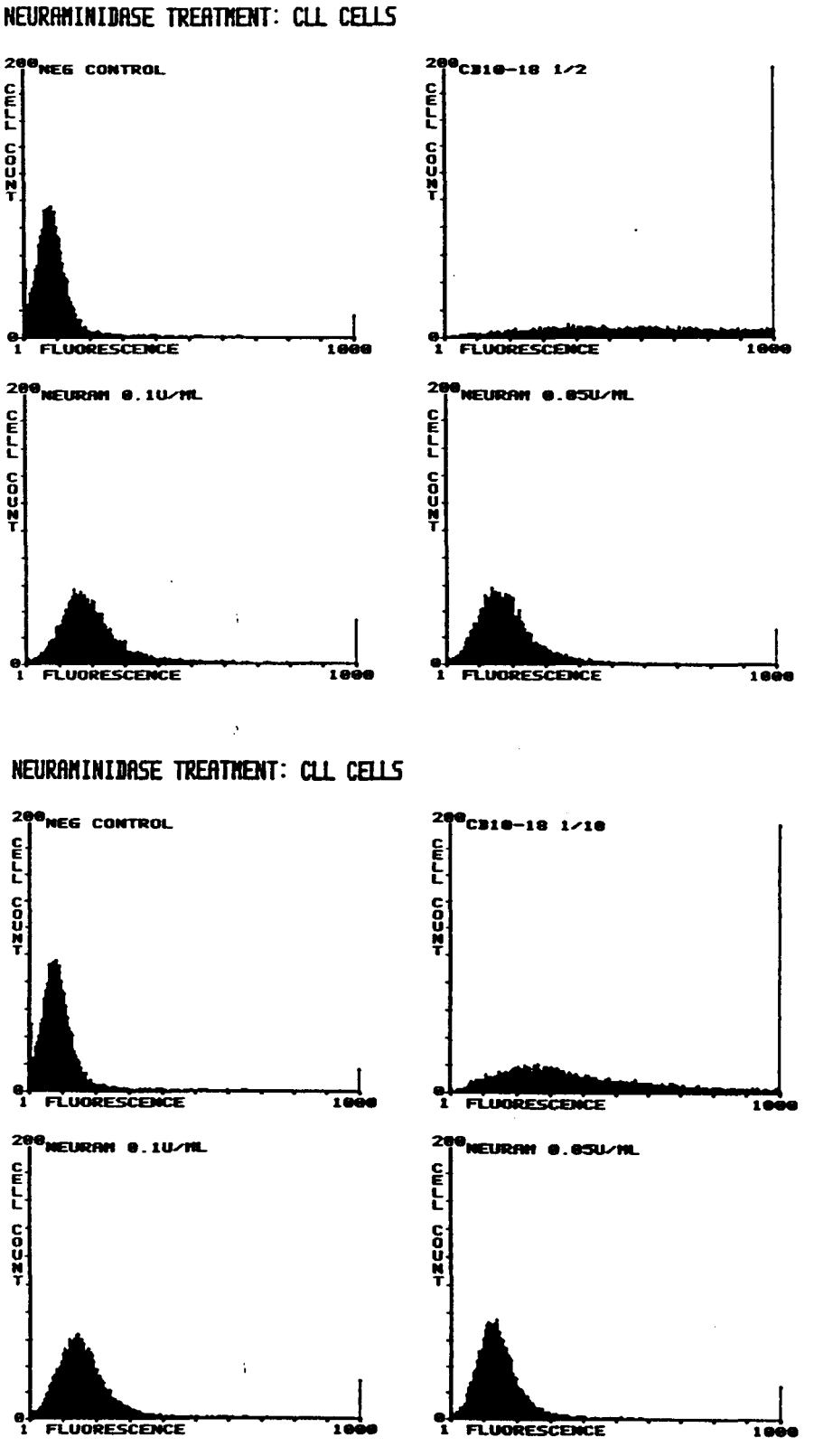


Figure 5.1.3b Cytofluorograph binding profiles of F36-CB10-18 at 1/2 and 1/10 dilutions to CLL cells following neuraminidase treatment at 0·1 and 0·05 U/ml.

Figure 5.1.4 shows the mean channel fluorescence values for F15-DD5-26 and F36-CB10-18 binding to CLL cells. Following enzyme treatment, near background binding levels were found, demonstrating complete removal of the autoantigenic epitope by neuraminidase.

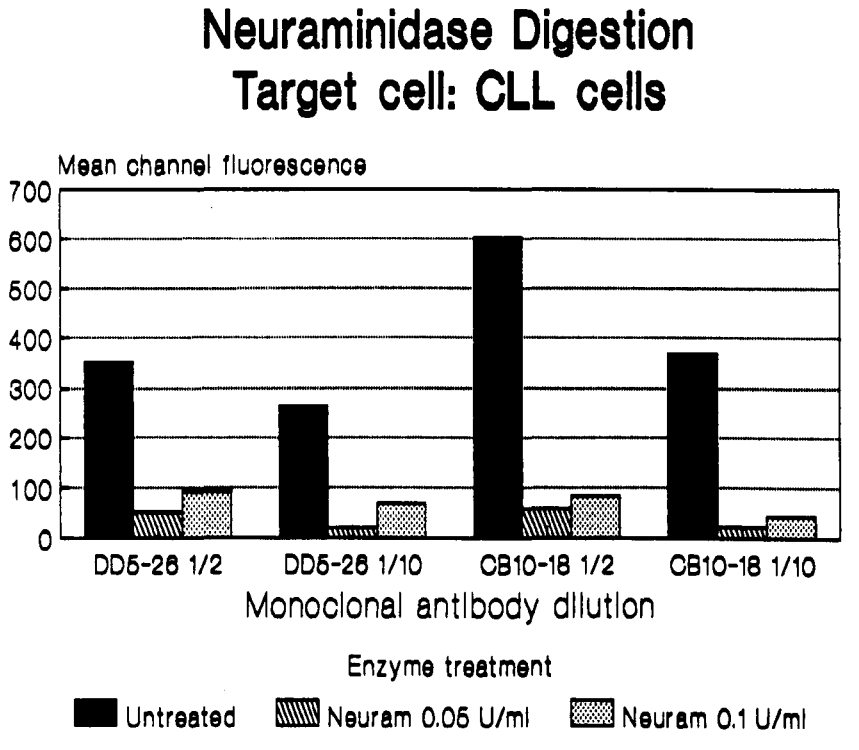


Figure 5.1.4 Cytofluorograph analysis of F15-DD5-26 and F36-CB10-18 binding at 1/2 and 1/10 dilutions to CLL cells following neuraminidase treatment at 0·1 and 0·05 U/ml.

ii) T and B cells; Monoclonal autoantibody binding to T cells was not consistently removed at these enzyme concentrations and B cell binding was unaffected (data not shown). Neuraminidase concentrations of 0·1 and 0·2 U/ml reduced F15-DD5-26 binding at 1/2 dilution to T cells by 19% and 28% respectively (Fig 5.1.5a). F36-CB10-18 binding at 1/2 dilution showed a 46%

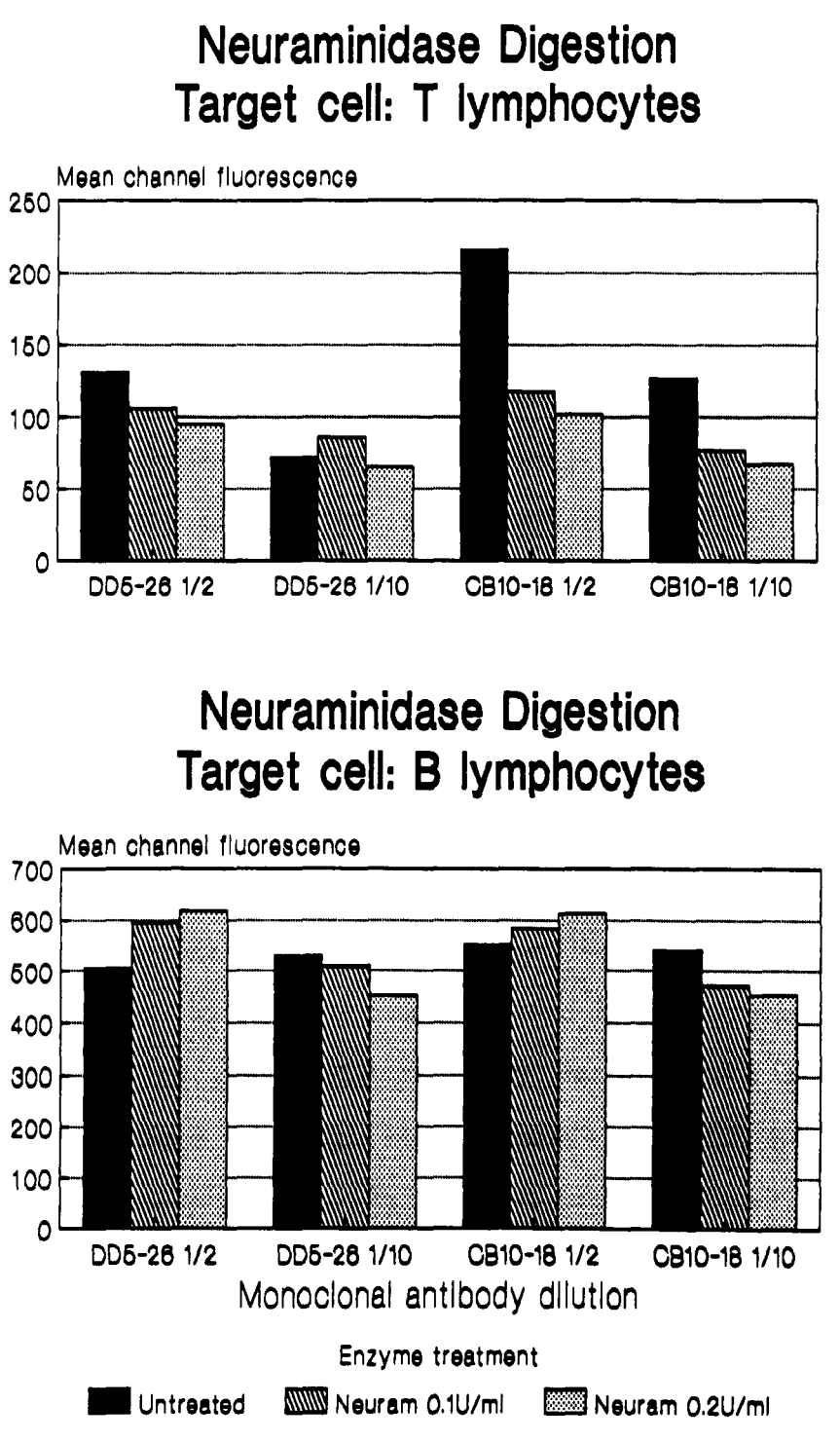


Figure 5.1.5 Cytofluorograph analysis of F15-DD5-26 and F36-CB10-18 binding at 1/2 and 1/10 dilutions to T cells (panel a) and B cells (panel b) following neuraminidase treatment at 0·1 and 0·2 U/ml.

and 53% reduction in mean channel fluorescence (Fig 5.1.5a). These enzyme concentrations produced no effect on ALA binding to B cells (Fig 5.1.5b).

The effect of neuraminidase treatment on autoantibody binding to T cells was variable. One experiment, using F36-CB10-18 at 1/2 and 1/10 dilutions showed a marked reduction in autoantibody binding using neuraminidase at 0·8, 0·4 and 0·2 U/ml (Fig 5.1.6a). Again, B cell binding was unaffected at these concentrations (Fig 5.1.6b). Although the B cell profiles showed some shift to the left with F36-CB10-18 at 1/2 dilution, there was no overall reduction in mean channel fluorescence at 1/2 or 1/10 dilutions.

These results indicate the involvement of a carbohydrate residue (sialic acid) in autoantibody binding to CLL and T cells, but not to B cells. The resistance of the B cell autoantigen to cleavage by neuraminidase may be caused by differing degrees of sensitivity, or be due to a distinct neuraminidase insensitive epitope. To investigate this further, T and B cells were tested using an increased enzyme concentration of 1·0 U/ml (Fig 5.1.7). As previously found, T cells showed a marked inhibition in autoantibody binding, with a mean reduction of 68% in mean channel fluorescence (Fig 5.1.7a). B cell binding showed a mean reduction of 35% following neuraminidase treatment at 1·0 U/ml (Fig 5.1.7b). Binding of the anti-RhD MAb, FOM-A, at 1/10 dilution was reduced following neuraminidase treatment using 1·0 U/ml for both T cells (Fig 5.1.7a) and B cells (Fig 5.1.7b), although little effect was found at 1/2 dilution.

The results suggest that autoantibody binding to CLL cells and T cells is dependant on a sialic acid defined epitope, whereas B cell binding is only partially dependant on this molecule.

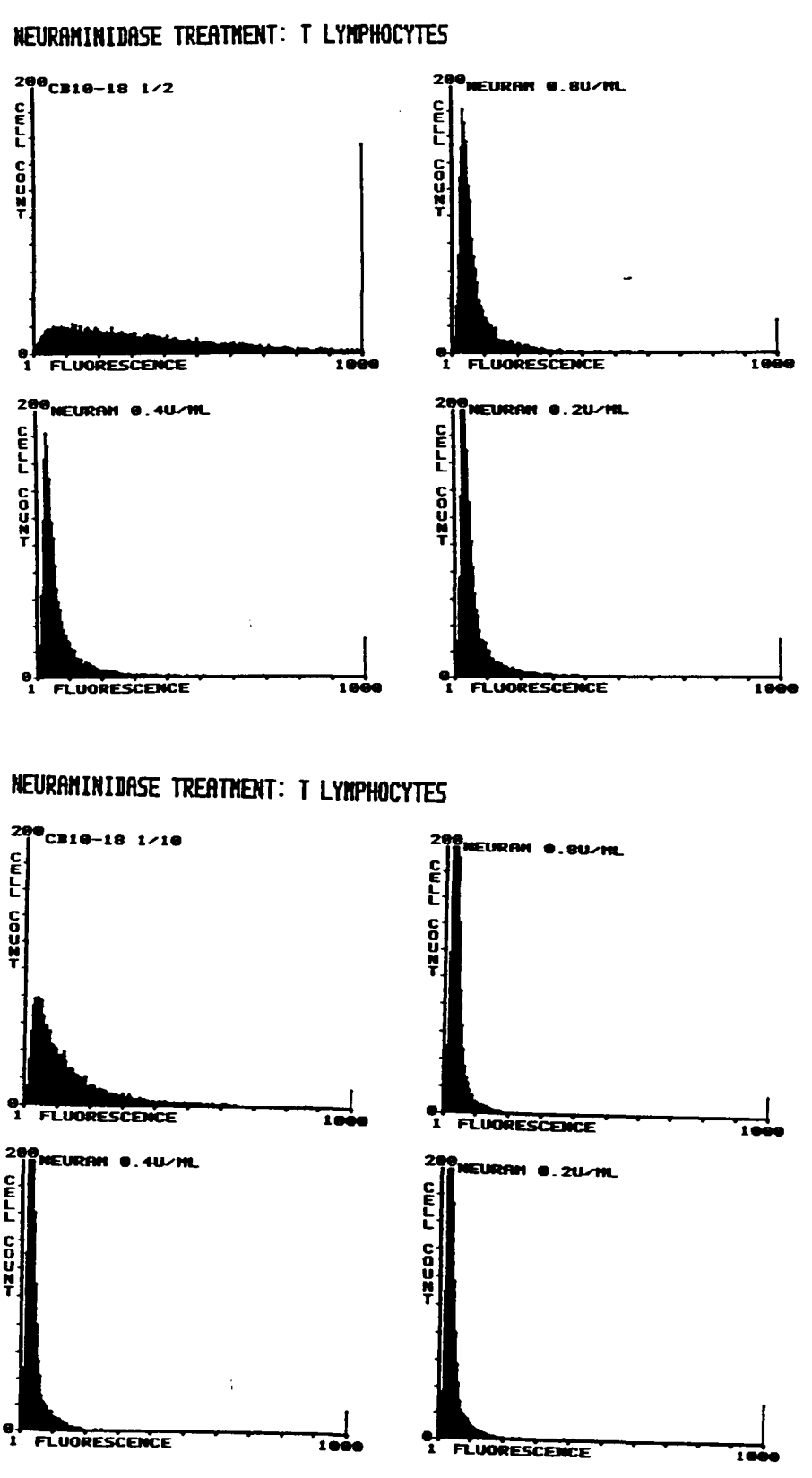


Figure 5.1.6a Cytofluorograph binding profiles of F36-CB10-18 binding at 1/2 and 1/10 dilutions to T cells following neuraminidase treatment at 0·8, 0·4 and 0·2 U/ml.

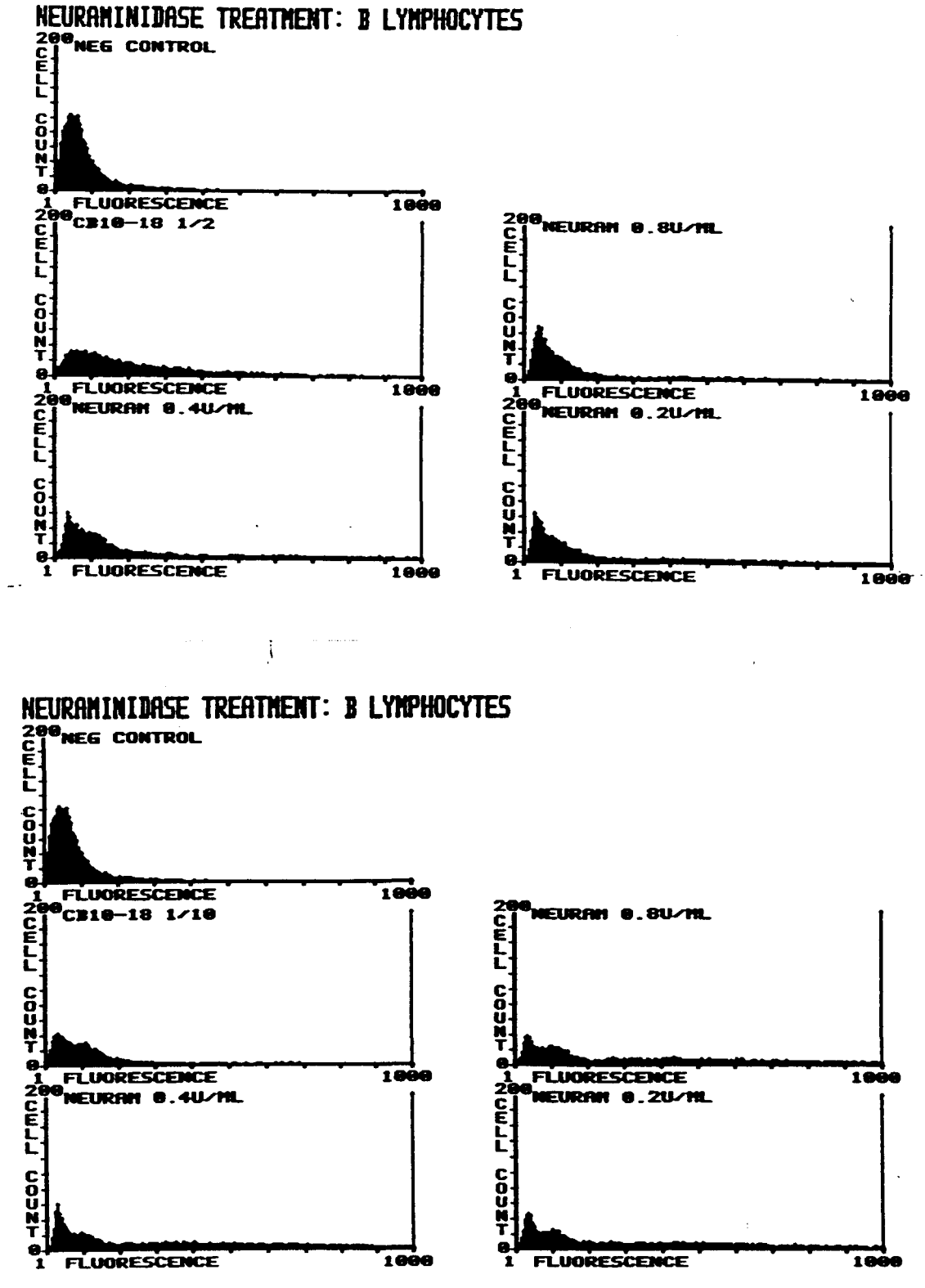


Figure 5.1.6b Cytofluorograph binding profiles of F36-CB10-18 binding at 1/2 and 1/10 dilutions to B cells following neuraminidase treatment at 0·8, 0·4 and 0·2 U/ml.

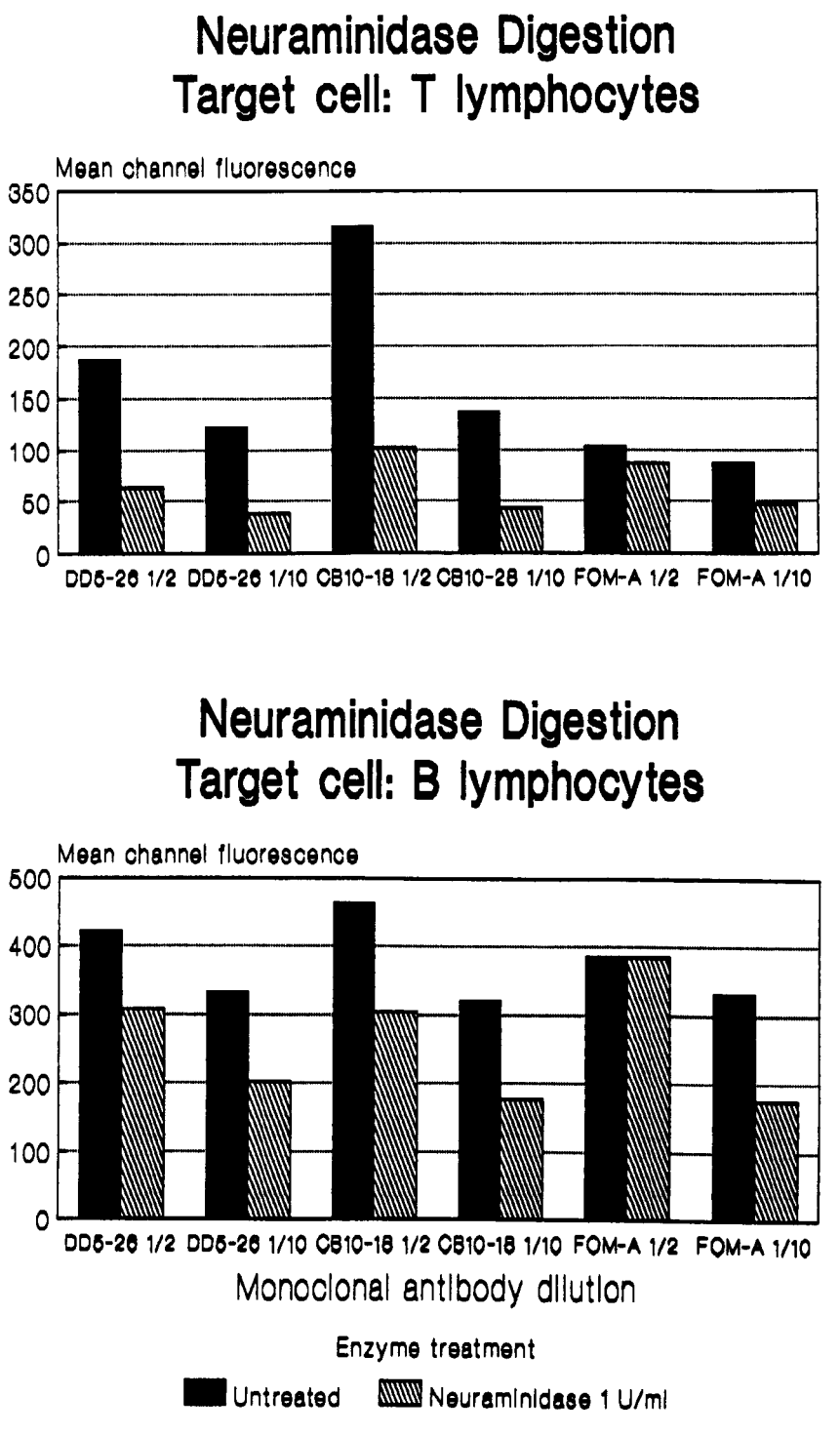


Figure 5.1.7 Cytofluorograph analysis of F15-DD5-26, F36-CB10-18 and FOM-A binding at 1/2 and 1/10 dilutions to T cells (panel a) and B cells (panel b) following neuraminidase treatment at 1·0 U/ml.

5.1.2 Effect of Papain digestion on ALA MAb binding:

a) Control studies

i) Cytotoxicity. Papain is a proteolytic enzyme which cleaves HLA class I molecules from the cell surface (Chapel and Welsh, 1972). The effect of papain treatment on T and B cells was tested using an HLA class I alloantisera specific for HLA-Bw6 by cytotoxicity. Papain at concentrations of 2·0mg/ml down to 0·125mg/ml produced high background lysis in the cytotoxicity assay (data not shown). Concentrations below 0·125mg/ml produced no reduction in titre with the HLA-class I alloantisera nor with the ALA MAbs (data not shown). Therefore the cytotoxicity method could not be used to determine the effect of papain on autoantibody binding.

ii) Fluorescence binding assay. The mouse IgG MAb W6/32, which recognises a monomorphic protein defined determinant on all HLA class I molecules was used as a positive control in a fluorescence binding assay. T cells treated with papain at 4·0mg/ml down to 0·25mg/ml were analyzed for W6/32 binding (Fig 5.1.8). Enzyme concentrations of 0·5 and 0·25mg/ml had little effect on HLA class I binding. At 1·0mg/ml a 35% reduction in mean channel fluorescence was observed, and at 2·0 and 4·0mg/ml an 82% and 93% reduction in binding were found respectively. Papain at 2·0mg/ml gave good cytofluorograph scatter profiles and at 4·0mg/ml had acceptable scatter profiles. Therefore these enzyme concentrations were selected for analysis of ALA binding.

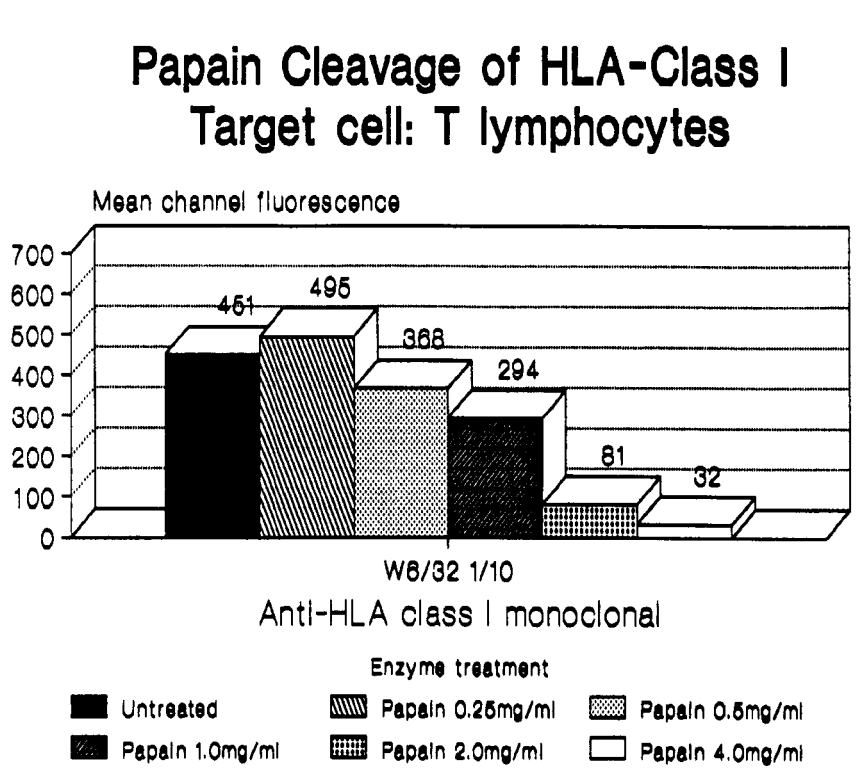


Figure 5.1.8 Cytofluorograph analysis of papain cleavage of HLA class I molecules from T cells using enzyme concentrations of 4·0mg/ml down to 0·25mg/ml with W6/32 (mouse IgG anti-HLA class I) at 1/10 dilution.

b) Cytofluorograph analysis of papain treatment on ALA binding:

Treatment of lymphocytes with papain at 2·0 and 4·0mg/ml was found to cause high non-specific background binding of the FITC-goat-anti-human-IgM alone (Fig 5.1.9).

Attempts were made to block this non-specific second layer binding with foetal calf serum (FCS) and human blood group AB sera (AB). Following enzyme treatment using papain at 2·0 and 4·0mg/ml, the cells were incubated with neat FCS or human AB serum, on ice for 20 minutes, as described in

Materials and Methods (Chapter 2.3c). Non-specific background binding of FITC-rabbit-anti-mouse-IgG (RaM) and FITC-goat-anti-human-IgM (GaH) were measured (Fig 5.1.10).

The results showed that there was no background binding to papain treated cells when using RaM-IgG. However, the high background binding seen with GaH-IgM was completely blocked following incubation in foetal calf serum. Blocking with human blood group AB serum only served to increased the background binding.

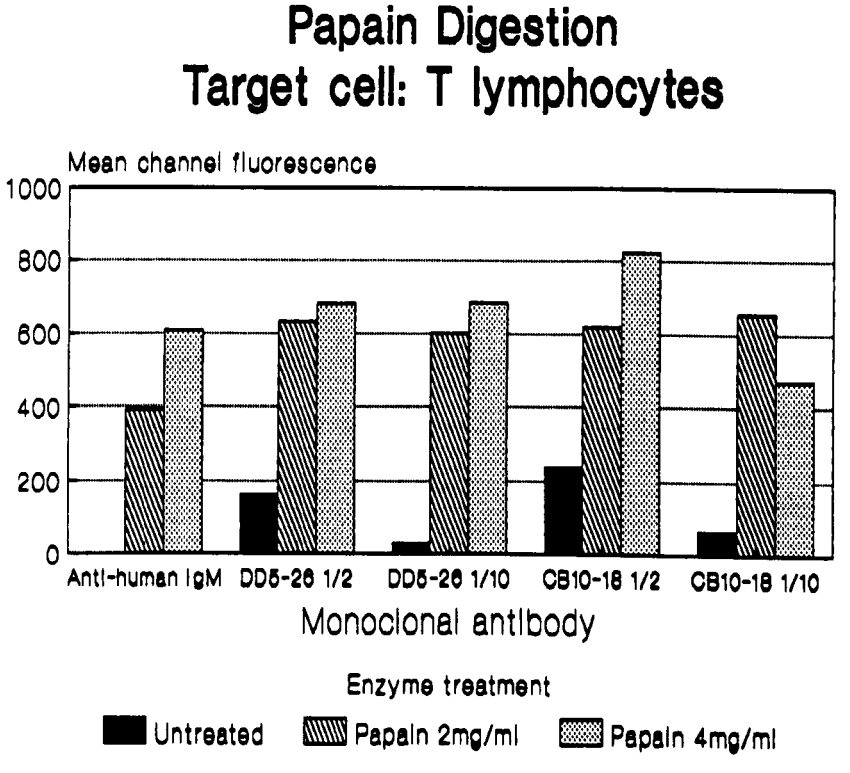


Figure 5.1.9 Cytofluorograph analysis of FITC-goat-anti-human-IgM background binding, F15-DD5-26 and F36-CB10-18 binding to T cells following papain treatment at 2·0 and 4·0mg/ml.

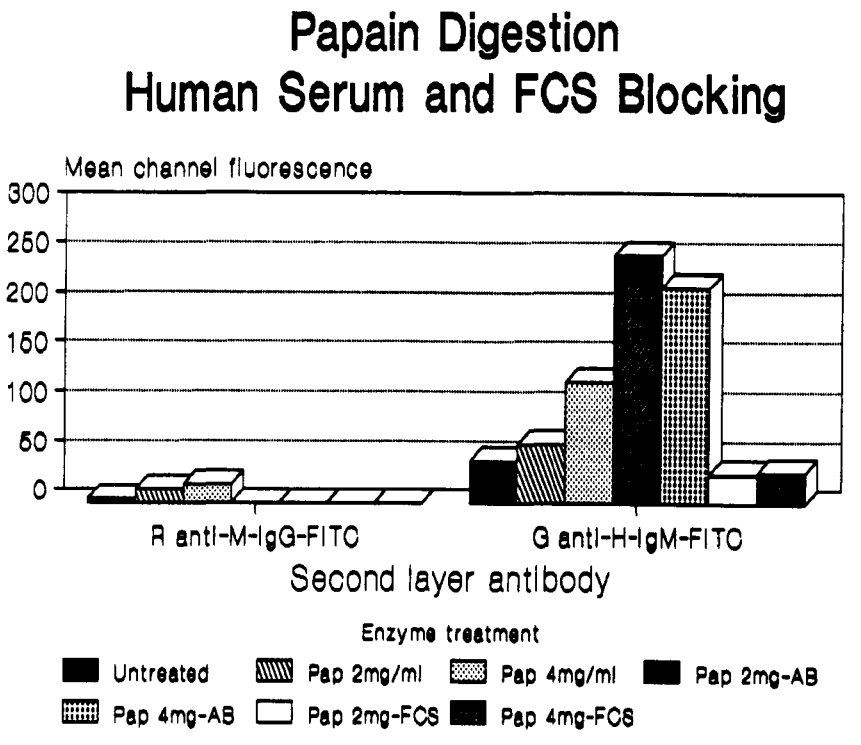


Figure 5.1.10 Cytofluorograph analysis of foetal calf serum (FCS) and human AB serum (AB) blocking on background binding of FITC-rabbit-anti-mouse-IgG and FITC-goat-anti-human-IgM to T cells following papain digestion at 2·0 and 4·0mg/ml.

Blocking with FCS enabled analysis of the effect of papain digestion on monoclonal autoantibody binding to lymphocytes to be determined. ALA binding following treatment of T cells with papain at 2·0mg/ml, and with B cells using papain at 2·0 and 4·0mg/ml were tested using F15-DD5-26, F36-CB10-18, and FOM-A (anti-RhD), at 1/2 and 1/10 dilutions (Fig 5.1.11).

ALA binding to T cells was partially removed following treatment with papain, with a reduction in mean channel fluorescence of 32% and 37% for

F15-DD5-26 and F36-CB10-18 respectively (Fig 5.1.11a). No effect of papain digestion was found for FOM-A binding to T cells. In parallel control studies, reactivity of PA2.6 (mouse IgG anti-HLA class I) was reduced by 78%, demonstrating papain activity.

Binding inhibition to B cells was only found at 1/10 dilution, with 35% and 46% reduction in F15-DD5-26 and F36-CB10-18 binding respectively (Fig 5.1.11b). There was also a 70% reduction in FOM-A binding to B cells at 1/10 dilution. Reactivity of the control antibody, PA2.6 (mouse IgG anti-HLA class I), was completely removed following papain digestion.

The results suggest that the autoantibody target antigen is possibly a glycoprotein molecule. However the effect of papain was less than that for neuraminidase.

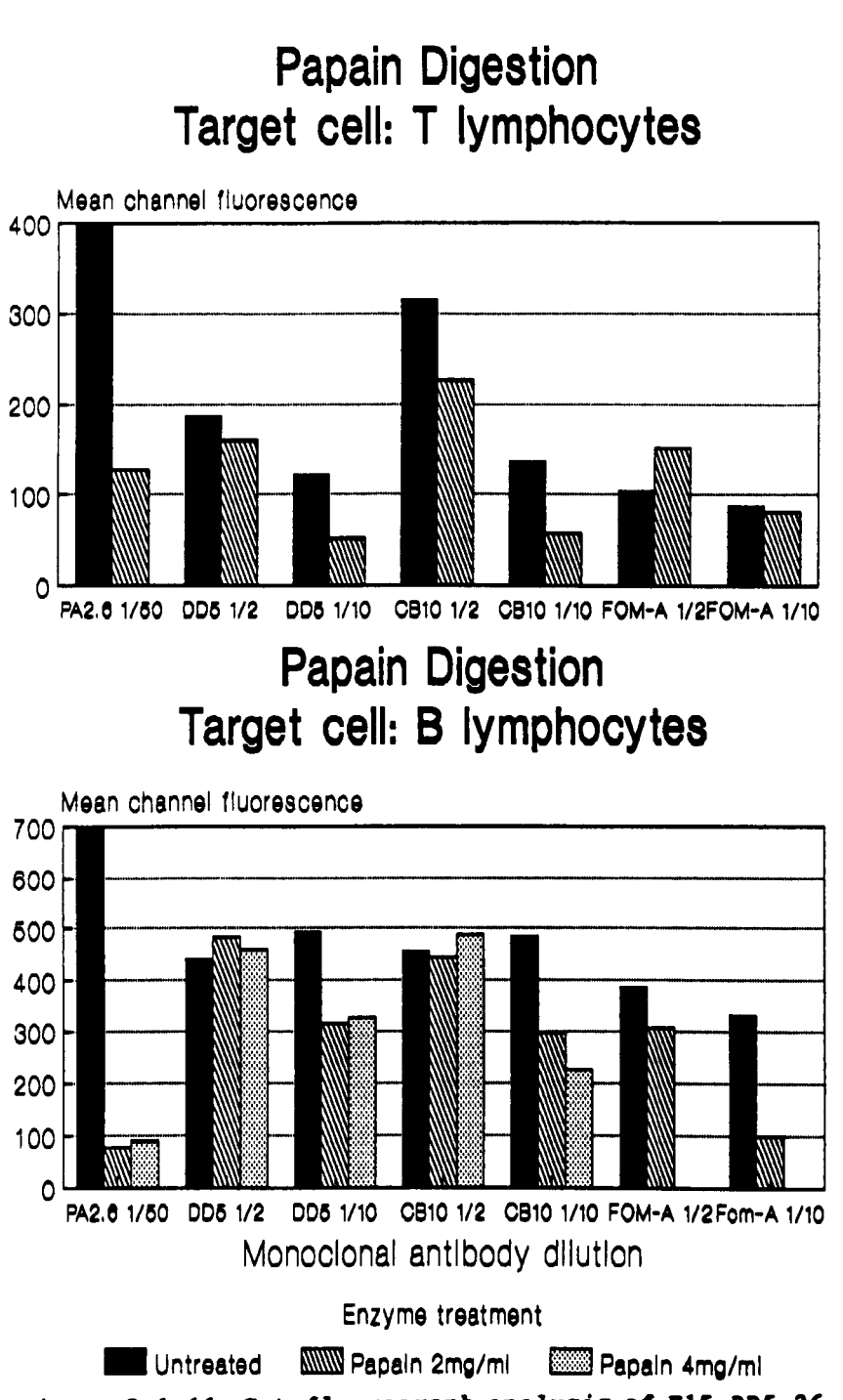


Figure 5.1.11 Cytofluorograph analysis of F15-DD5-26, F36-CB10-18 and FOM-A binding at 1/2 and 1/10 dilutions and PA2.6 (mouse IgG anti-HLA-class I control) to T cells (panel a) and B cells (panel b) following papain digestion at 2·0 and 4·0mg/ml. (Background binding of FITC-goat-anti-human-IgM in this and all subsequent enzyme digestion experiments have been blocked with FCS as previously described).

5.1.3 **Effect of Peptidase digestion on ALA MAb binding:**

The effect of peptidase digestion on autoantibody binding to T and B cell targets was performed using F15-DD5-26 and F36-CB10-18 at 1/2 dilutions (Fig 5.1.12). The results show peptidase at 2·0mg/ml produced no significant change in mean channel fluorescence.

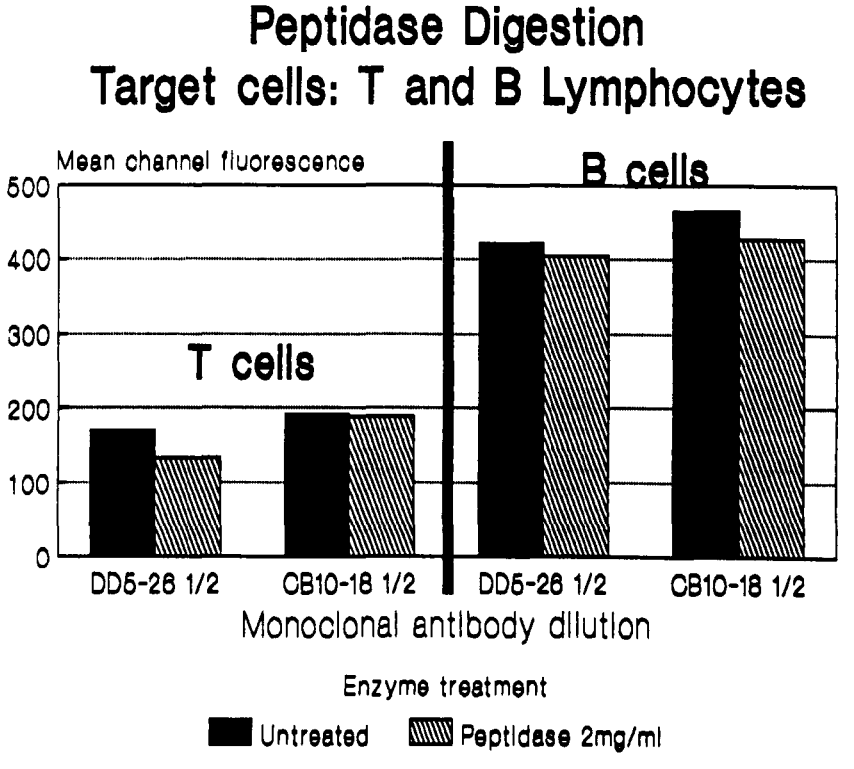


Figure 5.1.12 Cytofluorograph analysis of F15-DD5-26 and F36-CB10-18 binding at 1/2 dilution to T and B cell targets following peptidase digestion at 2·0mg/ml.

5.1.4 **Effect of Lipase digestion on ALA MAb binding:**

Lipase digestion at enzyme concentrations of 2·0 and 4·0mg/ml was tested against T and B cell targets using F15-DD5-26 and F36-CB10-18 at 1/2 and 1/10 dilutions (Fig 5.1.13).

F15-DD5-26 and F36-CB10-18 binding to T cells at 1/2 dilutions was slightly increased following lipase digestion at 2·0 and 4·0mg/ml (Fig 5.1.13a). At 1/10 dilution, no change in mean channel fluorescence values were found using lipase at 4·0mg/ml, and binding was increased with lipase at 2·0mg/ml.

Lipase digestion of B cell targets produced a small decrease in F15-DD5-26 and F36-CB10-18 binding at 1/2 dilution (Fig 5.1.13b), and further inhibition of binding at 1/10 dilution. To confirm this observation, a second experiment was performed using lipase at 2·0mg/ml using T and B cell targets with F15-DD5-26, F36-CB10-18 and FOM-A at 1/2 and 1/10 dilutions (Fig 5.1.14). No consistent inhibition of MAb binding was seen in this experiment with either T cells (Fig 5.1.14a) or B cells (Fig 5.1.14b).

The discrepancy of B cell binding between these experiments remains unresolved. There was no evidence of binding inhibition following lipase digestion of T cell targets in either experiment, and only a small reduction in ALA binding to B cells in one experiment. Therefore there is insufficient evidence to support cleavage of the autoantigenic epitope from T or B cells by lipase. Clearly, the experiments lack appropriate controls to demonstrate enzyme cleavage of lipid molecules from the cell surface. This was due to difficulty in locating MAbs known to bind a lipid defined antigenic determinant found on the cell surface of human lymphocytes.

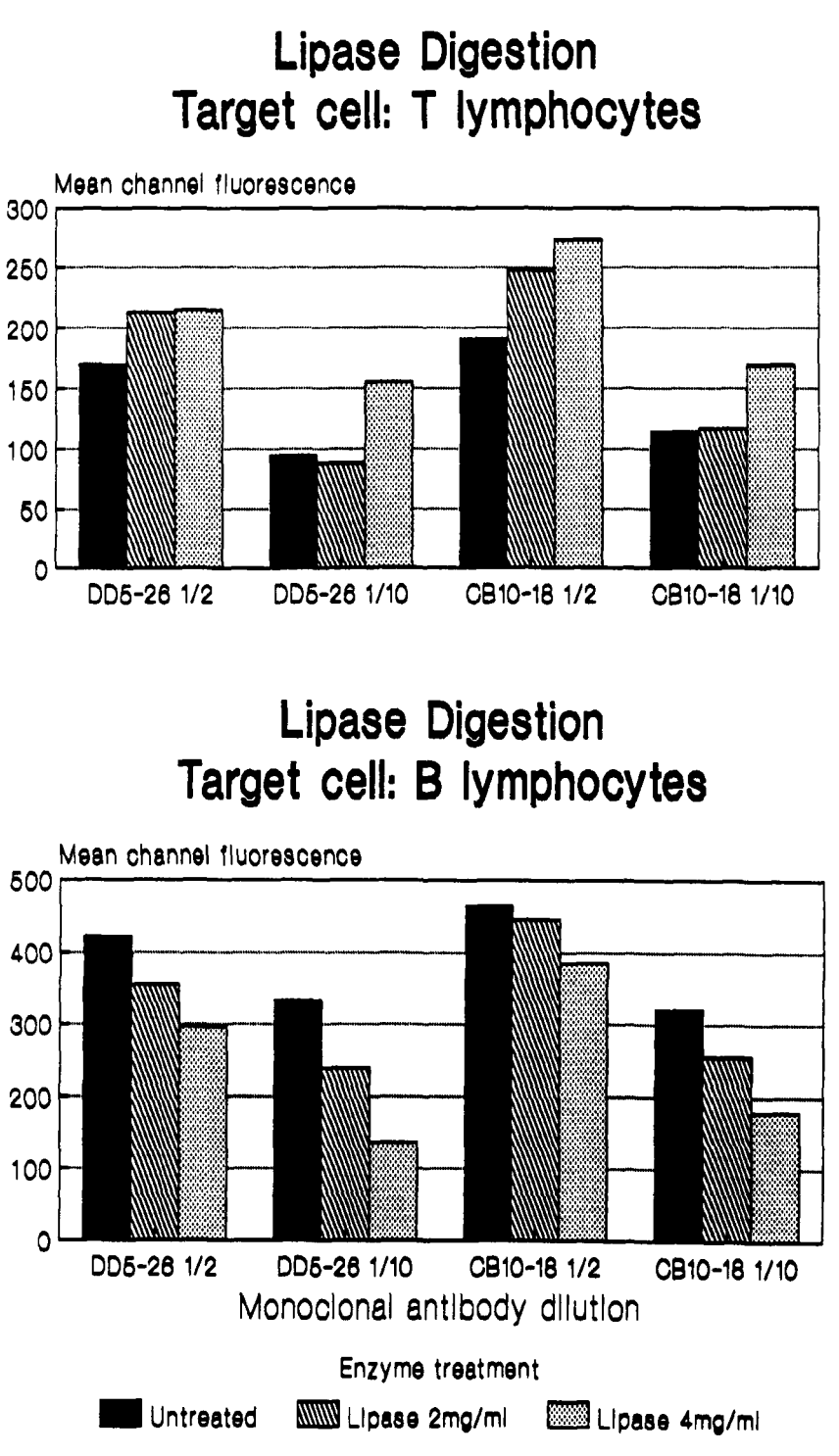


Figure 5.1.13 Cytofluorograph analysis of F15-DD5-26 and F36-CB10-18 binding at 1/2 and 1/10 dilutions to T cells (panel a) and B cells (panel b) following lipase treatment at 2·0 and 4·0mg/ml.

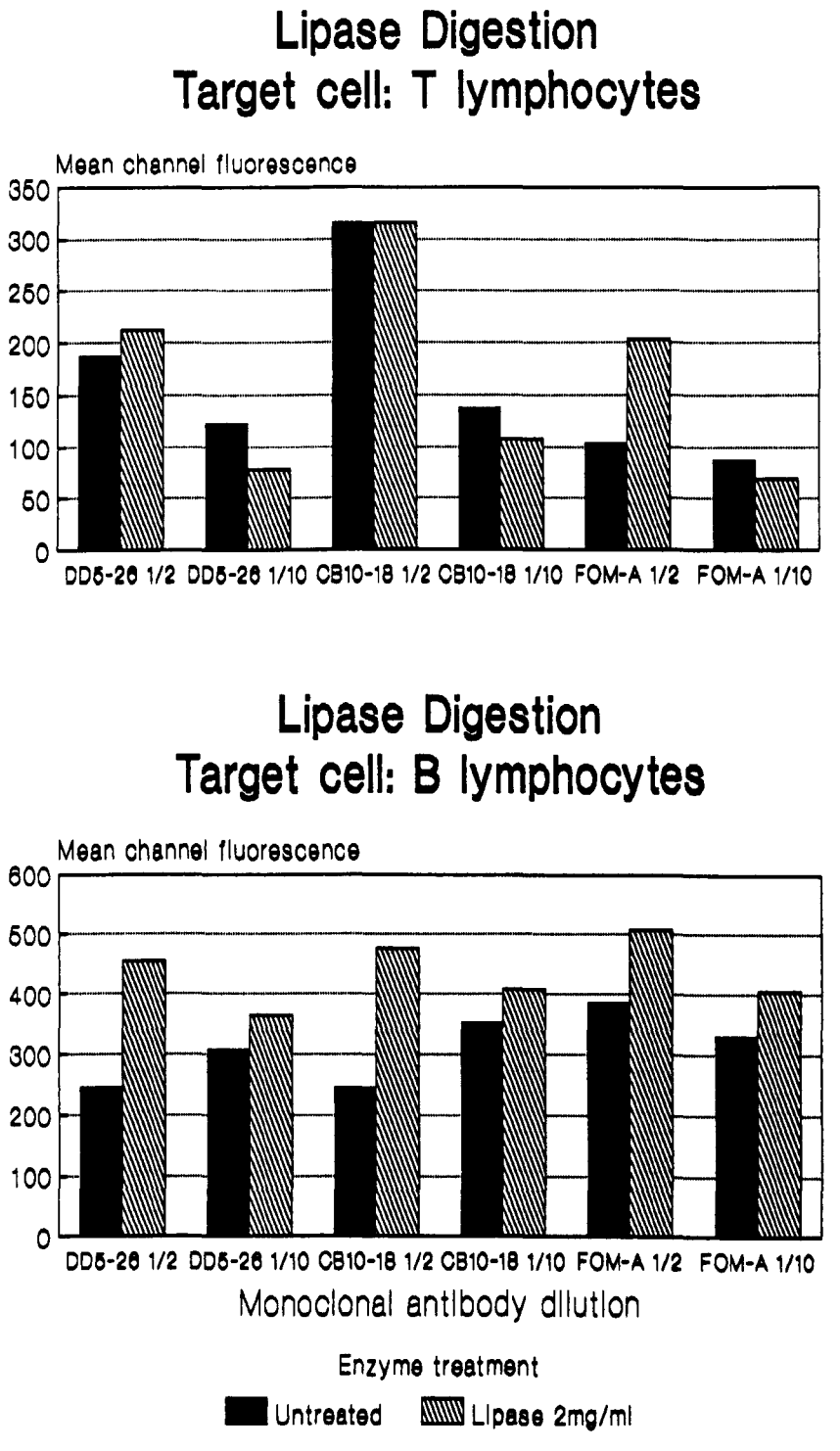


Figure 5.1.14 Cytofluorograph analysis of F15-DD5-26, F36-CB10-18 and FOM-A binding at 1/2 and 1/10 dilutions to T cells (panel a) and B cells (panel b) following lipase treatment at 2·0mg/ml.

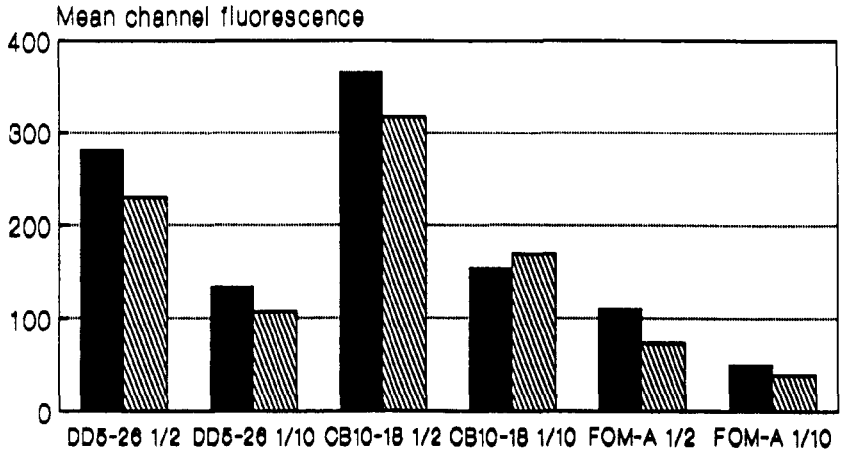
5.1.5 **Effect of Amylase digestion on ALA MAb binding:**

Amylase digestion of T and B cell targets was tested using enzyme concentrations of 2·0 and 6·25mg/ml using autoreactive MAbs (F15-DD5-26 and F36-CB10-18) and anti-RhD MAb (FOM-A) at 1/2 and 1/10 dilutions (Fig 5.1.15).

A small reduction in mean channel fluorescence was observed for T cells using amylase at 2·0mg/ml (Fig 5.1.15a). There was no change in mean channel fluorescence values for T cells using amylase at 6·25mg/ml, although the cytofluorograph scatter profiles were poor (data not shown).

There was no inhibition of autoantibody binding to B cell targets following amylase digestion at 2·0mg/ml (Figs 5.1.15b). As was seen for T cells, B cell targets treated with amylase at 6·25mg/ml had poor cytofluorograph scatter profiles. At 1/2 dilution there was increased B cell binding of F15-DD5-26 and F36-CB10-18 compared to the untreated controls (data not shown). However, at 1/10 dilution there was no change in binding levels.

As for the lipase digestion, no suitable control MAbs which bind an amylase sensitive carbohydrate epitope on the lymphocyte cell surface was found. Therefore amylase activity was not confirmed. However increased enzyme concentrations above 2·0mg/ml resulted in a poor cytofluorograph scatter profiles which could not be assessed.



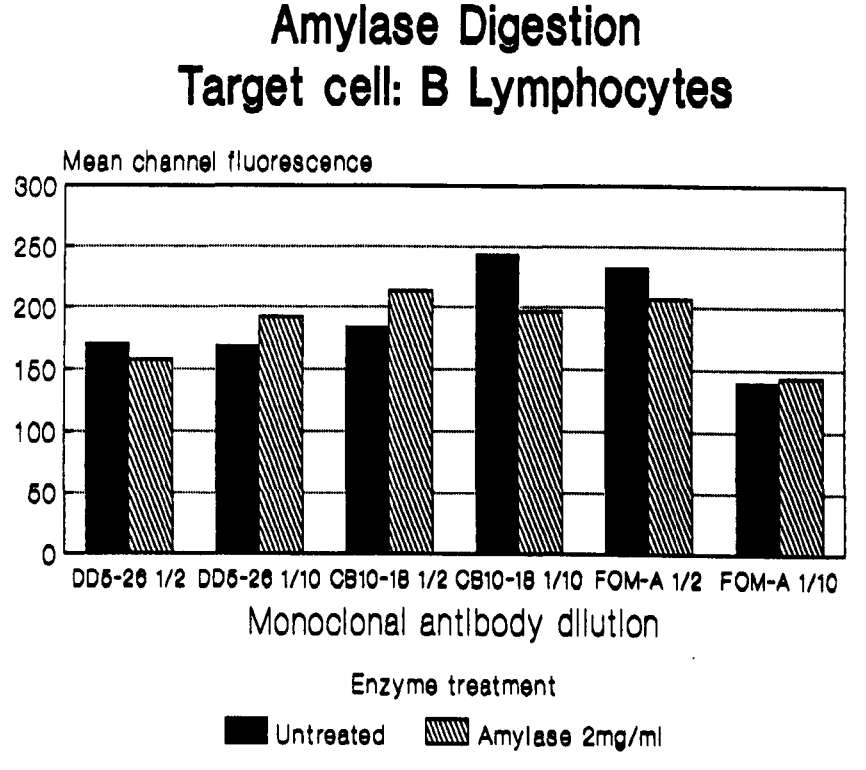


Figure 5.1.15 Cytofluorograph analysis of F15-DD5-26, F36-CB10-18 and FOM-A binding at 1/2 and 1/10 dilutions to T cells (panel a) and B cells (panel b) following amylase treatment at 2·0mg/ml.

5.1.6 **Effect of Trypsin digestion on ALA MAb binding:**

Trypsin digestion of T and B cell targets at a concentration of 2·5mg/ml was tested using F15-DD5-26, F36-CB10-18 and FOM-A at 1/2 and 1/10 dilution (Fig 5.1.16).

T cell binding was marginally reduced for F15-DD5-26 and F36-CB10-18 at 1/2 dilution, but not at 1/10 (Fig 5.1.16a). Binding of FOM-A (anti-RhD) to T cells was increased following trypsin digestion.

Trypsin digestion of B cell targets produced no significant change in mean channel fluorescence for F15-DD5-26, F36-CB10-18 or FOM-A at either 1/2 or 1/10 dilution (Fig 5.1.16b).

In parallel assays, binding of PA2·6 (mouse IgG anti-HLA class I) was reduced by only 10% following trypsin digestion. Increased trypsin concentrations produced poor cytofluorograph scatter profiles which could not be analyzed (data not shown).

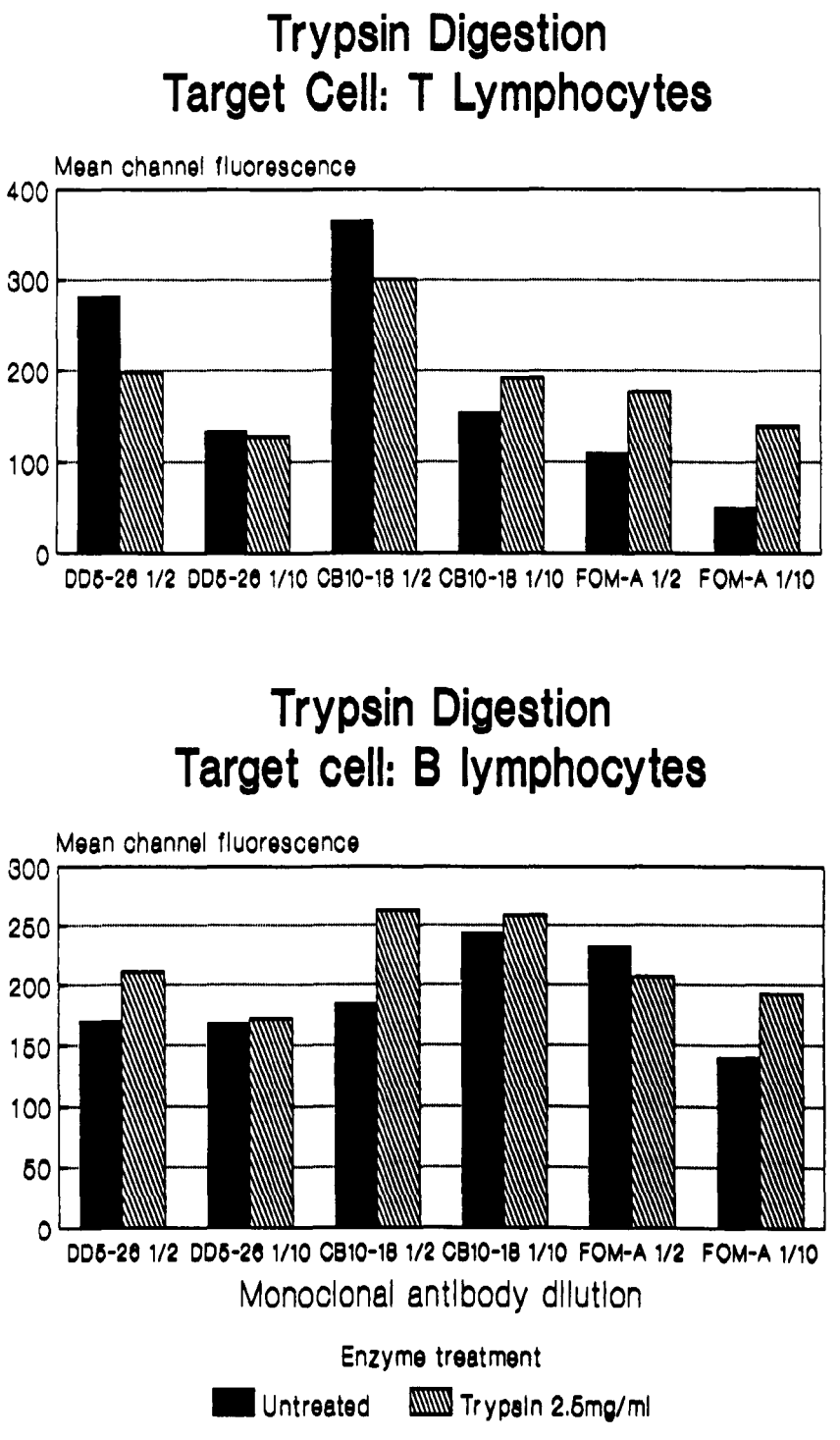


Figure 5.1.16 Cytofluorograph analysis of F15-DD5-26, F36-CB10-18 and FOM-A binding at 1/2 and 1/10 dilutions to T cells (panel a) and B cells (panel b) following trypsin treatment at 2·5mg/ml.

5.1.7 Discussion

The molecular nature of the epitopes recognised by ALA was investigated by enzymatic cleavage of cell surface molecules. The involvement of carbohydrate, protein and lipid residues in ALA binding were determined. The results showed that ALA binding to low affinity T and CLL cell targets was dependant on N-acetyl-neuramic acid (sialic acid), whereas high affinity binding to B cells was only partially dependant on this molecule. This indicates that the molecular nature of the autoantigen may vary on cells of different lineage. There appears to be a different distribution of autoantigenic epitopes on T and B cells giving rise to the different binding profiles. Therefore, the differences in T and B cell binding affinity found in cytotoxicity and binding assays may be explained by differing carbohydrate composition of the autoantigen.

Heterogeneity of carbohydrate residues on identical protein molecules present on cells of different lineage have been reported. Barclay et al have reported differential glycosylation of the Thy-1 molecule expressed on rat thymocytes and the brain (Barclay, Letarte-Muirhead and Williams, 1976). In addition they identified two types of Thy-1 molecule on thymocytes according to their ability to bind lentil lectin, which they termed Thy-1L+ and Thy-1L-. In both examples, Thy-1 molecules on thymocytes and brain had similar amino acid composition, but marked differences in carbohydrate compositions. In particular, sialic acid residues were found in small amounts on brain Thy-1 and in increased amounts on thymocyte Thy-1L+ and Thy-1L- molecules.

Differences in glycosylation of glycoproteins on rat T and B cells have also been reported (Standring et al, 1978). The leucocyte common antigen

(LCA) on rat thymocytes, T cells and B cells were found to have molecular weights of 150,000, 170,000 and 200,000 kD respectively. These differences were due to differential glycosylation of LCA. Again, sialic acid was identified as an abundant membrane molecule associated with differential glycosylation.

Moreover, evidence for the differential glycosylation of HLA-DR molecules has been found on cells of different lineage. Cowing (Cowing and Chapdelaine, 1983) demonstrated that HLA-DR molecules on normal resting B cells are unable to stimulate alloreactive T cells in a primary MLR, whereas HLA-DR on dendritic cells act as very potent stimulator of the MLR response. However, cleavage of sialic acid from the HLA class II molecules with neuraminidase restored the ability of B cells to stimulate a primary alloreactive response.

The digestion of T and B cell targets with papain also reduced ALA binding. These results, and those using neuraminidase, indicate the possibility of a glycoprotein molecule as the target molecule. The involvement of cell surface protein molecules could not be confirmed using peptidase or trypsin digestion.

Lipase digestion of lymphocyte cell surface molecules also produced no consistent difference in autoantibody binding. This supports the earlier findings where ALA binding was not inhibited by PtC or lipid-A. The lipase results add further evidence to exclude the involvement of lipid determinants as the lymphocytotoxic autoantibody target molecule.

The lack of suitable controls to demonstrate specific enzyme cleavage of

cell surface molecules in some experiments means that some results should be interpreted with caution. The methods used for these experiments were based on published data examining carbohydrate and protein contributions to various antigenic epitopes on HLA molecules (Sparrow, Vaughan and McKenzie, 1986). These reports also lacked appropriate controls to confirm specific enzyme activity. Attempts to increase enzyme concentrations from those used resulted in poor cytofluorograph scatter profiles which could not be analyzed. Therefore the maximum enzyme concentrations possible for these experiments were used indicating that lack of enzyme activity was unlikely.

5.2 Lectin Inhibition Studies to Determine ALA Specificities

Enzyme cleavage of lymphocyte cell surface molecules indicated that a sialic acid defined epitope is involved in autoantibody binding. Corroborative evidence for this was sought by binding inhibition studies using lectins to block carbohydrate determinants. Lectins are proteins or glycoproteins of non-immune origin which can bind and precipitate complex carbohydrates. The lectins used, their carbohydrate specificities, blood group specificities and mitogenic properties are shown in Materials and Methods (Table 2.3.4).

Purified T and B lymphocytes were pre-incubated with the appropriate lectin, then washed and tested for lymphocytotoxic autoantibody binding inhibition. The concentration of lectin used for this study (0·2mg/ml) was chosen to be about ten to twenty times that required in RBC agglutination assays, given by the manufacture.

5.2.1 Cytofluorograph analysis of lectin inhibition of autoantibody binding

Following lectin treatment of T and B cell targets, three different patterns of lymphocytotoxic autoantibody binding reactivity were found (summarised in Table 5.2.1). The first group produced no change in autoantibody binding, the second produced increased autoantibody binding and the third produced inhibition of autoantibody binding.

Group 1. Lectins which gave no change in autoantibody binding:

Ptilota plumosa, Sophora japonica, Ulex europaeus, Dolichos biflorus, Arachis hypogaea and Helix pomatia gave no change in lymphocytotoxic autoantibody binding after lectin treatment of T and B cell targets (Figs 5.2.1-5.2.6).

Table 5.2.1 Lectin Inhibition Results

Lectin	Inhibition results T cells 3			B cells		
	DD5	CB10	FOM	DD5	CB10	FOM
Group 1.						
Ptilota plumosa(Red marine algae)	-	-	-	-	-	-
Sophora japonica(Japanese pagoda)	-	-	-	-	-	-
Ulex europaeus (Gorse)	-	-	-	-	-	-
Dolichos biflorus (Horse gram)	-	-	-	-	-	-
Arachis hypogaea (Peanut)	-	-	-	-	-	-
Helix pomatia(Roman edible snail)	-	-	-	-	-	-
Group 2.						
Vicia faba (Fava bean)	*	*	*	*	*	*
Phaseolus vulgaris (Kidney bean)	*	*	*	(*)	(*)	*
Lens culinaris (Lentil)	*	*	-	-	-	-
Euronymus europaeus(Spindle tree)	-	*	-	-	(*)	-
Concanavalin A (Jack bean)	NA	*	*	-	-	*
Group 3.						
Triticum vulgaris (Wheat germ)	+	+	+	+	+	+
Mycoplasma gallisepticum	+	+	+	-	-	-

- *No change in autoantibody binding following lectin treatment.*

\+ *Inhibition of autoantibody binding following lectin treatment.*

* *Increased autoantibody binding following lectin treatment*

(*) weak increase.

NA Not assessed.

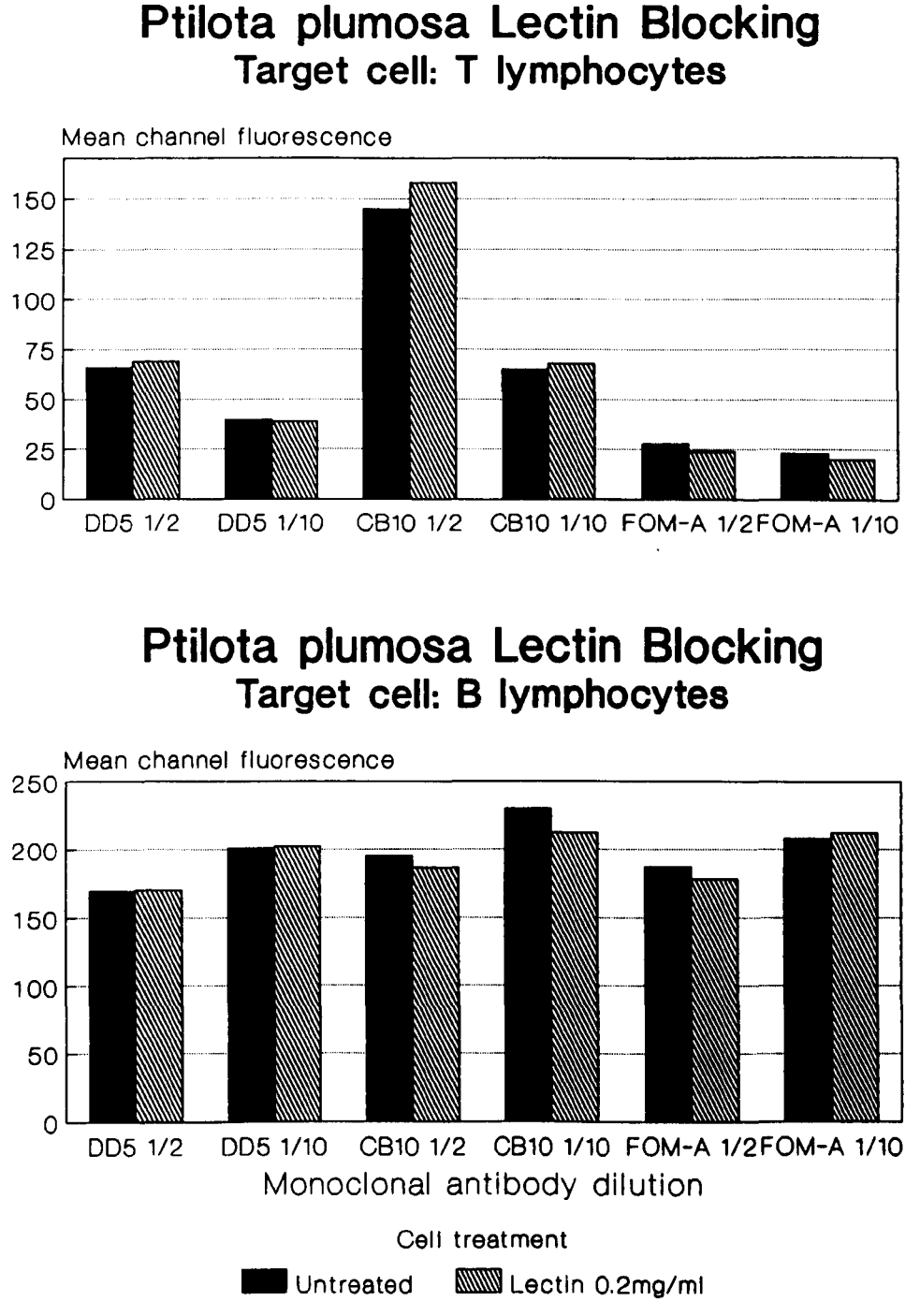


Figure 5.2.1 Cytofluorograph analysis of F15-DD5-26, F36-CB10-18 and FOM-A binding at 1/2 and 1/10 dilutions to T cells (panel a) and B cells (panel b) following Ptilota plumosa lectin treatment at 0·2mg/ml.

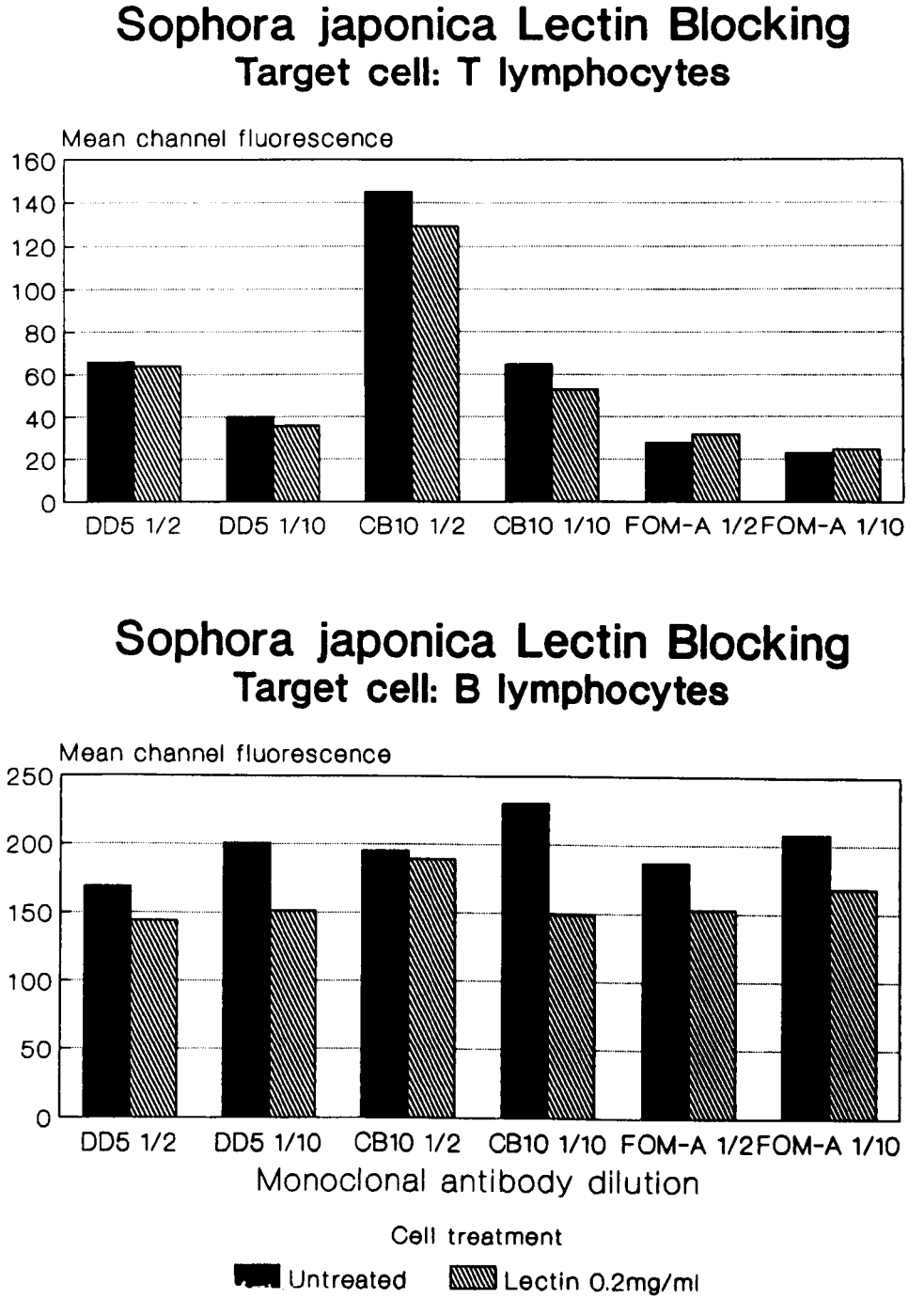
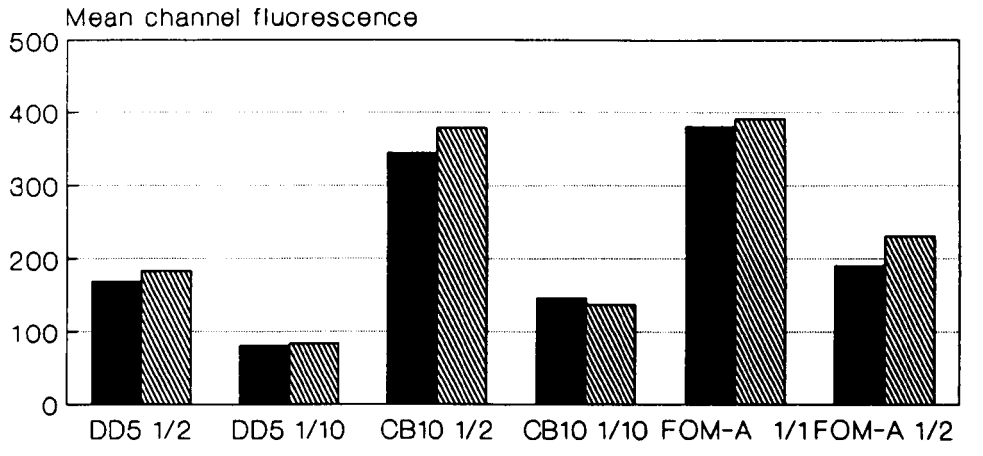


Figure 5.2.2 Cytofluorograph analysis of F15-DD5-26, F36-CB10-18 and FOM-A binding at 1/2 and 1/10 dilutions to T cells (panel a) and B cells (panel b) following Sophora japonica lectin treatment at 0·2mg/ml.



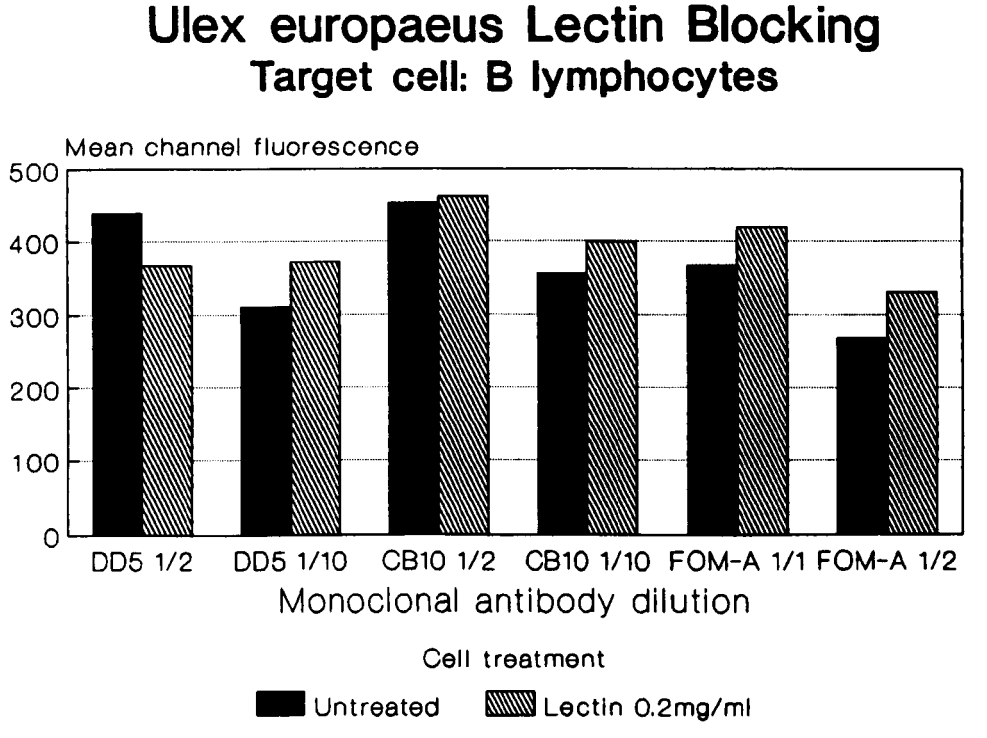
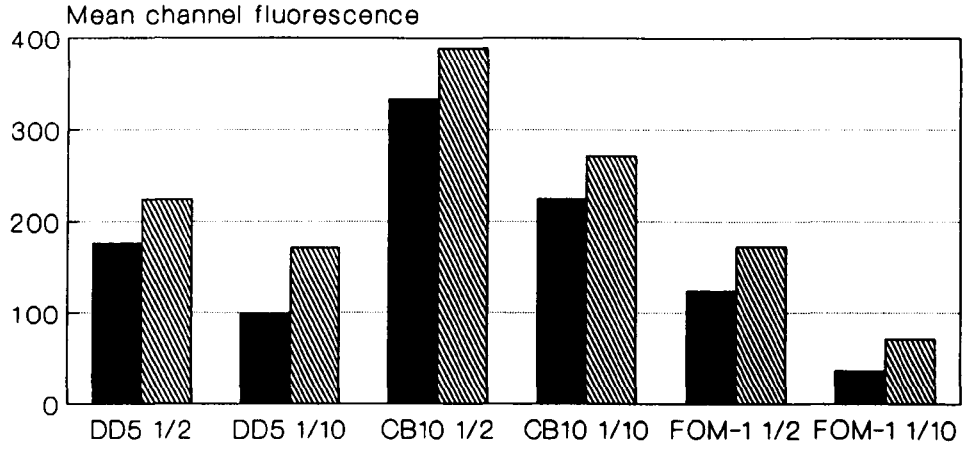


Figure 5.2.3 Cytofluorograph analysis of F15-DD5-26, F36-CB10-18 and FOM-A binding at 1/2 and 1/10 dilutions to T cells (panel a) and B cells (panel b) following Ulex europaeus lectin treatment at 0·2mg/ml.



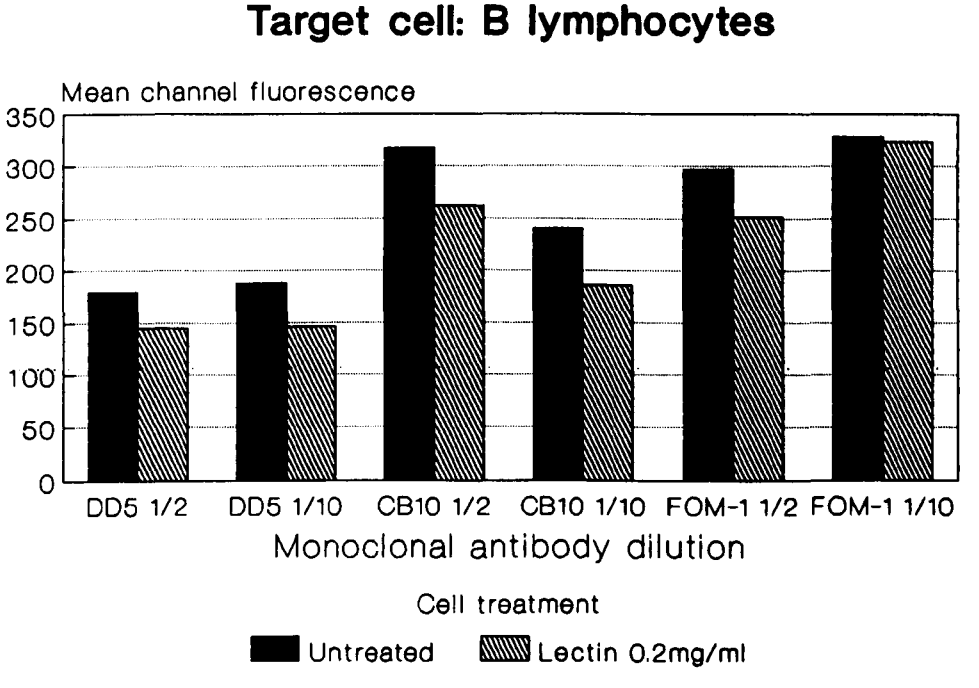
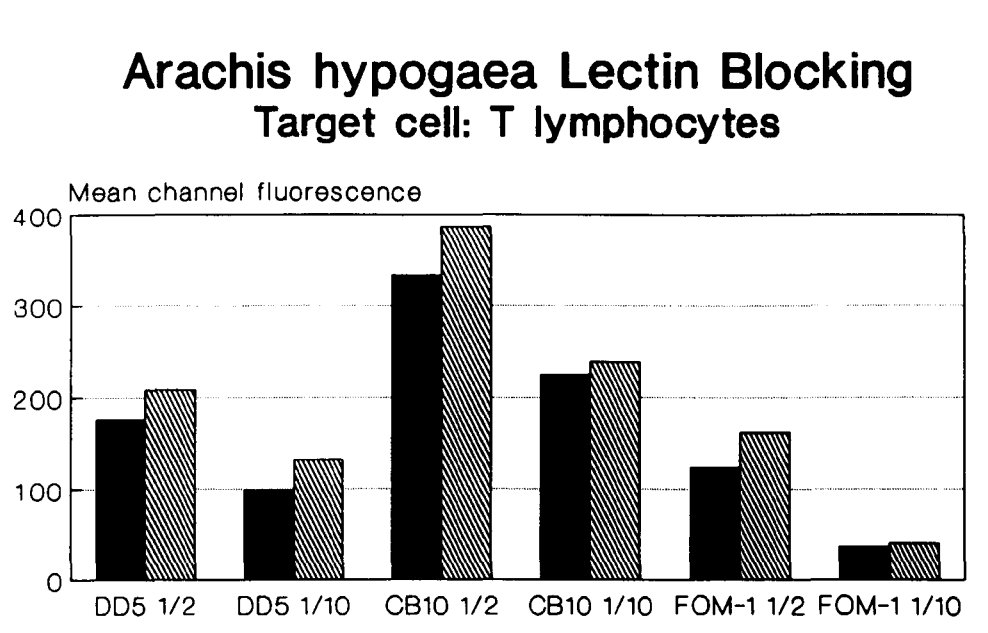


Figure 5.2.4 Cytofluorograph analysis of F15-DD5-26, F36-CB10-18 and FOM-A binding at 1/2 and 1/10 dilutions to T cells (panel a) and B cells (panel b) following Dolichos biflorus lectin treatment at 0·2mg/ml.



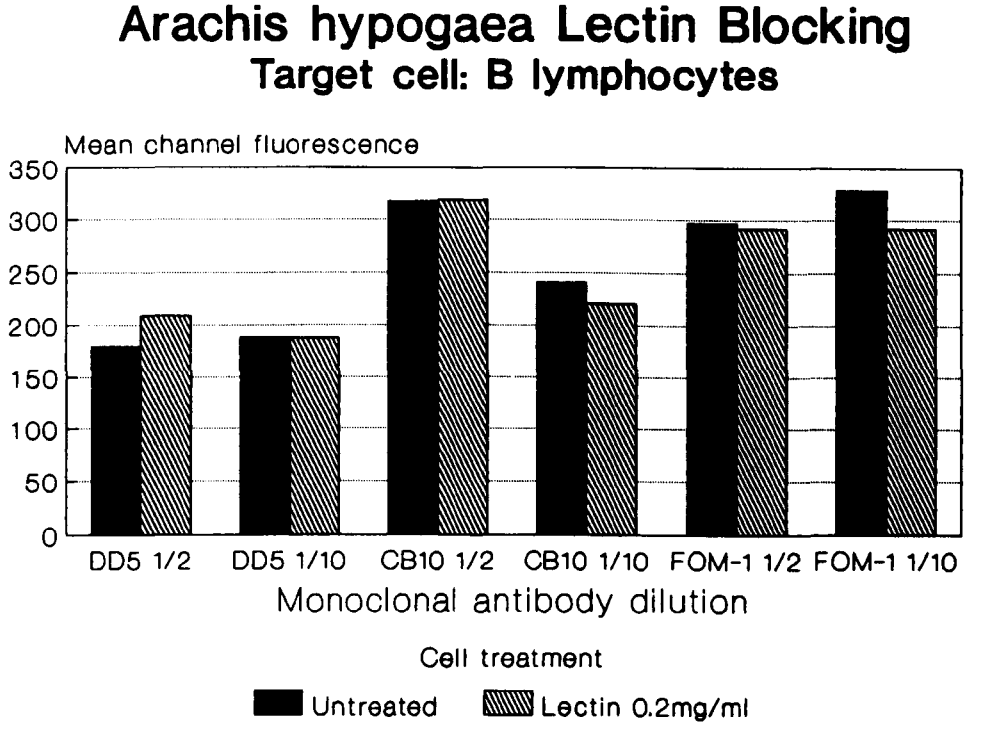
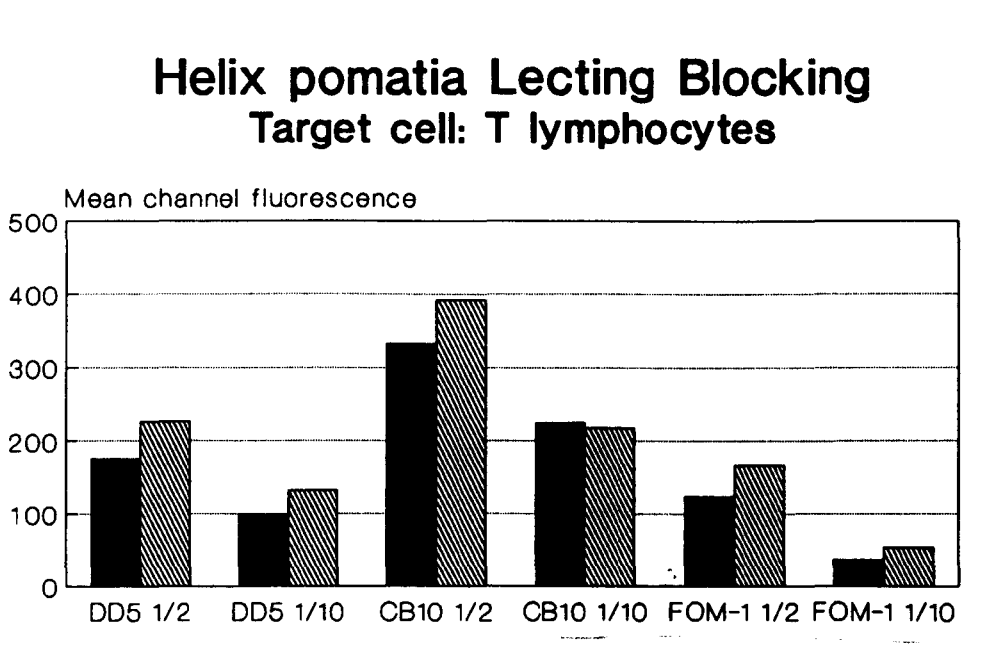


Figure 5.2.5 Cytofluorograph analysis of F15-DD5-26, F36-CB10-18 and FOM-A binding at 1/2 and 1/10 dilutions to T cells (panel a) and B cells (panel b) following Arachis hypogaea lectin treatment at 0·2mg/ml.



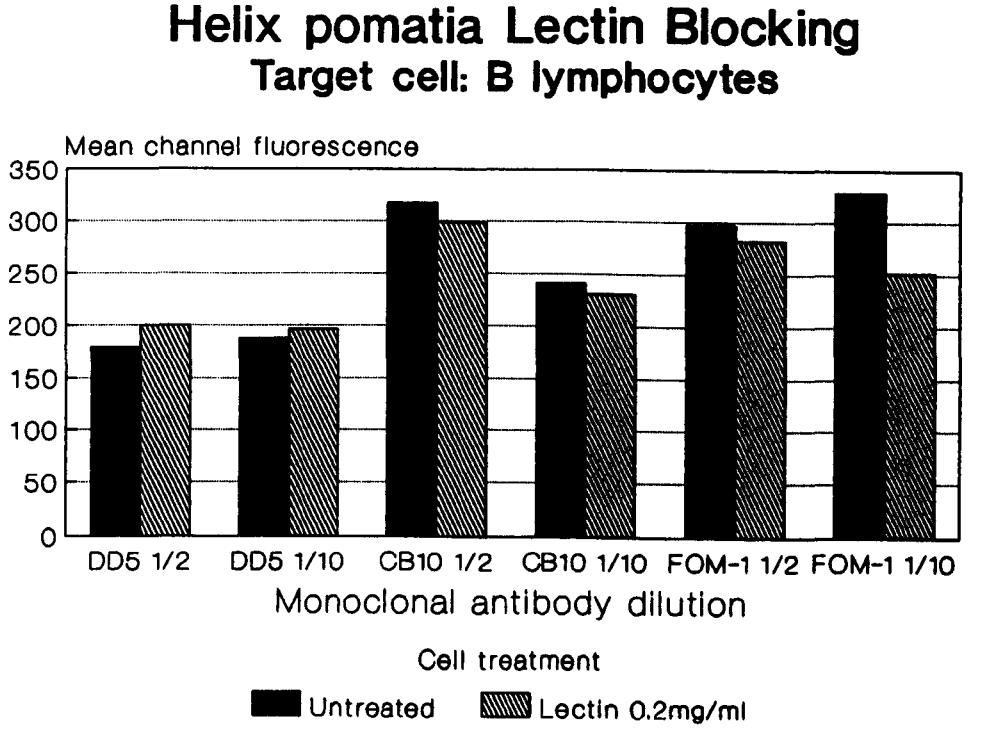
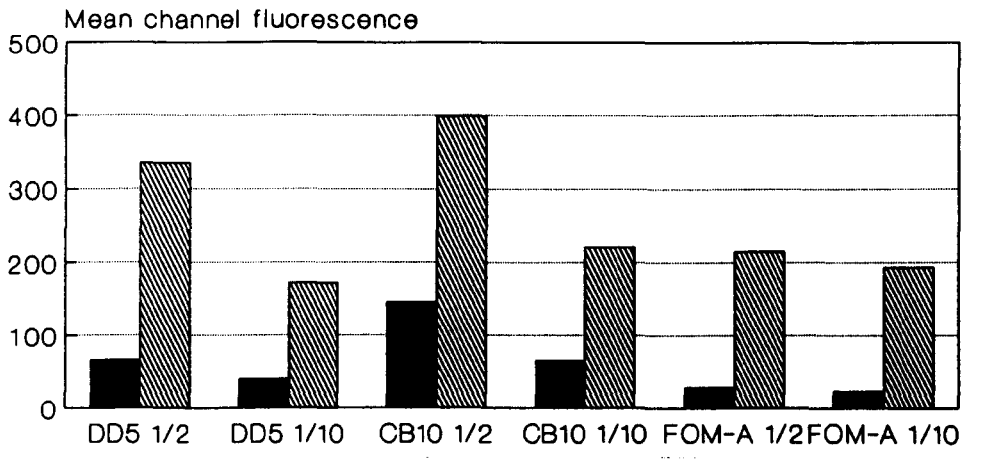


Figure 5.2.6 Cytofluorograph analysis of F15-DD5-26, F36-CB10-18 and FOM-A binding at 1/2 and 1/10 dilutions to T cells (panel a) and B cells (panel b) following Helix pomatia lectin treatment at 0·2mg/ml.

Group 2. Lectins which caused increased autoantibody binding:

The following lectins resulted in increased lymphocytotoxic autoantibody binding to T and/or B cells. Vicia faba and Phaseolus vulgaris produced increased binding to both T and B cells (Figs 5.2.7 and 5.2.8). Lens culinaris produced increased autoantibody binding to T cells alone (Fig 5.2.9), and Euronymus europaeus produced increased T cell binding (and marginal increased B cell binding) for F36-CB10-18 only (Fig 5.2.10). Concanavalin A produced poor T cell profiles, with greatly increased autoantibody binding (Fig 5.2.11a, F15-DD5-26 binding profiles were not assessable), and a small increase in autoantibody binding to B cells (Fig 5.2.11b).



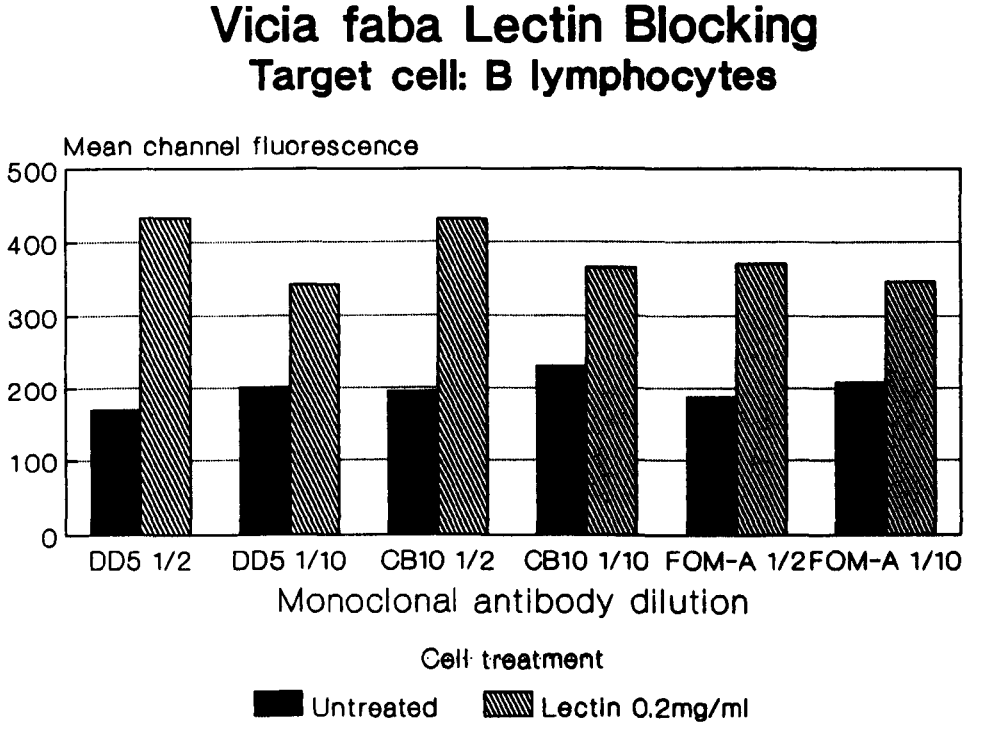
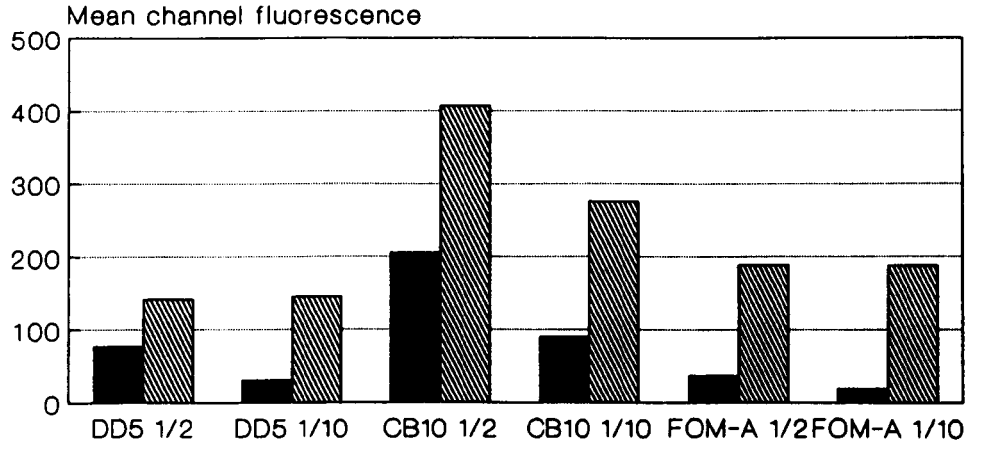
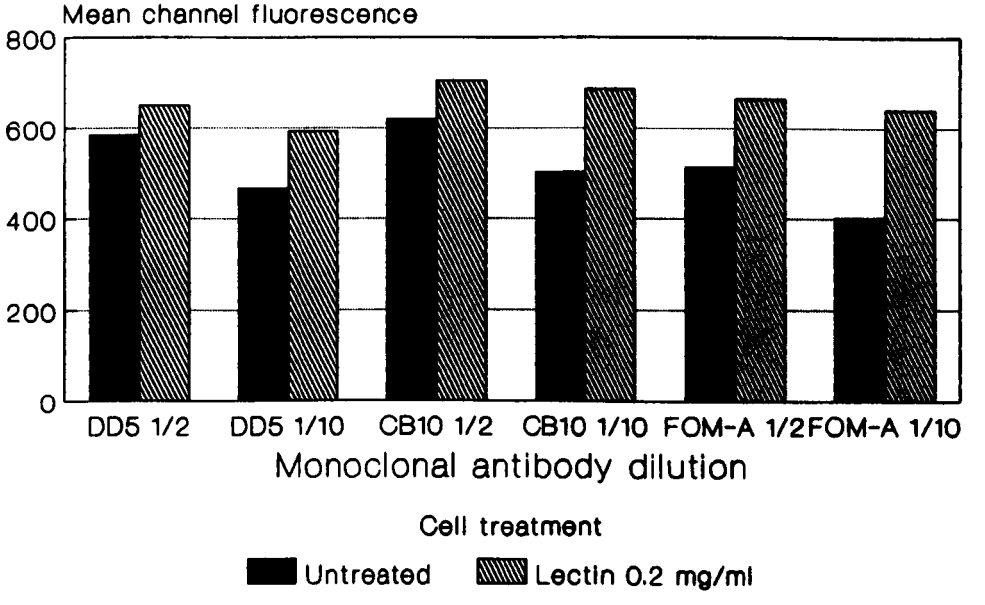


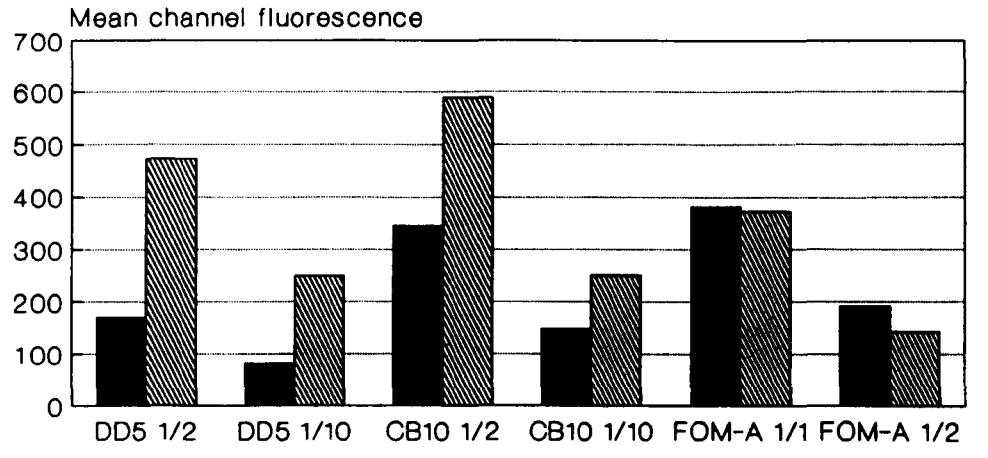
Figure 5.2.7 Cytofluorograph analysis of F15-DD5-26, F36-CB10-18 and FOM-A binding at 1/2 and 1/10 dilutions to T cells (panel a) and B cells (panel b) following Vicia fava lectin treatment at 0·2mg/ml.





(Lectin blocking of scale)

Figure 5.2.8 Cytofluorograph analysis of F15-DD5-26, F36-CB10-18 and FOM-A binding at 1/2 and 1/10 dilutions to T cells (panel a) and B cells (panel b) following Phaseolus vulgaris lectin treatment at 0·2mg/ml.



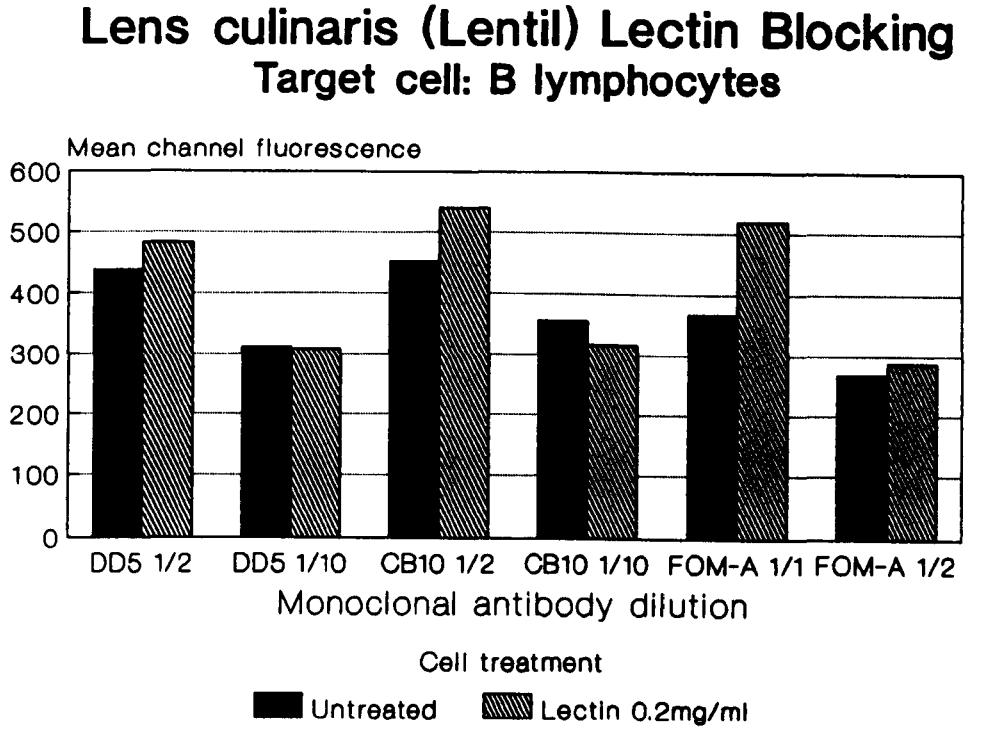
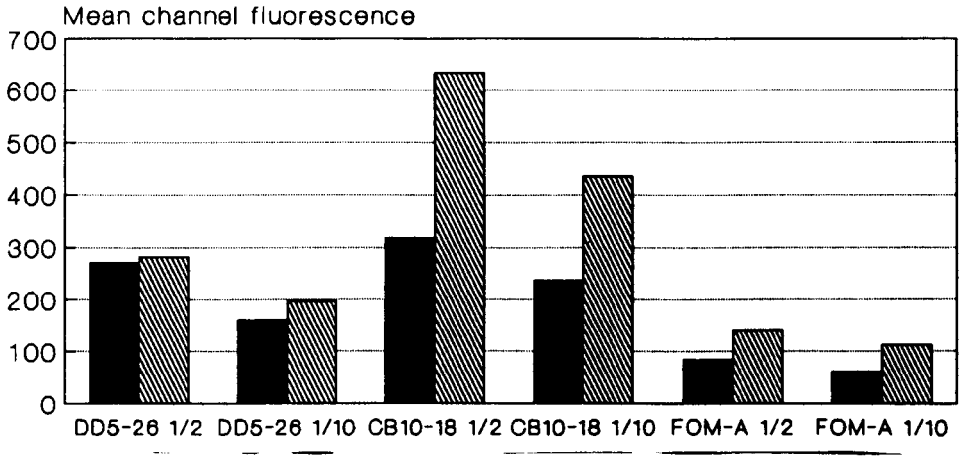


Figure 5.2.9 Cytofluorograph analysis of F15-DD5-26, F36-CB10-18 and FOM-A binding at 1/2 and 1/10 dilutions to T cells (panel a) and B cells (panel b) following Lens culinaris lectin treatment at 0·2mg/ml.



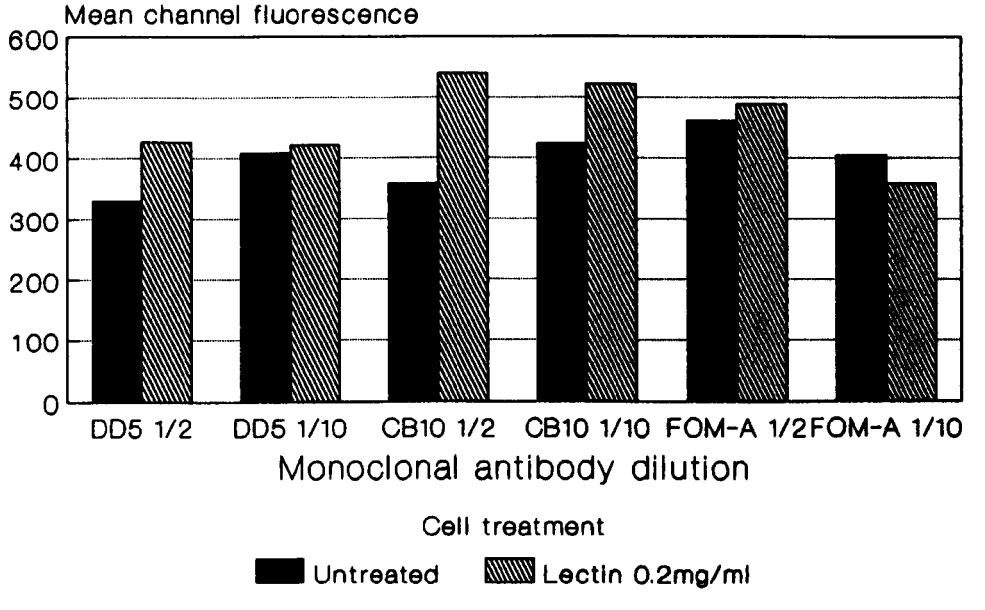
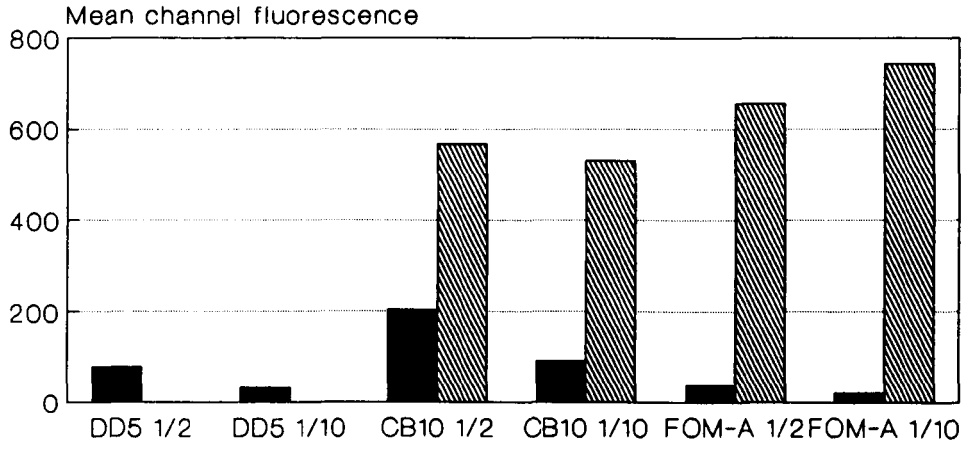


Figure 5.2.10 Cytofluorograph analysis of F15-DD5-26, F36-CB10-18 and FOM-A binding at 1/2 and 1/10 dilutions to T cells (panel a) and B cells (panel b) following Euronymus europaeus lectin treatment at 0·2mg/ml.



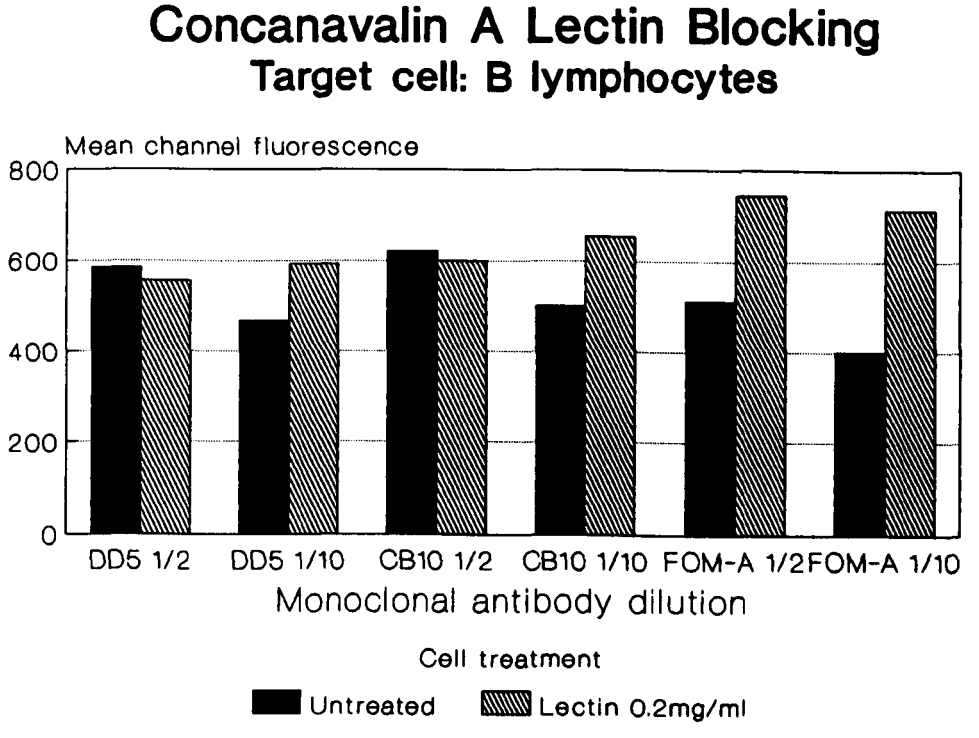
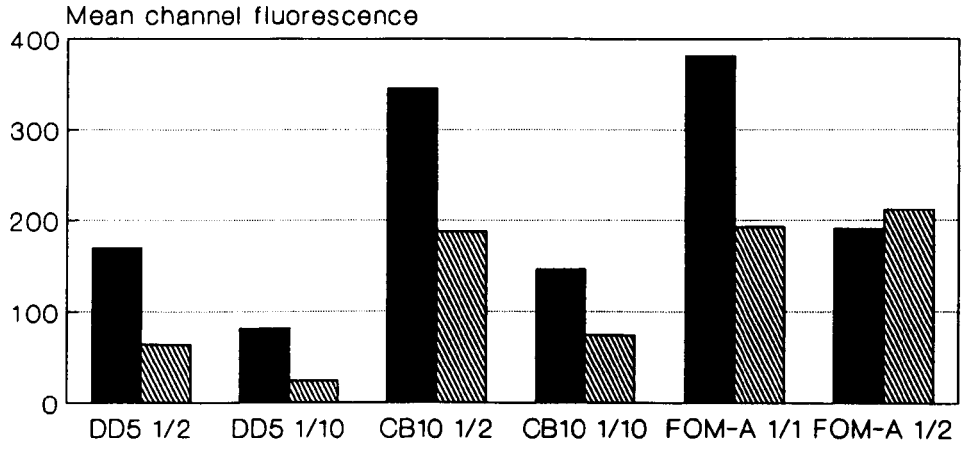


Figure 5.2.11 Cytofluorograph analysis of F15-DD5-26, F36-CB10-18 and FOM-A binding at 1/2 and 1/10 dilutions to T cells (panel a) and B cells (panel b) following Concanavalin A lectin treatment at 0·2mg/ml.

Group 3. Lectins which inhibited autoantibody binding:

The lectin from Triticum vulgaris (wheat germ), which binds sialic acid, strongly inhibited autoantibody reactivity against both T and B cell targets (Fig 5.2.12). A mean inhibition of 53% and 72% was found for autoantibody binding to T cells (low affinity target) and B cells (high affinity target) respectively. Binding of FOM-A (anti-RhD) to T and B cells was also inhibited. To confirm this observation, in a second independent experiment, a mean T cell binding inhibition of 35% and B cell binding inhibition of 69% was seen for F15-DD5-26, F36-CB10-18, and MAD (anti-RhD) (data not shown).

Mycoplasma gallisepticum produced 59% inhibition of autoantibody binding to T cell targets, but no inhibition with B cell targets (Fig 5.2.13).



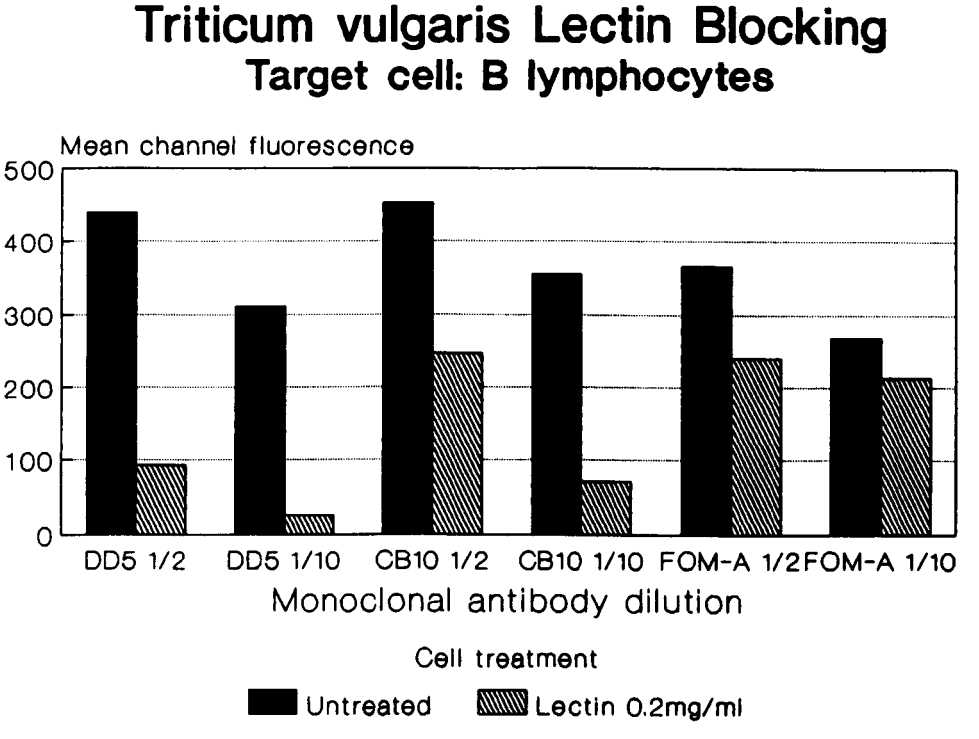
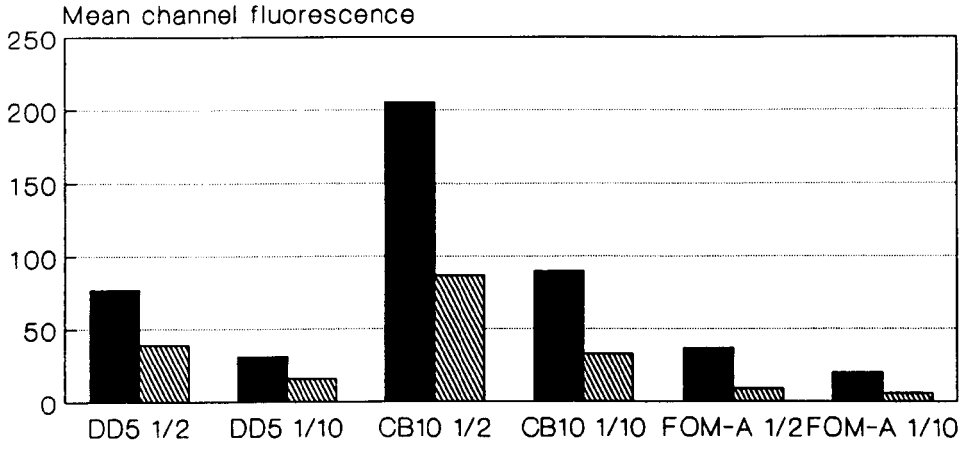


Figure 5.2.12 Cytofluorograph analysis of F15-DD5-26, F36-CB10-18 and FOM-A binding at 1/2 and 1/10 dilutions to T cells (panel a) and B cells (panel b) following Triticum vulgaris lectin treatment at 0·2mg/ml.



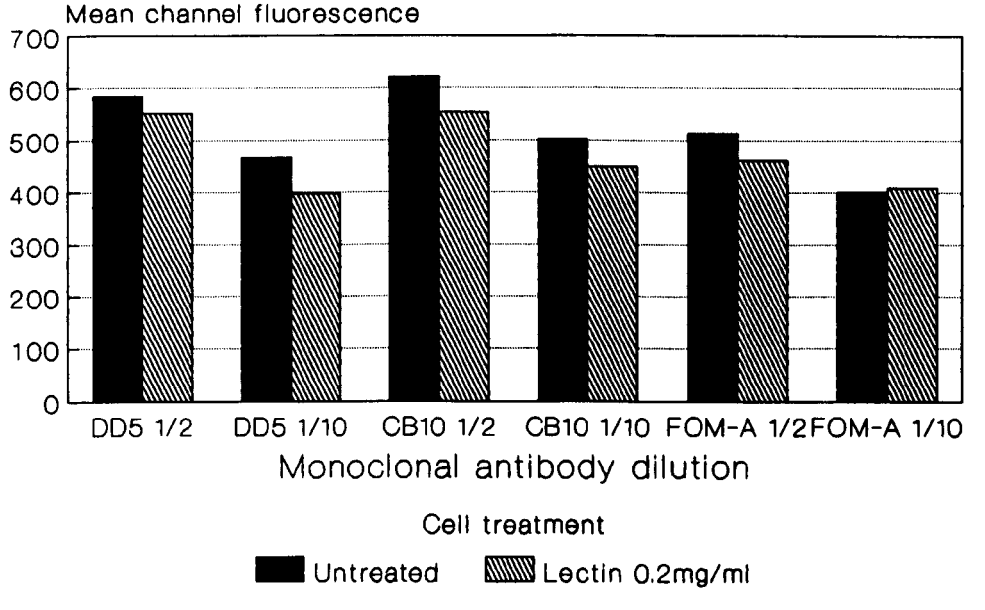


Figure 5.2.13 Cytofluorograph analysis of F15-DD5-26, F36-CB10-18 and FOM-A binding at 1/2 and 1/10 dilutions to T cells (panel a) and B cells (panel b) following Mycoplasma gallisepticum lectin treatment at 0·2mg/ml.

5.2.2 Lectin inhibition of autoantibody binding in cytotoxicity tests

Cytofluorograph binding inhibition studies indicated that pretreatment of T and B cells with lectin from Triticum vulgaris blocked monoclonal lymphocytotoxic autoantibody binding. In an attempt to apply this finding to block autoantibodies in the crossmatch test, reactivity was assessed following lectin treatment of cells in the cytotoxicity assay.

T and B lymphocytes were pretreated with different concentrations (serial dilutions from 10mg/ml to 0·015mg/ml) of lectin from Triticum vulgaris. The cells were then tested by cytotoxicity using autoreactive and anti-HLA antibodies and the titres were compared to untreated controls. Monoclonal lymphocytotoxic autoantibodies (F15-DD5-26, F36-CB10-18), anti-RhD (MAD), anti-HLA-A2 alloserum and a T and B cell lymphocytotoxic autoantibody from a renal dialysis patient (DV) were tested. Volumes of 1μl of each reagent were dispensed at serial dilutions from neat to 1/128 into the wells of microtest trays. Each serum dilution was tested with lectin treated and untreated T and B cells for cytotoxicity inhibition.

No change in cytotoxicity titres were found for any combination of lectin and antibody concentration with either T or B cells (data not shown).

5.2.3 Autoantibody binding to lectins

Cytofluorograph binding studies demonstrated increased binding of monoclonal autoantibodies to T and B cells coated with lectin from Vicia fava and Phaseolus vulgaris. This indicated that the polyreactive antibodies could bind the lectin on the cell surface. An alternative possibility is that the lectin is polyvalent, being able to bind both the cell membrane and IgM carbohydrate determinants. To investigate this

further, two tests were performed to analyze the nature of the lectin binding.

i) Lysis of lectin coated red blood cells by lymphocytotoxic autoantibodies and complement.

The method was followed as described in Materials and Methods (2.3 g-iv). Red blood cells were coated with lectin and allowed to agglutinate in 96 well microtitre plates. This was followed by the addition of lymphocytotoxic autoantibodies (F15-DD5-26 and F36-CB10-18) and anti-RhD (FOM-1) MAbs at doubling dilutions from 1/2 to 1/2048. To detect binding of the antibodies to lectin coated red cells, rabbit complement was added and following incubation the wells were examined for cell lysis (Table 5.3.1).

The results show that red blood cells coated with lectin from Phaseolus vulgaris were lysed in the presence of the autoreactive MAbs and rabbit complement (Table 5.3.1 panel a). F15-DD5-26 gave 100% lysis at 1/2 dilution and F36-CB10-18 to 1/8 dilution. No lysis was seen with FOM-1 nor in the PBS controls.

Blood group B RhD negative red cells coated with lectin from Euronymus europaeus were also lysed by F15-DD5-26 and F36-CB10-18 to 1/8 and 1/32 dilutions respectively (Table 5.3.1 B). This lectin produced increased binding of F36-CB10-18 to lymphocytes in cytofluorograph analysis. As the IgM levels of F15-DD5-26 (67μg/ml) are higher than F36-CB10-18 (42μg/ml), the increased titre of F36-CB10-18 can not be due higher to immunoglobulin levels. Therefore the results are more likely to relate to the antibody specificity, with the autoantibodies binding to the lectin. However, the

possibility of the lectin binding the IgM can not be ruled out.

Additional controls, consisting of untreated red cells with antibody plus complement (in the absence of lectin), and also red cells coated with lectin from Triticum vulgaris (which caused inhibition of autoantibody binding) resulted in weak red cell lysis at MAb dilutions of 1/2 for F15-DD5-26 and 1/4 with F36-CB10-18 (data not shown). The difference in red cell lysis between the controls and the presence of lectin from Phaseolus vulgaris and Euronymus europaeus were marked. Although the weak lysis of untreated red cells by autoreactive MAbs and complement indicated some binding, fluorescence binding assays analyzed on a cytofluorograph were completely negative (data not shown). In control studies, FOM-1 (anti-RhD) was found to bind specifically to RhD positive but not RhD negative red blood cells. The results may indicate weak low affinity binding of the autoreactive MAbs to red blood cells which is only detected in cytotoxicity assays as no washing steps are involved. This would be compatible with the hypothesis that the antibodies are polyreactive and bind with low affinity to multiple antigenic targets.

The results indicate that the antibodies can bind lectin coated red blood cells and support the cytofluorograph analysis data which showed increased binding of autoreactive MAbs to lectin coated lymphocytes. The higher titres of F36-CB10-18 despite lower immunoglobulin levels argue against the possibility that the lectin is binding IgM directly. However due to the weak background lysis in the negative controls, the results should be interpreted with caution.

	Monoclonal antibody dilution (reciprocal)										
	2	4	8	16	32	64	128	256	512	1024	2048
DD5-26	+	(+)	-	-	-	-	-	-	-	-	-
	+	(+)	-	-	-	-	-	-	-	-	-
CB10-18	+	+	+	(+)	-	-	-	-	-	-	-
	+	+	+	(+)	-	-	-	-	-	-	-
FOM-1	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-
PBS	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-

(A) Lysis of blood group A RhD negative red cells coated with lectin from Phaseolus vulgaris.

	Monoclonal antibody dilution (reciprocal)										
	2	4	8	16	32	64	128	256	512	1024	2048
DD5-26	+	+	+	(+)	-	-	-	-	-	-	-
	+	+	+	(+)	-	-	-	-	-	-	-
CB10-18	+	+	+	+	+	(+)	-	-	-	-	-
	+	+	+	+	+	(+)	-	-	-	-	-
FOM-1	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-
PBS	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-

(B) Lysis of blood group B RhD negative red cells coated with lectin for Euronymus europaeus.

Table 5.3.1 Autoantibody and complement mediated lysis of Phaseolus vulgaris (A) and Euronymus europaeus (B) coated red blood cells from blood group A and B RhD negative donors respectively. (+ = cell lysis, - = no cell lysis).

ii) Inhibition of lectin agglutination of red blood cells by lymphocytotoxic autoantibodies.

To confirm the formation of lectin/IgM complexes, the ability of the lymphocytotoxic autoantibodies to inhibit lectin agglutination of red blood cells was determined. The method was followed as described in Materials and Methods (Chapter 2.3-g-iii). Autoreactive MAbs (F15-DD5-26 and F36-CB10-18) and anti-RhD MAb (FOM-1) at serial dilutions from neat to 1/1024 were incubated with the appropriate lectin at 0·2mg/ml for one hour at 22°C, followed by the addition of 100μl of a 2% red blood cell suspension. The lectins selected for study were Phaseolus vulgaris, Ulex europaeus, Euronymus europaeus and Triticum vulgaris.

The results (Table 5.3.2) show inhibition of Phaseolus vulgaris lectin agglutination of red blood cells by F15-DD5-26 and F36-CB10-18 to dilutions of 1/8. FOM-1 gave no agglutination inhibition in this experiment but gave weak inhibition at neat only in subsequent tests (data not shown). Incubation of Phaseolus vulgaris in PBS-DAB alone gave no inhibition.

	Monoclonal antibody dilution (reciprocal)										
	N	2	4	8	16	32	64	128	256	512	1024
DD5-26	-	-	-	(-)	(+)	+	+	+	+	+	+
		-	-	(-)	(+)	+	+	+	+	+	+
CB10-18	-	-	-	(+)	(+)	+	+	+	+	+	+
	-	-	-	(-)	(+)	+	+	+	+	+	+
FOM-1	(-)	+	+	+	+	+	+	+	+	+	+
	(-)	+	+	+	+	+	+	+	+	+	+
PBS	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+

Table 5.3.2 Inhibition of red blood cell agglutination by lectin from Phaseolus vulgaris at 0·2µg/ml by autoreactive (F15-DD5-26 and F36-CB10-18) and anti-RhD (FOM-1) MAbs at dilutions neat to 1/1024 (+ = agglutination, - = no agglutination).

The lectins from Triticum vulgaris and Ulex europaeus (which caused no increase in MAb binding to lymphocytes) gave no inhibition of red cell agglutination (data not shown). However the lectin from Euronymus europaeus (which caused increased MAb binding to lymphocytes and MAb lysis of lectin coated red cells) also gave no inhibition of agglutination. This observation is not consistent with the interpretation of the previous results indicating the formation of MAb/lectin complexes using lectin from Euronymus europaeus. It is however possible that the formation of MAb complexes with this lectin does not block its ability to agglutinate red blood cells.

The data indicates the presence of Phaseolus vulgaris/MAb complexes which block the ability of the lectin to bind red blood cells. The results further support the suggestion that the autoantibodies bind the lectin from Phaseolus vulgaris on the cell surface.

iii) Lectin inhibition of autoantibody binding in the lymphocytotoxicity test

The above data indicates that the autoantibodies can bind some lectins on the cell surface of lymphocytes and red blood cells. In addition, inhibition of red cell agglutination showed that the autoantibodies form MAb/lectin complexes which block lectin activity. It is therefore possible that the formation of MAb/lectin complexes may also block lymphocytotoxic autoantibody reactivity.

Attempts were made to utilise the formation of MAb/lectin complexes through inhibition of autoantibody reactivity in the cytotoxicity test. F15-DD5-26, F36-CB10-18, FOM-1, anti-HLA-A2 alloserum, anti-HLA-Bw6 alloserum and serum from a patient with T and B cell lymphocytotoxic autoantibodies (DV) were tested at serial dilutions from neat to 1/128. The reagents were incubated with lectin from Phaseolus vulgaris, Triticum vulgaris, Ulex europaeus and Vicia fava at concentrations of 50 and 25μg/ml in PBS-DAB. As untreated controls MAbs were incubated with PBS-DAB alone. Volumes of 1μl were then dispensed into a microtest tray under paraffin oil and tested against T cell targets in a standard cytotoxicity assay. Cytotoxicity titres of the lectin treated and untreated antibodies were compared.

No inhibition of MAb cytotoxicity was found (data not shown). Additional experiments were performed using Vicia fava at serial dilutions from 500μg/ml to 3·8μg/ml with the above reagents using T cell targets. These also produced no cytotoxicity inhibition (data not shown).

The above results using cytofluorograph analysis, lectin coated red blood cell cytotoxicity and monoclonal autoantibody inhibition of lectin/red

blood cell agglutination all indicated the formation of lectin/MAb complexes. These complexes were able to inhibit lectin binding to red blood cells, however no inhibition of monoclonal autoantibody reactivity was observed in cytotoxicity tests. It is possible that the lectin formed complexes with the IgM antibodies by binding carbohydrate determinants without inhibiting the antibody binding site and/or blocking its ability to activate complement. Therefore these findings were not helpful for the differentiation of autoreactive antibodies and HLA antibodies in the crossmatch test using a cytotoxicity assay.

The dichotomy of effective blocking of autoantibody binding by lectin from Triticum vulgaris in cytofluorograph assays but not in cytotoxicity tests is also difficult to resolve. Results of both tests were reproducible, and yet the use of lectin inhibition to distinguish autoantibodies from alloantibodies by cytotoxicity does not work.

5.3 Sialic acid inhibition of autoantibody binding

The results of neuraminidase treatment of CLL cells, T and B cells and the inhibition of autoantibody binding by the lectin Triticum vulgaris are both consistent with the view that autoantibody binding is dependant on a sialic acid defined epitope. Therefore attempts were made to block autoantibody binding to T and B cells using sialic acid in cytofluorograph binding and cytotoxicity assays.

5.3.1 Cytofluorograph analysis of sialic acid inhibition of autoantibody binding

Fluorescence binding assays were performed with T and B cell targets using autoreactive MAbs (F15-DD5-26 and F36-CB10-18) and anti-RhD MAb (MAD) at 1/2 and 1/10 dilutions in the presence of sialic acid at 25 and 12·5mg/ml. Due to the low pH of the sialic acid (pH4·0), the untreated controls were also adjusted to pH4·0 accordingly (Fig 5.3.1).

The results show increased autoantibody binding to both T and B cells targets in the presence of sialic acid compared to the untreated controls. This indicates that there was no blocking of autoantibody binding in the presence of free sialic acid. However, unlike in previous observations, T cell binding at pH4·0 (negative control) showed higher autoantibody binding at 1/10 than at 1/2 dilution. This contrasts with the consistent observation of reduced binding to low affinity T cell targets following autoantibody dilution at pH7·2 (Chapter 4). Viability tests using eosin dye exclusion demonstrated that both the sialic acid treated and untreated cells were >95% viable (data not shown), therefore excluding the possibility of non-specific binding to dead cells.

The lack of inhibition by free (synthetic) sialic acid may be due to different conformations of sialic acid dependant epitopes present on membrane bound glycoprotein molecules compared to free sialic acid in solution. However the results indicate increased MAb binding to low affinity T cell targets at low pH. It is possible that autoreactive antibodies may bind negatively charged cell surface molecules, showing increased affinity at low pH due to the presence of these molecules in ionised form.

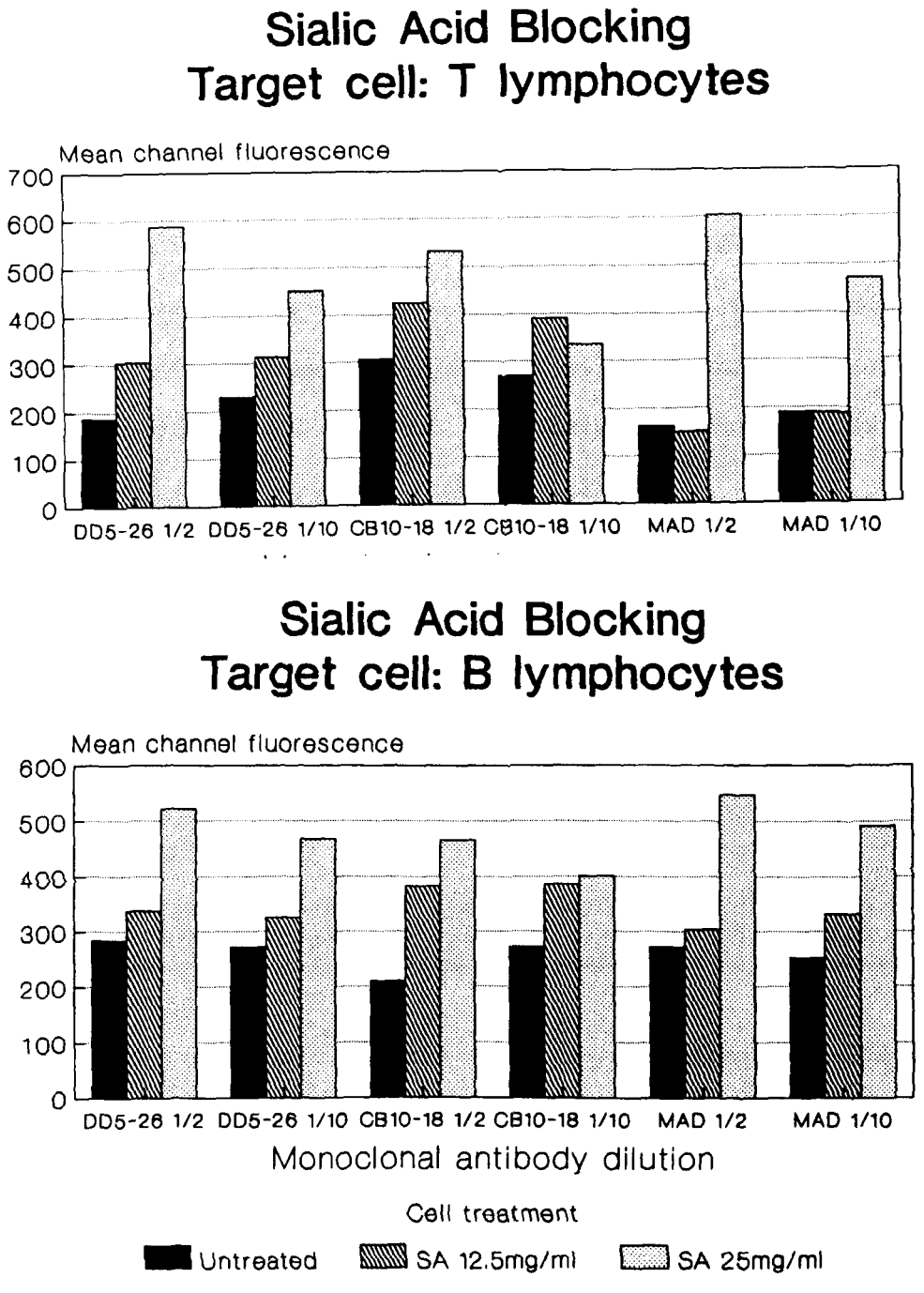


Figure 5.3.1 Cytofluorograph analysis of F15-DD5-26, F36-CB10-18 and MAD binding at 1/2 and 1/10 dilutions to T cells (panel a) and B cells (panel b) in the presence of free sialic acid at 12·5 and 25·0mg/ml.

5.3.2 Cytotoxicity analysis of sialic acid inhibition of autoantibody binding

MAbs were incubated with an equal volume of sialic acid at 8μg/ml in PBS-DAB or with PBS-DAB alone for 1 hour at 4°C. Volumes of 1μl were dispensed into the wells of a microtest tray and screened in a standard microlymphocytotoxicity test. Initial studies were performed using MAbs F15-DD5-26, F36-CB10-18 and MAD at 1/2 and 1/10 dilutions against a panel of four T and B cell targets. Cytotoxicity reactivity of treated and untreated MAbs were compared (Table 5.3.3).

MAb dilution	F15-DD5-26		F36-CB10-18		MAD	
	normal	sialic acid	normal	sialic acid	normal	sialic acid
T cells						
1/2	4/4*	4/4	4/4	4/4	4/4	4/4
1/10	4/4	0/4	4/4	2/4	4/4	0/4
B cells						
1/2	4/4	4/4	4/4	4/4	4/4	4/4
1/10	4/4	1/4	4/4	4/4	4/4	2/4

Table 5.3.3 Cytotoxic inhibition of autoreactive (F15-DD5-26 and F36-CB10-18) and anti-RhD (MAD) MAbs at 1/2 and 1/10 dilutions treated with sialic acid at 8mg/ml compared to untreated controls (No. of cells positive / No. of cells tested).*

No inhibition of cytotoxicity was found against T or B cell targets at 1/2 dilution. However at 1/10 dilution, all F15-DD5-26 and MAD reactivity and 2 of 4 tests using F36-CB10-18 were blocked against T cell targets. In addition, 3 of 4 tests using F15-DD5-26 and 2 of 4 tests using MAD were blocked against B cell targets. No B cell inhibition was found using F36-CB10-18.

Further tests were performed comparing titres of the above MAbs (sialic acid treated and untreated) at doubling dilutions from neat to 1/128 against a T and B cell target. Controls studies were performed using Terasaki Park medium at pH4 and pH7·2. No difference in cytotoxicity titres of the MAbs at pH4 and pH7·2 was found in the untreated controls. There was a mean reduction of between 2 and 3 doubling dilutions with sialic acid at 8μg/ml against both T and B cell targets (data not shown).

These results indicated that sialic acid could partially block MAb reactivity in the cytotoxicity test. Low affinity T cell targets were more effectively blocked than high affinity B cell targets. Furthermore, blocking of the lower affinity MAbs, F15-DD5-26 and MAD, was greater than that for the higher affinity MAb, F36-CB10-18.

In an attempt to optimise the sialic acid blocking, cytotoxicity titres of MAbs and alloantisera treated with different concentrations of sialic acid were compared. MAbs F15-DD5-26, F36-CB10-18, two anti-HLA alloantisera (Orford HLA-A2 and Clark HLA-B12) and serum from a patient with T and B cell autoantibodies (DV) were tested. The reagents were titrated at doubling dilutions from neat to 1/128 in duplicate. Each sample was treated with an equal volume of sialic acid at concentrations of 50mg/ml down to 1·56mg/ml at pH4 and pH7. Cytotoxicity titres against one T cell target were compared to the untreated controls (Table 5.3.4).

Sialic acid concentration	F36-CB10-18 dilution (reciprocal) N	2	4	8	16	32	64	128
50mg/ml (pH4)	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-
12.5	+	(+)	-	-	-	-	-	-
6.25	+	+	+	+	(+)	-	-	-
3.125	+	+	+	+	+	-	-	-
1.56	+	+	+	+	(+)	-	-	-
0	+	+	+	+	(+)	-	-	-
50mg/ml(pH7.2)	+	+	+	+	-	-	-	-
25	+	+	+	+	(+)	-	-	-
12.5	+	+	+	(+)	-	-	-	-
6.25	+	+	+	+	+	-	-	-
3.125	+	+	+	+	(+)	-	-	-
1.56	+	+	+	(+)	(+)	-	-	-
0	+	+	+	+	(+)	-	-	-

Sialic acid concentration	F15-DD5-26 dilution (reciprocal) N	2	4	8	16	32	64	128
50mg/ml (pH4)	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-
12.5	+	(+)	-	-	-	-	-	-
6.25	+	+	+	+	(+)	-	-	-
3.125	+	+	+	+	(+)	-	-	-
1.56	+	+	+	+	-	-	-	-
0	+	+	+	+	(+)	-	-	-
50mg/ml(pH7.2)	+	+	+	+	(+)	-	-	-
25	+	+	+	+	+	-	-	-
12.5	+	+	+	(+)	(+)	-	-	-
6.25	+	+	+	+	+	-	-	-
3.125	+	+	+	+	+	-	-	-
1.56	+	+	+	+	(+)	-	-	-
0	+	+	+	+	(+)	-	-	-

Table 5.3.4 Cytotoxicity titres of autoreactive MAbs F36-CB10-18 and F15-DD5-26 at doubling dilutions from neat to 1/128 against a T cell target, following pretreatment with sialic acid at pH4 and pH7·2 using concentrations of 50mg/ml to 1·56mg/ml, compared to untreated controls. + =80-100% lysis, (+) =20-79% lysis, - =negative, NT =not tested.

a)

Sialic acid concentration	Serum DV dilution (reciprocal) N	2	4	8	16	32	64	128
50mg/ml (pH4)	-	-	-	-	-	-	-	-
25	+	-	-	-	-	-	-	-
12.5	+	(+)	-	-	-	-	-	-
6.25	+	(+)	-	-	-	-	-	-
3.125	+	+	(+)	-	-	-	-	-
1.56	+	+	(+)	-	-	-	-	-
0	+	+	(+)	-	-	-	-	-
50mg/ml(pH7.2)	+	(+)	(+)	-	-	-	-	-
25	+	+	(+)	-	-	-	-	-
12.5	+	(+)	-	-	-	-	-	-
0	+	+	+	+	(+)	-	-	-

b)

Sialic acid concentration	Clark (B12) dilution (reciprocal) N	2	4	8	16	32	64	128
50mg/ml (pH4)	-	-	-	-	-	-	-	-
25	+	-	-	-	-	-	-	-
12.5	+	+	+	(+)	-	-	-	-
6.25	+	+	+	+	-	-	-	-
3.125	+	+	+	+	(+)	-	-	-
1.56	+	+	+	+	(+)	-	-	-
0	+	+	+	+	(+)	-	-	-
50mg/ml(pH7.2)	+	+	NT	NT	NT	NT	NT	NT
25	+	+	+	+	-	-	-	-
12.5	+	+	+	+	-	-	-	-
6.25	+	+	+	-	-	-	-	-
3.125	+	+	+	+	(+)	-	-	-
1.56	+	+	+	+	-	-	-	-
0	+	+	+	+	-	-	-	-

Table 5.3.5 Cytotoxicity titres of a T and B cell lymphocytotoxic autoantibody (DV, panel a) and a HLA-B12 alloantiserum (Clark, panel b) at doubling dilutions from neat to 1/128 against a T cell target, following pretreatment with sialic acid at pH4 and pH7·2 using concentrations of 50mg/ml to 1·56mg/ml, compared to untreated controls.

+ =80-100% lysis,

(+) =20-79% lysis,

- =negative, NT =not tested.

c)

Sialic acid concentration	Orford (A2) dilution (reciprocal) N	2	4	8	16	32	64	128
50mg/ml (pH4)	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-
12.5	+	+	-	-	-	-	-	-
6.25	+	+	+	+	-	-	-	-
3.125	+	+	+	+	-	-	-	-
0	+	+	+	+	-	-	-	-
50mg/ml(pH7.2)	+	+	+	(+)	-	-	-	-
25	+	+	+	(+)	-	-	-	-
12.5	+	+	+	(+)	-	-	-	-
6.25	+	+	+	(+)	-	-	-	-
3.125	+	+	+	(+)	-	-	-	-
0	+	+	+	(+)	-	-	-	-

Table 5.3.5c Cytotoxicity titres of anti-HLA-A2 alloserum (Orford) tested at doubling dilutions from neat to 1/128 against a T cell target, following pretreatment with sialic acid at pH4 and pH7·2 using concentrations of 50mg/ml to 1·56mg/ml, compared to untreated controls.

The results show that sialic acid concentrations of 50mg/ml at pH4 caused complete inhibition of cytotoxicity with both the autoreactive MAbs (F15-DD5-26 and F36-CB10-18), patient serum lymphocytotoxic autoantibody (DV), and the anti HLA-A2 (Orford) and B12 (Clark) alloantisera. At a sialic acid concentration of 25mg/ml reactivity of F15-DD5-26, F36-CB10-18 and Orford (HLA-A2) was completely removed, and residual activity with neat serum only was seen with DV (T and B auto) and Clark (HLA-B12). Using sialic acid concentrations of 12·5mg/ml, the titre of each reagent was blocked by two doubling dilutions. Sialic acid concentrations of 6·25mg/ml and below gave no change in cytotoxicity titres compared to the untreated control. No cytotoxicity inhibition using any concentration of sialic acid was seen at pH7·2.

The results indicated that sialic acid could inhibit cytotoxicity of both the monoclonal autoantibodies and the lymphocytotoxic autoantibodies in patient serum. However the effect was not specific to autoantibodies as inhibition of cytotoxic HLA class I alloantisera was also found. It was possible that the non-specific inhibition was due to blocking of complement activity.

To investigate this further a standard cytotoxicity test was performed using F36-CB10-18 at doubling dilutions against a T cell target, with the addition sialic acid added directly to the rabbit complement immediately before use. The equivalent of 1μl of sialic acid (at each of the above concentrations, pH4 and pH7·2) per 5μl of complement was used (Table 5.3.6).

Sialic acid concentration	F36-CB10-18 dilution (reciprocal) N	2	4	8	16	32	64	128
50mg/ml (pH4)	-	-	-	-	-	-	-	-
25	+	+	+	+	+	(+)	-	-
12.5	+	+	+	+	+	(+)	(+)	-
6.25	+	+	+	+	+	+	(+)	-
3.125	+	+	+	+	+	+	(+)	-
0	+	+	+	+	+	+	(+)	-
50mg/ml(pH7.2)	+	+	+	+	(+)	(+)	-	-
25	+	+	+	+	(+)	(+)	-	-
12.5	+	+	+	+	+	(+)	(+)	-
6.25	+	+	+	+	+	(+)	(+)	-
3.125	+	+	+	+	+	(+)	(+)	-
0	+	+	+	+	+	(+)	(+)	-

Table 5.3.6 Cytotoxicity titre of F36-CB10-18 at doubling dilutions from neat to 1/128 against a T cell target, with the addition of sialic acid (pH4 and pH7·2) from 50mg/ml to 3·125mg/ml added directly to the rabbit complement. The equivalent of 1µl of sialic acid per 5µl of rabbit complement was used.

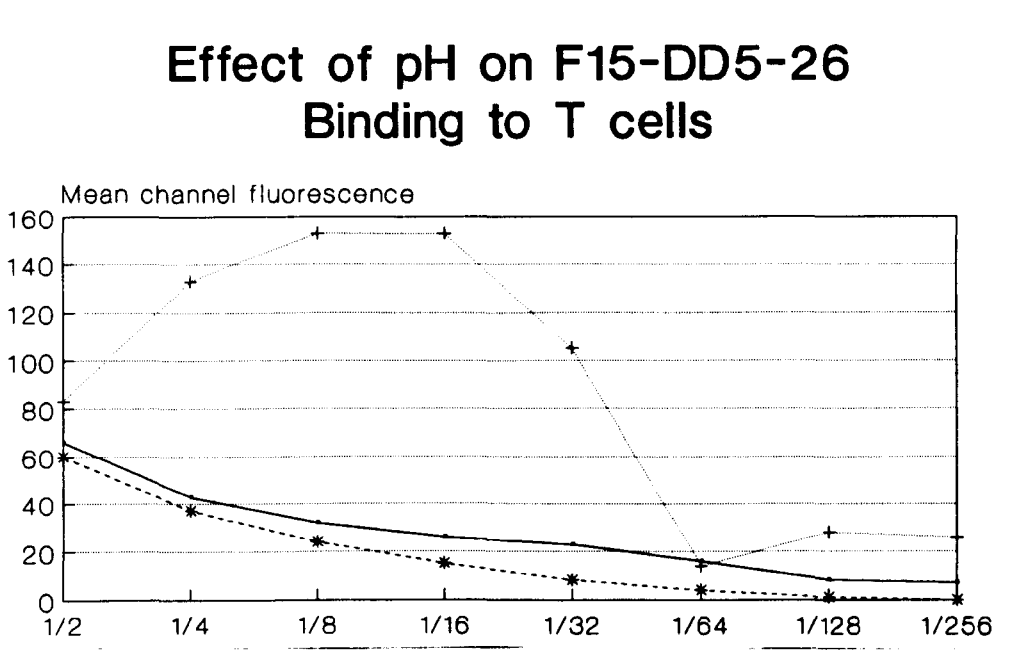
The results show complete inhibition of cytotoxicity using sialic acid at concentrations of 50mg/ml at pH4, when added directly to the rabbit complement. No difference in cytotoxicity titres was seen for the other sialic acid concentrations at pH4 and no inhibition was found using sialic acid at any concentration at pH7. The results therefore indicate that the cytotoxicity inhibition may be at the level of the complement and may not be due to antibody binding inhibition. It is therefore not possible to interpret lymphocytotoxic autoantibody binding inhibition results using sialic acid in cytotoxicity assays.

5.4 Effect of pH on autoantibody binding

Although neuraminidase cleavage of cell surface molecules and blocking with lectin from Triticum vulgaris indicated that autoantibody binding was to a sialic acid defined epitope, binding inhibition studies using free sialic acid gave increased autoantibody binding. Also increased binding to low affinity T cell targets was found in the low pH controls.

This suggests an alternative possibility, that polyreactivity of these antibodies are dependent on antigenic charge, specifically against negatively charged cell surface molecules. To investigate this possibility the influence of pH on MAb binding to low affinity T cell targets was examined. The fluorescence binding assay was used with F15-DD5-26 and F36-CB10-18 at doubling dilutions from 1/2 to 1/256, diluted in buffered saline at pH 4·0 (18ml of 0·2M sodium acetate mixed with 82ml of 0·2M acetic acid), pH 7·2 (PBS-DAB), and pH 9·6 (45ml of 0·1M sodium carbonate mixed with 55ml of 0·1M sodium bicarbonate) (Fig 5.4.1).

The results show a rapid fall in MAb titre at pH 7·2 and 9·6. However the low affinity T cell binding was reversed at pH 4·0 with both MAbs reactive to 1/32 dilution. These results confirm that low pH increases the MAb binding affinity to T cell targets, suggesting that binding may be to multiple negatively charged cell surface molecules in ionised form.



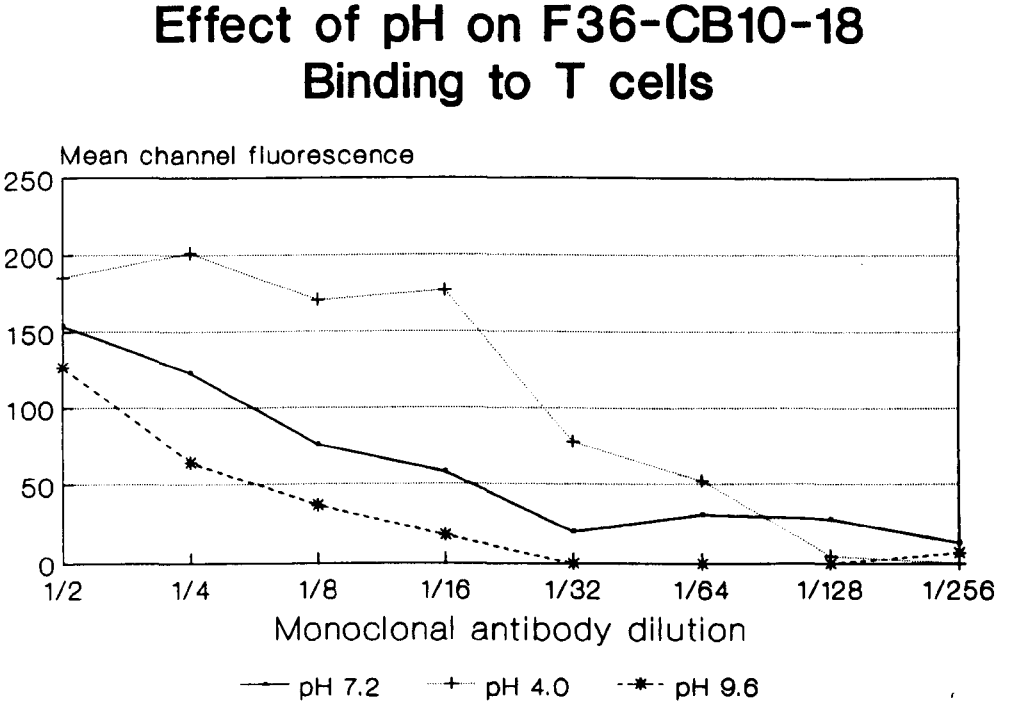


Figure 5.4.1 Cytofluorograph analysis of the effect of pH on F15-DD5-26 (panel a) and F36-CB10-18 (panel b) binding to low affinity T cell targets using MAbs at doubling dilutions (1/2 to 1/256) at pH4·0, 7·2 and 9·6.

Discussion

The lectin from Triticum vulgaris (wheat germ) binds N-acetyl-neuramic acid and N-acetyl-D-glucosamine. Pre-treatment of both T and B cells with Triticum vulgaris lectin strongly inhibited autoantibody and non-specific anti-RhD binding to lymphocytes in the fluorescence binding assay. Moreover, binding to high affinity B cell targets was inhibited by 70%. Lectin from Mycoplasma gallisepticum, specific for glycophorin, also inhibited T cell but not B cell ALA binding. No other lectin tested inhibited autoantibody binding. Glycophorin is a carbohydrate determinant on group O red blood cells. Red cell agglutination and cytofluorograph binding studies have shown that the autoantibodies produced in this study do not bind red cells of any major blood group. Therefore glycophorin alone is unlikely to constitute the autoantigen.

The binding inhibition by Triticum vulgaris is consistent with the neuraminidase sensitivity, as both techniques are specific for N-acetyl-neuramic acid. The results suggest that autoreactive antibodies bind a sialic acid dependent epitope. An alternative hypothesis is the polyreactivity of the autoantibodies is dependent on antigenic charge. Lymphocytotoxic autoantibodies may be capable of binding many negatively charged cell surface molecules. This view is supported by the finding that incubation of T cells with the autoantibodies at low pH alters the binding profile from low affinity to high affinity targets. Charge dependent polyreactive IgM antibodies with specificity for anionic phospholipids have been described (Mackworth-Young, 1990). The high binding to B cells and partial cleavage by papain are all consistent with the view that these are polyreactive antibodies, capable of binding multiple negatively charged antigens with no unique antigenic priming stimulus or specificity.

Other lectins, particularly lectin from Vicia fava and Phaseolus vulgaris caused an increase in ALA binding. This was not due to low pH as all lectins had neutral pH at 0·2mg/ml in PBS-DAB. It therefore appears that the autoantibodies bound some lectins attached to the cell surface. This finding is consistent with the view that lymphocytotoxic autoantibodies are capable of binding multiple types of molecules, due to their polyreactivity. Alternatively it is possible that the lectins are polyvalent and bind IgM directly. However the increased titre of F36-CB10-18 in lectin/red blood cell lysis and agglutination inhibition experiments compared to F15-DD5-26, despite lower immunoglobulin levels, argues against this.

We conclude that the characterisation of autoantibodies in renal dialysis patients, and comparison of these findings with descriptions of polyreactive IgM antibodies, demonstrate that they represent a similar group of antibodies. Low affinity binding of lymphocytotoxic autoantibodies may be associated with sialic acid dependant epitopes on T cells and CLL cells, and other negatively charged molecules found on different cell types.

Chapter 6

Immunochemistry

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6.9 Discussion

6.10 Immunoprecipitation

6.11 Inhibition of the mixed lymphocyte reaction (MLR)

6.12 Discussion

6.1 In-vivo production of lymphocytotoxic autoantibodies in nude mice

To produce high concentrations of human IgM, the in-vivo growth of heterohybridomas in ascites fluid of nude mice was attempted.

Twenty pristane treated eight week old MF1 nude mice were inoculated by an intraperitoneal injection of heterohybridoma cells. Ten mice each received $1{\cdot}5 \times 10^6$ F15-DD5-26 cells and ten mice received $1{\cdot}5 \times 10^6$ F36-CB10-18 cells. After one month, no mice injected with F36-CB10-18 showed signs of ascites production. However four of the ten mice inoculated with F15-DD5-26 had ascites production. A total of 39ml of ascites fluid was harvested from these mice. Ascites fluids were individually screened by cytotoxicity against a panel of 6 T cells, 5 B cells and 4 CLL cells at doubling dilutions from neat to 1/128. All gave positive cytotoxicity with each cell type, although the strength of reactivity and the panel reactive profiles were only equal to those found with tissue culture supernatants. The median cytotoxicity titres were 1/12 for T cells and CLL cells and 1/128 for B cells (data not shown). It was therefore felt that the ascites fluid offered no advantage compared to tissue culture supernatants as a source of IgM.

At the time of harvesting, heterohybridoma cells derived from two ascites fluids were returned to tissue culture in-vitro. Both clones continued to grow well, although one gave only weak cytotoxic B cell reactivity. It therefore appeared that some hybridoma cells had become unstable when grown in-vivo and had either failed to grow or lost antibody production. This finding may explain the unexpectedly low lymphocytotoxic autoantibody titres of the ascites fluid. The successful growth of some of the F15-DD5-26 clones compared to no growth of F36-CB10-18 clones probably reflects

the faster growth rate and improved stability of F15-DD5-26.

It was recognised that the surviving F15-DD5-26 antibody producing heterohybridomas may have adapted for in-vivo growth in nude mice. However, subsequent inoculation of nude mice with these cells, with the expectancy of improved results was not attempted.

6.2 IgM purification from tissue culture supernatant

Human IgM was present in F15-DD5-26 and F36-CB10-18 tissue culture supernatant at levels of between 40-70μg/ml. In the absence of equipment for preparative HPLC or affinity chromatography, a method for purification of IgM from large quantities of tissue culture supernatant was developed by combining ion exchange chromatography and molecular exclusion gel filtration. This enabled bulk extraction of immunoglobulin present at low levels in tissue culture supernatant. SDS-PAGE analysis and cytotoxicity testing of the purified immunoglobulin indicated that acceptable IgM yields and purity could be obtained.

6.2.1 Ion exchange chromatography

Bakerbond ABx 40μm is an ion exchange resin which exhibits high affinity for all immunoglobulin classes. The manufacturers specifications indicate that the resin does not bind the major contaminating proteins, albumin and transferrin, and other serum proteins found in tissue culture supernatants or ascites fluid. The resin is a silica based porous material with weakly anionic and cationic functional groups. The binding and elution of the immunoglobulin is based on manipulation of the pH and ionic strength of the buffers. The manufactures describe a one step purification procedure of immunoglobulin with an 80-99% purity from tissue culture media and ascites

fluid, with a 97% immunoglobulin recovery. This procedure may be performed using a solid phase 'slurry' technique, without the need for chromatography equipment for batch purification of immunoglobulin from tissue culture supernatant.

a) IgM purification using ABx 40μm ion exchange resin:

Manufactures specifications indicated 1·0g of ABx 40μm resin would bind 150mg of IgG. No information on the binding capacity of IgM was available. It was estimated that 1ml of ten times concentrated tissue culture supernatant would contain 0·5mg of IgM, therefore 3·0mg of resin would have sufficient binding capacity for 0·5mg of immunoglobulin. On the assumption that the resin binding capacity for IgM, being a larger molecule than IgG, may be lower, initial tests using 50mg of resin per 1ml of x10 concentrated tissue culture supernatant were used.

Immunoglobulin positive and negative fractions were separated from F15-DD5-26 ascites, F15-DD5-26 supernatant and F36-CB10-18 supernatant according to the protocol in materials and methods. The fractions were dialysed with PBS-DAB and concentrated to their original volumes (1ml) and then tested by cytotoxicity for lymphocytotoxic autoantibody reactivity against a panel of 3 T cells, 3 B cells and 1 CLL cell (Table 6.2.1).

Target cell		Median cytotoxicity titre (reciprocal) IgM positive fraction DD5asc	DD5spt	CB10spt	IgM negative fraction DD5asc	DD5spt	CB10spt
	O.D.	0.597	0.501	0.701	3.44	3.30	3.36
T		16	32	8	32	64	64
B		>128	>128	32	>128	>128	>128
CLL		16	16	4	64	128	32

O.D. = optical density (absorbance at 280nm).

Table 6.2.1 Cytotoxicity titres of IgM positive and negative fractions separated from 1ml of x10 concentrated tissue culture supernatant (spt) or neat ascites fluid (asc) using 50mg of ABx 40μm ion exchange resin.

Cytotoxicity results show that autoantibody titres were higher in IgM negative fractions than the IgM positive fractions for each cell tested. This indicates that the separation of IgM was incomplete. Ascites fluid was noted to produced a cloudy precipitate on dilution with MES buffer, with protein precipitating out of solution.

b) Determination of IgM binding capacity of ABx 40μm ion exchange resin

To determine the optimal quantity of ABx 40μm resin required to bind all the IgM present in tissue culture supernatant, 1ml volumes of x10 concentrated F15-DD5-26 supernatant was absorbed with 200, 400 and 800mg/ml of ABx 40μm ion exchange resin. The eluted IgM containing and IgM depleted fractions were dialysed in PBS-DAB, concentrated to 1ml final volume and tested by cytotoxicity against 3 T cells, 3 B cells and 1 CLL cell (Table 6.2.2).

ABx40μm (mg)	Target cell	Median cytotoxicity titre (reciprocal) IgM +'ve (O.D.)	IgM -'ve (O.D.)
200	T	16 (2.20)	0 (3.34)
	B	>128	8
	CLL	8	0
400	T	16 (3.05)	4 (3.23)
	B	>128	32
	CLL	16	2
800	T	16 (2.45)	0 (3.23)
	B	>128	8
	CLL	16	0

O.D.= optical density (absorbance at 280nm).

Table 6.2.2 Determination of the optimal quantity of ABx 40μm ion exchange resin required for the separation of IgM from 1ml of x10 concentrated F15-DD5-26 tissue culture supernatant.

The cytotoxicity results using T cells and the CLL cell targets showed complete absorbtion of reactivity from the IgM depleted fractions using 200 and 800mg/ml of resin. The residual T cell and CLL cell reactivity in the IgM depleted fraction using 400mg/ml was not in accordance with the results using lower and higher resin concentrations and remains unexplained. Residual B cell reactivity was found at all resin concentrations. This reflects the high sensitivity of B cell targets in cytotoxicity tests. The low B cell titres in IgM depleted fractions is probably a result of very low concentrations of residual IgM. These results demonstrated that most IgM contained in the tissue culture supernatant had been eluted in the immunoglobulin positive fractions.

c) SDS-PAGE analysis of IgM purity:

The high optical densities of the IgM positive fractions (Table 6.2.2)

indicated protein concentrations of between 1·6 and 2·2mg/ml. This was higher than expected for IgM alone and therefore suggested contamination with other proteins. To determine the immunoglobulin purity, IgM positive and negative fractions from F15-DD5-26 supernatant, separated using 200mg/ml ABx 40μm, were analyzed by SDS-PAGE under non-reduced and reduced conditions (Plate 6.2.1).

Lane 2, using 20μg of commercially purified IgM (non-reduced, Northeast Biomedical, Uxbridge), demonstrated pentameric IgM (molecular weight 900,000) which does not migrate into a 10% acrylamide gel, with several lower molecular weight contaminating protein bands. On reduction (lane 3), IgM heavy chains at molecular weight 70,000 (indicated) and several contaminating protein bands were found. The non-reduced IgM positive fraction from F15-DD5-26 (lanes 4 and 7) show high molecular weight IgM with three major contaminating protein bands between molecular weights 43,000 and 67,000. The reduced samples (lanes 5 and 8) show two major contaminating bands at molecular weights 67,000, with only weak IgM heavy chain bands (indicated). IgM light chains (molecular weight 23,000) have migrated off the lower end of these gels. The non-reduced and reduced IgM negative fraction (lanes 9 and 10) demonstrate the mass of contaminating protein depleted from the IgM positive fraction.

These results demonstrate that there were major contaminating protein bands present in both the commercial and F15-DD5-26 purified IgM preparations. The contaminating protein in F15-DD5-26 was of low molecular weight (between molecular weights 43,000 and 67,000) compared to the non-reduced pentameric IgM (molecular weight 900,000).

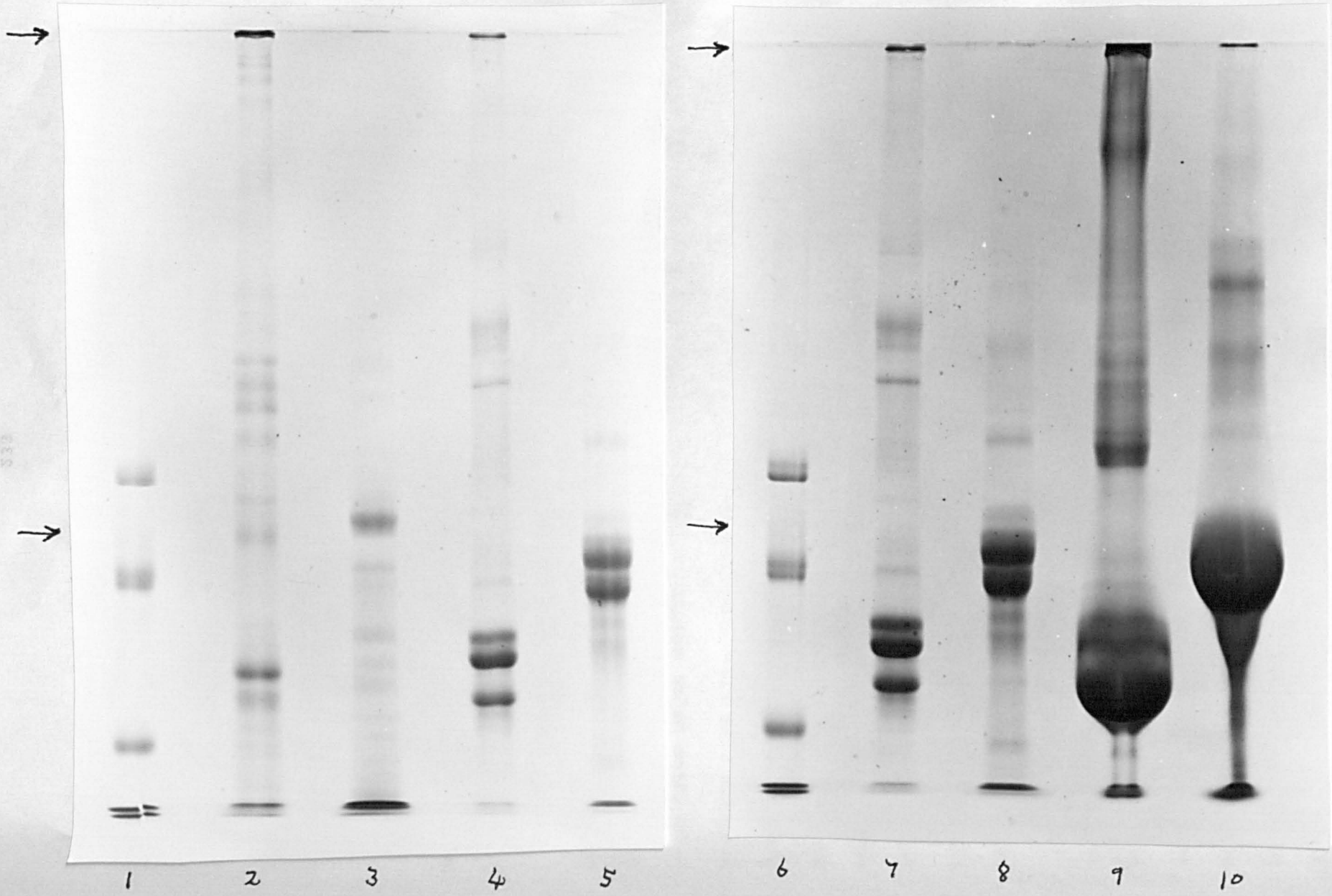
1
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Plate 6.2.1

SDS-PAGE analysis of purified IgM from F15-DD5-26 tissue culture supernatant using 200mg/ml of ABx 40μm ion exchange resin

Lane 1, low molecular weight markers (94k, 67k, 43k).

Lane 2, commercial IgM (20μg of protein), non-reduced.

Lane 3, commercial IgM (20μg of protein), reduced.

Lane 4, IgM positive fraction (30μg of protein), non-reduced.

Lane 5, IgM positive fraction (30μg of protein), reduced.

Lane 6, low molecular weight markers.

Lane 7, IgM positive fraction (60μg of protein), non-reduced.

Lane 8, IgM positive fraction (60μg of protein), reduced.

Lane 9, IgM negative fraction, non-reduced.

Lane 10, IgM negative fraction, reduced.

d) Further purification of IgM by ion exchange chromatography

The F15-DD5-26 IgM positive fractions pooled from 200, 400 and 800mg/ml of ABx 40μm ion exchange preparations were purified a second time by the same procedure. Re-purified IgM positive and negative fractions were analyzed by SDS-PAGE (Plate 6.2.2). Lanes 2 (non-reduced) and 3 (reduced) show the single step IgM purification of F15-DD5-26 used as a starting material. Lanes 4 (non-reduced) and 5 (reduced) show the repeated ion exchange purification step gave near pure IgM with only minor contaminating protein bands. Lanes 6 (non-reduced) and 7 (reduced) show the contaminating proteins were eluted in the IgM negative fraction.

This indicated that near pure IgM could be prepared from tissue culture supernatants using this method. However, the x2 purified IgM fraction contained only 120μg of protein, indicating a 24% immunoglobulin yield. Cytotoxicity screening using 3 CLL cells, 3 T cells and 3 B cell targets gave weak reactivity, with mean titres of 1/2, 1/4 and 1/16 respectively (data not shown). This confirmed that the IgM yield was very low. The x2 purified IgM negative fraction was completely negative in cytotoxicity tests against all cell types.

Plate 6.2.2

SDS-PAGE analysis of F15-DD5-26 IgM positive fractions purified x2 using ABx 40μm ion exchange resin

Lane 1, low molecular weight markers (94k, 67k, 43k).

Lane 2, purified x1, IgM positive fraction, non-reduced.

Lane 3, purified x1, IgM positive fraction, reduced.

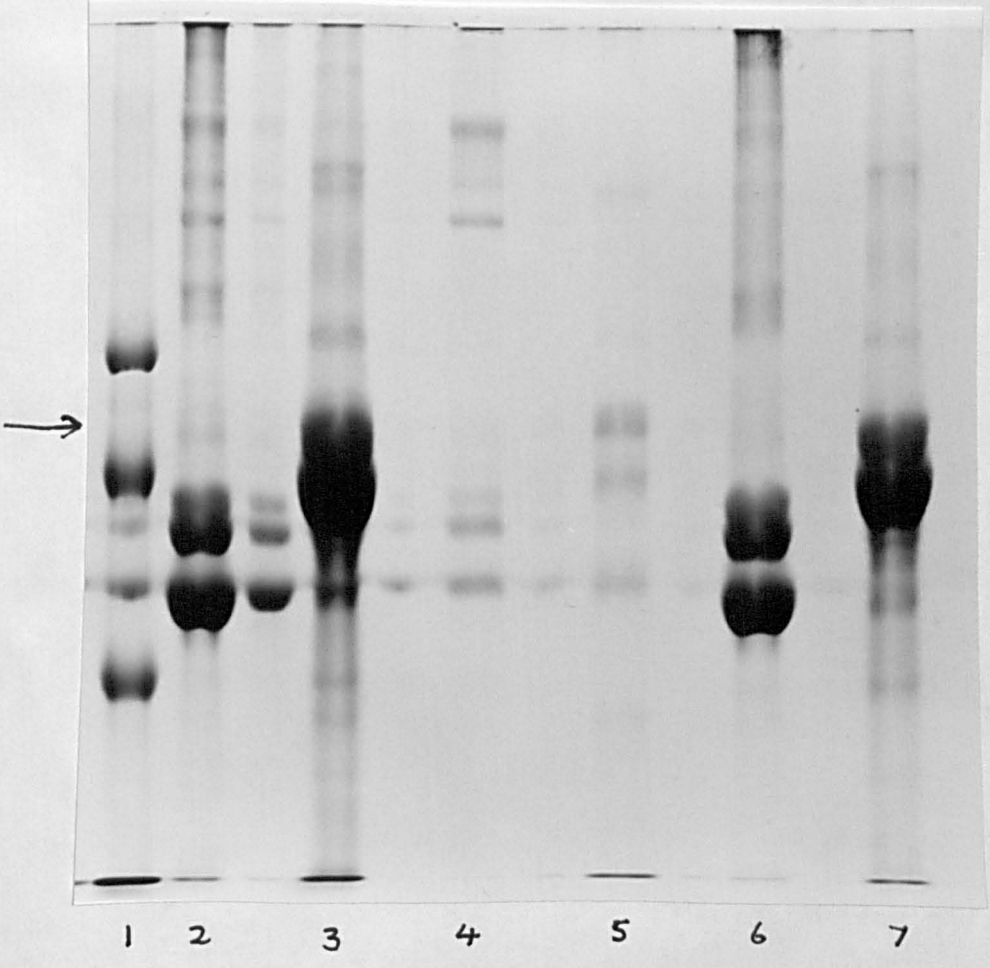
Lane 4, purified x2, IgM positive fraction, non-reduced.

Lane 5, purified x2, IgM positive fraction, reduced.

Lane 6, purified x2, IgM negative fraction, non-reduced.

Lane 7, purified x2, IgM negative fraction, reduced.

(Protein bands between lanes on this gel were due to inadvertent overloading of lane 2).



6.3 Purification of IgM by gel filtration

Preparative IgM purification from tissue culture supernatant using single step ion exchange chromatography with AB x 40μm resin gave good IgM recovery although two major contaminating protein bands were found on SDS-PAGE analysis. These bands were of low molecular weight compared to pentameric IgM. Therefore further IgM purification was obtained by molecular exclusion chromatography using Sephacryl S300 High Resolution gel filtration.

6.3.1 Column calibration

High molecular weight calibration markers, thyroglobulin (molecular weight 669,000), ferritin (molecular weight 440,000), catalase (molecular weight 232,000) and aldolase (molecular weight 158,000) were applied to a Sephacryl S300 gel filtration column to ensure appropriate separation of these molecules. Their elution profiles are shown in Figure 6.3.1.

The results demonstrate sequential elution of the calibration markers in descending order of molecular weight. The column gave complete separation of thyroglobulin from catalase and aldolase. This indicates that ascending gel filtration was successful at separating the calibration markers between molecular weights 669,000 and 232,000 and would therefore be appropriate for the separation of IgM (molecular weight 900,000) from the low molecular weight contaminating proteins (molecular weights between 43,000 and 67,000).

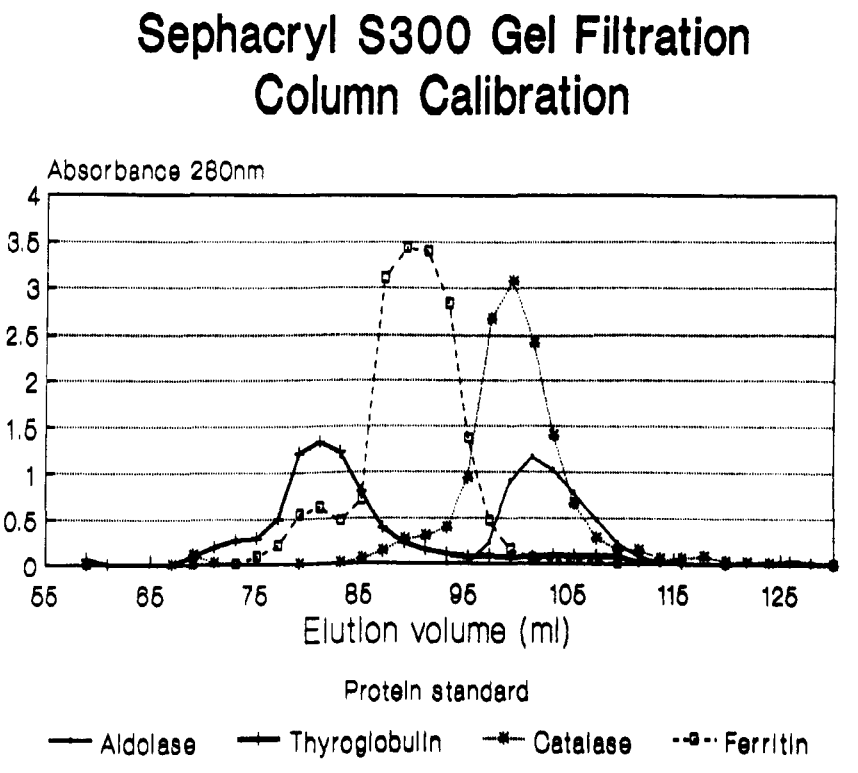


Figure 6.3.1 Calibration of Sephacryl S300 gel filtration column.

6.3.2 Purification of F15-DD5-26 IgM by gel filtration

The technique was then applied to the IgM positive fractions partially purified from F15-DD5-26 using ABx 40μm ion exchange chromatography. IgM in a final volume of 10ml PBS-DAB was prepared from 10ml of x10 concentrated F15-DD5-26 tissue culture supernatant by ion exchange chromatography (as above), giving a total protein yield of 8·36mg. Half of this (5ml) was loaded onto the Sephacryl S300 gel filtration column. The elution profile (indicated by absorbance at 280nm) is shown in Figure 6.3.2.

Three peaks were seen, the first peak being high molecular weight pentameric IgM and the subsequent peaks being the lower molecular weight

contaminating proteins. The total protein content in the IgM fractions (peak 1) was 1·12mg, indicating an overall 45% immunoglobulin yield from the original 100ml of tissue culture supernatant (containing about 5mg of IgM).

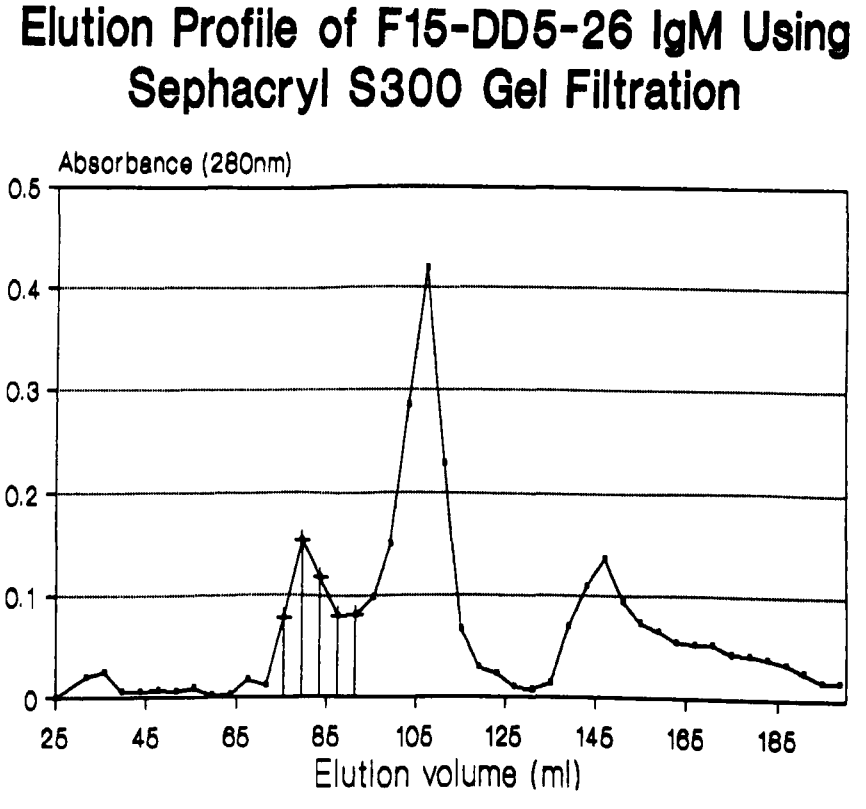
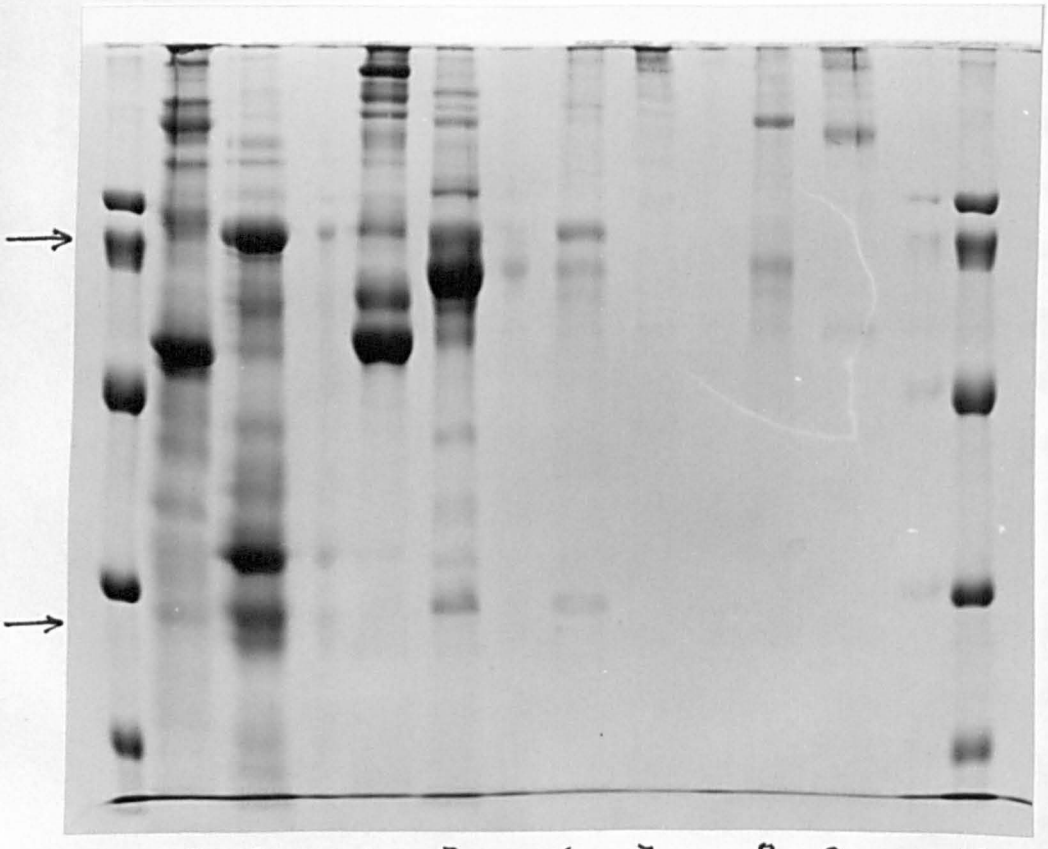


Figure 6.3.2 Sephacryl S300 gel filtration elution profile of the IgM positive fraction from 10ml of x10 concentrated F15-DD5-26 tissue culture supernatant, initially purified using ABx 40μm ion exchange chromatography. The hatched area denotes the fractions with lymphocytotoxic autoantibody reactivity.

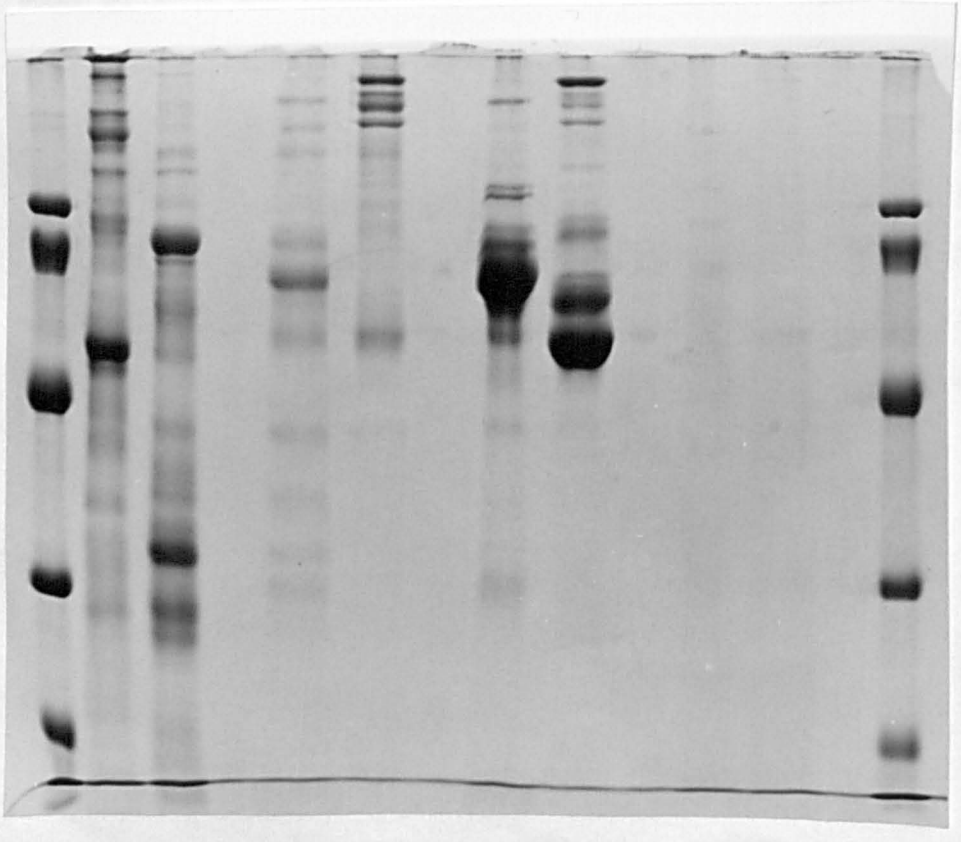
6.3.3 SDS-PAGE analysis of F15-DD5-26 IgM fractions purified using ABx 40μm ion exchange chromatography and molecular exclusion gel filtration

To confirm the purity of the IgM, fractions from the three peaks were analyzed by SDS-PAGE (Plate 6.3.3). Commercially purified IgM shows a large number of contaminating protein bands when non-reduced (lane 2) and reduced (lane 3) as previously described. IgM heavy chains (molecular weight 70,000) and light chains (molecular weight 23,000) can be seen in lane 3 (reduced).

As previously seen, IgM positive fractions purified from F15-DD5-26 supernatant by ion exchange chromatography, contains a number of contaminating protein bands when non-reduced (lane 4) and reduced (lane 5). However following further purification by gel filtration, IgM harvested from peak 1 (fractions 19-22) shows no contaminating bands when non-reduced (lane 7). F15-DD5-26 IgM heavy and light chains are seen in lane 6 (reduced) with a minor contaminating band (molecular weight approximately 67,000) located below the IgM heavy chain. This protein band was predominantly eluted in fraction 23, between peaks 1 and 2 as seen in lane 8 (reduced). The major contaminating protein bands were progressively eluted in fractions 25 (lanes 14 and 15) and fraction 27 (lanes 16 and 17) which correspond to the second peak, and fractions 35-40 (lanes 18 and 19) from the third peak.



1 2 3 4 5 6 7 8 9 10



11 12 13 14 15 16 17 18 19 20

Plate 6.3.3

SDS-PAGE analysis of IgM purified from 100ml of F15-DD5-26 tissue culture supernatant by ion exchange chromatography and gel filtration

Lane 1, low molecular weight markers (94k, 67k, 43k, 30k, 20k).

Lane 2, commercially purified IgM, non-reduced.

Lane 3, commercially purified IgM, reduced.

Lane 4, purified IgM (x1 ABx 40μm), non-reduced.

Lane 5, purified IgM (x1 ABx 40μm), reduced.

Lane 6, gel filtration fractions 19-22 (peak 1), reduced.

Lane 7, gel filtration fractions 19-22 (peak 1), non-reduced.

Lane 8, gel filtration fraction 23 (between peaks 1 & 2),reduced.

Lane 9, gel filtration fraction 23 (between peaks 1 & 2), non-reduced.

Lane 10, low molecular weight markers (94k, 67k, 43k, 30k, 20k).

Lane 11, low molecular weight markers (94k, 67k, 43k, 30k, 20k).

Lane 12, commercially purified IgM, non-reduced.

Lane 13, commercially purified IgM, reduced.

Lane 14, gel filtration fraction 25 (early peak 2), reduced.

Lane 15, gel filtration fraction 25 (early peak 2), non-reduced.

Lane 16, gel filtration fraction 27 (peak 2), reduced.

Lane 17, gel filtration fraction 27 (peak 2), non-reduced.

Lane 18, gel filtration fractions 35-40 (peak 3), reduced.

Lane 19, gel filtration fractions 35-40 (peak 3), non-reduced.

Lane 20, low molecular weight markers (94k, 67k, 43k, 30k, 20k).

6.3.4 Cytotoxicity screening of IgM purified from F15-DD5-26

The cytotoxicity titres of the original x10 concentrated tissue culture supernatants, the IgM positive and negative fractions purified using ABx 40μm ion exchange chromatography and the gel filtration fractions from the three peaks were screened by cytotoxicity using 5 T cells, 3 B cells and 1 CLL cell (Table 6.3.1).

	Median cytotoxicity titre (reciprocal)					
Target cell	x10 conc T.C Spnt	ABx 40 IgM +	ABx 40 IgM -	F19-22 peak 1	F25-29 peak 2	F35-40 peak 3
T cell	128	32	16	16	0	0
B cell	1024	>128	>128	64	2	0
CLL cell	64	8	2	8	0	0

Table 6.3.1 Cytotoxicity titres of IgM purified from 10ml of x10 concentrated F15-DD5-26 tissue culture supernatant by ion exchange chromatography and gel filtration.

The results show strong cytotoxic reactivity in the ABx 40μm IgM positive fraction. However strong residual reactivity was found in the IgM negative fraction indicating incomplete separation of IgM. Following gel filtration all the IgM cytotoxic reactivity was found in the first peak (fractions 19-22), with only very weak B cell reactivity in the second peak (fractions 25-29) and no reactivity in the third peak (fractions 35-40).

The results confirm that near pure IgM, with acceptable immunoglobulin yields could be purified from tissue culture supernatant using combined ion exchange chromatography and molecular exclusion gel filtration. Moreover, lymphocytotoxic autoantibody reactivity was isolated only in the purified IgM containing fractions. This confirms that autoantibody reactivity is due to IgM antibodies.

6.4 Purification of IgM from F36-CB10-18 tissue culture supernatant

The above procedure, combining ion exchange chromatography and molecular exclusion gel filtration was then applied for the purification of IgM from F36-CB10-18 tissue culture supernatant.

6.4.1 Purification of IgM from F36-CB10-18 using combined ion exchange chromatography and molecular exclusion gel filtration

IgM purified from 10ml of x10 concentrated F36-CB10-18 tissue culture supernatant using 400mg/ml of ABx40μm ion exchange resin was dialysed into a final volume of 10ml in PBS-DAB. 5ml of the IgM positive fraction was applied to a Sephacryl S300 gel filtration column as previously described. Two protein peaks were eluted from the column (Figure 6.4.1).

The first peak (fractions 17-21) contained high molecular weight pentameric IgM with 0·61mg of protein. This indicated a total IgM yield of 1·22mg from 100ml of supernatant which represents about a 30% recovery (assuming an original IgM concentration of 40μg/ml in 100ml of tissue culture supernatant).

Elution Profile of F36-CB10-18 IgM Using Sephacryl S300 Gel Filtration

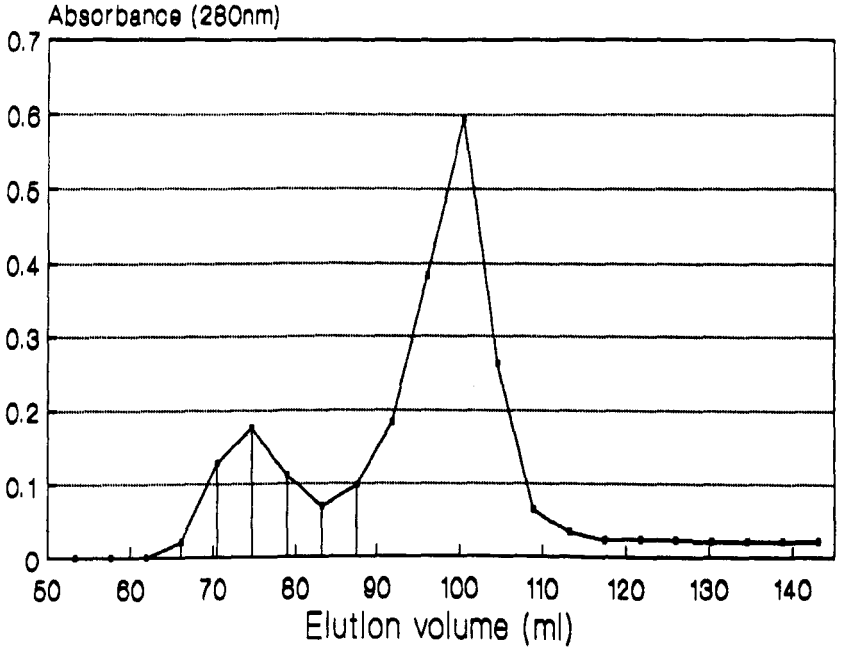
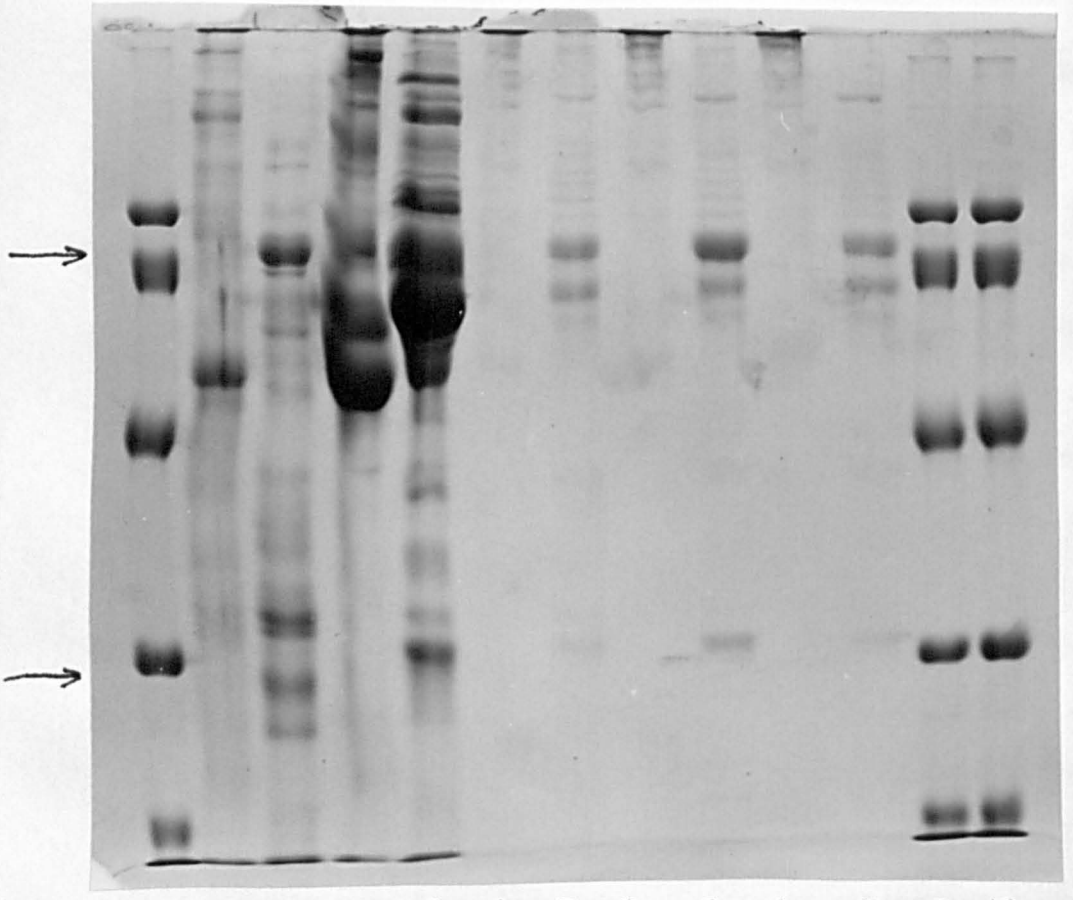


Figure 6.4.1 Sephacryl S300 gel filtration elution profile of the IgM positive fraction from 10ml of x10 concentrated F36-CB10-18 tissue culture supernatant, initially purified using ABx 40μm ion exchange chromatography. The hatched area denotes fractions with lymphocytotoxic autoantibody reactivity.

6.4.2 SDS-PAGE analysis of IgM purified from F36-CB10-18

IgM positive and negative fractions purified using 400mg/ml of ABx 40μm ion exchange resin and selected gel filtration fractions were analyzed by SDS-PAGE (Plate 6.4.1). As previously described, lanes 2 and 3 (non-reduced and reduced) show commercially purified IgM, with heavy and light chains visible in lane 3 (reduced). Lanes 4 and 5 (non-reduced and reduced) show the IgM positive fraction purified using ABx 40μm ion exchange chromatography, with IgM heavy and light chains indicated (lane 5, reduced). Gel filtration fractions 17, 18 and 21 from peak 1 (lanes 6,8 and 10, non-reduced) all demonstrated pentameric IgM which does not migrate into the 10% acrylamide gel, with little evidence of other contaminating protein bands. When reduced (lanes 7,9 and 11) IgM heavy chains (molecular weight 70,000) and light chains (molecular weight 23,000) were present. In addition a minor contaminating protein band was found at molecular weight 67,000, as was seen with F15-DD5-26 IgM purification. Again this contaminating protein band was predominantly eluted after the IgM, in fraction 25 (lanes 15 and 16) and fraction 26 (lanes 17 and 18). The major low molecular weight contaminating proteins from the ion exchange step were progressively eluted in the second peak (fractions 28-30, lanes 19-24). These results show that F36-CB10-18 gel filtration fractions eluted in peak 1 contained IgM, with no major contaminating proteins.


1 2 3 4 5 6 7 8 9 10 11 12 13

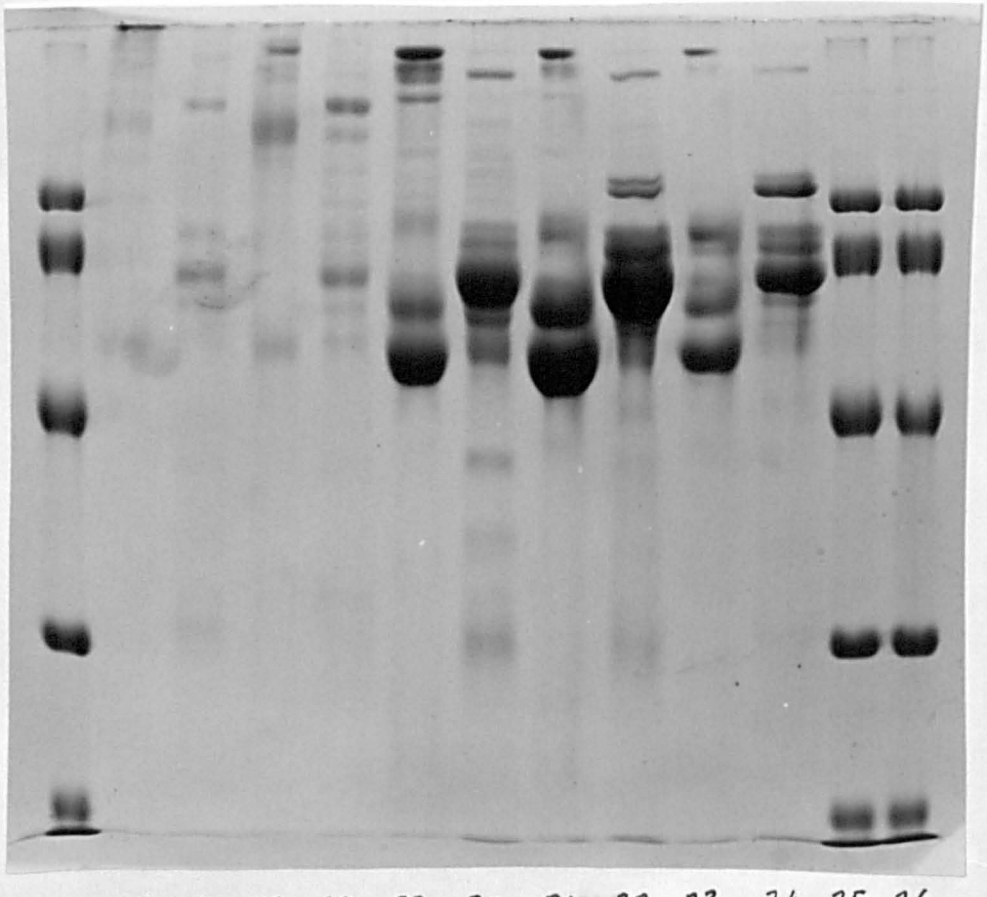

14 15 16 17 18 19 20 21 22 23 24 25 26

Plate 6.4.1

SDS-PAGE analysis of IgM purified from 10ml of x10 concentrated F36-CB10-18 tissue culture supernatant by ion exchange chromatography and gel filtration

Lane 1, low molecular weight markers (94k, 67k, 43k, 30k, 20k).
Lane 2, commercially purified IgM, non-reduced.
Lane 3, commercially purified IgM, reduced.
Lane 4, purified IgM (x1 ABx 40μm), non-reduced.
Lane 5, purified IgM (x1 ABx 40μm), reduced.
Lane 6, gel filtration fraction 17 (peak 1), non-reduced.
Lane 7, gel filtration fraction 17 (peak 1), reduced.
Lane 8, gel filtration fraction 18 (peak 1), non-reduced.
Lane 9, gel filtration fraction 18 (peak 1), reduced.
Lane 10, gel filtration fraction 21 (peak 1), non-reduced.
Lane 11, gel filtration fraction 21 (peak 1), reduced.
Lane 12, low molecular weight markers (94k, 67k, 43k, 30k, 20k).
Lane 13, low molecular weight markers (94k, 67k, 43k, 30k, 20k).

Lane 14, Low molecular weight markers (94k, 67k, 43k, 30k, 20k).
Lane 15, gel filtration fraction 25, non-reduced.
Lane 16, gel filtration fraction 25, reduced.
Lane 17, gel filtration fraction 26 (peak 2), non-reduced.
Lane 18, gel filtration fraction 26 (peak 2), reduced.
Lane 19, gel filtration fraction 28 (peak 2), non-reduced.
Lane 20, gel filtration fraction 28 (peak 2), reduced.
Lane 21, gel filtration fraction 29 (peak 2), non-reduced.
Lane 22, gel filtration fraction 29 (peak 2), reduced.
Lane 23, gel filtration fraction 30 (peak 2), non-reduced.
Lane 24, gel filtration fraction 30 (peak 2), reduced.
Lane 25, low molecular weight markers (94k, 67k, 43k, 30k, 20k).
Lane 26, low molecular weight markers.

6.4.3 Cytotoxicity screening of IgM purified from F36-CB10-18

IgM positive and negative fractions following purification from F36-CB10-18 tissue culture supernatant using 400mg/ml of ABx 40μm ion exchange resin and pooled fractions from the two gel filtration peaks (fractions 17-21 and 26-30) were tested by cytotoxicity using 3 T cells, 2 B cells and 1 CLL cell (Table 6.4.1).

Target cell	Median cytotoxicity titre (reciprocal)				
	x10 conc TCS	ABx 40um IgM +	ABx 40um IgM -	F17-21 peak 1	F26-30 peak 2
T cell	256	>128	8	128	0
B cell	768	>128	32	>128	2
CLL cell	64	64	2	128	0

Table 6.4.1 Cytotoxicity titres of IgM fractions purified from 10ml of x10 concentrated F36-CB10-18 tissue culture supernatant by ion exchange chromatography and gel filtration.

The results show strong cytotoxicity in the IgM positive fraction following purification using 400mg/ml of ABx 40μm resin, with relatively weak residual cytotoxicity in the IgM depleted fraction. Lymphocytotoxic autoantibody reactivity was eluted in the first gel filtration peak (fractions 17-21) containing the IgM. No T cell and CLL cell, and only weak B cell reactivity was found in the second peak (fractions 26-30). These data show good IgM purity and cytotoxic reactivity of F36-CB10-18 separated using combined ion exchange chromatography and gel filtration although the overall immunoglobulin yield was low (31%).

6.5 Bulk Purification of IgM from F15-DD5-26 and F36-CB10-18 tissue culture supernatant

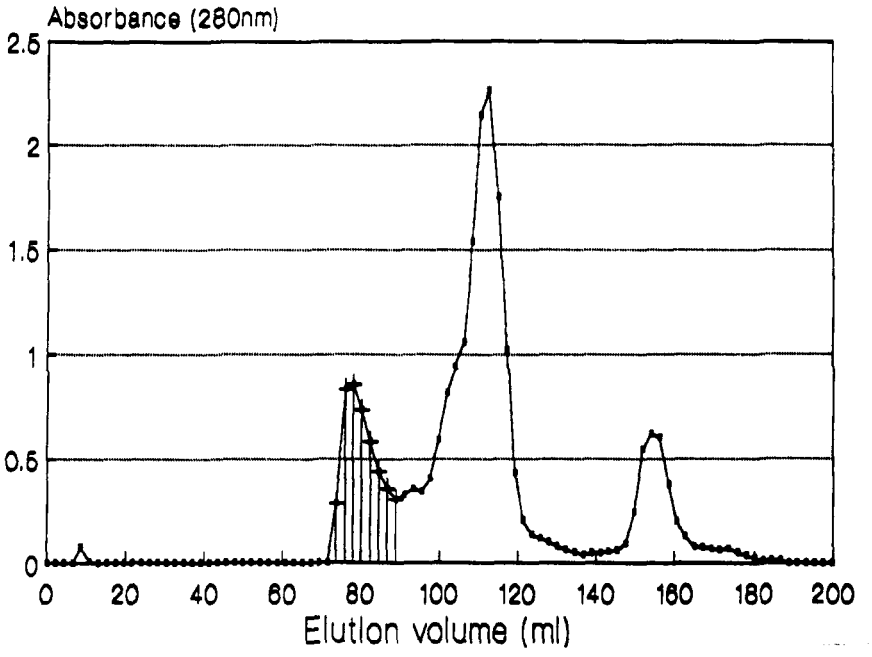
The use of Bakerbond ABx 40μm ion exchange resin combined with gel filtration on a Sephacryl S300 column demonstrated that milligram quantities of IgM could be separated from tissue culture supernatant with good immunoglobulin purity and an acceptable yield. The method was then scaled up for the separation of IgM from 1 litre quantities of F15-DD5-26 and F36-CB10-18. The cytotoxicity titres, immunoglobulin yield and purity were analyzed.

6.5.1 Bulk purification of IgM from one litre volumes of F15-DD5-26 and F36-CB10-18 tissue culture supernatants

One litre volumes of medium harvested from exhausted F15-DD5-26 and F36-CB10-18 cultures were concentrated to 100ml through a YM10 ultra filtration membrane by positive pressure at 15 P.S.I. Partially purified IgM was prepared, as above, using 40g of ABx 40μm ion exchange resin and dialysed in PBS-DAB to a final volume of 9ml. IgM positive fractions were passed through a Sephacryl S300 gel filtration column in three steps of 3ml volumes.

6.5.2 Sephacryl S300 Gel filtration profiles

The gel filtration elution profiles were similar to those previously found for each antibody (Figure 6.5.1).



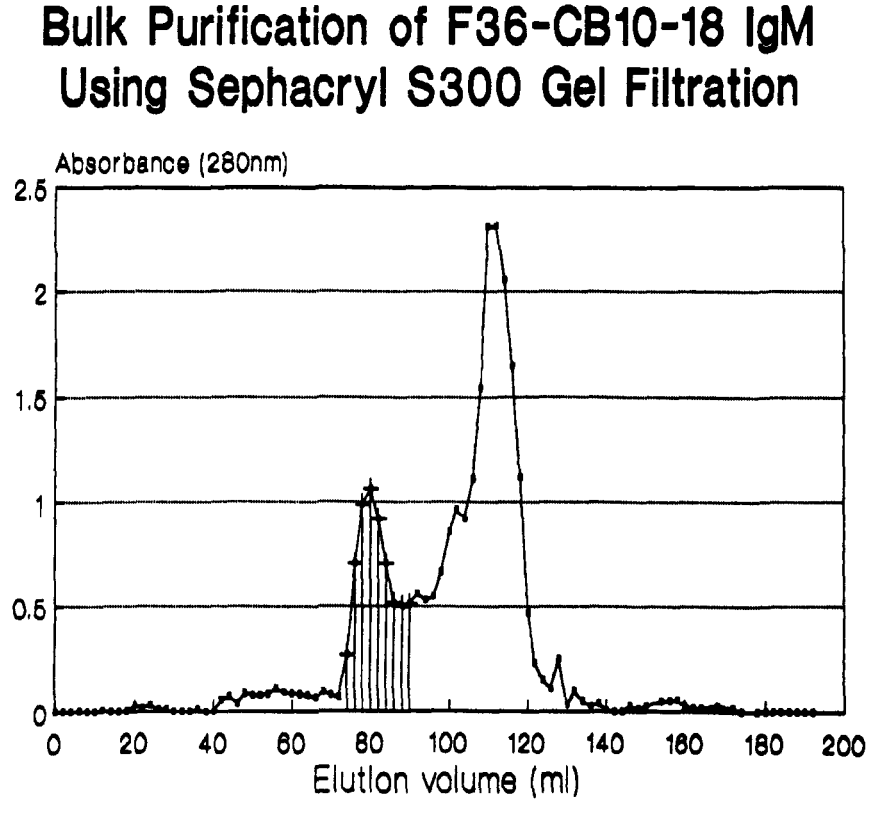


Figure 6.5.1 Sephacryl S300 gel filtration elution profile of the IgM fraction from 100ml of x10 concentrated F15-DD5-26 (panel a) and F36-CB10-18 (panel b) tissue culture supernatant, initially purified using ABx 40μm ion exchange chromatography. The hatched area denotes fractions with lymphocytotoxic autoantibody reactivity.

6.5.3 SDS-PAGE analysis of purified IgM

The final purity of IgM (gel filtration fractions pooled from peak 1) of F15-DD5-26 and F36-CB10-18 were analyzed using SDS-PAGE (Plate 6.5.1). Lanes 2 and 6 (non-reduced) show pentameric IgM from F15-DD5-26 and F35-CB10-18 respectively, which does not migrate into the 10% acrylamide gel. No contaminating protein bands were seen. Lanes 3 and 8 (reduced) show bands at molecular weights 70,000 and 23,000 corresponding to the IgM heavy and light chains for F15-DD5-26 and F36-CB10-18 respectively. Again, no major contaminating protein bands were present. The total purified IgM recovered (indicated by absorbance at 280nm) was 22·6mg for F15-DD5-26 and 17·14mg for F36-CB10-18, representing an approximate 34% and 41% yield for each antibody respectively. This indicated that the scaled up method for bulk purification of human IgM from tissue culture supernatant gave near pure immunoglobulin with an acceptable yield.

Plate 6.5.1

SDS-PAGE analysis of bulk purification of IgM from 100ml of x10 concentrated F15-DD5-26 and F36-CB10-18 tissue culture supernatant by ion exchange chromatography and gel filtration.

Lane 1, Low molecular weight markers (94k, 67k, 43k, 30k, 20k).

Lane 2, F15-DD5-26 purified IgM, non-reduced.

Lane 3, F15-DD5-26 purified IgM, reduced.

Lane 4, F15-DD5-26 purified IgM, biotinylated, non-reduced.

Lane 5, F15-DD5-26 purified IgM, biotinylated, reduced.

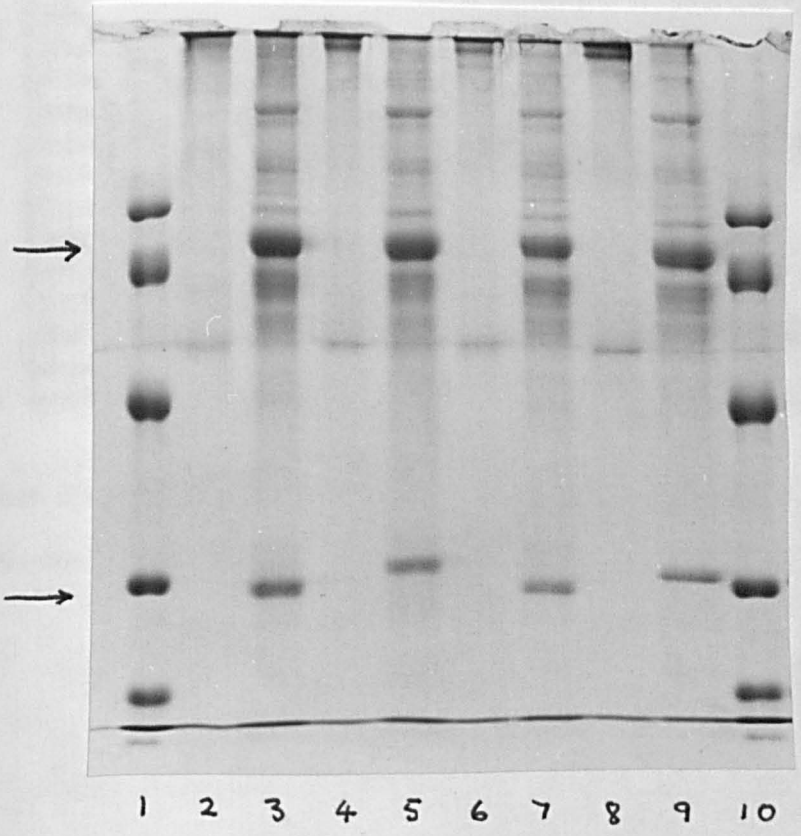
Lane 6, F36-CB10-18 purified IgM, non-reduced.

Lane 7, F36-CB10-18 purified IgM, reduced.

Lane 8, F36-CB10-18 purified IgM, biotinylated, non-reduced.

Lane 9, F36-CB10-18 purified IgM, biotinylated, reduced.

Lane 10, Low molecular weight markers (94k, 67k, 43k, 30k, 20k).



6.5.4 Cytotoxicity screening of purified IgM

Fractions corresponding to each peak for each antibody were pooled and tested for lymphocytotoxic autoantibody reactivity against one T cell, one B cell and one CLL cell (Table 6.5.1). The results revealed that strong lymphocytotoxic autoantibody reactivity was eluted in the IgM positive fractions following purification with ABx 40μm ion exchange chromatography, with only weak residual B cell reactivity in the IgM depleted fraction. Following further purification by gel filtration, lymphocytotoxic autoantibody reactivity was eluted in peak 1, with weaker residual B cell reactivity in the IgM depleted fractions (peaks 2 and 3).

Monoclonal antibody	Cytotoxicity titre (reciprocal) T cell	B cell	CLL cell
F15-DD5-26			
ABx 40 IgM +'ve	>256	>256	128
ABx 40 IgM -'ve	0	8	0
Peak 1 (IgM)	32	>256	16
Between peaks 1 & 2	4	32	4
Peak 2	0	0	0
Peak 3	0	0	0
F36-CB10-18			
ABx 40μm IgM +'ve	>256	>256	>256
ABx 40μm IgM -'ve	0	32	0
Peak 1 (IgM)	>256	>256	>256
Between peaks 1 & 2	2	32	4
Peak 2	0	16	0

Table 6.5.1 Cytotoxicity titres of IgM fractions purified from one litre volumes of F15-DD5-26 and F36-CB10-18 tissue culture supernatant.

6.6 Biotinylation of purified IgM

1mg quantities of purified IgM from F15-DD5-26 and F36-CB10-18 were directly biotinylated according to the method of Gretch (Gretch, Suter and Stinski, 1987). To confirm successful biotinylation without loss of antibody reactivity, binding of labelled and unlabelled MAbs were compared by a) cytotoxicity and conventional binding assays using FITC-anti-human-IgM, and b) biotin/FITC-avidin binding assays, against T and B cell targets.

6.6.1 Cytotoxicity and binding assay results:

A small reduction in cytotoxicity titre (one doubling dilution) was found with the biotinylated MAbs compared to the original unlabelled IgM (data not shown). A corresponding drop in mean channel fluorescence of 25% was found on cytofluorograph analysis using FITC-anti-human-IgM (data not shown). These data showed that the biotinylation procedure had not resulted in substantial loss of cytotoxicity and binding reactivity of the autoantibodies.

6.6.2 Binding profiles of biotinylated monoclonal antibodies:

FACScan profiles (logarithmic scale) of biotinylated MAbs, F15-DD5-26 and F36-CB10-18 tested at 1/10 and 1/20 dilutions against T and B cells are shown Figure 6.6.1.

T cell binding profiles (panel a) show successful biotinylation of both F15-DD5-26 and F36-CB10-18, with both antibodies giving similar profiles to those seen with FITC-anti-human-IgM. MAb F15-DD5-26 gave mean channel fluorescence values of 88 (76% positive) and 49 (54% positive) at 1/10 and 1/20 dilutions respectively. F36-CB10-18 gave higher binding with mean

channel fluorescence values of 227 (88% positive) and 178 (87% positive) at 1/10 and 1/20 dilutions respectively (background mean channel fluorescence values of 10 with 10% positive have been deducted from this data).

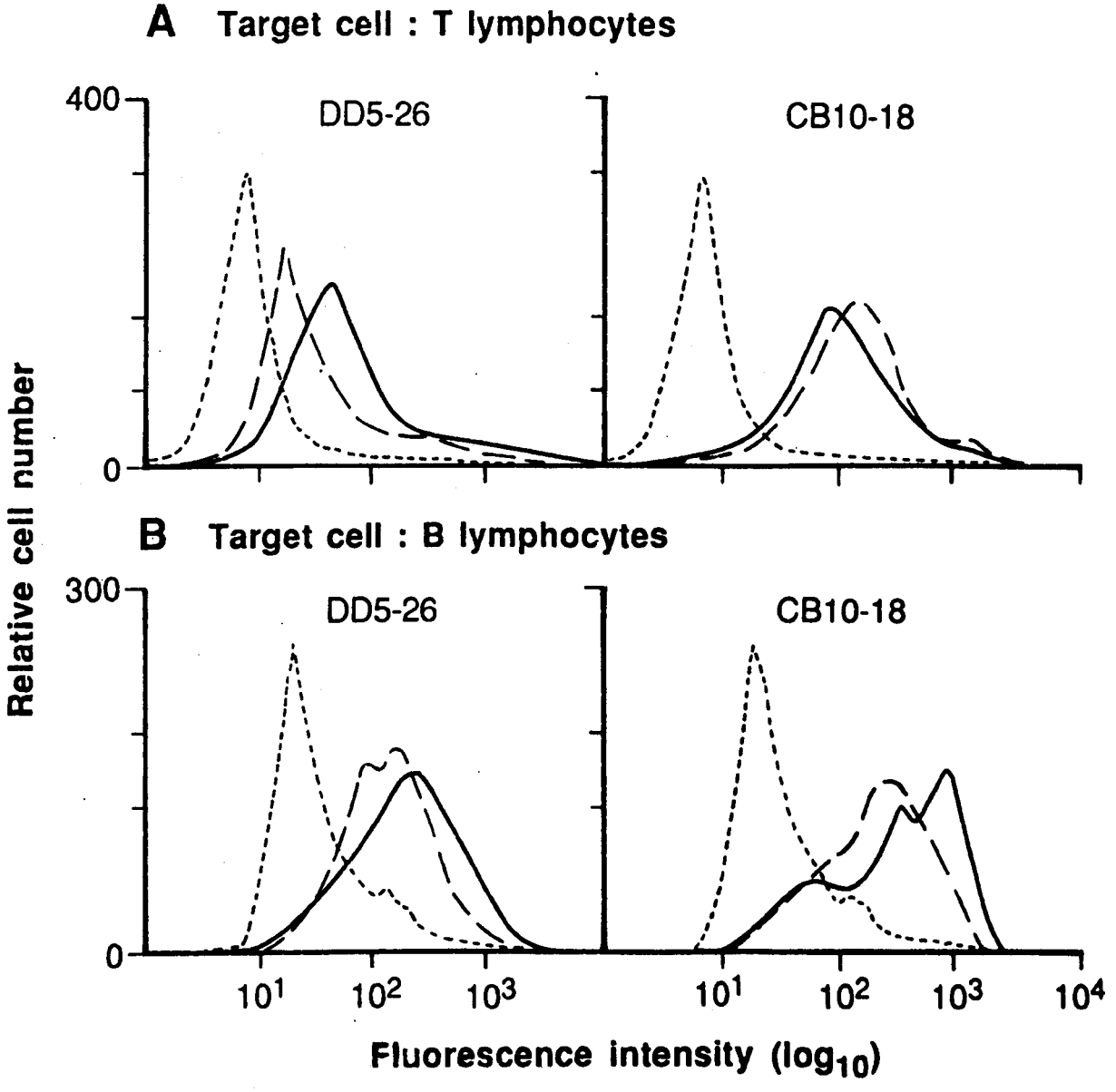


Figure 6.6.1 FACScan binding profiles of biotinylated F15-DD5-26 and F36-CB10-18 IgM at 1/10 and 1/20 dilutions against T cells (panel a) and B cells (panel b) in a FITC-avidin binding assay.

---- =untreated FITC-avidin alone, —— =MAb 1/10, — — =MAb 1/20.

B cell binding profiles (panel b) show higher mean channel fluorescence values of 295 (72% positive) and 199 (62% positive) for F15-DD5-26 at 1/10 and 1/20 dilutions respectively. F36-CB10-18 gave values of 518 (79% positive) and 339 (75% positive) at 1/10 and 1/20 dilutions respectively (background values of 61 with 12% positive have been deducted from this data).

The stronger binding of F36-CB10-18 compared to F15-DD5-26 may reflect more efficient biotin labelling of F36-CB10-18, or alternatively be due to its increased binding affinity. The results using directly biotinylated MAbs are in accordance with the cytotoxicity and cytofluorograph bindings assays which demonstrated low binding to T cells which was still further reduced at dilution, at antibody concentrations which give higher mean channel fluorescence with B cell targets. However a reduction in mean channel fluorescence using B cell targets was seen between 1/10 and 1/20 dilutions, indicating that the antibodies were not saturating.

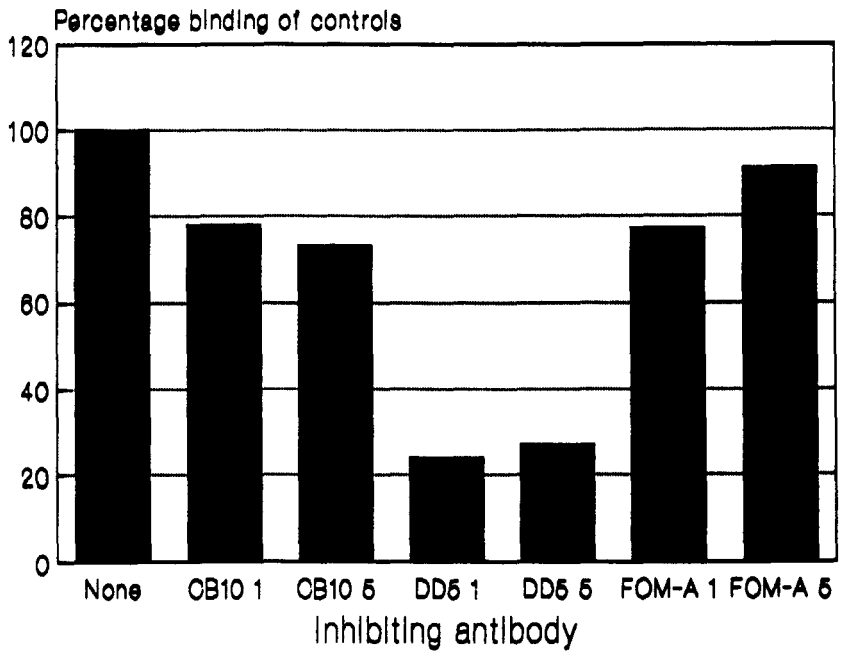
FACScan binding profiles for B lymphocytes using FITC-anti-human-IgM demonstrated two cell populations; surface IgM positive and surface IgM negative B cells (see Chapter 4). Binding of the autoreactive MAbs indicated that both cell populations were positive, with a shift of both peaks to the right. The FACScan profiles using directly biotinylated MAbs also correlate with these findings. Only one B cell peak was seen using FITC-avidin alone. This peak shifted to the right with the addition of the biotinylated MAbs. The positive B cell population was seen as a single peak, indicating that there is no discrete B cell subpopulation to which the autoantibodies are binding. However a 'shoulder' was seen with the F36-CB10-18 binding to B cells at 1/10 dilution, indicating some degree of

differential binding to B cell populations. This profile was not apparent for F15-DD5-26. The results are consistent with the view that autoantibodies bind both surface IgM positive and IgM negative B cells.

6.7 Competitive inhibition of lymphocytotoxic autoantibody binding:

Characterisation of the lymphocytotoxic autoantibodies thus far has indicated that they may be polyreactive, with broad specificities. The results have suggested that the autoreactive antibodies are capable of binding many negatively charged molecules on the cell surface. To investigate if the monoclonal lymphocytotoxic autoantibodies bind similar or distinct cell surface molecules, unlabelled IgM purified from F15-DD5-26 and F36-CB10-18, and FOM-A tissue culture supernatant were used to inhibit the binding of directly biotinylated MAbs. The experimental design is shown in Materials and Methods (Table 2.3.1).

The percentage binding of biotinylated MAb in the presence of each unlabelled inhibiting antibody were compared to the normal controls (PBS-DAB alone). Biotinylated F15-DD5-26 was strongly inhibited by unlabelled IgM purified from F15-DD5-26 (Fig 6.7.1 panel a). Equal inhibition was found using both 0·1mg/ml and 0·05mg/ml of biotinylated IgM with 24% and 27% binding of controls respectively. Inhibition of biotinylated F15-DD5-26 at 0·1mg/ml and 0·05mg/ml by F36-CB10-18 IgM gave 78% and 73% binding of controls respectively, indicating a degree of common specificity. However little inhibition was found with FOM-A tissue culture supernatant (approximately 0·05mg/ml) using biotinylated F15-DD5-26 at 0·1mg/ml or 0·05mg/ml (77% and 91% binding respectively).



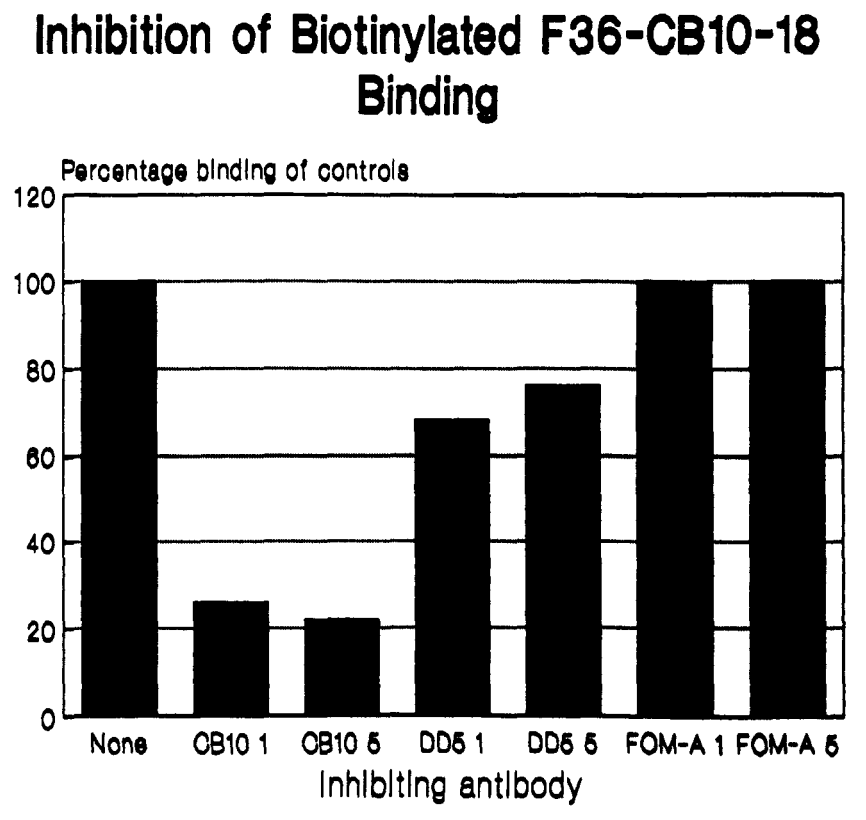


Figure 6.7.1 Competitive inhibition of biotinylated F15-DD5-26 IgM (panel a) and F36-CB10-18 IgM (panel b) at 0·1 and 0·05mg/ml, using unlabelled IgM purified from F15-DD5-26 and F36-CB10-18, and FOM-A tissue culture supernatant.

Similar results were found with inhibition of biotinylated F36-CB10-18 binding (Fig 6.7.1 panel b). Directly biotinylated F36-CB10-18 at 0·1mg/ml and 0·05 mg/ml was strongly inhibited by itself with 26% and 22% binding of controls respectively. A 68% and 76% binding inhibition was found using unlabelled F15-DD5-26 IgM and no inhibition was found using FOM-A tissue culture supernatant.

These results indicate that F15-DD5-26 and F36-CB10-18 give weak mutual inhibition, demonstrating that they share some degree of common binding specificity. However the strong 'auto-inhibition' by themselves (mean 75% inhibition) compared to the weaker 'cross-inhibition' (mean 26% inhibition) shows that they also bind distinct antigenic epitopes. F15-DD5-26 and F36-CB10-18 were selected as having different immunoglobulin light chain usage (lambda and kappa respectively) and therefore the differences in binding specificity are not surprising. However the cross inhibition of each other are consistent with them sharing some collective binding features. The lack of binding inhibition by FOM-A tissue culture supernatant may indicate that the non-specific binding is too distinct structures to those of F15-DD5-26 and F36-CB10-18. Alternatively, the lack of inhibition may be due to the lower binding affinity of FOM-A compared to the autoreactive MAbs. This latter view gains support from the small degree of inhibition of F15-DD5-26 which has low affinity compared to no inhibition of F36-CB10-18 which was selected for improved binding affinity. FOM-A tissue culture supernatant was used in this test as insufficient antibody was available for purification of IgM. However equivalent concentrations of IgM (approximately 0·05mg/ml) were used. It is therefore unlikely that the lack of inhibition by FOM-A was due to this factor.

6.8 Inhibition of biotinylated autoantibodies by patient serum:

The assumption that the monoclonal lymphocytotoxic autoantibodies F15-DD5-26 and F36-CB10-18 are the same as those found in the serum of renal dialysis patients has thus far been founded on correlations of the cytotoxicity profiles found using patients serum and the monoclonal antibodies. The monoclonal antibodies produced in this study have identical reactivity patterns to those in patients' sera.

The availability of biotinylated primary antibodies allows direct comparison of the specificity of autoantibodies in patients' sera and the autoreactive MAbs. Binding inhibition of biotin labelled monoclonal antibodies was tested with T and B cells using serum from; 1) normal individuals (blood group AB serum pooled from five untransfused males); 2) dialysis patient DV, with 100% T, B and CLL cell panel reactivity due solely to IgM lymphocytotoxic autoantibodies; and 3) dialysis patient EW, with 100% B cell panel reactivity due to IgM autoreactive antibodies.

6.8.1 Inhibition of autoantibody binding to T cells by patient sera:

The results show that T cell binding was strongly inhibited by normal AB serum (mean binding 44%) as well as that of serum DV (mean binding 52%) (Fig 6.8.1). Less binding inhibition was found with serum EW containing autoreactive B cell antibodies (mean binding 71%). These results indicate that lymphocytotoxic autoantibody binding to low affinity T cell targets could be blocked by normal serum as well as those with lymphocytotoxic autoantibodies.

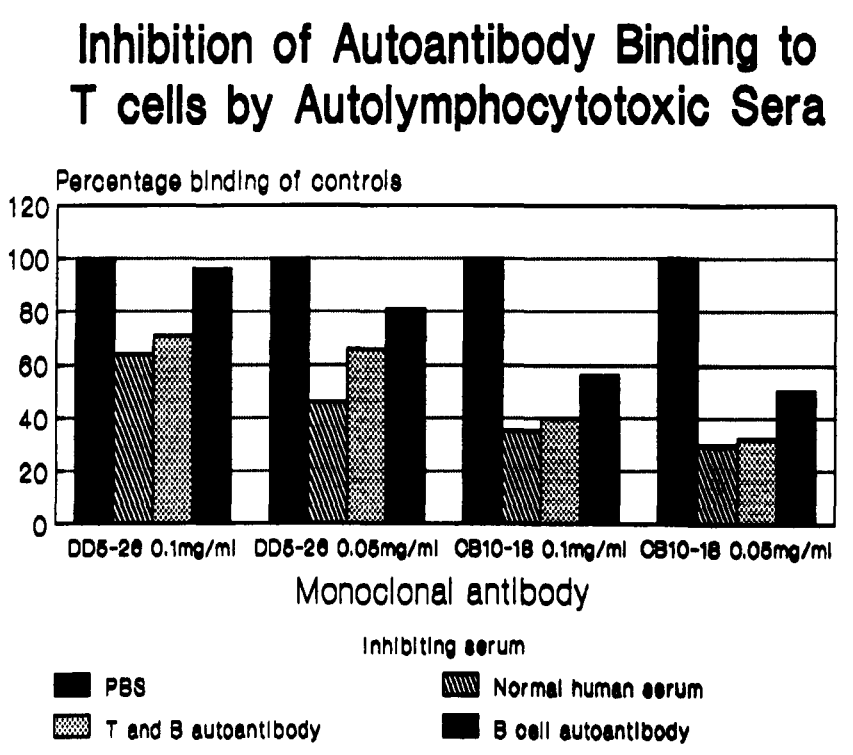
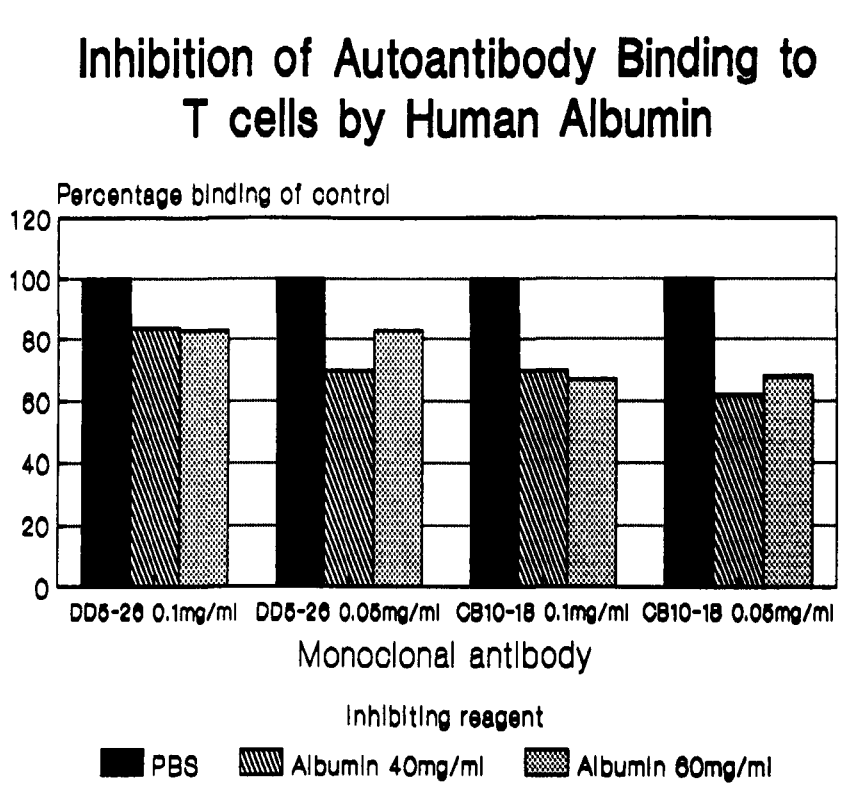


Figure 6.8.1 Percentage binding inhibition of biotinylated F15-DD5-26 and F36-CB10-18 IgM binding at 0·1 and 0·05mg/ml, to T cells by a) normal human serum, b) patient DV with T, B and CLL cell lymphocytotoxic autoantibodies and c) patient EW with B cell lymphocytotoxic autoantibodies only.

To investigate this further, the ability of purified serum proteins to inhibit autoreactive monoclonal antibody binding was tested. Human albumin at 40mg/ml (normal physiological concentration of serum albumin), and 60mg/ml (total serum protein level), and human IgM at 1·0mg/ml and IgG at 5·0mg/ml (within normal physiological range) were used to inhibit MAb binding (Fig 6.8.2).



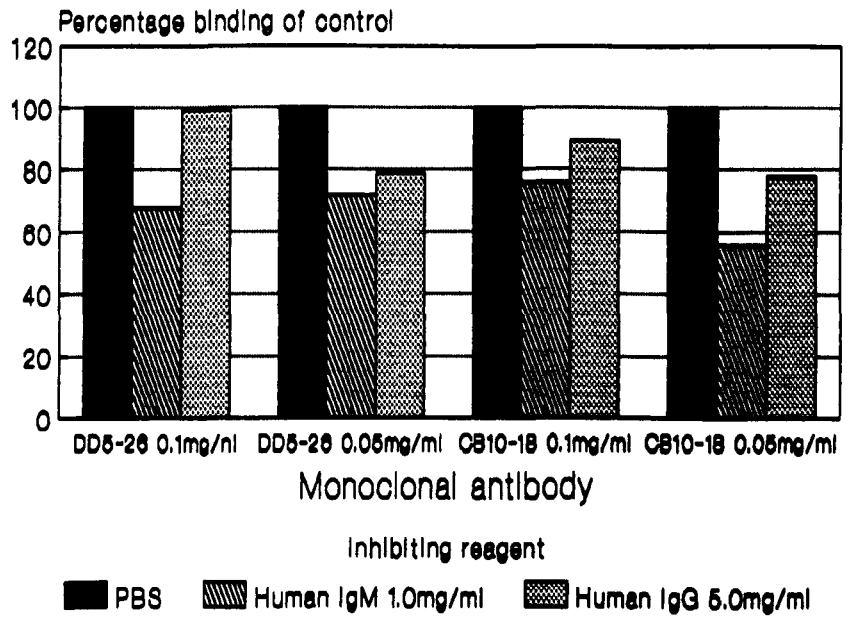


Figure 6.8.2 Competitive inhibition of biotinylated F15-DD5-26 IgM and F36-CB10-18 IgM at 0·1 and 0·05mg/ml against T cell targets, using human albumin at 40 and 60mg/ml (panel a) and human IgM (1·0mg/ml) and IgG (5·0mg/ml) (panel b).

T cell binding of F15-DD5-26 was inhibited by human serum albumin at both 40mg/ml and 60mg/ml with mean 77% and 83% binding respectively. This confirms that the low affinity T cell binding inhibition was non-specific. IgM and IgG also gave binding inhibition, with mean binding of 70% and 89% compared to the normal controls (PBS-DAB alone). F36-CB10-18 was similarly blocked by human albumin at 40mg/ml and 60mg/ml (mean 66% and 67% binding respectively) and by IgM and IgG (mean 66% and 83% binding respectively).

6.8.2 Inhibition of autoantibody binding to B cells by patients sera:

The same experiments were performed using high affinity B cell targets (Figure 6.8.3). In these tests only serum DV, with strong T and B cell lymphocytotoxic autoantibodies caused inhibition of MAb binding, with 50% and 62% binding for F15-DD5-26 and F36-CB10-18 respectively. The higher binding of F36-CB10-18 compared to F15-DD5-26 again correlates its higher binding affinity. No inhibition was found using normal AB serum (mean 102% binding), and weak inhibition was seen with serum EW (B cell autoantibodies), with 82% and 85% binding for F15-DD5-26 and F36-CB10-18 respectively.

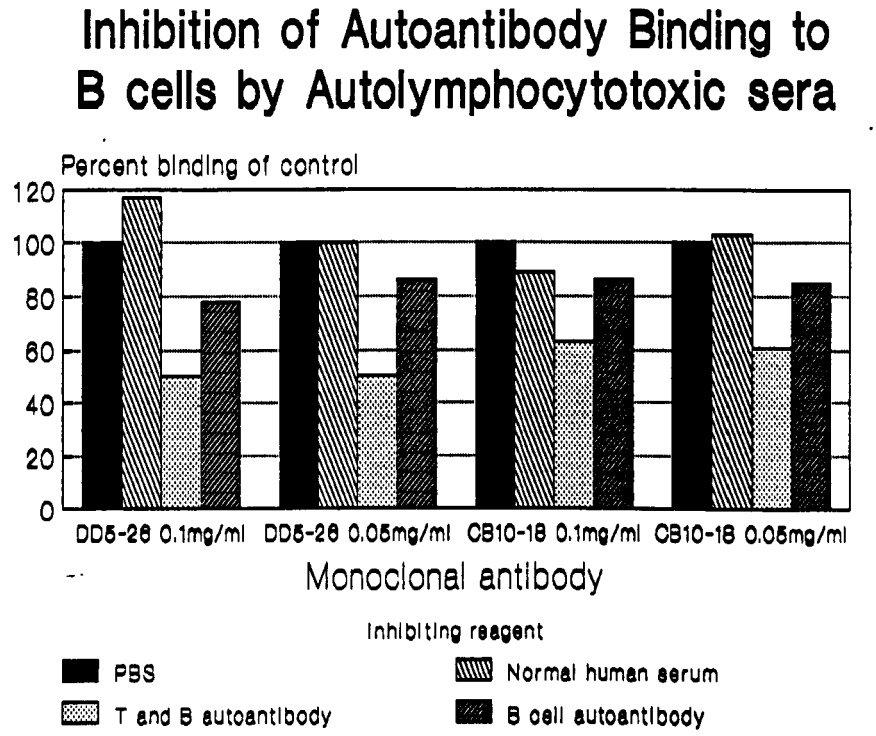
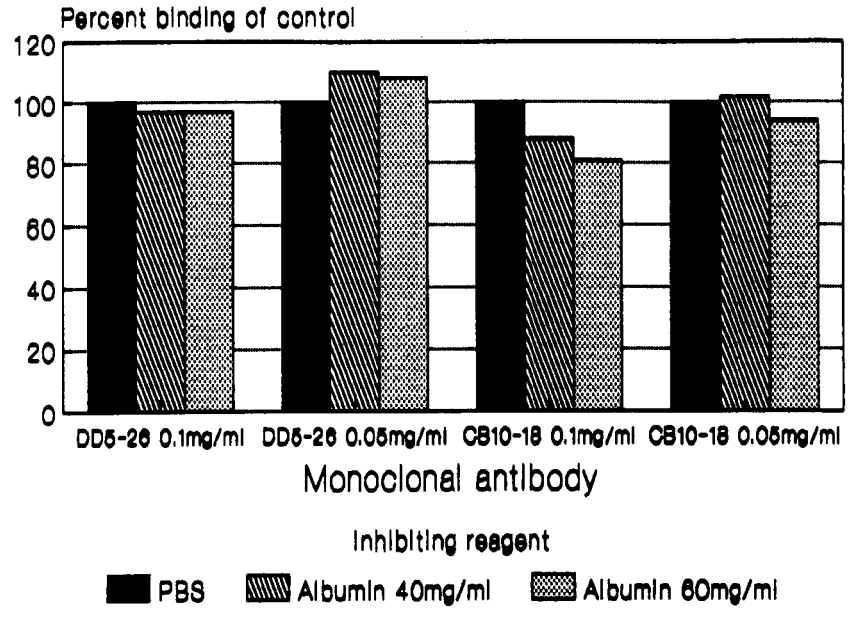


Figure 6.8.3 Percentage binding inhibition of biotinylated F15-DD5-26 and F36-CB10-18 IgM binding at 0·1 and 0·05mg/ml, to B cells by a) normal human serum, b) patient DV with 100% T, B and CLL cell lymphocytotoxic autoantibodies and c) patient EW with B cell lymphocytotoxic autoantibodies only.

No significant B cell binding inhibition was found with human albumin at 40mg/ml or 60mg/ml (mean 92% and 95% binding respectively) (Figure 6.8.4), nor with purified human IgM or IgG (mean 90% and 98% binding respectively). These results demonstrate that the monoclonal lymphocytotoxic autoantibody binding to B cells were only inhibited by alloantisera containing strong lymphocytotoxic autoantibodies.



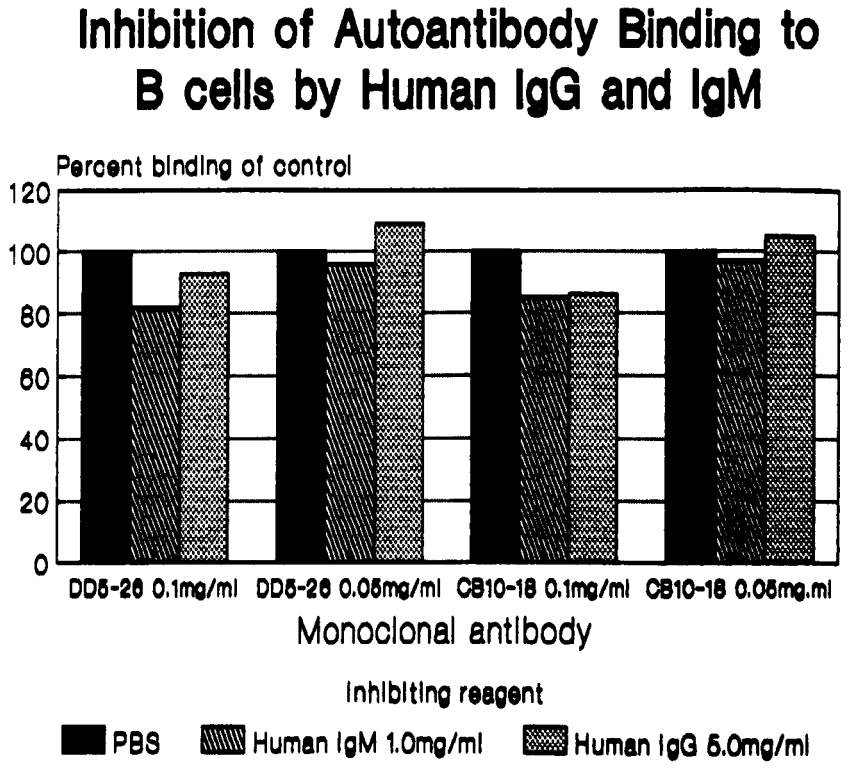


Figure 6.8.4 Competitive inhibition of biotinylated F15-DD5-26 IgM and F36-CB10-18 IgM at 0·1 and 0·05mg/ml, against B cell targets using human albumin at 40 and 60mg/ml (panel a) and human IgM (1·0mg/ml) and IgG (5·0mg/ml) (panel b).

6.9 Discussion

The results show that low affinity T cell binding can be non-specifically blocked using physiological concentrations of normal human serum and purified proteins (albumin, IgM and IgG). The apparent non-specific blocking by unlabelled human IgM using T cell targets may explain the discrepancy with Cicciarelli (Cicciarelli et al, 1980) who concluded that the autoantibodies are IgM-anti-IgM. However high affinity B cell binding is not inhibited by normal human serum, nor by purified human albumin or IgM and IgG at physiological concentrations. B cell binding was only inhibited by serum DV. This patient was selected to have strong (high titre) IgM lymphocytotoxic autoantibodies reactive with all T, B and CLL cell targets. The cross inhibition by autoantibodies from this patients serum indicates that they share a common specificity.

There was only weak binding inhibition with serum EW, containing only B cell lymphocytotoxic autoantibodies. The cytotoxicity of this serum with B cells alone is probably due to low antibody titre and affinity, being reactive with only high affinity B cell targets, but not low affinity T and CLL cell targets. Therefore EW was less able to block the stronger binding of the MAbs.

The data therefore supports the view that the human monoclonal autoantibodies produced in this study are the same as the autoantibodies found in renal dialysis patients' serum.

6.10 Immunoprecipitation

Attempts to immunoprecipitate the autoantigenic target molecule from the cell surface of the cell line K562 were unsuccessful (data not shown). Immunoprecipitation using IgM is known to be difficult. Therefore to improve the chances of success, affinity chromatography using the avidin-biotin technique was used (Wilchek and Bayer, 1988). High affinity interactions between these molecules (10^{15} M^{-1}) and the ability to introduce 'spacers' onto the biotin molecule to avoid steric hinderance, enables increased efficiency for antigen purification compared to conventional MAb-agarose affinity columns (Updyke, Nicolsen and A, 1984). Gretch (Gretch, Suter and Stinski, 1987) estimated that the use of the avidin/biotin based immunoaffinity system was 20-50 times more efficient at isolating antigen than the use of antibody linked to cyanogen-bromide activated sepharose.

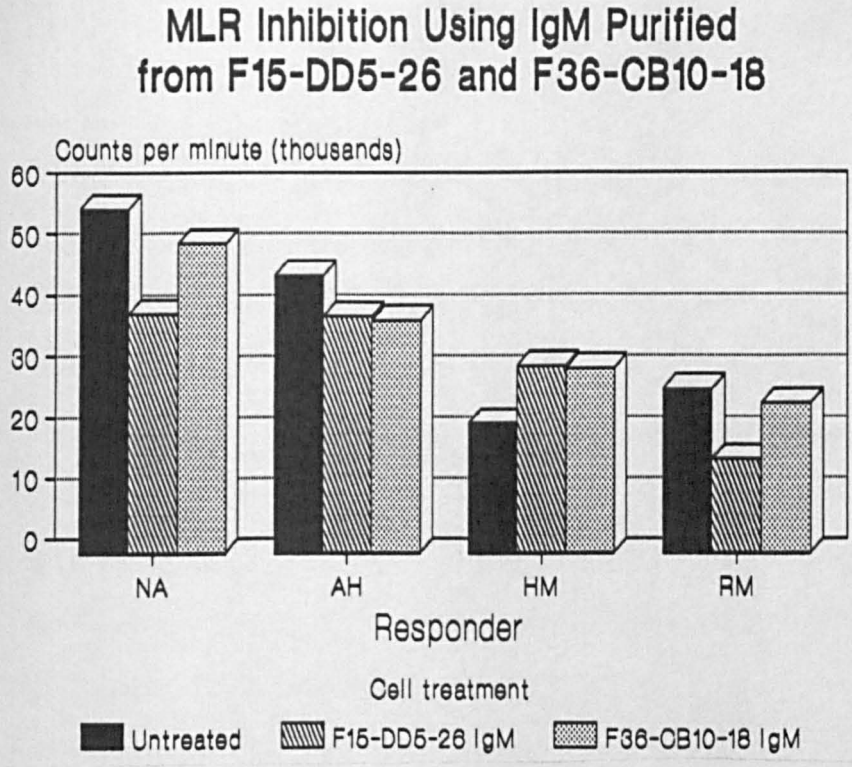
Autoradiography revealed no immunoprecipitation bands from detergent solubilised K562 cell membrane antigens. However, binding of biotin labelled IgM to the avidin-agarose matrix was confirmed as immunoglobulin light and heavy chains were eluted and identified following protein staining of the gel. This indicates that the method worked appropriately. One possible reason for the absence of an immunoprecipitation product may have been the susceptibility of the glycoprotein to acid hydrolysis following elution at low pH.

6.11 Inhibition of the mixed lymphocyte reaction (MLR)

Reports have postulated that lymphocytotoxic autoantibodies have an immunoregulatory role associated with enhanced graft survival (see Chapter 1). Some investigators have found that serum from patients with autoantibodies block MLR reactivity (Esquenazi et al, 1983; Spitalnik et al, 1984). To investigate if the monoclonal lymphocytotoxic autoantibodies cause inhibition of MLR responses, proliferation of alloreactive T cells were assessed with and without the addition of the MAbs.

MLR inhibition studies were initially performed using F15-DD5-26 and F36-CB10-18 tissue culture supernatant. These gave greatly enhanced proliferative responses in both the alloreactive and autologous combinations (data not shown). The increased proliferation was likely to be due to the presence of non-specific growth factors in the conditioned media.

The assays were repeated using IgM purified from tissue culture supernatant. Initially, 10μl of IgM at 0·5mg/ml in PBS-DAB was added to an MLR with four responder and six stimulator cell combinations. Untreated controls received 10μl of PBS-DAB alone. Median counts per minute of quadruplicate tests were calculated for each responder and stimulater combination following background (autologous control) subtraction. The levels of stimulation of MAb treated combinations were compared to the untreated controls. The results showed no consistent trend of inhibition or stimulation and therefore the data is summarised as the mean response of each cell against the six stimulator cells (Fig 6.11.1). There was no overall inhibitory or stimulatory effect of MAb treatment when compared to the normal untreated controls.



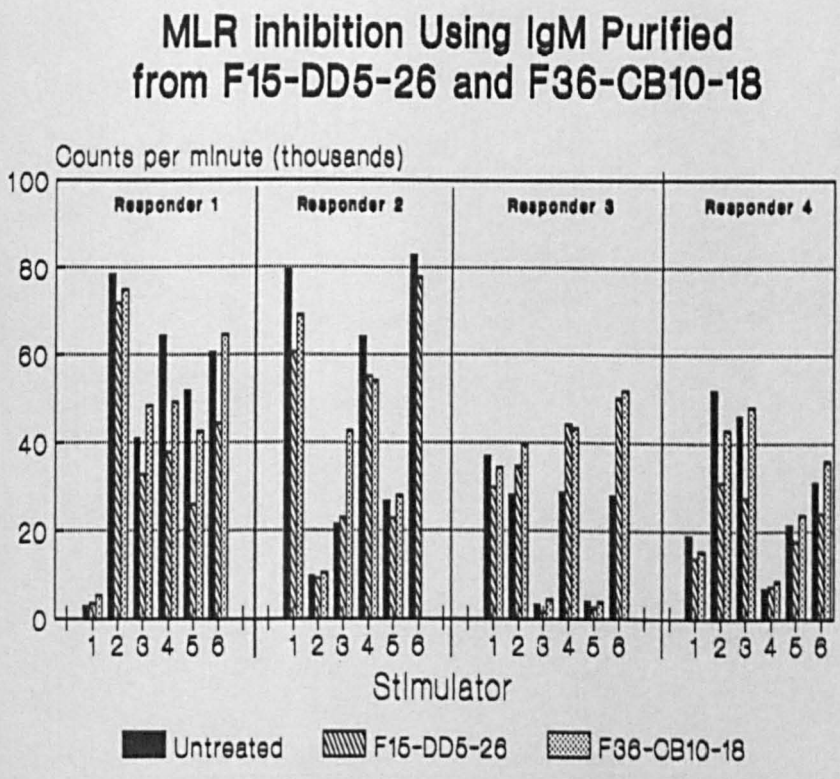
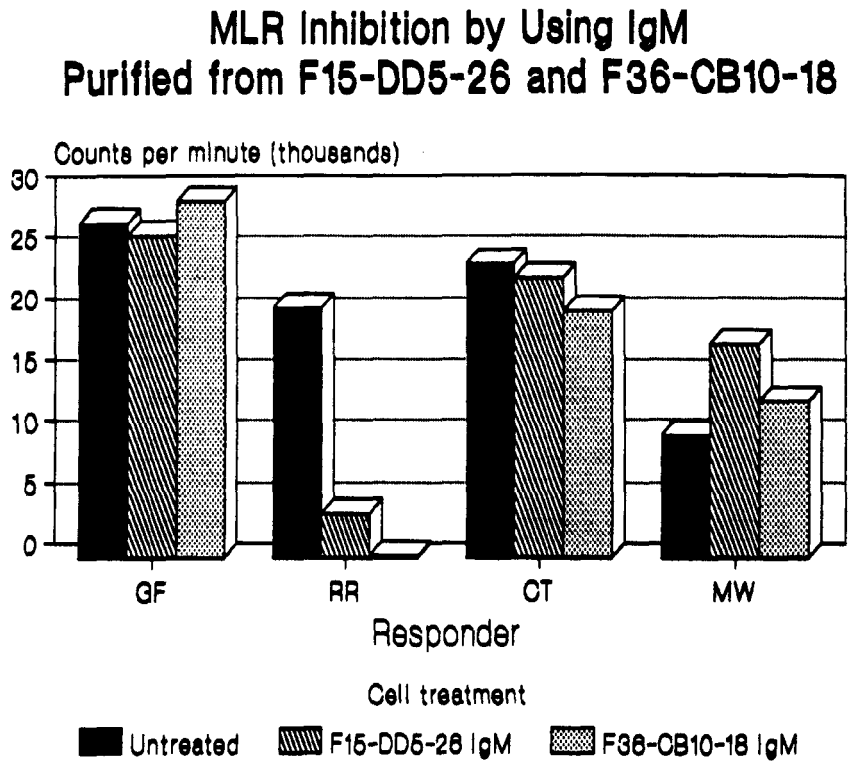


Figure 6.11.1 The effect of IgM purified from F15-DD5-26 and F36-CB10-18 at a final concentration of 0·05mg/ml on MLR responses of four normal individuals. Panel 'a' shows the mean counts for the four responders and panel 'b' shows the individual counts of each responder/stimulator combination.

Further combinations were tested, as above, using increased IgM concentrations (10μl at 1·0mg/ml) in an MLR with four responder and four stimulator cell combinations. Cells were derived from three normal individuals (GF, CT, MW) and one patient with X-linked hypogammaglobulin anaemia (RR) (Fig 6.11.2). Comparisons of responses of the three normal individuals (panel a) showed no effect of MAb treatment compared to the untreated controls. However both the response and stimulation values of the patient R.R. were markedly inhibited following MAb treatment. There was an 82% and 99% suppression of response (panel a) and 83% and 98% suppression of stimulation values (panel b) for F15-DD5-26 and F36-CB10-18 respectively. Although this patient had normal MLR responses in the untreated controls, he was found to have a deficient T cell response to PHA which was only 11% relative to the healthy controls (GF,CT,MW) (data not shown).



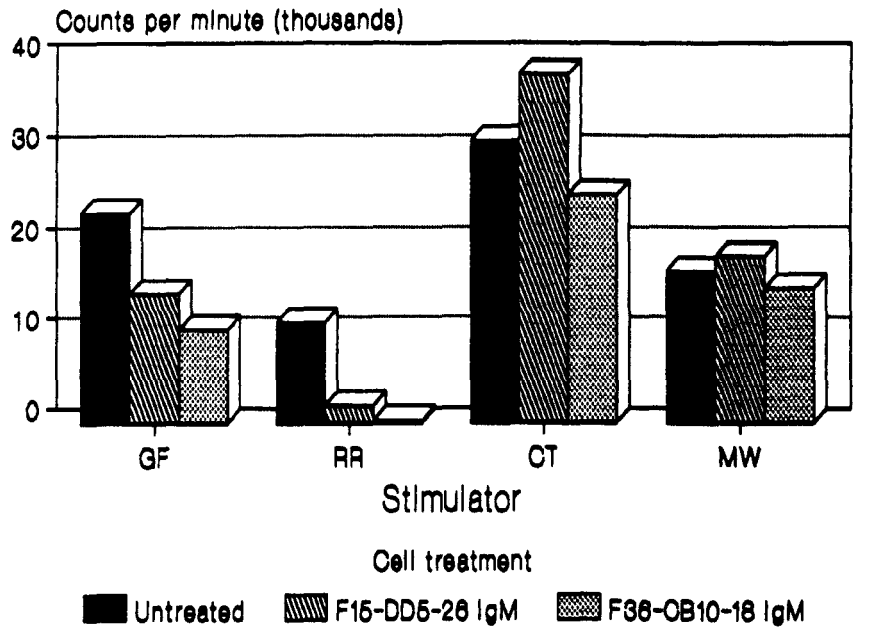
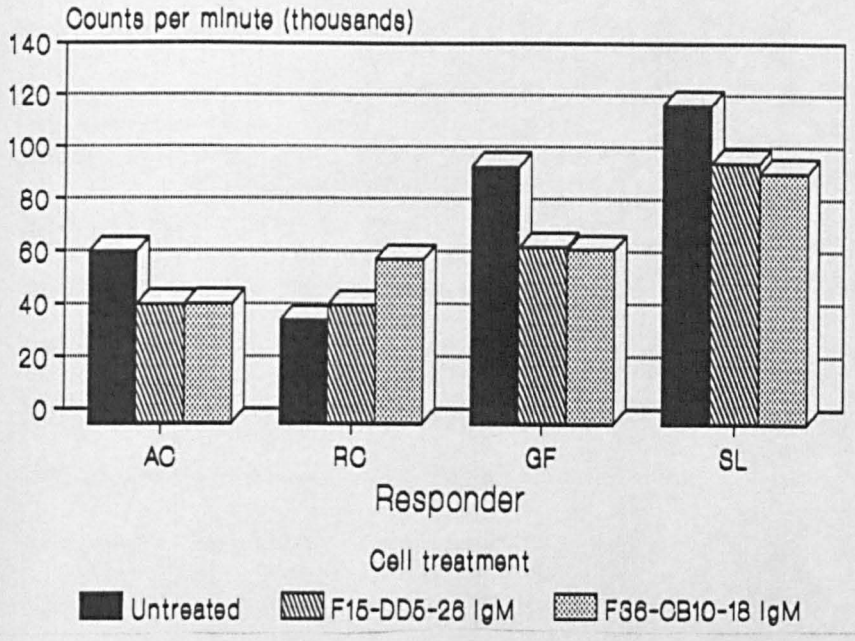


Figure 6.11.2 The effect of IgM purified from F15-DD5-26 and F36-CB10-18 at a final concentration of 0·1mg/ml on the MLR responses (panel a) and stimulation (panel b) of three normal individuals (GF, CT and MW) and one patient with X-linked hypogammaglobulin anaemia (RR).

A third MLR was carried out to investigate if the monoclonal autoantibodies caused inhibition of weak MLR combinations, but not strong alloreactive responses. An MLR with four responder and four stimulator cell combinations was carried out with the inclusion of a one haplotype matched sibling pair (AC and RC) (Fig 6.11.3). The double normalised relative response of this pair was 49% compared to a mean of 94% for the mismatched controls (data not shown). The addition of the MAbs gave only a small suppressive effect with both the weaker sibling pair combination and the strong mismatched unrelated controls.



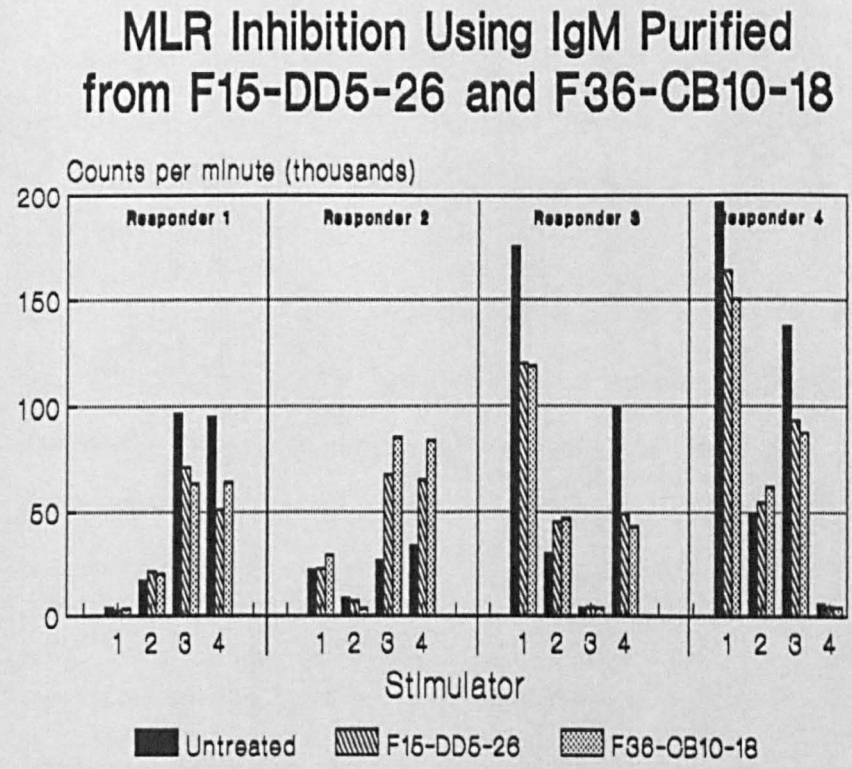


Figure 6.11.3 The effect of autoreactive (F15-DD5-26 and F36-CB10-18) human IgM MAbs on MLR responses of two unrelated individuals (GF, SL) and a one haplotype matched sibling pair (AC, RC). Panel 'a' shows the mean counts of the four responders and panel 'b' shows the individual counts of each responder/stimulator combination.

6.12 Discussion

Several reports have shown MLR inhibition caused by lymphocytotoxic antibodies in serum from renal dialysis patients (Sengar, Rashid and Harris, 1975; Miller et al, 1975). However, these studies are subject to question as the results may be explained by factors other than the presence of autoantibodies. For example HLA class II antibodies (Ettenger et al, 1978) and antiidiotypic antibodies induced following blood transfusion (Nagarkatti, Joseph and Singal, 1983; Haisa et al, 1989) inhibit MLR responses. Matsumoto demonstrated inhibition of MLRs and mitogen stimulated lymphocytes associated with sera containing cold B-cell cytotoxins (Matsumoto et al, 1983). Inhibition was strongest when the sera were lymphocytotoxic against both the responder and stimulator cells. They also demonstrated that inhibitory reactivity could be absorbed with spleen cells and was present in the IgM but not IgG and IgA fractions.

Primary alloreactive MLR responses are mediated through CD4 positive T cell recognition of HLA-DR, and -DQ (and -DP?) molecules on the stimulator cell surface. Many patients with autoantibodies also have additional HLA antibodies, which may be the true source of MLR inhibition. Furthermore, severe uraemia causes an immunosuppressive effect. This may act as an inherent source of error in the studies performed using serum and cells from renal dialysis patients. Post transplant serum may contain immunosuppressive drugs such as cyclosporine which would also non-specifically cause MLR suppression.

This study utilised purified IgM from lymphocytotoxic monoclonal autoantibodies. This has the advantage of being able to measure the effect of the autoantibodies without being influenced by other extraneous factors.

The results showed no consistent effect of the lymphocytotoxic autoantibodies on MLR reactivity of normal cells. This was true for both weak and strong alloreactive combinations. We therefore conclude that IgM lymphocytotoxic autoantibodies do not cause MLR suppression and it is unlikely that they perform an associated immunoregulatory role in-vivo.

Chapter 7

Determination of the immunoglobulin class and specificity of antibodies causing a positive crossmatch

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7.5 Case Presentation: A positive B cell crossmatch due to IgG anti-HLA-DQ antibody present at the time of transplantation in a successful renal allograft

7.5.1 Patient details

7.5.2 Serology

7.5.3 Immunohistology

7.5.4 Discussion

7.1 Introduction

It is well established that a positive crossmatch caused by lymphocytotoxic autoantibodies are not associated with early graft failure. However the clinical relevance of a positive crossmatch in the absence of autoantibodies is less clear. In highly sensitized patients, who may have multiple antibody populations, the precise specificity of the positive crossmatch against a particular donor is difficult to determine. It is possible that reports of varied transplant outcome when performed with a positive crossmatch may be due to different antibody specificities causing the positive crossmatch.

The aim of this chapter was to develop an in-vitro assay which could reliably define the specificity of donor reactive antibodies in highly sensitized patients with multiple antibody populations and in combination with the use of dithiothreitol (to reduce IgM), to define the antibody class and specificity in a series of positive crossmatch transplants.

7.1.1 Background studies

Previous studies had established that pre-incubation of T lymphocytes with allele specific non-cytotoxic mouse monoclonal antibodies directed against HLA class I antigens could specifically inhibit anti-HLA alloantibodies of the same specificity (Ellis, Taylor and McMichael, 1982). It was also established that broadly reactive MAbs (locus specific) could specifically inhibit alloantibodies without interfering with antibodies directed at unrelated determinants (Ellis et al, 1985).

Studies using HLA-Class I specific monoclonal antibodies directed against monomorphic determinants demonstrated locus specific cytotoxicity

inhibition of alloantisera. This method has been utilised to determine the antibody specificity of a series of transplants performed with a positive T and B cell crossmatch (Chapman et al, 1986a).

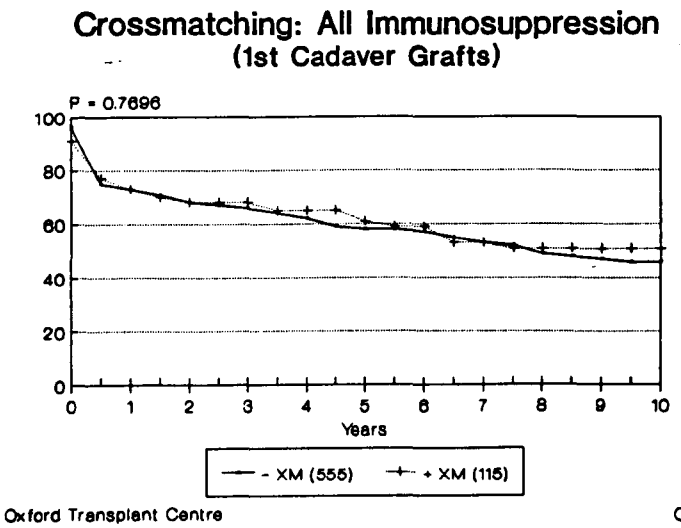
In this study, the technique of cytotoxicity inhibition of the T cell crossmatch using monomorphic anti-HLA class I monoclonal antibodies has been extended to define the specificity of the B cell crossmatch. This involved the identification of suitable non-cytotoxic monomorphic anti-HLA class I, DR and DQ locus specific MAbs and the development of a cytotoxicity inhibition assay which could define the specificity of the B cell crossmatch. Preliminary studies identified three suitable MAbs which under optimal conditions gave locus specific inhibition of a number of well characterised anti-HLA alloantisera. It was also established that the technique could distinguish multiple antibody populations present in many dialysis patients.

The technique of cytotoxicity inhibition, in combination with the use of dithiothreitol reduction was then applied to define the immunoglobulin class and specificity of donor reactive antibodies in 152 renal transplants performed with a positive crossmatch. A correlation was then sought with graft outcome to investigate the clinical relevance of the different antibody types.

7.1.2 Positive Crossmatch Transplants

Between 1978 and 1990, 232 renal transplants were performed with a positive crossmatch. This represents about 25% of all transplants carried out in Oxford (Figure 7.1.1). Actuarial one year graft survival of first grafts (panel a) was 73% (N=115) which was identical to transplants performed with

a negative crossmatch (N=555). Of 176 regrafts, approximately 2/3 (N=118) have been performed with a positive crossmatch (panel b). One year actuarial regraft survival was 65% for those with a positive crossmatch compared to 81% with a negative crossmatch. This demonstrated a higher initial loss although there was no difference in regraft survival at ten years.



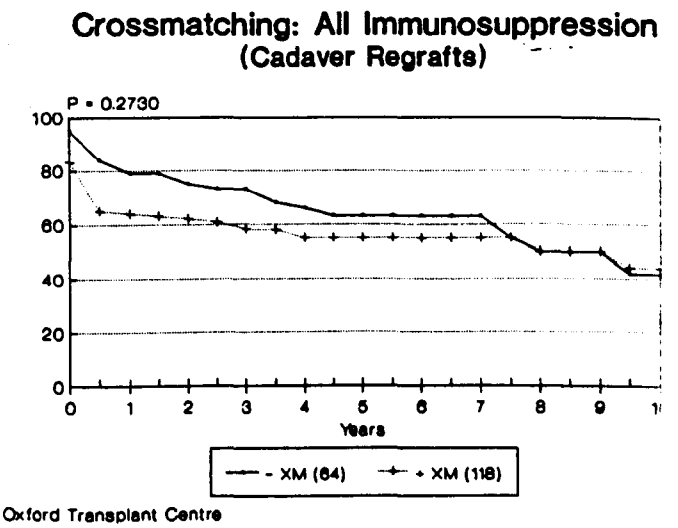


Fig 7.1.1 Actuarial graft survival of all renal transplants performed with a positive T and/or B cell crossmatch compared to those with a negative crossmatch in primary grafts (panel a) and regrafts (panel b).

Donor T and/or B cells and the appropriate recipient sera were available in 152 cases to determine the immunoglobulin class and specificity of the antibody causing the positive crossmatch. For the early transplants this was performed retrospectively using cryopreserved donor lymphocytes and stored recipient sera. More recently the study has been carried out prospectively using fresh donor cells.

7.1.3 Selection of serum samples for crossmatching

Serum samples were collected from patients at monthly intervals after acceptance on to the transplant waiting list, with additional samples taken 14 days following any blood transfusions. After transplantation, serum samples were collected daily for the first week and then at weekly intervals for three months, and monthly thereafter. The samples were stored at -30°C until testing using a selected panel of 40 peripheral blood lymphocytes and 30 B cells from patients with chronic lymphatic leukaemia (CLL), covering all the accepted HLA-ABC, DR and DQ alleles. Sera with the highest panel reactivity, representing different periods of sensitisation ("peak") and the most recent sample available ("current") were selected for crossmatching.

7.1.4 Immunosuppression

During the period of this study a number of different post transplant immunosuppression protocols were used. Between 1978 and 1985 patients received azathioprine in combination with low dose steroids (23 patients) or high dose steroids (one patient) (Morris et al, 1982). Between 1982 and 1985, 34 patients received cyclosporin, either alone or in combination with prednisolone (Morris et al, 1987), and from 1985 94 patients received triple therapy (Jones et al, 1988). Highly sensitised patients were given

prophylactic intravenous methylprednisolone at 500mg daily for the first three days. Subsequent rejection episodes were treated with a three day course of methylprednisolone at 500mg daily. More recently anti-thymocyte globulin (ATG) has been used and was administered in 22 patients and OKT3 in 3 patients. All patients in this study with a positive T cell crossmatch caused by HLA antibodies received cyclosporin with one exception who received azathioprine and low dose steroids.

7.2 Results of control studies

7.2.1 HLA Class I

Twenty HLA-class I typing reagents were tested against 60 B cell donors (Table 7.2.1). Fifty-nine of the 60 tests were blocked by one or more doubling dilution with PA2.6 alone, with 12 tests completely blocked. The average reduction in titre was 2.2 doubling dilutions. Blocking was further improved by the addition of the $F(ab')_2$ second layer antibody, with all 60 tests blocked by one or more doubling dilution, and 26 completely blocked. The average reduction in titre was 3.2 doubling dilutions.

7.2.2 HLA-DR

A total of 23 HLA-DR typing reagents were tested against 66 B cells (Table 7.2.1). Of the 66 tests, 41 were blocked by NDS-22 alone and 9 were completely blocked. The average reduction in titre was one doubling dilution. With the addition of the $F(ab')_2$ second layer antibody 64 B cells were tested. Blocking was improved, with all 64 tests blocked by one or more doubling dilutions and 33 completely blocked. The average reduction in titre was 3 doubling dilutions.

7.2.3 HLA-DQ

Fifteen HLA-DQ typing reagents were tested against 65 B cells (Table 7.2.1). Of 53 B cells tested using alloantisera specific for HLA-DQw1 (including the splits DQw5 and 6) and DQw3 (including the splits DQw7, w8 and w9), 50 were blocked by LEU-10 alone, and eleven tests were completely blocked. The mean reduction in titre was 2 doubling dilutions. With the addition of the $F(ab')_2$ second layer antibody 59 B cells were tested. Blocking was only marginally improved; 55 tests were blocked by at least one doubling dilution, 21 completely blocked and an average reduction in titre of 2.1 doubling dilutions. None of the 6 tests, using two sera with specificity for HLA-DQw2 were blocked by LEU-10 alone, nor with the addition of the $F(ab')_2$ second layer antibody (Table 7.2.1).

No. sera	MAb	Specificity	No. cell	MAb Alone Blocked[a]	MAb Alone Ave[c]	No. cell	MAb + $F(ab')_2$ Blocked	MAb + $F(ab')_2$ Complete[b]	MAb + $F(ab')_2$ Ave
20	PA2.6	HLA-ABC	60	59 (98)[d]	2.2	60	60 (100)	26 (43)	3.2
23	NDS-22	HLA-DR	66	41 (62)	1.0	64	64 (100)	33 (52)	3.0
13	LEU-10	HLA-DQw1,3	53	50 (94)	2.0	59	55 (93)	21 (38)	2.1
2	LEU-10	HLA-DQw2	6	0	-	6	0	-	-

[a] *The number of tests with a reduction in titre of at least 1 doubling dilution.*

[b] *The number of tests giving total inhibition (negative in neat serum).*

[c] *The average reduction in titre (number of doubling dilutions).*

[d] *Numbers in parentheses represent the percentage of tests blocked.*

Table 7.2.1 Cytotoxicity inhibition controls.

7.3 Positive crossmatch transplants

The positive crossmatch transplants have been analyzed on the basis of reactivity with donor T and B cells or B cells only. These groups were further subdivided according to the immunoglobulin class and specificity of the crossmatch and correlated with graft survival at six months and one year after transplantation.

T and B cell positive crossmatch transplants

A total of 87 transplants performed with a positive T and B cell crossmatch were analyzed for the immunoglobulin class and specificity of the antibody against donor cells. Overall graft survival at one year was 69%.

7.3.1 IgM non-HLA antibodies

a) Live related donors; Nine transplants were performed using related donors (Table 7.3.1). Of these, 4 were HLA identical and 5 were single haplotype matched. All the crossmatches were DTT reduced indicating an IgM antibody and all had a positive autologous crossmatch. Blocking studies using PA2.6 (anti- HLA class I) were performed in four cases which showed no cytotoxicity inhibition confirming the non-HLA nature of these antibodies. Of the nine transplants, eight (89%) were successful. The single graft loss (patient JM) was a non rejection failure following severe hypotension and subsequent renal vein thrombosis. In seven cases the crossmatch was positive at the time of transplantation (current) and all seven were successful.

Initials	TpN	TxDate	ImSup	TxN	Hap	PRA	Curr	DTT	PA2.6	Outcome
ST	290	20. 7.81	Low	1	2	99	+	Yes	No	Success
MS	389	26. 9.83	Low	1	2	100	+	Yes	No	Success
SW	436	25. 6.84	Low	1	2	100	+	Yes	NT	Success
AT	516	8. 7.85	Low	1	1	100	+	Yes	No	Success
DD	637	16. 2.87	Trip	1	1	75	167	Yes	NT	Success
RR	663	13. 7.87	Trip	1	2	85	+	Yes	NT	Success
AA	707	7.12.87	Trip	1	1		+	Yes	NT	Success
BP	762	26. 9.88	Trip*	1	1	70	+	Yes	No	Success
JM	882	8. 1.90	Trip*	3	1	100	4738	Yes	NT	NRF 5

Table 7.3.1 T and B cell positive crossmatch transplants caused by IgM non-HLA antibodies performed using live related donors.

Legend for Table 7.3.1 and subsequent tables.

***TpN** =transplant number. **TxDate** =transplant date.*

***ImSup** =immunosuppression protocol; **Low** =azathioprine with low dose steroids;*

CyA =Cyclosporin A alone; CYA+S =Cyclosporin plus low dose steroids;

*Trip =triple therapy; * =ATG; ^ =OKT3.*

***Hap** =number of haplotypes matched.*

***PRA** =Percentage panel reactive antibodies determined using a panel of 40 peripheral blood lymphocytes or purified T cells.*

***Curr** =number of days from the positive crossmatch to the time of transplantation; + =current positive with the most recent serum sample.*

***DTT** =dithiothreitol reduction; **Yes** =DTT reduced (IgM); **No** =not DTT reduced (IgG).*

***PA2.6** =Cytotoxicity inhibition by MAb PA2.6 (monomorphic HLA-class I); **Yes** =blocked; **No** =not blocked; **NT** =not tested.*

***Outcome** =graft outcome at the present time; **successful** =patient free from dialysis; **Rej** =graft failure due to rejection or **NRF** =non-rejection failure (return to dialysis or patient death) with the number of days to failure.*

b) Cadaver donor transplants, first grafts

Positive T and B cell crossmatches caused by IgM non-HLA antibodies were found with 26 first cadaver transplants (Table 7.3.2) and 22 (85%) were successful at six months. Of these, 13 were positive at the time of the transplant and 10 (77%) were successful at six months.

c) Cadaver donor transplants, regrafts

Of 22 regrafts performed with a T and B cell positive crossmatch, 20 (91%) were successful at six months (Table 7.3.3). Of these, four were positive at the time of transplantation and all were successful.

Initials	TpN	TxDate	ImSup	TxN	PRA	Curr	DTT	PA2.6	Outcome
AW	211	11.11.79	Low	1	100	86	Yes	No#	Success
AB	292	30. 7.81	Low	1	100	+	Yes	No	NRF 2254
PL	318	6. 4.82	CyA	1	100	+	Yes	No	Success
EW	348	22. 9.82	CyA	1	100	+	Yes	No	NRF 211
GP	368	17. 3.83	Low	1	100	+	Yes	No	REJ 2335
YD	413	8. 2.84	Low	1	95	1068	Yes	No	Success
RR	415	18. 2.84	Low	1	100	927	Yes	No	NRF 0
GF	457	23. 9.84	CyA	1	63	2298	Yes	No	Success
MC	467	24.10.84	CyA	1	63	257	Yes	No	REJ 356
DH	474	19.11.84	Low	1	100	+	Yes	No	NRF 71
WS	545	5.11.85	Trip*	1	100	456	Yes	No	REJ 302
DH	585	10. 6.86	Trip	1	88	799	Yes	No	Success
LC	607	17. 9.86	Trip	1	90	+	Yes	No	Success
RV	627	22. 1.87	Trip	1	100	1339	Yes	No#	Success
AT	691	18.10.87	Trip	1	43	996	Yes	No	Success
RE	693	21.10.87	Trip	1	100	2282	Yes	No	Success
RA	737	19. 5.88	Trip*	1	71	+	Yes	No	Success
FK	742	18. 6.88	Trip	1	90	123	Yes	No#	Success
JP	749	6. 7.88	Trip	1	100	736	Yes	No	Success
NK	766	26.10.88	Trip	1	98	+	Yes	No	Success
MH	779	5.12.88	Trip*	1	100	+	Yes	No	Success
SS	796	13. 2.89	Trip*	1	100	+	Yes	No	NRF 0
SO	799	14. 3.89	Trip	1	100	5136	Yes	No	Success
RJ	808	18. 4.89	Trip	1	100	+	Yes	No	NRF 0
SI	826	9. 6.89	Trip	1	87	+	Yes	No	Success
ED	913	14. 5.90	Trip	1	100	+	Yes	No	Success

Table 7.3.2 T and B cell positive crossmatch transplants caused by IgM non-HLA antibodies performed in first cadaver grafts.

 # =Also had a positive B cell crossmatch caused by an IgM non-HLA antibody present at the time of transplantation (not included in the B cell crossmatch analysis).

Initials	TpN	TxDate	ImSup	TxN	PRA	Cur	DTT	PA2.6	Outcome
DP	177	2. 1.79	Low	2	100	102	Yes	No	Rej 97
JS	241	3. 6.80	Low	2	100	1125	Yes	No	Success
LW	375	4. 5.83	CyA	3	100	1420	Yes	No	Rej 691
VH	444	11. 7.84	CyA+S	2	100	726	Yes	No	Success
SM	511	18. 6.85	CyA+S	2	100	2722	Yes	No	Success
EW	521	17. 7.85	Trip	2	100	1078	Yes	No	NRF 1379
KC	534	20. 9.85	Trip	2	100	1719	Yes	No	Success
JM	541	12.10.85	Trip	2	100	3230	Yes	No	Rej 0
SR	561	14. 1.86	Trip	2	100	259	Yes	No	Success
LW	568	25. 2.86	Trip	4	100	536	Yes	No	Success
MK	589	26. 6.86	Trip	2	96	1296	Yes	No	Success
SG	618	20.11.86	Trip	2	23	+	Yes	No	Success
RR	623	26.12.86	Trip	2	100	+	Yes	No	NRF 1048
SB	647	29. 3.87	Trip	3	88	3355	Yes	No	Success
ML	677	14. 8.87	Trip	2	100	2608	Yes	No	Success
ST	679	28. 8.87	Trip	2	83	+	Yes	No	Success
PM	684	10. 9.87	Trip	2	100	203	Yes	No	Success
JR	746	25. 6.88	Trip	2	100	395	Yes	No#	Success
EG	798	10. 3.89	Trip	2	100	1414	Yes	No	Success
PR	851	21. 9.89	Trip*	2	65	528	Yes	No	Success
VA	874	5.12.89	Trip*	3	87	1804	Yes	No	Success
RR	938	16. 8.90	Trip	3	100	+	Yes	No	Success

Table 7.3.3 T and B cell positive crossmatch transplants caused by IgM non-HLA antibodies performed in cadaver regrafts.

7.3.2 T and B cell positive crossmatch transplants caused by IgM HLA class I antibodies

Donor T and B cell reactive IgM HLA class I antibodies (DTT reduced and blocked by PA2.6) were found in 17 patients (Table 7.3.4). Six were first grafts and 5 (83%) were successful and 11 were regrafts and 7 (64%) were successful.

Initials	TpN	TxDate	ImSup	TxN	PRA	Cur	DTT	PA2.6	Outcome
CO	558	10. 1.86	Trip	1	100	1025	Yes	Yes-	Success
JB	674	29. 7.87	Trip	1	100	1239	Yes	Yes&	Success
JW	695	3.11.87	Trip	1	63	571	Yes	Yes	Success
TG	754	17. 8,88	Trip*	1	13	42	Yes	Yes	Success
JW	810	20. 4.89	Trip	1	65	345	Yes	Yes	Success
RH	820	26. 5.89	Trip	1	93	352	Yes	Yes	Rej 0
JB	434	7. 6.84	Low	2	100	912	Yes	Yes	Rej 8
WB	438	28. 6.84	CyA+S	2	100	1828	Yes	Yes	Success
CK	439	28. 6.84	CyA+S	2	100	1190	Yes	Yes	Success
CB	445	20. 7.84	CyA+S	3	100	1060	Yes	Yes	Rej 45
ED	449	20. 8.84	CyA+S	3	100	1000	Yes	Yes	Success
MM	506	24. 5.85	CyA+S	2	63	1228	Yes	Yes	Success
TL	523	19. 7.85	Trip	2	100	2025	Yes	Yes	Rej 83
DB	539	6.10.85	Trip	2	96	1921	Yes	Yes	Rej 4
TM	587	24. 6.86	Trip*	2	100	3905	Yes	Yes	Success
TB	642	8. 3.87	Trip*	2	100	573	Yes	Yes	Success
KW	653	6. 6.87	Trip	2	100	1591	Yes	Yes$	Success

Table 7.3.4 T and B cell positive crossmatch transplants caused by IgM anti-HLA class I antibodies.

- Also had a positive B cell crossmatch due to an IgG HLA-DQ antibody, present at the time of transplantation (see B cell crossmatch analysis).

$ Also had a peak reactive B cell crossmatch due to an IgM HLA-DQ antibody (see B cell crossmatch analysis).

& Also had a peak reactive B cell crossmatch due to an IgM HLA-DR antibody (see B cell crossmatch analysis).

7.3.3 T and B cell positive crossmatch transplants caused by IgG HLA class I antibodies

Of nine T and B cell positive crossmatch transplant caused by IgG HLA class I antibodies (blocked by PA2.6 and not reduced by DTT) only one (11%) functioned beyond 28 days (Table 7.3.1). All graft failures were due to rejection. In one case (RF, TP No.443) the crossmatch was positive at the time of transplantation using current serum. In this case the kidney donor and recipient were thought to be HLA identical, however retrospective analysis demonstrated a HLA-C locus incompatibility to which the recipient was sensitised. This transplant suffered a hyperacute rejection (Chapman et al, 1986c). (The patient has subsequently received a fifth transplant using a fully HLA identical donor and this transplant is successful with a creatinine of 177μmol/l one and a half years post transplant).

In addition there were a further four positive crossmatch transplants caused by IgG antibodies which were not blocked by PA2.6 (two cases) and cytotoxicity inhibition tests were not performed (two cases). All four of these transplants failed within 3 months.

Initials	TpN	TxDate	ImSup	TxN	PRA	Cur	DTT	PA2.6	Outcome	
DH	435	16. 6.84	CyA+S	1	100	2950	No	No	NRF	44
AL	463	12.10.84	CyA+S	1	100	1920	No	No	NRF	24
EB	491	23. 3.85	CyA+S	1	69	3283	No	Yes	Rej	7
RF	388	14. 7.83	CyA	3	100	720	No	NT	Rej	0
RF	443	10. 7.84	CyA+S	4	100	+	No	Yes	Rej	0
GH	446	1. 8.84	CyA+S	2	100	1035	No	Yes	Rej	0
RI	447	1. 8.84	CyA+S	2	94	830	No	NT	Rej	9
MD	448	6. 8.84	CyA+S	2	35	1410	No	Yes	Rej	28
BH	464	21.10.84	CyA+S	3	100	1760	No	Yes	Rej	9
BK	552	20.12.85	Trip	3	100	3089	No	Yes£	Rej	0
MB	592	17. 7.86	Trip*	2	100	1444	No	Yes	Rej	28
MJ	615	10.10.86	Trip*	2	100	311	No	Yes≠	Rej	8
EH	643	9. 3.87	Trip*	2	98	706	No	Yes	Success	

Table 7.3.1 T and B cell positive crossmatch transplants caused by IgG antibodies.

£ =also had a peak reactive B cell crossmatch due to an IgG HLA-DR antibody (not included in the B cell crossmatch analysis).

7.4 B cell positive crossmatch transplants

A total of 65 transplants performed with a positive B cell (negative T cell) crossmatch were analyzed. Overall one year graft survival for this group was 78%.

7.4.1 IgM non-HLA antibodies

a) Living related donors

Eight positive B cell crossmatch transplants were performed using living related donors (Table 7.4.1). All were single haplotype matched and were positive at the time of transplantation. An autologous crossmatch was positive in each case and all were caused by IgM antibodies. All transplants were successful with a follow-up time of between one and five years.

Initials	TpN	TxDate	ImSup	TxN	Hap	Cur	DTT	Outcome
AS	496	11. 4.85	CyA+S	1	1	+	Yes	Success
HA	498	15. 4.85	CyA+S	1	1	+	Yes	Success
FA	583	9. 6.86	Trip	1	1	+	Yes	Success
OA	621	8.12.86	Trip	1	1	+	Yes	Success
AM	628	26. 1.87	Trip	1	1	+	Yes	Success
AB	710	11. 1.88	Trip	1	1	+	Yes	Success
F	717	8. 2.88	Trip	1	1	+	Yes	Success
HM	836	24. 7.89	Trip	1	1	+	Yes	Success

Table 7.4.1 Positive B cell crossmatch transplants caused by IgM non-HLA antibodies using live related donors.

b) Cadaver donor transplants, first grafts

Of 17 primary transplants performed with a positive B cell crossmatch caused by IgM non-HLA antibodies 13 (76%) were successful at six months (Table 7.4.2). Of these, seven were positive using current serum and six (86%) were successful at six months.

Initials	TpN	TxDate	ImSup	TxN	PRA	Cur	DTT	Block	Outcome
OZ	278	29. 4.81	CyA	1	13	539	Yes	No	Success
MH	342	4. 9.82	CyA	1	87	+	Yes	No	Success
JP	354	19.10.82	Low	1	50	+	Yes	No	Success
AO	364	9. 2.83	Low	1	57	303	Yes	No	Rej 35
PH	441	6. 7.84	Low	1	36	+	Yes	No	Rej 718
DH	469	26.10.84	CyA	1	95	170	Yes	NT	Success
DH	597	5. 8.86	Trip	1	43	495	Yes	No	Success
AD	616	15.10.86	Trip	1	80	+	Yes	No	Success
CR	626	22. 1.87	Trip	1	53	79	Yes	No	NRF 44
MS	645	26. 3.87	Trip	1	97	408	Yes	No	Success
PR	726	7. 4.88	Trip	1	50	247	Yes	No	NRF 0
EH	745	24. 6.88	Trip	1	40	+	Yes	No	Success
EH	790	28.12.88	Trip	1	83	2612	Yes	No	Success
PJ	791	28.12.88	Trip	1	48	853	Yes	No	Success
PT	875	8.12.89	Trip	1	7	+	Yes	No	NRF 58
MB	892	6. 2.90	Trip	1	73	+	Yes	No	Success
CO	917	23. 5.90	Trip	1	51	76	Yes	No	Success

Table 7.4.2 Positive B cell crossmatch transplants caused by IgM non-HLA antibodies in primary cadaver grafts. (PRA =highest percentage reactivity of sera tested against a panel of 40 PBL's and 30 CLL's).

c) Cadaver donor transplants, regrafts

Of 18 regrafts performed with a positive B cell crossmatch caused by IgM non-HLA antibodies 16 (89%) were successful at six months (Table 7.4.3). Six crossmatches were positive using current serum taken at the time of transplantation and all were successful.

Initials	TpN	TxDate	ImSup	TxN	PRA	Cur	DTT	Block	Outcome
AA	158	29. 7.78	High	2	94	970	Yes	No	NRF 2802
HD	248	9. 7.80	Low	2	84	+	Yes	No	Success
BF	298	27. 9.81	Low	2	100	+	Yes	No	Success
JK	372	4. 4.83	Low	3	100	605	Yes	No	Rej 41
CO	442	6. 7.84	CyA+S	2	85	1155	Yes	No	Success
DF	451	28. 8.84	CyA+S	3	96	1595	Yes	No	Success
VA	462	11.10.84	CyA+S	2	100	2400	Yes	No	Rej 120
RC	468	25.10.84	CyA+S	2	100	1375	Yes	No	Success
JD	520	17. 7.85	Trip	2	41	1160	Yes	No	Success
BN	617	24.10.86	Trip	2	93	+	Yes	No	Success
GM	750	7. 7.88	Trip	2	43	+	Yes	No	Success
MH	765	26.10.88	Trip*	2	97	1402	Yes	No	Success
RF	834	21. 7.89	Trip*	2	57	898	Yes	No	Success
AL	853	21. 9.89	Trip*	2	33	+	Yes	No	Success
PW	852	21. 9.89	Trip	2	87	71	Yes	No	Success
MB	854	23. 9.89	Trip	2	NT	445	Yes	No	Success
LW	865	5.11.89	Trip^	3	97	1797	Yes	No	Success
SL	883	8. 1.90	Trip*	3	53	+	Yes	No	Success

Table 7.4.3 Positive B cell crossmatch transplants caused by IgM non-HLA antibodies in cadaver regrafts.

In addition two patients (one first graft and one regraft) had a positive B cell crossmatch which was not reduced by DTT and not blocked for HLA class I, DR or DQ indicating an IgG non-HLA antibody. Both transplants are successful beyond one year (data not shown).

7.4.2 Positive B cell crossmatch transplants caused by IgM HLA antibodies

Ten transplants were performed with peak positive - current negative B cell crossmatches caused by IgM HLA antibodies of which six (60%) were successful at six months (Table 7.4.4). Five were due to HLA class I antibodies (reactive with B cells only because of increased antigen expression compared with T cells (Coxe-Gilliand and Cross, 1980)) and four (80%) were successful.

Only one positive B cell crossmatch was due to IgM HLA-DR antibodies (blocked by NDS-22) and this graft is functioning beyond one year. The lack of positive crossmatches due to HLA-DR antibodies in this study is not unexpected because our policy has been not to transplant an HLA-DR mismatched kidney into a patient with a positive crossmatch where autoantibodies could not be demonstrated.

Four B cell positive crossmatch transplants were found to be caused by peak reactive IgM HLA-DQ antibodies (blocked by LEU-10). All were regrafts and only one (25%) was successful beyond one month.

Name	TpN	TxDate	ImSup	TxN	PRA	Cur	DTT	Block	Outcome
MJ	418	28. 2.84	Low	1	75	21	Yes	PA2.6	Success
JJ	644	26. 3.87	Trip	1	100	547	Yes	PA2.6	Success
ET	821	1. 6.89	Trip	1	77	360	Yes	PA2.6	Success
MB	594	27. 7.86	Trip*	2	100	1117	Yes	PA2.6	Success
BH	652	12. 5.87	Trip*	4	100	875	Yes	PA2.6	Rej 8
JB	674	29. 7.87	Trip	1	100	1239	Yes	NDS-22&	Success
GP	329	13. 6.82	Low	2	100	401	Yes	LEU-10"	Rej 0
RD	493	25. 3.85	CyA+S	2	99	2531	Yes	LEU-10	Rej 18
SB	629	29. 1.87	Trip	2	88	3436	Yes	LEU-10@	Rej 5
KW	653	6. 6.87	Trip	2	100	1591	Yes	LEU-10&	Success

Table 7.4.4 B cell positive crossmatch transplants caused by IgM HLA antibodies.

@ =also had a peak reactive positive T cell crossmatch due to an IgM non-HLA antibody (not included in the T cell analysis).

& =also had a peak reactive positive T cell crossmatch due to an IgM HLA-class I antibody (see T cell crossmatch analysis).

" =also had a current positive B cell crossmatch due to an IgM non-HLA antibody (not included in the analysis).

7.4.3 B cell positive crossmatch transplants caused by IgG HLA antibodies

Ten positive crossmatch transplants were caused by peak reactive IgG HLA antibodies and six (60%) were successful at six months. Five were directed at HLA class I (blocked by PA2.6) and three were successful at six months. One patient (MM, TpN 417) had poor function and the transplant failed at 14 months due to chronic rejection.

Two transplants were performed with peak reactive positive B cell crossmatches caused by IgG HLA-DR antibodies. In both situations the patients were highly sensitised for HLA-Class I and a donor was found with a negative T cell crossmatch. Patient RD also had a repeat HLA-DR mismatch with a previous donor. The class and specificity of the crossmatch was determined prospectively and the patients were treated prophylactically with a ten day course of OKT3. Patient RD also received consecutive immunosuppression with ATG. Despite these measures both patients suffered acute rejection. Patient JL failed at two months and patient RD, although free from dialysis at one year has never enjoyed good function. This patient has a creatinine of 547μmol/L 17 months post transplant.

Three transplants were performed with positive B cell crossmatches caused by IgG HLA-DQ antibodies. Two were present in the peak reactive serum only and one was successful. One patient (CO) had a positive crossmatch using current serum and this transplant was successful. This case study is discussed in detail in Chapter 7.5.

Name	TpN	TxDate	ImSup	TxN	PRA	Cur	DTT	Block	Outcome
MM	417	19. 2.84	Low	1	100	1040	No	PA2.6	Rej 425
MC	398	8.11.83	Low	2	92	575	No	PA2.6	Rej 0
GH	430	11. 4.84	CyA	2	60	2976	No	PA2.6	Success
DB	483	13. 2.85	CyA+S	3	94	3385	No	PA2.6	Rej 28
CP	795	10. 2.89	Trip*	2	100	1647	No	PA2.6@	Success
RD	822	1. 6.89	Trip^	3	100	1401	No	NDS-22	Success
JL	911	9. 5.90	Trip^	2	93	456	No	NDS-22	Rej 65
CO	558	10. 1.86	Trip	1	100	+	No	LEU-10&	Success
EM	582	2. 6.86	Trip	2	47	487	No	LEU-10	NRF 0
PF	625	31.12.86	Trip*	1	100	238	No	LEU-10	Success

Table 7.4.5 Positive B cell crossmatch transplants caused by IgG HLA antibodies.

^ =received OKT3 immunosuppressive therapy (patient RD also received ATG).

@ =also had a peak reactive positive T cell crossmatch due to an IgM non-HLA antibody (not included in the T cell analysis).

& =also had a peak reactive positive T cell crossmatch due to an IgM HLA-class I antibody (see T cell crossmatch analysis).

7.4.4 Analysis of a positive B cell crossmatch caused by HLA antibodies in primary and regrafts

No association was seen between the immunoglobulin class of the B cell HLA antibody and graft outcome. The six month graft success rates were 60% for both IgG and IgM, although two patients with IgG antibodies had poor function and one failed just beyond one year. However when the data are analyzed according to primary or regraft, all 7 primary grafts were functioning beyond one year compared to only 5/13 (38%) regrafts.

7.4.5 Discussion

The interpretation of the relevance of a positive serological crossmatch to renal allograft outcome has become considerably more complicated than previously believed. It is necessary to consider the specificity of the antibody, whether the antibody is in peak and current sera or in peak serum only, and the immunoglobulin class of the antibody. The specificity of the antibody may be HLA class I, HLA class II (HLA-DR, DQ or DP), or not directed at HLA at all, these latter antibodies being mostly autoreactive. The immunoglobulin class of the cytotoxic antibody can be IgG or IgM.

The favourable outcome of renal transplantation in patients with a positive crossmatch resulting from autoantibodies is not in question (Cross, Greiner and Whittier, 1976; Reekers et al, 1977; Ting and Morris, 1977), and this is true even when the current serum gives a positive crossmatch (Ting and Morris, 1983). On the other hand the relevance of a positive crossmatch due to HLA antibodies is not fully understood. The main reasons for this controversy is that the precise specificity of the antibody causing a positive crossmatch has not been defined in most studies, and in many studies the authors have assumed that the antibody is against HLA without any supporting data. For example, the distinction between HLA and non-HLA antibodies has been deduced by platelet absorption (Jeannet, Benzonana and Arni, 1981; Sirchia et al, 1979; Russ et al, 1987), performing an autologous crossmatch (Ettenger, Jordan and Fine, 1983; Cross, Greiner and Whittier, 1976) and varying the pre-complement incubation temperature (Ayoub et al, 1980; Fauchet et al, 1980; Iwaki et al, 1979). Cold reactive or non-platelet absorbed antibodies (presumed non-HLA) have been associated with good graft survival, whereas warm reactive or platelet absorbed antibodies (presumed HLA) have been associated with poor graft survival

(Iwaki et al, 1979; Ayoub et al, 1980; Rhodes et al, 1984; Sirchia et al, 1979). Unfortunately, these tests do not always distinguish non-HLA from HLA antibodies since many autoantibodies react at all temperatures (Deierhoi, Ting and Morris, 1983), platelet absorption does not distinguish between HLA class II and non-HLA antibodies, and patients who have autoantibodies may have HLA antibodies in addition.

As a more reliable test to distinguish HLA from non-HLA antibodies the cytotoxicity inhibition test has been developed with MAbs against HLA class I, DR, and DQ. Control studies have demonstrated locus specific inhibition of alloantisera specific for alleles of these loci (with the exception of HLA-DQw2), and the ability of the technique to define antibody specificities in highly sensitised patients that were undefined by conventional serology. In combination with the use of DTT, these techniques were applied to define the precise specificity and immunoglobulin class of 152 positive crossmatch transplants.

In this study the 1 year graft survival for all positive T and B cell crossmatch transplants was 69%, and this figure is similar to other reports (Cardella et al, 1982; Goeken and the Clinical Affairs Committee, 1985). However, when the data are divided into those caused by HLA class I and by non-HLA antibodies a different picture emerges. The non-HLA group (most were autoreactive) had a very high success rate of 88% at 6 months compared with only 50% for the HLA class I antibody group. Furthermore, when the HLA antibody group was broken down into those with IgM and IgG antibodies a striking difference was seen. The former group had a respectable success rate of 71% at 6 months, but in the latter group it was only 8%. These data strongly suggests that the specificity and immunoglobulin class of the peak

antibody that gives rise to a positive crossmatch is an important consideration in the decision whether or not to proceed with a transplant. It is not possible to make the broad generalisation that a peak positive T cell crossmatch can always be ignored, as some studies have suggested. Perhaps the early failures seen in many published studies are in patients with IgG HLA antibodies, and if this is so then a positive crossmatch caused by this type of antibody should be a contra-indication to transplantation. The number of patients in this study is relatively small and obviously an analysis of a larger number of patients is needed before one can be certain about this conclusion.

Several studies have indicated that the prognosis for success is lower in cadaver regrafts than primary grafts performed in the presence of a positive crossmatch (Kerman et al, 1985; Goeken and the Clinical Affairs Committee, 1985; Matas et al, 1984). Cardella also reported a lower regraft outcome, although this was not different from their regraft success rate in transplants performed with a negative crossmatch (Cardella et al, 1985). Overall the Oxford data shows a lower regraft success rate when compared with primary grafts. However, the results do not correlate with primary grafts and regrafts per se, but with the type of pregraft sensitisation. The cadaver regrafts have a high expectancy of success if the crossmatch is due to IgM non-HLA antibody, while those due to IgG HLA antibodies have failed whether they were primary grafts or regrafts.

The significance of a positive B cell crossmatch is also controversial. Because the early studies showed that graft survival in B cell positive crossmatch transplants was similar to, or even better than, grafts with a negative crossmatch (Fauchet et al, 1985; Myburgh et al, 1977; Ettenger et

al, 1976; Ting and Morris, 1977; D'Apice and Tait, 1979), many transplant units do not perform B cell crossmatches. On the other hand some centres avoid altogether transplanting any B cell positive crossmatch donors. The finding of an overall one year graft survival rate of 78% is in agreement with other studies that have demonstrated no deleterious effect of a positive B cell crossmatch. However, like the T cell crossmatches, when the positive B cell crossmatch transplants are divided according to specificity, those due to autoantibodies have a much higher success rate than those due to HLA antibodies for both primary grafts and regrafts. This was true whether the antibody was in the peak serum only or in the current serum as well. However, unlike the T cell crossmatch data there was no correlation between the immunoglobulin class of the B cell HLA antibody and graft outcome. The only striking correlation seen in the B cell positive crossmatch group due to HLA antibodies was with good primary graft survival (100%) but poor regraft survival (38%), and these results are in agreement with those of Reekers and Fluit (Reekers and Fluit, 1985). When the HLA antibody group was divided into those due to HLA-ABC, DR, and DQ antibodies the number of patients in each group were too small to draw firm conclusions. However, it may be relevant to note that two of five grafts with IgG HLA class I antibodies failed within 6 months, and a third graft failed at 15 months due to chronic vascular rejection.

There are few reports on the relevance of HLA-DR antibodies causing a positive crossmatch. This paucity of data may be because transplant units are reluctant to transplant a DR mismatched graft in the presence of a positive B cell crossmatch or, as suggested some time ago, most positive B cell crossmatches are not caused by HLA-DR antibodies but by non-HLA antibodies (D'Apice and Tait, 1980). In the studies where HLA-DR antibodies

have been implicated, most have shown a deleterious effect (Rhodes et al, 1984; Ayoub et al, 1980; Russ et al, 1987; Ting and Morris, 1981; Sirchia et al, 1979; Noreen et al, 1987), and in a report of hyperacute rejection by Ahern et al. (Ahern et al, 1982), and other similar reports (Dejelo and Williams, 1977; Mohanakumar et al, 1981; Berg and Moller, 1981) the antibody was present at the time of transplantation. In this study there were only two cases of a positive crossmatch due to IgG HLA antibodies present at the time of transplantation. One was an HLA-C locus antibody and the graft suffered hyperacute rejection (Chapman et al, 1986c). The other was an HLA-DQ antibody and this graft was successful (Taylor et al, 1987). In two cases a peak reactive IgG HLA-DR antibody was defined. Despite prophylactic immunosuppression with OKT3 and ATG both kidneys suffered acute rejection. One patient (JL) failed at two months and the second (RD) has poor function at one year. In the latter case the crossmatch had been negative for nearly four years. Although it is dangerous to draw conclusions from just two examples, it does appear that even prophylactic OKT3 and ATG can not control the memory response in patients sensitized to HLA-DR.

Another factor, the relevance of which is not fully understood is the interval between the last positive crossmatch and the transplant. Some investigators consider as little as two months appropriate (Cardella et al, 1982), whereas others require four months (Fuller, Forbes and Delmonico, 1985) or six months (Norman, Barry and Wetzsteon, 1985; Reed et al, 1987) before transplantation. However in a report by Palmer et al. (Palmer et al, 1989), where the antibodies causing a positive crossmatch were removed by extracorporeal immunoadsorption, the interval was only a few hours. By design, in this study the interval between a positive T and B cell

crossmatch caused by HLA antibodies was greater than one year (range 1.5 to 10 years) for all but one patient in whom it was 42 days.

Although many advances have been made in our understanding of the significance of a positive crossmatch in predicting renal graft outcome there are still many unanswered questions. It does appear that the specificity and immunoglobulin class of the antibody causing the positive crossmatch is an important consideration. In spite of the advances, many more well-defined studies are needed before we can say for certain which positive crossmatches are clinically relevant and which can be ignored, but some indications are strongly suggested in this study.

7.5 Case Presentation: A positive B cell crossmatch due to IgG anti-HLA-DQ antibody present at the time of transplantation in a successful renal allograft

7.5.1 Patient details

A 25 year old woman with end stage renal failure due to chronic pyelonephritis began haemodialysis treatment in 1983. She was nulliparous and had not previously been transfused. The patient first received elective platelet transfusions (3 units) (Chapman et al, 1986b), and then clinically necessary blood transfusions (16 units) between her acceptance on to the transplant waiting list (1983) and subsequent transplantation (1986). Figure 7.5.1 demonstrates the evolution of her cytotoxic antibodies over this period, and the association of these antibodies with the transfusions.

The patient was highly sensitised, with 100% panel reactive antibodies and consequently had a positive crossmatch with all potential donors. In January 1986 vascular access for haemodialysis was becoming difficult and peritoneal dialysis was contraindicated by previous abdominal surgery. She was therefore considered urgently for renal transplantation with a view to accepting a kidney with a positive T cell crossmatch with peak reactive but negative with current serum. A donor was found who fulfilled this criteria but in addition had a positive B cell crossmatch in serum taken on the day of transplantation. Because of the clinical urgency the decision was taken to transplant despite both the peak serum positive T cell and a current serum positive B cell crossmatch.

Immunosuppression after transplantation consisted of triple therapy with cyclosporin, azathioprine and prednisolone, with prophylactic use of

intravenous methylprednisolone 500mg daily for the first five days and for the treatment of rejection episodes thereafter. Following transplantation the graft functioned immediately with the production of 3,600ml of urine in the first 24 hours. Despite prophylactic use of methylprednisolone the serum creatinine did not fall rapidly and a total of four grams of methylprednisolone were used to treat acute rejection within the first nine days. Renal function subsequently improved such that on day 18 post transplant her creatinine was 162 μmol/l. She has suffered no further rejection episodes the patient is currently well with a plasma creatinine level of 196μmol/l four years post transplant.

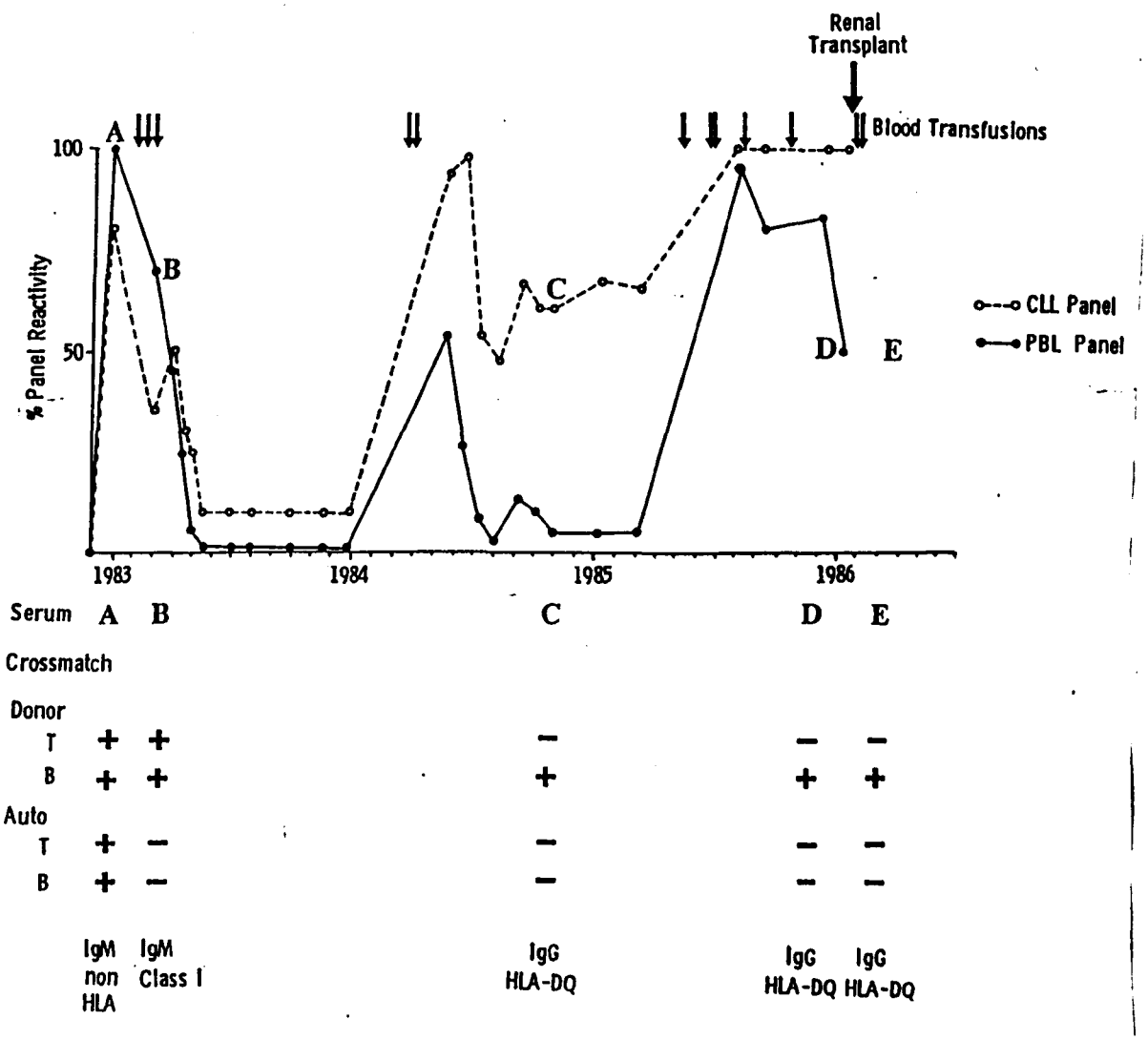


Figure 7.5.1 Panel reactivity of serum samples taken from the patient between 1982 and 1986. Timing of both platelet and blood transfusions are indicated by arrows. Sera A to E have been selected for further study with the results of the donor and autologous T and B cell crossmatches shown. The conclusions for the determined antibody class and specificity of the crossmatch as defind by further studies are given for each serum.

7.5.2 Serology

Cytotoxicity inhibition and DTT reduction studies were performed on selected sera, four taken before transplantation (sera A to D, Figure 7.5.1), and one after transplantation (serum E, Figure 7.5.1).

Serum A was taken before the first transfusions and was autoreactive, lysing both autologous T and B cells (Figure 7.5.1). The donor crossmatch was also positive with T and B cells, but could not be inhibited by PA2.6 (anti-HLA class I) and was entirely reduced by DTT (Table 7.5.1).

Serum B was taken after the first transfusion and was selected because autoreactivity could not be demonstrated, but the donor crossmatch was positive with both T and B cells. Both the T and B cell crossmatch was blocked by PA2.6 (Table 7.5.1) indicating antibody specific for donor HLA class I. This positive crossmatch was also reduced by DTT suggesting an IgM anti-HLA class I antibody.

Serum C reacted with 55% of the panel of CLL cells but only 5% of the PBL panel and was shown to be specific for HLA-DQw1 in panel tests (Chi^2 =21.63, [$p<0.001$ with Yates correction], $r=0.86$). Panel screening demonstrated that this serum was not reactive with HLA-DRw13 and DRw52, which were the other serologically defined HLA-D region antigens mismatched between donor and recipient (Table 7.5.2). Reactivity of this serum was inhibited from a titre of 1/32 to a titre of 1/4 on donor B lymphocytes by LEU-10 which is specific for HLA-DQ locus products (Table 7.5.1). The B cell crossmatch was not reduced by DTT, suggesting that the antibody was an IgG anti HLA-DQw1.

Serum D was taken on the day of transplantation and had a titre of 1/128 in the standard crossmatch with donor B lymphocytes. Despite broad panel reactivity with undefinable specificity, the donor crossmatch using serum D was not reduced by DTT and was inhibited by LEU-10 (Table 7.5.1). The donor and recipient were mismatched for HLA-DQw1, DRw13 and DRw52 but no inhibition of the B cell crossmatch was seen with NDS-22 (specific for HLA-DR) which inhibited known HLA-DR sera in parallel assays. We therefore concluded that the positive B cell crossmatch using serum taken on the day of transplantation was caused by an IgG anti HLA-DQ antibody. It was also possible to identify the same antibody at an unchanged titre using serum taken five days after transplantation (Table 7.5.1, serum E). In addition, lymph collected from the lymphocoele one month after transplantation revealed an IgG antibody specific for HLA-DQw1 (Chi^2=16.30 [$p<0.001$ with Yates correction], r=0.73), also with a titre of 1/128.

Serum	MAb	Cell	Titre of crossmatch			DTT	Conclusion
			Cell treatment				
			None	MAb alone	MAb+ F(ab')2		
A	PA2.6	T	1*	1	1	Yes	IgM autoantibody
B	PA2.6	T	16	1	neg	Yes	IgM HLA class I
	PA2.6	B	64	16	4	Yes	
	NDS-22	B	32	64	64		
	Leu-10	B	64	64	64		
C	PA2.6	B	32	32	32	No	IgG HLA-DQ
	NDS-22	B	16	16	16		
	Leu-10	B	32	4	8		
D	PA2.6	B	128	128	128	No	IgG HLA-DQ
	NDS-22	B	128	128	128		
	Leu-10	B	128	32	64		
E	PA2.6	B	128	128	128	No	IgG HLA-DQ
	NDS-22	B	128	128	128		
	Leu-10	B	128	64	64		

Table 7.5.1 Class and specificity of the donor crossmatch as demonstrated by DTT reduction and cytotoxicity inhibition

(=reciprocal of titre).*

Recipient type.		Donor type.	
HLA-A	1, 24(A9)	24(A9),	25(A10)
B	35, 44(B12)	7,	18
Bw	4, 6	6	
Cw	4, 5	7	
DR	4,	4,	w13(w6)
DRw	53,	53,	52
DQw	3,	3,	1

Table 7.5.2 Serological HLA tissue typing of donor and recipient. Donor HLA class II antigens mismatched for the recipient are underlined.

7.5.3 Immunohistology

Histopathology of the allograft (carried out by Dr M.Dunnill, Histopathology, John Radcliffe Hospital) demonstrated the presence of glomerular neutrophil polymorphs one hour after revascularisation, which were not seen in the biopsy taken immediately before transplantation. This glomerular infiltrate was associated with occasional foci of interstitial neutrophils. At day seven occasional neutrophils were still present within the glomeruli and focal adhesions between the glomerular tuft and Bowman's capsule were seen, while the interstitial tissue showed only mild focal lymphocyte infiltration. By day 19 the tubular morphology and interstitium were essentially normal though the glomeruli were still abnormal with capillary obliteration and mesangial expansion. These changes had largely regressed by three months at which time there was no interstitial fibrosis and only minor arterial intimal fibrosis. By seven months the major abnormalities were intimal fibrosis of the arteries with a 50% reduction in their lumen, and moderate diffuse interstitial fibrosis.

Immunoperoxidase studies of the early biopsies (carried out by Dr S.V.Fuggle, Nuffield Department of Surgery) revealed normal expression of HLA-DQ antigens before transplantation with weak staining of the glomerular endothelium and intertubular structures. This pattern was unchanged in the day 7 biopsy but at day 19 a focal area of proximal tubules were also expressing HLA-DQ antigens. Staining for monomorphic HLA-DR showed the same pattern with normal expression on glomerular endothelium and mesangium, intertubular structures and proximal tubules at day 0 and day 7, while there was a focal area of increased expression at day 19.

7.5.4 Discussion

It is generally accepted that a positive T and B cell crossmatch caused by antibodies to HLA class I antigens and present at the time of transplantation would result in rapid graft failure and probably hyperacute rejection. The question that has been addressed here is whether or not that holds true for antibodies specific for HLA class II antigens present in serum taken on the day of the transplant.

The major difficulty encountered in analysis of the positive B cell crossmatch is the exact definition of the antibody specificity. Identification of antibodies specific for HLA-class II antigens has usually relied upon indirect techniques such as segregation with HLA-DR in families, or tissue typed panels demonstrating definable HLA-DR polymorphisms (d'Apice & Tait 1980). However it is not usually possible to define specificities when a mixture of antibodies are present in a highly sensitised patient. The technique of cytotoxicity inhibition can dissect specificities contained within complex multispecific antisera and this has enabled the precise identification of the specificity of antibodies causing positive crossmatches against this particular kidney donor.

In this case presentation the patient had serum samples which gave positive crossmatches caused by three different antibodies. Prior to her first transfusion there was a positive T and B cell crossmatch with donor lymphocytes that was due to an IgM autoantibody. Following the initial platelet transfusions the patient developed an IgM anti-HLA class I antibody that was reactive with donor T and B cells. Positive crossmatches caused by both of these antibodies were associated with good outcome of renal transplantation (Chapter 7.1 and 7.3). The third antibody reactive

with donor lymphocytes developed after further blood transfusions, remained to the day of transplantation, and was still detectable in both serum and lymph with the functioning graft in-situ. This antibody was IgG and was reactive with B but not T lymphocytes from the donor. At one point conventional specificity analysis defined an anti-HLA-DQw1 antibody, while the positive B cell crossmatch was inhibited by a monoclonal antibody specific for HLA-DQw1 and DQw3 (LEU-10).

By analogy with the T cell crossmatch, this patient represents the most stringent test of the relevance of HLA-DQ specific antibodies in renal transplantation, since the positive crossmatch was present in serum taken at the time of operation. However the transplant did not suffer hyperacute rejection. The graft glomeruli were however infiltrated by neutrophil polymorphs within one hour, suggesting the presence of antibody mediated damage (Williams et al, 1968; Kincaid-Smith et al, 1969). Renal function was poor for the first week but by then the histology revealed little evidence of rejection. The immune response that did occur was sufficiently controlled for the graft to maintain reasonable function at three years. Study of the expression of HLA class II antigens in this renal allograft, using monomorphic monoclonal antibodies confirmed the presence of HLA-DQ in a normal distribution (Fuggle et al, 1986). Monomorphic HLA-DR expression was also normal in the initial biopsy but a focal increase in both HLA-DR and DQ was seen in the biopsy taken on day 19. It is therefore unlikely that lack of antigen expression in this particular kidney explained the absence of a catastrophic immune reaction.

The experience of this patient would suggest that successful renal transplantation can be performed despite the presence of circulating

cytotoxic IgG antibodies specific for HLA-DQ antigens expressed on the kidney. It is uncertain whether or not the relatively heavy triple therapy immunosuppression together with prophylactic methylprednisolone was relevant and it would be unwise to extrapolate from this single experience. It is possible that antibodies to other HLA class II antigens could have different implications, particularly if antigen density in the target organ is important. It is therefore important for these questions to be resolved using techniques that define, with reasonable certainty, the antigen specificity of antibodies causing positive B cell crossmatches.

Chapter 8. General Discussion

8.1 Strategies for transplanting highly sensitized patients

8.1.1 Locating a negative crossmatch donor

i) Beneficial matching
ii) The SOS scheme
iii) Acceptable mismatches

8.1.2 Identification of non-damaging positive crossmatches

i) Lymphocytotoxic Autoantibodies
ii) Peak positive/current negative crossmatches
iii) Desensitization

8.2 Crossmatch Sensitivity

8.3 Concluding remarks

8.1 Strategies for transplanting highly sensitised patients

The cytotoxicity crossmatch test, which identifies patients sensitized to a particular donor, has virtually eliminated the phenomenon of hyperacute rejection with immediate irreversible graft loss. A positive crossmatch forewarns the transplant team of a vigourous immune response against the graft and the transplant would therefore not be performed. Transplants performed with a negative crossmatch do not carry the risk of hyperacute rejection. Highly sensitized patients are those who have antibodies reacting with >85% of potential donors (Claas, Van Leeuwen and Van Rood, 1989; Klouda et al, 1987). It is extremely difficult to find a suitable crossmatch negative donor for these patients, consequently they accumulate on transplant waiting lists (Lamm, Madsen and Fjeldborg, 1987; Pfaff, Vaughn and Sanfilippo, 1987). The successful transplantation of these patients poses a major challenge for transplant centres.

Several strategies have been developed to achieve this aim. They consist of two main approaches; A) to improve the chances of finding a negative crossmatch donor, and B) identify circumstances under which a positive crossmatch can be safely ignored.

8.1.1 Locating a negative crossmatch donor

i) Beneficial matching: This scheme, administered through the United Kingdom Transplant Service (UKTS), Eurotransplant (Hendriks et al, 1987) and others (Opelz, 1987b; Carpenter et al, 1989; Pfaff, Vaughn and Sanfilippo, 1987) has enabled many successful transplants. HLA matched donors are selected to minimise the chances of the recipient having donor specific HLA antibodies. Matching on a national and international basis improves the chances of finding a compatible donor. However due to the

extreme polymorphism of the human MHC, the probability of finding a matched donor, even using national registries is still remote.

ii) The SOS scheme

To increase the likelihood of locating a negative crossmatch donor, sera are sent to all the transplant centres throughout the country and crossmatched against all donors (Klouda et al, 1987). In the event of a serum giving a negative crossmatch, the kidney is despatched to the appropriate transplant centre. Nationally 83 transplants were performed with a 57% one year success rate. Similarly, Opelz (Opelz, 1988a) has reported 100 transplants with a 71% first and 68% regraft success at one year. In this study, emphasis for the need of minimum HLA matching criteria (1 HLA-A, 1 B and 1 DR match) was made with an associated 78% success.

iii) Acceptable mismatches

Acceptable mismatches are identified by screening sera against a panel of selected individuals who differ from the recipient by only one allele (Claas, Van Leeuwen and Van Rood, 1989). This enables the identification of antigens to which the recipient is not sensitized. Altogether 120 patients have been transplanted with a 70% four year graft survival (F.Claas, Eurotransplant Meeting, September, 1990). Interestingly the acceptable mismatches appear to consist of the non-inherited maternal haplotype (NIMA) in 50% of patients, and both antigens in 30% of patients (Claas et al, 1988). This is proposed to reflect tolerance to maternal antigens acquired during foetal development.

8.1.2 Identification of non-damaging positive crossmatches

i) Lymphocytotoxic Autoantibodies

It is well established that lymphocytotoxic autoantibodies are not damaging, even when present at the time of transplantation (see Chapters 1 and 7). Techniques used to identify and distinguish autoantibodies from alloantibodies include:

a) positive autologous crossmatch and absorption with autologous PBLs or LCLs;

b) weak or negative CLL reactivity;

c) not platelet absorbed;

d) K562 reactivity and absorption;

e) low optimal reactivity temperature;

f) the antibody class is usually IgM; and

g) they are not inhibited by HLA monoclonal antibodies.

ii) Peak positive / current negative crossmatches

Many centres believe that transplants can be performed without regard to previous sensitization provided the current serum is negative. Graft survival equal to those with a negative crossmatch has been reported (see Chapter 1). However others consider this acceptable for first but not regrafts and some correlate success with the presence of anti-idiotypic antibodies in the current serum. Studies presented in this thesis indicate that the antibody class and specificity of the crossmatch are important in identifying those with a higher propensity to fail, which is independent of primary or regraft status.

The long time interval between the positive crossmatch and transplantation in many cases raises the question as to the physiological role of antibody

in the rejection process. Acute graft loss may be due to cellular mechanisms initiated by T cells and the immunoglobulin class may act as a marker for sensitization at a cellular level. The production of IgM does not require T cell priming and therefore immunological memory is absent, whereas IgG production is T cell dependant and hence indicates prior T cell activation. Therefore sensitization at a cellular level is associated with a secondary response which can not be controlled by conventional immunosuppression. The association of good primary but poor regraft survival in other studies may be due to the increased likelihood of T cell priming and IgG production following graft rejection. We have noted that sensitization through blood transfusion alone in uraemic dialysis patients is frequently associated with only IgM production.

The significance of the B cell crossmatch is still open to debate. Data presented here confirm that whilst overall acceptable graft survival has been achieved, weak HLA class I and HLA class II antibodies are associated with poor regraft survival. A recent publication by Kapurran, Lindholm and Moller (Karuppan, Lindholm and Moller, 1990) using similar techniques, is in close accordance with these findings. They found no overall difference with IgG versus IgM in 47 patients transplanted with a current weak positive B cell crossmatch. Nevertheless poor graft survival was associated with class I antibodies (50% successful at one year, N=22) most of which were IgG.

iii) Desensitization

There remains a group of patients (those with current anti-HLA antibodies) whom it is difficult to transplant. Some centres have attempted desensitization protocols before transplantation. Attempts to desensitize

through the generation of suppressor cells following repeated leucocyte transfusion in conjunction with cyclosporin have proved unsuccessful (Weir et al, 1988). However, circulating antibodies can be removed by plasmaphoresis (Fauchald et al, 1990; Backman et al, 1989) or extracorporeal immuno-adsorption (Palmer et al, 1989; Fehrman et al, 1990) in conjunction with intensive immunosuppression.

It is interesting to compare the acceptable outcome of transplants with donor reactive IgG antibodies converted to negative following their removal, to the unacceptable graft survival in patients who have lost their antibodies naturally. This paradox is difficult to resolve. It is possible that the administration of pregraft cyclophosphamide and relatively heavy post transplant immunosuppression in desensitized patients is a relevant factor. Desensitization appears to be most effective in patients with low antibody titres (Fauchald et al, 1987; Esnault et al, 1990) some of whom may lose their antibodies spontaneously (Okazaki et al, 1987). Thus, those patients successfully transplanted may represent a selected group. Furthermore, care was taken to avoid the main specificities to which the recipients were sensitized (Thick, 1987; Taube et al, 1989). A combination of these factors may be sufficient to avoid uncontrolled graft rejection.

Concurrent use of prophylactic ATG in other studies may also be a relevant factor. However this level of immunosuppression carries inherent risks associated with increased morbidity and mortality. Therefore careful selection of clinically suitable patients is important (Palmer et al, 1989). Moreover, the use of ATG (either prophylactic or to treat rejection episodes) in our own study did not rescue the kidney from irreversible destruction.

8.2 Crossmatch Sensitivity

There are two schools of thought concerning the value of the crossmatch test in clinical transplantation. Some feel more sensitive techniques such as the AHG-CDC (Johnson et al, 1987) and flow cytometry (Talbot et al, 1990) are necessary. Improved regraft survival has been associated with a negative crossmatch using these more sensitive assays (Kerman et al, 1990; Iwaki et al, 1987). Flow cytometry also detects non-complement fixing antibody isotypes and is more sensitive for the detection of HLA-antibodies than AHG-CDC and CDC (Rodey et al, 1987). However few reactions associated with increased morbidity are due to the non-cytotoxic IgG 4 isotype (Talbot et al, 1989). The use of immunomagnetic bead separation techniques also renders cells more responsive (Vartdal et al, 1986) and increases antibody titres (Povlsen, Graugaard and Kissmeyer-Nielsen, 1988; Vartdal et al, 1987).

However increased sensitivity is often associated with decreased specificity resulting in many false positives (Thistlethwaite et al, 1987; Mahoney et al, 1990). Transplants which would otherwise have been successful may then be excluded. Moreover, centres which report significant correlations with graft outcome frequently use only current sera for crossmatching (Johnson et al, 1987). The association of early graft failure with a positive flow cytometry crossmatch also correlates with previous sensitization and regraft status (Mahoney et al, 1990). It is therefore possible that the technique detects subliminal sensitization which would normally be detected in peak reactive sera tested in a conventional cytotoxicity crossmatch.

8.3 Concluding remarks

It remains impossible to draw a general consensus on the role of donor specific sensitization and renal transplant outcome. While a current positive cytotoxicity crossmatch due to IgG HLA-class I antibodies would results in hyperacute rejection their significance in other situations is unclear. There are many varied crossmatch techniques and policies governing the selection of donor and recipient pairs. However this study has allowed some insight into the significance of pregraft antibodies by defining the precise specificity and correlating their presence with graft outcome.

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