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The Human T Cell Response to Class II Major Histocompatibility Antigens.

A thesis submitted for the degree of

Doctor of Philosophy

to the Faculty of Science,

University of London

by

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March 1990

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Abstract.

Human T cell responses against allogeneic class II major histocompatibility complex (MHC) antigens were studied at the clonal and population levels using two *in vitro* approaches:

1. Human T cell clones (TCC) with specificity for HLA-DR2 were generated to study the fine specificity and activation requirements of individual alloreactive T cells. Eight alloreactive CD4+ve TCC were studied and found to be heterogeneous in their properties. Five out of the eight TCC were found to be cytotoxic, and a variety of patterns of reactivity were seen in proliferation assays against peripheral blood mononuclear cell (PBMC) or B-lymphoblastoid cell line (B-LCL) stimulators. In some cases non-responsiveness against B-LCL or PBMC stimulators could be explained by a requirement for exogenous interleukin 2.

The HLA class II specificities of 4 TCC were studied in detail. Novel polymorphisms of HLA-DR2 were found which could not be detected by serological or restriction fragment length polymorphism analysis. The allogeneic determinants recognized by alloreactive T cells were found to depend on more than the HLA class II molecules themselves. Analysis of linear amino acid sequence could not explain the specificity of all the TCC tested. In addition, alloreactive TCC could discriminate between identical HLA class II molecules expressed on cells of different lineage, and associated with different HLA haplotypes. These results support the concept that alloreactive TCC recognize allogeneic MHC molecules complexed with peptides.

2. The frequencies of HLA class II specific cytotoxic T lymphocyte precursors (CTLp) were compared in number of responder:stimulator combinations using limiting dilution analysis (LDA). Alloreactive

CTLp frequencies were studied in PBMC populations enriched for CD4+ve T cells by immunomagnetic depletion of CD8+ve T cells. Variation in alloreactive CD4+ve CTLp frequency was seen, with high (1/1880) and low (1/38987) CTLp frequencies being obtained when limiting dilution cultures were assayed against B-LCL targets. The variation in alloreactive CD4+ve CTLp frequencies were confirmed in further experiments using murine L cell transfectants expressing DR molecules, as target cells. CTLp frequencies from 1/1257 to <1/100,000 were obtained using DR+ve L cells, whereas CTLp frequencies from 1/867-1/11267 were obtained using B-LCL targets in the same experiments. In six out of eight allogeneic combinations tested CTLp frequencies obtained using the DR+ve L cells was significantly lower than when B-LCL targets were used.

The variation in CTLp frequencies against multiple HLA class II antigens expressed on B-LCL or against single HLA class II antigens expressed on murine L cell transfectants suggest that there is individual variation in the cytotoxic T cell repertoire against HLA class II antigens.

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Acknowledgements.

I am indebted to my supervisors, Dr. C.E.M. Sharrock and Professor J. R. Batchelor for their advice, encouragement and support during these studies. I also wish to thank Dr. R.I. Lechler for much helpful discussion. My colleagues (past and present) in the Department of Immunology, RPMS have to provided a friendly working environment. In particular I wish to acknowledge the following: S. Sidhu for teaching me the art of cell culture; the Tissue Typing lab for providing HLA types and phelobotomy services; Dr. M. Ritter, for introducing me to Immunology through her DCP course; Drs P.D. Mason and R.N.S. Heard for advice on Macintosh computers; Ms M. Daley for invaluble help with flow cytometry, and everyone in the T cell lab for putting up with me during this study.

Thanks also go to those mentioned in the text who contributed to these studies by generously supplying reagents or advice.

I am extremely grateful to the trustees of the Nan Dimond Fund for providing financial support during my time at the RPMS. Heartfelt thanks go to my friends, Gordon Mehrtens and Russell Shaw for helping me in my darkest hour. Finally, I would like to thank my parents for their constant support and encouragement.

Abbreviations .

ABS	AB serum.
B-LCL	B-lymphoblastoid cell line.
CD	Cluster of differentiation antigen.
CPM	Counts per minute.
^{51}Cr	Chromium 51.
CTL	Cytotoxic T lymphocyte.
CTLp	Cytotoxic T lymphocyte precursor.
EBV	Epstein-Barr virus.
FCS	Fetal Calf serum.
FITC	Fluorescein isothiocynate conjugate.
GM	Growth medium.
GVHD	Graft versus host disease.
HLA	Human leucocyte antigen.
HM	Hepes buffered medium.
HTC	Homozygous typing cell.
IFN	Interferon.
LDA	Limiting dilution analysis.
MAb	Monoclonal antibody.
MHC	Major histocompatibility complex.
MLC	Mixed lymphocyte culture.
MLR	Mixed lymphocyte reaction.
%RR	Percentage relative response.
PBMC	Peripheral blood mononuclear cells.
PLT	Primed lymphocyte typing test.
RFLP	Restriction fragment length polymorphism.
rIL1	Recombinant interleukin 1.
rIL2	Recombinant interleukin 2.
rIL4	Recombinant interleukin 4.
SD	Standard deviation.
SI	Stimulation index.
TCC	T cell clone.
TCL	T cell line.
^{3}H-TdR	Tritiated thymidine.

Chapter 1
Introduction

1.1 Discovery of the Major histocompatibility complex.

The major histocompatibility complex (MHC) is a group of genes, present in all higher vertebrates (reviewed by Klein 1986a), which code for molecules which play a vital role in T cell recognition of foreign antigens. An elegant analogy between the recognition function of the MHC and radio waves was suggested by Medawar in 1985, "...the MHC has much the function of a carrier wave in radio transmission: this is the wave to which a radio set is tuned and a radio signal is generated by an amplitude, frequency or phase modulation of the carrier wave."

Major histocompatibility complex antigens were discovered as a result of the strong immunological reaction caused by transplanting tissue between genetically different (allogeneic) individuals of the same species. The mechanisms by which MHC molecules can stimulate both antigen specific T cell recognition or alloreactive T cell recognition has been the subject of much intense study. The recent crystallographic resolution of HLA-A2 (Bjorkman et al. 1987a, 1987b) has provided a structural model to investigate MHC function. It is interesting to compare the excitement with which this analysis was greeted by immunologists (Townsend and McMichael 1987) to the comparative lack of response to Peter Gorer's discovery 51 years earlier, of blood group antigens on mouse erythrocytes (Gorer 1936). One of these antigens was the first antigen characterized of what was later to become the histocompatibility-2 (H-2) complex of mice, the first MHC to be discovered.

Gorer's work linked together discoveries on blood group antigens and tumour transplantation that had been made in the early part of this century. The discovery of the H-2 complex was dependent on genetic analysis made possible by the development of inbred mouse strains. Inbred strains are potent immunological tools, because genetic identity of

individuals (of the same sex) within a strain, allows immunological responses (or any other response) to be measured against a uniform genetic background.

Inbred mouse strains had been available commercially since the beginning of the 1900s (reviewed by Klein 1986a). Dealers attempted to keep marketable properties such as coat colour or tail length, by mating related individuals. Such inbreeding was practiced with brother x sister pairs for as many as 12 generations. Inbred mouse strains were of interest to geneticists such as Little, who was studying the inheritance of coat colour in mice. He wished to determine whether the coat colour genes could be fixed in a mouse line by inbreeding. In 1909 he started a line that was the ancestor of the DBA/1 and DBA/2 lines. Subsequently Little developed two further strains, one derived from black mice, the C57BL strain, and one derived from albino mice, the A strain. Further inbred mouse strains resulted from the efforts of other geneticists such as Bagg, who developed the BALB/C strain (Bagg albino), and Strong who developed the CBA and C3H strains by crossing Balb/C mice with DBA mice.

In the early 1900s cancer research was focused on what defect caused cancer cells to grow out of control. At that time it was not clear whether malignancy was caused by the cancer cells themselves, by a transferable agent, or by another defect in the body. In a bid to answer this question many investigators excised tumours and transplanted them into new healthy hosts, with variable results. In some mice the transplanted tumour grew and could be transferred successfully to other animals, on other occasions the tumours grew in one host but not in another, finally there were some which failed to grow at all. Loeb and Jensen discovered independently that the ability of tumours to grow in mice depended on

the "race" or strain of the mice (reviewed by Little, 1941). For example in 1908 Loeb observed that tumours arising in a strain of Japanese mice (JW strain) would only grow in Japanese mice but not in other wild mice, native to Philadelphia. These early observations were followed up by Tyzzer, who used a more systematic genetic approach. He produced F1 and F2 hybrids between JW mice and common mice and found that tumours excised from JW mice could grow in the F1, but not the F2 hybrids. This suggested to Tyzzer that tumour susceptibility could not be explained by Mendel's Law (Tyzzer 1909).

Tyzzer's work came to the attention of Little, who suggested a hypothesis to explain Tyzzer's findings. Little proposed that the trait of tumour susceptibility was not controlled by one but by several loci and that the number of susceptible individuals in the F2 generation (f') could be predicted from the formula, $f'=(3/4)^n$, where n is the number of susceptibility genes. According to this formula, if a large number of genes controlled tumour susceptibility, then only a small number of individuals would be susceptible to the tumour in the F2 generation. Thus large numbers of mice would be required to detect susceptible individuals, and Tyzzer's results could be explained on the basis of inadequate sample size. Little and Tyzzer collaborated and demonstrated that Little's hypothesis was correct. They mated susceptible Japanese mice and non-susceptible wild mice and showed that tumour susceptibility among the offspring, was controlled by more than 15 genes (Little and Tyzzer 1916). The nature of the tumour susceptibility genes however remained unknown, until Gorer linked them to blood group antigens some 20 years later.

Human blood group antigens were discovered by Landsteiner in 1901. Landsteiner noticed that mixing the sera of certain individuals with red

cells from different individuals resulted in clumping of the red cells (haemagglutination). Landsteiner separated sera and erythrocytes from a number of unrelated individuals and mixed them in a criss-cross fashion on a glass slide. On the basis of haemagglutination patterns obtained from these experiments, Landsteiner was able to define three blood group antigens A, B and C (later called O). Cells from group A individuals were agglutinated by serum from group B individuals and vice versa. Cells from group C, were not agglutinated by any sera, whilst group C sera was able to agglutinate both group A and group B cells. Later, work in 1910 by von Dungern and Hirszfeld demonstrated that human blood group antigens were inherited. The presence of genetically controlled, distinct blood group antigens between individuals explained the haemolysis effects observed by Landois in 1875, when he transfused blood between different animal species and also non-identical individuals of the same species (reviewed by Grabar 1984).

A relationship between blood group antigens and tumour transplantation was proposed by J.B.S. Haldane in 1933. Haldane had become interested in tumour transplantation after visiting Littles laboratory in the United States. Haldane proposed that genetically controlled tumour resistance factors were similar to blood group antigens, and that the failure of transplanted tumours to grow was due to a destruction mechanism similar to the destruction of incompatible blood following blood transfusion. At Haldane's prompting, Peter Gorer set out to test this hypothesis.

Gorers first task was to identify blood group antigens in mice, in which the bulk of work on tumour transplantation had been done. Previous attempts at defining blood groups in mice had failed (reviewed by Gorer 1936a), Gorer succeeded by developing new techniques. After

unsuccessful experiments using normal sera from mice, rabbits, horses and fowl, Gorer found his own serum (group A) was able to discriminate between erythrocytes from three inbred mouse strains. The strains Gorer used, A, CBA and C57BL, were souvenirs from Haldane's visit to America. Gorer's serum agglutinated erythrocytes of strain A strongly, those of strain CBA weakly and those of C57BL not at all. In some simple crossing experiments Gorer was able to show that the property of red cells being agglutinated by his serum was a dominant Mendelian trait (Gorer 1936a). Immunization of rabbits with mouse erythrocytes produced antisera able to recognize three antigens (Gorer 1936b). Antigen I was shared by A and CBA strains, antigen II was expressed strongly in A and weakly in CBA, and antigen III was expressed in all three strains. Antigen II was clearly important as it could be used to discriminate between mice of distinct genetic backgrounds.

The role of antigen II in determining tumour susceptibility was investigated by transplantation of a sarcoma that had originated spontaneously in an A strain mouse, into mice of different strains. (Gorer 1937a). The sarcoma grew in all of the A strain mice but not in the C57 mice. The F1 hybrid of C57 x A, and the F1 x A backcross were all susceptible. The proportion of susceptible mice in the F1 x C57 backcross and the F2 generation suggested that the susceptibility was controlled by two genetic loci.

These results with tumour susceptibility confirmed what others had found before Gorer, that the tumour susceptibility was controlled by genetic factors. The major breakthrough that Gorer made was the demonstration that one of the tumour resistance genes was the same gene that coded for antigen II. When Gorer typed his parental and hybrid animals with anti-II serum he discovered that all of the mice that lacked

antigen II rejected the tumour. Of mice which had survived, about half had the antigen. The conclusion from these experiments was that one of the two genes coding for resistance was also coding for antigen II. This was supported by the finding that the sera of mice that rejected the tumour contained antibodies specific for antigen II.

Thus antigen II could be detected by xenogeneic (human) and allogeneic (mouse) antibodies, and more importantly was shared by normal and malignant tissue.

On the basis of these results Gorer formulated an immunological theory of transplantation (Gorer 1938). The hitherto mysterious tumour resistance (or susceptibility) factors were, according to Gorer, antigens that were shared by malignant and non-malignant tissues. Individuals of the same species or of different inbred strains differed in these antigens and when tissues were exchanged between such individuals, the antigens elicited an immune response that resulted in the destruction (rejection) of the transplant. He favoured a mechanism by which allogeneic transplanted tissue was rejected by the action of antibodies.

The significance of Gorers results was lost to many of his contemporaries. It was only after the second world war that the importance of his findings were realized in connection with the work of Peter Medawar. Medawar showed in 1944, that there was a distinct histological difference between skin grafts exchanged between two allogeneic rabbits (allografts) and skin grafts exchanged between different parts of the body, in the same rabbit (autografts). The rejection process was rapid for the allogeneic skin grafts. In the first week after transplantation, auto and allografts were indistinguishable: they healed in and re-established blood supply. In the second week, allografts became inflamed, swollen and their dermis infiltrated with masses of lymphocytes and

monocytes. Blood vessels were destroyed in the areas of infiltration, and the whole graft began to degenerate. The destruction of the graft was complete by the end of the third week, and the wound healed by ingrowth of the host epidermis. In contrast autografts remained unchanged.

If a second allograft was transplanted into the same recipients, accelerated rejection took place, the degenerative changes indicative of rejection occurring during the first week. Apparently the first graft had sensitized the recipient, which then rejected the second graft in an accelerated fashion. In addition sensitization was systemic, since no matter on what part of the body the graft was placed, it was always rejected in an accelerated fashion. The characteristics of the rejection process; accelerated rejection of second grafts, specificity and systemic character of sensitization were considered by Medawar as evidence for the immunological nature of the allograft rejection (Medawar 1944).

Further experimentation by Medawar (1946) demonstrated that intradermal injections of allogeneic leucoytes also had a sensitizing effect on subsequent skin grafts, leading him to propose that rejection of skin grafts was due to recognition of antigens similar to those described earlier by Gorer (1936) in mice. These antigens were common to both skin and leucocytes. Gorer and Medawar, however favoured different mechanisms for rejection of transplanted tissue. Gorer considered that rejection was a humoral process, whereas Medawar favoured cellular mechanisms of rejection (reviewed by Medawar 1985).

Gorer was able to extend the genetic analysis of blood group antigens and confirm the validity of his theories after World War II, using congenic mouse strains. These are lines of inbred mice first developed by Snell, which differ from sister lines by a small portion of the genome. Snell used a complex experimental protocol involving repeated

backcrossing and measurement of resistance to transplanted tumours, to derive congenic lines which differed from sister lines only at loci determining tumour resistance (Snell 1948). His aim was to produce lines different from each other at only the tumour resistance locus, and no other. This proved to be impossible. Upon analysis of his lines, Snell found that some of them retained not only tumour resistance but also some other marker gene of the donor strain. For example the congenic line A.CA, which originated from the inbred strain A and an outbred stock CA carrying the mutation fused tail (Fu), was not only resistant to A-strain tumours but also displayed the shortened, crooked tails caused by the Fu gene. Subsequent genetic tests demonstrated that the visible marker (Fu) was linked to the resistance gene, and allowed the resistance gene to be placed into a linkage group.

Gorer was able to test some of Snell's congenic lines with the antisera produced by mice which had rejected tumour transplants. Analysis of the A.CA line revealed that in addition to the fused tail and resistance gene , the line also inherited the allele coding for the absence of antigen II from the CA stock. Snell and Gorer then showed that the resistance factor inherited by A.CA and CA was most probably an allele at the locus coding for the antigen II (Gorer et al. 1948) and lead Gorer to propose presciently that "There may be a long series of alleles at this locus." Snell suggested that the tumour resistance factors be called histocompatibility (H) genes, using the prefix "histo" because the genes controlling tumour resistance were likely to be the same as those determining resistance to tissue transplants in general. Individual genes were to be distinguished by serial numbers (Snell 1948). Later Gorer and Snell followed this proposal by christening the first identified histocompatibility gene (antigen II), histocompatibility-2, later abbreviated to H-2. The assignment of H-2 to a

linkage group subsequently allowed mapping of H-2 to chromosome 17, more than 2 decades later (Miller and Miller 1972).

Further genetic analysis of congenic lines in tumour grafting and skin grafting experiment revealed the presence of two types of H gene (reviewed by Graff and Bailey 1973). On the basis of the speed of allograft rejection, H genes could be divided into those which had a major (strong) effect and those which has a minor or weak effect. Later studies revealed that all the major H genes formed a complex occupying a single region of chromosome 17, the same region into which the original gene for antigen II was placed. When a similar region was discovered in other species, H-2 was referred to as the major histocompatibility complex (MHC) of mice.

The work of Gorer and Snell pioneered two different approaches to histocompatibility testing. Gorer looked at the problem of transplantation immunity from the humoral aspect. He utilized antibodies produced in one individual against the MHC antigens of another individual. In order to analyze the antibodies he used serological methods, such as the haemagglutination assay and later the cytotoxic test developed in collaboration with O'Gorman (Gorer and O'Gorman 1956). He was followed in this serological approach by many others (reviewed by Amos et al. 1955, Gorer et al. 1959) whose studies demonstrated that:

a) the H-2 molecules were antigenically complex, each consisting of a large number of determinants.

b) many of the inbred strains differ in their H-2 antigens.

c) the H-2 complex consists of several linked genes coding for separate, serologically detectable molecules.

Gorers work with antisera set the pattern for the discovery and analysis of the MHC in humans, the HLA complex, a discovery based on extensive use of serological methods. Gorer also made several contributions to

clinical transplantation. Firstly, he was the first to demonstrate a immunological mechanism for transplant rejection. Second, his typing of tumours to determine the success of the transplant, has clear analogy with the present day clinical tissue typing for organ or bone marrow transplants. Third, he devised the cytotoxicity test (Gorer and O'Gorman 1956) which, in it's modern miniaturised form, is used in tissue typing laboratories all over the world. These major contributions originated from his luck in choosing mouse erythrocytes for analysis (Humphrey 1984). Mouse erythrocytes unlike those of human and other species (e.g. cattle, dog, horse and rabbit) express what are now termed class I MHC antigens, without which the erythrocyte agglutination assay would not have revealed the H-2 system.

Snell's "histogenetic" approach was based on cellular immunity. Snell measured the *in vivo* compatibility of tissues from different individuals, by transplanting tumour or skin grafts. The analysis of congenic mice strains allowed him to postulate the presence of histocompatibility (H) genes, which determined the fate of transplanted tissue. These tests and the *in vitro* correlates; the MLR and cell mediated lymphocytotoxicity (CML) test, revealed the great complexity of the chromosomal region containing the H genes. It became apparent that each mouse strain possessed a characteristic cluster of polymorphic H genes, which were inherited together as a haplotype on the same parental chromosome. In addition the cellular approach pioneered by Snell also revealed the presence of minor histocompatibility genes which did not map to the H-2 complex, and could not be detected by antisera.

1.2 Discovery of the human leucocyte antigen (HLA) complex

Leucopaenia is a haematological disorder that results in the reduction in the number of circulating leucocytes and is now known to be caused by diseases marked by bone marrow infiltration (aplastic anaemia, leukaemia) or by drugs which act via the same mechanism (reviewed by Wells 1984). In Paris in the 1950s the causative mechanism of leucopaenia attracted the interest of Jean Dausset. He wondered whether the low numbers of leucocytes in leucopaenic patients, could be due to the destructive effects of leucocyte specific autoantibodies and complement. To test whether leucopaenic patients develop leucocyte-specific antibodies he mixed together serum from one patient with leucocytes obtained from the bone marrow of another individual. Massive clumps were observed, which indicated that like erythrocytes, leucocytes could be agglutinated by antibodies (leucoagglutinins) present in blood. By using this test Dausset was able to find leucoagglutins in the blood of most leucopaenic patients. The leucoagglutinins could not be autoantibodies, as the group of patients he studied had received multiple blood transfusions. All the antibodies he could detect were specific for the white blood cells of the blood donors rather than the patients own leucocytes (Dausset 1954).

Rose Payne, working at Stanford in the United States, was also interested in the possible immunological causes of leucopaenia, and following the lead of Dausset, was able to demonstrate leucoagglutinins in the sera of leucopaenic patients. She also observed that leucoagglutinins could be found in the sera of other patients, besides those with leucopaenia. The one factor all these patients had in common was a history of multiple transfusions. Many of the patients developed a characteristic febrile (high fever) reaction after six or more transfusions. These observations suggested that the leucoagglutinins had nothing to do

with leucopaenia, and that they were antibodies against alloantigens expressed on the transfused white blood cells. Later she observed that febrile reactions could occur after the first transfusion, but only in women who had given birth to several children. This observation indicated that the mothers were producing antibodies against the white blood cells of their foetuses. In 1958 she obtained convincing evidence that pregnancies immunized the mothers against foetal leucocyte antigens inherited from their father (Payne and Rolfs 1958).

These same conclusions had also been reached independently by Jon van Rood in Leiden, Holland. Van Rood followed up an observation made with sera obtained from a mother who had given birth to twins and developed an adverse reaction to transfused blood despite matching for blood group antigens. Like Payne, van Rood was able to show that the serum from this woman was able to agglutinate leucocytes from the donor. Since this woman had given birth to several children , van Rood reasoned that she had developed leucoagglutinins in response to paternal antigens of the foetus. Later, he proved that multiple pregnancies immunized mothers against leucocytes leaked from the foetus into the mothers circulation (van Rood et al. 1958).

Dausset had meanwhile characterized leucocyte antigens as distinct entities. He typed a large panel of leucocyte donors and noticed that leucocytes of three donors were rarely agglutinated, which suggested that these individuals lacked the antigen that was otherwise frequent in the French population. Dausset designated this antigen MAC (after the first letters of the surname of the three individuals) and by typing monozygotic and dizygotic twins he proved that it was genetically controlled (Dausset 1958). He also demonstrated in a manner reminiscent of Gorer's experiments with antigen II, that transfusion of blood from MAC+ve into

MAC-ve individuals resulted in MAC-specific antibodies. MAC antigen is now known as HLA-A2.

Van Rood introduced an important innovation to resolve the complex reactivity pattern obtained from post-transfusion sera or sera obtained from pregnant women. He reasoned that statistical analysis was needed to determine reactivity patterns of the sera, and since these calculations were laborious, he programmed a computer to perform them. The result of this was that Van Rood and colleagues were able to define leucocyte antigen 4* controlled by a single locus with at least two alleles, 4a and 4b. (Van Rood and van Leeuwen 1963). Payne also realized the importance of statistical analysis and enlisted the help of Walter Bodmer to analyse complex antibody reactivity patterns. One year later than van Rood (Payne et al. 1964) they were also able to demonstrate a leucocyte antigen locus, with at least three alleles: LA1, LA2 and a blank (absence of LA1 and LA2).

At the beginning of the 1960s three systems of leucocyte antigens had been defined, Dausset's MAC, van Rood's 4a/4b, and Payne-Bodmer's LA. The relationship between these antigens was unclear and a combination of new typing techniques and international collaboration was needed to resolve them.

New typing techniques were developed to overcome the deficiencies of leucoagglutination methods which were technically unreliable and extremely tricky to set up. The new techniques were based on the cytotoxicity mediated by complement upon meeting MHC specific antibodies bound to cells.

In the lymphocytotoxicity test, or as it was commonly known the cytotoxic test, serial dilutions of antibodies were mixed with cells and Guinea pig complement (Gorer and O'Gorman 1956). The mixtures were

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* They originally discovered three others which did not withstand further statistical analysis.

incubated and the number of dead cells determined using dyes such as trypan blue or eosoin. These tests were originally carried out in test tubes which meant that testing large numbers of antisera and cells was cumbersome.

Two important innovations allowed the cytotoxicity test to be used for typing human leucocyte antigens. The first was the discovery that rabbit serum was a good source of complement for reactions involving human alloantibodies (Walford et al. 1964). Guinea pig serum which was the source of complement for analysis of the H-2 system, was found not to work for all human alloantibodies.

The second important innovation was the miniaturisation of the test (Terasaki et al. 1964). This was achieved by developing plastic plates with small wells, reducing dramatically the amount of sera required, and also making screening more convenient as the whole plate could be screened on an inverted microscope. This advance resulted in increased sensitivity of the test, accelerated its execution and made screening of large panels of cells and antisera possible.

The different reagents and techniques used in individual laboratories for typing histocompatibility antigens, meant that direct comparison of typing results was impossible. The initiation of International Histocompatibility Workshops which gathered together all the leading investigators for exchange of reagents, results and techniques, proved to be a turning point in the definition of the human MHC system. The first Histocompatibility Workshop was organized by Amos and held in Washington in 1964. The workshop was held for the purpose of comparing a series of different approaches to histocompatibility testing. At this workshop, no correspondence between the data obtained by different workers was seen, which was not surprising given the different

techniques and reagents used. However by standardizing reagents and international collaboration, it was established at the second Histocompatibility Workshop held in Leiden in 1965, that the antigens defined by different techniques were the same. This workshop set the pattern for subsequent workshops where exchange of reagents, results and techniques lead to the definition of new histocompatibility antigens. It was at this meeting that both Dausset (1965) and van Rood (1965) proposed that most of the antigens were controlled by a single complex of loci analogous to the H-2 complex of the mouse. Evidence for these proposals came from a study by Batchelor and Chapman (1967). They carried out family studies and demonstrated the inheritance of distinct parental MHC antigens in the offspring, either in positive association (on the same haplotype) or in "repulsion" (on the other haplotype).

Large scale family studies formed the main component of the third Histocompatibility Workshop meeting held in Torino in 1967. In this workshop all the heads of the major tissue typing laboratories were made to type the same set of blood samples from large Italian families, each using their own methods. The results were pooled and analyzed by computer and it was concluded that there was one major complex of genes coding for human leucocyte antigens. The next problem was standardizing the nomenclature for these antigens, as different laboratories had given this complex different designations, a problem foreseen by Payne and Bodmer (1964), and one that was to plague the study of histocompatibility antigens in subsequent years. A diplomatic solution was reached in that a new nomenclature was to be used, the complex was now known as the "HLA" or human leucocyte antigen complex. In subsequent Histocompatibility Workshops serological definition of HLA loci began in earnest.

1.3 Discovery of HLA class II genes

By 1975, the combined efforts of many workers in Histocompatibility Workshops, had serologically defined three HLA loci, HLA-A, HLA-B and HLA-C, each with multiple alleles. The complexity of the HLA system was increased when it was discovered that there was another set of molecules which mapped to the MHC but which had different functional effects when compared to the known serologically defined molecules. In 1977 Klein, suggested calling these two distinct molecules class I and class II, a designation which was supported by later studies revealing the different structures of the two kinds of molecules.

The HLA class II region or HLA-D region was originally mapped on the basis of the mixed lymphocyte reaction (MLR) which results from the co-culture of lymphocytes from genetically dissimilar individuals. Two groups (Bach and Hirschorn 1964, Bain et al. 1964) independently demonstrated that incubation of mixed lymphocyte cultures (MLC) for 3-4 days resulted in T cell blast transformation, and subsequent rapid cell division. Early MLR studies measured the response of populations of cells from both individuals. Later techniques such as mitomycin C treatment (Bach and Vyononow 1966) or X-irradiation (Kasakura and Lowenstein 1965) were used to inactivate one population and thus make the reaction unidirectional. MLR stimulating determinants were found to map to a locus of the HLA region (Amos and Bach 1968) which was distinct from the HLA loci then known to be coding for serologically detectable antigens (Yunis and Amos 1971). A further refinement to the MLR test came from the use of homozygous typing cells (HTC), stimulator cells which were homozygous for a particular MLR activating gene. During the 1975 International Histocompatibility Workshop six new alleles controlling

MLR determinants (Dw types) were provisionally defined using this method, and assigned to a new locus, HLA-D.

Evidence that class II antigens in man could be detected serologically came from studies principally by van Leeuwen and van Rood. Van Leeuwen and her colleagues found that MLR could be blocked using antisera produced by cross immunization of individuals that were identical at the serologically detectable loci (van Leeuwen et al. 1973). They also showed that only a proportion of the lymphocytes could be stained with the antisera, an observation that can now be explained by the limited tissue distribution of class II antigens. More detailed characterization of such antisera lead van Rood to propose a new series of antigens (van Rood et al. 1975). A major problem of analyzing the determinants suggested by the studies of van Leeuwen and van Rood was that their antisera only reacted with a small proportion of peripheral blood lymphocytes. This problem was overcome by the development of methods for enrichment of B cells allowing analysis of sera detecting class II antigens (as reviewed by Bodmer 1977). This lead to the definition of a new series of allelic determinants after collaborative studies for the 7th International Histocompatibility Workshop in 1977. The specificity of putative class II specific antibodies was resolved by sorting antibodies on the basis of typing reactions on the same panel of individuals and in selected families. The antigenic determinants defined by these antibodies seemed to be governed by a locus residing in the vicinity of HLA-D but possibly not identical with D. The uncertainty about whether the new locus was identical with D, lead to the designation of "DR" or D-related antigens.

The specificity of HLA class II antisera was improved by absorbing out anti-class I antibodies using class I positive, class II negative cells such as platelets. This advance led to the subsequent identification of many of the

DR alleles. There were however, antibodies which did not fit in the DR series, although they reacted with HLA-controlled determinants. Tosi and colleagues typed a mutant B cell line, (Daudi) which lacked HLA class I, with a number of anti-DR sera (Tosi et al. 1978). They defined two determinants which they called DC1 and DC2, which were in linkage disequilibrium with DR.

A number of groups then identified further non-DR determinants such as MB (Duquesnoy et al. 1979), MT (Park et al. 1980), LB (Termijtelen et al. 1980) and DS (Goyert and Silver 1981). Most of these determinants were clearly associated in a complex way with the original DR determinants, but the nature of this association, as well as the relationships between the different systems remained a subject of controversy.

Subsequently many biochemical studies and the collaboration of the groups in Histocompatibility Workshops made order out of the chaos. For example biochemical studies clearly demonstrated that some determinants from the DC, MT and LB series were identical, and defined a locus distinct from DR (Shackelford et al. 1981). The new locus was named DQ at the 9th Histocompatibility workshop (Albert et al. 1984), with serological definition of three antigens. DQ1, DQ2 and DQ3. The DR and DQ series show a marked degree of linkage disequilibrium, that is, certain alleles of the two series tend to occur together , more often than would be expected from their individual gene frequencies.

Two of the MT series (MT2 and MT3) of determinants were shown to be carried by the same molecules as those carrying the DR determinants. These determinants were shown to be supertypic (public) determinants, or determinants shared between different haplotypes. For example MT2 was shown to associate with DR3, DR5, DR6 and DR8, whereas MT3 associated

with DR4, DR7 and DR9. These determinants were renamed DRw52 and DRw53 respectively at the 9th International Histocompatibility Workshop in 1984 (Albert 1984).

Meanwhile a third antigenic system had been defined (Shaw et al. 1980) and was called SB (secondary B cell antigens). These were discovered by secondary stimulation of mixed lymphocyte cultures between responders and stimulators matched for class I, DR and DQ determinants. SB antigens were distinct from other class II antigens as recombinants between SB and the rest of the HLA complex could be detected. The SB designation was changed to DP after the 9th Histocompatibility Workshop.

The relationship between DR (and other) antigens and the Dw specificities, defined by T cell responses, was to become an obsession of many laboratories in the 1980s. Studies using T cell clones, biochemical techniques and monoclonal antibodies illustrated the complexity of the HLA-D region but clear answers to the relationship between serological and T cell defined polymorphisms, were not revealed until the 10th Histocompatibility Workshop. For the first time the reactivity patterns of T cell clones on a panel of homozygous B-LCL were correlated with the polymorphisms detected by serological typing, biochemical studies, and RFLP analysis. The main conclusions from the study were that the majority of T cell defined Dw types could be distinguished on the basis of polymorphism in the DRß molecule and that alloreactive T cell clones were able to define unique polymorphisms not defined by other means (discussed in section 1.4.2).

1.4 Polymorphism of HLA class II gene products

Study of the polymorphism of HLA gene products is important for understanding the structural complexity of HLA and how this relates to function. Despite the polymorphism of the HLA system, it is striking that

there are many examples of diseases that have a strong association with HLA antigens (Batchelor 1984). Many of these disease associations were originally defined with serological reagents which may not have revealed the full extent of HLA polymorphism. More precise definition of HLA polymorphisms has in some cases revealed more significant disease associations (reviewed by Batchelor 1984).

Investigation of HLA polymorphisms is also important for matching donors and recipients for clinical transplantation, as post-transplant survival times have been shown to be improved with HLA class II matching (reviewed by Morris et al. 1987). This may be especially important in the case of bone marrow transplantation, where prevention of graft versus host disease (GVHD) in HLA matched but unrelated donors is a major problem (Kaminski et al. 1989).

1.4.1 Serological methods

Serological methods were originally used to define antigens mapping to the MHC of mouse (Gorer 1936) and man (Dausset 1952). In spite of the development of more sensitive molecular biology and biochemical techniques, the lymphocytotoxicity test (Gorer and O'Gorman 1954, Terasaki et al. 1964) remains the major method of HLA typing. The main advantage of serological tissue typing is that it is a cheap, quick and reliable technique (tissue types can be obtained in one day), allowing the study of many samples. Comparison of results between laboratories is also convenient, as antisera can be exchanged easily by post between laboratories for typing of local HLA variants.

Anti-sera for HLA typing is usually obtained from either multiparous women or patients who have had blood transfusions. The major drawback of these anti-sera, is they contain HLA-specific antibodies which are heterogeneous in terms of immunoglobulin subtype and specificity.

The complex reactivity patterns produced by such antisera can only be resolved by statistical analysis. The first step of this analysis to screen a number of antisera on a large panel of unrelated individuals. to group together antisera with similar reactivity patterns. Secondly antisera with similar reactivity patterns are screened on a panel of previously typed individuals, to determine their specificity. Although antisera have a long storage life, it is difficult to obtain repeat batches of antisera, so screening of new antisera has to be carried out on a regular basis.

An indication of the polymorphism defined by serological reagents, is shown in the list of HLA antigens in Table 1.1. Collaborative studies for the 10th International Histocompatibility Workshop assigned 24 HLA-A , 50 HLA-B, 11 HLA-C, 18 HLA-DR, 9 HLA-DQ and 6 HLA -DP antigens. The relative decrease in the number of new HLA class I specificities defined by serology suggests that such techniques are reaching the limits of their sensitivity (Bodmer 1989).

The demonstration that immortalized lines of antibody producing cells could be generated by somatic cell hybridization (Kohler and Milstein 1975), appeared to answer the HLA serologists prayers. The use of homogenous antibodies produced by a single B cell clone would overcome many of the problems produced by the heterogeneous and complex antisera. However fourteen years on from this discovery, many clinical laboratories are still using polyclonal antisera reagents. The reason for this is that few mouse antibodies have been produced which recognize polymorphic determinants of HLA i.e. determinants which are specific for an allelic HLA gene product . The majority of antibodies produced have been against monomorphic determinants, that is determinants shared by

TABLE 1.1 COMPLETE LISTING OF RECOGNIZED HLA SPECIFICITIES

A	B	C	D	DR	DQ	DP
A1	B5	Cw1	Dw1	DR1	DQw1	DPw1
A2	B7	Cw2	Dw2	DR2	DQw2	DPw2
A3	B8	Cw3	Dw3	DR3	DQw3	DPw3
A9	B12	Cw4	Dw4	DR4	DQw4	DPw4
A10	B13	Cw5	Dw5	DR5	DQw5 (w1)	DPw5
A11	B14	Cw6	Dw6	DRw6	DQw6 (w1)	DPw6
Aw19	B15	Cw7	Dw7	DR7	DQw7 (w3)	
A23 (9)	B16	Cw8	Dw8	DRw8	DQw8 (w3)	
A24 (9)	B17	Cw9 (w3)	Dw9	DR9	DQw9 (w3)	
A25 (10)	B18	Cw10 (w3)	Dw10	DRw10		
A26 (10)	B21	Cw11	Dw11 (w7)	DRw11 (5)		
A28	Bw22		Dw12	DRw12		
A29 (w19)	B27		Dw13	DRw13 (w6)		
A30 (w19)	B35		Dw14	DRw14 (w6)		
A31 (w19)	B37		Dw15	DRw15 (2)		
A32 (w19)	B38 (16)		Dw16	DRw16 (2)		
Aw33 (w19)	B39 (16)		Dw17 (w7)	DRw17 (3)		
Aw34 (10)	B40		Dw18 (w6)	DRw18 (3)		
Aw36	Bw41		Dw19 (w6)			
Aw43	Bw42		Dw20	DRw52		
Aw66 (10)	B44 (12)		Dw21			
Aw68 (28)	B45 (12)		Dw22	DRw53		
Aw69 (28)	Bw46		Dw23			
Aw74 (w19)	Bw47		Dw24			
	Bw48		Dw25			
	B49 (21)		Dw26			
	Bw50 (21)					
	B51 (5)					
	Bw52 (5)					
	Bw53					
	Bw54 (w22)					
	Bw55 (w22)					
	Bw56 (w22)					
	Bw57 (17)					
	Bw58 (17)					
	Bw59					
	Bw60 (w40)					
	Bw61 (w40)					
	Bw62 (15)					
	Bw63 (15)					
	Bw64 (14)					
	Bw65 (14)					
	Bw67					
	Bw70					
	Bw71 (w70)					
	Bw72 (w70)					
	Bw73					
	Bw75 (15)					
	Bw76 (15)					
	Bw77 (15)					
	Bw4					
	Bw6					

alleles of a particular locus. eg. anti-DR, or anti-DQ. Although there are well characterized antibodies against subtypes of HLA class I antigens (López de Castro 1989), there are few monoclonal antibodies with specificity for polymorphic determinants on HLA class II molecules (reviewed by Marsh and Bodmer 1989).

The reason for the lack of success in producing allele-specific reagents may be due to species differences between mouse and man, that is, mouse B cells may preferentially recognize xenogeneic determinants common to all class II molecules rather than allogeneic determinants which differ between class II molecules.

One way to focus the mouse immune system onto polymorphic portions of human class II molecules, is to express human molecules in mouse cells by gene transfection (Germain and Malissen 1986). Antibodies against HLA-DP have been made using this technique (Heyes et al. 1986).

There may however be structural reasons why antibodies detecting polymorphic determinants of HLA class II molecules, cannot be made. For instance, nucleotide sequencing of HLA-DR alleles reveals that modules of sequence are shared between alleles (Bell et al. 1989). The presence of these modules in antibody accessible sites (Brown et al. 1988) on the class II molecule could explain the difficulties in generating allele specific anti-DR monoclonal antibodies. In addition many of the amino acid residues which are polymorphic between DR alleles, are situated in the antigen binding site and are unlikely to be accessible to antibody (Brown et al. 1988).

1.4.2 Cellular Methods.

1.4.2.1 Homozygous typing cells (HTC).

One method that has been used extensively to define D-region determinants has been the homozygous typing cell (HTC) test (Mempel et

al. 1973), which is based on the mixed lymphocyte reaction. Homozygous typing cells are derived from two sources. The first is from donors who have come from a consanguineous union (between related individuals), resulting in homozygosity for all HLA region genes. Alternatively HTC are derived from families where the parents share one haplotype. All possible combinations from the family are typed by MLR, and the offspring who neither stimulates or is stimulated by cells from the parents is presumed to be homozygous. These cells can then be used as a source of HTC stimulators for typing unrelated responder individuals in primary MLR. The use of this technique has allowed definition of determinants of the HLA-D region. Such determinants are usually referred to as Dw types, as the nature of the determinants is ill defined and may involve the cumulative T cell reactivity to multiple class II differences (Bach 1985). As with serologically defined determinants, nomenclature battles have also been fought over T cell- defined determinants, some groups referred to these as lymphocyte defined determinants (LD, Segall et al. 1980). A common nomenclature based on Dw types was agreed after the 10th Histocompatibility workshop (1989).

The use of HTC for defining HLA class II polymorphisms has a number of disadvantages. Firstly, because HTC are used in primary MLR, it is sometimes difficult to distinguish between positive and negative typing responses, as all unrelated cells will provide a proliferative response of some sort. Thus an arbitrary cut off has to be selected. This is easy when the response is clearly biphasic but is more difficult when there is no clear biphasic response. Secondly, the responses to HTC may represent an aggregate of responses associated with DR, DQ, DP, DRw52 or DRw53 (Bach et al. 1984). Finally, HTCs are difficult to find in an outbred population.

1.4.2.2. Primed lymphocyte typing (PLT).

The primed lymphocyte typing (PLT) test (Sheehy et al. 1975) involves HLA typing of family members and selecting pairs sharing one haplotype (usually parent child combination). In each pair one member is used as a responder and the other as a stimulator, primary MLR is induced and the responding cells are frozen when they are in the resting phase (usually 10 days after initiation of MLR). At the time of typing the frozen cells (PLT reagents) are thawed and restimulated in secondary MLR by a panel of cells from unrelated individuals. Since the cells have been primed, proliferative responses can be assayed after 48 hours rather than after 5-6 days as for HTC. Responses at 48 hours against antigens involved in the priming will be strong, whereas responses against antigens not involved in the priming will be minimal.

The PLT has similar problems to the HTC test in that the PLT reagents will consist of multiple clones of cells recognizing different HLA class II determinants. PLT reagents have however been able to show the presence of determinants not detected by serological typing. Segall and colleagues (1980) demonstrated that apparently HLA identical individuals could stimulate each other in MLR (Segall et al. 1980), and that the stimulating determinants could be defined by PLT reagents. This is one of many examples of T cells recognizing determinants not recognized by serological reagents and demonstrates the power of T cell reagents to analyze functional polymorphism of HLA class II molecules. The complex relationships between DR types and Dw types are summarized in Table 1.2.

1.4.2.3 T cell clones.

PLT and HTC responses are likely to consist of reactivity to multiple HLA class II antigens. A homogeneous population of T cells capable of long term growth and with specificity for single HLA class II gene

Table 1.2. Summary of HLA Class II haplotypes. (Modified from Flomenberg et al. 1989).

HLA-DR		HLA-Dw	HLA-DRw52/53	HLA-DQ	HLA-B	Ethnic group
DR1		Dw1		DQw5 (DQw1)	B35	
		Dw20		DQw5 (DQw1)	B14	Jewish
DR2	DRw15	Dw2		DQw6 (DQw1)	B7	
	DRw15	Dw12		DQw6 (DQw1)	Bw52	Japanese
	DRw16	Dw21		DQw5 (DQw1)		
	DRw16	Dw22		DQw7 (DQw3)		Warao Indian
DR3	DRw17	Dw3	DRw52	DQw2	B8	
	DRw17	Dw3 variant	DRw52	DQw2	B18	
	DRw18	Dw new	DRw52	DQw4	Bw42	Black
DR4		Dw4	DRw53	DQw7 (DQw3)	B44	
			DRw53	DQw8 (DQw3)	Bw62	
		Dw10	DRw53	DQw8 (DQw3)	B38	Jewish
		Dw13	DRw53	DQw7 (DQw3)		
			DRw53	DQw8 (DQw3)		
		Dw14	DRw53	DQw8 (DQw3)	Bw60	
		Dw15	DRw53	DQw4		Japanese
DR5	DRw11	Dw5	DRw52	DQw7 (DQw3)		
	DRw11	DB2	DRw52	DQw1		
	DRw12	DB6	DRw52	DQw7 (DQw3)		
DRw6	DRw13	Dw18	DRw52	DQw6 (DQw1)		
	DRw13	Dw18	DRw52	DQw1		
	DRw13	Dw19	DRw52	DQw6 (DQw1)		
	DRw14	Dw9	DRw52	DQw5 (DQw1)		
	DRw14	Dw16	DRw52	DQw7 (DQw3)	Bw62	American Indian
DR7		Dw7	DRw53	DQw2		
		Dw17	DRw53	DQw2	B44	
		Dw11	blank	DQw9 (DQw3)	Bw57	
		Dw11	DRw53	DQw9 (DQw3)	Bw57	
		DB1	DRw53	DQw7 (DQw3)	B13	
DRw8		Dw8.1		DQw4		Caucasian
		Dw8.2		DQw4		American Indian
		Dw8.3		DQw1		Oriental
		Dw8.3		DQw7 (DQw3)		Dutch
DR9		Dw23	DRw53	DQw9 (DQw3)		Oriental
DRw10		Dw new		DQw5 (DQw1)		Jewish
DR Bon		Dw new		DQw1		French

products, is desirable for understanding the contribution of individual DR, DQ or DP molecules to a particular Dw haplotype.

A crucial development in this regard was the discovery that supernatants derived from T cells activated by mitogen (Morgan et al 1976) or allogeneic stimulator cells (Gillis et al. 1977) could support the long term *in vitro* growth of T cells. The active constituent of these supernatants was found to be T cell growth factor (TCGF) later named interleukin 2 (IL2). This discovery was of great importance for human studies, where ethical considerations mean that *in vitro* rather *in vivo* experiments have to be performed.

Alloreactive T cell clones with specificity for HLA class II determinants were first reported by Bach et al (1979). In this study the technique of limiting dilution was used to clone T cell blasts from a primary mixed lymphocyte culture. The limiting dilution technique involves serially diluting a cell suspension from the responder cell population until a concentration of one cell/ well or less is reached (reviewed by Lefkovits and Waldmann 1984). Thus the dilution of cells becomes the limiting factor. At the limiting dilution many of the wells contain no cells at all, and the majority of those that do contain cells, have no more than one cell. To allow T cell growth, each culture well also contains irradiated allogeneic stimulator cells and T cell growth factors. After a certain incubation time, individual culture wells are screened for T cell growth, wells positive for T cell growth are presumed to contain the progeny of a single cell.

The other main T cell cloning technique used in the late 1970s and early 1980s was the soft agar technique (Kornbluth and Dupont 1980), first used in the mouse system (Fathman and Hengarter 1978). In this technique a layer of feeder cells is grown on the bottom of a petri dish,

then X-irradiated. The feeder cells are then overlayed with a highly concentrated (dense) agar solution containing medium and IL2. On top of this another layer of less dense (soft agar) is poured containing the cells to be cloned. The density of cells in the top agar layer is such that individual cells are clearly separated from one another. When they divide, individual colonies are formed which can then be picked out and transferred separately into liquid medium with IL2. This method never achieved popularity for human studies, probably because of the many technically difficult steps involved and the low cloning efficiency (reviewed by Schwartz 1982).

The results from a number of studies have demonstrated that alloreactive T cell clones recognize a complex array of specificities; some correlating with known serological specificities (Zeevi et al. 1982, Rosen-Bronsen et al. 1986); others which could discriminate between subsets or "splits" of the serologically defined determinants. (Eckels and Hartzman 1982, Pawelec and Wernet 1980, Fraser and Strominger 1987). Large scale studies for the 10th Histocompatibility Workshop designated 17 DR-associated and 16 DQ-associated polymorphisms defined by T cell clones, in addition to those defined by serological typing (Flomenberg 1989).

Thus alloreactive T cell clones are valuable reagents for the study of HLA-D region. They are highly specific, alloreactive T cell clones can distinguish between DR molecules that can differ in only 1 amino acid (Flomenberg 1989), a difference not detected by serological typing. The use of clonal T cell reagents also allows more confident interpretation of third party reactivity, which can be ascribed to genuine cross-reactivity. The major advantage of using T cell clones to define polymorphisms of HLA class II antigens is that they will define functional determinants important in alloreactive or antigen-specific T cell responses.

The disadvantages of T cell clones are the same as for any other cells requiring long term culture, that is loss of function due to aging (Pawelec et al. 1988) or due to mycoplasma infection (Rosen-Bronson and Eckels 1987). In addition the fragile nature of T cell clones, means that exchange of these reagents between laboratories is difficult.

1.4.3 Two dimensional (2-D) gel electrophoresis.

The polymorphism of protein chains from HLA class II molecules can be studied by two dimensional (2-D) gel electrophoresis. This technique allows characterization of protein chains, in one direction chains can be distinguished on the basis of net charge whilst in the other direction chains are distinguished on the basis of molecular weight. The technique is very sensitive; the polymorphic nature of the DRß chains and the non-polymorphic nature of DRα chains, were first revealed by 2-D gels (Charron and McDevitt 1979).

In brief, the technique involves metabolically labelling HLA class II molecules with radioactive isotopes, then immunoprecipitating the labelled protein chains using HLA class II specific monoclonal antibodies. The immunoprecipitates are run on 2-D gels and a series of spots can be visualized which represent various species of mature and immature protein chains, which separate out on the basis of their net charge and their molecular weight. Polymorphism between HLA class II molecules is revealed by the relative positions of spots representing α and ß chains for individual molecules.

The main disadvantages of the technique are that multiple time-consuming steps are involved in preparing samples for analysis, and that a large number of metabolically active cells is needed initially. Amino acid differences which do not result in resultant net change in charge, cannot be resolved by 2-D gels (Lee et al. 1987).

1.4.4. Molecular genetic analysis of HLA class II antigens.

1.4.4.1 Restriction fragment length polymorphism (RFLP) analysis.

Polymorphism of the HLA class II antigens can also be studied at the genetic level by analyzing restriction fragment length polymorphisms (RFLP) of genomic DNA. These polymorphisms take the form of characteristic banding patterns which are produced when the genomic DNA is cut with restriction enzymes and then hybridized with allele specific probes eg. DRß or DQß. RFLP analysis has been used extensively to examine HLA and disease associations. The use of RFLP analysis to study of diseases with known HLA associations, has in many cases produced RFLP patterns with significantly increased incidence in disease populations (Cohen et al. 1984, Heard et al. 1989) These patterns are likely to reflect the greater precision with which RFLP analysis can type HLA polymorphisms (see below), rather than the presence of disease-specific DNA sequences.

Recently RFLP typing has become more applicable to routine tissue typing as banding patterns generated with certain enzyme:probe combinations have been shown to correlate with broad DR or Dw types (Bidwell 1988).

The RFLP technique has certain advantages. Many DNA samples may be stored for simultaneous analysis. In addition it is possible to analyze samples with multiple DNA probe: enzyme combinations using aliqouts of the same DNA sample. The long storage times of DNA may prove useful in retrospective studies when new disease associations come to light. The disadvantages of RFLP analysis are that, firstly the banding patterns obtained do not necessarily represent polymorphism in the coding sequence and thus the expressed product of HLA class II genes. Secondly the technique takes much longer than serological typing.

However, recent technical developments have increased the sensitivity of the technique. Allele-specific oligonucleotide DNA probes can be used to type cDNA sequences which have been amplified by the polymerase chain reaction (PCR).

1.4.4.2. Nucleotide sequencing.

Nucleotide sequencing of cDNA has allowed examination of genetic polymorphism for HLA class II alleles. The rapid progress made in this area has been illustrated by the nucleotide sequencing of DR alleles. The DRα gene was the first HLA class II gene to be sequenced in 1982 (Lee et al. 1982). Since then, the DRß chain genes from the most frequently occurring haplotypes of DR1-8 have been sequenced, most of these being obtained in the last two years (Bell et al. 1987, Bell 1989). The reason for the recent acceleration in the number of HLA class II sequences determined, has been the development of the PCR technique, which allows rapid expansion of specific sequences. The comparison of sequence data between DRß chain genes has demonstrated that some DR alleles have modules of sequence homology, suggesting common evolutionary origin (Bell et al.1989, Gorski et al. 1989). The comparison of DRß sequences has also revealed that there are three regions of hypervariability in the first domain ($ß_1$) at amino acid positions 9-14, 26-34 and 67-74 (see Section 1.6).

1.5 Genetic map of the HLA region

A series of genetic studies have mapped the HLA region to chromosome 6 (reviewed by Klein 1986). The genes and their protein products are classified as follows: class I genes (HLA-A, HLA-B, HLA-C) , class II genes (HLA-DR, HLA-DQ and HLA-DP) and class III genes, which code for components of the complement system (factor B, C2, C4A, C4B). The genes for tumour necrosis factor (TNF) and 21-hydroxylase also map

to the HLA region. A recent map of the HLA region is shown in Figure 1.1, and an expanded map of the HLA class II region is shown in Figure 1.2.

The HLA class II region consists of at least 16 genes. Within the DR subregion, the DRα gene has been established as being non-polymorphic across a large number of haplotypes (Bell et al. 1989). Two expressed DRß loci have been identified. The DRß1 locus which appears to be the most important single HLA class II locus for determining Dw types (Flomenberg 1989) and serological types (Bell et al. 1987). The other expressed DR locus in most haplotypes is either DRß3 or DRß4. DRß3 codes for DRw52 which is present on DR3, DR5, DR6 and DR8 haplotypes and is said to be "supertypic" as it is shared between these haplotypes. DRß4 codes for DRw53, a supertypic antigen shared by DR4, DR7 and DR9 haplotypes. The DR2 haplotypes are an exception to this rule as they appear to form their own family, with an invariant DR molecule shared by all DR2 haplotypes (Lee et al. 1987). The genomic organisation of HLA-DR2 has still to be determined. DR1 haplotypes also lack either DRw52 or DRw53 determinants, although RFLP analysis suggests that DR1 may be related to DRw52 (Bell et al. 1987).

The DQ subregion consists of one set of expressed α and ß chains, both of which are polymorphic. In addition there are a pair of DQ loci which are non-polymorphic, and apparently not expressed at the cell surface.

The DP subregion has an α:ß pseudogene pair and and a pair of expressed α and ß genes. The expressed α and ß genes are both polymorphic, although the extent of the polymorphism has yet to be realized (Bugawan et al. 1989). No polymorphism has been recognized at either the DNα or DOß locus, although the detection of specific mRNA for both loci suggests gene expression.

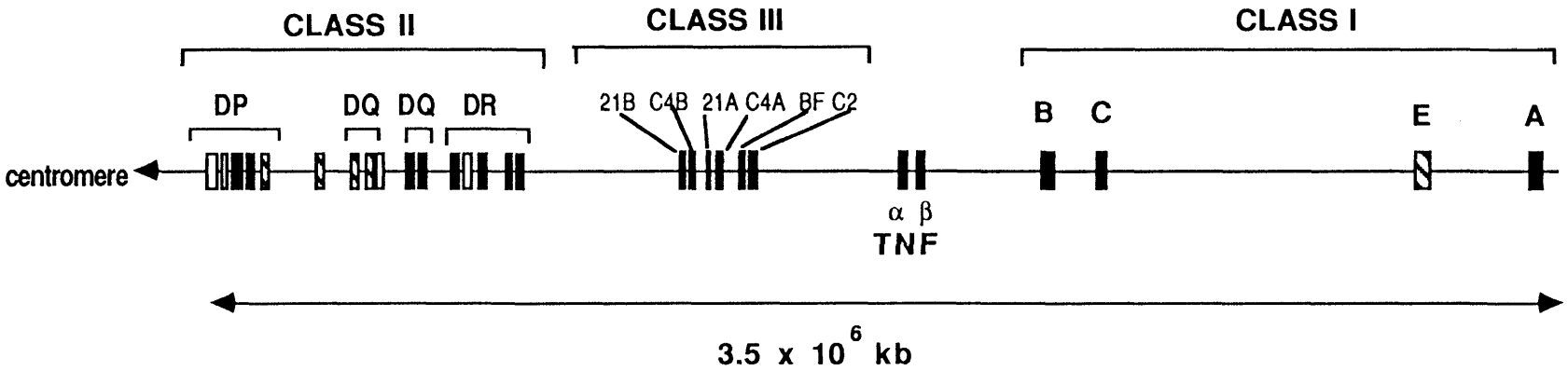


Figure 1.1 Physical map of the HLA region (adapted from Bodmer, 1989) . Full shading signifies that the gene is expressed, no shading that it is a pseudogene, and cross-hatching that mRNA has been detected, but as yet no cell surface product has been identified.

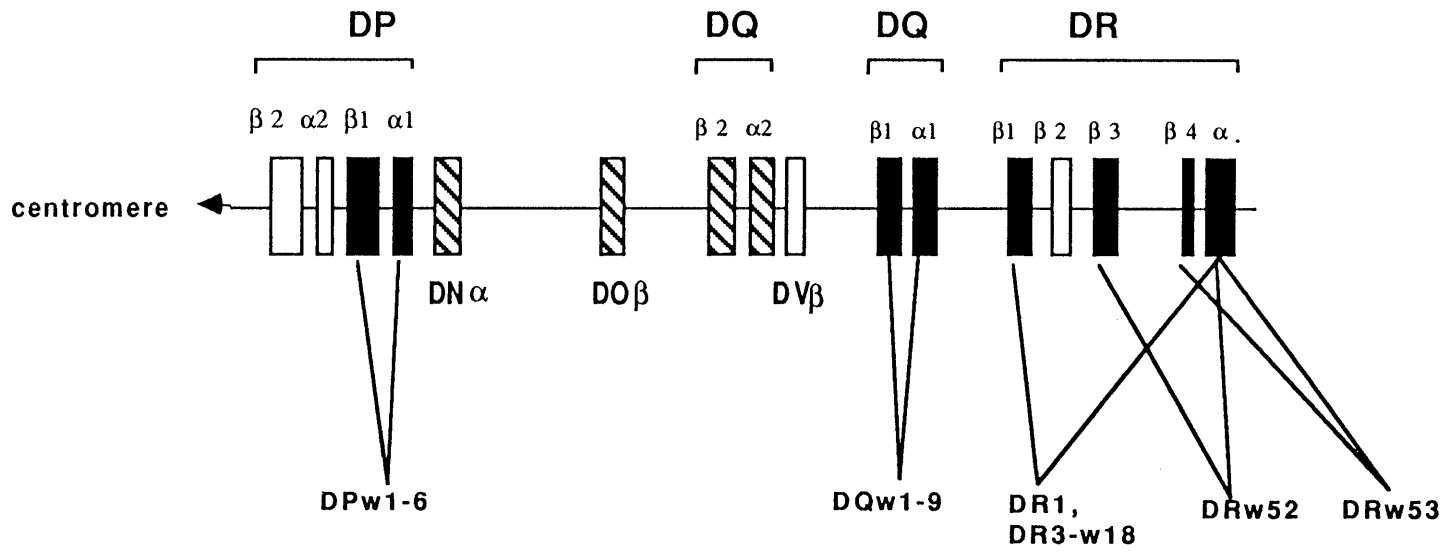


Figure 1.2. Genes and serologically defined products of the HLA Class II region (adapted from Bodmer 1989). Full shading indicates that the gene is expressed, no shading that it is a pseudogene and cross-hatching indicates that mRNA has been detected but no cell surface molecule has yet been identified. The number of serologically defined antigens which were assigned in the Xth Histocompatiblity workshop are shown for each locus. At the time of writing no nomenclature had been agreed for the two DRß chains expressed on DR2 haplotypes.

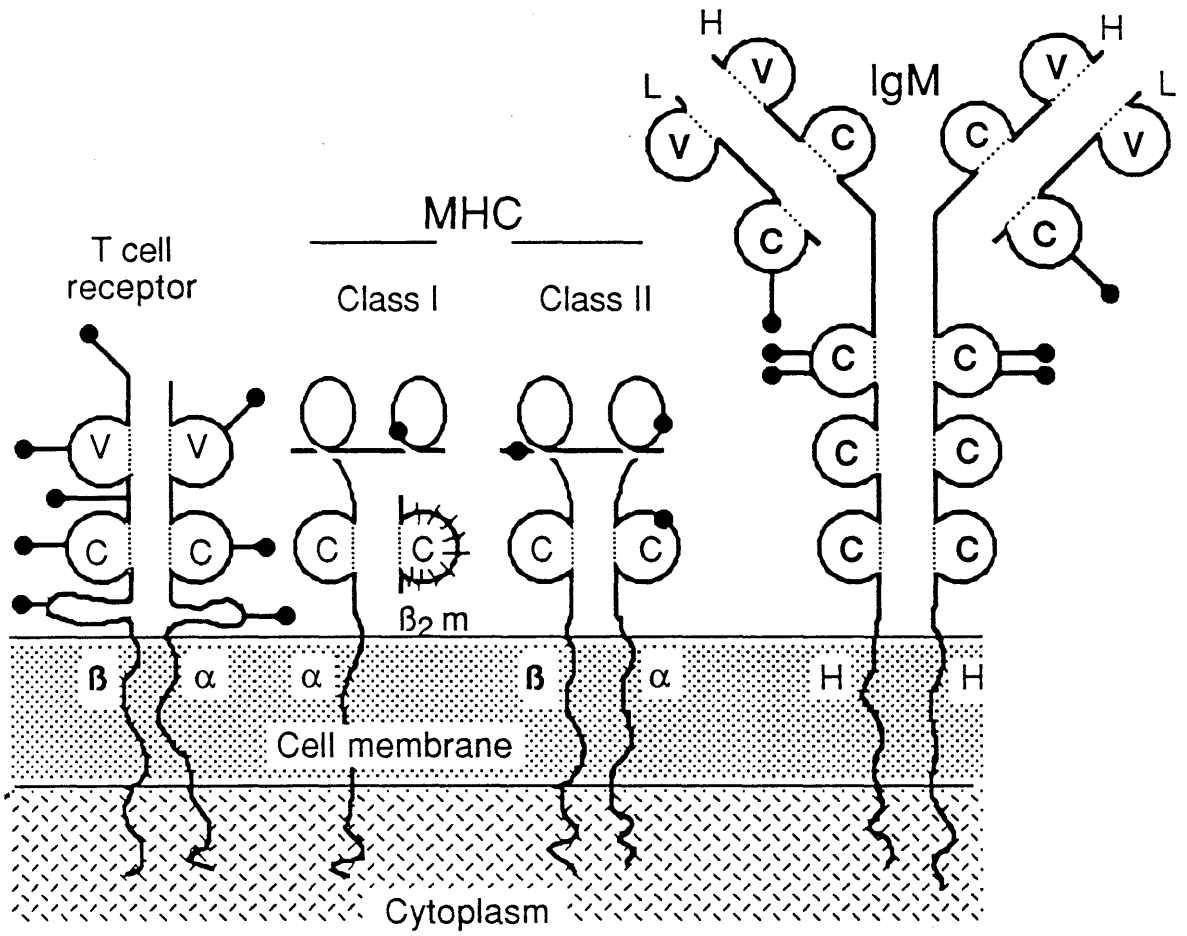
1.6 Structure of HLA class II molecules.

The HLA class II molecules are heterodimers consisting of alpha and beta chains with domain-like structures similar to that of immunoglobulin molecules (see Figure 1.3). A schematic diagram of HLA class II molecule structure is shown in Figure 1.4. Each molecule consists of two membrane distal domains (α1 and ß1), two membrane proximal domains (α2 and ß2), a transmembrane portion and a cytoplasmic tail. Three of the four domains have centrally placed disulphide bridges. The ß2 and α2 domain, nearest the membrane have strong sequence homology with immunoglobulin constant region genes. The transmembrane region is connected to the extracellular domain by a hydrophilic-connecting peptide, while the transmembrane region itself is characteristically hydrophobic, ending with a group of positively charged amino acids. The cytoplasmic tail varies in length depending on the isotype (DR, DQ, DP).

The best characterised HLA class II molecule, HLA-DR, has a ß chain which is 237 amino acids long. This consists of a first domain (ß1) of 94 amino acids, a second domain (ß2) of 84 amino acids, a cytoplasmic tail of 18 amino acids, a hydrophobic transmembrane region of 21 amino acids and a connecting peptide of 10 amino acids .

The crystallographic structure of HLA-A2 is represented in Figure 1.5. The two external domains of HLA class I molecules, α1 and α2, are folded to form a groove whose sides are two parallel α helical segments. The floor of this groove is lined by an eight stranded ß pleated sheet also formed from the α1 and α2 domains. This groove-like structure sits on ß-pleated structures formed from the α3 and ß2 microglobulin domains. Bjorkman and colleagues proposed that the groove formed between the two α helices, was the binding site for antigen.

Figure 1.3 Molecules belonging to the immunoglobulin gene superfamily. (modified from Williams 1985). The diagram shown below is reproduced with permission from the RPMS "Immunology Update for Clinicians" course booklet.



..... = Disulphide bond
—●— = Carbohydrate side chain

C = Immunoglobulin, constant domain
V = .. variable ..

C = Domain with homologies to Ig, constant domain
V = .. variable ..

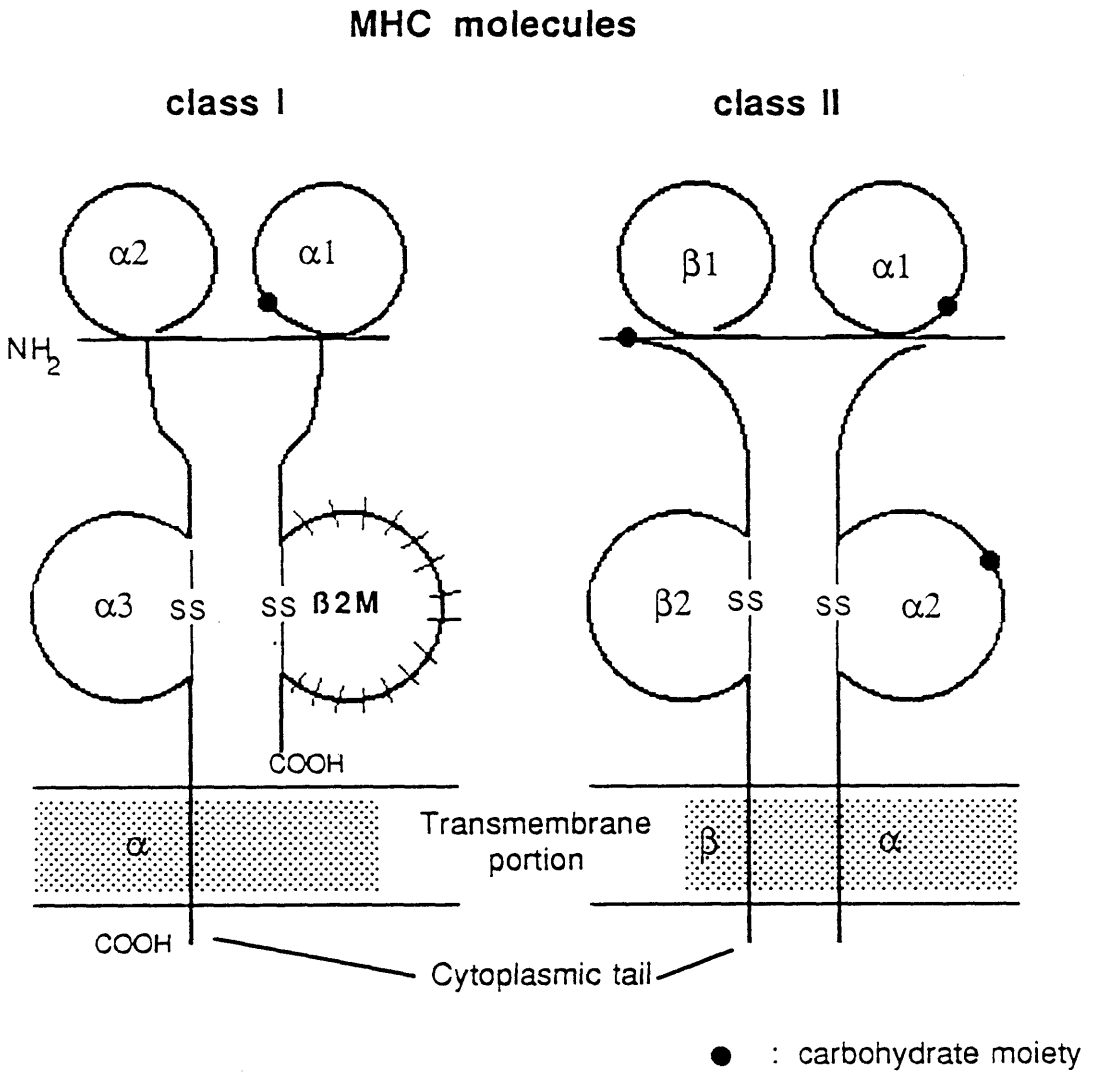


Figure 1.4. Structure of MHC class I and class II molecules (diagram modified from "Immunology update for clinicians " with permission from the Dept. Immunology, RPMS). MHC class I molecules are formed from the association of a single chain (alpha chain or heavy chain) of 43kD with ß2 microglobulin (12kD). The alpha 1 and alpha 2 domains are highly polymorphic and form the peptide binding site for MHC class I molecules (see Figure 1.5). The alpha 3 domain is relatively non-polymorphic and associates with ß2 microglobulin which is invariant within a species. The MHC class II molecule is a heterodimer consisting of an alpha chain (30-33kD) and a beta chain (25-30kD). The alpha chain is invariant in HLA-DR molecules, but is polymorphic in DQ and DP molecules. The beta1 domain is polymorphic in all HLA class II isotypes and together with the alpha 1 domain is thought to form the peptide binding site (see FIgure 1.5). The beta2 and alpha 2 domains are relatively non-polymorphic.

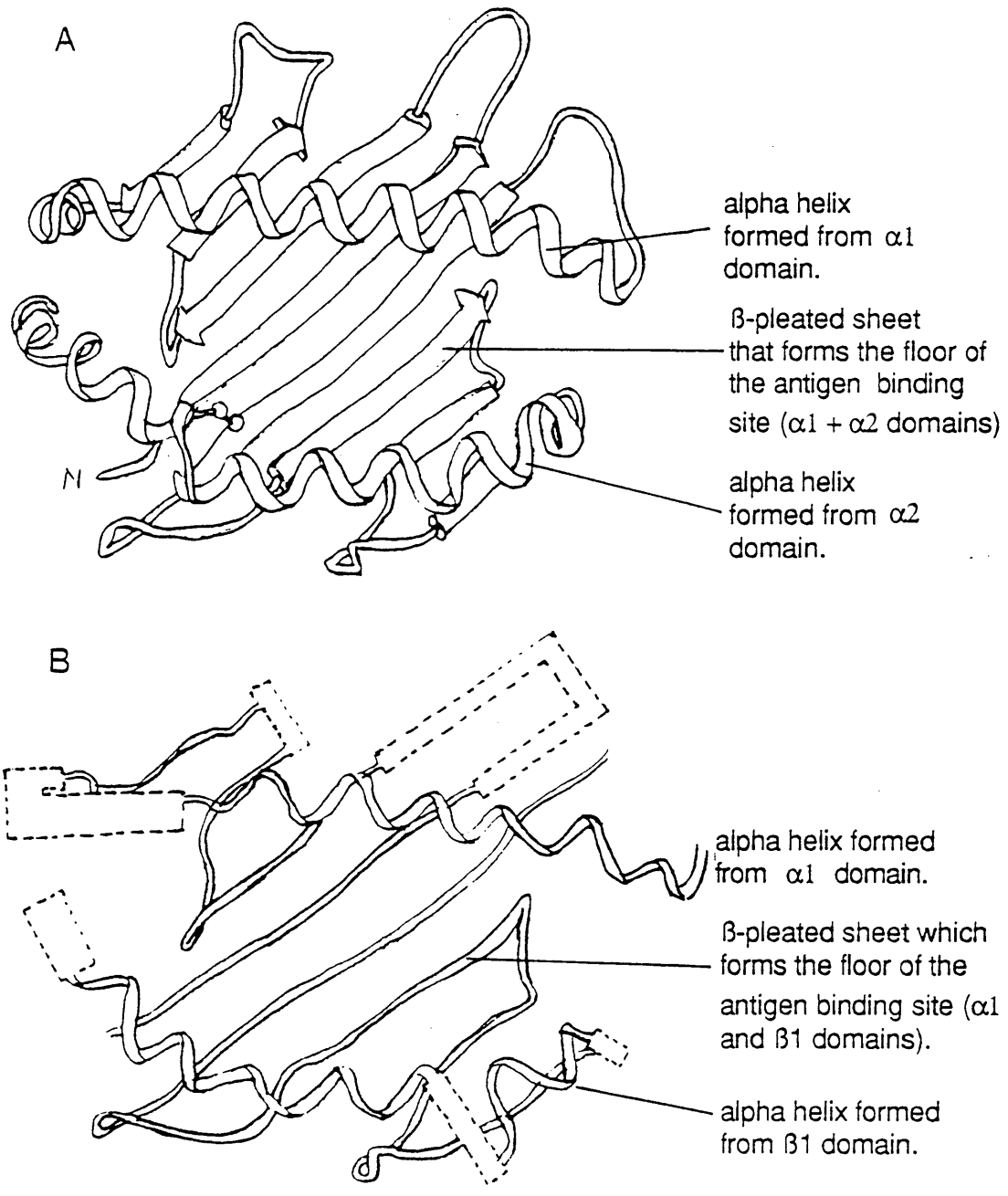


Figure 1.5. Three dimensional structure of HLA-A2 molecule and theoretical structure of HLA class II molecule, as seen from above. The 3-D structures of HLA class I (a) and HLA class II (b) are represented as ribbon diagrams (modified from Bjorkman et al. 1987a and Brown et al. 1988). The model for HLA class II structure has been predicted from amino acid sequence homologies between MHC class II molecules and MHC class I molecules, the boxed regions on the class II molecule indicate regions which lack homology with MHC class I molecules.

1.7 Function of HLA class II molecules[§] .

1.7.1 MHC restriction

The MHC complex in man and mouse were discovered as the result of the strong immune responses to allogeneic MHC molecules. It seems unlikely however that a highly polymorphic complex of genes evolved simply for the purpose of ensuring graft rejection between individuals. A physiological function for the MHC gene products was discovered in 1974 when Zinkernagel and Doherty demonstrated that cytotoxic T lymphocytes only recognized viral antigens in association with self MHC molecules. They showed that T cells from mice primed with lymphocytic choriomenigitis virus (LCMV) were only able to lyse LCMV-infected fibroblasts when they shared at least one H-2 haplotype (Zinkernagel and Doherty 1974a). Thus the virus-specific CTL were restricted in their recognition to syngeneic H-2. They suggested that this was due to the cytotoxic T cells recognizing "altered self", or H-2 molecules which had been altered by association with viral antigens.

Prior to Zinkernagel and Doherty's demonstration of MHC restriction for virus-specific CTL, another model had been proposed for the requirement of histocompatible antigen presenting cells. This model was based on the observations made by a number of groups which had shown that generation of antigen-specific antibody responses required the presence of histocompatible antigen presenting cells. (Rosenthal and Shevach 1973, Katz et al. 1973, Feldmann et al. 1974). Katz and colleagues suggested that the requirement for histocompatible antigen presenting cells was due to the interaction between two MHC molecules. Only cells sharing the same MHC molecules (in a like-like fashion) could interact

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[§] Many of the functional studies described in this section have been done in mice. These studies have been described in most cases without mention of mouse MHC nomenclature. The human studies described always include HLA nomenclature.

(Katz et al. 1973). Zinkernagel and Doherty were able to disprove this model and show that viral recognition by T cells could only occur in the context of self MHC molecules (Zinkernagel and Doherty 1974b). They reasoned that if the like-like interaction (intimacy) model was correct, then every sensitized T cell clone of an F1 hybrid should recognize the viral antigen and H-2 molecule of either parental type. Such clones should therefore be able to kill both parental targets.

The alternative proposal was that T cells recognized virus associated with H-2 molecules, and that T cells from an F1 animal would comprise two populations of T cells each recognizing virus in association with one parental type.

To test these proposals they took advantage of the observation that when LCMV-primed T cells were inoculated into lethally irradiated, virus infected recipients, they home equally well to lymphoid tissues of syngeneic or allogeneic recipients, but continue to proliferate only in syngeneic recipients. Thus further T cell replication was not triggered by free virus, but was dependent on T cell exposure to histocompatible, virus infected targets.

To test the two proposals explaining MHC restriction, LCMV-primed T cells from an F1 donor ($H\text{-}2^k$ x $H\text{-}2^d$) were inoculated into irradiated virus-infected recipients with different H-2 haplotypes. Clonal expansion of donor cells with specificity for virus+ $H\text{-}2^k$ occurred only in recipients of the $H\text{-}2^k$ type, and not in those possessing $H\text{-}2^d$. The sensitized T cells were shown to be of donor origin. These results argued against the intimacy model as this predicted that sensitized F1 T cells should proliferate in both $H\text{-}2^k$ or $H\text{-}2^d$ recipients. Instead Zinkernagel and Doherty suggested that the virus-infected F1 hybrids contained at least two populations of T cells, each recognizing a complex of virus and H-2 of one

parental type. This might be mediated through two T cell receptors, recognizing H-2 and virus as separate entities (dual recognition hypothesis) or one T cell receptor recognizing H-2 molecules altered by association with viral antigens (altered-self hypothesis). Shortly afterwards MHC restriction of cytotoxic T cells was confirmed for hapten-modified cells (Shearer et al. 1975) and minor histocompatibility antigens (Bevan et al. 1975, Gordon et al 1975).

Zinkernagel and Doherty showed that MHC restriction of LCMV specific T cells mapped to the class I region of the H-2 complex (Zinkernagel and Doherty 1975). The requirement for syngeneic antigen presenting cells to generate T helper cells was later reinterpreted as a demonstration of MHC restriction. This MHC restriction was mapped to the class II or I-A region (Katz et al. 1975, Erb and Feldmann 1975).

MHC restriction was confirmed for human T cell responses; virus specific cytotoxic T cells recognized virus only in association with HLA class I (McMichael et al. 1977, Rickinson et al. 1980), and antigen-specific T helper cells recognized antigen only in association with HLA class II molecules (reviewed by Thorsby et al. 1982).

The development of monoclonal antibodies specific for phenotypic markers on human T cells (Reinherz et al. 1979) allowed the definition of subpopulations with distinct functional properties; OKT4 (now CD4) positive T cells belonged to the T helper subset whereas OKT8 (now CD8) positive T cells belonged to the cytotoxic T cell subset. Many studies however have demonstrated that there is considerable functional overlap between each subset, and it is now established that CD4 and CD8 are markers of class I and class II restriction respectively and not phenotypic markers for cell function (Meuer et al. 1982).

1.7.2 Antigen processing and presentation.

A large body of work supports a view that both class I and class II MHC molecules present processed antigens in the form of peptides to antigen specific T cells (Babbit et al. 1985, Buus et al. 1986, Townsend et al. 1986, Guillet et al. 1987). MHC class I and class II restricted T cells appear to recognize peptides generated by different intracellular pathways (reviewed by Long 1989).

The requirement of an intracellular processing step before soluble antigens could be presented by MHC class II molecules was first suggested by Ziegler and Unanue (1981). They demonstrated that presentation of Listeria monocytogenes bacteria by macrophages could only occur after a time-lag of between 30 and 60 minutes. Paraformaldehyde fixation of macrophages after this time had no effect on presentation to T cells, whereas fixing the macrophages before exposure to bacteria resulted in lack of presentation. These results suggested that the antigens recognized by helper T cells were not intact but rather degraded intracellularly before exposure on the surface of the antigen-presenting cells. Shimonkevitz and colleagues provided direct support of this hypothesis as ovalbumin enzymatically digested *in vitro* could be presented by cells metabolically inactivated by glutaraldehyde, whereas the untreated native ovalbumin was not recognized (Shimonkevitz et al. 1983).

Although T helper responses had been shown to be MHC class II restricted (see Section 1.7.1) it was not known whether antigenic fragments bound directly to MHC class II molecules. Watts et al. (1984) were able to demonstrate that MHC class II molecules could bind processed peptides of antigen but not intact antigen, in the absence of antigen presenting cells. Direct peptide binding to MHC molecules was later shown by functional

recognition of fluorescently-labelled peptides bound to detergent solubilized MHC (Babbit et al. 1985).

Morrison et al. (1981) demonstrated that influenza specific CTL recognized viral antigens derived from different sources depending on whether they were MHC class I restricted or class II restricted. MHC class I restricted CTL recognized viral antigens derived from an endogenous source and antigen presentation was found to require protein synthesis. In contrast MHC class II restricted CTL recognized viral antigens derived from an exogenous source and were dependent on the activity of lysosomes.

It has been proposed that the differences in antigen processing in different intracellular compartments can explain whether an antigen is recognized by class I or class II restricted T cells (Germain 1986). Peptides associating with MHC class II molecules are generated by endocytosis and degradation of exogenous antigen in endosomes, where they meet newly synthesized class II molecules; whereas peptides associating with MHC class I molecules are derived from endogenous proteins degraded in the endoplasmic reticulum.

There are however, exceptions to this model. For example measles virus-specific CTL recognize endogenously synthesized viral proteins associated with HLA class II gene products (Jacobson et al. 1989). It has been suggested that differences in the route of cell infection between measles virus and influenza virus may explain the differences in antigen processing and presentation to class II restricted CTL (Long and Jacobson 1989). In addition, some class I-restricted CTL can be stimulated with peptides derived from soluble antigens, which are presented in the context of MHC class I molecules (Staerz et al. 1987). These studies suggest that

there may be some overlap between processing pathways for class I and class II restricted recognition.

Antigen processing for class II specific T cells may depend additionally on the non-polymorphic invariant chain (Ii) which is associated intracellularly with the class II α and ß chains. Stockinger et al. (1989) demonstrated that transfection of an Ii gene into MHC class II positive murine fibroblasts, transferred the ability to process native antigen. MHC class II positive fibroblasts lacking Ii were unable to process native antigen although they were able to present peptides of the same antigen.

The three dimensional structure of HLA-A2 contains a single antigen binding site (Bjorkman et al. 1987a). An intriguing aspect of the crystal structure of HLA-A2 was the finding of an area of electron density in the binding site, which was consistent with the presence of a bound peptide. This finding suggests that both class I and class II MHC molecules may be normally occupied by peptides derived from self-proteins. This notion is supported by peptide binding studies using radio-labelled peptides and purified MHC class II molecules. Buus et al. (1988) have shown that only a small proportion (5-10%) of MHC molecules bound radio-labelled exogenous peptide after incubation under saturating concentrations. This result may be explained by a high occupancy of MHC molecules with endogenous peptides, which is due to the slow dissociation rates of MHC and peptide complexes (Buus et al. 1986).

1.7.3 HLA class II and disease.

It striking that in a system as polymorphic as the HLA system (see Table 1.1) that certain antigens are over represented in disease populations, when compared to normal controls of the same ethnic group. For example strong associations have been shown between HLA-DR2 and Goodpastures syndrome (Rees et al. 1978), multiple sclerosis (Compston

and Batchelor 1978) and narcolepsy (Langdon et al. 1984). These and many other HLA and disease associations led to the proposal that there may be genetic susceptibility to disease, and that this might be controlled by HLA genes acting as immune response (Ir) genes.

The first indications that disease susceptibility might be influenced by hereditary factors, came from studies testing the response of outbred guinea pigs to bacterial antigens, in the early 1900s. The influence of hereditary factors was inferred by the transmission of resistance or susceptibility traits from parent to offspring in families (reviewed by Klein 1986). Later, variation in response to bacterial antigens was seen between inbred mouse strains (Gorer and Schütze 1938). The involvement of H-2 genes in the determination of disease susceptibility became apparent when it was found that the resistance of certain inbred mouse strains to virally-induced leukaemia segregated with the H-2 complex (Lilley et al. 1964).

The antigenic complexity of pathogens may result in multiple genes controlling the immune response. The development of synthetic peptides in the early 1960s, allowed antibody responses to antigens with few homogenous epitopes to be determined (Humphrey 1984). Humphrey and Sela demonstrated that certain strains of rabbit were unable to make antibodies against synthetic polypeptides (reviewed by Humphrey 1984). McDevitt and Sela (1965) confirmed these results using two inbred mouse strains and later McDevitt and Chinitz (1969) were able to show that immune responses against the polypeptides were linked to the H-2 haplotypes of the mouse strains used. These studies suggested the existence of immune response (Ir) genes controlling the antibody response to synthetic antigens. Subsequent analysis of the immune response to polypeptides in a large number of strains and recombinants, mapped the Ir genes to the H-2 region (McDevitt et al. 1972).

This work has an obvious parallel with human disease, with susceptibility linked to possession of particular HLA class II antigens. However studies to prove this association have been restricted for two main reasons. Firstly, genetic studies are more difficult in the human, as there are no inbred strains as there are in the mouse. Secondly in many cases the aetiology of the diseases is not known, so immune responses to defined antigens cannot be investigated.

Perhaps the clearest example of human Ir gene control has been the study of human T cell responses to streptococcal cell wall (SCW) antigen in the Japanese population (reviewed by Sasazuki et al. 1989). In these studies high and low primary T cell responses to SCW antigen were seen in population studies. Low responders were shown to be over represented in individuals with DR2. The lack of response in primary lymphocyte cultures was not due to a defect in antigen presentation , as strong primary responses in low responders could be seen when anti-DQ antibodies were added to the culture system. Sasazuki and colleagues suggested that the effect of the anti-DQ antibodies was to prevent the action of DQ-restricted suppressor cells (Sasazuki et al. 1989). At present it is difficult to determine the significance of these findings in relation to disease susceptibility as studies measuring the immune response to defined antigens in normal human populations have been scarce.

Another reason to doubt that HLA class II genes could influence disease susceptibility through an Ir gene effect is the fact that in diseases associated with HLA polymorphisms, there is no single HLA polymorphism amongst the patients.

This might be explained in several ways. First, as discussed earlier (Section 1.4) the full extent of HLA class II polymorphism may not have been realized. The products of the DP region in particular, are ill defined

(Bugawan et al. 1989). It is possible that HLA class II variants not detectable by current methods may be in linkage disequilibrium with known alleles. Such variants may show a much higher correlation with a particular disease. This has been shown in a study of insulin-dependent diabetes mellitus (IDDM) in caucasoid populations (Todd et al. 1987). IDDM had been shown in earlier studies to be strongly associated with the presence of two serologically defined antigens DR3 and DR4. The maximum disease susceptibility correlated with the presence of both DR3 and DR4 (Svejgaard et al. 1983). HLA class II genes from IDDM patients and controls were sequenced and it was found that a single amino acid residue at position 57 of the DQß chain determined susceptibility to IDDM. DQß alleles that were positively associated with IDDM had either alanine (Ala), valine (Val) or serine (Ser) at position 57. Aspartic acid (asp) is present at position 57 in all the DQ alleles which were neutral or negatively associated with disease.

This correlation was strengthened by a similar observation for the I-Aß (murine homologue of DQß) sequences found in the non-obese diabetic (NOD) mouse strain. The I-Aß chain of NOD mice is unique amongst mouse strains as it has serine at position 57, Aß chains from resistant mouse strains have Asp at this position.

Another important conclusion from the study of Todd et al (1987) was the finding that for full disease susceptibility, Asp negative residues were required at position 57 for both MHC haplotypes, heterozygosity appeared to confer resistance to IDDM. The requirement of two class II alleles for full disease susceptibility may well apply to other diseases. It should be noted however, that despite the precise definition of the HLA class II antigens associated with IDDM, there was still no absolute correlation between HLA class II and IDDM.

An alternative explanation for the lack of absolute correlation of disease susceptibility with HLA class II molecules, is that other genetic and non-genetic factors may influence disease susceptibility. For example genetic factors such as the sex of the patient or enzyme isotypes may be important (reviewed by Batchelor 1984). Environmental influences on disease susceptibility have been inferred from the lack of absolute concordance for incidence of HLA associated diseases in monozygotic twin pairs. For example Ebers et al. (1984) could only find 30% concordance for incidence of multiple sclerosis in monozygotic twin pairs.

HLA class II gene products play a direct role in the pathogenesis of certain autoimmune diseases (reviewed by Feldmann 1989). HLA class II antigens have been shown to have to have a restricted distribution, being mainly expressed on macrophages, dendritic cells and B cells. However expression of MHC class II antigens can be induced on cells which do not express class II under normal circumstances. This "aberrant" expression of MHC class II antigens has been shown by histological studies to be common to tissue undergoing immune attack. This was first seen in the rat, with expression of rat MHC class II antigens being shown on tissue from GVHD sites (Mason et al. 1981, Lampert et al. 1981). Shortly afterwards, HLA class II antigens were shown to be expressed on tissue being attacked in an autoimmune fashion which lead to the proposal that "aberrant" expression of HLA class II played a direct role in pathogenesis of autoimmune diseases (Bottazzo et al. 1984). A series of studies have supported this proposal, particularly with regard to autoimmune thyroiditus. These studies have demonstrated that the class II molecules expressed by thyrocytes from autoimmune thyroids are functional; these molecules are able to present peptides of influenza haemagglutinin to T cell clones (Londei et al. 1985) and stimulate T cells cloned from

autoimmune thyroid glands (Londei et al. 1987). The "aberrant" expression of HLA class II on thyrocytes is likely to be mediated primarily by gamma interferon (reviewed by Feldmann 1989).

However aberrant expression of MHC class II may not induce auto immune pathogenesis in all diseases. Recent studies have demonstrated that organ-specific expression of MHC class II molecules in transgenic mice does lead to development of autoimmune diseases, however there were no lymphoid infiltrates characteristic of tissues undergoing autoimmune attack (Sarvetnick et al. 1988, Lo et al. 1988).

In addition to involvement in the pathogenesis of autoimmune disease, HLA class II molecules may play a role in immunoregulation of normal immune responses. Autoimmunity may result from a breakdown of this mechanism. Evidence that HLA class II molecules may be involved in immunoregulation via T cell-T cell (T-T) interactions has come from a number of sources. Recognition of high doses of influenza peptide in the absence of antigen presenting cells has been shown to induce antigen-specific tolerance in T cell clones (Lamb et al. 1983). The induction of tolerance was shown to be dependant on HLA class II molecules expressed on the T cell clones, as tolerance could not be induced in the presence of anti-HLA class II specific monoclonal antibodies (Lamb and Feldmann 1985).

It has been shown that under some circumstances, HLA class II positive T cells can process and present soluble antigens. Lanzavecchia has demonstrated that CD4+ve T cell clones can present gp120 from HIV to CD4+ T helper cells (Lanzavecchia et al. 1989). MHC class II restricted CTL have also been shown to recognize soluble antigens presented by B cells (Shinohara et al. 1989). On the basis of these observations it has been suggested that class II restricted CTL may form a population of

immunoregulatory cells which can suppress antigen specific T or B cell responses by mediating lysis of effector cells (Simpson 1989, Lanzavecchia 1989).

HLA class II genes may be involved in disease development in three ways. First, they may determine susceptibility to disease, either in isolation or in combination with non-HLA genes. Second, they may be involved in the pathogenesis of autoimmune disease, by inducing the activation of auto-antigen reactive T cells. Third they may help to regulate the immune response by serving as recognition structures for cytotoxic/non cytotoxic immunoregulatory T cells.

1.8 The nature of alloreactivity.

1.8.1 The frequency of alloreactive T cells.

Alloreactivity or the T cell response to allogeneic MHC molecules is characterized by a high frequency of responding cells. This characteristic was quantitated in studies in the rat, which determined the number of responding blasts appearing after mixed lymphocyte culture (Wilson and Nowell 1971) or at the site of GVHR (Ford et al. 1975).

The development of limiting dilution analysis has allowed quantification of precursor cells rather than mature responding cells. For limiting dilution assays, serial dilutions of lymphocyte suspensions are made, and a number of replicate culture wells (usually 24-60) are plated for each dilution step. The culture conditions will vary according to the specific effector function being assayed e.g. lymphokine production, cytotoxicity, proliferation. After a suitable incubation time, the contents of the wells are assayed for appropriate effector function and assuming that culture conditions are optimal, the only factor which should determine whether a culture well is positive for a particular effector function, is the presence of a specific precursor cell.

If culture conditions are such that only a single specific cell type is limiting then the probability of obtaining a precursor cell in any given well can be estimated from the Poisson distribution. The frequency of the specific precursor in the whole population can be calculated from the zero order equation of the Poisson distribution: $\mu=-\ln Fo$, where Fo is the fraction of negative cultures, e is the base of the natural logarithm and μ is the average number of precursor cells per culture. When there is on average only one precursor cell per culture, it follows that $\mu=1$ and thus $Fo= e^{-1}=0.37$. The frequency of specific precursors can be extrapolated from Fo=0.37 on the semi-logarithmic plots of experimental data (Figure 1.6). If only a single cell type is limiting (single hit kinetics) the experimental data should provide a good fit to a straight line which intercepts the graph at the origin (intercept of 1.0 on the Y axis).

The technique of limiting dilution is particularly valuable in human studies where the frequency of functional subsets of T cells can be determined using PBMC without the requirement for tissue samples. The technique has been used to determine frequencies of lymphokine producing T cells (Kees et al. 1984, Theobald et al. 1989), virus-specific CTL (Borysiewicz et al. 1988, Hickling et al. 1987, Schmid et al. 1988) and antigen specific proliferative T cells (Van Oers et al. 1978, Gebel et al. 1983, Brett et al. 1987). The most popular use of the technique has been for the detection of alloreactive CTLp.

The maximum frequencies of alloreactive CTLp obtained in a number of human LDA studies are shown in Table 1.3. The main conclusion from these studies was that there is a high frequency of alloreactive CTLp in humans, although variation in CTLp frequency may occur depending

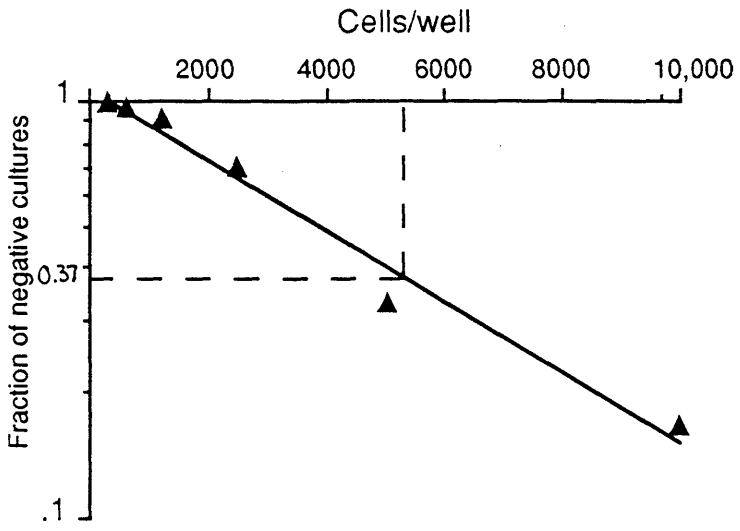


Figure 1.6. Semi-logarithmic plot of results from a theoretical limiting dilution assay. The line fitted by linear regression passes close to the origin indicating experimental results consistent with single hit kinetics. The frequency of specific precursors can be extrapolated from Fo= 0.37, resulting in a frequency estimate in this case, of 1 in 5400. Minimum chi-squared and maximum likelihood methods do not require graphs to estimate frequency.

on the allogeneic combinations used (Sharrock et al. 1987, Zhang et al. 1989). Different experimental protocols* have produced a range of alloreactive CTLp frequencies (1/66-1/5000#) in peripheral blood, which overlap with CTLp frequencies (1/399-1/666) obtained in earlier studies in the mouse (Lindahl and Wilson 1977, MacDonald et al. 1980). Alloreactive CTLp frequencies in the mouse were found to be 10-100 fold higher than virus-specific CTLp frequencies determined in non-immunized mice (MacDonald et al. 1980, Askonas et al. 1980). In humans there is an apparent overlap between frequencies obtained for alloreactive CTLp and virus specific CTLp (Tables 1.3 and 1.4). In the majority of studies however, CTLp frequencies were determined using seropositive individuals, so the LDA assays were measuring the frequencies of primed T cells and not precursor cells. When seronegative individuals have been used, CTLp frequencies have been virtually undetectable (Borysiewicz et al. 1988). In addition, different experimental protocols have been used for detection of alloreactive CTLp and virus-specific CTLp so direct comparison of CTLp frequencies may not be valid.

1.8.2 Generation of the alloreactive T cell repertoire.

For many years after the discovery of MHC restriction (Zinkernagel and Doherty 1974), it was not clear whether T cell recognition was mediated through two receptors, one for MHC and one for antigen, or a single receptor with specificity for both MHC and antigen. Support for the single receptor hypothesis came from studies by Hünig and Bevan (1980) who demonstrated that CTL clones specific for MHC A plus antigen X, could cross-react on MHC B plus antigen Y, but were not able to recognize other combinations of MHC and antigen.

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* See Table 4.1.

The maximum frequency for untreated PBMC is shown from Table 1.3.

Table 1.3. Frequencies of alloreactive CTLp in man

Responder Cells	CTLp Freq[1]	Reference
PBMC	1/ 5000	Sharrock et al.(1987)
PBMC	1/ 870	Bryan et al. (1983)
PBMC	1/ 733	Rosenkrantz et al.(1985)
PBMC	1/ 1200	Kaminski et al. (1988)
PBMC	1/ 3322	Bryan et al. (1983)
PBMC	1/2049	Zhang et al. (1989)
PBMC	1/ 900	Burns et al. (1984)
PBMC	1/ 500	Deusch et al. (1986)
T cells*	1/ 240	Kabelitz et al. (1985)
T cells*	1/ 2010	Moretta et al. (1983)
T cells*	1/ 66	Kabelitz et al. (1987)
T cells*	1/ 535	Kabelitz et al. (1987)
CD4+veT cells	1/ 1620	Kabelitz et al. (1987)
CD4+ve T cell	1/ 11600	Moretta et al. (1983)
CD8+ve T cells	1/ 190	Kabelitz et al. (1985)
CD8+ve T cells	1/ 710	Moretta et al. (1983)

[1]Maximum frequency obtained in publication cited. Pheotype and specificity of CTLp detected, not determined in the majority of studies.

Table 1.4. Frequencies of virus specific CTLp in man.

Virus	CTLp frequency[1]	CTLp Phenotype	Reference
Cytomeglovirus	1/5000-20,000#	CD8	Borysiewicz et al. 1988
Epstein Barr virus	1/540	CD8	Borgault et al. 1989
Epstein Barr virus	1/17622	CD4	Borgault et al. 1989
Herpes simplex virus1	1/4510-7915	CD4	Schmid et al. 1988
Influenza (A/Texas/77)	1/3564	CD8	Borgault et al. 1989
Influenza (A/Texas/77)	1/35703	CD4	Borgault et al. 1989
Varicella-zoster virus	1/15,873* -500,000	CD8	Hickling et al. 1987

[1] Ranges represent maximum and minimum frequencies in a number of individuals.
CTLp frequency in seronegative individuals was < 1:500,000.
* Maximum frequency obtained from individual after recent challenge with Zoster. An asymptomatic seropositive individual produced a CTLp frequency of 1:34,483.

Further support for the one receptor hypothesis came from Kappler et al (1981), who made a hybrid between T cells with different MHC restriction and antigen specificities. In functional tests the hybrid retained the original specificities with no new specificities being generated. Thus there was no mixing between MHC specificities and antigen specificities, a result consistent with MHC and antigen being recognized by a single receptor.

The results of Hünig and Bevan also suggested that alloreactive T cells come from the same population as antigen specific T cells. It is now known that T cell recognition of antigen in the context of MHC is mediated through clonally variable T cell receptors consisting of α and ß polypeptide chains (Hedrick et al. 1987, Yanagi et al. 1987). Many studies have subsequently shown that alloreactive and antigen specific T cell clones can utilize the same T cell receptor genes (Rupp et al. 1985, Fink et al. 1986). Definitive proof for this concept has come from the demonstrations that transfection of a single pair of α and ß T cell receptor genes into a T cell line, was sufficient to transfer donor T cell specificity for both MHC plus antigen, and allogeneic MHC (Dembic et al. 1986).

Many studies have shown that the antigen-specific repertoire of mature T cells is heavily influenced by the microenvironment encountered in the thymus during early T cell development (reviewed by Sprent et al. 1989, Marrack and Kappler 1988). Immature bone marrow derived T cells enter the thymus where they undergo a series of ill-defined differentiation and selection events, before emerging into the periphery as mature T cells with full helper or cytotoxic effector function. The repertoire of mature T cells exported from the thymus appears to be influenced by both positive and negative selection events. The concept of positive selection, appears to account for the MHC restriction of T cells. A

number of studies using radiation bone marrow chimeras have shown that immature T cells are selected to recognize exogenous antigens in association with the same MHC molecules encountered in the thymus (reviewed by Sprent et al. 1989). There is selection against T cells (negative selection) which react to thymic MHC molecules in the absence of exogenous antigen. The best evidence for such selection has come from studies with transgenic mice which have demonstrated deletion of specific T cell receptor families as a result of transgene expression (reviewed by Marrack and Kappler 1988).

Does thymic education affect the alloreactive T cell repertoire? A genetic influence on the cytotoxic T cell repertoire to allogeneic MHC class I molecules has been shown by Sherman (1980), who demonstrated that TCCs derived from a responder: stimulator combination differing by only one MHC class I antigen, exhibited at least 50 different T cell receptor (TCR) specificities. Multiple reactivity patterns were obtained when the TCCs were tested on large panels of stimulator cells with mutated MHC class I molecules, and each reactivity pattern was interpreted as a unique TCR specificity. The repertoire to alloantigens represented by the reactivity patterns appeared to be controlled by the H-2 types of the mice as the relative proportions of individual patterns differed between mice strains differing only at the MHC loci (Sherman 1982). In addition inheritance of one set of parental T cell reactivity patterns was seen in F1 hybrids between two different mouse strains. Novel T cell reactivity patterns were also seen in the F1 hybrids, suggesting that in outbred populations, there may be individual variation in the T cell repertoire against alloantigens.

Sherman's results have been confirmed at the molecular level by more recent studies. Garman et al. (1986) analyzed T cell receptor V region gene

usage in bulk cultures of alloreactive T cells and found significant biases of V gene usage according to the strain of mouse used. Bill et al. (1989) were able to show that 37 patterns of T cell receptor gene usage could be obtained from T cell hybridomas specific for the same MHC class II molecule.

A direct influence of the thymus on the selection of the alloreactive T cell repertoire has been shown by Gress et al. (1986) using radiation bone marrow chimeras. The cytotoxic T cell response to bm6 molecules (mutant K^b) was influenced by the MHC type of the thymus and the MHC genotype of the T cell. Homology at the K^b loci was required for generation of cytotoxicity against bm6. K^b T cells maturing in an allogeneic thymus or allogeneic T cells maturing in a K^b thymus were unable to mount a cytotoxic T cell response against bm6 cells. It is not clear however, whether such mechanisms contribute to development of alloreactive T cell repertoires in normal individuals, as cytotoxic T cell responses could be made against bm6, in all mouse strains tested. It would be interesting to see if there was a quantitative difference in CTLp frequency against bm6 molecules in mice of different strains and also whether different T cell receptor specificities were generated according to the strain.

The main conclusions that can be drawn from studies of the alloreactive T cell repertoire in mice are:

1. There are genetic differences in the alloreactive T cell repertoire between different mouse strains. The thymus has been shown to influence the alloreactive T cell repertoire in chimeric mice, but how thymic education determines T cell receptor specificities for allogeneic MHC is not known.

2. The same α: ß T cell receptor can recognize both self-MHC plus antigen, and allogeneic MHC in the absence of exogenous antigen. In the majority of studies no correlation has been observed between any individual T cell receptor V genes and reactivity to a particular MHC molecule. An exception to this is the striking correlation observed between expression of Vß17a T cell receptor genes in T cell hybridomas and reactivity to a mouse H-2 class II molecule I-E plus an unknown B cell product (Kappler et al. 1987, Marrack and Kappler 1988). However it is not clear whether this correlation is due to recognition of a B cell-derived "superantigen" which binds specifically to I-E molecules (White et al. 1989).

3. Alloreactive TCCs specific for either a single MHC class I or class II antigen express a diverse array of T cell receptors suggesting that multiple determinants can be found on a single MHC molecule. The nature of the allogeneic determinants that are recognized by T cells is discussed below.

1.8.3 Models for recognition of allogeneic MHC molecules.

Two major models of allogeneic MHC recognition have been proposed for explaining the high frequency of alloreactive T cells (see Figure 1.7).

1. Alloreactive T cells recognize "interaction antigens" consisting of allogeneic MHC complexed with self and non-self cell surface components (Matzinger and Bevan 1977). Each MHC molecule can give rise to multiple "interaction antigens" from the complexes formed between MHC and cell surface components. This results in the stimulation of a large number of alloreactive T cells with different fine specificities.

2. The majority of alloreactive T cells are recognizing determinants on the MHC molecule itself i.e. a "naked" molecule, present at high density on the cell surface (Bevan 1984). Since antigen specific T cells recognize a complex of both antigen and MHC, and not all MHC

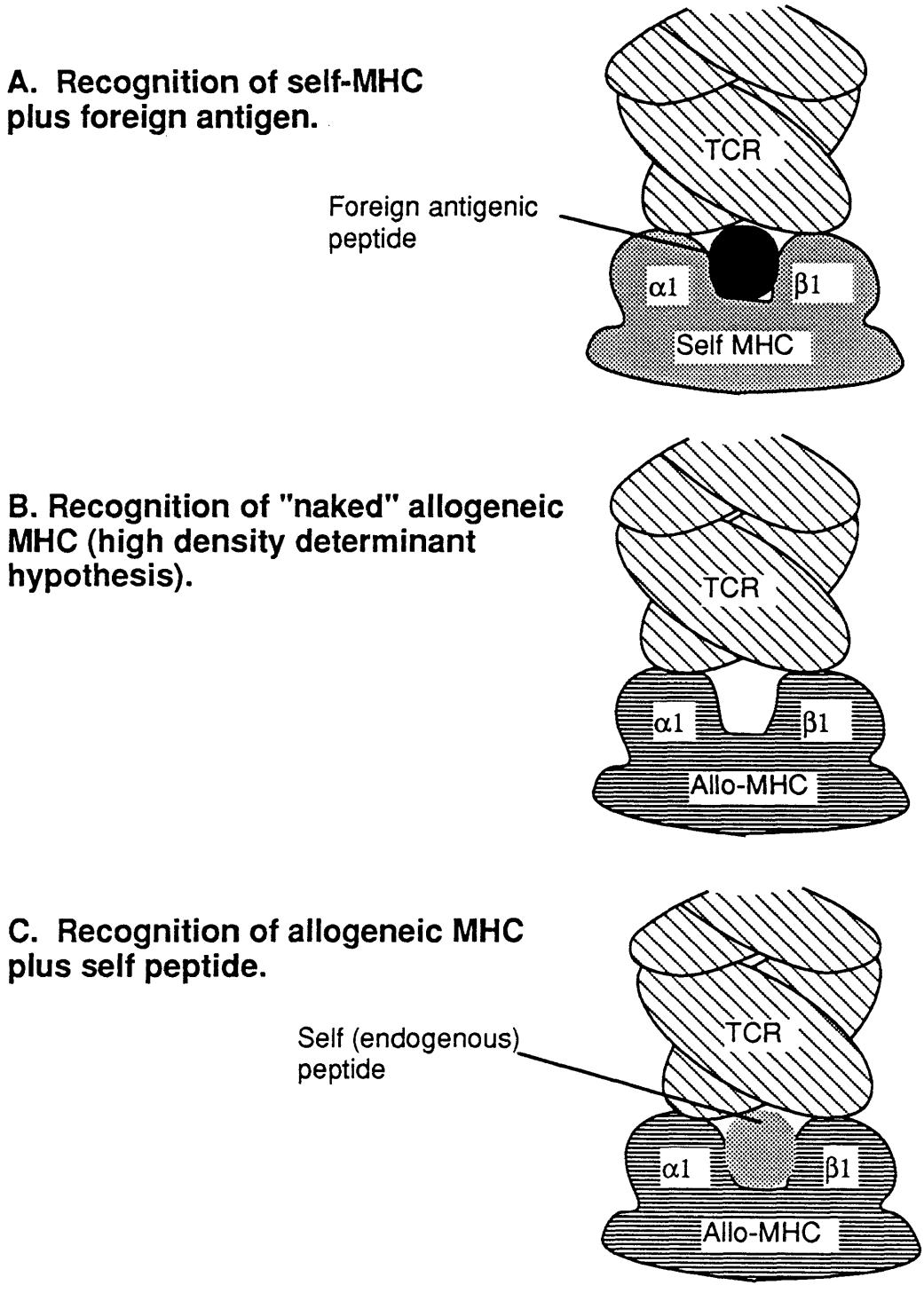


Figure 1.7. Models for T cell recognition of allogeneic MHC (adapted from Davis and Bjorkman 1988) (A). Model for antigen-specific T cell recognition. The T cell receptor (TCR) binds a complex of self-MHC (hypothetical antigen binding site shown) and foreign antigenic peptide. (B) Alloreactive T cells recognize "naked" allogeneic MHC without peptide. Such determinants are present at high densitiy on the cell surface resulting in stimulation of many alloreactive T cells (Bevan 1984). Only some MHC molecules will bind antigenic peptide resulting in a lower density of determinants available for antigen-specific T cells. (C) Alloreactive T cells recognize allogeneic MHC associated with self-peptides. MHC molecules are normally bound by self-peptides, generated during catabolism of self-proteins. Mulitple allogeneic determinants formed from the association of allogeneic MHC plus self peptides, and resulting in stimulation of multiple populations of alloreactive T cells (Matzinger and Bevan 1977, Kourilsky et al. 1987, Marrack and Kappler 1988).

molecules will be associated with antigen at any one time, the number of available determinants for antigen specific T cells will be low. Thus only high affinity antigen-specific T cells will be stimulated.

In contrast all the MHC molecules will be available for recognition by alloreactive T cells resulting in a high density of determinants. This will allow stimulation of a large number of T cells as T cells of low and high affinity for allogeneic MHC molecules can be stimulated.

Matzinger and Bevan (1977) originally suggested that minor histocompatibility antigens might associate with MHC to stimulate alloreactive T cells. However no convincing evidence for the involvement of minor antigens in allogeneic MHC has emerged. The work of Langhorne and Lindahl (1982) argues against a role for minors, as no difference in CTLp frequency could be seen against the same H-2 molecules in association with different minor histocompatibility antigens.

Subsequently the Matzinger and Bevan model has been modified to take into account the peptidic nature of antigens associating with MHC molecules. This modified model proposes that alloreactive T cells recognize a complex of MHC and endogenous peptide (Kourilsky et al. 1987, Marrack and Kappler 1988).

Although it is difficult to totally disprove the "high determinant" density hypothesis, a number of recent studies suggest that MHC-bound peptides play a role in the recognition of allogeneic MHC molecules.

Firstly, the slow rate rates of dissociation observed for antigenic peptides complexed to MHC molecules in a cell free system (Buus et al. 1987), and the likely presence of a bound peptide in purified crystals of HLA-A2 suggest that MHC molecules are normally occupied by peptides. Buus et al. (1988) have suggested that around 95% of MHC molecules may be occupied by endogenous peptides.

Secondly, some alloreactive T cells are able to discriminate between the same allogeneic MHC molecule expressed on cells of different lineage (Manca et al. 1985, Minami et al. 1987, Marrack and Kappler 1988). Marrack and Kappler suggested that such cell lineage specificity could be best explained by differences in the array of peptides generated by different cell types, thus different allogeneic determinants will be formed according to cell type.

Further evidence for recognition of allogeneic MHC molecules complexed with peptides has been suggested by the recent study by Lombardi et al. (1989a). In this study, the majority of HLA-DR1 specific T cell clones tested were able to respond to HLA-DR1 expressed on either human B-LCL or murine L cell transfectants. Both cell types were able to process and present intact influenza virions to an influenza haemagglutin-specific T cell clone. Differential effects were seen however, when DR1+ B-LCL and DR1+ L cells were treated with glutaraldehyde, which prevents antigen processing. Alloreactive TCC recognition of B-LCL was not affected by glutaraldehyde whereas recognition of the DR1+ L cells was abolished. The glutaraldehyde-fixed L cells were able to present peptides of influenza haemagglutin to a haemagglutinin-specific TCC, demonstrating that the loss of recognition was not due to a defect in the HLA class II molecule. These results suggest that the anti-DR1 reactive TCCs recognized a complex of MHC and peptide, in which the peptide was derived from human proteins processed by the L cells.

The most direct evidence for peptide involvement in recognition of allogeneic MHC molecules has come from a recent study by Heath et al. (1989). A number of murine TCCs specific for a MHC class I molecule, Kb, could not recognize the same molecule when it was expressed on a

human cell. Specific recognition was reconstituted by pulsing the human cells with cytoplasmic extracts from the mouse cells. The predominant component of the cytoplasmic extracts was found to be a 15 amino acid peptide, which suggests that the TCC were recognizing an allogeneic determinant formed from allogeneic MHC complexed with murine peptides.

1.9. Present Study.

The present study is an investigation into the factors influencing human T cell responses to allogeneic MHC class II antigens. Alloreactive T cell responses have been studied at the clonal and the population level, in order to define the allogeneic determinants recognized by individual alloreactive T cells and to determine whether there is variation in the cytotoxic T cell repertoire against HLA class II antigens. The results of this study are presented and discussed in the following chapters.

Chapter 2.

Materials and Methods.

2.1. Blood donors and HLA typing.

Peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers in the Department of Immunology, RPMS, London, and who were aged between 22 and 58 years. HLA phenotypes of PBMC and B-lymphoblastoid cell lines (B-LCL) were determined by serological typing (Tissue Typing laboratory, Department of Immunology, RPMS) using anti-sera from the 10th Histocompatibility Workshop. Dw types (Segall et al. 1981) were assigned by cellular methods using T cell clones (G. Lombardi et al 1989b, Sharrock et al. 1989) and homozygous typing cells (W. Ollier, London Hospital, London). In some cases Dw types were assigned on the basis of restriction fragment length polymorphism (RFLP) studies after the method of Bidwell et al. (1988). RFLP studies were performed by Paul Brookes of the Tissue Typing laboratory, Department of Immunology, RPMS.

2.2. Culture conditions.

2.2.1. AB serum.

Pooled human AB serum was kindly provided by Mr John Davies, Regional Blood Transfusion Laboratory, Oxford, England. The serum was prepared using a protocol devised by S. Sidhu, Department of Immunology, RPMS, London. Pooled serum was heat-inactivated at 56^0C for 30 minutes, then cooled to room temperature. Fats were precipitated by ultra-centrifugation (ultra-centrifuge model JH-1, Beckman); the serum was spun at 10,000 RPM for 1 hour at 10^0C. The clarified serum was carefully decanted, and filtered through glass fibre paper (Whatman, grade G/F). The serum was sterilized before use, by pumping the serum through 0.8μ and 0.22μ filters (Sartorius). The AB serum was stored at -20^0C.

2.2.2. Fetal calf serum.

Fetal calf serum (FCS) was purchased from a variety of sources (Flow labs, Imperial and Sera-lab). All batches were heat-inactivated as for human AB serum, and tested for their ability to support growth of B-LCL and murine L cells for at least three weeks before routine use.

2.2.3. Tissue culture medium.

The T cell culture medium (TCM) consisted of RPMI 1640, buffered with 0.23% sodium bicarbonate (Flow laboratories, Irvine, Scotland) and supplemented with 100 I.U. penicillin (Flow), 100μg/ml streptomycin (Flow) and 10% (v/v) human AB serum. Serum-free RPMI 1640 was used to wash cells.

For B-lymphoblastoid cell lines (B-LCL), the tissue culture medium used was similar to TCM except that 10% (v/v) fetal calf serum was used, instead of human AB serum. In addition an anti-mycoplasma antibiotic, Tylocine (Flow) was added at 1%v/v. This medium was referred to as B-LCL culture medium (BCM).

Hepes buffered-medium (HM) was used when cells were to be manipulated outside the incubator for long periods e.g. immunomagnetic depletions (section 2.3.6). HM consisted of RPMI 1640, buffered with 20mM Hepes (Flow) and 0.075% sodium bicarbonate (Flow), and supplemented with penicillin and streptomycin as for TCM.

2.2.4. Mycoplasma testing.

T cell clones and other cell lines were tested for mycoplasma by Gillian Webb (Department of Immunology, RPMS) using a nucleic acid hybridization assay kit (Gen-Probe Inc., California, U. S. A.). Briefly, the protocol for the assay was as follows. Supernatants from test cultures were aspirated, and mixed with a mycoplasma-specific DNA probe (^{3}H-TdR labelled) under hybridizing conditions.

The amount of specific hybridization was measured by the amount of ^{3}H-TdR (CPM) detected by ß-scintillation spectroscopy.

The % hybridization was calculated from the following formula:

$$\% \text{ hybridization} = \frac{\text{Sample CPM-Background CPM}}{\text{Total counts CPM of probe}}$$

Cultures positive for mycoplasma were those with % hybridization greater than or equal to 0.4%. Negative controls were always less than 0.2% and positive controls were greater than 30%.

Mycoplasma positive cultures were cleared of mycoplasma using BM-cycline antibiotics BM1 and BM2 (Boehringer Mannheim, Mannheim, West Germany). Cell cultures were initiated with BM1 at a dose of 10µl antibiotic per 10 mls tissue culture medium. After 3 days the cells were washed and resuspended in fresh medium and BM2 antibiotic (10µl/ml), and there after cultured with alternating cycles of BM1 and BM2 for a total of 2-3 weeks. The cells were retested for mycoplasma infection after treatment.

2.2.5 Laminar flow hoods and incubation conditions.

All cells to be cultured were handled asceptically in laminar flow hoods. There were separate laminar flow hoods for mycoplasma-free cultures (as determined by hybridization test) and mycoplasma contaminated cultures.

All cultures were incubated at 37^{0}C in a 5% CO_2 in air atmosphere (Hereus Christ). Separate incubators were used for mycoplasma-free and mycoplasma-contaminated T cell cultures. In addition, T cell cultures were maintained in separate incubators from B-LCL or murine L cell lines.

2.3. Cell separation techniques.

2.3.1 Peripheral blood mononuclear cells.

Peripheral blood mononuclear cells (PBMC) were isolated using density centrifugation on Ficoll-Hypaque (Pharamcia, Milton Keynes, England) density gradients after the method of Bøyum (1964). Blood was drawn from healthy individuals by venous puncture and placed into 20ml sterile universal containers (Sterilin, Teddington, England) containing 200 i.u. of preservative free heparin (Leo Labs Ltd., London, England). The blood was diluted 1:1 with RPMI 1640 before layering slowly onto Ficoll-Hypaque. Approximately 10 mls of diluted blood was layered onto an equal volume of Ficoll-Hypaque in a 20ml sterile universal container . The tubes were centrifuged at 800g for 20 minutes at 18^0C. After centrifugation the lymphocyte-rich interface was carefully removed using a Pasteur pipette and transferred to a fresh 20ml universal container, which was then topped up with RPMI 1640 before 10 minutes centrifugation at 400g. The resulting pellet was resuspended in fresh RPMI 1640 medium and centrifuged at 200g for 5 minutes. This step was repeated to remove any contaminating platelets.

2.3.2. Cell enumeration.

Cells in suspension were enumerated using Neubauer haemocytometers (Weber, England). Cell suspensions were examined by phase contrast microscopy (Olympus model BH-2) under 6000x magnification. The Neubauer counting chamber examined under the light microscope consisted of a grid divided into 25 squares. Cells in each of the 25 squares were counted and the total was multiplied by 10^4 to give the number of cells per ml of the suspension. Live cells were clearly distinguishable from dead cells by virtue of their bright appearance under

the microscope with blue haze around the cell membrane. In contrast dead cells had a dull appearance and had a grey colouration.

2.3.3. Monocytes.

Monocyte-enriched populations were obtained by adherence to plastic (Koller et al. 1973). PBMC were resuspended in HM containing 10% AB serum at a cell concentration of $2x10^6$ cells/ml, and incubated in 6 cm diameter plastic petri dishes (Falcon) for 1-2 hours. Non-adherent cells were removed with 5 rinses of warm RPMI 1640 medium, before 2mls of ice-cold competition reagent (CR) was added. The CR used was a modified version of a reagent described by Kumagai et al (1978) and consisted of phosphate buffered saline (PBS, Oxoid) with 0.05% EDTA and 10% human AB serum. Plates with CR were kept at 4^0C for 30 minutes, after which time most adherent cells had detached from the plastic and could be harvested using a Pasteur pipette. Any remaining adherent cells were removed by gently scraping the plates with the rubber end of a 1ml syringe plunger, followed by further rinses with cold CR. Between 5-12% of PBMC were adherent and these monocyte-enriched fractions were typically 80% CD14 positive and 90% HLA-DR positive as assessed by flow cytometry.

2.3.4. T cells.

T cell enriched populations were obtained by passaging PBMC through nylon wool columns (Julius, Simpson and Herzenberg 1973). These were prepared by packing 0.7g of acid washed and combed nylon wool (Fenwal, USA) into a 10ml syringe barrel (Monoject, England), then replacing the plunger. The column was sterilized by autoclaving before use. A sterile 21g needle (Monoject, England) was placed on the syringe barrel and sealed with a rubber bung. The column was filled with RPMI 1640 medium and any air bubbles were removed by working the nylon wool with the end of

a Pasteur pipette. The column was then resealed with the plunger and incubated at 37^{0}C. for one hour. During this incubation period, PBMC were depleted of monocytes by adherence to plastic as described previously (section 2.3.3). At the end of the incubation, excess medium was drained from the column and PBMC suspension (10^7/ml) was added to the top of the column drop by drop. An additional 1ml of medium was slowly added to the top of the column before the column was sealed and incubated for one hour at 37^{0}C. The bung and the piston were removed from the column and the non-adherent cells (T cell enriched fraction) were eluted from the column using 50 mls of warm RPMI 1640 medium, added at a rate of one drop per second. Cell purity was typically more than 90% using anti-CD3 antibody.

Alternatively >90% pure populations of T cells were obtained by overnight adherence of PBMC to deplete monocytes, then two cycles of immunomagnetic depletion (section 2.3.6) with a "cocktail" of monoclonal antibodies which included anti-DR (L243), anti-B cell (CD22) and anti-natural killer cell (CD56).

2.3.5. B cells

Purified B cells were obtained by positive selection of CD22+ve cells from PBMC using flow cytometry (Epics CS, Coulter Electronics, Luton, England). PBMC were incubated with CD22 antibody, followed by incubation with FITC-sheep anti-mouse antibody (see section 2.6.2 for full method). PBMC contained on average, 13% CD22+ve cells, and of these approximately 10% were selected by the EPICS cell sorter. A representative fluorescence profile is shown in Figure 2.1. Cells to the right of line 1 were CD22 positive (13.61% of total cells), and of these all the cells to the right of line 2 were selected by flow cytometry (10.72% of total). The Epics cell sorter was operated by Ms. Maria Daly (Department

of Immunology, RPMS), further details of the procedures are described in section 2.6.

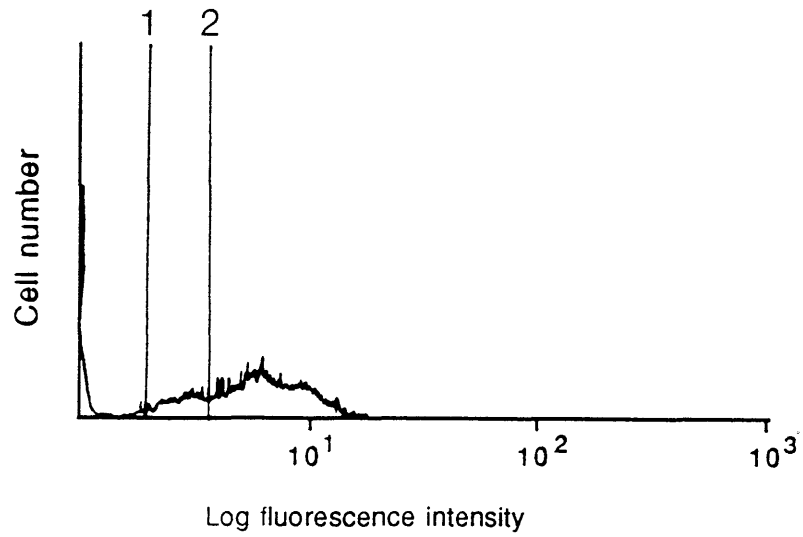


Figure 2.1. Fluorescence profile of CD22 positive cells isolated from peripheral blood.

2.3.6. CD4+ve T cell enriched fractions.

CD4+ve T cell enriched PBMC fractions for use in limiting dilution analysis (see section 2.5.2) were obtained by immunomagnetic depletion of CD8+ve T cells (Gaudernack et al. 1986).

PBMC were obtained by Ficoll-Hypaque gradient centrifugation as described (section 2.3.1). PBMC at a concentration of 2x 10^7/ml in HM were dispensed into a 10ml test tube and cooled in an ice/water mixture. Anti-CD8 antibody coated magnetic beads (HLA Class I Dynabeads, Dynal, Oslo, Norway) were mixed with PBMC at a ratio of 100µl of beads to 10^7 of PBMC. The bead/PBMC mixture was then left on a Rock-N-Roller (Model A257, Denley, England) for 30 minutes at 4^0C. to allow rosetting of beads with CD8 positive T cells. The contents of the tube were resuspended gently in 5mls of ice-cold HM, and a magnet applied to the side of the tube. Non-rosetted cells in suspension, were removed using a Pasteur pipette and dispensed into a clean tube. The rosetted population was washed twice more using the magnet and cold HM to free any remaining non-

rosetted cells. The non-rosetted population was washed, resuspended in HM and subjected to two more cycles of immunomagnetic depletion using fresh antibody coated beads. Efficiency of depletion was determined for every experiment by determining %CD8+ve T cells remaining in the depleted populations.

The simultaneous removal of CD8 and NKH1 positive cells for some LDA experiments was achieved by immunomagnetic depletion using an indirect method. PBMC were resuspended in ice-cold HM and saturating amounts of anti-CD8 (Dakopatts, High Wycombe, England) and anti-HNK1 (Becton Dickinson, Cowley, England) antibodies then left for 30 minutes on ice. After two washes with cold HM, immunomagnetic depletion was carried out as before, except that magnetic beads coated with sheep anti-mouse immunoglobulin antibody (Dynabead M450, Dynal) instead of anti-CD8, were used. The efficiency of depletion was determined by flow cytometry (see section 2.6.).

Purifed CD4+ve T cells were also obtained by positive selection of CD4+ve T cells using flow cytometry. T cell enriched fractions (see section 2.3.4.) were incubated with CD4 antibody, followed by incubation with FITC-sheep anti-mouse antibody (see section 2.6.2 for full method). CD4+ve T cells and CD4-ve T cells were collected from the flow cytometer, CD4+ve T cells being obtained with>99.4% purity. The negatively selected CD4-ve population was used as a source of CD8+ve T cells.

2.4. Cell culture techniques.

2.4.1. Lymphokines.

2.4.1.1. Human recombinant interleukin 2 (rIL2)

Human recombinant IL2 (rIL2) was obtained from Boehringer Mannheim (Mannheim, West Germany). Stock rIL2 (200u/ml) was

stored at -20^{0}C until required for use. Recombinant IL2 was diluted with TCM to provide working dilutions of between 2.5 and 20u/ml (v/v).

2.4.1.2. Human recombinant interleukin 4 (rIL4).

Human rIL4 (150,000 units/ml) was a gift from Genzyme, USA and diluted for use in RPMI1640. Aliqouts of this preparation were stored at -20^{0}C and were thawed as required. The biological activity of this preparation was determined by Mark Larché (Department of Immunology, RPMS, London).

2.4.1.3 Human recombinant interleukin 1 (rIL1).

Human rIL1α and rIL1β were provided by Glaxo Group Research (Greenford, England) and were diluted in RPMI1640 before storing at -20^{0}C. The biological activity of these preparations were determined in thymocyte assays by Nick Smithers (Glaxo Group Research, Greenford, England).

2.4.2. Generation of B-lymphoblastoid cell lines.

B-lymphoblastoid cell lines (B-LCL) were generated by incubating PBMC with Epstein-Barr virus (EBV) containing tissue culture supernatant from mycoplasma free marmoset cell line B-958 (method and cell line provided by Dr Frances Gotch, Oxford, England).

The supernatant was prepared by culturing B-958 cell line in 75cm^3 tissue culture flasks (Falcon) until a density of approximately 10^6/ml had been reached. At this point, the lids of the tissue culture flasks were sealed and the cultures incubated at 37^{0}C. for one week. The contents of the tissue culture flasks were decanted into sterile universal containers before centrifuging at 800g for 10 minutes. Cell-free supernatant was obtained by filtration (0.45μ), and was stored at -70^{0}C.

In order to EBV transform B cells, PBMC were pelleted by centrifugation (200g for 5 minutes) before resuspending in 1ml of EBV-containing supernatant ($5x10^6$-$10x10^6$ cells/ml). The PBMC-supernatant suspension

was incubated at 37^0C for 1 hour. The PBMC were washed in RPMI 1640 once, before resuspending in BCM (10^6 cells/ml) and dispensing into 24 well linbro plates (1ml/well). PHA (Wellcome, Beckenham, England) was added to each well at a dose of 2μg/well. The cultures were incubated at 37^0C. for ten days by which time T cell death had occurred. One ml of fresh BCM was added per well, and the cultures continued for a further 7 days. Spent medium (1ml) was removed from the cultures and 1ml of fresh culture medium added. The cultures were continued in 24 well plates until the wells became confluent, at which point the contents of all the wells were pooled and transferred to $25cm^3$ tissue culture flasks. B-LCL were generally established within 4-6 weeks, and were HLA typed before use.

Homozygous B-LCL defining subtypes of HLA-DR2 were provided by other laboratories as follows: Akiba, RML, WT18 (Dr J. Bodmer, ICRF, London), PGF and AZH (Professor A. McMichael, Institute of Molecular Medicine, Oxford). Selected homozygous B-LCL from the 10th Histocompatibility Workshop panel were also used, details of these can be found in the Table 6.2. All B-LCL were tested for their ability to stimulate polyclonal T cell lines, before use in T cell specificity experiments.

B-LCL were cultured in suspension in 25 or 75 cm^3 tissue culture flasks (Falcon) at a maximum density of $5x10^5$ cells/ml of BCM. The cells were fed with fresh BCM every 3-4 days.

2.4.3. Murine L cell transfectants.

The murine fibroblast (L cell) transfectants used in this study are listed in Table 2.1. They were cultured as monolayers in Dulbeccos modified Eagles medium (DMEM) supplemented with penicillin-streptomycin, l-glutamine and the appropriate selection drugs (see Table 2.1). When the L cell monolayers became confluent (every 3-4 days) the adherent cells were

Table 2.1. Murine L cell transfectants.

Name	HLA Class II.	Selection Media	Origin
Dap 3	None	None	R. Sekaly and E. O. Long, NIH, USA.
.5-3.1	DR1 Dw1	HAT[1]	R. Sekaly and E. O. Long, NIH, USA.
LDR2a	DR2 Dw2	HAT	D. Wilkinson and J. Trowsdale, ICRF, London.
LDR2b	DR2 Dw2	HAT	D. Wilkinson and J. Trowsdale, ICRF, London.
B20C	DR4 Dw14	G418	L. Barber and R.I. Lechler, RPMS, London.
RGT1	DRw11 Dw5	MXH[2]	R. Greenlaw and R.I. Lechler, RPMS, London.

More details of the transfectants can be found in the following references:
5-3.1, Lechler et al (1988), LDR2a and LDR2b, Wilkinson et al. (1988).

1 Hypoxanthine, aminopterine and thymidine.

2 Mycophenolic acid, xanthine and hypoxanthine.

detached using trypsin-EDTA (0.05% trypsin, 0.02% EDTA, Flow) and subcultured at a 1:4 dilution. The L cell monolayers were prepared for trypsin treatment by washing the cells free of excess serum with several rinses of DMEM. Trypsin-EDTA was then added to each flask of transfectants (2mls of trypsin/25cm^3 flask) and these were incubated at 37^{0}C until the L cells had detached from the plastic. The trypsin was inactivated by adding 10 mls of BCM. The cell suspension was decanted from the tissue culture flask, centrifuged at 200g for 5 minutes , and the cell pellet resuspended in fresh RPMI 1640. The cells were washed twice more in fresh RPMI 1640 by centrifugation at 200g for 5 minutes.

For proliferation assays the L cells were obtained by trypsin treatment of the monolayers on the day of the assay. Before use in the proliferation assays L cells were inactivated with mitomycin C (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan). L cells were pelleted by centrifugation (200g for 5 minutes), resuspended in 100µl of GM and mitomycin C added at a dose of 50µg/ml. The L cells were then incubated for one hour at 37^{0}C and washed three times in RPMI 1640 before use.

2.4.4. T cell cloning.

The protocol used for generating alloreactive T cell clones was derived from Sharrock and Sidhu (unpublished observations). The T cell clones described in this study were derived as follows. Ten million responder JRB PBMC were co-cultured with ten million X-irradiated (3000 rads, Caesium source) stimulator SM PBMC§ in a 25 cm^2 tissue culture flask (Falcon), in a total volume of 10 ml of TCM. The cells were harvested after six days, and T cell blasts were enriched by ficoll-hypaque density centrifugation. The T cell blasts were restimulated with fresh irradiated SM PBMC at a responder: stimulator ratio of 1:2, with culture conditions

§ Freshly isolated PBMC were used throughout the cloning procedure.

as before. This secondary (2^0) MLC was fed with fresh TCM supplemented with 20u/ml IL2 after three days of culture and cultured for another three days before being stimulated with irradiated SM PBMC again. The tertiary (3^0) MLC was fed with fresh TCM and rIL2 (20u/ml) after three days of culture, and cultured for a further four days before cloning.

Viable T cell blasts were obtained from the 3^0 MLC by Ficoll-Hypaque density centrifugation (section 2.3.1) and resuspended in TCM at 60 cells/ml. Both the T cell blasts and the stimulator cells were dispensed into Terasaki wells (Cel-cult, Sterilin) using Hamilton repeating syringes (model 1750 for 10µl volumes, model 1725 for 5µl volumes). These syringes were sterilized by rinsing with a 70% ethanol in water solution (6 rinses) followed by further rinsing with RPMI 1640 (6 rinses). Irradiated stimulator PBMC (10^4 cells/well) were dispensed into Terasaki wells in a volume of 10µl of TCM with 20/ml rIL2, prior to the addition of the T cell cloning cell suspension at 5µl per well. The T cell cloning suspension was resuspended at regular intervals. "Dummy" plates containing T cell blasts only, were set up to check for clumping.

After 7 days of culture, the Terasaki wells were screened for the presence of proliferating T cells using an inverted microscope (Wild Heerbrugg, Switzerland) under a 150x magnification. Wells were scored positive if T cell blasts occupied more than one third of the culture well area. Positive wells were noted and left for a further three days for clonal expansion and to eliminate false positives. The contents of individual positive Terasaki wells were transferred using a Pasteur pipette to flat well microtitre plates (Flow), with 10^5 PBMC in 200µl of GM plus rIL2. Further expansion of T cell clones was achieved by subsequent transfer to 48 well plates (Costar) containing 10^6 PBMC/well, then to 24 well linbro plates (Sterilin)

containing $2x10^6$ PBMC/well. T cell clones were cryopreserved (section 2.4.6) and stored in liquid nitrogen until required.

2.4.5. Growth of T cell clones.

T cell clones (TCC) were grown by stimulation with irradiated SM PBMC (freshly isolated or from cryopreserved stocks) at a TCC: stimulator ratio of 1:2. Recombinant IL2 was added at a dose of 20u/ml. The cultures were inspected every day using an inverted microscope at 150x magnification. When culture wells became confluent with T cell clones the contents of individual culture wells were resuspended and half the volume dispensed into new wells. Fresh GM and rIL2 was added to all wells.

T cell blasts were usually observed on days 3-4 post PBMC stimulation, with peak proliferation occurring on day 4-5. A six fold increase in cell number was achieved with fast growing T cell clones by day 5. After this time T cells gradually returned to resting morphology with a decrease in size and a decrease in growth rate.

Restimulation with fresh PBMC occurred when the cultures were observed to have resting morphology (days 5-7). For restimulation, T cell clones were washed twice with RPMI 1640 then resuspended in TCM before addition of fresh irradiated SM PBMC and 10u/ml rIL2§ . T cell clone cultures were occasionally cleared of debris by Ficoll-Hypaque centrifugation, either before PBMC stimulation or 3-4 days after PBMC stimulation.

2.4.6. Cryopreservation of Cells.

The cell freezing mixture consisted of 40% RPMI 1640, 10% Dimethylsuphoxide (DMSO, BDH, Poole, England) and 50% FCS. This was

§ Some time after the initiation of T cell cloning cultures , it was found that the rIL2 concentration could be decreased from 20u/ml to 10u/ml with no signifcant effect on proliferation.

cooled in an ice bath for at least 30 minutes before use. Cells to be frozen were washed once in RPMI 1640 before resuspending in pre-cooled freezing mixture (1-5x10^6 cells/ml). This suspension was dispensed into pre-cooled plastic vials (Nunc, Denmark). The vials were parcelled in paper towels and wedged into a polystyrene box, lined with paper towels. The box was left overnight in a -70^{0}C freezer. The following day the frozen vials were transferred to liquid nitrogen for long term storage.

When required, cryopreserved cells were thawed rapidly in a 37^{0}C waterbath. The thawed cell suspension was removed from the vials using a pasteur pipette and then dispensed drop by drop into a sterile tube containing 9 mls of RPMI 1640. The cells were washed twice in RPMI 1640 before use.

2.5 T cell function assays.

2.5.1 Proliferation assays.

2.5.1.1 Assay conditions.

The proliferative response of the T cell clones to irradiated stimulator cells was assessed by measuring thymidine ([^{3}H-TdR] , Amersham International, England.) uptake. The proliferation assays were set up in round-bottom 96 well microtitre plates (Falcon) with 10^4 responder T cell clones per well. The T cell clones were co-cultured with either X-irradiated 2x10^4 B-LCL or 5-10x10^4 PBMC in a final volume of 200 µl of GM. The radiation doses used for PBMC and B-LCL were 3000 rads and 10,000 rads respectively. B cells were irradiated with 1000 rads as in murine studies (Frohman and Cowing 1985).

The proliferative response of T cells to mitomycin C treated L cell transfectants (see Section 4.c.) was measured by culturing 10^4 T cells with 3x10^4 L cells in 96 well flat bottom plates (Flow). All proliferation assays were incubated for 72 hours with 1µCi/well of ^{3}H-TdR present for the last

18 hours. The contents of the culture wells were harvested onto glass-fibre paper (Tritertek, Skatron, Norway) using a semi-automatic cell harvester (Skatron) and the amount of ^{3}H-TdR uptake determined by β-scintillation counting (Model 1216 Rackbeta, LKB Wallac).

2.5.1.2 Data analysis.

Results from proliferation assays were expressed in four ways:

1. Mean counts per minute (CPM)± the standard deviation (SD), of triplicate wells for individual experiments.

2. Delta (Δ) CPM or the mean CPM of experimental wells with the background CPM subtracted. Standard deviation for these results was expressed as a % of the total experimental CPM. This was used to eliminate differences in background counts when data from a number of experiments were compared. ΔCPM was calculated in two ways depending if the proliferation assays were performed in a) the absence or b) the presence of rIL2:

a) ΔCPM= (CPM of TCC +stimulator cell)-(CPM of stimulator cell only).

b) ΔCPM= (CPM of TCC+stimulator cell+ IL2)-[(CPM of TCC plus IL2) +(CPM of stimulator cell only)].

When T cell clones were tested with stimulator cells in the presence of rIL2, a positive response was scored if the conditions shown below were satisfied:

[(Mean CPM of T cell clone+stimulator+IL2)-2SD] > [(Mean CPM of T cell clone+ IL2)+2SD].

3. Stimulation index (SI) was used to provide a rough guide to significant proliferation. A positive response to any given stimulator cell was defined as one where the calculated S.I. was greater than 10, in

accordance with the guide-lines laid down for the T cell study for the 10th Histocompatibility Workshop. (Flomenberg et al. 1989).

Stimulation index was calculated from the following formula:

$$\text{Stimulation index (S.I.)} = \left(\frac{\Delta\text{CPM (TCC+stimulator)}}{\text{(CPM of TCC+medium)}} \right).$$

4. Percentage relative response (%RR) was used to compare pooled data from PBMC or B-LCL panel studies, as it was impossible to test all the TCC against all cells in a panel in one experiment. In order to overcome variations in S.I. between experiments and to allow comparison of the results, all proliferative responses were expressed as a percentage of the positive control (SM PBMC or SM B-LCL). Only results where the SI was >10 for the positive control were analyzed. The percentage relative response (%RR) was calculated from the following formula:

$$\%\ \text{RR} = 100\text{x} \left(\frac{\text{(}\Delta\text{CPM of clone+stimulator)}}{\text{(}\Delta\text{CPM. of clone+positive control)}} \right).$$

In the 10th Histocompatibility Workshop studies significant proliferation in panel studies was scored if %RR was greater than 25% (Flomenberg et al. 1989). In the current study positive responses were scored if the %RR of any TCC:stimulator combination exceeded the maximum %RR obtained with the negative control.

2.5.1.3. Monoclonal antibody blocking of proliferative responses.

In some experiments the effect of monoclonal antibodies on T cell clone proliferative responses was assessed by adding antibodies at the beginning of the culture period. The culture conditions were similar to those used

for proliferation assays except that stimulator cells were incubated with monoclonal antibodies for 30 minutes at 37^0C before the addition of the T cell clones. All antibodies used were added to microtitre wells in a volume of 50µl. When the antibody used was a tissue culture supernatant, the maximum concentration used for blocking experiments was 25% (50µl of supernatant in 200µl final volume). Purified antibodies were diluted in GM and used at a range of doses from 1-10µg/ml. Antibodies in ascites were also diluted in GM and were used at a maximum dilution of 1:100.

The inhibitory effect of monoclonal antibodies was expressed as % inhibition and was calculated from the following formula:

$$\%\text{inhibition}=100\text{x}\left(\frac{(\text{CPM in absence of antibody- CPM in presence of antibody})}{(\text{CPM in absence of antibody})}\right).$$

2.5.2. Limiting dilution assays.

2.5.2.1. Limiting dilution cultures.

The limiting dilution protocol used was a modified version of the protocol first decribed by Sharrock et al (1987). The level of reproducibility of this system has been established in previous studies (Sharrock et al. 1987, Kaminski et al. 1989). Limiting dilution (LD) cultures were set up in 96 well plates with v-bottoms (Sterilin). Graded numbers of freshly isolated PBMC or CD8 depleted PBMC (3,125-10,000) were co-cultured with $5\text{x}10^4$ irradiated (3000 rads) stimulator PBMC. Stimulator cells were prepared from cryopreserved stocks. 30 replicates were set up for each responder cell concentration. On days 3 and 6 the LD cultures were fed with TCM containing 5% rIL2 (10u/ml) and the cultures assayed for cell mediated lysis of B-LCL targets on day 10.

In some assays limiting dilution conditions were used to assess the effect of immunomagnetic depletion of bulk populations. In these experiments, responders at 10^4/well were mixed with $5x10^4$ irradiated stimulators. The cultures were fed with GM+rIL2 on days 3 and 6, as for the LD cultures and assayed for CTL activity on day 10.

2.5.2.2. *Statistical Analysis.*

Culture wells for LDA experiments were scored as positive if the counts exceeded the mean plus 3 standard deviations of the control wells (spontaneous release). Results from limiting dilution assays were analyzed using a computer program based on the Glim 3.12 statistics package (Royal Statistical Society, London). The computer program was developed in collaboration with Vic Aber (RPMS statistician) and was used;

A. To estimate CTLp frequency using the maximum likelihood method (De St. Groth 1985).

B. To provide 95% confidence limits for the CTLp frequency estimate.

C. To fit a straight line based on the experimental data, with a y-intercept of 1.0. Such a line is indicative of single hit kinetics or the titration of a single cell type (Leftkovits and Waldmann 1986). The "goodness of fit" between the experimental points and the straight line generated was determined by the χ^2 statistic. P values (n-1 degrees of freedom) >0.05 were consistent with single hit kinetics.

Linearity of the experimental data was also assessed using regression analysis which allowed determination of the best straight line regardless of the intercept value. Points for the best fit straight lines and the correlation coefficent r (Campbell 1974b) were calculated using the Statworks 1.2 software package (Cricket Software, Philadelphia, USA) for the Apple Macintosh. Data was considered linear if the p value (n-2 degrees of

freedom) for r was <0.05. Experiments with non-linear results were rejected.

Experimental data was represented graphically as semi-logarithmic plots of responder cell number (horizontal axis) versus the fraction of negative wells (vertical axis). The lines shown were fitted to the experimental data by the maximum likelihood computer program (for lines with a y-intercept of 1.0).

CTLp frequencies were considered significantly different from each other if their 95% confidence limits were non-overlapping.

2.5.3. Cell-mediated cytotoxicity assays.

2.5.3.1. Target cells.

2.5.3.1.1 Phytohaemagglutinin stimulated T cell blasts (PHA blasts).

For preparation of PHA blasts, PBMC were prepared from fresh whole blood (section 2.3.1) or cryopreserved stocks as described (section 2.4.6) and resuspended at a concentration of 10^6 cells/ml of GM. This suspension was dispensed into a 24 well linbro plate in aliqouts of 1ml/well. Purified PHA (Wellcome) was added to each culture well at a dose of 2µg/ml. T cell blasts were observed within 3-4 days and the cultures were split and fed with fresh GM and IL2 as described for the T cell clones (section 2.4.5). The cultures were continued until the PHA blasts were required as targets (maximum of 7 days).

When required for cytotoxicity assays PHA blasts were harvested from the culture wells and washed three times before use.

2.5.3.1.2 B-Lymphoblastoid cell lines (B-LCL).

B-LCL were cultured as described (section 2.4.2). B-LCL were washed twice before use in cytotoxicity assays.

2.5.3.1.3 Murine L cell transfectants.

These were cultured as described (section 2.4.3). For cytotoxicity assays, L cells were trypsinized the day before the assay, resuspended in BCM and left as cell suspensions in 12 cm petri dishes (Falcon) until required for ^{51}Cr labelling.

2.5.3.1.4 Target cell lines sensitive to natural killer and lymphokine activated killer activity.

An HLA negative target cell line, K562, sensitive to natural killer mediated lysis, was used in some cytotoxicity assays. This cell line was grown in suspension in $75cm^3$ tissue culture flasks and passaged every 2 days. The presence of lymphokine activated killers (LAK) was determined using Daudi B-LCL. This cell line lacked HLA Class I but expressed HLA-DR6, and was cultured using the same culture conditions as for B-LCL (section 2.4.2).

2.5.3.2. Labelling target cells.

The cell-mediated lysis of the target cells described above was investigated by labelling the cells with Chromium-51 (^{51}Cr) isotope (as described by Brunner et al 1968) and determining the amount of isotope released from the targets after co-incubation with cytotoxic effector cells. A predetermined number of cells were pelleted in a 10 ml test tube. The supernatant was decanted and the cell pellet was resuspended and labelled with ^{51}Cr (Amersham, Bucks., England) using an isotope activity of $200\mu ci/10^7$ target cells. The target cells were incubated for 1 hour at 37^0C, with gentle flicking of the tube every 15 minutes to resuspend any pelleted cells. The targets were washed three times before resuspending in GM at a concentration of 10^5 cells/ml. Targets were used at a concentration of $2x10^4$ cells/ml when TCC effector numbers were limiting

or when LD cultures were assayed against multiple targets in split well analysis.

2.5.3.3. Cell mediated cytotoxicity assay for T cell lines and T cell clones.

T cell lines (TCL) generated by mixed lymphocyte culture or T cell clones (TCC) were cultured for 6-7 days before testing in cytotoxicity assays. These effectors were washed twice in RPMI 1640 before resuspending in culture medium. Effector cell concentrations varied from $6x10^5$-$5x10^6$/ml. The effectors were added to v-well microtitre plates in 100 µl volumes, with 3 replicates for each effector:target combination. When required, a range of effector:target ratios was obtained by serial dilution (three-fold dilution) of the top effector concentration.

^{51}Cr labelled targets were added to the experimental and control wells. Controls for the CTL assay consisted of 6 wells for total release (targets incubated with 1% Triton-X) and 6 wells for spontaneous release (targets in absence of effectors). The cultures were incubated for 4 hours at 37^0C then centrifuged (200g for 5 minutes) before the removal of 100 µl of supernatant from each well. The amount of ^{51}Cr released into the supernatants was determined using a gamma spectrometer (LKB 1260 Multigamma).

Specific lysis for each effector:target combination was based on the mean CPM for triplicate wells, and determined using the following formula:

$$\% \text{ specific lysis} = 100x\left(\frac{(\text{experimental release-spontaneous release})}{(\text{total release-spontaneous release})}\right)$$

2.5.3.4. Cell mediated cytotoxicity assay for limiting dilution assay cultures.

LDA cultures were incubated for 10 days before testing in CML assays. Before the assay, 150 µl of supernatant was removed from each culture

well. The tissue culture plates put on a micro-plate shaker (Tritertek) for 2 minutes before adding 100 µl of the target cell suspension (10^5 cells/ml). All other conditions were identical to the cytotoxicity assay for TCL and TCC, except that number of replicates for spontaneous release was between 24 and 36.

2.5.3.5. Monoclonal antibody blocking of cell mediated lysis.

The specificity of cytotoxic cultures was investigated in the ^{51}Cr assay by incubating CTL targets with appropriate monoclonal antibodies (MAb) for 30 minutes at 37^0C, before adding the effector cells.

The % inhibition was calculated from the following formula:

$$\% \text{ inhibition} = 100x \left(\frac{(\% \text{ specific lysis no MAb} - \% \text{specific lysis plus MAb})}{\% \text{specific lysis no MAb}} \right).$$

2.6. Indirect immunofluoresence and flow cytometry

2.6.1. Source and specificity of monoclonal antibodies used.

The source and specificity of Mab used are shown in Table 2.2 Some antibodies used for antibody blocking experiments were dialysed to remove sodium azide. Visking tubing was prepared, by boiling for 30 minutes. After allowing to cool, the tubing was rinsed with PBS several times, both inside and out. The tubing was sealed at one end using plastic clips (Mediclip, England). The antibody was dispensed into the tubing which was then sealed. The antibodies were dialysed by immersing the tubing overnight in 10 litres of PBS at 4^0C. The PBS was agitated using magnetic stirring apparatus (Model 553, Chemlab, England). The next day, the dialysis tubing was immersed in 10 litres of fresh PBS for a further 6 hours. The dialysed antibody was removed from the tubing using a needle and syringe, and filter sterilized using a 0.22µ filter (Flow).

Table 2.2. Monoclonal antibodies used for functional and phenotypic analysis.

Antibody	Antigen	Specificity	Source
OKT3	CD3	T cells	ATCC[1], USA
Leu3a	CD4	Subset of T cells	Becton-Dickinson, UK
DAKO-T8	CD8	Subset of T cells	Dako, UK
YFC 118.88	CD11 (LFA-1)	Leucocytes	S. Cobbold, Cambridge, UK.
FMC17	CD14	Monocytes	Sera-lab, UK
RFB4	CD22	B cells	Sera-lab, UK
2A3	CD25 (TAC)	Activ. T & B cells	Becton-Dickinson, UK.
RR1	CD54 (ICAM-1)	Activ T cells, B cells, dendritic cells, macrophages.	W. Makgoba, RPMS, UK.
Leu19	CD56 (NKH-1)	NK cells and subset of T cells.	Becton-Dickinson, UK
TS2/9	CD58 (LFA-3)	Leucocytes, epithelial cells.	W. Makgoba, RPMS, UK.
W6/32	HLA class I	Nucleated cells.	Sera-Lab, U.K.
L243	HLA-DR	B cells, macrophages, dendritic cells, activ. T cells.	ATTC, USA
Genox 3.53	HLA-DQ1	As above.	ATTC, USA
B7/21	HLA-DP	As above.	ATTC, USA

[1] Hybridomas obtained from American Type Culture Collection, Maryland, U.S.A.

2.6.2. Indirect immunofluoresence staining.

Cells for phenotypic analysis were pelleted in LP3 tubes (Luckhams) at cell numbers between $2x10^5$-10^6. The supernatants were discarded and the cell pellet resuspended in 20µl of MAb (neat tissue culture supernatant or ascites diluted 1:1000) was added. Normal human serum was added to prevent non-specific binding through Fc receptors. The cell suspensions were left on ice for 30 minutes. After two washes with cold PBS supplemented with 5% FCS, the cells were resuspended in 10µl of fluorescein isothiocynate (FITC) conjugated Sheep anti-mouse IgG MAb (Amersham), used at 1:10 dilution in PBS. The suspensions were left for a further 30 minutes at 4^0C in the dark. The cells were washed three times and resuspended 200µl of ice-cold PBS containing 1% paraformaldehyde. The samples were stored in the dark at 4^0C until required for analysis.

Cells to used in functional assays, were handled under aseptic conditions during all flow cytometry procedures. All reagents used were sterile and all immunofluorescent labelling was carried out in laminar flow hoods. After labelling the cells were resuspended in ice-cold sterile PBS with 5% AB serum and kept on ice until required for cell sorting.

2.6.3. Flow cytometry.

Flow cytometry was used for phenotypic analysis of cell populations and for positive selection of specific cell types. Cells to be analyzed were labelled by indirect immunofluorescene as described in section 2.6.2. Phenotypic analysis was carried out using the EPICS Profile flow cytometer. Cells were analyzed for size and antigen density by forward light scatter and log fluorescence intensity respectively. Electronic gating (bit-map gating) was used to isolate individual populations of cells for analysis. For example lymphocytes from PBMC could be selected for analysis on the basis of their size.

Negative controls (cells stained with FITC-sheep anti-mouse antibody only) were run through the EPICS, an electronic gate was set above the non-specific background fluorescence. Any immunofluorescently labelled cell with fluorescence exceeding this level had positive expression of the specific antigen. Fluorescence histograms of cell number (abcissa) versus log fluorescence intensity (ordinate) were generated by computer integration of the number of positive cells within the electronic "window".

Mean cell fluorescence intensity of a cell population provided an indication of the density of antigens on individual cells. A high figure relative to the background indicated an antigen expressed at high density on the cell surface. The mean cell fluorescence intensity could also be used to compare antigen expression on different cell types, although this was only valid if this was done in the same experiment.

The procedure for cell sorting was similar to the one described for phenotypic analysis, except that the cells were always handled under asceptic conditions. Cells to be sorted were labelled with MAb by indirect immunofluorescence (section 2.6.2). Negative controls (same as above) were run through the fluorescence activated cell sorter (EPICS CS) so that appropriate electronic gating could be set, to detect cells positive for the antigen of interest. Positive cells were collected by the EPICS and deposited into test-tubes containing RPMI 1640. The cells were washed once with RPMI 1640 and resuspended in growth medium before use.

Chapter 3.

Factors influencing recognition of HLA class II antigens by alloreactive T cell clones.

3.1 Summary

The factors influencing human T cell recognition of HLA class II antigens were investigated using alloreactive CD4+ T cell clones (TCCs) generated against DR2 (DRw15) DQ1 (DQw6) Dw12 stimulator PBMC.

The TCC characterized were found to be heterogeneous in their properties, five out of eight TCC were cytotoxic, and there was variation in the ability of individual clones to proliferate against PBMC or B-LCL stimulator cells. One TCC was able to proliferate to both PBMC and B-LCL whereas the others appeared to have a preference for either PBMC or B-LCL only. In most cases non-responsiveness against B-LCL or PBMC could be explained by the activation requirements of individual TCCs, as non-responsiveness could be overcome by the addition of exogenous interleukin 2 (IL2). For one TCC the requirement of exogenous IL2 for proliferative responses to B-LCL could be explained by the requirement for autocrine induction signals found in the adherent fraction of PBMC but absent from B-LCL.

The HLA class II specificity patterns of four TCC were studied and were shown to be complex. Additional polymorphism in HLA-DR2 was revealed, which was not evident from serological or RFLP typing. Two TCC reacted against DR2 B-LCL only, whereas the other two TCC reacted against subsets of the DR2 cells tested. Cross-reactivity on non-DR2 cells was also observed.

The specificity of two TCC which reacted against DR2 (DRw15) B-LCL only, could be correlated with linear amino acid sequences found only in DR2 molecules. This is discussed in relation to the hypothetical model of MHC class II structure. The cross-reactivity shown by one TCC on DR2 B-LCL and DR5 B-LCL could not be explained on the basis of linear amino

acid sequence. This suggests that factors besides the MHC class II molecule , contribute to the allogeneic MHC determinants recognized by T cells.

Additional patterns of T cell reactivity support this view. One TCC was able to distinguish between between identical DR molecules associated with different HLA haplotypes. The failure of DR2 specific TCC to respond to allogeneic DR2 molecules expressed on all cell types suggests that allogeneic determinants may be cell-lineage specific.

Taken together these results argue against alloreactive T cells recognizing allogeneic MHC class II molecules only. The patterns of reactivity observed were consistent with the concept that alloreactive T cells recognize a complex of allogeneic MHC and bound peptide.

3.2. Introduction.

The definition of functional polymorphisms associated with HLA class II antigens is of importance not only for allowing more precise matching of HLA types for transplantation (reviewed by Morris et al. 1987) but also for understanding the mechanisms behind HLA associated diseases (reviewed by Batchelor 1984). HLA-DR2 is of interest because it's strong associations with diseases such as Goodpastures syndrome (Rees et al. 1978), multiple sclerosis (Batchelor and Compston 1978) and narcolepsy (Langdon et al. 1984).

HLA-DR2 was originally defined serologically, at the 7th Histocompatibility Workshop (reviewed by Bodmer 1977), and has subsequently been shown to consist of two serologically defined subtypes, DRw15 and DRw16 (Friedel et al. 1987, Batchelor et al. 1989).

Additional complexity among DR2 haplotypes has been revealed by cellular typing reagents which have defined D-region types (Dw types) or lymphocyte defined determinants (LD) among different ethnic groups (Table 3.1). For example, the DRw15 types detected by antisera can be further subdivided into Dw2, present in caucasian populations, and Dw12 ,present at high frequencies in Japanese populations. The DRw16 antigen group consists of non-Dw2/Dw12 variants such as LD-AZH, LD-MN-2, LD-tb24 and LD-5a defined by cellular typing of different ethnic populations (Table 3.1). The relationship between these subtypes was clarified after the 10th Histocompatibility Workshop resulting in the division of DRw16 into Dw21 (LD-AZH, MN-2, tb-24) and Dw22 subtypes (LD-5a).

A number of biochemical studies have demonstrated that all cells which are typed as HLA-DR2, express two types of DRß molecules, ßa and ßb (Sasazuki et al. 1985, Jacobson et al. 1985, Lee et al. 1987, Wu et al. 1987). The ßb chain always migrates to the same position on 2-D gels regardless

of DR2 subtype, whereas the position of the DRßa chain varies according to the DR2 subtype. Sasazuki et al. (1985) were the first to propose that the non-polymorphic DRßb chain shared by DR2 subtypes might be similar to the DRw52 and DRw53 antigens, which are said to be "supertypic" or shared by a number of DR haplotypes. e.g. DRw52 is found on DR3, DR5 and DR6 haplotypes. Thus the non-polymorphic DRßb chain can be said to be "supertypic" to all DR2 subtypes (Sasazuki et al. 1985, Lee et al. 1987).

Nucleotide sequencing of DR2 genes has revealed that the ßb genes of Dw21/Dw22 subtypes are a hybrid of sequences found in Dw2/Dw12 DRßa and DRßb genes (see Figure 3.1). This finding has lead to the proposal that DRßb genes of Dw21/Dw22 subtypes have arisen from a recombination event between DRßa and DRßb genes of Dw2/Dw12 subtypes (Lee et al. 1987, Wu et al. 1987). The differences in nucleotide sequence between the DRßb genes of different DR2 subtypes does not however result in differences in the net charge of the ßb protein chains, as resolved by 2-D gels.

Alloreactive TCCs are highly sensitive reagents for investigating functional polymorphisms of HLA class II antigens. Many studies have shown that the HLA class II determinants recognized by alloreactive TCCs also function as MHC-restriction elements for antigen-specific TCCs (Sasazuki et al. 1985, Jacobson et al. 1985, Zeliszweski et al. 1987).

In this chapter, a panel of alloreactive TCCs have been used to investigate the polymorphism of HLA class II antigens associated with HLA-DR2 haplotypes and to determine the factors which influence alloreactive T cell responses. The results for this chapter are divided into three sections, the first dealing with general properties of the TCCs generated, the second with their specificity and the final section with their requirements for activation.

Table 3.1 Serological and cellular subtypes of HLA-DR2.

Serological Subtypes	**T cell defined subtypes** (pre-10th Workshop nomenclature)[1]	**B-LCL**[2]	**Notes** (reference)
DRw15	Dw2	PGF, Schu	Most frequent DR2 subtype in caucasian populations (Thorsby and Piazza 1975).
	Dw12	Akiba, E4181234	Most frequent subtype in Japanese population (Sasazuki et al. 1980).
DRw16	Dw21 (LD-AZH)*	AZH	Discovered in Israeli population (Cohen et al. 1984).
	Dw21 (LD-MN-2)*	MN-2	Discovered in American caucasian population (Reinsmoen et al.1984).
	Dw21 (LD-tb24)*	WT18	Discovered in French caucasian population (Friedel et al 1984).
	Dw22 (LD-5a)	RML	Discovered in S.American Indians. In linkage with DQw3 (Layrisse et al. 1979)

[1]Defined by primed lymphocyte typing reagents (PLT) or homozygous typing cells (HTC).
[2] B-LCL defining Dw types. Full HLA types of these B-LCL can be seen in table 2 of the appendix.
*2-D gel electrophoresis and cDNA sequencing have shown that these subtypes are homologous in their DRß gene products.

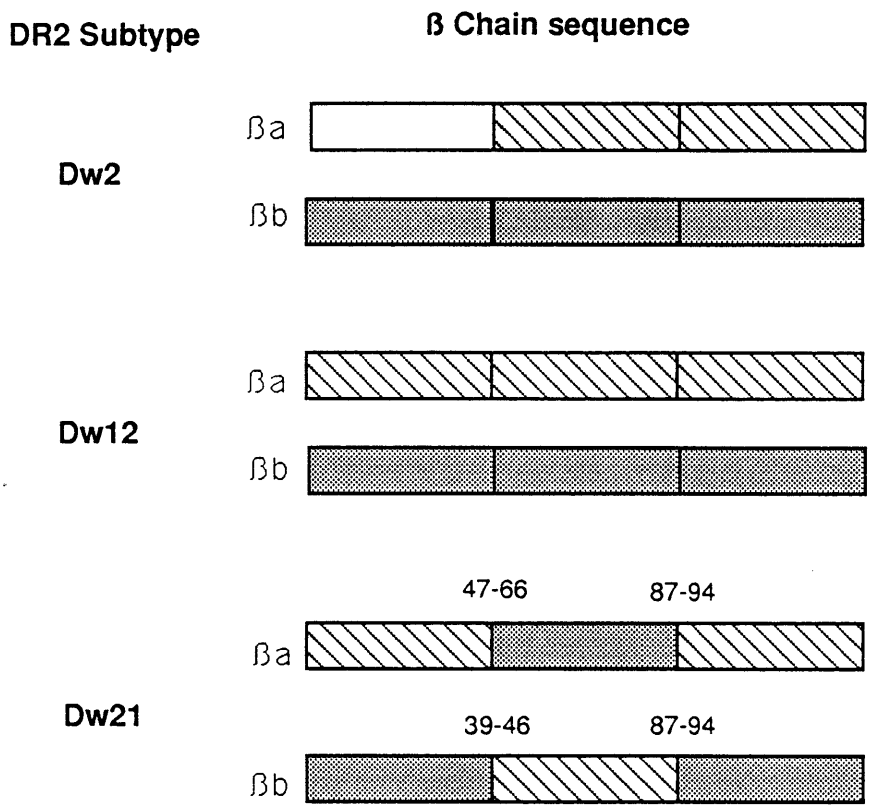


Figure 3.1 Relationship between DRß genes of DR2 subtypes. Diagram is modified from Lee et al. 1987. DR2 haplotypes express two DRß chain genes; DRßa and DRßb. The sequence homologies between 1st domain nucleotide sequences are indicated by the respective shading. Dw22 sequences are not shown as they are identical to Dw21. The Dw21/Dw22 subtypes are thought to have arisen by a recombination event between Dw12ßa and Dw12ßb genes. The numbers above the boxes indicate possible break-points for the cross-overs (Lee et al. 1987).

3.3 Results

3.3.1. General properties of alloreactive T cell clones.

3.3.1.1 SM stimulator cells express the DRw15 Dw12 subtype of DR2.

The stimulator cells for the T cell cloning were from an oriental individual SM, who was HLA typed as HLA A11,24 B13,15 DR2 (DRw15) DQw6. RFLP typing of SM DNA using Taq1 restriction enzyme and DRß DNA probes confirmed the serological typing data for HLA class II antigens, in that SM cells were shown to be homozygous for HLA-DRw15. The banding pattern obtained with SM DNA (Figure 3.2) is identical to those obtained for DNA from DRw15 cell lines (Schu, MGAR), with bands at 13, 1.6 and 1.3 Kb. This banding pattern is distinct from those obtained with DRw16 B-LCL (AZH, DEM, RML).

The serologically defined HLA-DRw15 can be subdivided into Dw2 and Dw12 subtypes on the basis of cellular typing. In order to determine the Dw subtype of SM cells, SM PBMC were typed using HTC reagents. SM PBMC were typed as non-Dw2, "probably Dw12" using HTC typing (N. Reinsmoen, personal communication).

RFLP typing of SM B-LCL using Taq1 restriction enzyme and DQß DNA probes confirmed that these cells were expressing the Dw12 subtype of HLA-DRw15. In Figure 3.3a it can be seen that SM B-LCL produces a banding pattern distinct from B-LCL typed as Dw2 (MGAR, PGF), Dw21 (AZH) and Dw22 (RML). The single 2.7 kb band obtained with SM DNA was characteristic of Dw12 B-LCL (Bidwell 1988). The results in Figure 3.3b obtained using the same Taq1: DQß combination show the banding pattern for SM more clearly and additionally show that the pattern is distinct from those obtained with DR8, DR6, DRw11, DR4 and DRw16 B-LCL.

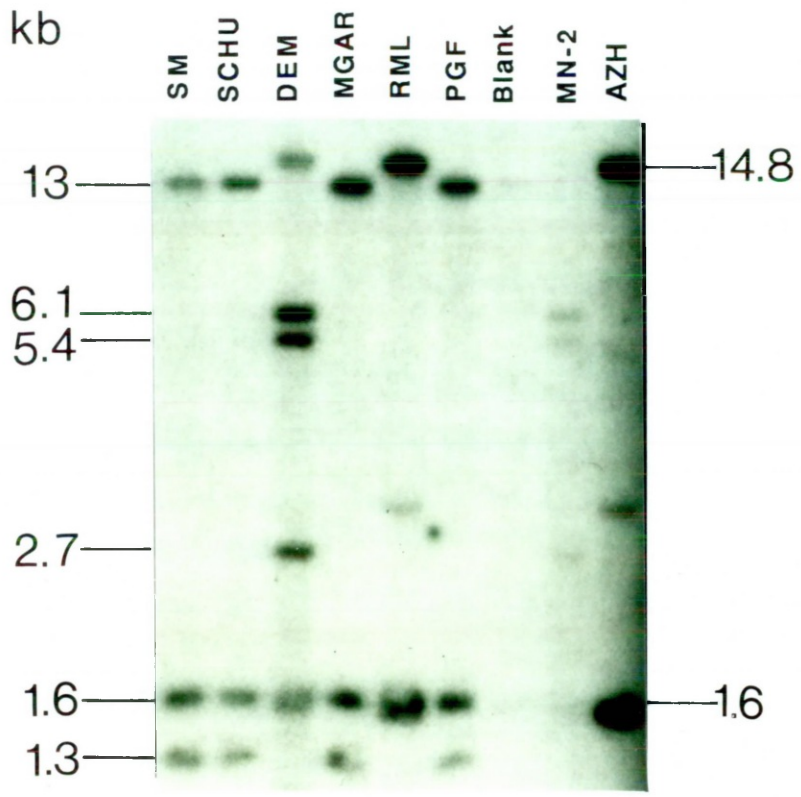


Figure 3.2. SM B-LCL types as DRw15 by RFLP analysis. Taq1 digests of genomic DNA from SM-B-LCL were southern blotted and hybridised with a DRß locus specific probe. DRw15 B-LCL (SCHU, MGAR, PGF) and DRw16 B-LCL (DEM, AZH, RML and MN-2) were also typed using the same reagents. DRw15 B-LCL are characterized by bands at 13, 1.6 and 1.3 Kb (Bidwell et al. 1988).

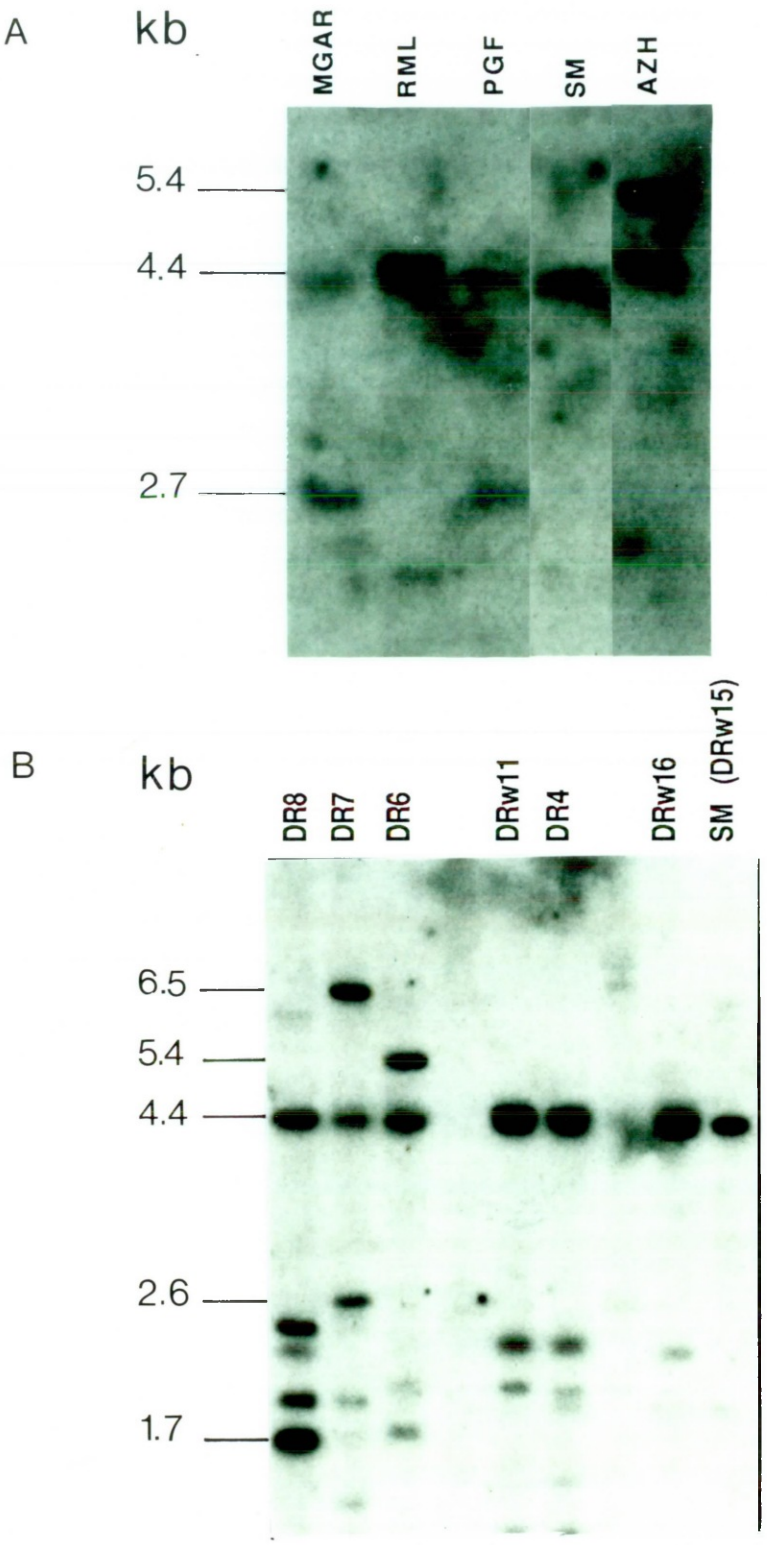


Figure 3.3. SM B-LCL types as Dw12 by RFLP analysis. (A) RFLP patterns of DR2 subtypes-Dw2 B-LCL (MGAR, PGF), SM B-LCL, Dw21 B-LCL (AZH) and Dw22 B-LCL (RML). SM B-LCL lacks the 2.7 Kb band associated with Dw2 B-LCL and has a different banding pattern from either Dw21 or Dw22 B-LCL. The results shown in (A) come from the same filter used in Figure 3.2, and some lanes have been removed for clarity. (B) DQß typing patterns obtained with DR8, DR7, DRw11, DR4 and DRw16 B-LCL compared with SM B-LCL. SM B-LCL is the only cell line to have the banding pattern indicative of the Dw12 subtype (Bidwell et al. 1988).

The RFLP data was supported by functional data, SM cells were unable to stimulate an alloreactive TCC (DAF10) which was specific for a DQw6 subtype associated with Dw2*. Representative results for TCC DAF10 are shown in Table 3.2. DAF10 was shown to react to all cells typed as DRw15 Dw2, but was unable to react to any of the cells typed as DRw15 Dw12. No reactivity was seen against SM B-LCL or PBMC, confirming that SM cells were not of the Dw2 subtype.

3.3.1.2. Generation of alloreactive T cell clones.

Alloreactive TCCs were derived from a TCL primed against SM PBMC. T cell blasts isolated from a T cell line were cloned using limiting dilution. The method used, is summarized in Figure 3.4 and has been found to favour the generation of CD4+ve alloreactive TCCs (Sharrock, Sidhu, Lombardi and Deacock unpublished observations).

The results in Table 3.3 show that the cloning protocol used was suitable for the efficient generation of alloreactive TCCs. There were a total of 303 out of 1170 wells plated at 0.3 cells/well which were scored as positive for T cell growth 10 days after cloning. The results in Table 3.3 also demonstrate the increase in plating efficiency when the plates were screened on day 10 as opposed to day 7. The majority (190/303) of the positive cultures at 0.3 cells/well were confluent by day 10 and these were transferred to 96 well microtitre plates containing fresh irradiated SM PBMC.

* Sharrock and Sidhu, Xth Histocompatibility workshop studies.

Table 3.2 SM cells do not express HLA-DQ molecules associated with HLA-DRw15 Dw2 haplotypes.

Cell[a]	HLA TYPE[b]			T Cell Clone Proliferation[c]	
	DR	DQ	Dw	Δ CPM	(%SD)
PGF B-LCL	w15	w6	2	6297	(5)
SCHU B-LCL	w15	w6	2	7570	(8)
AKIBA	w15	w6	12	-1621	(5)
E418 B-LCL	w15	w6	12	-3236	(2)
SM B-LCL	w15	w6	12	128	(4)
AZH B-LCL	w16	w5	21	-111	(3)
WT18 B-LCL	w16	w5	21	-1157	(20)
DF PBMC	w15,w11	w6, w7	2,nd*	12036	(2)
DPR PBMC	w15,w13	w6,6	2,nd	14634	(9)
ML PBMC	w15,4	w6,7	2,4	13188	(4)
RIL PBMC	w15,4	6,7	2,4	17757	(8)
RH PBMC	w15,7	w6,nt	2,nd	15670	(6)
SM PBMC	w15	w6	12	199	(39)
SS PBMC	w11,4	w7,w8	nd,DKT2	147	(25)

Experiments performed in collaboration with Dr. G.Lombardi, RPMS.

[a] Stimulator B-lymphoblastoid cell lines (B-LCL) were tested at stimulator:responder ratio of 2:1, peripheral blood mononuclear cell (PBMC) stimulators were tested at 10:1. Significant proliferation is boxed.

[b] For HLA Class I types of cell lines see Appendix.

[c] Proliferation of DQw6 specific T cell clone DAF10, measured by thymidine incorporation of triplicate cultures. Δ CPM=(Mean CPM of T cell clone+stimulator)-(Mean CPM of stimulator only). %SD= Standard deviation as a % of (Mean CPM of clone+stimulator). Results are pooled from several experiments, each responder:stimulator combination was tested at least three times.

* nd=not determined.

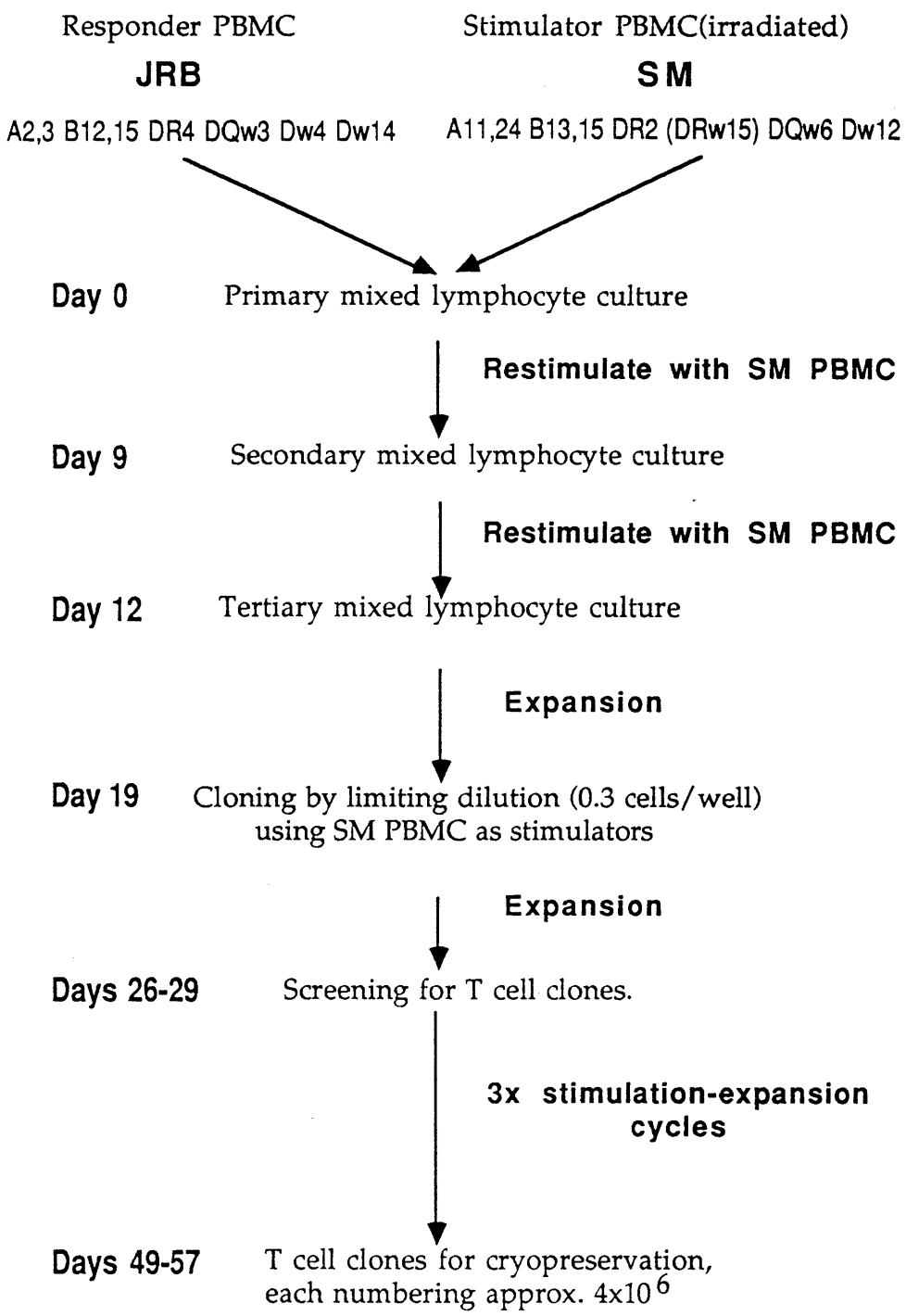


Figure 3.4. Summary of T cell cloning protocol.

Table 3.3 Efficiency of T cell cloning using limiting dilution method.

Dilution (cells/well)	Positive* wells day 7	%Positive# wells day 7	Positive wells day 10	%Positive wells day 10
100.0	60/60	100.0	60/60	100.0
10.0	109/120	90.8	120/120	100.0
3.0	18/120	15.0	72/120	60.0
0.3	132/1170	6.6	303/1170	25.9

T cell growth was observed in 167 out of 190 wells in the 96 well microtitre plates and the 77 fastest growing clones were restimulated with SM PBMC in 48 well, then 24 well tissue culture plates. Seventy TCCs survived these transfers and were frozen for storage in liquid nitrogen. Eighteen TCCs were thawed and cell numbers expanded by weekly stimulation with SM PBMC and rIL2. Eight of the fastest growing TCCs were selected for functional analysis. In addition the TCL used for the T cell cloning was included for comparative purposes in some specificity experiments.

It was not proven that the TCC isolated from limiting dilution of the TCL were derived from a single cell. The probability of clonality was however, greater than 88% according to the Poisson distribution§ (Campbell 1979a).

* Wells were considered positive if T cell blasts occupied >1/3 of the area of the Terasaki well.

% positive wells for each cell concentration = number of positive wells divided by number of wells plated per concentration.

§ Probability that cultures obtained were the progeny of more than one cell was 12%.

3.3.1.3 Phenotypic analysis of T cell clones.

The CD4 and CD8 phenotypes of eight TCC were determined by indirect immunofluorescence and flow cytometry. The results shown in Table 3.4 demonstrate that all eight TCCs tested were CD4+ve and CD8-ve, as in all cases the percentage of CD4 positive cells exceeded 90% and the percentage of CD8 positive cells were close to control values. No significant difference in the level of CD4 expression was observed between individual TCC, as can be seen in the FACS profiles shown in Figure 3.5. In contrast to the TCC, the TCL consisted of a mixture of CD4 positive (59.5%) and CD8 positive cells (44.5%). The CD8+ve cells in the TCL could be divided into distinct populations of low and high CD8 expressing cells, comprising 26.6% and 16.6% of the total respectively (Figure 3.5 e).

Table 3.4 Phenotypic analysis of alloreactive T cell clones.

Cells	% Positive cells[1]		
	Control[2]	CD4	CD8
SM PBMC	0.8	45.1	16.7
TCL[3]	0.2	59.5	44.6
3A1	0.2	96.1	0.2
4C3	0.1	97.9	1.2
7D5	1.8	92.6	0.7
8C1	0.4	95.4	0.5
9A10	0.4	99.2	0.7
9E9	0.5	98.7	0.4
9F6	1.9	91.3	1.8
18C2	0.6	97.6	0.4

[1] % positive cells determined by indirect immunofluorescence and flow cytometry. All T cell clones were tested at least twice with the same antibodies.

[2] Negative controls consisted of cells stained with fluoresceinated sheep anti-mouse antibodies only.

[3] TCL is the T cell line that T cell clones 3A1-18C2 were derived from. Two populations of CD8+ cells were observed (see figure 2 and results 3.2) : a population expressing a low density of CD8 (26.6%) ; and a population expressing a high density of CD8 (16.6%)

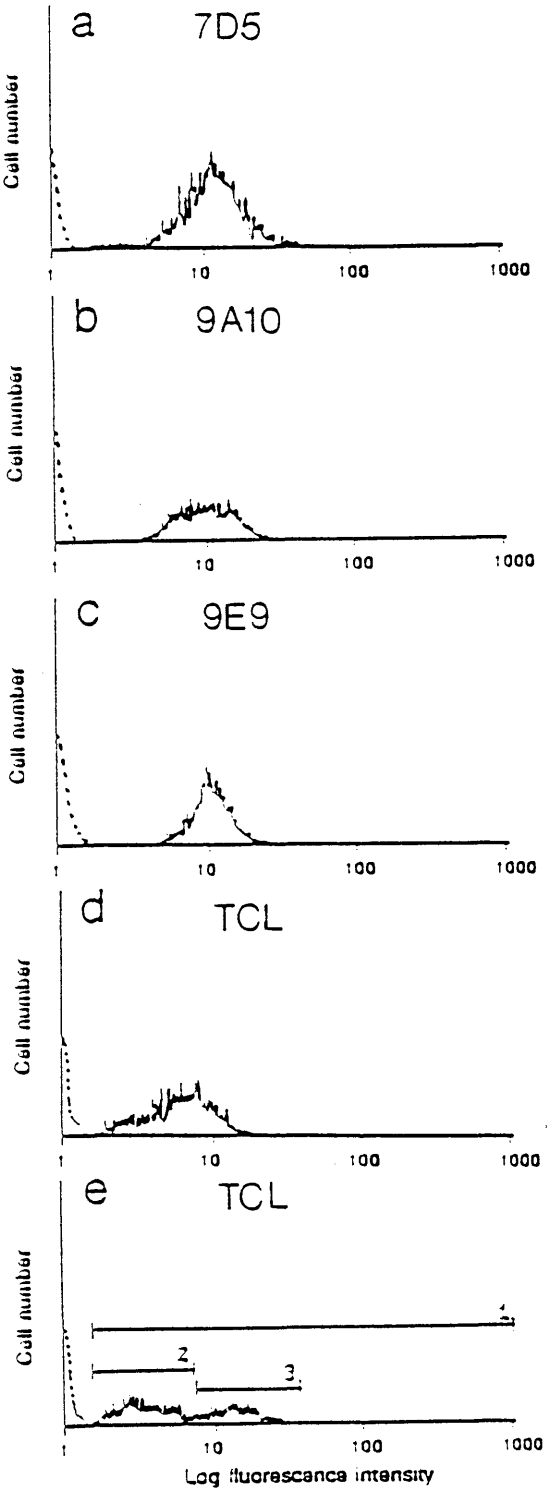


Figure 3.5. CD4 and CD8 expression of alloreactive T cell clones and a T cell line. The expression of CD4 on either T cell clones (a,b,c) or the T cell line (d) from which the T cell clones were derived, was determined by flow cytometry 6 days after PBMC stimulation. The fluorescence profiles shown (solid lines), were all obtained in the same experiment. The fluorescence profiles obtained for CD8 expression on T cell clones (not shown) were indistinguishable from the negative controls (dotted lines). The T cell line (e) contained 44.5% CD8+ve cells (1), with two distinct populations of low (2) and high (3) CD8 expressing cells. Negative controls (dotted lines) were cells stained with second layer fluoresceinated antibody only.

3.3.1.4 Five out of eight CD4+ve alloreactive T cell clones are cytotoxic.

The results in Figure 3.6 demonstrate that five out of eight CD4+ve TCCs (3A1, 4C3, 7D5, 9E9 and 18C2) were cytotoxic, being able to mediate lysis of SM B-LCL targets. This cytotoxicity was specific for SM B-LCL as no cytotoxicity for third party B-LCL (Madura), autologous B-LCL (JRB) or an NK sensitive target cell line (K562) was observed. In contrast the polyclonal TCL which consisted of CD4+ve and CD8+ve T cells, was able to mediate significant lysis of third party B-LCL, K562 and autologous B-LCL.

Two TCC 9A10 and 9F6 which were shown to be non-cytotoxic on SM B-LCL or SM PHA blast targets (Figure 3.7a and b) were however, able to lyse T cell hybridoma targets secreting anti-CD3 antibodies.

3.3.1.5 Heterogeneity in proliferative responses to B-LCL and PBMC among CD4+ve alloreactive T cell clones.

The results in Table 3.5 show that distinct patterns of reactivity were observed among the alloreactive TCCs when tested in proliferation assays using PBMC or B-LCL stimulators. Analysis of median values for stimulation index# revealed four distinct patterns of reactivity. TCCs such as 4C3 generally exhibited poor proliferative responses to B-LCL and weak responses to PBMC. In contrast there were TCCs, such as 7D5, which proliferated strongly to both PBMC and B-LCL. Other TCCs appeared to preferentially proliferate against either PBMC or B-LCL; TCCs 18C2 and 9E9 proliferated strongly to B-LCL but not to PBMC; whereas TCCs 9A10 and 9F6 proliferated strongly to PBMC, and not to B-LCL. The observed differences in proliferative responses between individual TCC did not correlate with functional cytotoxicity.

Stimulation index= $\frac{\text{(CPM of clone+ stimulator cell)-(CPM of stimulator cell alone)}}{\text{(CPM of clone only)}}$

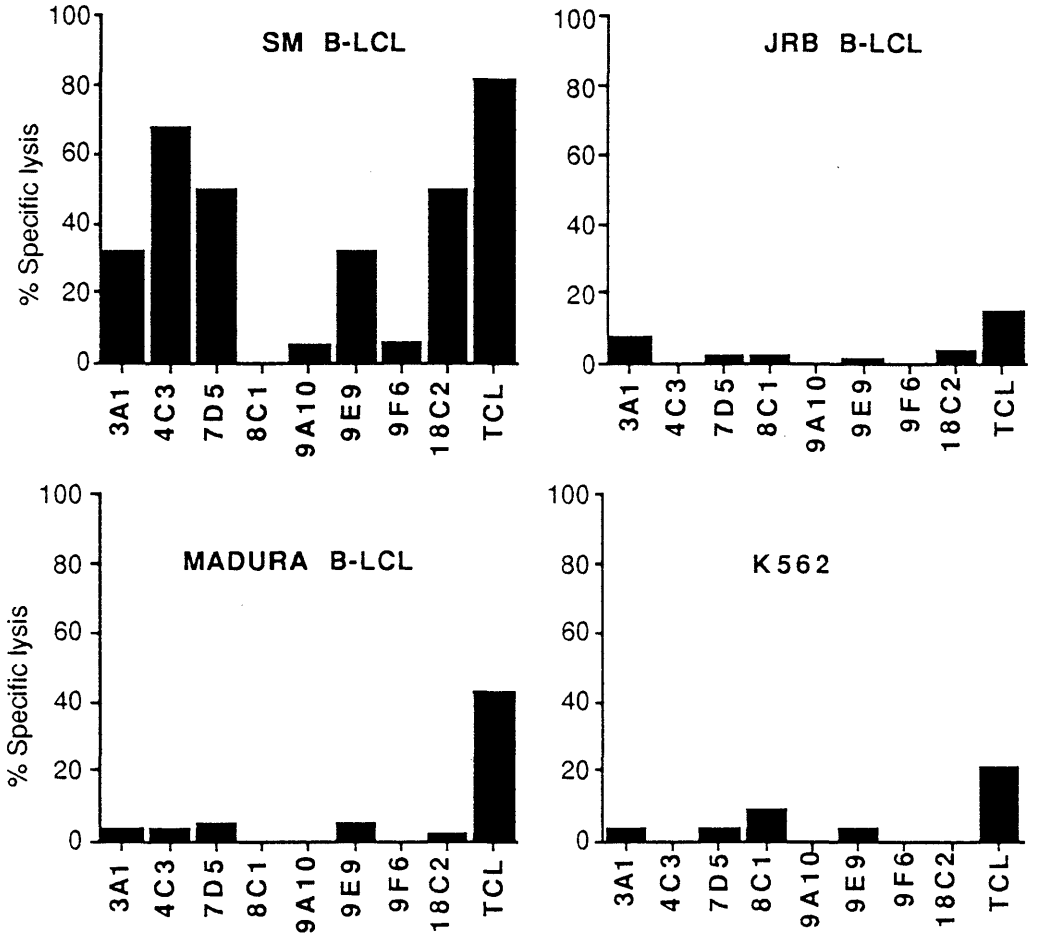


Figure 3.6. Five out of eight CD4+ve alloreactive T cell clones are cytotoxic. T cell clones were tested for cytotoxicity in chromium release assays at an effector: target ratio of 20:1. In addition the T cell line (TCL) from which the clones were derived was also tested. The clones were tested against targets from the original stimulator (SM B-LCL), autologous targets (JRB B-LCL),third party (Madura B-LCL), and an NK sensitive target (K562). Specific lysis figures were calculated from the means of triplicate cultures. Standard deviations were always less than 15%.

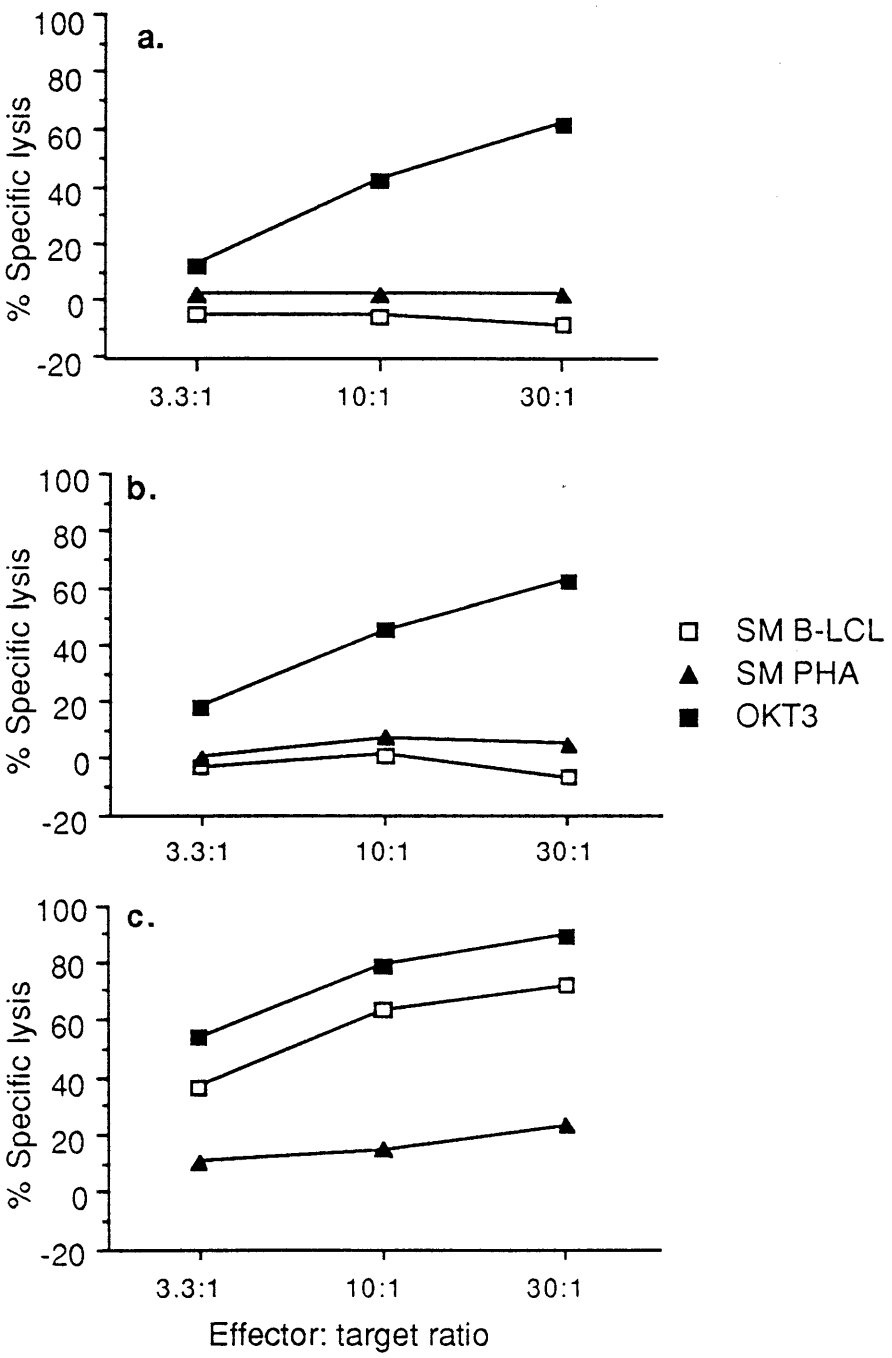


Figure 3.7. Non-cytotoxic alloreactive T cell clones can lyse a murine hybridoma secreting anti-CD3 antibodies. T cell clones 9A10 (a), 9F6 (b) and 4C3 (c) were tested in CTL assays 7 days post-PBMC stimulation. CTL targets were SM B-LCL, SM PHA blasts and OKT3 hybridoma cells. Negative % specific lysis figures indicate experimental counts which were lower than the spontaneous release counts.

Table 3.5 Heterogeneity in proliferative responses to B-LCL and PBMC among CD4+ alloreactive T cell clones.

Clone	CTL?	STIMULATION INDEX (N)*			
		B-LCL		PBMC	
4C3	YES	6.4	(8)	5.8	(8)
9E9	YES	**21.1**	(12)	2.9	(6)
18C2	YES	**19.9**	(12)	4.5	(6)
3A1	YES	8.5	(5)	**40 0**	(2)
8C1	NO	2.5	(8)	**14.1**	(4)
9A10	NO	2.2	(8)	**22.9**	(16)
9F6	NO	2.4	(14)	**83.0**	(15)
7D5	YES	**43.5**	(8)	**91.9**	(14)
TCL	YES	**81.8**	(10)	**159 0**	(8)

In order to investigate whether the differential responses of individual TCC against PBMC and B-LCL were due to sub-optimal doses of stimulator cells, TCC were tested at stimulator to responder (S:R) ratios from 0.1:1 to 10:1. SM B-LCL were found to be more potent stimulator cells than PBMC at low S:R ratios, with optimum proliferation responses being obtained at a S:R ratio of 1:1 for TCC 18C2, 7D5 and the TCL (Figure 3.8). The failure of TCC 9F6 and 9A10 to proliferate against PBMC and B-LCL stimulator cells (Table 3.5) was not due to a sub-optimal B-LCL dose, as TCC9A10 and 9F6 were unable to respond to B-LCL at any S:R ratio tested (Figure 3.8).

It is likely that the poor responses of TCC18C2 to PBMC were due to a sub-optimal S:R ratio, as the results in Figure 3.8 suggest that significant responses might be obtained using higher doses of PBMC.

* Median stimulation index from pooled data (see appendix for all data). n=number of experiments. SI greater than 10 (in bold type) indicate significant proliferation (Plätke et al. 1989). Stimulator: responder ratios used were 1:2 for B-LCL and 1:10 for PBMC.

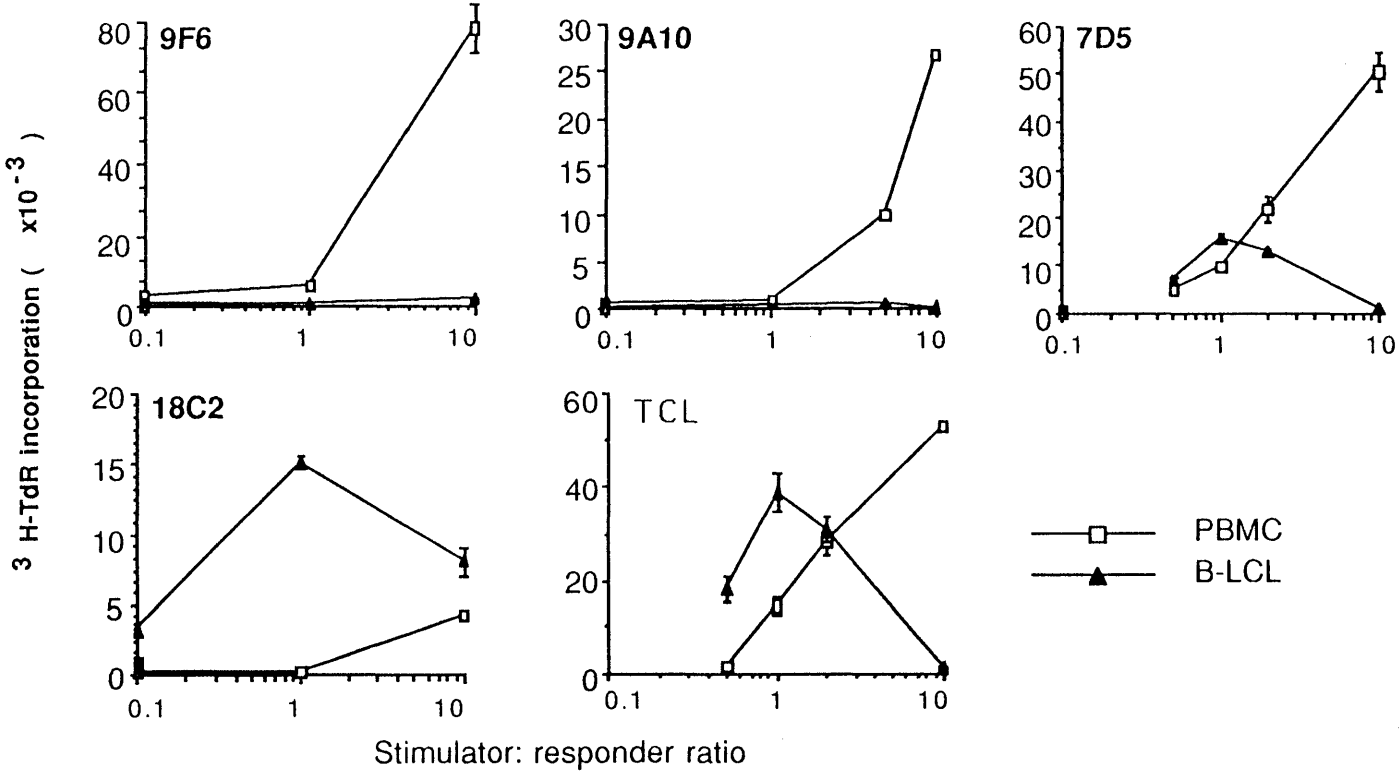


Figure 3.8. Efficiency of peripheral blood mononuclear cells and B-lymphoblastoid cell lines as stimulator cells for alloreactive T cell clones. Four T cell clones (9F6, 9A10, 7D5 and 18C2) were tested in proliferation assays 6-7 days post-PBMC stimulation. Ten thousand T cell clones were incubated with 1000-10,000 irradiated SM B-lymphoblastoid cell lines or SM peripheral blood mononuclear cells.

3.3.1.7 HLA-DR2 (DRw15) subtype specific proliferation of alloreactive T cell clones.

Preliminary studies were carried out to determine the DR2 subtype specificity of TCC 7D5, 9E9 and 18C2 using B-LCL expressing different subtypes of DR2. All of these clones were able to react to B-LCL serologically defined as DRw15 as shown in Table 3.6. None of the clones were able to proliferate against B-LCL expressing the DRw16 split of DR2 or against autologous JRB B-LCL. Non-responsiveness against the DRw16 +ve B-LCL was not due to a defect in the B-LCL as they were all able to stimulate the polyclonal TCL.

TCC 3A1 appeared to proliferate weakly to SM B-LCL, however proliferative responses of this clone were variable, with significant proliferation to SM B-LCL being obtained in only 1 out of 5 experiments.

The inability of some TCC (3A1, 4C3, 8C1, 9A10, 9F6) to respond to SM B-LCL was not due to a defect specific to SM B-LCL, as these TCC were similarly unable to respond to any of the other DR2 B-LCL tested.

3.3.1.8 Specific T cell clone responses to B-LCL and PBMC in the presence of exogenous IL2.

TCC 3A1 and 4C3 were unable to proliferate against SM B-LCL (Table 3.5) but were able to mediate specific lysis of SM B-LCL in CML assays (Figure 3.6). These results suggested that the inability of these TCC to proliferate against SM B-LCL was due to defects in activation and not in recognition. The results in Table 3.7 demonstrate that the inability of some TCC to proliferate against SM B-LCL was overcome by addition of exogenous rIL2. DRw15 specific augmentation of proliferative responses above the background (TCC+ IL2 only) was seen for TCC 3A1, 4C3 and 9F6. No augmentation was seen for TCC8C1 or 9A10.

Table 3.6 . HLA-DR2 subtype specificity of alloreactive T cell clones.

		^{3}H-TdR incorporation into T cell clones, ΔCPM (% SD)[1]																	
B-LCL	DR (Dw)	3A1		4C3		7D5		8C1		9A10		9E9		9F6		18C2		TCL	
Akiba	15 (12)	320[2]	(7)	396	(17)	17427*	(11)	-115	(11)	212	(9)	30089	(3)	-129	(9)	5051	(19)	32809	(7)
PGF	15 (2)	64	(27)	-343	(5)	17105	(9)	-122	(18)	187	(10)	36462	(5)	-101	(30)	1754	(8)	26939	(3)
SM[3]	15 (12)	2089	(18)	588	(12)	9614	(10)	112	(2)	124	(5)	8505	(9)	139	(2)	2424	(10)	20664	(18)
AZH	16 (21)	-166	(24)	-105	(19)	285	(10)	-192	(10)	-49	(13)	265	(8)	11	(10)	-80	(9)	26019	(7)
MN-2	16 (21)	234	(11)	224	(19)	465	(14)	-6	(6)	231	(11)	268	(26)	202	(22)	96	(12)	26293	(18)
WT18	16 (21)	-267	(11)	282	(7)	133	(7)	-832	(5)	-15	(33)	-200	(7)	-386	(31)	-100	(2)	19483	(17)
RML	16 (21)	169	(28)	-32	(4)	348	(26)	59	(2)	70	(37)	125	(42)	20	(7)	16	(5)	19003	(14)
JRB[4]	4 (4), 4 (14)	167	(15)	-15	(11)	665	(27)	59	(41)	77	(18)	1357	(29)	29	(60)	-87	(10)	767	(21)

[1] Delta (Δ) CPM = (Mean CPM of T cell clone + B-LCL)-(Mean CPM of B-LCL only). % SD= standard deviation of mean expressed as a % of the total CPM.
[2] CPM for T cell clones in absence of B-LCL was <277 CPM.
* Positive reactions underlined (stimulation index>10).
[3] Original stimulator for T cell clones.
[4] B-LCL autologous to T cell clones

Table 3.7. Some CD4+ve T cell clones require exogenous IL2 for proliferation against DR2 (DRw15) B-lymphoblastoid cell lines.

		^{3}H-TdR incorporation into T cell clones, ΔCPM (% SD)#									
B-LCL	DR (Dw)	3A1		4C3		8C1		9A10		9F6	
PGF	w15 (2)	16839	(4.7)	19255	(10)	-2684	(13)	-143	(4.8)	6080	(5.3)
Akiba	w15 (12)	14394	(6)	22488	(4)	-6168	(4)	509	(4)	15782	(7)
SM*	w15(12)	19583	(11)	15428	(9)	-9294	(15)	1660	(6)	6279	(12)
AZH	w16 (21)	-1327	(3)	-5092	(26)	NT	NT	2423	(2)	1178	(6)
MN-2	w16 (21)	-1410	(16)	-6790	(10)	-46	(3)	-327	(6)	847	(2)
WT18	w16 (21)	-1958	(6)	-2019	(3)	-4618	(7)	2099	(8)	1244	(5)
RML	w16 (22)	-6868	(23)	-10557	(9)	-4264	(17)	-152	(1)	-2782	(4)
JRB+	4 (4, 14)	-2087	(5)	-8576	(10)	-7201	(2)	-1139	(8)	-1007	(6)

ΔCPM= (mean CPM of T cell clone + B-LCL+ rIL2)-(mean CPM of T cell clone+rIL2). Significant values are underlined. %SD=% standard deviation of mean.

* Original stimulator for T cell clones.

+ Autologous to T cell clones.

Table 3.8 Recombinant IL2 augments T cell clone responses to PBMC.

	T cell clone proliferation (mean CPM thymidine incorporation±SD)[1]								
	3A1	**4C3**	**7D5**	**8C1**	**9A10**	**9E9**	**9F6**	**18C2**	**TCL**
PBMC	707± 156	1491±397	4004± 600	1625±336	2483±245	1377±283	2012± 20	387± 55	17889±1565
PBMC+IL2*	16304± 242	21083±1581	13872±1855	28167± 620	23466±1722	9614±724	28661± 1958	7288± 815	48873±2157
IL2	11140± 277	8517±1165	3952± 56	21804±480	5303±357	2713± 53	12222± 1200	1877±475	20076±922
MEDIUM	74± 24	79± 20	76± 11	105± 30	64± 16	584± 35	274± 102	105± 6	140± 36

[1] Mean thymidine incorporation of triplicate cultures each containing 10^4 T cell clone or T cell line (TCL) in the presence of $5x10^4$ PBMC. T cell clones or lines were tested 6-7 days after the last stimulation with PBMC. Data is from a single experiment and is representative of at least three seperate experiments for each T cell clone.

* Recombinant IL2 used at 10u/ml (v/v).

The addition of rIL2 to TCC cultured with sub-optimal numbers of PBMC also had an augmenting effect. The results in Table 3.8 demonstrate that the presence of rIL2 resulted in significant augmentation of TCC responses above control values (clone plus rIL2 only, or clone plus PBMC only). The results in Table 3.9 show that for at least 2 of the clones tested, the augmentation of PBMC responses was specific. Augmentation by rIL2 was seen for TCCs 4C3 and 9A10 only in the presence of SM PBMC, and not to other allogeneic PBMC.

Table 3.9. Recombinant IL2 specifically augments T cell clone proliferation to PBMC.

Donor	DR (Dw)	Thymidine incorporation (CPM±SD)	
		4C3	9A10
SM	w15 (12)	45033± 1021	66483± 800
GW	1 (1), 3 (3)	8368± 294	28684± 3393
TR	4 (-), 5 (-)	5420± 165	19532± 1787
YK	6 (-), 1 (-)	4110± 221	19546± 2466
SK	6 (-)	3079± 166	17326± 3216
MK	7 (-)	22960± 1914	21554± 2244
RG	w11 (-*), 8 (-)	3409± 1490	23066± 647
IL2		16833± 1310	19882± 2265
Medium		643± 55	769± 334

* Ten thousand TCC cultured with 50, 000 PBMC stimulators plus rIL2 (10u/ml). Results were considered significant for any TCC+PBMC combination if the CPM-2SD was greater than the CPM+2SD of the IL2 control.

3.3.1.9. Summary of the properties of alloreactive T cell clones.

The properties of the alloreactive TCCs which were characterized are summarized in Table 3.10.

Table 3.10. Summary of general properties of alloreactive T cell clones.

Clone/line	Phenotype	CTL*	Proliferation§			
			PBMC		B-LCL	
			-IL2	+IL2	-IL2	+IL2
3A1	CD4	Y	+	✔	-	✔
4C3	CD4	Y	-	✔	-	✔
7D5	CD4	Y	++	✔	++	✔
8C1	CD4	N	+/-	✔	-	✖
9E9	CD4	Y	-	✔	++	✔
18C2	CD4	Y	-	✔	++	✔
9A10	CD4	N	+	✔	-	✖
9F6	CD4	N	++	✔	-	✔
TCL	CD4 CD8	Y	++	✔	++	✔

* Cytotoxicity determined with SM B-LCL.

§ Proliferation in absence and presence of rIL2. ++ indicates strong proliferation, + indicates significant proliferation, +/- weak, generally not significant proliferation, - no significant proliferation , ✔ augmentation in presence of rIL2, ✖ no significant augmentation in the presence of rIL2.

3.3.2 Specificity of alloreactive T cell clones.

3.3.2.1 Alloreactive cytotoxic T cell clone recognizes HLA-DR molecules expressed on HLA-DRw15 +ve B-LCL.

The specificity of one cytotoxic TCC, 4C3 was determined in CML assays using a panel of B-LCL. The results in Table 3.11 demonstrate that TCC 4C3 was only able to mediate lysis of B-LCL typed as DRw15, with no lysis of B-LCL typed as DR1, DR3, DR4, DRw11, DR6 or DR7. TCC 4C3 was also able to lyse DRw15 PHA blasts, although with slightly lower efficiency than for B-LCL targets. No lysis of PHA blasts typed as DR1,7 or DR4,6 was seen.

Table 3.11. T cell clone 4C3 mediates lysis of DR2 (DRw15) target cell lines.

Target cell line	HLA DR (Dw)	Specific lysis[a]
WT100BIS B-LCL	1 (1)	-8.0
SSR PHA blasts	1, 7 (1, nt[b])	8.0
PB B-LCL	1, 2(1, 2)	<u>74.0</u>
PGF B-LCL	w15 (2)	<u>80.0</u>
DP B-LCL	w15 (2)	<u>73.0</u>
AKIBA B-LCL	w15 (12)	<u>79.0</u>
SM B-LCL	w15 (12)	<u>78.0</u>
SM PHA blasts	w15 (12)	<u>70.0</u>
ML B-LCL	w15 (2),4 (nt)	<u>63.0</u>
AZH B-LCL	w16 (21)	4.5
MN-2 B-LCL	w16 (21), 4 (nt)	0.0
RML B-LCL	w16 (22)	-7.0
VAVY B-LCL	3 (3)	3.0
CS B-LCL	3 (nt), 4 (nt)	2.0
JRB B-LCL	4 (4),4 (14)	5.0
ND PHA blasts	4 (4),w13 (19)	5.0
SID B-LCL	4 (KT2), 5 (5)	5.0
BM21 B-LCL	5 (5)	-3.0
TD B-LCL	6 (nt), 6 (nt)	4.0
FR B-LCL	7 (nt)	4.0

[a] T cell clone 4C3 was tested at an effector: target ratio of 30:1 on all targets, except SM PHA blasts where an effector: target ratio of 50:1 was used. Positive reactions (Specific lysis >10%) are underlined.

[b] nt=not tested.

The DR2 subtype specificity of TCC 4C3 as determined by CML assays, matched the results obtained in proliferation assays using rIL2 (Table 3.7) and suggest that the augmentation seen with rIL2 was specific.

TCC 4C3 was shown to be recognizing a determinant associated with the DR molecule. The results shown in Figure 3.9 demonstrate that an anti-DR monoclonal antibody, could inhibit the lysis of SM B-LCL, whereas antibodies against DQ, DP or HLA class I were unable to inhibit lysis.

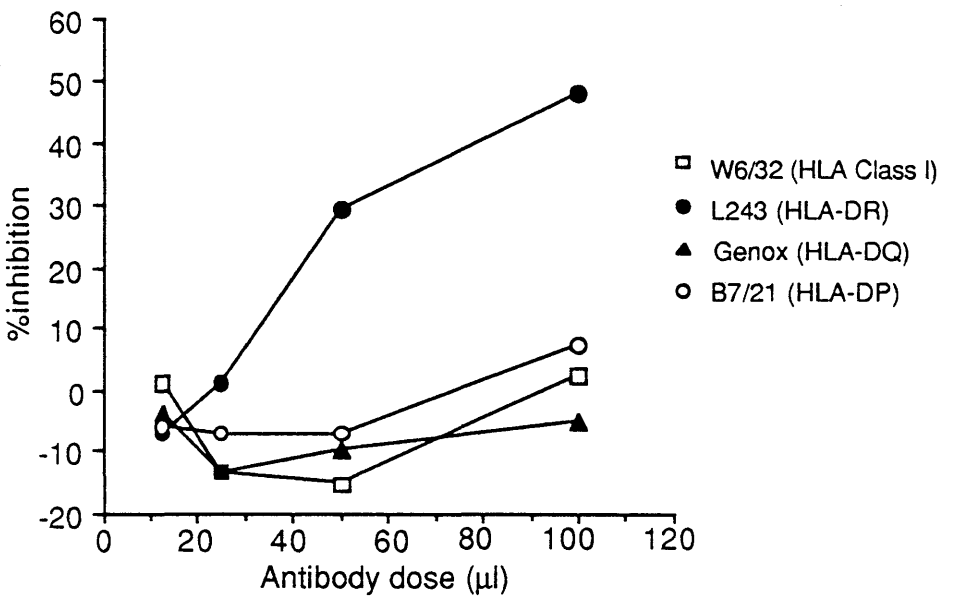


Figure 3.9. Cytotoxic T cell clone is HLA-DR specific. ^{51}Cr labelled SM B-LCL targets were incubated with monoclonal antibodies in the form of neat tissue culture supernatant (L243, Genox 3.53, B7/21) or ascites diluted 1:10 (w6/32). TCC 4C3 was added to provide an effector:target ratio of 20:1. Negative inhibition indicates an increase in specific lysis in the presence of the antibody. Specific lysis of SM B-LCL by 4C3 in the absence of antibody was 55%.

3.3.2.2 Additional heterogeneity amongst HLA-DR2 subtypes detected by alloreactive T cell clones.

The specificity of three TCCs, all of which has been shown to react to SM PBMC, was determined using a panel of 21 HLA typed PBMC (DR1-DR8). Three different patterns of reactivity were seen suggesting recognition of three different determinants associated with DR2. In Table 3.12 it can be seen that TCC9F6 and TCC7D5 were able to respond to some but not all of the DR2 (DRw15) Dw2 PBMC tested, indicating additional polymorphism of DR2 not detected by serology or Dw typing by RFLP. TCC 9A10 was only able to respond to the original stimulator SM PBMC out of all the DR2 PBMC tested, suggesting recognition of an allogeneic determinant absent from any DRw15 Dw2 PBMC.

Two of the TCC were also able to react to non-DR2 PBMC. TCC9F6 was able to cross-react on SS PBMC (DR4, DRw11), although this response was generally weaker than for DR2 PBMC. No basis for this cross-reactivity could be determined from the available serological typing data. In addition to recognizing SM PBMC, TCC 9A10 produced significant responses against GK PBMC (DRw11,w13), but not to any other DRw13 or DRw11 PBMC on the panel. The serological typing data for SM and GK indicated that they only had HLA-DQw6 antigens in common (see Table 6.2), although recognition of DQw6 by TCC 9A10 was unlikely as 9 other DQw6+ve PBMC failed to stimulate TCC 9A10.

Table 3.12. Additional heterogeneity detected in DR2 subtypes by alloreactive T cell clones.

Stimulator (ethnic origin)[b]	HLA TYPE[a]				Reactivity[c]		
	DR	DQ	DRw52/ DRw53	Dw	9A10	9F6	7D5
NF (c)	1	w5		1	-	-	ND[#]
GW (c)	1,w17	w5,w2	52	1,ND	-	-	-
PB (c)	w15,1	w5,w6		2,1	-	++	++
SM (o)[d]	w15	w6		12	++	++	++
ML (c)	w15,4	w6,w8	53	2,4	-	++	++
RIL (c)	w15,4	w6,w8	53	2,4	-	-	-
DP (n)	w15,w13	w5	52	2, ND	-	-	-
RH (c)	w15,7	w6, w2	53	2, ND	-	++	++
SF (c)	w17	w2	52	ND	-	ND	ND
CG (c)	4	w8	53	ND	-	-	-
TR (c)	4,w11	w8	53	ND	-	-	-
SS (ac)	4,w11	w2,w8	53	DKT2,ND	-	+	-
ND (c)	4,w13	w8,w6	53	4,18	-	-	-
GL (c)	w11	w7	52	ND	-	-	ND
JM (c)	w11	w7	52	5	-	-	-
GK (c)	w11,6	w7,w6	52	ND	+	-	-
EK (c)	w11,7	w7,w2	52,53	ND	-	-	-
RG (c)	w11,w8	w7,w4	52	ND	-	-	-
YK (o)	w13,1	w6,w5	52	ND	-	-	-
SK (o)	w13	w6	52	ND	-	-	-
MK (c)	7,7	w2	53	ND	-	-	-

[a] Determined by serological typing and RFLP typing. All DR2 (DRw15)+ve cells are enclosed by the box.

[b] Ethnic origin; C= Caucasian, O=Oriental, AC=Asian Caucasian, N=Negroid.

[c] Data is pooled from several experiments, reactivity is determined on the basis of % relative response of positive control (see Appendix Table 6.3). Positive reactivity (++) or (+) was scored if %RR for any stimulator cell was >than the negative controls (TR, ND, CG) in three repeat experiments. ++= %RR>50% in three repeat experiments, + indicates %RR>22%, but <50%, - indicates negative reactions consistently <22%.
The maximum %RR for the negative controls was 22%.

[d] donor of stimulator PBMC for all T cell clones.

[#] ND =not determined.

NB. T cell clone 9A10 was negative on a further 4 DRw15 Dw2 PBMC and 1 DRw16 Dw21 PBMC

3.3.2.3 HLA-DR and DP specificity of alloreactive T cell clones revealed by monoclonal antibody inhibition.

The distinct reactivity patterns produced by individual TCC when assayed on panels of HLA typed PBMC suggested that different allogeneic determinants were being recognized. Monoclonal antibody blocking experiments were carried out to determine whether the different patterns of reactivity could be explained by recognition of different HLA class II molecules. The results shown in Figure 3.10 demonstrate that 7D5, 9F6 and 18C2 recognize allogeneic determinants associated with DR molecules. Significant inhibition of proliferation was seen for both TCC in the presence of an anti-DR monoclonal antibody, whereas anti-DQ or anti-DP monoclonal antibodies had no significant effect.

The cross-reactivity of TCC9F6 on SS PBMC (DR4,5) was shown to be due to a cross-reaction on the DR molecule, as anti-DR monoclonal antibody was able to block the proliferation of 9F6 against either SM PBMC or SS PBMC.

In contrast to the other TCC tested, TCC 9A10 was only able to react against SM PBMC out of all the DR2 PBMC tested. TCC9A10 also produced different antibody blocking results from the other TCC tested, proliferation being inhibited by both anti-DR and anti-DP antibodies, but not anti-DQ antibodies.

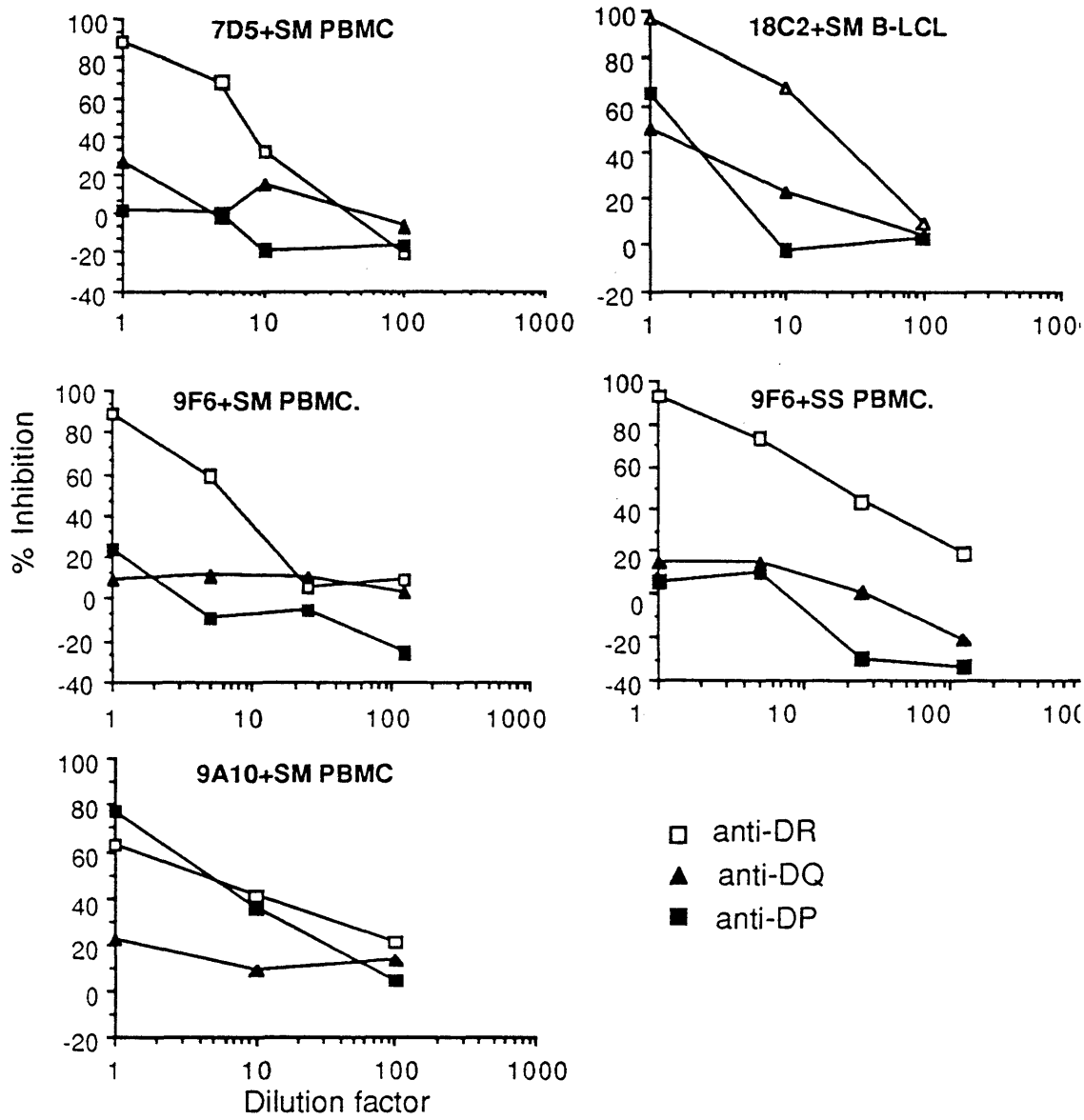


Figure 3.10. Monoclonal antibody inhibition of alloreactive T cell clone proliferation. Results are from separate experiments and are representative of at least two repeat experiments for each T cell clone. Stimulator cells (SM PBMC/B-LCL) were incubated with monoclonal antibodies for 30 minutes prior to adding T cell clones. Dilutions were made from the top dose of antibody added (50µl of supernatant in 200µl total volume). T cell clone 9F6 was also tested on SS (DR4,5) PBMC in the presence of monoclonal antibodies.

3.3.2.4 Alloreactive T cell clones recognize supertypic and subtypic determinants associated with DR2.

The specificities of three DR-specific TCCs were determined on a panel of 38 B-LCL defining all the major subtypes of DR1 to DR8. The results in Table 3.13 show two different patterns of reactivity for the TCCs tested. TCC 4C3* and 18C2 appeared to recognize a subtypic determinant or a determinant specific for the DR molecules expressed on DRw15 (Dw2 and Dw12) B-LCL, as significant reactions were only seen against DRw15 B-LCL, and not to B-LCL expressing subtypes of DR1,DR3, DR4, DR5, DR7 and DR8.

In contrast TCC 9F6* recognized a "supertypic" determinant or a determinant shared between DRw15 B-LCL (Dw12, some Dw2) and DRw11 Dw5 B-LCL. This cross-reaction confirmed the responses of TCC9F6 to some DR5 cells on the PBMC panel in Table 3.12.

3.3.2.5 An alloreactive T cell clone can distinguish between subtypes of DRw15 Dw2 on the basis of haplotype.

Cells typed as DRw15 Dw2 could be further subdivided into two types on the basis of the reactivity of TCC9F6. This subdivision correlated with the HLA haplotype of the DRw15 Dw2 +ve cells , as when the reactivity pattern of TCC 9F6 was compared to the full HLA class I and class II types of Dw2 Cells tested, reactivity was seen to correlate with expression of an HLA A3 B7 DR2 Dw2 haplotype (Table 3.14). No reactivity was seen to DRw15 Dw2 associated with other haplotypes.

* Tested in the presence of rIL2.

Table 3.13. Alloreactive T cell clones recognize supertypic and subtypic determinants associated with HLA-DR2..

B-LCL	HLA TYPE			Reactivity#		
	DR	DQ	Dw	9F6	18C2	4C3
NF	1	w5	1	-	NT	NT
SA	1	w5	1	-	-	-
WT100BIS	1	w5	1	-	-	-
PGF	w15	w6	2	+	+	+
DO208915	w15	w6	2	-	+	+
MGAR	w15	w6	2	-	+	+
DAF	w15, w11	w6	2,ND	-	+	+
SCHU	w15	w6	2	+	+	+
AKIBA	w15	w6	12	+	+	+
E4181324	w15	w6	12	+	+	+
SM	w15	w6	12	+	+	+
AZH	w16	w5	21	-	-	-
MN-2	w16, 4	w5,w8	21,4	-	-	-
WT18	w16	w5	21,1	-	-	-
RML	w16	w7	22,1	-	-	-
SF	3	w2	ND	-	-	-
BOLETH	4	w8	4	-	-	-
YAR	4	w8	10	-	-	-
PE117	4	w8	14	-	-	-
JRB	4	w8	4,14	-	-	-
JHAF	4	w7	13	-	-	-
KT2	4	w8	KT2	-	NT	NT
SID	4,w11	w8	KT2, 5	+	-	NT
SWEIG	w11	w7	5	+	-	-
BM21	w11	w7	5	+	-	-
BM16	w11	w7	5	+	-	-
JVM	w11	w7	NEW	-	-	NT
TISI	w11	w7	NEW	-	-	NT
HOR	w13	w6	19	-	-	-
ABO	w14	w5	9	-	-	-
AMALA	w14	w7	16	-	-	NT
CBG-IB	w13	w5	18	-	-	-
MOU	7	w2	17	-	-	-
LBUF	7	w2	DB1	-	-	-
LUY	8	w7	8.3	-	-	-
OLGA	8	w4	8.2	-	-	-
TAB	8	w6	8.3	-	-	-
BTB	8	w4	8.1	-	-	-

Reactivity data pooled from several experiments, each clone:stimulator combination was tested at least three times. Reactivity was calculated from % relative response in each experiment (see Appendix Table 6.4 for actual values).
Positive responses (boxed) were those with %RR > than the negative control (JRB) in three repeat experiments. ++ indicates %RR>50%, + indicates %RR<50% but >28%, negative responses (-) were those not satisfying these criteria. TCC 4C3 and 9F6 were tested in the presence of rIL2.

Table 3.14. An alloreactive T cell clone can discriminate between subtypes of HLA-DRw15 Dw2 on the basis of haplotypes.

Cell	HLA TYPES§					Reactivity of T cell clones¶		
	A	B	DR	DQ	Dw	9F6	18C2	4C3
ML	2,3	7,44	w15,4	w6,3	2,4	+	nd*	+
PB	3,11	7,35	w15,1	w6,w5	2,1	+	+	+
PGF	3	7	w15	w6	2	+	+	+
RH	1,3	7,40	w15,7	w6,2	2,nd	+	nd	+
SCHU	3	7	w15	w6	2	+	+	+
DAF	2,30	44,35	w15,w11	w6,	2,nd	-	+	+
Do208915	25	18	w15	w6	2	-	+	+
DPR	2,33	44,63	w15,w13	w6,w6	2, nd	-	nd	nd
MGAR	26	8	w15	w6	2	-	+	+
RIL	2,3	27,44	w15,4	w6,3	2,4	-	nd	nd

§ Determined by serological typing using Xth Histocompatibility workshop anti-sera and RFLP typing.

¶ Pooled data from proliferation assays for TCC 9F6 and TCC 18c2. Data from TCC 4C3 is pooled from CML assays and proliferation assay data. TCC 4C3 and TCC 9F6 were tested for proliferation in the presence of rIL2 (see Table 3.7). Reactivity of TCC 9F6 to Dw2 B-LCL is boxed.

* nd= not determined.

3.3.2.6 T cell defined polymorphisms of HLA-DR2 do not always correlate with linear amino acid sequence.

TCCs 18C2, 4C3 and 9F6 all recognized determinants associated with HLA-DR molecules (Figure 3.10). In order to determine whether the specificity of the TCC studied was due to direct recognition of DR molecules, amino acid sequences of DRß chains were analyzed for motifs correlating with the reactivity patterns of the TCC.

TCC 9F6 recognized an allogeneic determinant shared between DR molecules of Dw2, Dw12 and Dw5 subtypes. This reactivity could be explained by recognition of an amino acid motif shared by Dw2 DRßa, Dw12 DRßa and Dw5 DRß1 molecules (Figure 3.11). Further evidence for recognition of this motif in the third hypervariable region (HVR) comes from the negative reactions seen against DwNew B-LCL which have DRß1 chains which differ from Dw5 ß1 chains only at positions in the third HVR. TCC9F6 was however unable to respond to Dw21 and Dw22 B-LCL, which also have the motif in their DRßa chains which are actually more homologous to Dw5 DRß1 chains than Dw2/Dw12 DRßa chains (see Figure 3.1, and Figure 3.11). Thus no correlation could be seen between linear amino acid sequence and the specificity of TCC9F6.

The reactivity patterns of TCC 4C3 and 18C2 could be explained by recognition of an allogeneic determinant shared between DRß molecules of Dw2 and Dw12 subtypes but absent from all other DRß alleles. The specificity cannot be explained by recognition of Dw2 or Dw12 DRßa chains as these share sequence homology with Dw5 ß1 chains (see Figure 3.11). It can be seen in Figure 3.12 that Dw2 and Dw12 subtypes of DR2 have DRßb chains which are identical, with amino acids at positions 67, 70 and 71 (3rd HVR) being unique to DR2 subtypes. However the DRßa chain of Dw21 and Dw22 subtypes also contain the same amino acid residues (see Figure 3.1). The recognition of Dw2 and Dw12 B-LCL but not

		Amino acid position													Reactivity
		1st HVR					2nd HVR					3rd HVR			
Dw	ß chain	9	10	11	12	13	28	30	31	37	38	67	70	71	TCC 9F6
Dw2	ßa	Q	Q	D	K	Y	H	D	I	D	L	F	D	R	+
Dw12	ßa	-	-	-	-	-	-	G	-	N	V	-	-	-	+
Dw5	ß1	E	Y	S	T	S	D	Y	F	Y	V	-	-	-	+
DwNew	ß1	E	Y	S	T	S	D	Y	F	Y	V	I	-	E	-
Dw21	ßa	-	-	-	-	-	-	G	-	N	V	I	Q	A	-
Dw21	ßb	W	-	P	-	R	D	Y	F	S	V	-	-	-	-

Figure 3.11. An alloreactive T cell clone defines a polymorphism of HLA-DR2 which cannot be explained by linear amino acid sequence. Sequences were derived from Lee et al. 1987 (Dw2, Dw12 , Dw21), Tieber et al. 1986 (Dw5) and Bell et al. 1987 (DwNew). Amino acids are represented by the single letter code (see below) and dashes indicate homology with Dw2 ßa sequence. The boxed region indicates the predicted epitope for TCC9F6.

Key for code

G=glycine, D=aspartic acid, T=threonine, R=arginine, F= phenylalanine, L=leucine, Q=glutamine, K=lysine, Y=tyrosine, E= glutamic acid, C=cysteine, H= histidine, N=asparagine, V=valine, I=isoleucine, S=serine, M=methionine, W=tryptophan.

		Amino acid position													
		1st HVR					2nd HVR					3rd HVR			Reactivity
Dw /DR	ß chain	9	10	11	12	13	28	30	31	37	38	67	70	71	4C3/18C2
Dw2	ßb	W	Q	P	K	R	D	Y	F	S	V	I	Q	A	+
Dw12	ßb	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Dw21	ßb	-	-	-	-	-	-	-	-	-	-	F	D	R	-
DR3 DRw17	ß1	E	Y	S	T	S	-	-	-	-	-	L	-	K	-
Dw5	ß1	E	Y	S	T	S	-	-	-	-	-	F	D	R	-
DwNew	ß1	E	Y	S	T	S	-	-	-	-	-	-	D	E	-
DRw8	ß1	E	Y	S	T	G	-	-	-	-	-	F	D	R	-
Dw21	ßa	Q	-	D	-	Y	H	G	I	N	-	-	-	-	-

Figure 3.12 The specificity of some alloreactive T cell clones can be explained by linear amino acid sequence. Sequences obtained from Lee et al. 1987 (Dw2, Dw12, Dw21), Gorski et al. 1989 (DR3), Tieber et al. 1986 (Dw5), Bell et al. 1987 (DwNew, DR8). Sequence homology with Dw2 DRßb is represented by a dash.

other B-LCL can only be explained if sequences in the 1st, 2nd and 3rd HVR are considered together.

3.3.2.7 Failure of murine L cell transfectants expressing HLA-DR2 gene products, to activate proliferation or cytotoxicity of alloreactive T cell clones.

Analysis of amino acid sequences of allelic DRß genes predicted that TCC 4C3 and 18C2 might recognize determinants on the DRßb gene products. Murine L cells transfected with either DR2 ßa or ßb genes (Wilkinson et al. 1987) were used in an attempt to map the specificity of DR2 specific TCC to either DRßa or DRßb gene products. The results shown in Table 3.15 demonstrate that the murine L cell transfectants used, were unable to stimulate any of the DR2 specific alloreactive TCCs tested. Two TCC (4C3 and 9F6) which could proliferate to DR2 B-LCL in the presence of rIL2, were unable to respond to the DR2+ L cells under the same conditions. Similarly TCC 18C2 which could respond to DR2+ B-LCL in the absence of rIL2, was also unable to respond to DR2+ L cells.

DR2 specific TCC which were able to mediate specific lysis of DR2+ B-LCL were unable to mediate lysis of DR2+ L cell transfectants in either 4 hour or 7 hour CTL assays. Representative results from a 7 hour CML assay are shown in Figure 3.13. There was significant lysis of both B-LCL and PHA blast targets, however no lysis of the DR2+ L cell transfectants was seen.

The negative results obtained with the transfectants were not due to a low level of DR expression, as the transfectants express HLA-DR at a level approaching that seen on B-LCL and peripheral blood monocytes (Figure 3.14).

Table 3.15. Murine L cell transfectants expressing HLA-DR2 gene products fail to stimulate DR2- specific alloreactive TCCs.

Stimulator	Thymidine incorporation of T cell clone , ΔCPM (%SD)∞											
	4C3				9F6				18C2			
	-IL2		+IL2		-IL2		+IL2		-IL2		+IL2	
PGF B-LCL§	-463	(13)	29647	(14)	944	(42)	16324	(13)	4011	(7)	7105	(3)
LDRßa*	-981	(10)	-7500	(4)	-338	(25)	-1500	(10)	-955	(22)	-2816	(4)
LDRßbΩ	31	(15)	529	(9)	-959	(3)	253	(9)	-1196	(13)	-3066	(12)

∞Delta CPM = (Mean CPM of clone+Stimulator cell)-(Mean CPM of stimulator cell only). For responses against stimulator cells in the presence of rIL2 (10u/ml) , T cell responses to rIL2 in the absence of stimulator cells were subtracted. %SD is the standard deviation of the Mean CPM of clone+Stimulator cell, expressed as a %. 10,000 TCC were cultured together with 20,000 B-LCL or murine L cell stimulator cells. Significant proliferation is underlined.

§ DR2 ßa and ßb genes from PGF have been shown to be identical to the genes transfected into the murine L cells (Wilkinson et al. 1987).

* Murine L cell transfectant expressing DRßa genes which are polymorphic between DR2 subtypes.

ΩMurine L cell transfectant expressing DRßb genes which are electrophoretically constant between DR2 subtypes.

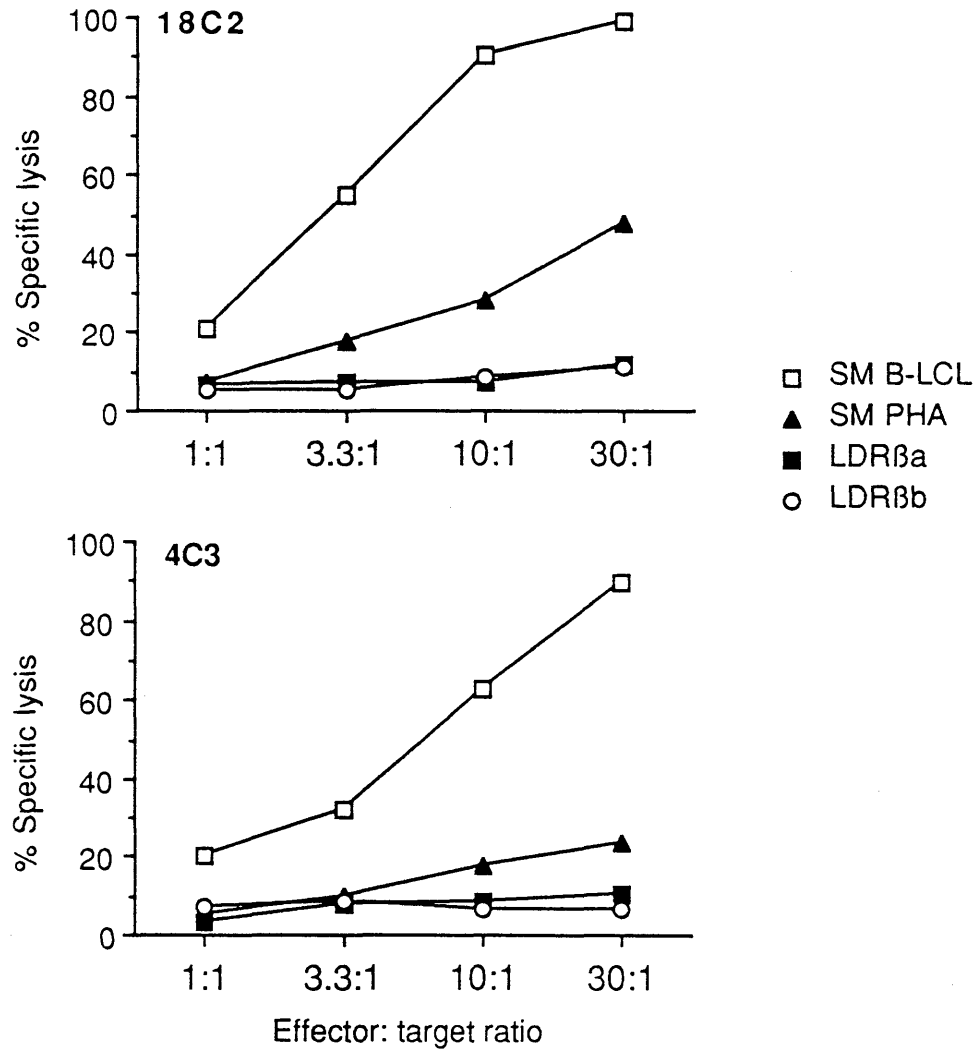


Figure 3.13. Failure of HLA-DR2 specific T cell clones to lyse murine L cell transfectants expressing products of HLA-DR2 genes. T cell clones 4C3 and 18C2 were tested in cytotoxocity assays on days 6-7 post-PBMC stimulation. Target cells were: SM B-lymphoblastoid cell line (□) and SM phytohaemmagglutinin blasts (▲) derived from the original stimulator individual for the T cell clones; mouse L cells expressing the DRßa chain which is polymorphic between DR2 subtypes (LDRßa ■) and mouse L cells expressing the DRßb chain which is non-polymorphic between DR2 subtypes (LDRßbO). T cell clones were mixed with target cells at theindicated effector: target ratios and incubated for 7 hours.

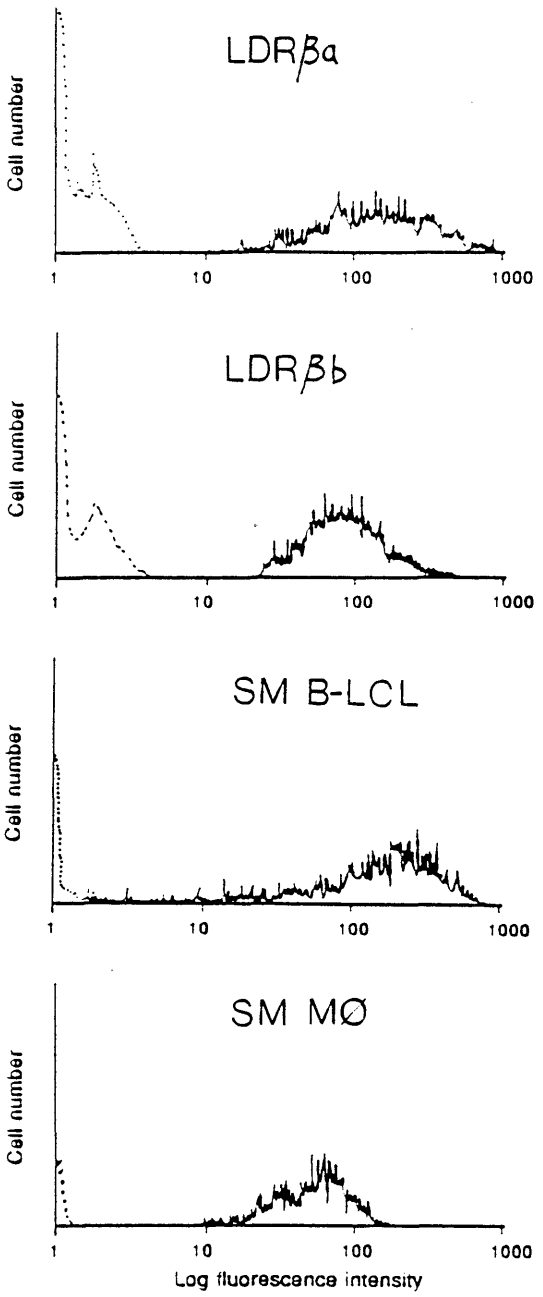


Figure 3.14. Flow cytometric analysis of cell surface HLA-DR expression. Murine L cell transfectants (LDRßa and LDRßb), SM B-lymphoblastoid cell line and adherent monocytes (from donor SM) were analyzed for expression of HLA-DR by indirect immuno-fluorescence labelling with L243 monoclonal antibody (solid lines). Negative controls were labelled with second layer fluorescent antibodies only (dotted lines).

3.3.3 Activation requirements of alloreactive T cell clones.

3.3.3.1 T cell clones which fail to proliferate in response to B-lymphoblastoid cell lines cannot be induced to respond by addition of interleukin 1 or interleukin 4.

TCCs 9A10 and 9F6 were unable to respond to B-LCL from the original stimulator (SM) in proliferation assays although both TCC were able to proliferate in response to SM PBMC (see Table 3.5). Specific responses to SM B-LCL could be obtained for TCC 9F6 but not TCC 9A10, in the presence of rIL2 (Table 3.7). TCC 9F6 and 9A10 were not dependant on exogenous IL2, as proliferative responses to PBMC could be obtained in the absence of exogenous IL2. In an attempt to characterize the activation requirements of these TCC further, the effect of other lymphokines which regulate T cell activation and growth was assessed.

Several studies have demonstrated an enhancing role for IL1 in T cell activation (reviewed by Mizel 1987). The presence of exogenous soluble IL1 had no effect on the activation of 9A10 and 9F6. The results in Figure 3.15 demonstrate that addition of either rIL1α or rIL1ß at doses from 1000-0.1pg/ml (100-0.1u/ml) were not sufficient to induce TCC 9A10 and 9F6 to respond to B-LCL, although the optimum dose of IL1 for other functional assays lies in this range (Nick Smithers personal communication). Similar results were obtained with PBMC, in that no significant augmentation of PBMC responses were seen for either TCC in the presence of exogenous rIL1α or IL1ß. In addition no responses were seen to rIL1α or rIL1ß when added to the TCCs in the absence of stimulator cells.

Interleukin 4 has been shown to be a T cell growth factor for some human TCCs (Spits et al. 1987). The results in Figure 3.15 demonstrate that rIL4 was not a growth factor for TCC 9A10 or 9F6, and was

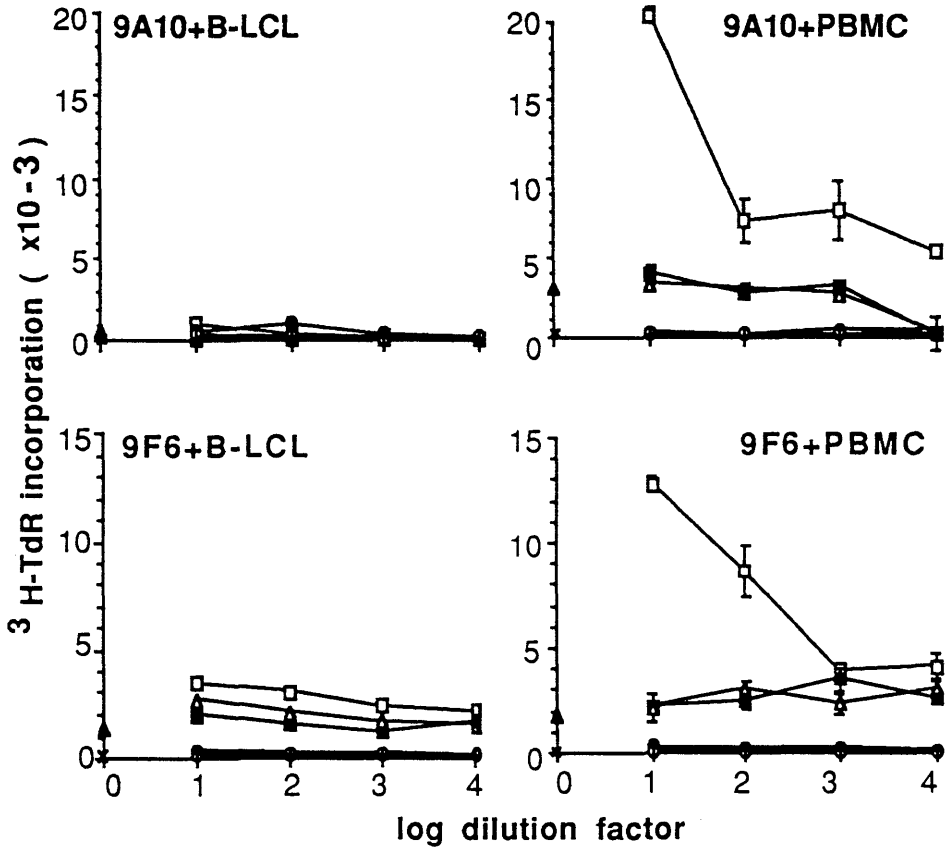


Figure 3.15. T cell clones which are non-responsive to B-lymphoblastoid cell lines cannot be induced to respond by interleukin1 or interleukin 4. T cell clones were cultured with either B-LCL or PBMC at a stimulator: responder ratio of 1:1 or 1:5 respectively. Interleukin 1 alpha or beta were titrated from a maximum dose of 1000pg/ml and interleukin 4 was titrated from a maximum dose of 150u/ml. Background counts of irradiated B-LCL and PBMC were 1041±192 and 50±15 CPM respectively. T cell clones were tested 6 days post-PBMC stimulation.

unable to induce either of the TCC to respond to B-LCL. In contrast, rIL4 was able to augment responses of both TCC against PBMC in a dose-dependent fashion. The augmentation of PBMC responses is likely to be due to the action of rIL4 on the PBMC stimulators, as no TCC responses were seen to rIL4 alone or to rIL4 plus B-LCL.

3.3.3.2 T cell clones respond to the adherent fraction of PBMC.

In order to characterize the HLA class II expressing cells which stimulated PBMC-reactive TCCs, PBMC were fractionated into plastic-adherent and non-adherent subpopulations. Between 5-12% of the total PBMC were recovered as adherent cells and phenotypic analysis of this fraction (Table 3.16) revealed an enrichment for HLA class II expressing cells (90%), the majority of which were monocytes (80%). The adherent fraction also contained B cells (7.5%) and T cells (4.6%). The non-adherent population had a similar composition of cells to unfractionated PBMC, with no significant decrease in the proportion of HLA-DR expressing cells. There were however slight decreases in the proportions of monocytes and B cells when compared to untreated PBMC.

Table 3.16. Phenotypic analysis of adherent and non-adherent fractions from peripheral blood.

	mean % positive cells ±SD (n)[a].			
Cell population	**DR**	**CD22**	**CD3**	**CD14**
PBMC	23.1±7.0 (8)	13.2±2.7 (6)	59.6±11.0 (7)	16.45± 8.9 (4)
Non-adherent cells	23.7±5.0 (6)	10.2±2.0 (6)	59.3±9.6 (7)	12.68± 3.8 (4)
Adherent cells	90.0±8.4 (6)	7.5±4.0 (4)	4.6±3.7 (5)	80.85±11.8 (4)

[a]. mean % of cells positive for antigens detected by indirect immunofluorescence over n experiments , SD=standard deviation of the mean. Negative controls (cells stained with second layer fluorescent antibody only) were always <3%.

A series of experiments demonstrated that alloreactive TCC responses against PBMC were due to stimulation by cells found in the adherent fraction of PBMC. Representative results from experiments designed to find the optimal cell dose of adherent cells are shown in Figure 3.16. The adherent cells were found to be more efficient stimulator cells than the untreated PBMC at a low cell dose (10^4). Optimal T cell responses were obtained using adherent cells at a dose of between $1x10^4$-$2x10^4$, however T cell responses against adherent cells at higher cell doses (greater than $5x10^4$) were variable. High cell doses were inhibitory to T cell responses in 3 out of 6 experiments, although adherent cells were always more potent stimulator cells than PBMC at low cell doses. Cells in the non-adherent fraction were always less efficient than untreated PBMC despite a similar proportion of DR expressing cells.

The results shown in Figure 3.17 demonstrate that cells contained in the adherent fraction of PBMC were the most potent stimulator cells for all alloreactive TCCs tested (panels a, b, d, e). Adherent cells elicited greater proliferative responses than untreated PBMC, B-LCL and the non-adherent fraction when tested at the same cell dose. The superior stimulatory ability of adherent cells was maintained even in the presence of rIL2, which augmented the proliferative response of TCC9A10 against PBMC and NAD but not B-LCL (e).

The potent stimulator cell type(s) found in the adherent fraction of PBMC were not of B cell origin. Results from a representative experiment shown in Figure 3.18 demonstrate that resting B cells purified from peripheral blood by flow cytometry, were unable to stimulate any of the TCC tested. No responses were seen against the B cells in three experiments, despite strong responses against the PBMC tested at the same cell dose.

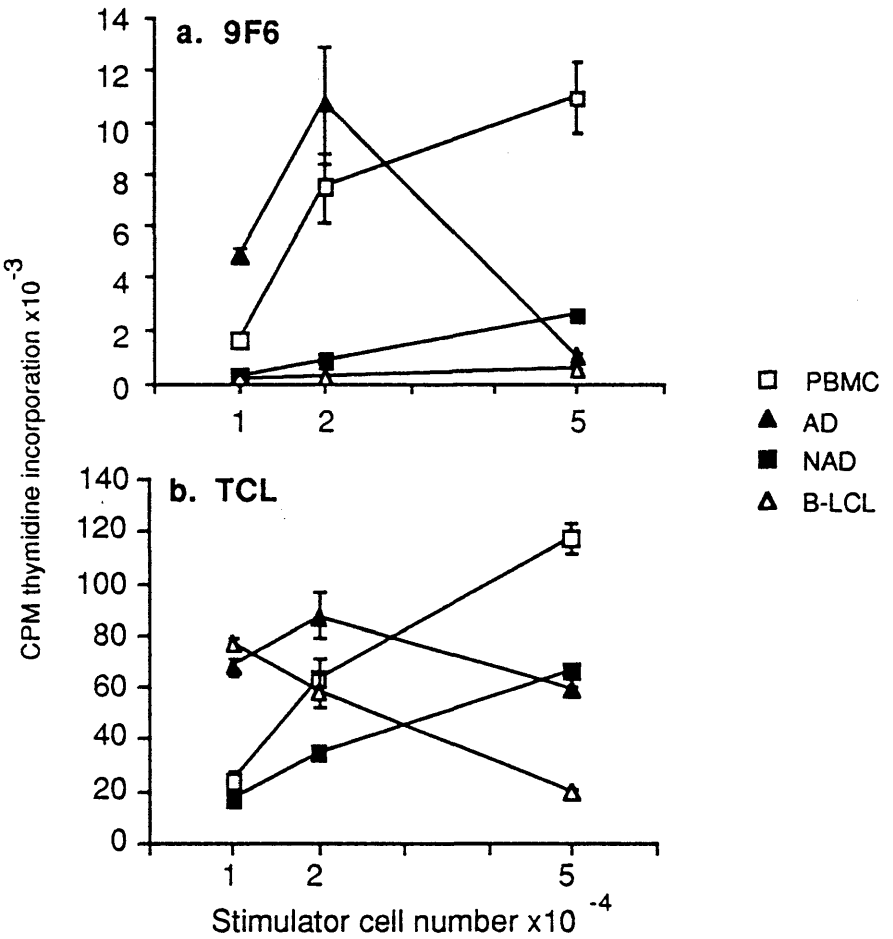


Figure 3.16. Cells in the adherent fraction of PBMC are potent stimulators of alloreactive T cell clones and lines at low stimulator cell doses. T cell clone 9F6 (a) or a T cell line (TCL, b) were cultured at 10^4 cells/well with varying numbers of stimulator PBMC, adherent cells (AD), non-adherent cells (NAD) and B-LCL, all derived from the same donor (SM). Points plotted represent the mean of triplicate cultures, with vertical bars representing the standard deviation. Response of 9F6 and TCL to medium alone was 59±35 and 532±76 respectively. Background thymidine incorporation of irradiated PBMC-derived stimulator cells was <100 CPM and for B-LCL was <600 CPM

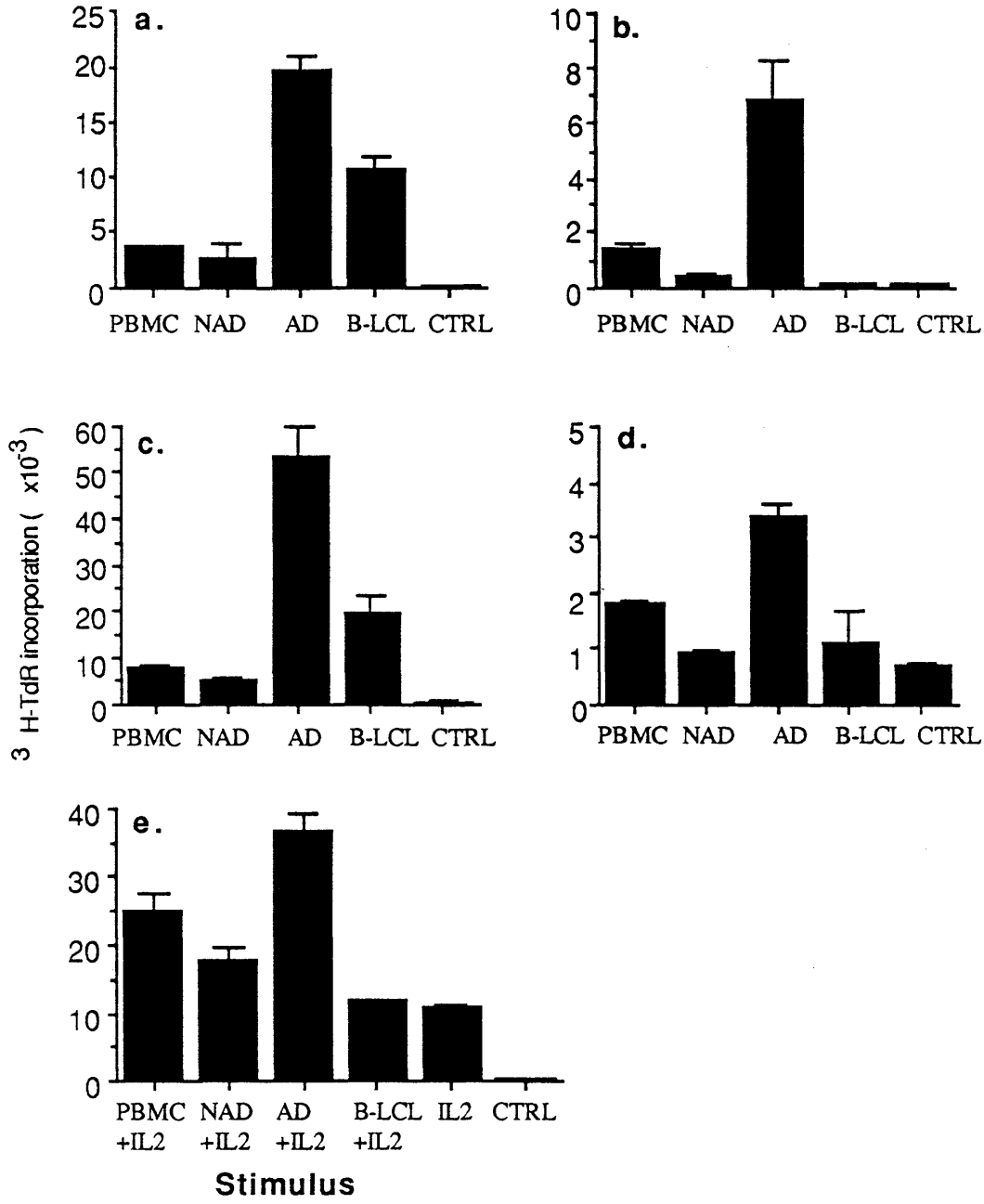


Figure 3.17. Plastic-adherent cells from peripheral blood are potent stimulators of alloreactive T cell clones. T cell clones (TCC) 7D5 (a), 9F6 (b), 9A10 (d), and T cell line (c) were tested on different cell types derived from donor SM. T cell clone 9A10 was additionally tested in the presence of 5u/ml rIL2 (e). The columns plotted, represent the mean of triplicate cultures containing 10,000 T cell clone/line and 20,000 irradiated stimulator cells. Horizontal bars represent the standard deviation of the mean. Cells tested were PBMC, non-adherent fraction of PBMC (NAD), plastic-adherent fraction of PBMC (AD), and B-LCL. Control wells (CTRL) contained T cell clone/line in absence of stimulators.

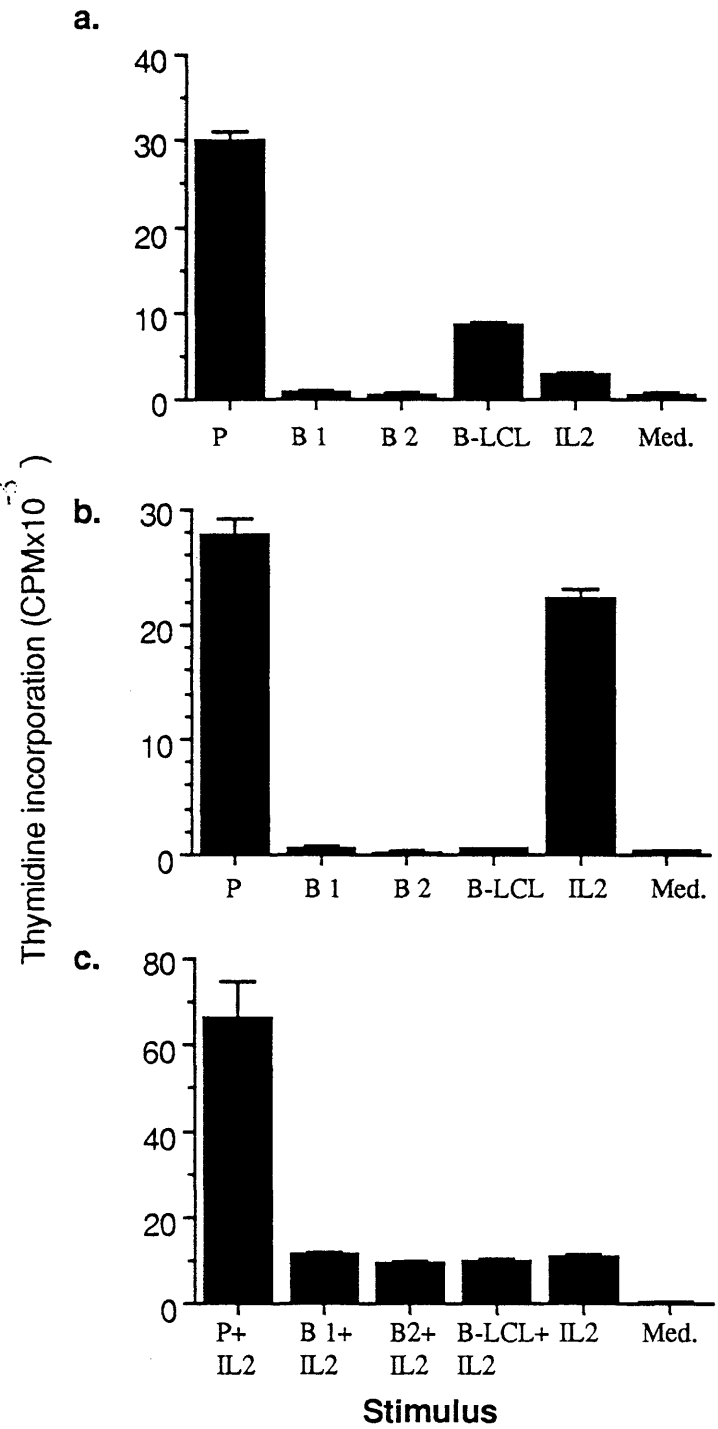


Figure 3.18. Purified B cells are unable to stimulate alloreactive T cell clones. T cell clones 7D5 (a), 9F6 (b) and 9A10 (c) were tested for proliferation against the following cell types: peripheral blood mononuclear cells at 5x10 4cells/well (P), B cells at 5x10^4 cells/well (B1), B cells at 1x10^4 cells/well (B2), and B-lymphoblastoid cell line at 1x10^4 cells/well (B-LCL). All T cell clones were tested at 1x10^4 cells/well on days 5-7 post-PBMC stimulation. T cell 9A10 was always tested in the presence of 5u/ml rIL2. Data comes from a single experiment, and is representative of three separate experiments.

TCCs able to react to PBMC, were able to respond to enriched populations of DR expressing cells in the virtual absence of allogeneic T cells. These results suggest that the failure of TCC9A10 and 9F6 to respond to B-LCL, despite being able to respond to PBMC was not due to a requirement for factors provided by allogeneic T cells, and may be due to differences in activation signals provided by B-LCL and adherent cells. These differences could be overcome however, by the presence of exogenous IL2, which was able to induce TCC 9F6 to respond against B-LCL (Table 3.7). TCC 9A10 was however unable to respond to B-LCL even in the presence of rIL2 (Table 3.7, Figure 3.17, 3.18). These results suggest that the failure of TCC 9A10 to respond to B-LCL was due to a defect in recognition and not activation,

3.3.3.3 B-lymphoblastoid cell lines express higher levels of HLA-DR, DQ, DP, ICAM-1 and LFA-3 than plastic-adherent cells from peripheral blood.

Molecules from the LFA family and their ligands have been shown to be important in mediating T cell activation through adhesion and signalling, with the LFA-3 and ICAM-1 molecules being important at the level of the antigen presenting cell (reviewed by Makgoba et al. 1989). The results shown in Figure 3.19 demonstrate that non-responsiveness to SM B-LCL was not due to inadequate expression of cell adhesion molecules. B-LCL expressed higher levels of HLA-DR, ICAM-1 and LFA-3 but lower amounts of LFA-1, when compared to adherent cells from PBMC. In addition comparison of the level of expression of HLA class II molecules between B-LCL and adherent cells, indicated that the non-reactivity of TCC 9A10 to B-LCL was not due to absence of any HLA class II molecule, as HLA-DR, DQ and DP molecules were expressed at higher levels on B-LCL than on peripheral blood monocytes (Figure 3.20).

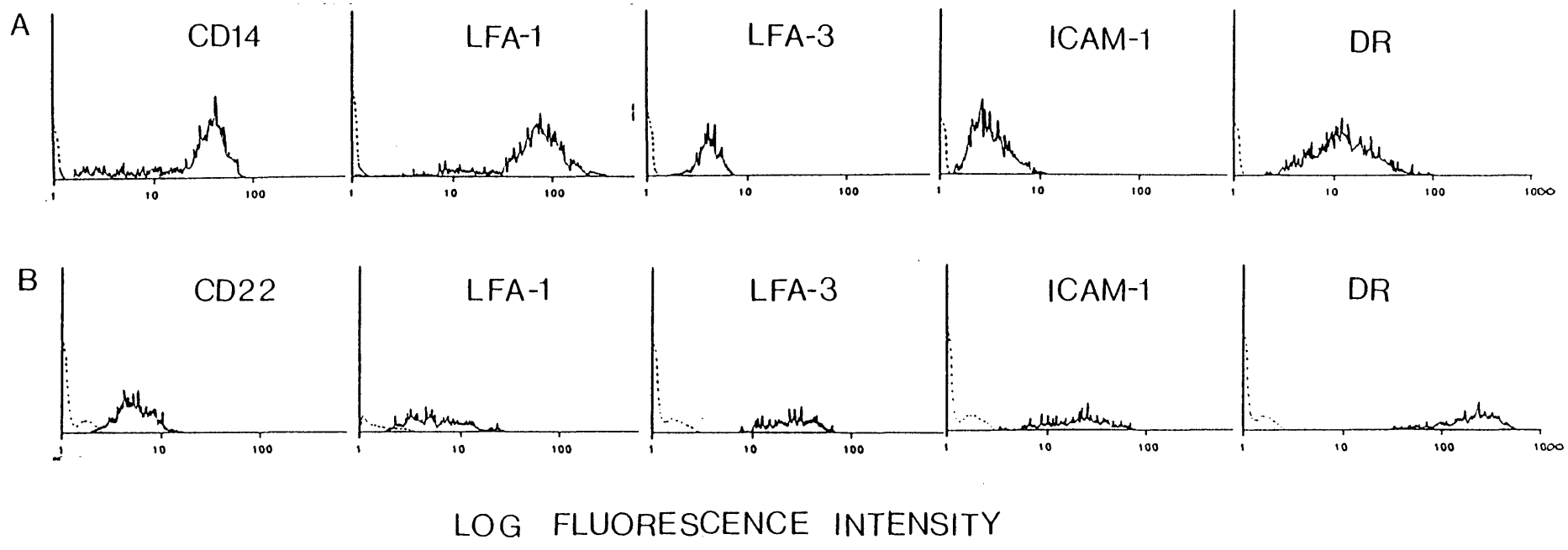


Figure 3.19. B-lymphoblastoid cell lines (B-LCL) express higher amounts of ICAM-1, LFA-3 and HLA-DR than plastic-adherent cells from peripheral blood. Adherent cells (A) or B-LCL (B) were subjected to phenotypic analysis using indirect immunofluorescence and flow cytometry. Both cell types were derived from the same donor SM. The cells were stained with monoclonal antibodies against: cell lineage markers CD14 (monocyte) and CD22 (B cells); HLA-DR; and the adhesion molecules LFA-1, LFA-3, and I-CAM-1. The fluorescence profiles are shown as solid lines. The negative control represented by the dotted lines, were cells stained with second layer fluorescent antibodies only.

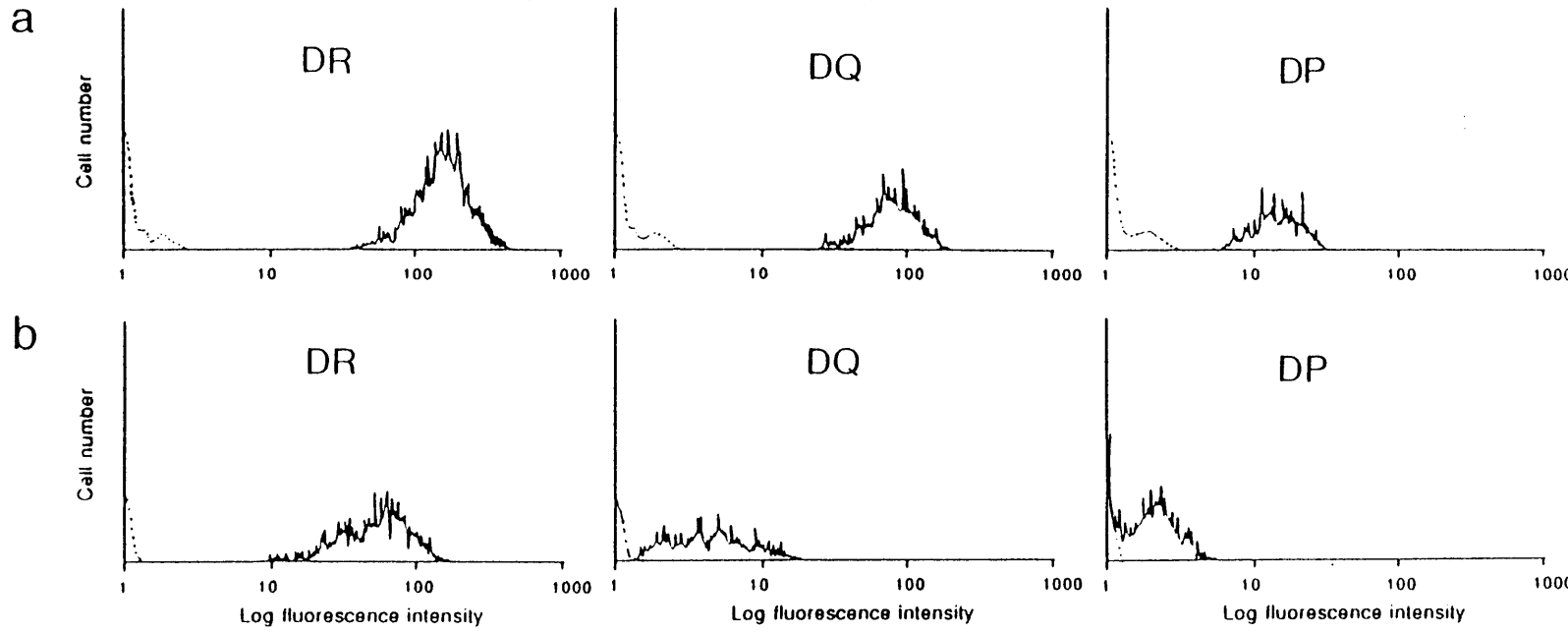


Figure 3.20 Comparison of expression HLA Class II gene products on SM B-lymphoblastoid cell line and adherent cells from peripheral blood. Level of expression of HLA Class II molecules on SM B-lymphoblastoid cell line (A) and adherent cells (B) from peripheral blood was determined using monoclonal antibodies against DR (L243), DQ1 (Genox), and DP (B7/21). Cells were analyzed by flow cytometry and fluorescence profiles (solid lines) shown were obtained in the same experiment, although each cell/antibody combination was tested at least three times. Negative controls are represented by dotted lines.

3.3.3.4 B-LCL are poor accessory cells for T cell clone response to PHA.

The non-responsiveness of TCC 9A10 and 9F6 to B-LCL, compared to PBMC, may be due to differences in accessory signals provided by the two stimulator cell populations. The results in Figure 3.21 demonstrate that B-LCL are poor accessory cells for PHA activation of TCC 9A10, when compared to either PBMC. No response was seen to either SM B-LCL or to WT B-LCL, although weak, non-specific stimulation of TCC 9A10 was seen when PHA was added to either SM B-LCL or WT B-LCL (DR1). The weak accessory function of B-LCL in PHA activation was also observed with TCC 4C3 and TCC 9F6 (Figure 3.21 b and c). No TCC response was seen to PHA alone.

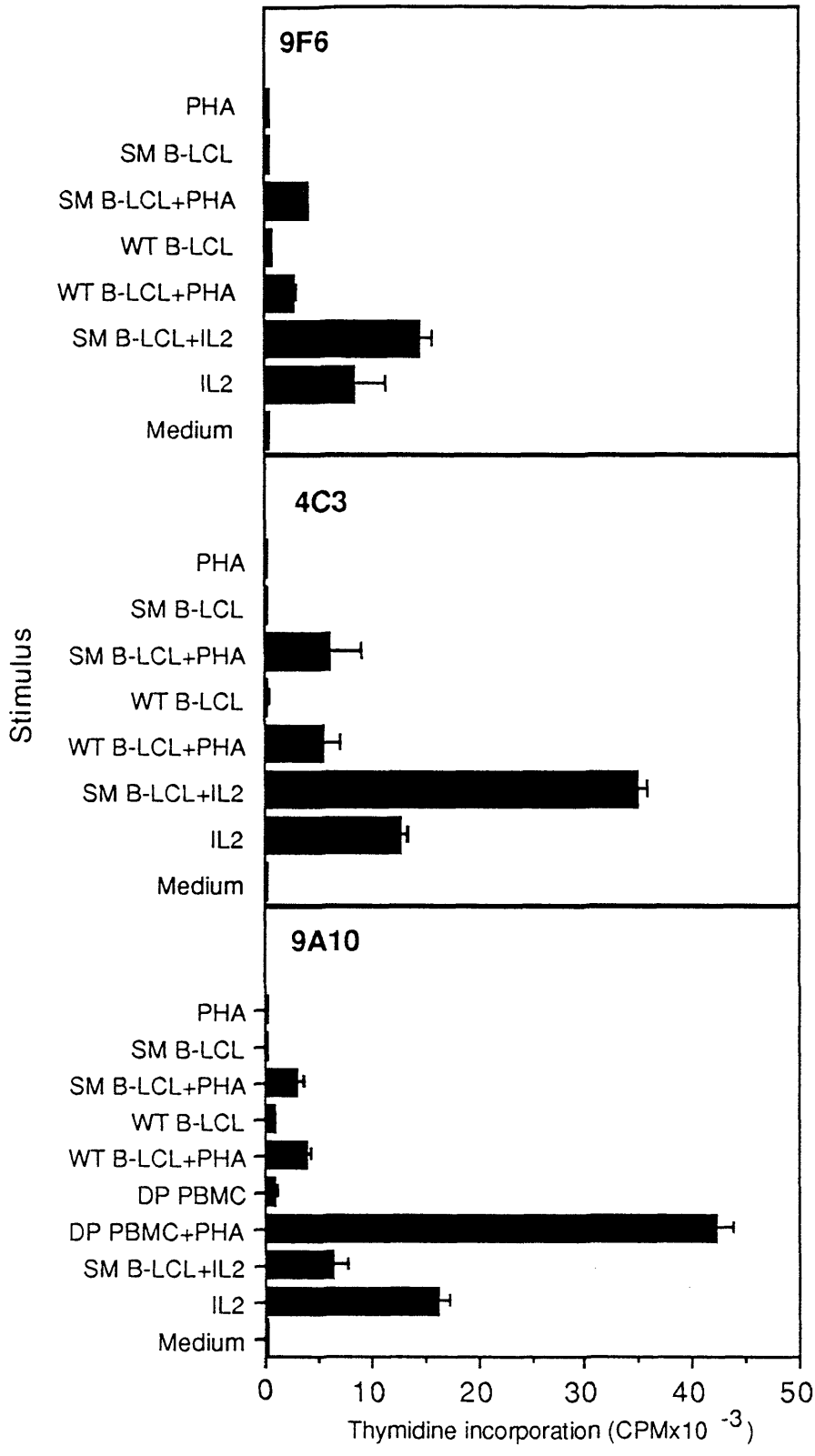


Figure 3.21. B-LCL are poor accessory cells for PHA activation of alloreactive T cell clones. Ten thousand T cell clones were cultured with $2x10^4$ DR2+B-LCL (SM) or $2x10^4$ DR1+ B-LCL (WT). PHA was added at 2μg/ml, rIL2 was added at 5u/ml. DP PBMC had been shown not to stimulate TCC 9A10, and was used at $5x10^4$. Thymidine incorporation for irradiated B-LCL alone was<200 CPM and for irradiated PBMC alone was <60 CPM.

Table 3.17 Properties of CD4+ve alloreactive T cell clones.

CLONE	CTL?	Proliferation[1]										Specificity[4]	
		PBMC		B-LCL		B cells#		AD cells[2]		DR2 L cells[3]			
		-IL2	+IL2	-IL2	+IL2	-IL2	+IL2	-IL2	+IL2	-IL2	+IL2	Antibody Blocking	Panels
3A1	Y	+	✔	-	✔								Dw2 and Dw12?
4C3	Y	-	✔	-	✔					-	✖	DR	Dw2 and Dw12
7D5	Y	++	✔	++	✔	-	✖	++	✔			DR	Some Dw2 and all Dw12
8C1	N	+/-	✔	-	✖								
9E9	Y	-	✔	++	✔								Dw2 and Dw12?
18C2	Y	-	✔	++	✔					-	✖	DR	Dw2 and Dw12?
9A10	N	+	✔	-	✖	-	✖	++	✔			DR & DP	Dw12?
9F6	N	++	✔	-	✔	-	✖	++	✔	-	✖	DR	Some Dw2, Dw12 and Dw5

[1] Proliferation in absence and presence of rIL2. ++ indicates strong proliferation, + indicates significant proliferation, +/- weak, generally not significant proliferation, - no significant proliferation , ✔ augmentation in presence of rIL2, ✖ no significant augmentation in the presence of rIL2.

#CD22+ B cells purified by flow cytometry .

[2]Plastic-adherent cells from peripheral blood. >80% CD14 +ve.

[3]Murine L cell transfectants expressing HLA-DRw15 (DR2).

[4]Determined with monoclonal antibody blocking experiments and PBMC & B-LCL panels. Question marks indicate putative specificities.

3.4 Discussion

3..4.1. The specificity of alloreactive T cell clones.

The cultures obtained after limiting dilution of the primed lymphocyte culture had a high probability of clonality and were referred to as clones in this study, although no formal proof of clonality was obtained. The clones were however stable with regard to phenotype, function, and specificity over the course of study. In addition preliminary analysis of the T cell receptor genes of two TCC (4C3 and 9F6) suggest clonality at the level of the T cell receptor (C. Sharrock unpublished observations). The properties and specificity of the TCCs studied are summarized in Table 3.17.

The stimulator PBMC for the TCC were typed as HLA DR2 (DRw15) Dw12 on the basis of serological typing, HTC typing and RFLP analysis. Although, ultimately confirmation of these typing results will require nucleotide sequencing of HLA class II genes from SM.

Recent studies have shown that cross-reactions of some alloreactive TCC on HLA-DR1 and DR4, could be explained by sequence homology in the third hypervariable region (HVR) of DR1 and DR4 ß chain molecules (Seyfried et al. 1988, Lombardi et al. 1989b). In this section the specificity of the alloreactive TCCs will be discussed with reference to published amino acid sequence data and the hypothetical structural model of MHC class II molecules (Brown et al. 1988).

The majority of the TCC characterized in the current study were able to react to both Dw2 and Dw12 stimulator cells, despite being derived from a MLC primed against Dw12 PBMC. Cross-reactive recognition of Dw2 and Dw12 cells appears to be common among DR2-reactive alloreactive TCCs (Reinsmoen and Bach 1987) and DR2-restricted, antigen specific TCCs (Jacobson et al. 1985, Sasazuki et al. 1985 and Wilkinson et al. 1988).

Such T cell cross-reactivity may be explained on the basis of MHC class II structure. All DR2+ve cells express two types of DRß molecules, DRßa and DRßb. In all the studies of antigen-specific TCCs (see above), T cell reactivity has mapped to the DRßa chains which differ between Dw2 and Dw12 subtypes at amino acid positions 30, 37 and 38. According to the hypothetical model of MHC class II structure (Brown et al. 1988), these amino acid residues are situated on the ß-pleated sheet which forms the floor of the antigen binding groove (Figure 3.22). The fact that either Dw2 or Dw12 cells can present measles antigen (Jacobson et al. 1985), Tetanus toxoid antigens (Sasazuki et al. 1985) or M.Leprae antigens (Wilkinson et al. 1988) to TCCs, suggests that the amino acid differences between Dw2 and Dw12 have no effect on the binding of these antigens.

It is not clear whether the alloreactive TCC studied recognized the DR2 ßa or ßb chains. Unsuccessful attempts were made to map the DRß chain specificity of the alloreactive TCC using murine L cell transfectants expressing either DRßa or DRßb gene products. The possible reasons for the lack of T cell response to murine L cell transfectants is discussed in section 3.4.2.

A complex pattern of reactivity was observed with TCC9F6, which was able to cross-react on serologically distinct DR2 and DR5 antigens. This cross-reaction was clearly demonstrated when TCC9F6 was tested on a large panel of B-LCL in the presence of rIL2. The results from proliferation assays were not so convincing, as TCC 9F6 was only able to respond weakly to one (SS PBMC) out of 6 DR5+ve PBMC tested. The weak responses against SS PBMC suggest that TCC9F6 recognizes DR5 with lower affinity than DR2 and the negative results obtained with PBMC may be explained by use of sub-optimal numbers of PBMC. In the case of the B-LCL panels, the higher levels of HLA class II expressed by the B-LCL, combined with

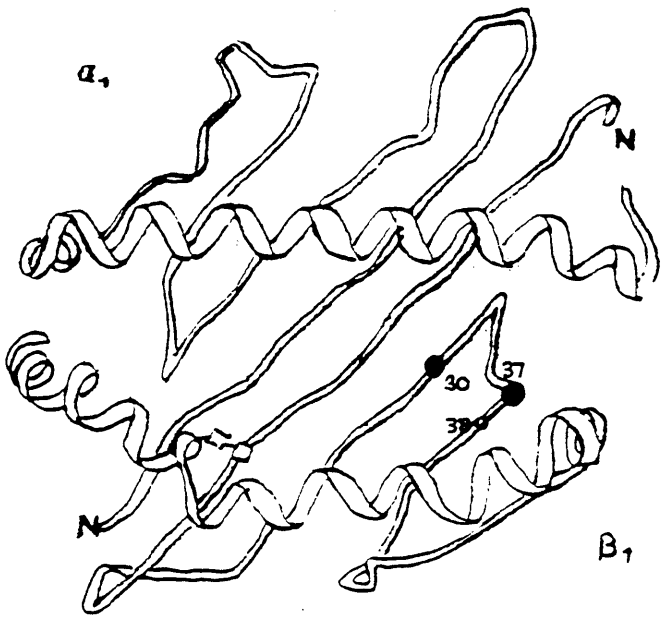


Figure 3.22. Amino acid residues which differ between Dw2 and Dw12 subtypes as represented on the hypothetical structural model of MHC Class II. (diagram modified from Brown et al. 1988). Dw12 ßa chains differ from Dw2 ßa chains at amino acids 30, 37 and 38. Amino acids represented by filled circles are postulated to point upwards from the floor of the antigen binding groove. Sequences obtained from Lee et al. 1987.

the presence of rIL2 may lower the stringency of activation allowing clear positive responses against DR5 B-LCL to develop.

The recognition of Dw2, Dw12 and Dw5 B-LCL by TCC9F6 could be explained by shared amino acid sequence in the third HVR of Dw2ßa, Dw12ßa and Dw5ß1 chains. This is an attractive proposal as amino acids in this region have been shown to have an important role in alloreactive (Cairns et al. 1985, Lombardi et al. 1989b) and antigen specific (Rothbard et al. 1988) T cell responses. The recognition of amino acids in the third HVR cannot be the sole reason for the observed specificity of 9F6 for two reasons.

First, the amino acid sequence shared between Dw2 DRßa, Dw12 DRßa and Dw5 DRß1 chains, was also present on DRßb chains of Dw21 and Dw22 B-LCL. In fact, the Dw21 and Dw22 DRßb chains have greater similarity to Dw5 DRß1 sequences than either Dw2 or Dw12 DRßa chains, sharing sequences in the 2nd and 3rd HVRs (Figure 3.11).

Second, TCC9F6 did not react against all cells typed as DR2 Dw2, reactivity was only seen to cells expressing the HLA A3 B7 DR2 Dw2 haplotype. The discrimination seen between Dw2 subtypes associated with different haplotypes was unlikely to be due to polymorphism between DRß genes.

Amino acid sequences for DRß genes from A3 B7 DR2 Dw2 haplotypes have been shown to be identical to DRß genes from other Dw2 haplotypes (Wu et al. 1987, Locke et al. 1987, Lee et al. 1987, Hurley et al. 1989). Further evidence for identity between Dw2 DRß chains has come from 2-D gel electrophoresis studies which demonstrated that the B-LCL used in the current study (Table 3.13), had identical DRß chains (Knowles et al. 1989).

The reactivity of TCC4C3 could also be explained by recognition of amino acids in the third HVR of Dw2 and Dw12 DRßb chains. However this stretch of amino acid residues is also present in the third HVR of

DRßa chains from Dw21 and Dw22 chains. DRßb chains from Dw2/Dw12 differ from DRßa chains from Dw21/Dw22 at seven positions. Differences occur at positions 9, 11, 13, 28, 30 , 31 and 37, although the substitutions at positions 9 and 37 are conservative, not resulting in a change in amino acid charge. All these residues are predicted to lie in the ß-pleated sheet which forms the floor of the antigen binding groove of MHC class II molecules (Figure 3.23) and as such are unlikely to interact directly with T cell receptors (Brown et al. 1988).

A number of recent studies have demonstrated that alloreactive TCCs which can discriminate between DR molecules differing by amino acids in the peptide binding site, are common (Reinsmoen and Bach 1989, Weyand and Goronzy 1989, Gorski et al. 1989, Lombardi et al. 1989b). It is possible that amino acid substitutions at sites in the peptide binding groove may affect the conformation of the DR molecule resulting in an epitope unique to Dw2 and Dw12 DR molecules. This may be mediated through direct interactions between amino acids in the ß-pleated sheet and the alpha-helical portions of the class II molecule. An alternative but not mutally exclusive possiblity is that differences in the petide binding site may change the spectrum of peptides bound, resulting in the absence of specific petides which contibute to the ligands recognized by TCC4C3 and 18C2.

In order to definitively map the amino acid residues which are important in the recognition of the alloreactive TCC described, a site-directed mutagenesis approach is needed. Future studies to be carried out in collaboration with Dr. P. Travers (Birkbeck College, University of London) will use such an approach using DR2 genes mutated at selected amino acid residues and transfected into HLA class II negative human cell lines.

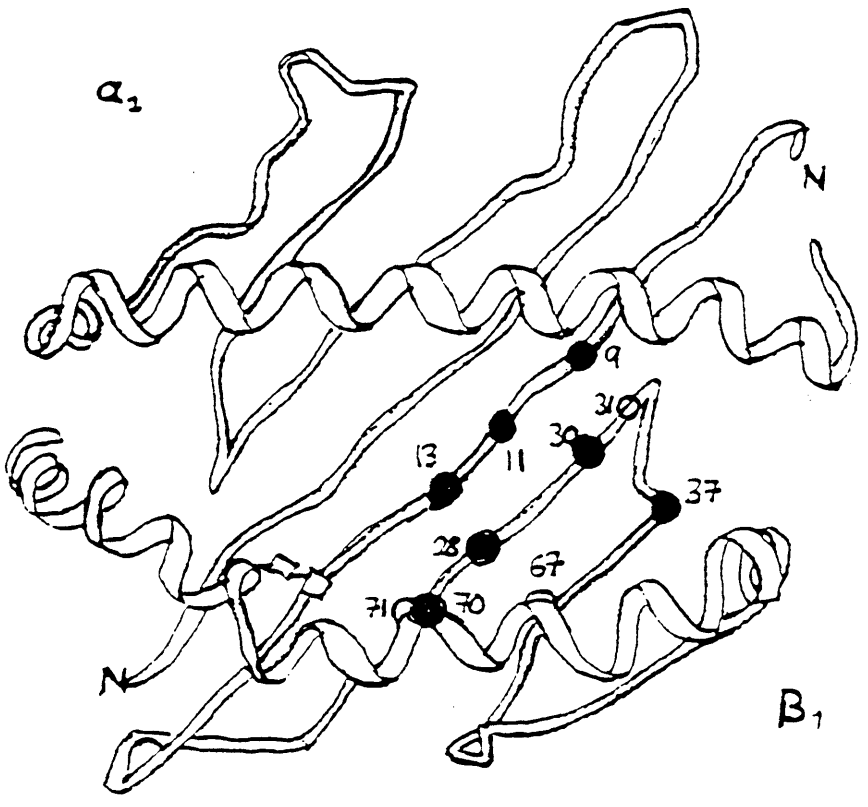


Figure 3.23. Amino acids in the first domain of DRß chains which influence reactivity of alloreactive T cell clones (diagram modified from Brown et al. 1988). TCC 18C2 and 4C3 recognize only Dw2 and Dw12 B-LCL, and may be recognizing a determinant on DRßb chains, influenced by amino acids at positions 11, 13, 28, 30, 31, 67, 70 and 71. Dw21 and Dw22 ßa chains are homologous to Dw2/Dw12 ßb chains at positions 67,70 and 71 , but are different at positions 9, 11, 13, 28, 30, 31 and 37. The changes at positions 9 and 37 are conservative changes not resulting in change in charge. Amino acid residues represented by filled circles are postulated to point upwards, whereas empty circles represent amino acid residues postulated to point into the antigen binding site.

TCC 9A10 has a unique pattern of reactivity among the alloreactive TCCs tested, recognizing a determinant expressed on only 2 out 26 PBMC (SM and GK PBMC) tested and being inhibited by both DR and DP antibodies. The finding that anti-DP antibody could block specific T cell proliferation was a striking finding as DP specific TCCs are comparatively rare, only 3 out of 100 TCC tested in the 10th histocompatibility workshop were DP specific as determined by monoclonal antibody blocking and testing on B-LCL panels (Flomenberg et al. 1989). DP typing of SM PBMC, GK PBMC and the rest of the PBMC panel used, is needed to determine if the specificity of 9A10 can be assigned to DP antigens. The most informative approach is likely to be DP typing using sequence specific probes, which can reveal at least 15 polymorphisms of DP compared to the 6 antigens detected by serological typing (Bugawan et al. 1989). The paucity of good serological reagents for DP typing, suggests that TCC9A10 has potential as a typing reagent. DP reactivity might also explain why TCC9A10 has weaker responses to PBMC stimulator cells than other TCC (Table 3.5), as DP molecules are expressed on PBMC at much lower levels than either DR or DQ molecules (see Figure 3.20). The possible reasons why TCC 9A10 does not respond to B-LCL expressing high levels of HLA-DP will be discussed in section 3.4.2.

The antibody blocking results could be explained in two ways. First TCC9A10 is recognizing a DP molecule common to both GK and SM PBMC. In this case the inhibitory effect of anti-DR antibody L243, which is DRα chain specific, may be attributable to shared antibody epitopes between DR α and DP α chains. The second possibility is that TCC9A10 is recognizing a novel molecule formed from the pairing of DRα and DPβ chains. The antibody blocking results using the anti-DR and the anti-DP specific monoclonal antibodies can thus be explained by antibody recognition of DRα and DPβ chains respectively. Further antibody

blocking studies using a larger panel of monoclonal antibodies in combination with biochemical studies to try to isolate DPα:DPß heterodimers, will distinguish between these two possibilities.

3.4.2 Cell lineage and haplotype specific recognition of HLA class II molecules.

The lack of complete correlation between allelic amino acid sequences and the specificity of the TCC tested, suggests that other factors besides polymorphism in the primary MHC structure are important in alloreactive T cell recognition. This proposal is supported by cell lineage and haplotype specific patterns of reactivity observed with the alloreactive TCC studied.

None of the TCCs tested were able to respond to murine transfectants expressing the products of HLA-DR2 genes. In addition, one TCC 9A10 was unable to respond to SM B-LCL under any of the experimental conditions used, although responses to PBMC and the adherent fraction of PBMC from the same donor were seen. These patterns of reactivity are reminiscent of previous studies which have demonstrated that alloreactive T cells can discriminate between apparently identical MHC molecules expressed on different cell types (Manca et al. 1983, Minami et al. 1987, and Marrack and Kappler 1988).

The cell lineage of the allogeneic antigen presenting cell could affect alloreactive T cell responses in three ways:

1. Cells which express HLA class II molecules may differ in their ability to provide accessory signals required for T cell activation. (Mueller et al. 1989).
2. Cells may differ in their expression of adhesion molecules, which are critical when there is a low level of expression of HLA class II (Altmann et al. 1989).

3. Alloreactive T cells recognize a ligand which is dependent on allogeneic MHC and bound peptides (Marrack and Kappler 1988). There may be differences in the peptides generated by different cell types due to catabolism of cell lineage specific proteins or differences in antigen processing abilities.

Previous functional studies using the L cell transfectants argue against the lack of either accessory signals or human adhesion molecules being the cause of the non-responsiveness seen using alloreactive TCCs. Wilkinson and colleagues had shown that HLA DR molecules were expressed on the L cells at levels comparable to human DR2+ve B-LCL (Wilkinson et al. 1988), a finding which has been confirmed in this study. The murine L cell transfectants had also been shown to be able to present whole antigen and peptides derived from M.Leprae bacteria to DR2-restricted TCCs, and to be potent stimulators in primary MLR (Wilkinson et al. 1988, Altmann et al. 1989).

Thus the most likely explanation for the lack of response against the L cell transfectants was the absence of the allogeneic determinants recognized by the TCC tested. This might be due to TCC recognition of species specific allogeneic determinants formed from complexes of human peptides and allogeneic HLA class II molecules. It has been shown that the ability of some TCC to recognize DR 1 expressed on L cell transfectants is due to processing and presentation of human proteins during the culture period (Lombardi et al. 1989a). The non-responsiveness of the TCC used in this study may be due to specificity for human peptide and HLA combinations which cannot be generated by murine L cell transfectants. The most direct method for testing this proposal is to try to induce recognition of the DR2 L cell transfectants by furnishing the DR2 molecules with human peptides. This kind of approach has been recently used by Heath et al. (1989) who demonstrated that some MHC class I (K^b)

specific murine CTL were unable to recognize K^b expressed on human transfectants, and that recognition of human targets could occur if the targets were pulsed with cytoplasmic extracts from mouse cells prior to CML assays.

Cell-lineage specificity of recognition may explain the reactivity pattern of TCC 9A10, which appears to discriminate between identical HLA class II molecules expressed on PBMC and B-LCL from the same donor. The non-responsiveness was not due to low level of HLA class II or lack of known adhesion molecules, as all HLA class II molecules, and ICAM-1 and LFA-3 were expressed at high levels on B-LCL. It is possible that despite high levels of expression of HLA class II molecules , B-LCL may fail to activate TCC 9A10 due to absence of some unknown accessory cell function. The absence of response even in the presence of rIL2 makes this possibility unlikely. Another possibility is that the high levels of HLA class II on B-LCL might have induced a state of clonal anergy at the stimulator cell numbers used. However no response was ever seen to B-LCL even at low stimulator cell numbers. The most likely explanation for the cell lineage preference of TCC 9A10 is that the B-LCL lack the allogeneic determinant recognized by this clone. This might occur in two ways:

1. Differences in protein catabolism mechanisms between B-LCL and peripheral blood monocytes might result in the display of different allogeneic MHC+peptide complexes.

2. Immortalisation of SM B cells by EBV may have affected the allogeneic determinant recognized by TCC9A10 either through changes in the HLA class II molecules or through loss of a gene product important in the generation of the allogeneic determinant.

Further studies using a variety of antigen presenting cells expressing the same HLA class II molecules will help to distinguish between these

possibilities. In addition experiments similar to the ones described to reconstitute recognition of murine L cells transfectants could be performed. SM B-LCL could be pulsed with cytoplasmic extracts from adherent cells to investigate the role of cell lineage specific peptides.

As discussed in section 3.4.1, no correlation could be seen between amino acid sequence and the specificity of TCC9F6, although the degree to which differences in amino acid residues may affect the tertiary structure of MHC molecules is not known. These findings suggested that the allogeneic determinant recognized by TCC9F6 was dependent on more than the primary structure of the MHC molecule itself. This notion was supported by the finding that TCC 9F6 can discriminate between the same DR molecule on the same cell type, but associated with different HLA haplotypes. Whether the recognition is determined by HLA class I genes per se or is a function of other genes on the haplotype will require further genetic analysis using HLA typed families.

One hypothesis to explain the specificity of TCC9F6 might be that the specific allogeneic determinant recognized by this TCC is a complex formed by DR molecules and peptides derived from proteins specifically expressed on A3 B7 DR2 haplotypes. Such recognition would also explain the inability of TCC 9F6 to recognize DR2 L cell transfectants as these do not express any other human genes. The most obvious candidates for peptides which might be polymorphic between HLA haplotypes would be those derived from the HLA class I molecules. Recent studies have demonstrated that HLA class I (Clayberger et al. 1987) and HLA class II peptides (de Koster et al. 1989) can be recognized in a specific fashion by alloreactive TCCs.

The results obtained in this chapter suggest that the alloreactive TCC studied were not specific for allogeneic HLA class II molecules only. Firstly, alloreactive TCCs were not always able to respond to cells

expressing high levels of HLA class II molecules. Alloreactive T cell responses were affected by the cell lineage of the allogeneic antigen presenting cell and the HLA haplotype associated with the HLA class II molecules. Secondly, analysis of the primary structure of MHC molecules could predict the specificities of some TCC, only if residues from the alpha helical region and antigen binding site were considered together. The specificity of these clones is unlikely to be due to to direct recognition of the MHC molecule. For other TCC no correlation between primary MHC class II structure and specificity could be determined.

These findings argue against the concept that alloreactivity is due to recognition of MHC molecules only, and tend to favour a model of alloreactivity that involves T cell recognition of peptides complexed to allogeneic MHC molecules. It remains to be seen whether the patterns of reactivity seen with the TCC in this study are present at high frequency at the population level.

3.4.4. Activation requirements of alloreactive T cell clones.

In studying the specificity of CD4+ TCCs, it was noted that the TCCs were heterogeneous in their functional characteristics and their activation requirements (summarized in Table 3.17). For example the TCCs studied could be broadly divided into cytotoxic and non-cytotoxic TCCs. Whether the cytotoxic TCCs obtained from this study were derived from true cytotoxic precursors (CTLp) or acquired their cytotoxicity *in vitro* is not known, although non-cytotoxic TCCs did not become cytotoxic under the culture conditions used. Non-cytotoxic TCCs could be induced to become cytotoxic if OKT3 hybridoma cells are used as targets, probably by a mechanism similar to that previously demonstrated using antigen specific CD4+ve TCCs (Spits et al. 1985) or CD4-ve CD8-ve TCCs (Londei et al. 1989). It would be interesting to study the expression of CD28 on these

TCC as a recent study has proposed that absence of CD28 correlates with cytotoxicity in the CD4+ve T cell subset (Takada et al. 1988).

The principal stimulating cell population in PBMC was found in the plastic adherent fraction. Such fractions are highly enriched for HLA class II expressing cells, and were shown to consist mainly of monocytes and B cells. It was likely that dendritic cells (DC) were also present in the adherent cell populations (van Hooris et al. 1982). Whether DC were the primary stimulator cells for the TCC tested is not known. Previous studies have demonstrated that monocytes and B cells were poor stimulator cells in primary MLR compared to DC (van Hooris et al. 1982, McKenzie et al. 1989). One important difference between previous studies and the results in section 3.3.3 was that the response of "primed" T cells were investigated, whereas previous studies have investigated the responses of "resting" T cells in primary MLR.

It was unlikely that B cells contained in the plastic adherent fraction, contributed to the stimulation of TCC. Purified resting B cells were unable to stimulate any TCC even in the presence of rIL2. It has been suggested that resting B cells are poor stimulators of both alloreactive and antigen-specific T cells because they lack co-stimulatory activities which together with MHC class II, activate T cells (Kreiger et al 1985, Weaver et al 1988). An alternative explanation is that B cells lack the allogeneic determinants that are recognized by the TCC (Minami et al. 1985). It may be possible to distinguish between these possibilities in further experiments by using activated B cells as stimulators.

The variation in proliferative responses observed with the TCC tested could be explained by individual TCC differing in their requirements for activation. TCC 9F6 was unable to proliferate in response to B-LCL despite strong proliferation against PBMC and monocyte-enriched populations from the same donor. Specific responses to B-LCL were seen

however if rIL2 was added to the culture system. These results suggest that the B-LCL expressed the allogeneic ligand recognized by TCC 9F6, but were unable to stimulate due to a defect in T cell activation. The poor responses of TCC to PHA in the presence of B-LCL suggests that B-LCL may lack some "accessory" function which is expressed by the adherent fraction of PBMC. This notion is supported by the failure of B-LCL to present nickel to nickel-specific TCCs (Kapsenberg et al. 1989), and by the loss of specific proliferation observed, when TCC are grown using B-LCL as feeder cells (Lundin et al. 1988, Pawelec et al. 1988).

B-LCL were able to stimulate some TCC, suggesting that non-responsiveness was determined at the level of the individual TCC. For example TCC 9E9 and 18C2 produced an opposite pattern of reactivity to TCC9F6, as they were able to respond to B-LCL but not PBMC. Responses to PBMC were seen however in the presence of rIL2. The strong proliferation against B-LCL argues against an absolute requirement for exogenous IL2. The most likely explanation is that these TCC are of low affinity and were unable to respond to PBMC because sub-optimal stimulator: responder ratio. Such a possibility could be addressed by increasing the range of cell doses in future experiments.

The "accessory" signals required for proliferation of some TCC may involve the induction of IL2 production (Meuer et al.1986). Some TCC such as 4C3 were clearly dependent on exogenous IL2 for proliferation against both PBMC and B-LCL whereas others such as 7D5 could proliferate to both PBMC and B-LCL stimulator cells without a requirement for exogenous IL2. Determination of the lymphokines produced by TCC with different patterns of reactivity may allow correlation of autocrine production with the patterns of proliferative responses observed.

IL1 (Lowenthal et al. 1985) and IL4 (Spits et al. 1987) have both been shown to be involved in T cell activation. Recombinant IL1 had no effect on the proliferation of TCC either in the absence or presence of stimulator cells suggesting that soluble rIL1 was not required for the activation of these TCC. Recent studies however suggest that induction of T cell autocrine production and proliferation requires synergy between IL1 and IL6 (Holsti and Raulet 1989, Houssiau 1989).

Recombinant IL4 was able to augment TCC proliferation to PBMC, although no proliferation was seen in response to rIL4 alone or in the presence of B-LCL. Although TCC responses to PBMC and IL4, and PBMC and IL2 were not compared directly, the mechanisms by which rIL2 and rIL4 augment proliferative responses to PBMC are likely to be different. Interleukin 4 unlike IL2 is able to increase the levels of MHC class II expression on both monocytes (Alavara-Gracia et al. 1989) and B cells. (Roehm et al. 1984). Further testing of the effect of lymphokines on alloreactive TCC is required in the light of the recent demonstrations that IL3 (Londei et al. *in press*) and IL7 (Londei et al. 1989) can act as growth factors for T cell subsets.

The elucidation of the signals required for the activation of individual TCCs will require more sophisticated approach than the experiments described in section 3.3.3. One approach will be to identify the cell surface molecules which are important in T cell activation as an increasing number of cell surface molecules have been shown to have a signalling function. Phenotypic analysis of different cell types may explain differences in their ability to act as stimulator cells. In order to define true cell-lineage specific recognition by alloreactive TCCs, it is important to distinguish between non-responsiveness due to lack of "accessory" function or lack of the specific ligand. The use of cytoplasmic dyes to

measure calcium flux may help to determine whether T cell receptors are triggered by allogeneic MHC molecules on different cell types.

3.5. Conclusions.

The small panel of human CD4+ve alloreactive TCC characterized in this chapter were heterogeneous in their specificity and their functional properties. The main conclusions made from the results obtained were as follows:

1. Alloreactive TCC can define novel polymorphisms of HLA-DR2, which are not detected by serological reagents, polyclonal T cell reagents or RFLP analysis.

2. Alloreactive TCC responses depended on more than the expression of HLA class II molecules. The specificity patterns obtained suggest that allogeneic determinants are complex and support the concept that MHC-bound peptides play a role in alloreactivity.

3. Alloreactive CD4+ve TCC were heterogeneous in their activation requirements. The addition of rIL2 could overcome non-responsiveness against allogeneic stimulator cells and suggests that TCC differ in their requirements for signals that induce autocrine proliferation.

Chapter 4.

Human cytotoxic T cell response to HLA class II antigens.

4.1 Summary

Limiting dilution assays (LDA) were used to compare the frequencies of HLA class II specific cytotoxic T lymphocyte precursors (CTLp) between a number of HLA-mismatched responder and stimulator combinations. The frequency of alloreactive CTLp was studied in peripheral blood mononuclear cell populations which had been enriched for CD4+ve T cells by immunomagnetic depletion of CD8+ve T cells. The CTL generated in the LDA system were shown to be specific for the HLA class II antigens expressed on B-lymphoblastoid and murine L cell transfectant targets.

Sixteen allogeneic combinations were initially tested in LDA using B-lymphoblastoid cell lines (B-LCL) as target cells. In some combinations, high alloreactive CD4+ve CTLp frequencies (1/1880) were obtained which were not significantly different from the frequencies of alloreactive CTLp detected in untreated PBMC populations (1/2133). In other combinations alloreactive CTLp frequencies in CD4-enriched PBMC were found to be at least twenty-fold lower than in the starting, non-fractionated PBMC.

Significant variation was found in alloreactive CTLp frequencies against the same set of HLA class II antigens when different individuals were used as responders (1/4951 to 1/34837). There was also a significant difference in alloreactive CTLp frequencies against different sets of HLA class II antigens when the same responder individual was used (1/1475 to 1/38987). These results could not be explained by differential target sensitivity and no correlation between degree of HLA-mismatch and CTLp frequencies could be seen.

The variation in the frequencies of HLA class II specific CTLp, was confirmed in further experiments using murine L cell transfectants expressing HLA-DR gene products, as target cells. CTLp frequency

differences were found between individuals against the same DR+ve transfectants (1/1257 to <1/100,000) and within individuals against different DR+ve transfectants (1/7646- to 1/100,000). For 2 out of 8 responder: stimulator combinations high CTLp frequencies were obtained, and no significant difference was seen between CTLp frequencies obtained using either B-LCL or murine L cell transfectant targets. For 6 out of 8 combinations the CTLp frequencies detected using the DR+ve L cell targets were low or undetectable, whilst the corresponding CTLp frequencies obtained using B-LCL targets were high. These results were not due to xenogeneic responses or differences between the transfectant targets.

The variation in HLA class II specific CTLp frequencies against either multiple HLA class II antigens expressed on B-LCL or single HLA-DR antigens expressed on murine L cells, suggests that there is individual variation in the cytotoxic T cell repertoire to HLA class II antigens.

1. Introduction

HLA class II and HLA class I reactive T cells can be distinguished on the basis of the phenotypic markers, CD4 and CD8 respectively (Meuer et al. 1982). For many years CD4 and CD8 were thought to divide human T cells into two functional subsets, with CD8+ve CD4-ve positive T cells comprising the cytotoxic/suppressor subpopulation, and the CD4+ve CD8-ve T cells the helper/inducer population (Reinherz et al. 1979). It is becoming clear however, that there is considerable functional heterogeneity within each of the two subsets. For example CD8+ve T helper cells have been described (Moretta et al. 1984), and there have been many reports of CD4+ve T cell clones and lines with specific cytotoxic activity for viruses (Schmid, 1988; Jacobson et al.1989), auto-antigens (Weber and Buurman, 1988; De Berardinis et al.1988) and HLA class II antigens (Bach et al. 1979)

It is not clear whether CD4+ve CTL have a physiological role *in vivo* . The demonstration that influenza virus specific CD4+ve T cell clones could acquire specific cytotoxicity after prolonged *in vitro* culture (Fleischer 1984) suggested that many virus-specific CD4+ CTL may have arisen as a result of *in vitro* selection, and that CD4+ve CTL did not have a role as anti-viral effector cells *in vivo*. This has been supported by studies of CTL specific for VSV (Hickling et al. 1987), EBV (Wallace et al. 1985) and influenza (Borgault et al. 1989) which have shown that the virus-specific CTL isolated from short term *in vitro* cultures are exclusively of the CD8+ve T cell subset. This is not the case for all viruses however, as CD4+ve CTL specific for measles virus (Jacobson et al. 1989) and HSV1 (Schmid 1988) have been isolated from short-term in vitro cultures. The preferential activation of either CD4+ or CD8+ve CTL in anti-viral responses may be a reflection of differences in the route of virus

infection (Long and Jacobson 1989) and subsequent antigen processing (Morrison et al.1986, Germain, 1986).

The role of CD4+ve CTL in allograft rejection is also unclear. CD4+ve T cells appear to make a minimal contribution to the primary *in vitro* CTL response against allogeneic MHC (Reinherz et al. 1979). Limiting dilution studies of alloreactive CTLp in CD4+ve T cell populations have demonstrated CTLp frequencies which are several fold lower than those found in the CD8+ve subset (Moretta et al.1983, Kabelitz et al.1985). On the basis of these studies it was suggested that the predominance of CD8+ve T cells in the primary cytotoxic response against allogeneic MHC molecules was due to the low precursor frequency of CD4+ve CTL.

Significant levels of HLA class II-specific cytotoxicity can be obtained after primary mixed lymphocyte culture, if CD8+ve T cell activation is negated by matching for HLA class I antigens (Feighery and Stastny 1979; Breuning et al.1984, Albrechtsen et al.1979) or by depletion of the CD8+ve T cell subset (Reinherz et al.1979). These studies suggest that the presence of CD8+ve T cells and not CD4+ve CTLp frequency, was the limiting factor in development of HLA class II specific CTL effectors.

Sharrock et al. (1987) have demonstrated that there are differences in CTLp frequencies between individuals against the same alloantigens, and that there is variation in CTLp frequencies within individuals against different alloantigens. Recently Zhang et al. have reported similar findings for CTLp shown to be specific for single HLA class I antigens (1989). The aim of the study described in this chapter was to determine whether there was variation in cytotoxic T cell responses against HLA class II antigens.

Table 4.1. Studies of alloreactive CTLp frequencies in man.

Responder Cells	Stimulator Cells (Number)	Fillers Cells[1]	Max Freq[2]	Targets	Culture Period	IL2 source	Reference
PBMC	PBMC (5x104)	Yes	1/ 5000	PHA blasts	7 days	TCGF[3]	Sharrock et al.(1987)
PBMC	PBMC/Spleen (10^5)	Yes	1/ 870	PHA Blasts	6 days	TCGF	Bryan et al. (1983)
PBMC	PBMC ($5x10^4$)	Yes	1/ 733	PHA blasts	10 days	TCGF	Rosenkrantz et al.(1985)
PBMC	PBMC ($5x10^4$)	No	1/ 1200	PHA blasts	10 days	5u/ml r IL2	Kaminski et al. (1988)
PBMC	PBMC/Spleen (10^5)	No	1/ 3322	PHA blasts	6 days	TCGF	Bryan et al. (1983)
PBMC	PBMC ($5x10^4$)	No	1/2049	PHA blasts	7 days	30u/m rIL2	Zhang et al. (1989)
PBMC	B-LCL ($1x10^4$)	No	1/ 900	B-LCL	10 days	TCGF	Burns et al. (1984)
PBMC	T Cell clones ($5x10^4$)	No	1/ 500	T Cell Clones	10 days	5ng/ml rIL2	Deusch et al. (1986)
T cells*	PBMC/Spleen (10^5/$5x10^4$)	No	1/ 240	Con A blasts	10 days	25u/mlrIL2 + MLC supt[4]	Kabelitz et al. (1985)
T cells*	Spleen (10^5)	No	1/ 2010	PHA blasts	14-18 days	TCGF	Moretta et al. (1983)
T cells*	B-LCL (200)	No	1/ 66	B-LCL	10-12 days	25u/ml rIL2 + MLC supt.	Kabelitz et al. (1987)
T cells*	PBMC (10^5)	No	1/ 535	Con A blasts	10-12 days	25u/ml rIL2 + MLC supt.	Kabelitz et al. (1987)
CD4+ T cells#	PBMC (10^5)	No	1/ 1620	Con A blasts	10 days	25u/ml rIL2 + MLC supt.	Kabelitz et al. (1985)
CD4+ T cells+	Spleen (10^5)	No	1/ 11600	PHA blasts	14-18 days	TCGF	Moretta et al. (1983)
CD8+ T cells#	PBMC (10^5)	No	1/ 190	Con A blasts	10 days	25u/ml rIL2 + MLC supt.	Kabelitz et al. (1985)
CD8+ T cells+	Spleen (10^5)	No	1/ 710	PHA blasts	14-18 days	TCGF	Moretta et al. (1983)

[1]Irradiated autologous filler cells

[2]Maximum frequency obtained in publication cited. [3]TCGF obtained by delectinated supernatants harvested 48 hours after PHA activation of PBMC.

* Purified by E-rosetting with sheep erythrocytes.

[4]Supernatant harvested from mixed lymphocyte supernatants after 48 hours.

Negative selection using antibody and complement

+ Purified by flow cytometry

4.3 Results

4.3.1 Analysis of limiting dilution culture conditions.

The culture conditions used for the limiting dilution assay had been established in collaboration with C. Sharrock and E. Kaminski and did not form part of these studies (Sharrock et al. 1987, Kaminski et al. 1988). The main difference between the systems used in the current study and previous studies, was the use of B-LCL as target cells instead of PHA blasts. B-LCL were chosen as targets because they constitutively express high levels of all HLA class II molecules and as such were likely to be more sensitive targets for detection of HLA class II specific CTL (see Figure 3.13). In addition, the availability of a large panel of homozygous B-LCL from the Xth Histocompatibility Workshop allowed greater flexibility in experimental design.

The results in Figure 4.1 and 4.2 from a representative experiment demonstrate that the culture conditions used were suitable for limiting dilution analysis of CTLp. The number of positive wells and the magnitude of specific lysis in individual wells both decreased as responder cell numbers became limiting. Between $1.7x10^5$-$2.4x10^5$ cells/well were generated from a starting number of $1x10^4$/well, after the 10 day incubation period. Significant cytotoxicity could be observed with responder cells as low as 625 cells/well (Figure 4.1). These results produced a semi-logarithmic plot which was a good fit to a straight line through the origin (χ^2=8.11, p>0.05). Straight line plots were obtained for all LDA experiments when judged by regression analysis however not all lines passed through the origin[§] (see Table 6.5 in Appendix). The average intercept for all experiments was 1.08±0.22. These results indicated that the culture system used was limiting for alloreactive CTLp.

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§ Lines intercepting the origin (Y =1.0) were obtained in 30 out of 58 experiments.

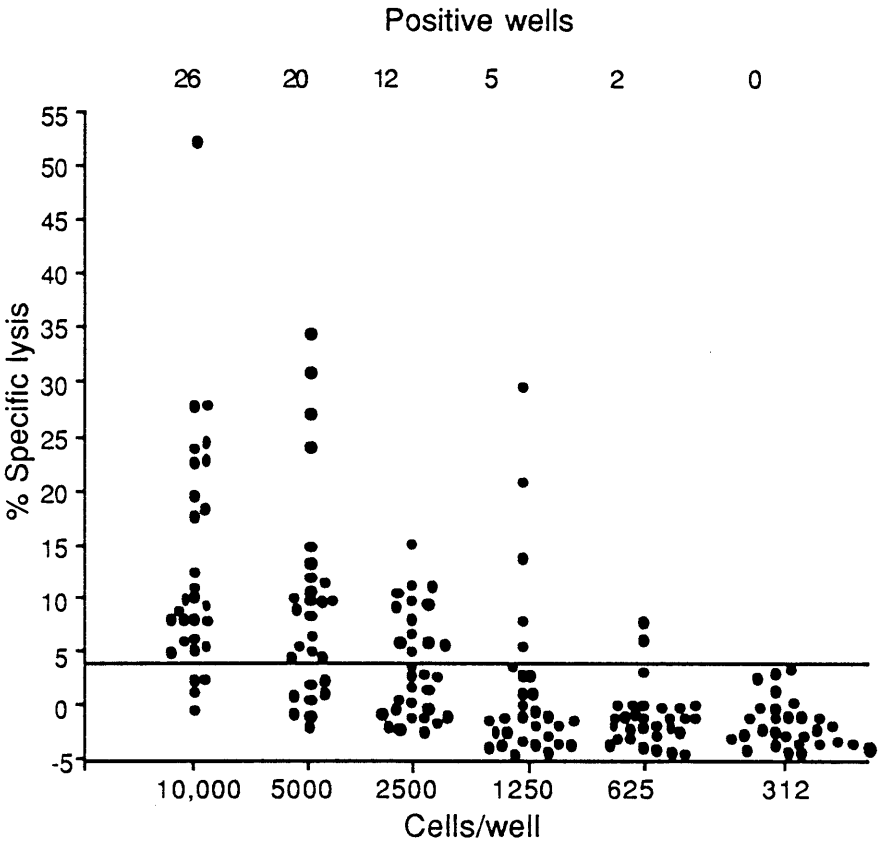


Figure 4.1 Cytotoxic activity of limiting dilution cultures. Limiting numbers of PBMC responders from JM (HLA A1,2 B8,18 DRw11 DQw7 DRw52) were cultured with $5x10^4$ stimulator PBMC from MR (HLA A3,25 B7,8 DRw15 DQw6). Points plotted represent the % specific lysis of MR B-LCL mediated by individual culture wells. The cut-off point for positive cultures (mean +3 SD of control wells) is shown by the horizontal line. The number of positive wells for each concentration are shown at the top of the figure.

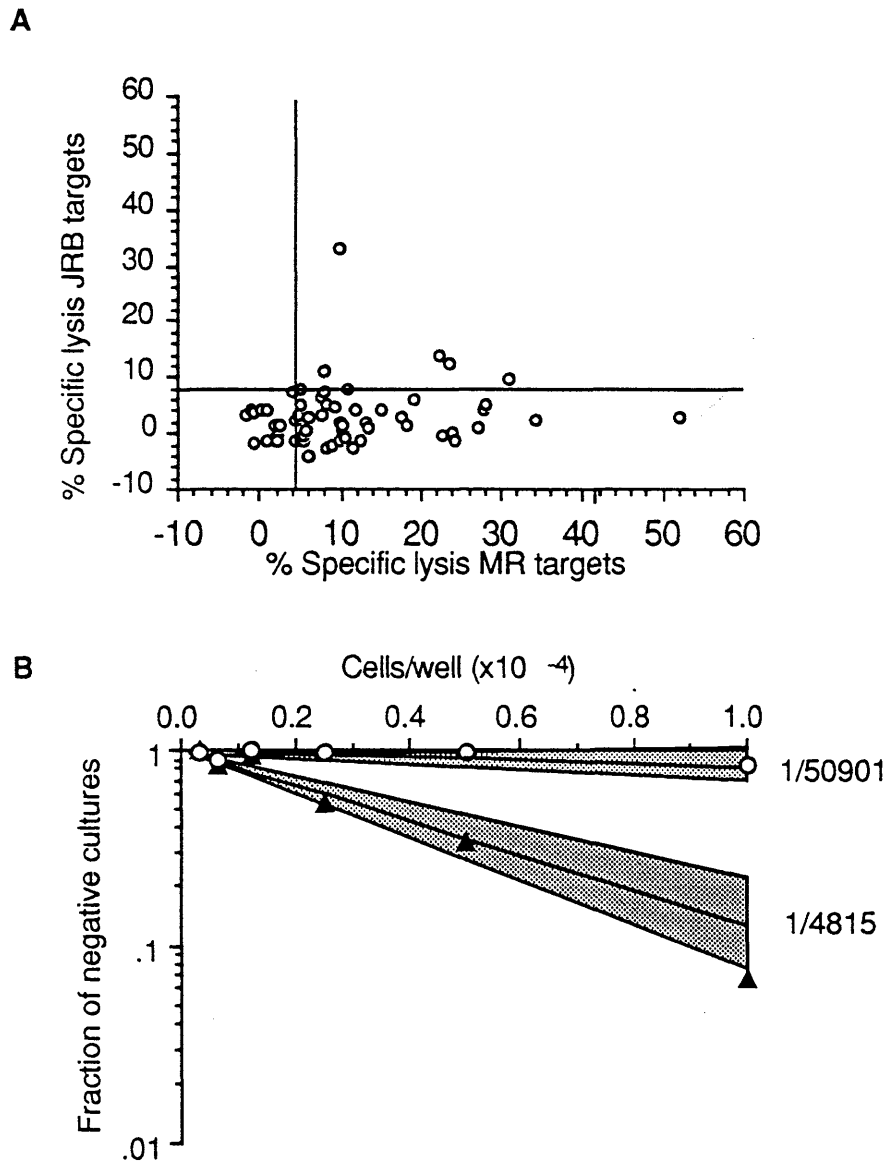


Figure 4.2 Split well analysis of limiting dilution assay cultures. LDA cultures between responder JM (HLA A1,2 B8,18 DRw11 DQw7) and MR (HLA A3,25 B7,8 DRw15 DQw6). At the end of the culture period the contents of individual wells were split and assayed against stimulator (MR) and non-stimulator targets (JRB, HLA A2,3 B62,44 DR4 DQw8). The specific lysis results for 60 individual wells were plotted on a scatter diagram, shown in panel A. The cut-off point (mean+3SD of controls) for scoring positive cultures is shown by a line for each target. The frequencies of alloreactive CTLp were determined against both stimulator targets (MR ▲) and non-stimulator targets (JRB ○), as shown in the semi-logarithmic plots in panel B. The shaded areas represent the 95% confidence limits.

The system was shown to be specific by split well analysis as shown in Figure 4.2a and 4.2b. The majority of culture wells were cytotoxic for the B-LCL targets of the stimulator only, although a minority of the wells were able to react on both stimulator and non-stimulator B-LCL. CTLp frequencies against the stimulator B-LCL were ten-fold higher than against the non-stimulator B-LCL, and were significantly different as shown by non-overlapping 95% confidence limits.

4.3.2 Immunomagnetic depletion of CD8+ve T cells from peripheral blood mononuclear cell populations.

The activation of CD8+ve CTL in primary mixed lymphocyte culture has been shown to have a detrimental effect on the development of CD4+ve class II specific CTL (Feighery and Stastny 1979, Reinherz et al. 1979). In order to overcome this effect, PBMC were depleted of CD8+ve T cells using anti-CD8 coated magnetic beads (Leivestad et al. 1986) PBMC and CD8-depleted PBMC were subjected to phenotypic analysis for every LDA experiment. The results from a representative experiment are shown in Figure 4.3. The depletion procedure was seen to be efficient with between 0.26 to 3% CD8 +ve T cells remaining after immunomagnetic depletion in twelve experiments, furthermore no increase in the proportion of CD8+ve T cells was seen in the CD8-depleted population after 10 days of culture. Total cell numbers recovered from such cultures were similar to those observed for untreated PBMC cultures. Between 1.1-3.1x10^5 cells/well were obtained from a starting cell number of 1x10^4 CD8-depleted PBMC/well after 10 days culture. There was an increase in the proportion of cells expressing NK cell markers (detected by Leu19 antibody) in both the PBMC and the CD8-depleted PBMC populations after 10 days of culture.

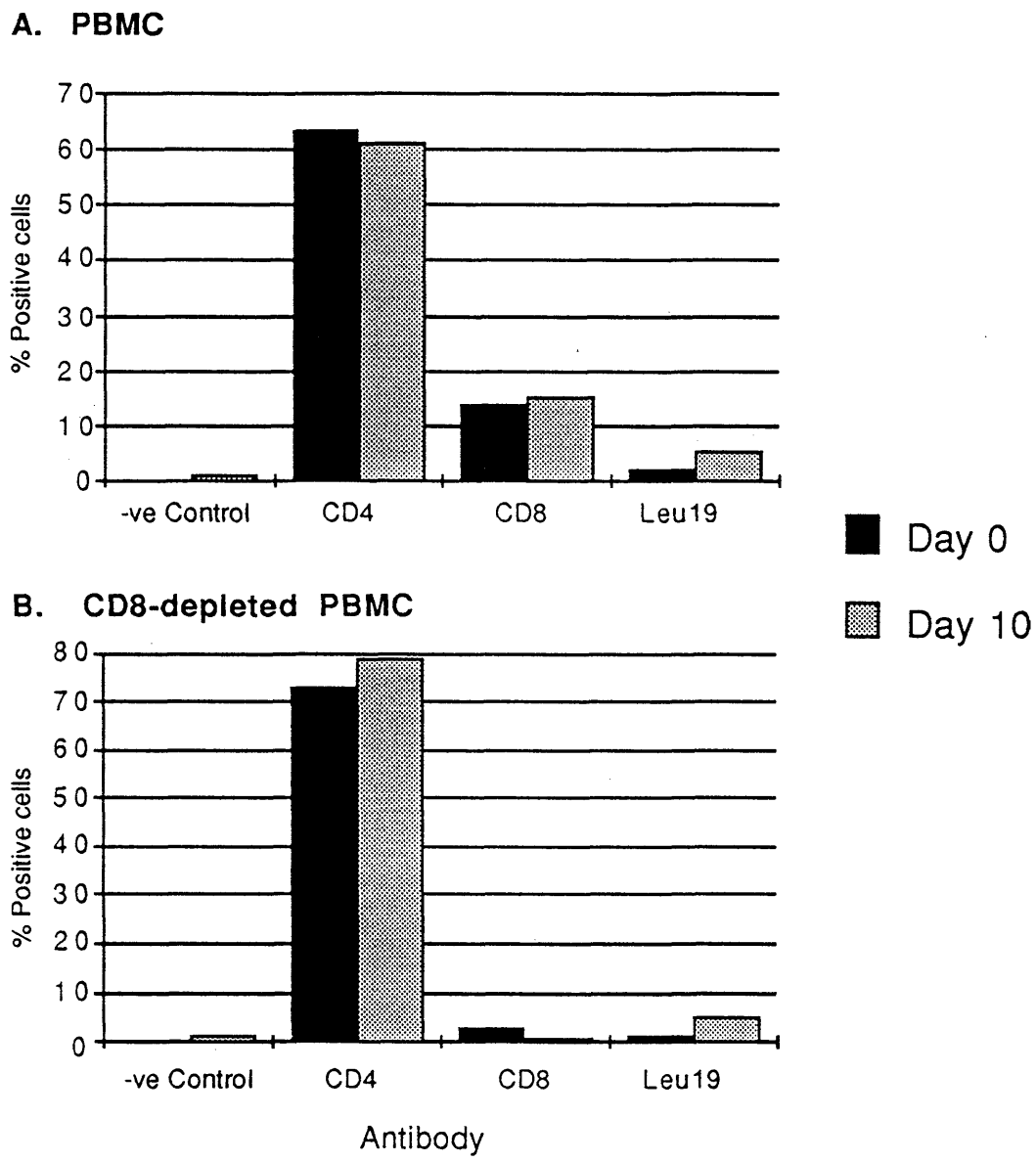


Figure 4.3 Phenotypic analysis of LDA cultures. PBMC (a) or CD8-depleted PBMC (b) were stained with anti-CD4, anti-CD8 and anti-NKH1 (leu19) antibodies on days 0 and 10 of culture. The % of positive cells stained with each antibody was determined by flow cytometry. Negative controls for PBMC and CD8-depleted PBMC were always less than 1.5%.

4.3.3 Immunomagnetic depletion of NKH1 positive cells abrogates lysis of Daudi targets but does not affect lysis of allogeneic targets.

The number of NKH-1 positive cells (detected by Leu19 antibody) expanded in both the CD8 depleted PBMC and untreated PBMC populations after culture (Figure 4.3). Since NKH1 positive cells mediate non-MHC restricted cytotoxicity, the role of NKH1 cells in mediating cytotoxicity of allogeneic B-LCL was investigated by subjecting PBMC already depleted of CD8+ve cells, to an additional immunomagnetic depletion using an antibody against NKH-1# (Leu19, see Table 2.2). The cytotoxic activity of depleted and undepleted PBMC populations after LDA culture against the same stimulator, was investigated in the presence of varying concentrations of rIL2. The results in Figure 4.4a, demonstrate that the untreated PBMC were highly cytotoxic against allogeneic B-LCL targets, and targets which are susceptible to non-specific killing such as K562 and Daudi. No significant killing was observed against autologous B-LCL targets.

CD8-depleted PBMC exhibited significant lysis of the stimulator target, but at a markedly reduced level, indicating that the bulk of the cytotoxic activity in untreated PBMC was mediated by CD8+ve T cells. The weak, but significant lysis mediated by CD8-depleted PBMC was consistent; specific lysis of between 11-26% was observed in three further experiments using different responders and targets from those of Figure 4.4. In the same experiments specific lysis of between 25-48% was observed in the untreated PBMC. Reduced lysis of K562 targets was also seen in CD8 depleted-PBMC, although there was no significant change in lysis against Daudi targets.

NKH1+ ve cells were always<1% after immunomagnetic depletion.

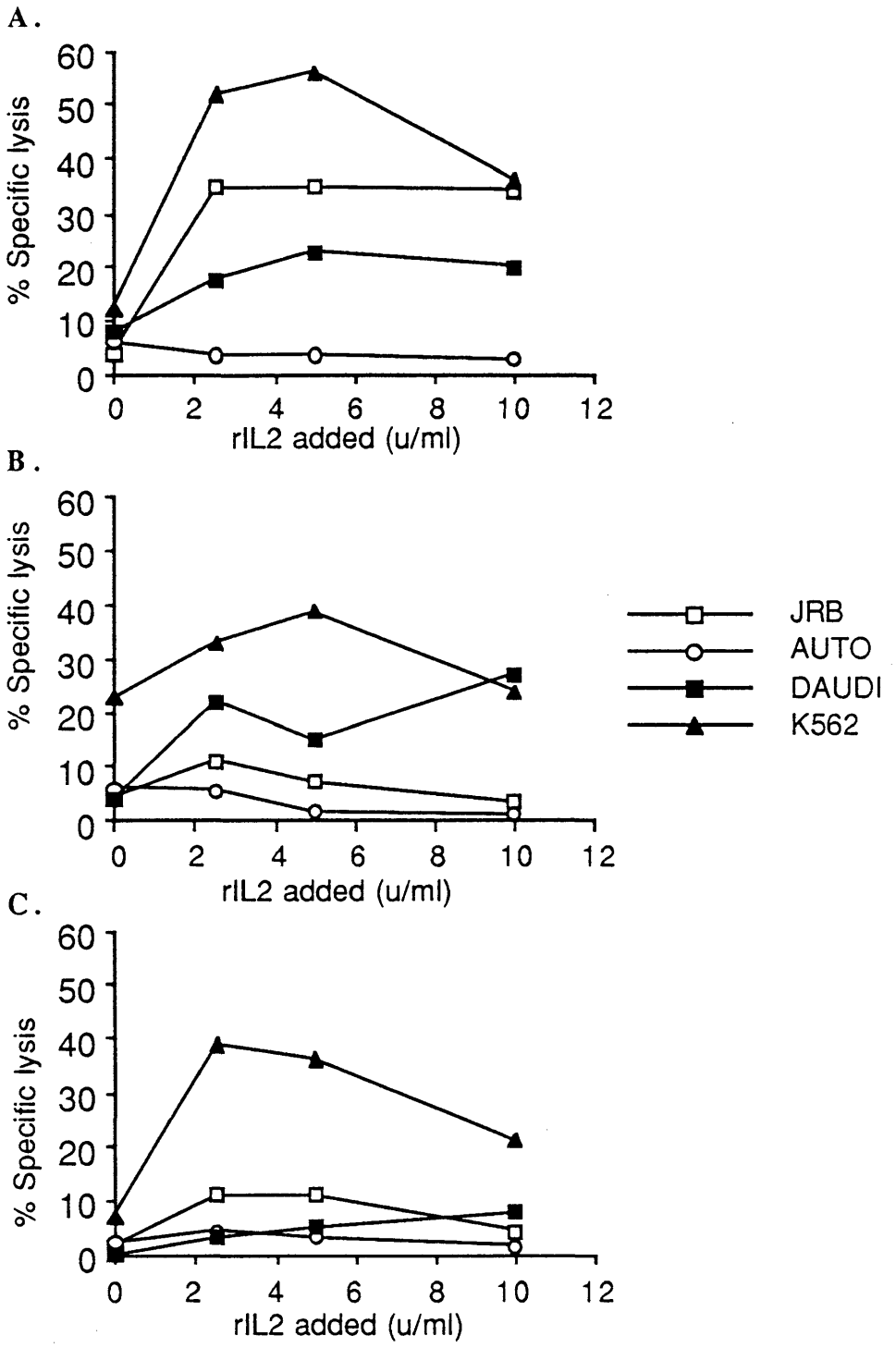


Figure 4.4 Depletion of NKH1+ (leu19+) cells abolishes lysis of Daudi target cells but does not affect specific lysis of allogeneic B-LCL targets. PBMC (a), CD8-depleted PBMC (b) and CD8+NKH1-depleted PBMC (c) from MB (HLA A1, B8 DR3 DQw2 DRw52) were cultured with irradiated allogeneic JRB PBMC (HLA A2,3 B44, 62 DR4 DQw8 DRw53). The cultures were fed with GM and varying concentrations of rIL2 on days 3 and 6, before being assayed for cytotoxic activity on day 10. Specific lysis was calculated from the mean of six replicate cultures.

When PBMC were depleted of both CD8+ve and NKH1+ve cells, the cytotoxicity against Daudi targets was lost, whilst lytic activity was retained against both K562 and allogeneic stimulator targets. There was no significant difference between specific lysis of allogeneic B-LCL mediated by either CD8-depleted (b) or CD8 plus NKH-1 (c) depleted PBMC. These results demonstrate that removal of NKH1+ve cells removes cells able to lyse Daudi targets, but not T cells able to lyse allogeneic targets. In addition these results indicate that the killing of allogeneic B-LCL targets under these conditions was specific, as no significant lysis of either autologous B-LCL (Figure 4.4 a,b, and c) was ever seen despite the presence of cells able to lyse K562 and Daudi targets.

The addition of rIL2 to the PBMC cultures (Figure 4.4a) resulted in augmentation of the cytotoxicity against K562, Daudi, and allogeneic B-LCL targets, but not autologous B-LCL targets. A similar effect was also seen with the CD8 depleted PBMC (Figure 4.4b) and the CD8 and NKH1+ depleted PBMC (Figure 4.4c). The optimum concentration of rIL2 to induce maximum cytotoxicity was found to be between 2.5-5u/ml in four separate experiments.

4. 3. 4 CD8-depleted PBMC mediate HLA class II specific lysis

The specificity of CTL generated from mixed lymphocyte cultures using CD8-depleted PBMC and untreated PBMC as responders, was investigated using split well analysis. The results shown in Figure 4.5b demonstrate that CTL derived from the CD8-depleted PBMC population are specific for HLA class II antigens. A highly significant correlation was observed between lysis of two allogeneic B-LCL targets sharing only HLA class II antigens. There was no correlation in specific lysis of the two HLA class II-matched targets when PBMC (containing both CD4+ve and CD8+ve cells) were used as responder cells (see Figure 4.5a).

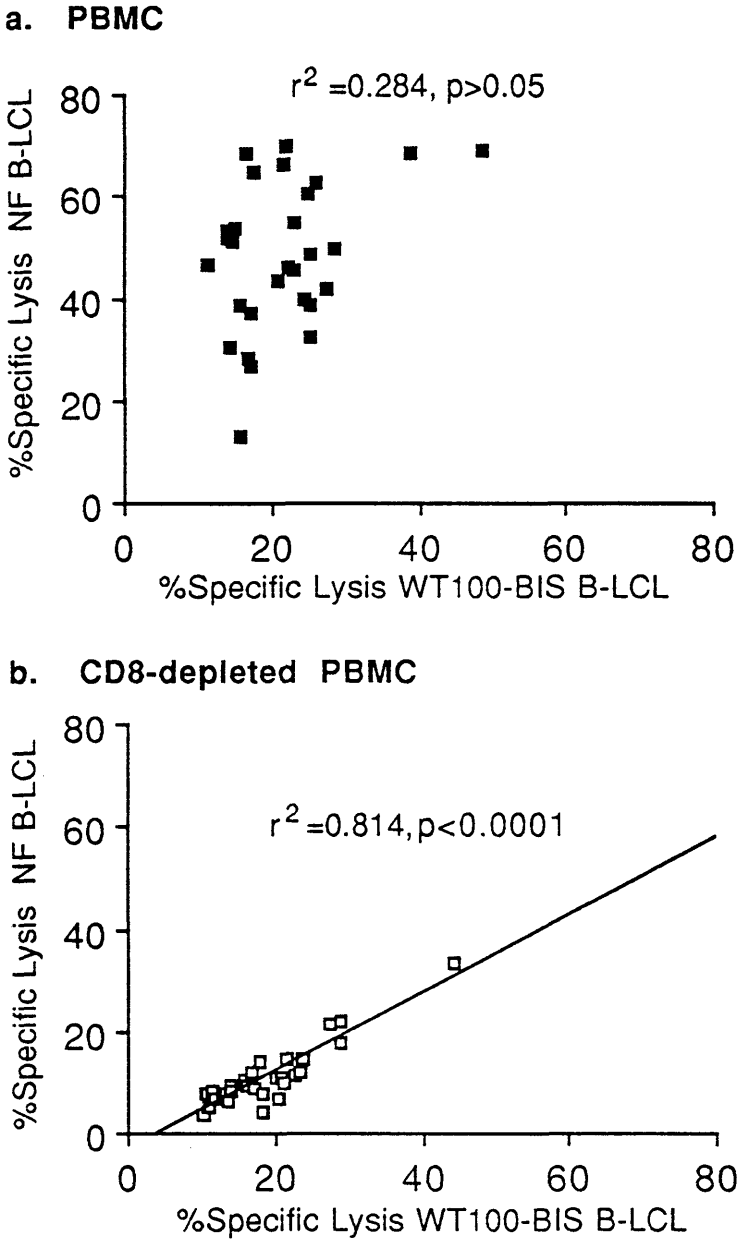


Figure 4.5 Split well analysis of PBMC and CD8-depleted PBMC cultures against allogeneic targets matched for HLA Class II. PBMC (a) and CD8-depleted PBMC (b) from SF (HLA A1,26 B7,12 DR3 DQw2 DRw52) were set up in primary MLC against NF PBMC (HLA A32 B14,15 DR1 DQw5). The cultures were incubated for 10 days under LDA conditions (see materials and methods) before the contents of individual wells were split and tested for CTL activity against NF B-LCL (stimulator) and WT100BIS B-LCL (HLA A11 B35 DR1 DQ1). The points plotted represent specific lysis results from individual wells Significant correlation occurs with a probability of <0.05.

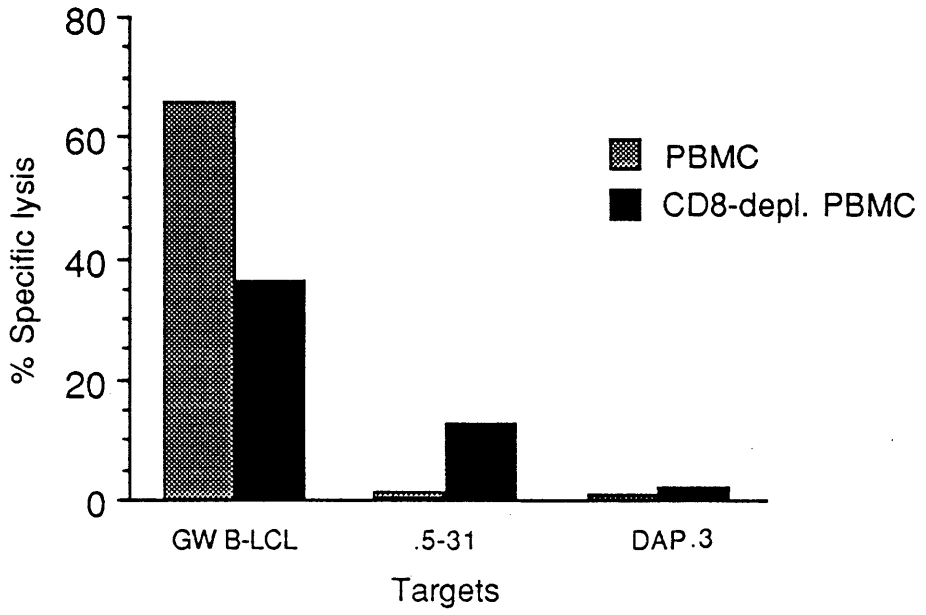


Figure 4.6. CD8-depleted PBMC mediate specific lysis of murine transfectants expressing HLA-DR1. PBMC (a) or CD8-depleted PBMC (b) from TD (HLA A1,2 B35 DRw13 DQw5 DRw52) were set up in primary MLC against GW PBMC (HLA A1,31 B8,51 DR1,3 DQw5,w2 DRw52). These cultures were tested for cytotoxic activity after 10 days of culture (see materials and methods). The target cells used were stimulator B-LCL (GW), DR1-expressing murine transfectant (5-3.1) and HLA negative parent L cell line (DAP. 3). Specific lysis was calculated from the mean of six replicate cultures after a seven hour chromium release assay.

A more stringent test for the presence of HLA class II specific CTL was to assay CD8-depleted PBMC cultures against targets expressing only HLA-DR antigens. In Figure 4.6 it can be seen that PBMC and CD8-depleted populations can both lyse stimulator B-LCL targets, however only the CD8-depleted population was able to lyse a murine L cell transfectant expressing HLA-DR1. This lysis was specific as there was no lysis of the untransfected (HLA negative) parental L cell line, DAP. 3. These results confirm the data of Figure 4.5, in that the CTL generated after MLC were predominantly HLA class I specific, whereas CD8 depletion of PBMC prior to MLC results in the development of CTL which are HLA class II specific.

4. 4. 5. Variation in CD4+ve CTLp frequency depending on the allogeneic responder:stimulator combination used.

Having shown that the depletion of CD8+ve T cells from PBMC allows development of HLA class II specific CTL, limiting dilution analysis was used to estimate the frequency of alloreactive CTLp in CD8-depleted PBMC. As shown in Table 4.2 a large range of CTLp frequencies (1/1016-39587) was found among the different responder: stimulator MLC combinations used. When autologous combinations (NF vs NF, SF vs SF in Tbale 4.2) were used CD4+ve CTLp frequencies were found that were close to the theoretical limits of detection (1/100,000). Ten out of 16 of the allogeneic combinations produced CTLp frequencies significantly different from the autologous controls.

In contrast to the CD4+ve CTLp frequencies, the CTLp frequencies obtained with untreated PBMC (presumably CD8+ve CTLp) had a much smaller range of between 1/2006 to 1/6296 (Table 4.3). In some combinations the high frequencies of CD4+ve CTLp obtained were similar to the CTLp frequencies obtained from the same allogeneic combinations using untreated PBMC (see SF vs NF, MB vs JRB in Table 4.2).

Table 4.2. Alloreactive CD4 +ve CTLp frequencies in unrelated individuals.

Responder & HLA type[a]		Stimulator & HLA type		Frequency[b] (range)	
NF[c]	DR1 DQw5 Dw1	TD	DRw13 DQw5 DRw52	1/1016	(779-1327)
NF[c]	DR1 DQw5 Dw1	JRB	DR4 DQw8 DRw53 Dw4,14	1/3635	(2906-4544)
NF[c]	DR1 DQw5 Dw1	SF	DR3 DQw2 DRw52	1/3902	(3031-5023)
NF[c]	DR1 DQw5 Dw1	MK	DR7 DQ2 DRw53	1/34837	(20003-60588)
NF[c]	DR1 DQw5 Dw1	NF	DR1 DQw5 Dw1 (autologous)	1/75008	(33865-166132)
MB	DR3 DQw2 DRw52	JRB	DR4 DQw8 DRw53 Dw4,14	1/1475[d]	(1124-1936)
MB	DR3 DQw2 DRw52	MK	DR7 DQw2 DRw53	1/4951	(3828-6402)
MB	DR3 DQw2 DRw52	SF	DR3 DQw2 DRw52	1/26778*	(16389-43754)
MB	DR3 DQw2 DRw52	SM	DRw15 DQw6 Dw12	1/38987*	(23038-65976)
SF	DR3 DQw2 DRw52	NF	DR1 DQw5 Dw1	1/1880[e]	(1481-2420)
SF[f]	DR3 DQw2 DRw52	BR	DRw15 DQw6 Dw2	1/3064	(2379-3945)
SF[f]	DR3 DQw2 DRw52	JRB	DR4 DQw8 DRw53 Dw4,14	1/5989	(4605-7789)
SF[f]	DR3 DQw2 DRw52	JM	DRw11 DQw7 DRw52 Dw5	1/8340	(6198-11221)
SF[f]	DR3 DQw2 DRw52	MK	DR7 DQw2 DRw53	1/12174	(8805-16833)
SF	DR3 DQw2 DRw52	SM	DRw15 DQw6 Dw12	1/39587*	(19659-79715)
SF[f]	DR3 DQw2 DRw52	SF	DR3 DQw2 DRw52 (autologous)	1/93702	(42037-208867)
SD	DR3,4 DQ2,3 DRw52,53	NF	DR1 DQw5 Dw1	1/2182*	(1716-2774)
JM	DRw11 DQw7 DRw52 Dw5	MR	DRw15 DQw6 Dw2	1/38949*	(23038-65976)

a) HLA phenotypes obtained by serological typing and RFLP analysis.
b) Frequency of CTLp in CD8 and NKH-1-depleted PBMC (frequencies marked with * were obtained from PBMC depleted of CD8+ve cells only) , range represents 95% confidence limits for frequency estimate.
c) Frequencies obtained in the same experiment.
d) Frequency obtained from CD8-depleted PBMC vs JRB in the same experiment=1/1096 (856-1403). Results from two further experiments for MB vs JRB were 1/1262 (988-1612) and 1/2611 (2054-3319).
e) Frequency obtained from CD8-depleted PBMC vs NF in same experiment=1/2273 (1676-2420). Results from two further experiments for SF vs NF were 1/701 (539-920) and 1/1430 (1118-1829).
f) Frequencies obtained in the same experiment.

Table 4.3. Frequency of alloreactive CTLp in unrelated individuals.

Responder & HLA type[a]		Stimulator & HLA type		Frequency[b]	(range)
MB *	A1 B8 DR3 DQw2 DRw52	SM	A11,24 B13,15 DRw15 DQw6 Dw12	1/2006	(1562-2577)
MB *	A1 B8 DR3 DQw2 DRw52	SF	A1,29 B7,12 DR3 DQ2 DRw52	1/2098	(1650-2667)
MB	A1 B8 DR3 DQw2 DRw52	GW	A1, 31 B8,51 DR1,3 DQ1,3 DRw52	1/2623	(2059-3342)
MB *	A1 B8 DR3 DQw2 DRw52	JRB	A2,3 B12,15 DR4 DQw8 DRw53 Dw4,Dw14	1/2672	(2099-3401)
MB	A1 B8 DR3 DQw2 DRw52	SD	A1, 11 B8, 35 DR3,4 DQw2,w8 DRw52 DRw53	1/6296	(4742-8360)
SF *	A1,29 B7,12 DR3 DQw2 DRw52	NF	A32 B14, 15 DR1 DQw5 Dw1	1/2133	(1638-2779)
SF *	A1,29 B7,12 DR3 DQw2 DRw52	MK	A1,9 B12,17 DR7 DQw2 DRw53	1/2611	(2054-3319)
SF	A1,29 B7,12 DR3 DQw2 DRw52	MB	A1 B8 DR3 DQw2 DRw53	1/5141	(3984-6834)
JM *	A1,2 B8 DRw11 DQw7 DRw52 Dw5	MR	A3,25 B7,8 DRw15 DQw6	1/4815	(3736-6205)

a) HLA phenotypes deterimined by serological typing.
b) Frequency of CTLp in PBMC with 95% confidence limits shown in brackets.
* Combinations where CD4+ CTLp were determined in the same experiment, see table 1.

In other combinations the frequencies of CD4+ve CTLp were up to 20 fold lower than those obtained from CD8 depleted PBMC (see SF vs SM, MB vs SM Table 4.2).

There was no significant difference between the CTLp frequencies obtained with CD8-depleted PBMC or with PBMC depleted of both CD8+ve and NKH1+ve cells, suggesting that NKH-1 effectors did not contribute to the lysis of allogeneic B-LCL targets (see footnotes d and e in Table 4.2).

There was no correlation between CTLp frequency and degree of HLA mismatch, as high and low CTLp frequencies were obtained from combinations mismatched by three HLA class II antigens. In addition if CTLp frequencies were divided into two groups according whether the responder:stimulator combination had three or two HLA class antigen mismatches, no significant difference[§] was found between the two groups.

There was considerable variation in the CD4+ve CTLp frequencies obtained between different individuals against the same allogeneic HLA class II antigens, as can be seen in Table 4.2. Three individuals (MB, SF, and NF) produced significantly different CD4+ve CTLp frequencies against MK. In addition there was variation in CTLp frequencies produced by any given individual against different allogeneic HLA class II antigens. This was not simply due to variation between experiments, as significant frequency differences could be obtained in the same experiment (see CTLp frequencies obtained using NF or MB in Table 4.2) and the frequencies within one responder: stimulator pair were reproducible over a number of experiments (see footnotes d and e in Table 4.2).

The specificity of the limiting dilution system was demonstrated by split-well analysis. The scatterplots in Figure 4.7 show that the majority of

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[§] $P>0.05$ (Mann Witney test)

alloreactive CD4+ve CTL detected in limiting dilution cultures, were specific for the HLA class II antigens of the stimulator. There was a negligible contribution from CTL specific for autologous B-LCL (Figure 4.7a), no CTLp frequency against autologous B-LCL targets could be determined (Figure 4.8). The results in Figure 4.7b show that third party-reactive CTL were also in the minority. The majority of limiting dilution cultures tested, lysed the B-LCL (DR4) which shared class II antigens with the stimulator, but did not lyse the third party B-LCL (DR7). It can be seen in Figure 4.8 that the frequency of alloreactive CTLp specific for the DR4 B-LCL was at least 8 fold higher that the frequency for third party specific CTLp (Figure 4.9).

4.3.6 High frequency of alloreactive CTLp in purified populations of CD4+ T cells.

A high frequency of alloreactive CTLp was obtained using CD4 positive T cells purified by flow cytometry, demonstrating that the high CTLp frequencies shown in Table 4.2 were not due to an artifact of the immunomagnetic depletion procedure. The results depicted in Figure 4.10, show that CTLp frequencies obtained from CD4-enriched populations derived by either immunomagnetic depletion or by flow cytometry, were not significantly different. CTLp frequencies for the negatively selected CD4-ve T cell population (CD8+ve) were significantly higher than for the positively selected CD4+ve T cell population, although there was no significant difference in CTLp frequency when compared to the starting, untreated PBMC population. There was also a significant difference between CD8-depleted PBMC and the untreated PBMC.

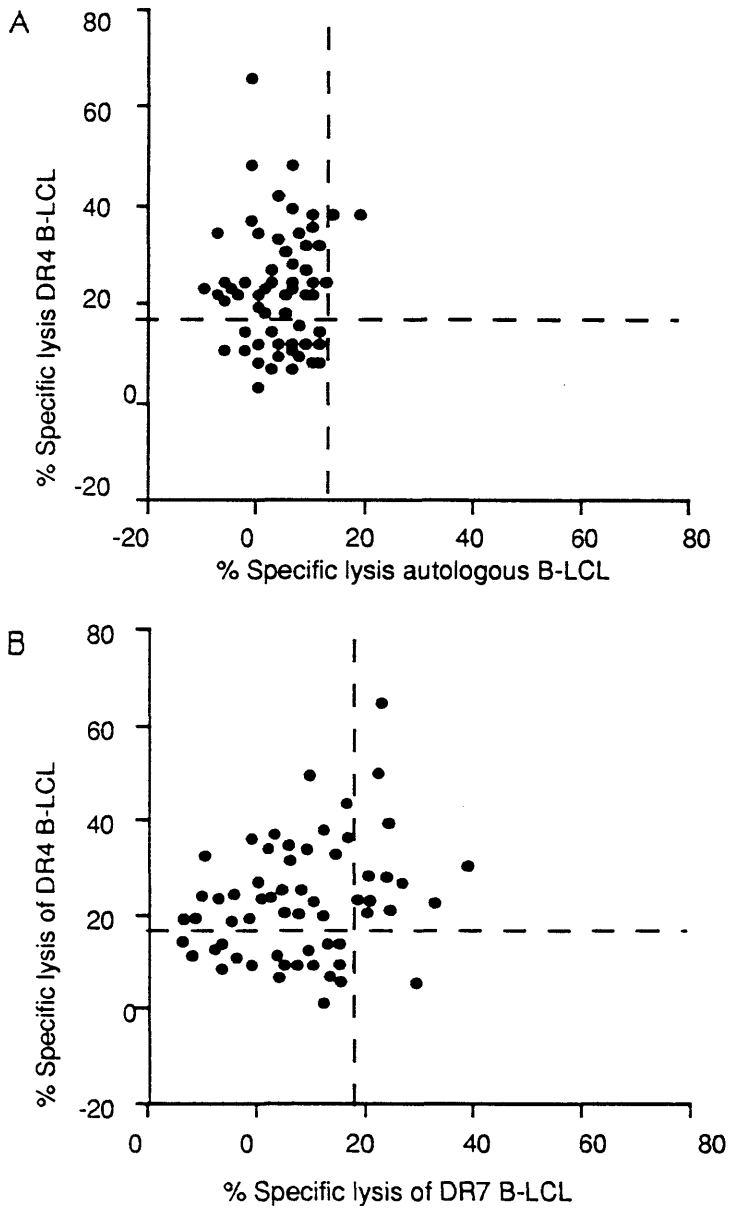
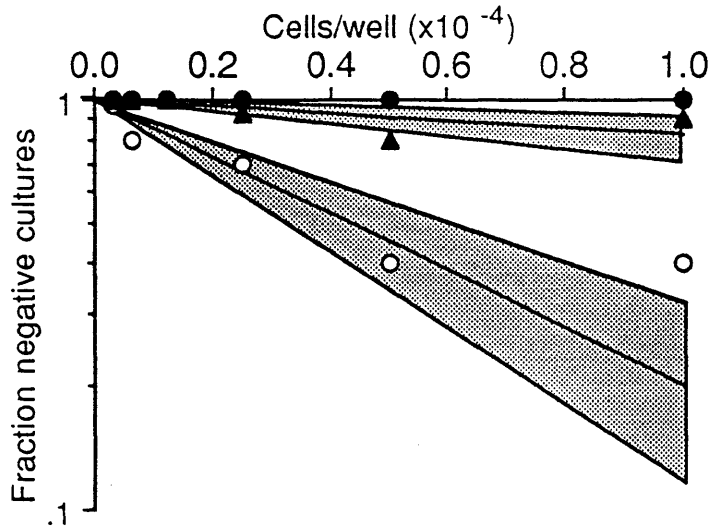


Figure 4.7 Specificity of limiting dilution cultures primed against HLA-DR4 as determined by split well analysis. LDA cultures were set up from 10,000 CD8-depleted PBMC from MB (HLA A1 B8 DR3 DQw2 DRw52) and 50,000 stimulator PBMC from JRB (HLA A2,3 B44, 62 DR4 Dw4,14 DQw8 DRw53) At the end of 10 days the contents of 60 culture wells were each split into 3 aliqouts, and assayed against DR4 B-LCL (PE117, HLA A24, B60, DR4 Dw14 DQw8 DRw53), autologous B-LCL (MB), and third party B-LCL (LBUF, HLA A30, B13, DR7 DQw2 DRw53). Each point plotted represents a single culture, and points above the dotted lines (mean + 3SD of control) were scored as positive for cytotoxicity. Target cell number was reduced from 10,000 to 2000 targets/well for this experiment.



Targets	Frequencies (95% confidence limits)
o DR4 B-LCL	f=1: 4973 (3815-6482)
▲ Third party B-LCL (DR7)	f=1: 40535 (22387-73395)
● Autologous B-LCL	Not detectable.

Figure 4.8. Specificity of LDA cultures primed against HLA-DR4 determined by split well analysis. LDA cultures were set up using CD8-depleted PBMC from MB (HLA A1 B8 DR3 DQw2 DRw52) and stimulator PBMC from JRB (HLA A2,3 B44,62 DR4 DQw8 DRw53). At the end of 10 days culture the contents of individual culture wells were split three ways and assayed against DR4 B-LCL (PE117, HLA A24 B60 DR4 DQw8 DRw53), autologous B-LCL (MB), and third party B-LCL (LBUF, HLA A30 B13 DR7 DQw2 DRw53).

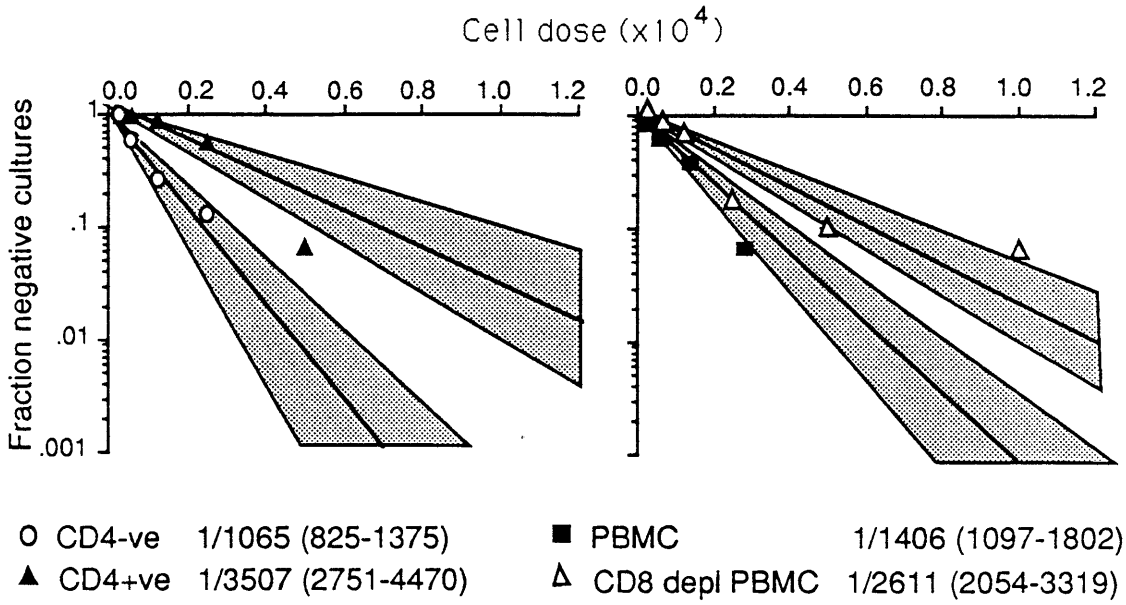


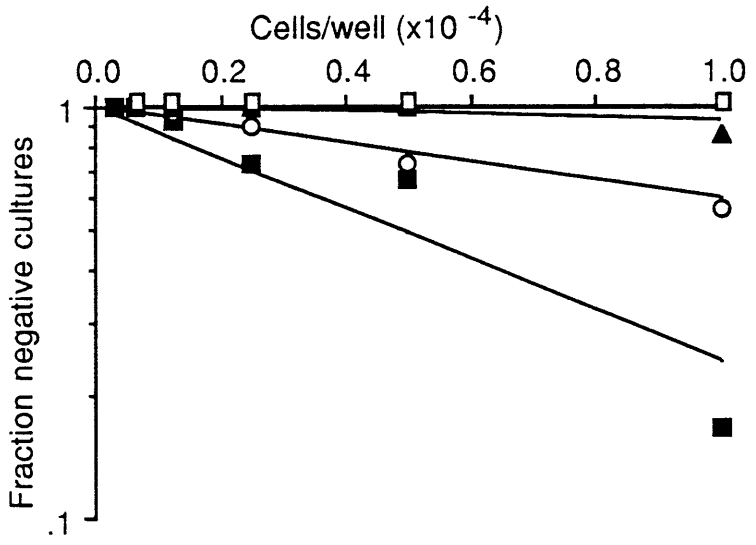
Figure 4.9. Frequency analysis of alloreactive CTLp in CD4-enriched cell populations. Results shown are from a single experiment. Limiting dilution cultures were set up between responder, MB (HLA A1 B8 DR3 DQ2) against stimulator JRB (HLA A2,3 B15 DR4 DQ3). The responder cells were : CD4-enriched T cell populations obtained by flow cytometry (see materials and methods); CD4-ve T cells obtained by flow cytometry; CD4 enriched populations obtained by immunomagnetic depletion of NKH1+ and CD8+ cells from PBMC; the starting (untreated) PBMC population. All cultures were set up on the same day, using freshly isolated MB PBMC. CTL assays were performed after 10 days incubation using JRB B-LCL targets. CTLp frequencies and 95% confidence limits are shown above.

4.3.7 High frequencies of alloreactive CTLp recognizing HLA-DR gene products expressed on murine L cell transfectants.

The interpretation of CTL frequency data obtained from HLA class II mismatched MLC combinations is difficult as multiple class II target antigens could be recognized on a B-LCL target. For instance HLA-DR, DQ, DP and DRw52 or DRw53 molecules could serve as target antigens. This problem can be overcome by the use of murine L cell transfectants expressing HLA class II gene products as CTL targets, allowing detection of CTLp specific for single HLA class II molecules. The results in Figure 4.10 demonstrate the LDA system used was sensitive enough to detect CTLp specific for HLA-DR1 expressed on a murine L cell transfectant. There was no specificity for xenogeneic determinants as no CTLp could be detected using untransfected mouse fibroblasts as targets (Figure 4.11). The frequency of HLA class II CTLp detected using the DR1+ve L cell target was lower than when the DR1+B-LCL target was used, but was significantly higher than the CTLp frequency obtained against third party B-LCL targets (Figure 4.10).

In order to investigate the cytotoxic T cell repertoire to HLA-DR molecules, alloreactive CD4+ve CTLp frequencies were determined in unrelated individuals, using both B-LCL and DR+1ve L cell transfectants as targets. The responder and stimulator individuals selected (Table 4.4), were nearly all homozygous for HLA class II antigens (as determined by serological typing and RFLP analysis). In all cases the B-LCL and L cell transfectant targets used for CML assays were HLA class II matched with the stimulator PBMC.

The results in Table 4.5 demonstrate a large variation in CTLp frequencies when murine L cell transfectants were used as targets, with frequencies ranging from 1/1257 to less than 1/100,000. The range of CTLp



Targets		Frequency (95% confidence limits)
■	NF B-LCL	f=1: 7051 (5364-9268)
o	531 L cell	f=1: 19795 (13386-29272)
▲	MK B-LCL	f=1: 142595 (53808-377885)
□	DAP-3 L cell	Not detectable

Figure 4.10. Frequency of CD4+ CTLp specific for HLA-DR1 expressed on a murine L cell transfectant. PBMC from SF (HLA A1,29 B7,12 DR3 DQw2 DRw52) were depleted of CD8+ and NKH1+ cells, before setting up in LDA cultures against EM PBMC (HLA A2,11 B35,40 DR1, DR3, DQw5,DQw2 DRw52). CTLp frequencies were determined against : DR1+ B-LCL (NF A32 B14,15 DR1 DQw5), DR1+ murine L cell transfectant (.5-3.1), HLA negative parent murine L cell line (DAP.3) and third party B-LCL (MK HLA A1,9 B12,17 DR7 DQw2 DRw53).

frequencies detected using B-LCL as targets was smaller and ranged from 1/867-1/11,267.

There was however, no absolute concordance between the CTLp frequencies obtained within one allogeneic combination using B-LCL and DR+ve L cell targets. The results could be divided into two groups. For 2 out of the 8 responder:stimulator combinations (NF vs JM and SM vs JM), high CTLp frequencies were obtained using L cell targets, with no significant difference from the CTLp frequency obtained using B-LCL targets in the same experiment. For the other 6 combinations CTLp frequencies obtained using L cell transfectant targets were low or undetectable, whilst the corresponding frequencies obtained using B-LCL were high. The patterns observed were reproducible in repeat experiments (see group III CTLp frequencies in Table 4.5).

Individual variation in CTLp frequencies could clearly be observed using DR+ve L cell targets. One individual (SM) produced high CTLp frequency against DR5+ve L cells, however the CTLp frequencies against DR4+ve or DR1+ve L cells were undetectable in the same experiment. Similarly, a low but significant CTLp frequency for MB was obtained using DR4+ve L cells, however no CTLp frequencies could be detected using the DR1+ve or DR5+ve L cells. Variation between individuals was confirmed by the demonstration that SM could produce a high CTLp frequency against the DR5 transfectant whereas MB was unable to generate a cytotoxic T cell response against the same DR5 transfectant. These results were obtained in the same experiment using the same batch of DR5 stimulator PBMC and the same batch of DR5 L cell targets.

The variation in CTLp frequencies observed could not be explained by differences in class II expression between target cells. All transfectants had stable expression of the transfected HLA-DR genes, although the levels of HLA class II expressed differed between transfectants.

Table 4.4. HLA typing data of allogeneic combinations used for determining CTLp frequencies against murine L cell transfectants.

Group[1]	Combinations[2]	HLA Type[3]					
		A	B	DR	DQ	DRw52 /53	Dw
I	R: SF	1,29	7,12	3	w2	52	nd[4]
(anti-DR1)	S: EM	2,11	35,49	1,3	w2,w5	52	1,nd
	R: MB	1	8	3	w2	52	nd
	S: NF	32	14,15	1	w5		1
	R: SM	11,24	13,15	w15	w6		12
	S: NF	32	14,15	1	w5		1
II	R: MB	1	8	3	w2	52	nd
(anti-DR4)	S: JRB	2,3	15,44	4	w8	53	4,14
	R: SM	11,24	13,15	w15	w6		12
	S: JRB	2,3	15,44	4	w8	53	4,14
III	R: NF	32	14,15	1	w5		1
(anti-DRw11)	S: JM	1,2	8	w11	7	52	5
	R: SM	11,24	13,15	w15	w6		12
	S: JM	1,2	8	w11	7	52	5
	R: MB	1	8	3	w2	52	nd
	S: JM	1,2	8	w11	7	52	5

[1]Group 1 combinations consisted of responders (R) primed against HLA-DR1 Dw1 stimulators (S) then assayed against HLA-DR1 Dw1 B-LCL or transfectants expressing HLA-DR1 Dw1. Similarly in Group 2 combinations responders were primed against HLA-DR4 Dw14 stimulators then assayed against HLA-DR4 Dw14 B-LCL or DR4 Dw14 transfectant targets. In group III combinations, responders were primed against DRw11 stimulators and were assayed against HLA-DRw11 Dw5 B-LCL or DRw11 Dw5 transfectant targets. All B-LCL targets were HLA Class I incompatible with stimulators.

[2]HLA types determined by serology and RFLP.

[3]ND=not determined

Table 4.5 High frequency of alloreactive CD4+ CTLp against murine L cell transfectants expressing HLA-DR gene products.

Group	Resp.	CTLp frequency (95% confidence limits)[1]			
		DR1 Dw1 B-LCL		**DR1 Dw1 Transfectant**	
I [2]	SF	1/7051	(5364-9268)	1/19795	(13386-29272)
	SM#	1/9454	(7021-12729)	<1/100,000	
	MB*	NT$		<1/100,000	
		DR4 Dw14 B-LCL		**DR4 Dw14 Transfectant**	
II [3]	MB*	1/1100	(846-1428)	1/22174	(12629-38932)
	SM#	1/11267	(8220-15443)	<1/100,000	
III[4]		**DRw11 Dw5 B-LCL**		**DRw11 Dw5 Transfectant**	
	NF	1/ 867	(674-1114)	1/1257	(984-1605)
	SM_a#	1/8886	(6637-11896)	1/7646	(5783-10110)
	SM_b	1/7673	(5802-10148)	1/9683	(6890-13602)
	MB_a*	1/2294	(2347-3820)	<1/100,000	
	MB_b	1/10406	(7656-14144)	1/55735	(29960-103686)

[1] All PBMC were depleted of CD8 and NKH1+ cells before setting up LDA cultures. Results are from single experiments except for SM and MB, where CTLp frequencies were determined in two separate experiments for each responder. SMb and MBb CTLp frequencies were obtained from the same experiment, using the same stimulator cells and the same targets for assay.

[2]SF anti-DR1 LDA cultures assayed against NF B-LCL (HLA Class II only, matched with Stimulator EM, see table 3). SM and MB anti-DR1 cultures were assayed against WT100BIS B-LCL (Class II only, matched with stimulator NF). All LDA cultures were assayed against .5-31 transfectant expressing HLA-DR1 Dw1 only.

[3]Target B-LCL was PE117 (matched only at HLA Class II with stimulators) and transfectant target was B20C (expresses HLA-DR4 Dw14 only).

[4]Target B-LCL was Sweig (matched only at HLA Class II with stimulator) and transfectant target was RGT1 (expresses HLA-DRw11 only).

$ NT=not tested.

Results obtained from the same experiment.

* Results obtained from the same experiment.

The fluorescence profiles of the transfectants and corresponding HLA-DR matched B-LCL are shown in Figure 4.10. Transfectants RGT1 (DRw11) and B20C (DR4) had levels of HLA-DR expression comparable to that seen on their corresponding B-LCL, whereas .5-31 (DR1) had a considerably reduced level of DR expression when compared to DR1 B-LCL.

All transfectants had been shown to express functional DR gene products, as all could stimulate alloreactive T cell clones (Lombardi et al. 1989a, Lombardi and Sidhu, unpublished observations), and antigen specific T cell clones (Lechler et al. 1988, Lamb, O'Hehir and Bal, unpublished observations). The HLA-DR1 transfectant, despite a relatively lower level of HLA-DR expression has been shown to stimulate alloreactive T cell clones with the same efficiency as DR1 B-LCL (Lombardi et al. 1989a), and has also been shown to be a potent stimulator in primary MLR responses (Lombardi et al. 1990).

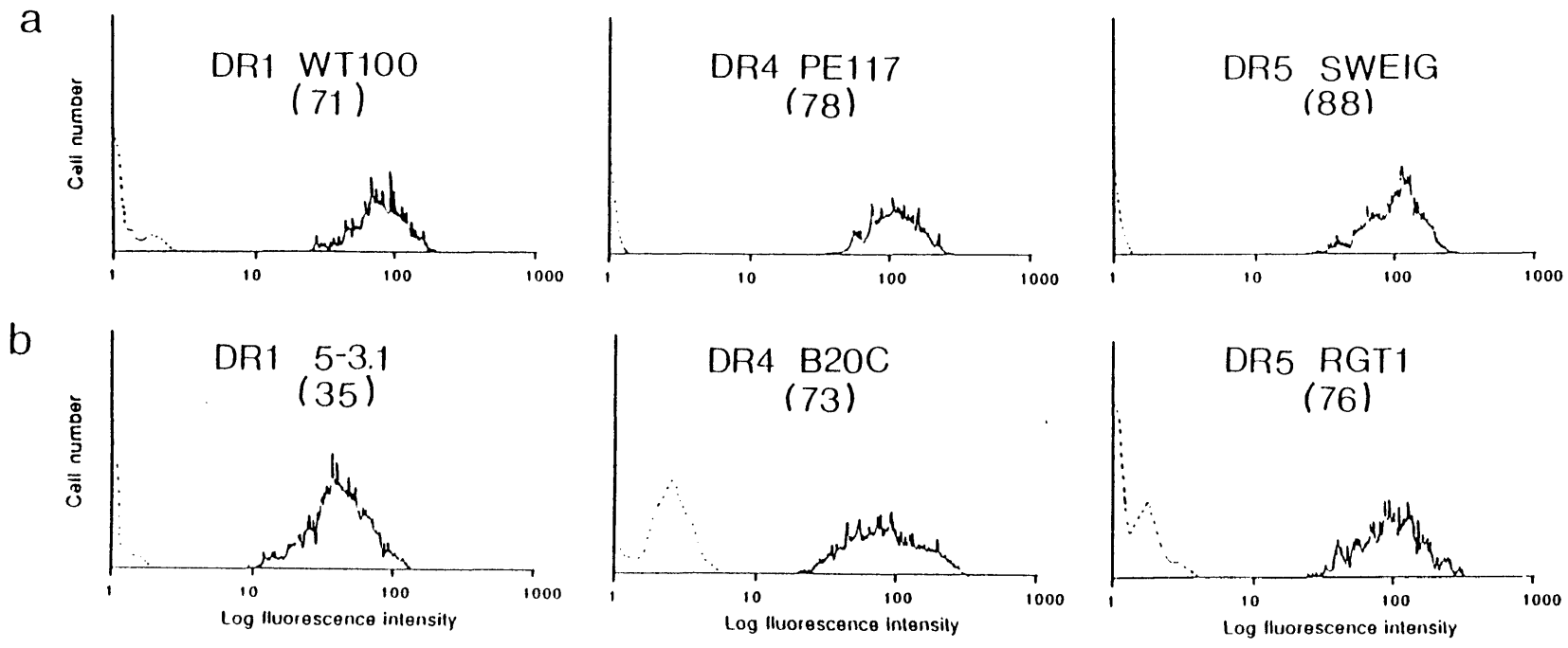


Figure 4.11. Expression of HLA-DR on B-lymphoblastoid cell lines and murine L cells transfected with HLA-DR genes. Cells were stained with anti-HLA-DR monoclonal antibody L243, and were analyzed by indirect immunofluorescence and flow cytometry. Fluorescence profiles are shown for (a) B-LCL and (b) murine L cells transfected with HLA-DR genes Dotted lines represent negative control of cells stained with second layer fluoresceinated antibody only, whilst solid lines represent cells stained with L243. Mean fluorescence intensity in brackets, provides a measure of cell surface expression on a per cell basis. Results were obtained in the same experiment, stable expression was observed for all cells between experiments.

4.4 Discussion

4.4.1. Experimental conditions.

In this study high frequencies of HLA class II-specific CTLp were found using a LDA system able to detect CD4+ve CTL. Successful detection of CD4+ve CTLp was achieved as a result of two modifications to previous LDA protocols. First, the conditions for functional maturation of CD4+ve CTL were optimised by immunomagnetic depletion of CD8+ve T cells. Second, the sensitivity of CTL detection was increased using B-LCL as targets for the CTL assay. B-LCL constitutively express high levels of HLA gene products (HLA class I and II) and adhesion molecules (LFA-3, ICAM-1), which may contribute to their sensitivity as targets for detecting alloreactive CTL (Kabelitz et al. 1987).

High frequencies of alloreactive CD4+ve CTLp were obtained in some allogeneic combinations in the current study and these were in contrast to previous studies (Moretta et al.1983, Kabelitz et al.1985) in which frequencies of alloreactive CD4+ve CTLp were reported to be several fold lower than alloreactive CTLp frequencies in unseparated T cell populations containing both CD4+ and CD8+ T cells. The discrepancy between previous studies and the current study may be explained by differences in experimental protocols. Both previous LDA studies used mitogen-activated T cells blasts as targets and these have been shown to be relatively insensitive targets for detecting HLA class II specific CTL (Albrechtsen et al. 1979, and Figure 3.13) In addition, only a small number of allogeneic combinations were used in these studies[1] and the current study demonstrates that there is considerable variation in alloreactive CTLp frequency (1/1016-1/38987), depending on the allogeneic combination used. It was unlikely that the observed variation in CTLp

[1] Three allogeneic combinations were tested by Moretta et al. (1983) and only one was tested by Kabelitz et al. (1985).

frequencies resulted from variation in assay conditions between experiments. The CTLp frequencies for two responder: stimulator combinations were found to be reproducible in three repeat experiments (Table 4.3). The possible reasons for variation in CTLp frequencies between different responder:stimulator combinations will be discussed in section 4.4.2.

The use of allogeneic B-LCL as CML targets to detect HLA class II specific CTL could result in detection of HLA-restricted EBV-specific CTL. These were unlikely to be present in the LDA cultures for two reasons. Firstly, the responders were primed against alloantigens using PBMC, thus avoiding the priming of any EBV-specific CTL. Secondly, Borgault et al. (1989) have demonstrated that even under optimal priming conditions the frequency of CD4+ve EBV-specific CTLp was thirty times lower than the frequency of CD8+ve EBV specific CTLp.

It is possible that allogeneic B-LCL may be lysed non-specifically by NK-like populations generated as a result of MLC (Seeley and Golub al.1978). NKH-1 is a marker expressed on the majority of NK cells and a subset of T cells (Lanier et al.1986), however depletion of NKH-1 positive cells from CD4-enriched PBMC abolished the lysis of Daudi but not K562 targets. These results might be explained by the heterogeneity of K562 killers in terms of cell surface markers (Lanier et al. 1987) and improved results may be obtained by depletion using a cocktail of antibodies against several NK markers. The additional depletion of NKH-1+ve cells from PBMC, after CD8 depletion, had no effect on the specific lysis of allogeneic B-LCL targets (Figure 4.4) and no significant differences in CD4+ve CTLp frequencies were seen when compared to populations depleted of CD8+ve cells only . These results suggest that distinct cell populations were responsible for the lysis of K562 cells (NK sensitive target), Daudi cells (LAK sensitive target) and allogeneic B-LCL targets. The existence of

separate effector populations has been suggested by studies utilizing cold target competition experiments which have demonstrated that K562 effectors are distinct from alloreactive CTL effectors (Seeley et al.1979). It is also likely that K562 and Daudi effectors are utilizing different lytic mechanisms to the alloreactive CTL generated after primary culture (Berke and Rosen, 1988).

Specific lysis of allogeneic B-LCL targets in this study (Figures 4.6, 4.8), and previous studies (Reinherz et al.1979, Gromo et al.1987) was seen to be generally lower in bulk lymphocyte cultures of CD4+ve T cells when compared to cultures of untreated PBMC or CD8+ve T cells. Moretta et al. (1983) suggested that such findings might be explained by the low frequency of CTLp among the CD4+ve T cell subset and the high frequencies of CTLp in the CD8+ve T cell subset. By performing limiting dilution assays on polyclonally activated CD4+ or CD8+ populations and assaying for non-specific, lectin-dependent cytotoxicity, Moretta et al. (1983) found that all the CD8+ve T cells were cytotoxic whereas a much lower proportion of CD4+ve T cells was cytotoxic, with frequencies of between 1/38-1/84 (2.6-1.2%) being obtained.

The recent demonstrations of phenotypic markers for CD4+ve CTL and CD8+ve CTL suggest that differences in cytotoxic response may be due to the different activation requirements of CD4 and CD8 CTL, rather than differences in CTLp frequency per se.

Morimoto et al. (1987) were able to divide human CD8+ve T cells iinto two subsets according to expression or non-expression of a marker designated S6F1. CTLp were found almost exclusively in the S6F1-ve population which constituted 42% of the total CD8+ve T cells population. In an analogous study Takada et al. (1989) demonstrated that absence of CD28 (leu 8) antigen correlated with cytotoxicity in the human CD4+ve T cell subset. CD28-ve T cells were found to make up 15-30% of the total T

cell population. If on average CD4+ve T cells constitute 70% of the total T cell pool then one might predict that between 11-21%# of all T cells would be CD4+ve CTLp compared to 18%* of all T cells being CD8 +ve CTLp. If such figures are representative, then the predominance of CD8+ve CTL after primary MLC may not be due to differences in precursor frequencies, but due to differences in activation requirements between the two subsets. As discussed in section 3.4.3, it should be possible to examine differences in activation requirements between T cell populations at the molecular level. Such studies will be important for examining the role of different T cell subsets in relation to allograft rejection and disease.

The limiting dilution system described in this study was shown to be specific for HLA class II antigens. The specificity CD4+ve CTL effectors generated in the limiting dilution cultures could be determined by assaying the contents of individual culture wells against a number of different target cells. For successful determination of CTLp frequencies in split well analysis experiments, it was found that reduced number of CTL targets/well had to be used (Figure 4.7). This was likely to be due to the reduction in the effector: target ratio when LD cultures were split into aliquots.

There was little evidence for the presence of CTL able to mediate lysis of autologous B-LCL. No CTLp frequencies against autologous B-LCL could be found in split well experiments, and the "autoreactive" CTLp frequencies obtained in other experiments (Table 4.3) were close to the limits of detectability. Similarly CTLp frequencies against third party B-LCL were also found to be low in this study (Figure 4.8, Figure 4.10). However it may be more suitable to use autologous B-LCL as a measure of

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% CTLp in CD4 subset x % CD4+ T cells in whole T cell population= 15% x 70% or 30%x70%

* % CTLp in CD8 subset x % CD8+ T cells in whole T cell population= 42% x 30%

specificity in limiting dilution cultures because it is difficult to obtain third party targets which do not share any HLA class II antigens with the stimulator. For example the LD cultures which were able to lyse both DR4 B-LCL and DR7 B-LCL (Figure 4.7), may be recognizing DRw53 which is common to both DR4 and DR7 haplotypes. In addition, apparently distinct HLA class II molecules may also share determinants which can be recognized by the same alloreactive T cells. The presence of such shared determinants has been suggested by the high frequency of cross-reactive TCC clones recognizing HLA-DR molecules (Eckels et al. 1989).

In conclusion, the limiting dilution assay decribed in this chapter was found to have a high degree of specificity and reproducibility and as such was suitable for investigating whether there was individual variation in CTLp frequencies against HLA class II antigens.

4.4.2. Individual variation in the cytotoxic T cell repertoire for allogeneic HLA class II antigens.

The primary aim in this study was to study the cytotoxic T cell repertoire to HLA class II antigens in unrelated individuals. Limiting dilution analysis is a powerful means of studying the T cell repertoire as the specificity of a large number of clonally derived T cells can be studied at the population level with minimum *in vitro* selection.

The frequency of alloreactive CTLp was determined in sixteen different responder:stimulator combinations and significant variation in CTLp frequency was found between individuals against the same set of allogeneic HLA class II molecules expressed on B-LCL, although these results were not confirmed in the same experiment. In addition individual variation in CTLp frequencies could be demonstrated against different allogeneic HLA class II antigens in the same experiment e.g. SF vs BR, SF vs JRB, SF vs MK and SF vs SM in Table 4.2. It is possible that

the variation in observed CTLp frequencies may result from variation in other parameters such as differential sensitivity of targets to lysis. Although this possibility was not investigated in the current study, it should be noted that the same B-LCL target cell could result in both low and high CTLp frequencies (MK and SF B-LCL in Table 4.2). The results obtained demonstrate that there was considerable variation in HLA class II specific CTLp frequency amongst the HLA mismatched combinations tested, and suggests that the cytotoxic T cell response against HLA class II antigens in some mismatched combinations will be weak or undetectable.

In order to examine the basis for high alloreactive CD4+ve CTLp frequencies it was important to define the specificity of the CTL generated in the LDA cultures. This was difficult to determine when using B-LCL as target cells as there will be at least 3 HLA class II molecules (DR, DQ and DP) expressed on a homozygous B-LCL and up to 8 class II molecules (DR, DQ, DP, DRw52 or DRw53) may be expressed in a heterozygous cell line. Further complexity may arise from the expression of hybrid class II molecules formed from isotyped-mismatched chains. For example hybrid DRα : DQß molecules have been shown to be expressed on B-LCL (Lotteau et al. 1987). Thus it was not possible to distinguish between high CTLp frequencies which were due to recognition of multiple class II antigens on B-LCL or high CTLp frequencies due to recognition of single HLA class II antigens.

In an attempt to examine the cytotoxic T cell response to HLA-DR antigens only, murine L cells transfectants expressing HLA-DR1 , DR4 or DR5 (DRw11) gene products were used as target cells. Limiting dilution cultures (CD8-ve, NKH1-ve) were stimulated with allogeneic PBMC and then assayed for cytotoxic activity using either B-LCL targets (matched for HLA class II antigens) or L cell targets (matched for HLA-DR only). All transfectants used had been shown to stimulate both alloreactive and

antigen-specific T cell clones. When transfectants were used as targets for the limiting dilution assays a large variation in the HLA class II specific CTLp frequencies were observed with CTLp frequencies ranging from 1/1257 to <1/100,000. The corresponding range of B-LCL frequencies obtained in the same experiments was 1/867-1/11267. In 2 out of 8 of the allogeneic combinations, the same high CTLp frequencies were obtained using both L cell transfectants and B-LCL. In contrast for 6 out of 8 combinations the CTLp frequencies detected were lower or undetectable with L cells compared to B-LCL. The variations in frequencies between the L cell transfectants could not be explained by variation in xenogeneic responses as the same responder was able to produce high and low CTLp frequencies, against DR5 and DR4 L cells respectively in the same experiment. No responses were seen against untransfected (DR-ve) murine L cells. In the small number of combinations tested high CTLp frequencies were produced only against DR5 + L cells. This was not due to a unique property of this transfectant as high and low CTLp frequencies could be produced against this transfectant in the same experiment.

The variation in CTLp frequencies obtained using the transfectants suggest that there is individual variation in the cytotoxic T cell repertoire against HLA class II antigens. The low CTLp frequencies obtained using L cell transfectants might be explained in three possible ways:

1. They represent a genuine variation in DR-specific CTLp frequency. The high frequencies obtained using the B-LCL may represent the composite CTLp frequency against HLA-DR, HLA-DQ , HLA-DP, HLA-DRw52, HLA-DRw53 etc.,

2. The majority of CTL generated after primary MLC are DR specific but are unable to recognize DR molecules expressed on xenogeneic cells. Thus the perceived decrease in CTLp frequency is actually due to a defect of recognition associated with the DR+ L cells, rather than actual decrease in

CTLp frequency per se. The short culture period (7 hours) required for CTL assays make it likely that the majority of HLA class II molecules expressed on a transfectants will be associated with peptides of murine lineage. Lombardi et al. (1989a) have suggested that the majority of cytotoxic T cell clones able to recognize DR1+ve L cells in proliferation assays, are unable to do so in CML assays, because of a requirement for processed human proteins. Thus CTL able to recognize DR+ve murine L cell transfectants may either be recognizing allogeneic determinants not affected by xenogeneic peptides, or recognizing MHC-bound peptides conserved between species.

3. The majority of alloreactive CTL generated are of low affinity unable to react to allogeneic MHC molecules expressed on xenogeneic cell types because of the absence of human adhesion molecules.

In order to determine the specificity of limiting dilution cultures split well analysis would be required. The contents of split culture wells would have to be assayed against multiple targets, each sharing only a single HLA class II specificity in common with the stimulator. If the predominant specificity is for DR antigens one should see a good correlation between the stimulator B-LCL and DR matched targets, and no correlation between the stimulator B-LCL and either DQ matched or DP matched targets. Such an approach will be difficult in practice as it will require transfectant targets expressing the same HLA-DR, HLA-DQ or HLA-DP antigens as the stimulator B-LCL.

The latter two hypotheses could account for the low CTLp frequencies obtained in 6/8 allogeneic combinations, when using L cell transfectants. However, to account for high CTLp frequencies one would have to predict that in any particular individual , the response against certain HLA class II antigens is not cell lineage specific, or that CTL have a higher affinity for some allogeneic HLA class II molecules than others.

It is conceivable that selection of the T cell repertoire could account for any one of the possible explanations above, perhaps negative selection in the thymus can delete T cells which can cross-react on certain allogeneic DR molecules, leading to a "hole" in the T cell repertoire. Or perhaps positive selection for self-reactive T cells also selects cells which cross-react with high frequency and high affinity on certain allogeneic MHC molecules, overcoming requirements for species-specific adhesion molecules or species-specific peptides. Before such speculation can be put to the test, an increased number of responder:stimulator combinations need to be tested. For example CTLp frequencies against DR4 could be determined in a large panel of individuals of the same HLA type. The results of such experiments will determine whether there is any correlation between CTLp frequency and the responder:stimulator combinations used.

Lechler and colleagues (1990) have recently suggested that alloreactive T cells can recognize MHC molecules by two mechanisms which are dependent on the structural features of responder and stimulator MHC molecules. It was suggested that allogeneic combinations could be divided into two categories on the basis of similarity or dissimilarity between MHC molecules, especially at amino acid residues thought to form T cell receptor contact sites (Lombardi et al. 1989b, Brown et al. 1988).. Thus combinations of DR molecules can be split into two categories as seen in Figure 4.12. In the first category (Figure 4.12a), the DR sequences have identity at T cell contact residues , whereas in the second category (Figure 4.12b) the DR sequences are dissimilar. For category 1 combinations, recognition of allogeneic molecules may occur in a manner similar to self-restricted recognition of antigen (Lombardi et al. 1989b). Differences between self-MHC and allogeneic MHC in the amino acids forming the ß-pleated sheet of the antigen binding groove may result in a

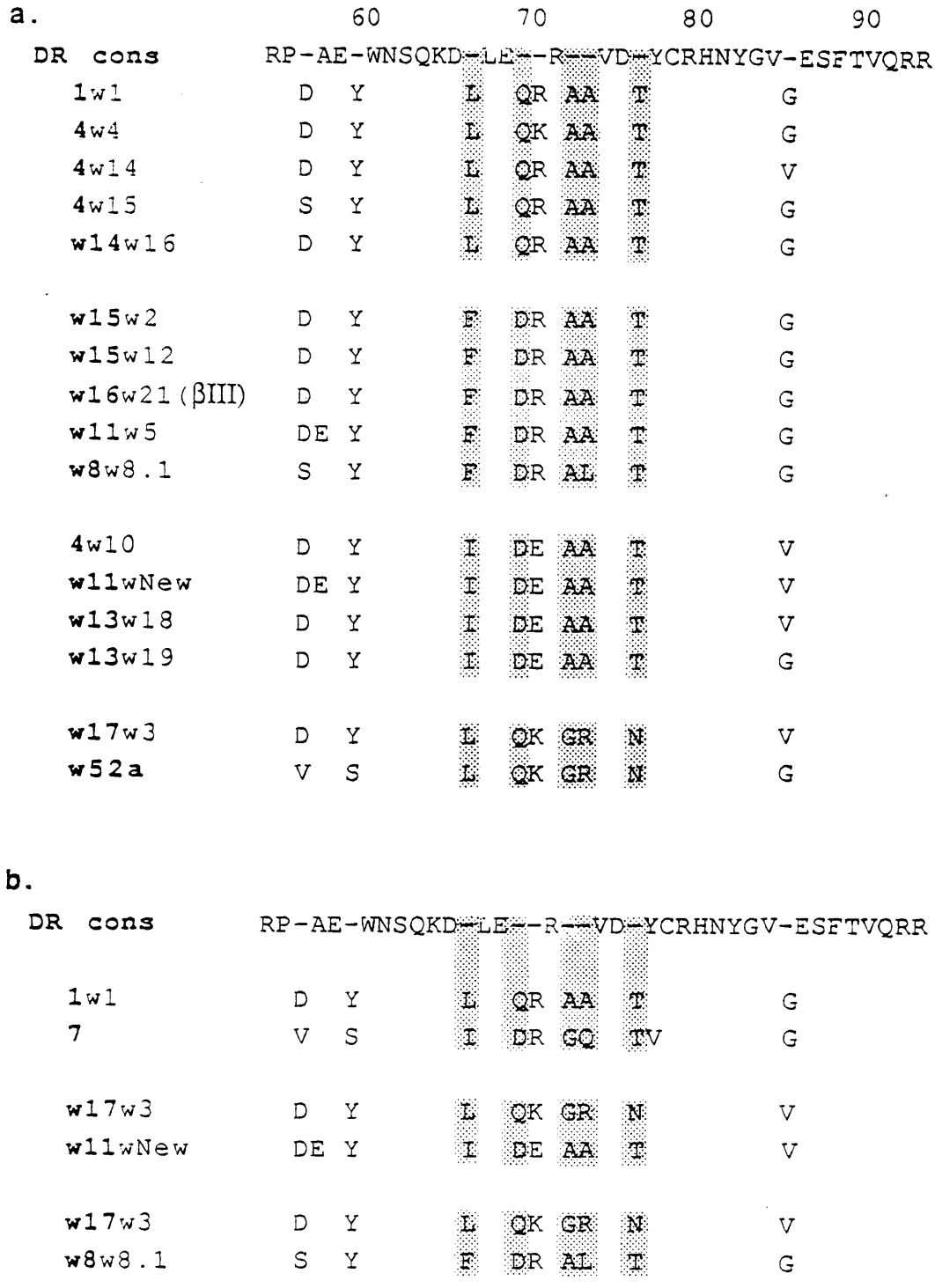


Figure 4.12. Allogeneic combinations can be divided into two categories on the basis of DR beta chain amino acid sequence (reproduced with permission, from Lechler et al. 1990). Amino acid sequences from carboxy-terminal half of multiple DRß1 domains are presented. Only residues differing from the DRß concensus sequence are shown. In category (a) , all the combinations shown share amino acid residues at positions which are thought to form T cell contact sites (boxed). In category (b) examples of allogeneic combinations are shown, which differ in their DRß1 domains at the postulated T cell contact positions (boxed).

different set of self-peptides binding to allogeneic MHC molecules. Thus, such complexes would be seen as novel determinants by self-restricted T cells.

In category 2 combinations where there is dissimilarity between the MHC molecules, alloreactivity will be due to T cells cross-reacting on allogeneic MHC molecules. These may be T cells which have escaped negative selection because of low affinity for self-MHC and may by chance, have greater affinity for allogeneic MHC molecules. In such cases the specificity of the T cell is predicted to be primarily for the MHC molecule and not for any MHC-bound peptides.

One prediction from this hypothesis is that allogeneic combinations in which the responder:stimulator DR molecules are similar to each other (category 1), will produce the highest precursor T cell frequencies. This may result from the activation of many self-MHC restricted T cells which recognize complexes of allogeneic MHC and peptide with high affinity.

Thus the variation in HLA class II specific CTLp frequencies demonstrated in this chapter might be determined by the HLA class II molecules expressed by the responder and stimulator. The use of murine L cell transfectants as target cells for LDA cultures, offers the means by which this proposal can be tested, as CTLp frequencies against individual DR antigens can be determined. The experiments performed with the transfectants were too few in number for any definite conclusions to be drawn. Only one category 1 combination was tested, whereas seven category 2 combinations were tested. The category 1 combination produced a high CTLp frequency , whereas for 6 out of 7 of the category 2 combinations, low or undetectable CTLp frequencies were obtained. A larger number of combinations need to be tested before these results can be interpreted.

4.5 Conclusions.

In conclusion the studies in this chapter have shown that there is extensive variation in the cytotoxic T cell response to HLA class II antigens among unrelated individuals. These results suggest that there is individual variation in the cytotoxic T cell repertoire to HLA class II antigens.

Chapter 5.

General discussion.

The results of chapter 3 demonstrate the complexity of allogeneic determinants recognized by a small panel of alloreactive T cell clones. Two T cell clones defined novel polymorphisms associated with HLA-DR2, which were not detected previously by serological or polyclonal cell populations. The use of T cell clones as HLA typing reagents has however, usually been limited to the laboratory of origin. The fragile nature of T cell clones, and the expertise required to generate and maintain them, have prevented widespread use. It may be possible to overcome these limitations by immortalizing human T cell clones either through infection with T cell specific viruses (Green et al. 1989), or by fusing T cells with tumour cell lines to generate T cell hybridomas (Kappler et al. 1981).

The polymorphic determinants recognized by individual alloreactive T cell clones was shown to depend on other factors besides polymorphism in the primary structure of HLA class II molecules. Alloreactive T cell clones could discriminate between identical HLA class II molecules on different cell types or associated with different HLA haplotypes. These patterns of reactivity are consistent with a model of allogeneic MHC recognition involving MHC-bound peptides. It is not clear however, how peptides may influence allorecognition. There are two main possibilities:

1. The primary specificity of alloreactive T cells is for conformational determinants on the MHC molecule which are dependent on the type of peptide bound. This is a modified version of the high determinant density hypothesis for alloreactivity , first proposed by Bevan (1984).

2. The specificity of alloreactive T cell clones is analogous to T cell recognition of antigens in that there is specificity for both peptide and MHC as first proposed by Matzinger and Bevan (1977).

Two recent studies support the notion that peptides play an important role in determining the three dimensional structure of MHC molecules.

Townsend et al. (1989) demonstrated that a mutant murine cell line with a low level of MHC class I (D^b) molecules could be induced to express D^b at levels recognized by CTL, if the cell lines were pulsed with high doses of a nucleoprotein peptide known to bind to D^b. They suggested that assembly, transport to the cell surface and the 'native" conformation of MHC class I molecules was influenced by bound peptide.

Mellins et al (1989) have suggested that peptides may influence the conformation of HLA-DR molecules. They studied B-LCL mutants which failed to present whole Tetanus Toxoid (TT) antigen to DR3 restricted T cell clones but were able to present peptides of TT to the same T cell clones. High levels of class II expression could be detected on the mutants using HLA-DR specific (monomorphic) monoclonal antibodies, however the mutants were unable to bind HLA-DR3 specific monoclonal antibodies. This was not due to mutation in the DR3 gene itself as identical nucleotide sequences were obtained for DR3 genes isolated from wild type and mutant B-LCL. In contrast to wild type DR3 molecules, DR3 molecules isolated from mutant B-LCL, were unstable and dissociated readily under reducing conditions.

These results suggest that the mutant DR3+ve B-LCL were unable to generate peptides which stabilized the class II molecule and contributed to conformational determinants recognized by antibodies. It might be predicted that such mutant B-LCL express "naked" MHC molecules free of endogenous peptides. Interestingly, Pious, Mellins and colleagues (1989[§]) have reported the preliminary finding that 3 out of 4 DR3-specific alloreactive T cell clones failed to react to the mutant B-LCL, whilst all were able to react to wild type DR3+ B-LCL. This raises the possibility that

[§] Reported at 7th International Congress of Immunology, July 1989, Berlin.

some alloreactive T cell clones may recognize MHC determinants not dependent on the presence of bound peptide.

T cell recognition of conformational MHC determinants which depend on bound peptide could account for cell lineage specific recognition patterns (Marrack and Kappler 1987) and the ability of peptides to inhibit or enhance alloreactive T cell responses (Eckels et al. 1989). Such a hypothesis may explain why in some cases, the allogeneic determinants recognized by alloreactive TCC cannot be deduced from the linear amino acid acid sequences of HLA class II molecules (as shown in chapter 3).

The main argument against alloreactive T cells recognizing conformation determinants on the MHC molecule, is that such a concept contradicts the dogma of antigen specific recognition, namely that T cell receptors have specificity for both antigenic peptide and MHC. Thus one might predict that any given alloreactive T cell clone will have specificity for an unknown peptide plus allogeneic MHC. The evidence that alloreactive T cells have specificity for peptides bound to allogeneic MHC is mostly indirect and is based on cell lineage specific patterns of recognition and the loss of recognition due to amino acid substitutions in the peptide binding site. As discussed above these results are subject to an alternative interpretation.

Two studies have demonstrated that some alloreactive T cell clones can recognize MHC molecules expressed in their native form on allogeneic cells, or peptides derived from the same MHC molecule but presented by autologous antigen presenting cells (Clayberger et al. 1987, De Koster et al. 1989). One interpretation of these studies is that the specificity of the alloreactive T cell clones is determined by peptides of HLA molecules. However the allogeneic peptide plus self MHC complex could equally well be mimicking conformational determinants normally recognized on the native allogeneic molecule.

So far there has been no direct demonstration that the specificity of human alloreactive TCC is determined by peptides derived from proteins other than HLA peptides. It may be difficult in practice to identify specific peptides recognized by alloreactive T cell clones from the array of endogenous peptides which are bound to HLA molecules.

Determining how alloreactive T cells recognize allogeneic HLA class II molecules will depend on defining the precise interaction between T cell receptors and HLA class II molecules. Studies of the crystal structure of HLA-DR1 are in progress and are likely to provide insights into how HLA class II structure can contribute to this interaction. Subtle differences have already been detected between the crystal structures of two HLA class I molecules, A2 and Aw68 (Bjorkman et al. 1987, Garrett et al. 1989). Such differences are more likely to occur between different HLA class II molecules and it may be difficult to propose a general model for T cell recognition of HLA class II molecules. For instance DRα chains are invariant, whereas DQα and DPα are polymorphic. Alloreactive T cells which are specific for DR may be predominantly recognizing determinants on the DRß chain, whereas DQ specific T cells may be recognizing determinants contributed by both DQα and DQß chains. Thus the T cell receptors of DR specific and DQ specific T cell clones may be binding to different parts of the HLA class II molecule.

The study of alloreactive T cell clones allows definition of the specificity of HLA class II specific T cell recognition at the clonal level, however such populations may not offer a true reflection of the T cell repertoire for HLA class II antigens as they represent an *in vitro* selected population.

In chapter 4, limiting dilution analysis was used to study the cytotoxic T cell response to HLA class II antigens at the population level by determining the frequency of CTLp. Variation in HLA class II specific

CTLp frequency was demonstrated against either complete HLA class II haplotypes expressed on B-LCL, or against single HLA-DR antigens expressed on murine L cell transfectants. Although variation in human HLA Class I specific CTLp frequencies have been observed in previous studies (Sharrock et al. 1987, Kaminski et al. 1988, Zhang et al. 1989), it is not clear how the variation is generated. There are two possibilities:

1. The cytotoxic T cell repertoire against allogeneic MHC molecules is under genetic control. As described in detail in section 1.8.2, such an effect has been shown for allogeneic MHC class I antigens using inbred mice (Sherman 1982, Gress et al. 1986). In contrast, the studies of Zhang et al (1989) have suggested that human cytotoxic T cell responses against MHC class I antigens are not under genetic control. They demonstrated significant differences in alloreactive CTLp frequencies between 3 out of 4 pairs of monozygotic twins when assayed against the same set of HLA class I antigens. It remains to be seen whether the same is true for cytotoxic T cell responses against HLA class II antigens.

2. Environmental antigens could influence alloreactive precursor frequencies by continually priming and expanding antigen specific populations of T cells during an individual's life time. Thus the vigorous nature of alloreactivity may be explained by the responses of primed antigen specific "memory cells " which are able to cross-react on allogeneic MHC molecules. Studies in mice (MacDonald et al. 1980) and human (Vanderkerckoeff et al. in press) have shown that *in vivo* priming with alloantigens can increase CTLp frequencies, however a number of studies have shown that priming of antigen-specific T cells is unlikely to contribute to increasing alloreactive T cell responses. For example Wilson and Fox (1971) demonstrated that there was no difference between the number of proliferating T cells in MLR when obtained from normal rats or from rats kept under germ-free conditions.

A stronger argument against the role of environmental antigens influencing alloreactive T cell precursor frequency is the demonstration by Merckenschlager et al. (1988) that there is no difference in the frequency of alloreactive T cells between phenotypically defined "memory" populations (CD45R-ve) and "naive" T cell populations (CD45R+ve). These studies suggest that around half of the alloreactive T cells which proliferate in response to HLA class II molecules have been primed. In light of the results of Zhang et al. (1989, see above) it would be interesting to determine whether the findings of Merckenschalger et al. could also be repeated for alloreactive cytotoxic T cells.

The question of how variation in alloreactive CTLp frequencies is generated remains unresolved. The results obtained by Zhang et al. (1989) if confirmed, argue against genetic control of T cell responses against alloantigens, however there is a lack of convincing evidence for any environmental influences on alloreactive T cell responses. It is possible that variation in alloreactive T cell responses between individuals may reflect unique patterns of reactivity influenced by a combination of both environmental and genetic factors.

As discussed in section 4.4.2. the variation in CTLp frequencies against single HLA class II antigens may be explained by structural features of HLA-DR molecules, and is thus determined by the responder: stimulator combinations used (Lechler et al. 1990). The results obtained in chapter 4 do not exclude this possibility, however it might be difficult to test these proposals in outbred human populations. The complex patterns of TCC reactivity obtained in chapter 3 suggest that other genes in addition to HLA class II genes will influence alloreactive T cell responses.

One of the driving forces behind studies on the nature of alloreactivity is the prospect of preventing rejection of allogeneic tissue in clinical transplantation. In chapter 4, high frequencies of alloreactive CD4+ve

CTLp were demonstrated, raising the possibility that such cells may be involved in graft rejection, especially in cases where there is matching for HLA class I antigens. A recent study has demonstrated that high CTLp frequencies in HLA matched unrelated donors could predict the severity of GVHD (Kaminski et al. 1989), and that the high CTLp frequencies correlated with the degree of HLA-mismatch. In chapter 4, low and high HLA class II specific CTLp frequencies could be obtained in HLA-mismatched combinations. These results taken together with demonstrations of variation in HLA class I CTLp frequencies (Sharrock et al. 1987, Zhang et al. 1989), suggest the possibility that some HLA mismatches may be less harmful than others.

The patterns of specificity demonstrated in Chapter 3, with only a small panel of alloreactive TCCs, suggest that there are likely to be multiple ways in which T cells can recognize HLA class II antigens. The demonstration of variation in HLA class II specific CTLp frequencies suggests that patterns of T cell specificity may differ between individuals. The future challenge will be to find out whether the effector cells mediating graft rejection have predominant patterns of T cell specificity, and whether the activation of these cells can be prevented *in vivo*.

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Appendix

Table 6.1 HLA types of PBMC panel.

PBMC Donor (ethnic origin)[1]	HLA TYPE[2]						
	A	B	C	Dw	DR	DQ	DRw52/ DRw53
NF (c)	32	14,15		1	1,1	w5,w5	
GW (c)	1,31	8,51	5	1,ND	1,w17	w5,w2	52
SSR (c)	1,3	17,27		1,ND	1,7	w5,w2	53
EM (c)	2,11	35,40		1,ND	1,w17	w5,w2	52
PB (c)	3,11	7,35		2,1	w15,1	w5,w6	
SM (o)	11,24	13,15	3	12	w15	w6	
ML (c)	2,3	7,44		2,4	w15,4	w6,w7	53
RIL (c)	2,3	27,44	5	2,4	w15,4	w6,w7	53
DPR (n)	2	44	4	2,ND	w15,w13	w6	52
MR (c)	3,25	7,8		2	w15	w6	
RH (c)	1,3	7,40		2,ND	w15,7	w6, w2	53
SF (c)	1,29	7,44		3	w17, w17	w2	52
CG (c)	2,24	18,44	3	ND	4	w8	53
TR (c)	2,3	15	3	ND	4,w11	w8, ND	53
SS (ac)	3	27,35		DKT2, ND	4,w11	w2,w8	53
ND (c)	2	15,44	3,5	4,18	4,w13	w8,w6	53
GL (c)	2	12,18		ND	w11,w11	w7,w7	52
JM (c)	1,2	8,18		5	w11,-	w7,-	52
GK (c)	10,28	14,16		ND	w11,6	w7,w6	52
EK (c)	9,29	12,21		ND	w11,7	w7,w2	52,53
RG (c)	2	5,40		ND	w11,w8	w7,w4	52
TD (c)	1,2	35	4	ND	w13	w5	52
YK (o)	24,30	7,44		ND	w13,1	w6,w5	52
SK (o)	11	40,44		ND	w13	w6	52
MK (c)	1,9	12,17		ND	7,7	w2	53

[1]Ethnic origin; C= Caucasian, O=Oriental, AC=Asian Caucasian, N=Negroid.
[2]Determined by serological typing and RFLP typing.

Table 6.2. HLA types of all B-LCL used.

B-LCL	HLA TYPE							
	A	B	C	Dw	DR	DQ	DP	DRw52/53
NF	32	14,15		1	1	w5		
SA*	24	7	7	1	1	w5	4	
WT100BIS*	11	35	4	1	1	w5	1	
GW	1,31	8,51			1,3	w5,w2		52
PB	3,11	7,35		1,2	1,w15	w5,w6		
PGF	3	7		2	w15	w6		
DO208915*	25	18		2	w15	w6	2,4	
MGAR*	26	8	7	2	w15	w6	4	
DAF	2,31	35,44		2,5	w15, w11	w2,w6		
ML	2,3	7,44		2,4	w15,4	w6,8		
DP	2,24	7,40		2	w15	w6		
MR	3,25	7,8		2	w15	w6		
SCHU*	3	7	7	2	w15	w6		
AKIBA	24	52		12	w15	w6		
E4181324*	1	52		12	w15	w6	2,4	
SM	11,24	13,15		12	w15	w6		
AZH	24	56		21	w16	w5		
MN-2	2	7		21,4	w16, 4	w5,w8		
WT18	2	27		21,1	w16	w5		
RML*	2	51		22,1	w16	w7		
SF	1,29	7,12			3	w2		52
MB	1	8			3	w2		52
SD	1,11	8,35			3,4	w2,8		52,53
BOLETH*	2	15	10	4	4	w8	4	53
YAR*	26	38		10	4	w8	4	53
PE117*	24	60	10	14	4	w8	4	53
JRB	2,3	15,44		4,14	4	w8		53
JHAF*	31	51		13	4	w7	3	53
KT2					4	w8		5
SID	3	27,35		KT2, 5	4,w11	w8		53
SWEIG*	29	61	2	5	w11	w7		52
BM21*	1	41		5	w11	w7		52
BM16*	2	18	7	5	w12	w7	2	52
JM	1,2	8		5	w11	w7		52
JVM*	2	18	5	NEW	w11	w7	2	52
TISI*	24	35	4	NEW	w11	w7		52
HOR*	33	44		19	w13	w6	4	52
TD	1,2	35	4		w13	w5		52
FR	2,9	13,40			6	w5		52
ABO*	2	18	7	9	w14	w5	4	52
AMALA*	2	62	9	16	w14	w7		52
CBG-IB*	1	62	9	18	w13	w5		52
MK	1,9	12,17			7	w2		53
MOU*	29	44		17	7	w2	2	53
LBUF*	30	13	6	DB1	7	w2		53
LUY*	2	51		8.3	8	w7	1,4	52
OLGA*	31	62	1,10	8.2	8	w4	3	52
TAB*	2	46	11	8.3	8	w6		52
BTB*	2	27	1	8.1	8	w4	4	52

* B-LCL from Xth histocompatibility workshop panel. (Flomenberg et al. 1989)

Table 6.3. Reactivity of alloreactive T cell clones on PBMC panels.

Stimulator (ethnic origin)[3]	HLA TYPE=				Reactivity[4]			
	DR	DQ	DRw52/ DRw53	Dw	9A10	9F6	7D5	TCL
NF (c)	1,1	w5,w5		1	1	1	ND#	1
GW (c)	1,w17	w5,w2	52	1,3	2	1	1	22
PB (c)	w15,1	w5,w6		2,1	1	**200**	**100**	**100**
SM (o)*	w15	w6		12	**100**	**100**	**100**	**100**
ML (c)	w15,4	w6,w8	53	2,4	1	**114**	**120**	**78**
RIL (c)	w15,4	w6,w8	53	2,4	1	8	1	**82**
DP (n)	w15,w13	w5	52	2, ND	6	7	15	**55**
RH (c)	w15,7	w6, w2	53	2, ND	1	**166**	**100**	**84**
SF (c)	w17, w17	w2	52	3	1	ND	ND	4
CG (c)	4	w8	53	ND	1	5	2	9
TR (c)	4,w11	w8	53	ND	3	12	1	3
SS (ac)	4,w11	w2,w8	53	DKT2,ND	1	**46**	3	20
ND (c)	4,w13	w8,w6	53	4,18	1	12	3	15
GL (c)	w11,w11	w7,w7	52	5	1	9	ND	6
JM (c)	w11	w7	52	5	1	16	1	**30**
GK (c)	w11,6	w7,w6	52	5, ND	**45**	14	6	**24**
EK (c)	w11,7	w7,w2	52,53	ND	6	6	4	**26**
RG (c)	w11,w8	w7,w4	52	5, 8.1	5	1	2	18
YK (o)	w13,1	w6,w5	52	ND	1	1	1	5
SK (o)	w13	w6	52	ND	1	1	1	2
MK (c)	7,7	w2	53	ND	1	1	1	**23**

[3]Ethnic origin; C= Caucasian, O=Oriental, AC=Asian Caucasian, N=Negroid.

[4]Data is pooled from several experiments. Positive reactivity (in bold type) is determined on the basis of % relative response of positive control (see appendix). Positive reactivity was scored if %RR for any stimulator cell was > negative control (ND, TR or CG) in three repeat experiments. The maximum %RR obtained for the negative controls was 22%. ++ indicates %RR>50%, + indicates %RR>22% but <50%.

ND =not determined.

* Donor of stimulator PBMC for all T cell clones.

NB. T cell clone 9A10 was negative on a further 4 DRw15 Dw2 PBMC and 1 DRw16 Dw21 PBMC.in one experiment.

Table 6.4. Reactivity of alloreactive T cell clones on B-LCL panels.

B-LCL	HLA TYPE			%RR[5]		
	DR	DQ	Dw	9F6	18C2	4C3
NF	1	w5	1	1	NT	NT
SA	1	w5	1	1	1	1
WT100BIS	1	w5	1	1	1	5
PGF	w15	w6	2	**95**	**72**	**124**
DO208915	w15	w6	2	22	**93**	**78**
MGAR	w15	w6	2	17	**115**	**132**
DAF	w15, w11	w6	2,5	21	**62**	**100**
SCHU	w15	w6	2	**100**	**162**	**113**
AKIBA	w15	w6	12	**108**	**138**	**145**
E4181324	w15	w6	12	**120**	**113**	**100**
SM	w15	w6	12	**100**	**100**	**100**
AZH	w16	w5	21	19	26	19
MN-2	w16, 4	w5,w8	21,4	19	13	1
WT18	w16	w5	21,1	20	20	15
RML	w16	w7	22,1	3	7	1
SF	3	w2		1	3	2
BOLETH	4	w8	4	26	8	1
YAR	4	w8	10	15	8	5
PE117	4	w8	14	21	9	5
JRB	4	w8	4,14	28	26	14
JHAF	4	w7	13	13	1	1
KT2	4	w8	KT2	1	NT	NT
SID	4,w11	w8	KT2, 5	**161**	19	NT
SWEIG	w11	w7	5	**190**	3	19
BM21	w11	w7	5	**172**	9	11
BM16	w11	w7	5	**198**	1	1
JVM	w11	w7	NEW	24	15	NT
TISI	w11	w7	NEW	24	12	NT
HOR	w13	w6	19	29	8	4
ABO	w14	w5	9	27	3	1
AMALA	w14	w7	16	28	6	NT
CBG-IB	w13	w5	18	18	10	1
MOU	7	w2	17	24	24	1
LBUF	7	w2	DB1	1	15	1
LUY	8	w7	8.3	1	3	6
OLGA	8	w4	8.2	1	6	9
TAB	8	w6	8.3	1	3	1
BTB	8	w4	8.1	12	5	8

[5]%RR=% relative response. to SM B-LCL. Calculated from the following formula:
%RR= {(Δ CPM of T cell clone + Stim B-LCL) / (Δ CPM of T cell clone+SM B-LCL).} x 100.
Δ CPM = CPM of (T cell clone + B-LCL) minus background CPM.
Positive responses were scored for B-LCL if %RR exceeded the %RR obtained for JRB B-LCL in three repeat experiments. %RR for JRB varied between 1 and 28 (n=16). The results shown are representative of three repeat experiments for each T cell clone: stimulator combination.

Key for Appendix Table 6.5 for LDA.data

Frequency 1 (freq 1) was obtained by maximum liklihood method. The χ^2 value indicates whether the experimental data was consistent with single hit kinetics. P1>0.05 suggests that the data was a good fit to a straight line through the origin (Y=1.0). Frequency 2 (Freq2) was extrapolated from the straight line fitted to the experimental data (linear regression). The correlation coefficient ,r provides a measure of how well the experimental data fits to the straight line (P2<0.05 for linearity). The Y axis intercept for this fitted line is shown in the last column. ND= not detectable (<1/100,000).

Table 4.2 Alloreactive CD4+ CTLp

Resp	Stim	Target	Freq1	Range	χ^2	P1	Freq 2	r	P2	Intercept
NF	TD	TD	1/1016	778-1827	7.9	<0.05	1/2338	0.957	<0.05	0.86
NF	JRB	JRB	1/3635	2906-4544	69.7	<0..05	1/3597	0.83	<0.05	1.6
NF	SF	SF	1/3902	3031-5023	2.9	>0.05	1/5161	0.99	<0.001	1.02
NF	MK	MK	1/34837	20003-60588	41	<0.05	1/91396	0.985	<0.01	1.01
NF	NF	NF	1/75008	33805-100132	3.11	>0.05	1/48188	0.97	<0.05	1.04
MB	JRB	JRB	1/1475	1124-1936	5.43	>0.05	1/1279	0.98	<0.05	1.3
MB	JRB	JRB	1/1262	988-1612	39.7	<0.05	1/1029	0.973	<0.01	0.59
MB	JRB	JRB	1/2611	2054-3319	14.6	<0.05	1/2492	0.89	<0.05	0.81
MB	JRB	JRB	1/1096	856-1403	4.37	>0.05	1/1024	0.97	<0.05	1.03
MB	MK	MK	1/4951	3828-6402	17.3	<0.05	1/2084	0.971	<0.05	1.36
MB	SF	SF	1/26778	16389-43754	3.85	>0.05	1/33291	0.938	<0.01	0.999
MB	SM	SM	1/38987	23038-65976	3.09	>0.05	1/44853	0.98	<0.001	0.989
SF	NF	NF	1/1880	1461-2420	4.19	>0.05	1/1832	0.99	<0.01	1.34
SF	NF	NF	1/701	534-920	9.35	>0.05	1/600	0.989	<0.05	0.98
SF	NF	NF	1/1430	1118-1829	5.62	>0.05	1/1300	0.96	<0..05	1.37
SF	NF	NF	1/2273	1676-2420	2.12	>0.05	1/2002	0.99	<0.001	1.1
SF	BR	BR	1/3064	2379-3945	10.5	<0.05	1/2327	0.99	<0.01	1.18
SF	JRB	JRB	1/5989	4605-7789	3.78	>0.05	1/6440	0.99	<0.001	1.02
SF	JM	JM	1/8340	6198-11221	23.4	<0.05	1/7158	0.9	<0.05	0.94
SF	MK	MK	1/12174	8805-16833	2.07	>0.05	1/12845	0.93	<0.01	0.96
SF	SM	SM	1/39587	19659-79715	3.15	>0.05	1/21559	0.87	<0.05	0.9
SF	SF	SF	1/93702	42037-208867	8.59	>0.05	1/46837	0.893	<0.05	1.01
SD	NF	NF	1/2182	1716-2774	19.5	<0.05	1/1832	0.971	<0.001	1.34
JM	MR	MR	1/38949	23038-65976	21.4	<0.05	1/100000	0.908	<0.01	1.02
JM	MR	JRB	ND							

continued.....

Results for Table 4.3

Resp	Stim	Target	Freq1	Range	χ^2	P1	Freq 2	r	P2	Intercept
MB	SM	SM	1/2006	1562-2577	16.7	<0.05	1/2400	0.8	<0.01	0.706
MB	SF	SF	1/2098	1650-2667	11.8	>0.05	1/2291	0.915	<0.05	1.14
MB	GW	GW	1/2623	2059-3342	10.8	>0.05	1/2662	0.95	<0.05	1.06
MB	JRB	JRB	1/2672	2099-3041	16.9	<0.05	1/2672	0.95	<0.05	1.23
MB	SD	SD	1/6296	4724-8360	3.48	>0.05	1/8799	0.98	<0.001	1.047
SF	NF	NF	1/1222	937-1592	8.01	>0.05	1/1446	0.985	<0.05	0.899
SF	MK	MK	1/2611	2054-3319	14.2	<0.05	1/3049	0.937	<0.05	1.18
SF	MB	MB	1/5141	3984-6634	8.11	>0.05	1/4141	0.99	<0.001	0.99
JM	MR	MR	1/4815	3736-6205	8.11	>0.05	1/55841	0.986	<0.05	1.16
JM	MR	JRB	1/50901	28077-92281	24.9	<0.05	1/55841	0.96	<0.05	1.01

Results for Figure 4.8

MB	JRB	PE117	1/4973	3815-6482	25	<0.05	1/9387	0.83	<0.05	0.79
MB	JRB	LBUF	1/40535	22387-73395	6.7	>0.05	1/91022	0.92	<0.05	1.01
MB	JRB	MB	ND							

Results for Figure 4.9

MB CD4+ (FACS)	JRB	JRB	1/3507	2751-4470	16.19	<0.05	1/2461	0.96	<0.01	1.53
MB CD4+ (CD8 depl)	JRB	JRB	1/2611	2054-3319	14.6	<0.05	1/2492	0.88	<0.05	0.81
MB CD4- (FACS)	JRB	JRB	1065	825-1375	8.75	>0.05	1/1006	0.987	<0.01	1.2
MB PBMC	JRB	JRB	1406	1097-1802	7.1	>0.05	1/1214	0.97	<0.05	1.5

Results for Figure 4.10

SF	EM	NF	1/7051	5364-9268	12.6	<0.05	1/41070	0.946	<0.01	1.5
SF	EM	5-3.1 L	1/19795	13386-29272	2.44	>0.05	1/16925	0.996	<0.001	1.03
SF	EM	MK	1/142595	53308-377885	4.1	>0.05	1/73078	0.89	<0.05	1.02
SF	EM	DAP3	ND							

Results for Table 4.5

NF	JM	SWEIG	1/867	674-1114	2.79	>0.05	1/1030	0.99	<0.05	0.94
SM	JM	SWEIG	1/7673	6637-111896	10.98	<0.05	1/6613	0.98	<0.001	1.15
SM	JM	SWEIG	1/8886	5802-10148	22.5	<0.05	1/2881	0.95	<0.05	1.3
MB	JM	SWEIG	1/10406	7556-14184	18.9	<0.05	1/11664	0.814	<0.05	0.94

Results for Table 4.5

Resp	Stim	Target	Freq1	Range	χ^2	P1	Freq 2	r	P2	Intercept
MB	JM	SWEIG	1/2994	2347-3820	31.3	<0.05	1/3613	0.93	<0.05	0.7
MB	JRB	PE117	1/1100	846-1428	1.9	>0.05	1/1143	0.989	<0.05	0.85
SM	JRB	PE117	1/11267	8220-15443	33	<0.05	1/4428	0.91	<0.05	1.6
SF	EM	NF	1/7051	5364-9268	12.6	<0.05	1/4107	0.946	<0.01	1.5
SM	NF	WT100	1/9454	7021-12729	15.8	<0.01	1/5610	0.92	<0.01	1.28
MB	NF	WT100	Not tested							
NF	JM	RGT1	1/1257	989-1605	12.4	<0.05	1/906	0.98	<0.01	0.93
SM	JM	RGT1	1/7646	5783-10110	11.7	<0.05	1/5292	0.959	<0.01	1.24
SM	JM	RGT1	1/9683	6890-13607	6.19	>0.05	1/13147	0.89	<0.05	0.99
MB	JM	RGT1	1/55735	29960-103686	2.62	>0.05	1/59352	0.88	<0.05	0.99
MB	JM	RGT1	ND							
MB	JRB	B20C	1/22174	12629-38932	4.3	>0.05	1/16416	0.952	<0.05	1.03
SM	JRB	B20C	ND							
SF	EM	5-31	1/19795	13386-29272	2.4	>0.05	1/16925	0.966	<0.001	1.03
SM	NF	5-31	ND							
MB	NF	5-31	ND							

Publications associated with this thesis.

Kaminski, E., Hows, J., Man, S. , Brookes, P., Mackinnon, S., Hughes, T., Avakian, O., Goldman, J.M. and Batchelor, J.R. (1989). Prediction of graft versus host disease by frequency analysis of cytotoxic T cells after unrelated bone marrow transplantation. Transplantation **48**, 608-613.

Man, S., Lechler, R.I., Batchelor, J.R. and Sharrock, C.E.M. (1990) Individual variation in the frequency of HLA class II specific cytotoxic T lymphocyte precursors. Eur. J. Immunol. *In press.*