INVESTIGATION OF THE MECHANISM OF THE BENEFICIAL EFFECT OF BLOOD TRANSFUSION ON RAT RENAL ALLOGRAFT SURVIVAL

by

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DECLARATION

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Marshall, H.E., Bolton, E.M., Gracie, J.A., Cocker, J.E., Sandilands G.P. & Bradley J.A. (1990) FcR blocking activity in serum of actively enhanced rat renal allograft recipients due to IgG anti-class II MHC alloantibody. Immunology, **69**, 379-384.

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SUMMARY

In the rat renal allograft model, pre-operative administration of donor strain blood is sufficient to produce long-term renal allograft survival even in the absence of adjunctive immunosuppression. The precise mechanisms underlying this enhancing effect are unclear but possible mechanisms include elimination or clonal deletion of alloreactive lymphocytes, removal of passenger leucocytes, blocking of effector cells by antibody or antigen and the induction of alloantigen-specific suppressor cells.

Rejecting rat renal allografts are characterised by a heterogeneous mononuclear cell and it was therefore important to determine whether pre-operative blood transfusion affected the infiltration of allografts. A detailed analysis was made of the pattern and phenotype of mononuclear cells infiltrating rejecting DA strain allografts in unmodified PVG recipients and non-rejecting DA allografts from PVG recipients which had been actively enhanced by injection of 1 ml of DA blood intravenously 7 days before transplantation. Excised grafts were stained with a range of monoclonal antibodies to rat cell surface antigens on day 1, 3, 5 and 7 post transplantation. Paradoxically pre-operative blood transfusion caused accelerated cellular infiltration of the graft and the rapid disappearance of graft interstitial dendritic cells. The phenotype of cells found within both enhanced and rejecting grafts was similar except for a reduced number of $CD8^+$cells and IL-2 receptor positive cells in the enhanced compared with rejecting grafts at day 5 after transplantation. However,there was no concomitant decrease in levels of in vitro cytotoxicity, since

graft infiltrating cells and splenocytes from transfused animals had comparable levels of both specific and non-specific cytotoxicity to those found in rejecting animals.

Class I and class II MHC antigen expression within enhanced and rejecting grafts was studied since the distribution and density of MHC expression may influence the vulnerability of the graft to host effector responses. Unexpectedly it was found that enhanced grafts underwent an accelerated and extensive induction of both donor class I and class II MHC antigens. This suggested that pre-operative blood transfusion initially causes sensitisation of the recipient resulting in an accelerated immunological response to the allograft, but that this is rapidly and effectively suppressed by immunoregulatory mechanisms.

The possibility that humoral factors may play a role in the beneficial effect of blood transfusion on graft survival was then investigated. Because of reports suggesting an association between serum Fc blocking activity and renal allograft survival, Fc blocking activity was measured in the serum of unmodified and transfused PVG recipients of a DA renal allograft. Serum harvested on day 5 from actively enhanced PVG recipients of a DA renal allograft was shown to specifically inhibit erythrocyte-antibody (EA) rosette formation with donor strain, but not third-party strain, splenocytes, while the levels of EA rosette inhibition in day 5 serum from rejecting rats was markedly lower. This FcR-blocking activity was present in enhanced serum fractions, prepared by discontinuous density gradient centrifugation, which corresponded to the 7S peak. Purified IgG prepared from enhanced serum was also found to inhibit EA rosette

formation with donor splenocytes, and absorption of the IgG preparations with donor-strain erythrocytes failed to abrogate EA rosette inhibition. Further experiments, in which absorbed IgG from enhanced animals was tested for FcR-blocking activity against splenocytes of defined major histocompatibility complex (MHC) subregion specificities, established that FcR blocking activity was mediated by IgG alloantibodies directed against donor MHC class II antigens.

This finding suggested that there might be important differences in the nature and timing of the alloantibody response to a renal allograft in rejecting and enhanced recipients. A sequential analysis was therefore made of the antibody response to donor class I and class II MHC antigens in unmodified PVG rats bearing a rejecting graft and transfused PVG recipients bearing an actively enhanced graft. There was a dichotomy in the antibody response to class I and class II MHC antigens. Enhanced recipients had barely detectable post transplantation levels of anti-class I antibody while unmodified recipients made a substantial anti-class I response which increased steadily after transplantation . On the other hand, enhanced animals had high levels of anti-class II antibody in their serum in the first 7 days after transplantation and the rejecting animals did not produce comparable levels of anti-class II until day 10 after transplantation. Cytotoxic antibody levels in rejecting animals were directed mainly at class I MHC antigens and rose steadily after transplantation. Conversely,cytotoxic antibodies in enhanced animals were directed at class II antigens and were present at low levels in the first 10

days after transplantation. Therefore in this strain combination enhancement correlated with high levels of anti-class II and low levels of anti-class I MHC antibodies.

The ability of serum from transfused and unmodified PVG recipients,5 days after transplantation with a DA kidney to inhibit the DA to PVG MLR was tested. Serum and purified IgG from enhanced animals showed greater inhibition of the MLR than rejecting serum or IgG.However there was also considerable inhibition of the third-party MLR. Since the proliferative response in the MLR is generally held to be a response to class II MHC antigens it seems likely that the greater inhibition of the MLR in enhanced animals is due to anti-class II antibodies.

The finding that blood transfusion primes the recipient to develop a high anti-class II alloantibody response to a subsequent renal allograft raises the possibility that the anti-class II antibody may contribute to the beneficial effect on graft survival. The ability of serum from actively enhanced rats during the early post-transplantation period to inhibit the MLR is in keeping with this suggestion, but in vivo adoptive transfer studies will be necessary to further substantiate this hypothesis.

ABBREVIATIONS

ADCC - Antibody dependent cellular cytotoxicity

ARCO - Antigen reactive cell opsonisation

BSA - Bovine serum albumin

ConA - Concanavalin A

CyA - Cyclosporin A

DAB - Dulbeccos A and B

DST - Donor specific transfusion

DTH - Delayed type hypersensitivity

EA - Erythrocyte antibody

FcR - Fc receptor

FCS - Foetal calf serum

GvH - Graft versus host

HLA - Human leucocyte antigen

IgG - Immunoglobulin G

IL-1 - Interleukin 1

IL-2 - Interleukin 2

LNC - Lymph node cell

MLR - Mixed lymphocyte reaction

NK - Natural killer

RES - Reticuloendothelial system

RT - Room temperature

TcR - T cell receptor

TDL - Thoracic duct lymphocyte

PLN - Popliteal lymph node

CHAPTER 1

INTRODUCTION AND REVIEW OF THE LITERATURE

1.1.0 The development and current status of renal transplantation

Renal transplantation is the treatment of choice for patients suffering from end-stage renal failure. It is not only cost effective in comparison to haemodialysis, but more importantly provides a much improved quality of life. However, successful clinical renal transplantation is a relatively recent phenomenon and has only been widely achieved over the last two decades.

In 1951 David Hume in Boston embarked on a series of cadaver kidney transplants. Although these operations were considered to be technically successful, unfortunately nearly all grafts were rapidly rejected. Another Bostonian, Dr. Joseph Murray, avoided the immunological problems associated with transplants between genetically different individuals by successfully transplanting kidneys between identical twins. This success heralded the first of many subsequent transplants between identical twins, but the problems of immunological rejection between non-identical individuals remained.

Hope for the seemingly intractable problem of graft rejection came with the discovery by Schwartz and Dameshek (1959) that a purine analogue, (6-MP) could effectively suppress the immune response of rabbits to human serum albumin. This exciting finding was followed up by Calne in Great Britain (1960) and Zukoski,Lee & Hume in the United States (1960) who independently found that 6-mercaptopurine would prolong the survival of renal allografts in dogs. In 1961 azathioprine, a derivative of 6-MP was synthesised and was found to

be a less toxic, but equally effective immunosuppressant. (Calne, Alexandre and Murray, 1962). Clinical trials of azathioprine began and in 1963 the first drug-treated recipient of a cadaver kidney to survive for one year was reported (Murray, Merrill,Harrison et al, 1963). For the next twelve years azathioprine in combination with corticosteroids was the accepted immunosuppressive therapy in most renal transplant units. In the 1970's the optimism which had pervaded the transplant world in the previous decade began to fade with the realisation that the immunosuppressive therapy available could not guarantee successful transplantation between unrelated individuals. One year cadaveric graft survival rates quoted in 1974 were still of the order of 50%, little better than several years previously (Opelz, Mickey and Terasaki, 1974). Tissue matching of the donor and recipient for histocompatibility antigens improved graft survival,but even well-matched grafts were sometimes rejected.

The next important advance was the discovery by Sandoz Ltd. of a new drug cyclosporin A (CyA) which was found to have remarkable immunosuppressive properties (Borel, 1976). Again, it was Calne who was the first to demonstrate the effectiveness of this new drug as an immunosuppressive agent in renal transplantation. (Calne, White, Thiru et al, 1978). The introduction of CyA has heralded a new era in clinical transplantation with 1 year cadaveric renal allograft survival rates of around 80% being widely reported (Morris, 1988). CyA either alone or in combination with steroids and/or azathioprine is now the standard immunosuppressive therapy in nearly all transplant units. Recently a novel fungal metabolite FK-506 was discovered (Kino,Hatanaka,Hashimoto et al,1987) and found to be a more potent

immunosuppressive agent than CyA . It has been found to inhibit T cell activation and lymphokine production in vitro, and to suppress rejection of skin and heart grafts in rats and kidney grafts in dogs (Kino, Hatanka, Miyata et al,1987; Thomson,Stephen,Woo et al(1989); Inamura,Nakahara, Kino et al,1988; Ochiai,Nagata, Nakajima et al,1987). However there are no published data to date on the use of FK-506 on human renal transplant patients. Despite the advances in immunosuppression, immunological rejection remains a major problem. Furthermore, the non-specific immunosuppression currently used results in an increased susceptibility to viral and other types of infection as well as an increased incidence of some types of malignancy . Moreover in the case of CyA, nephrotoxicity may be a serious side effect.

An important empirical observation in clinical renal transplantation was that transfusion with random donor blood prior to transplantation significantly improved graft survival (Opelz, Sengar, Mickey et al 1973). This initial observation was subsequently confirmed in several transplant centres and has led to the ubiquitous use of pretransplant transfusion for recipients of cadaver kidneys. Following on from this,Salvatierra(1980)introduced donor specific transfusion in an attempt to achieve better graft survival in one-haplotype matched living related recipients . The mechanism of the beneficial effect of blood transfusion on allograft survival remains unclear despite intensive study in this field. It is clear, however, that a better understanding of this important clinical observation may provide an insight into methods of further improving renal allograft survival.

The overall aim of this thesis is to examine the mechanism of prolonged allograft survival following donor specific blood transfusion using a rat renal allograft model. The rat model allows the immune response to defined histocompatibility differences to be examined, using inbred strains of rats. Furthermore the rat unlike the mouse has sufficiently large vessels such that anastamosis of the renal vessels may be performed with the aid of a dissecting microscope. While one must exercise caution in extrapolating the findings in an animal model, to the clinical situation one can hopefully gain information about, and a greater understanding of the "transfusion effect". This in turn may allow the development of strategies for inducing specific unresponsiveness in transplant recipients.

1.2.0. The blood transfusion effect in clinical transplantation

The beneficial effect of blood transfusion on renal allograft survival was not reported until 1973 (Opelz et al, 1973). Interestingly,however, Medawar, whilst preforming skin grafting experiments in rabbits, found that rabbits which had received massive transfusions of homologous blood did not reject their grafts(Medawar,1946). Subsequently Kearns and Reid (1949) reported the success of a father to son skin graft in which prior transfusion had occurred. The first report of a "donor specific" transfusion effect came from Halasz, Orloff and Hirose (1964) who showed in a canine renal allograft model that the subcutaneous injection of donor blood 10 and 5 days prior to transplantation prolonged graft survival . In

1967 Dossetor, MacKinnon, Gault et al reported the first case of a successful renal transplant in man following donor-recipient cross circulation. However, when Opelz et al (1973) compared kidney graft survival between patients who had received blood transfusions (from blood group compatible but otherwise random donors) prior to transplantation,with those who had not, they found that transfusion had a beneficial effect on survival. Furthermore, the same authors (1974) suggested that patients who had received whole blood or packed red cells (but not frozen cells) had improved graft survival and this beneficial effect correlated with the amount of blood received. In a study of over 1300 cadaver transplants, graft survival at one year was 42% for recipients who had never been transfused and 71% for those who had received more than 20 units of blood (Opelz and Terasaki, 1978). The general finding that blood transfusion had a beneficial effect on renal allograft survival was confirmed by other renal transplant centres, although there was disagreement over the amount and type of transfusion which gave the maximum benefit for graft survival along with the minimum risk of sensitisation (the development of cytotoxic antibodies to the graft donor). In a retrospective and prospective study of patients receiving only one transfusion prior to transplantation, 1 year graft survival rates were 73% and 70% respectively compared with only 26% in the non-transfused group (Persijn, D'Amaro and van Rood,1984). Other groups favoured the administration of between two and five transfusions and noted that maximum benefit was obtained if transplantation was carried out within three months of the transfusion protocol (Hourmant,Soulillou, Bui-Quang,1979; Werner-Favre, Jeannet, Harder et al,1979).

1.2.1 Blood components required for a transfusion effect

The demonstration that blood transfusion improved allograft survival led to many investigations aimed at determining which of the cellular or other components of blood were responsible for the beneficial effect. A number of experiments have addressed this question in man, but this has been most extensively studied in animal models (see section 1.9.2).

Some studies tried to achieve a transfusion effect with purified platelets which in man carry class I,but not class II major histocompatibility antigens. It had previously been shown in monkeys that platelet transfusion improved renal allograft survival without inducing cytotoxic antibodies (Borleffs, Neuhaus, van Rood et al, 1982). The three major studies carried out in man have produced conflicting results. Chapman, Fisher, Ting et al,1985 together with Pallardo, Montoro, Moll et al,1985 did not find any beneficial effect of platelet transfusion on graft outcome, but noted a significant degree of sensitisation. On the other hand,Betuel, Cantarovich, Robert et al (1985), noted a beneficial effect on graft survival and a minimum degree of sensitisation. Such contradictory findings may reflect differences in the purity of the platelet populations used. Other studies have looked at the role of leucocytes in the beneficial effect of blood transfusion. Freeze thawing of blood, which decreases the number of viable leucocytes was found to decrease the transfusion

effect: one year survival for patients receiving packed cells or whole blood was 53% compared with only 20% for patients receiving frozen cells (Opelz and Terasaki, 1974).

1.2.2. Donor specific transfusion

The earlier animal work on donor specific transfusion (DST) suggested that such a protocol in living related transplants might increase graft survival. While this was not considered necessary in HLA-identical or one-haplotype matched pairs with low reactivity in mixed lymphocyte culture (MLC), it was thought that it might be beneficial for pairs in which there was high MLC reactivity. Cochrum, Salvatierra and Belzer (1974) had previously shown that the latter group had a poorer renal allograft prognosis. This led Salvatierra et al (1980) to administer a course of donor specific transfusion in an attempt to achieve better graft survival in one-haplotype matched living related recipients with high MLC reactivity. The main problem with this approach was sensitisation and 13 of the 45 potential living related recipients developing either a T cell or a warm B cell crossmatch (discussed later). However, graft survival was excellent in the 30 patients who were transplanted: 95% had functioning grafts at one year and a low incidence of rejection episodes (Salvatierra, Iwaki,Vincenti et al, 1981a). Furthermore the transfusion effect was found to persist for at least four years after transplantation with 82% graft survival, which was comparable to that achieved with HLA-identical living related transplant recipients (Salvatierra,

Melzer, Potter et al, 1988). It is unclear at present whether DST confers any advantage over cyclosporin in one-haplotype matched living related transplants and this will depend on the results of a prospective study of these two groups(Salvatierra,Melzer,Vincenti et al,1987).

Before discussing the animal models which have been studied to address the possibility of developing strategies of specific immunosuppression, it is first necessary to look at the tissue antigens responsible for graft rejection and the mechanisms which are thought to bring about graft destruction.

1.3.0. The Major Histocompatibility Complex (MHC)

1.3.1. Discovery of the human MHC

The Major Histocompatibility Complex (MHC) is a complex of genes which encode cell surface glycoproteins which are the major transplantation antigens. Incompatibility between individuals for MHC antigens is the major barrier to successful transplantation. Moreover MHC antigens are also vital recognition molecules of the immune system and some description of their structure and function is necessary to understand their role in rejection. All vertebrates and some invertebrates possess an MHC and there is a high degree of homology with respect to immunogenetics and biochemistry.

Studies of the histocompatibility antigens of man began with the discovery by Jean Dausset in 1958 of antibodies to white blood cells in the sera of burned and multiply transfused patients (Dausset, 1958). Using such antisera he detected the first leucocyte antigen named 'Mac' on a panel of leucocyte donors. In 1958 van Rood and Payne independently discovered that cytotoxic antibodies to foetal leucocytes were present in the sera of multiparous women (van Rood, Eernisees and van Leeuwen, 1958; Payne and Rolfs, 1958). Using a leuco-agglutination test to define antigenic specificities two allelic systems were described: Group 4 (van Rood and van Leeuwen, 1963) and 'LA' (Payne,Tripp,Weigle et al, 1964). In 1968, Kissmeyer-Nielsen, Svejgaard and Hauge, described two allelic series of antigens coded by two distinct loci Human Leucocyte Antigen-A (HLA-A) (equivalent to LA) and HLA-B (equivalent to group 4). A third locus HLA-C was not established until 1972 (Svejgaard,Nielsen,Ryder et al 1972).

The HLA-D locus was designated when it was found that lymphocytes from different individuals proliferated when cultured together (Bach and Hirschkorn, 1964) even when HLA identical. The locus determining the mixed leucocyte reaction (MLR) was mapped outside the known HLA regions and designated HLA-D (Thorsby and Piazza, 1975). Later it was shown that D-region antigens could also be detected serologically, each being associated with an HLA-D determinant and hence termed HLA-DR (D-related) (Bodmer and Bodmer, 1978).

Expression of the HLA genes is co-dominant so that each cell expresses on its surface two sets of antigens, one inherited from each parent. HLA genes are usually inherited en bloc by the offspring from each parent, each set of genes being termed the haplotype.

1.3.2. The Genetic Map of HLA

I will describe in brief the maps of man, mouse and rat in the following section since many of the experiments to be described are in animal models. In particular, a description of the rat MHC is given since the work in this thesis has been carried out in the rat renal allograft model.

The MHC of man is located on the short arm of chromosome six. The class I antigens of man are encoded by the B,C and A loci which are located on the opposite end of the chromosome from the centromere. Telomeric to HLA is the TCA (T cell system A) region which codes for the antigens equivalent to mouse Qa or Tla which have been found on a subset of human T lymphocytes.

The class II antigens are encoded by three main subregions, namely DP, DQ and DR, though two other regions DN and DO have been described recently. The DP region codes for the α1 and ß1 genes of the DP class II molecule as well as two pseudogenes A2 and B2. DP is thought to be analagous to the H region of the rat, but has no homologue in mouse.

The DQ subregion has two sets of alpha (A1 and A2) and 2 sets of beta (B1 and B2) genes. The 1 and ß1 genes are expressed but the 2 and ß2 genes are not. The HLA-DR chain is encoded by a single locus (α1) while the ß chain is coded for by 2 genes ß1 and ß2, both of which are usually expressed.

The class III genes map between HLA-DR and HLA-B and code for C2, Factor B, C4, 21 OH and TNF α and ß. The genetic map of HLA can be seen in Fig 1.1

1.3.3 Discovery of the mouse MHC

In 1936 Peter Gorer showed that a rabbit anti-mouse serum reacted with red cells from some but not other mouse strains (Gorer, 1936). The determinant detected by this antibody was termed antigen II and was found to be controlled by an autosomal dominant locus which was termed histocompatibility-2 or "H-2"(Gorer,1937). He hypothesised that tumour grafts exchanged between mice elicited an immune response in the host due to an incompatibility between the host and donor for antigen II(Gorer,1938). The establishment of congenic strains of mice by Snell (1948) ,allowed Gorer to show that tumour grafts exchanged between mice differing at H-2 were rejected and the locus was termed the major histocompatibility complex (MHC),(Gorer,Lyman and Snell,1948).

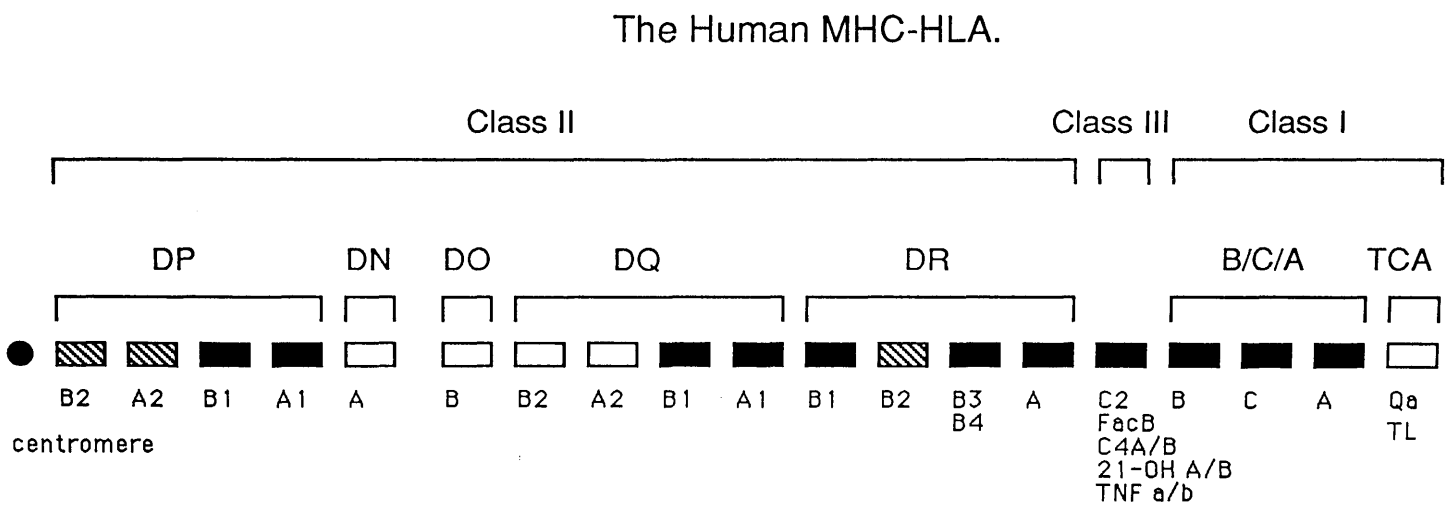


Figure 1.1 Genetic map of HLA (based on Bodmer et al 1988). Expressed genes are represented by filled boxes, pseudogenes by hatching and undetermined genes are blank.

1.3.4. The genetic map of H-2

The MHC of the mouse was mapped to chromosome 17 (Gorer et al,1948). The H-2K,H-2D and H-2L genes encode the class I antigens which were originally defined serologically (Bach, Widmer, Bach et al, 1972). The K and D/L regions which defined the H-2 complex are located at opposite ends of the H-2 region. These genes are highly polymorphic with more than 60 class I genes identified in the wild population (Duncan, Wakeland and Klein, 1979). The Tl and Qa loci map telomeric to the D/L region and code for the thymic leukemia antigen (TL antigen) found on thymocytes and the Qa antigens found on subpopulations of lymphocytes.

The immune response to certain synthetic polypeptides was found to map to a region between H-2K and H-2D (McDevitt and Tyan, 1968;McDevitt and Benaceraff 1972), and the genes encoded by the region termed immune response genes (Ir genes). It was also found that MLR activity mapped to this region which became known as the I region (Klein,1975). This region codes for the class II antigens which have been detected by both cellular and serological means. The I region can be subdivided into I-Q and I-E which code for two different class II antigens. The I-E antigen is encoded by two genes Eα and Eß: Eß is coded for by the I-A subregions while Eα is coded by I-E. The I-A subregions coded for the α and ß chains of the I-A antigen. The nature of Aß2 and Eß2 is unknown while Aß3 has been shown to be a pseudogene (Widera and Flavell, 1985).

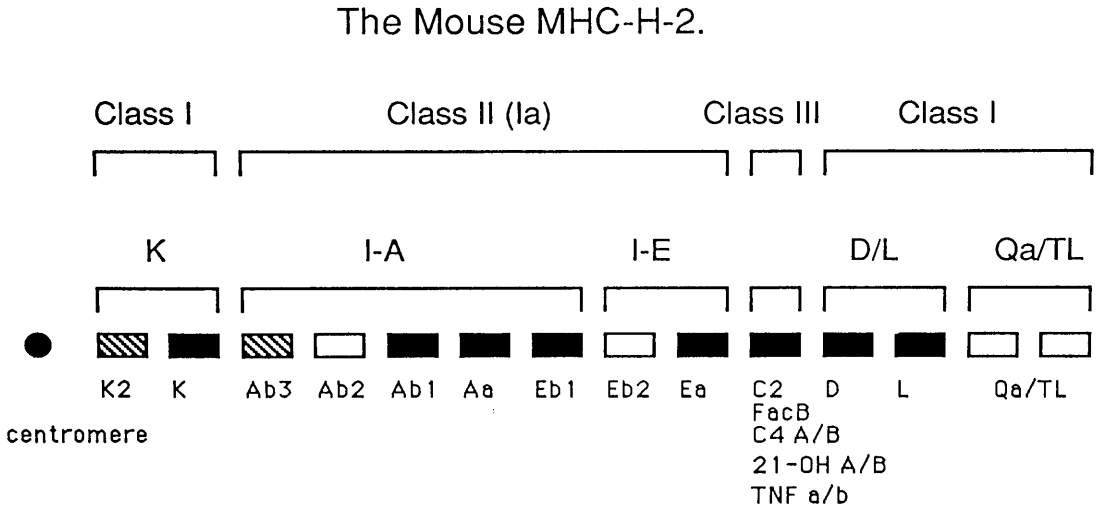


Figure 1.2 Genetic map of H-2 adapted from Bodmer (1986). Expressed genes are represented by filled boxes, pseudogenes by hatching and undetermined genes are blank.

The class III genes are located between the I-E and D/L loci and code for serum complement components C2, factor B and C4. The genetic map of the H-2 complex can be seen in figure 1.2.

1.3.5. The genetic map of RT1

The genetics of the MHC are less well defined for the rat than man and mouse. The rat MHC was designated RT-1 in 1978 (Gill,Kunz & Misra,1987) and has provisionally been placed on rat chromosome 14 (Oikawa,Yoshida,Saitoh et al 1983). A number of loci have been identified, though their position relative to the centromere is still unknown. The class I antigens are coded for by the RT1.A and E loci which are thought to be analagous to the H-2K and H-2D loci respectively. Several other class I antigens have been defined serologically and mapped to the RT1.A region namely F1 and Pa.

Antigens encoded by the G and C regions have properties similar to the TL and Qa antigens of the mouse (Gill et al, 1987). The class II loci, RT1.B and RT1.D are homologous to the I-A and I-E regions of the mouse (Blankenhorn and Cramer, 1985). Polymorphism arises in the RT1.B and D ß chains (Natori,Ohashi,Inomata et al 1983) rather than the α chains which are highly conserved (Wallis and McMaster, 1984). A third class II locus, RT1.H has been identified to the RT1.A side of RT1.B and may be the rat equivalent of HLA-DP (Walters,Locker,Kunz et al 1987a).

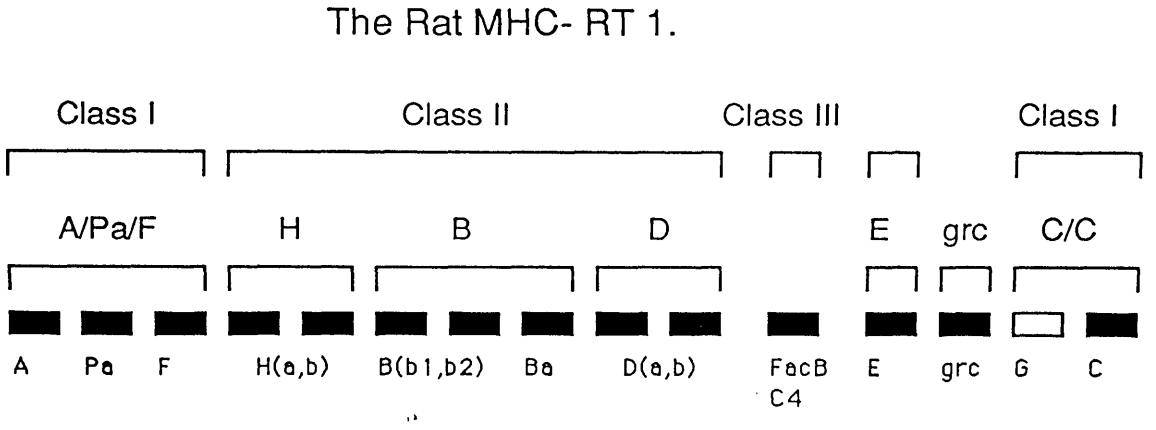


Figure 1.3 Genetic map of RT1 (based on Gill et al 1987, Watters et al 1987 and Wurst et al 1988). Expressed genes are represented by filled boxes and undetermined genes are blank.

So far the only class III genes to have been itentified are those encoding factor B (Wurst, Rothermel and Gunther 1988) and C4 (Walters,Locker, Kunz et al 1987b). The genetic map of the rat MHC can be seen in Figure 1.3.

1.4.0. Histocompatibility Antigens

1.4.1. Structure of MHC antigens

The antigens encoded by the MHC have been subdivided on the basis of structure and function into class I, class II and class III antigens (Klein,1975). The class I and class II antigens of mouse and man have the same basic structure (Nathenson, Uehera,Ewenstein et al,1981; Kauffman, Auffray, Korman et al,1984). The structure of a rat class I antigen has only just been described and appears to be similar to man and mouse(Radojcic,Stranick, Locker et al,1989) but a complete sequence for a class II gene has yet to be described.

Class I antigens are cell surface glycoproteins comprised of two chains: the α chain (approx 45 kd) is highly polymorphic and associated non-covalently with a non-variable light chain (ß2 microglobulin) which is not encoded by the MHC(Nathenson et al,1981). The heavy chain is composed of three extracellular domains (α1, α2, α3), a transmembrane part and an intracytoplasmic tail. Such molecules are said to be expressed on all nucleated cells, but have been difficult to detect on normal cells such as hepatocytes, corneal endothelium and syncitial trophoblasts (Daar, Fuggle & Fabre 1984; Lew,Lillehoj,Cowan et al 1986; Lala, Chatterjee-Hasrouni,Kearns et al 1983).

Class II antigens are heterodimetric glycoproteins comprised of an chain (33kd) and a ß chain (28kd) which forms two extracellular domains (α1, α2, ß1, ß2) both of which are linked to a transmembrane region and an intracellular tail(Kaufman et al, 1984).

Class II antigens have a more restricted tissue distribution being expressed constitutively on B cells, monocytes, dendritic cells and some endothelial cells and being induced on activated T cells.

The class III products comprise the serum complement components C2, C4 and factor B; the enzyme 21 hydroxylase and tumour necrosis factor α and ß. In the rat, only C4 and factor B have so far been identified (Gill et al, 1987). These factors unlike those coded by the MHC do not function as alloantigens.

1.4.2. The role of MHC antigens in immune recognition

The class I and class II MHC antigens , apart from serving as transplantation antigens, act as restriction elements in the recognition of foreign antigens thereby allowing the immune system to distinguish self from non-self(Schwartz,1985). T lymphocytes recognise foreign antigens in the context of self MHC; CD8+ T cells are restricted by class I antigens and will kill virus infected cells only if the cell is expressing the appropriate 'self' class I antigen (Zinkernagel and Doherty, 1979). $CD4^+$ cells are restricted by class II products (Bach,Bach and Sondel,1976) and function in a number of cell interactions such as T-B cell co-operation (Katz,Graves,Dorf et al, 1975) and in the generation of cytotoxic responses (Cantor and Boyse,

1975a,b).Although $CD4^+$ T cells generally mediate "helper" function and $CD8^+$ T cells have "cytotoxic" function, the correlation between phenotype and function is not strict(Meuer,Hussey, Penta et al,1982;Swain,1983).T cells, unlike B cells do not recognise native antigens but recognise processed peptides in association with MHC antigens (Grey and Chestnut, 1985; Townsend ,Rothbard, Gotch et al 1986). In the case of CD4+ cells antigen must be presented by class II positive accessory cells (dendritic cells or macrophages). Allorecognition was thought to involve direct recognition of foreign MHC, but recent studies have shown that allorecognition may involve recognition of endogenous peptides in the groove of the foreign MHC molecule(reviewed by Lechler, Lombardi, Batchelor et al,1990). This would explain the unusually high precursor frequency of alloreactive cells first described by Skinner and Marbrook (1976) since each peptide MHC combination could stimulate a separate T cell population.

1.4.3. Minor histocompatibility (mH) antigen

Minor histocompatibility antigens are coded for by a region outwith the MHC . The observation that grafts exchanged between HLA identical individuals are rejected, albeit somewhat slower than in HLA mismatched individuals,has been taken as evidence of a weaker or minor histocompatibility system. However the fact that skin grafts between MHC identical mouse strains can be rejected almost as rapidly as those between MHC incompatible strains argues that these minor antigens are not necessarily weak transplantation antigens. (Loveland

and Simpson, 1986). The precise nature of minor histocompatibility antigens (mH) is enigmatic but over forty loci encoding allelic forms of mH antigens have been found throughout the genome in mice (Bailey, 1975). They do not appear to evoke antibody responses and so cannot be defined serologically. However they have been found to elicit T cell responses in vitro and such recognition is MHC restricted (Loveland and Simpson, 1986). The best defined mH antigens are the H-Y antigens which are expressed by male but not female mice, as detected by rejection of male skin grafts by female mice.

The only evidence that mH antigens are on the cell surface is that target cells are killed in cytotoxicity assays and proliferative responses are restricted by class I or class II antigens. The immunogenicity of mH antigens is likely to depend on a number of factors including the host's immune response genes, and the number of antigens similar to self (the response to which would most likely be suppressed), the immunogenicity of the donor tissue and any tissue specific antigens (Loveland and Simpson, 1986).

1.4.4. Tissue specific antigens

Relatively little is known about the possible role that tissue specific antigens may play in graft rejection. An antigen system has been described on vascular endothelial cells (VEC) and 78% of recipients of HLA identical, living related renal allografts who irreversibly reject their allografts develop antibody to their donors' vascular endothelial cell specific antigens (Cerilli and

Brasile, 1988). Vascular endothelial cell antigens are found in high concentrations throughout the renal vasculature particularly the peritubular capillaries and veins. There is evidence from pedigree studies that vascular endothelial cell antigens segregate in association with parental HLA haplotypes and this supports the vascular endothelial cell antigen system being a part of or closely linked to the major histocompatibility complex. Eluates from rejecting renal allografts have been analysed for cytotoxic antibodies. Sera were found after absorption with T and B lymphocytes to react with renal cells supporting the existance of unique renal cell antigens and a possible role in rejection (Cerilli and Brasile, 1988).

1.5.0. Normal distribution of Class I and Class II antigens in organ grafts

The distribution of class I and class II MHC antigens varies between organs and species studied.Most species have strong class I expression on vascular endothelial cells (which may be important targets of the rejection response). Renal parenchymal cells such as the renal tubules have been shown to be weakly positive for class I antigens in both rats and humans(Hart and Fabre, 1981a; Daar, Fuggle , Fabre et al, 1984). The myocardial cells of the heart have consistently been shown to be class I negative, while the interstitial cells are positive in both the rat and human (Daar et al, 1984, Milton and Fabre, 1985). The interstitial dendritic cells

found in all grafts except those of nervous tissue are strongly class II positive and are thought to be important in sensitising the host to donor antigens (see section 1.6.1). In the human and other species the vascular endothelial cells of the kidney are normally class II positive but in the rodent have been found to be class II negative. In the rat the epithelial cells of the proximal convoluted tubules are class II positive (Hart and Fabre, 1981b) while in humans tubular expression of class II is variable (Fuggle,Errasti, Daar et al 1983) and in the mouse is negative (Benson, Colvin and Russell, 1985).

1.5.1 Induction of class I and class II antigens in organ grafts

MHC expression is influenced by the presence of various lymphokines. In particular the and ß interferons produced by a number of cell types have been found to induce class I antigens only (Fellous, Nir,Wallach et al, 1982; Wong, Clark-Lewis, Harris et al, 1984). Gamma interferon , produced by activated T cells,strongly induces both class I and class II antigens (Wong et al, 1984; Basham, Smith, Lanier et al, 1984). During graft rejection, infiltrating cells release MHC inducing lymphokines which upregulate class I and class II MHC antigens on donor tissue. This MHC induction is likely to have a major effect on the development and amplification of the rejection response.

After grafting, an increase in class I expression has been found in the parenchyma and vascular endothelium of the kidney (Milton, Spencer and Fabre, 1986a), on the myocardial cells of the heart (Milton and Fabre, 1985) exocrine cells of the pancreas (Steiniger et

al, 1985) and hepatocytes in the liver (Settaf,Milton, Spencer et al, 1988) during rejection. Induction of class I is more rapid in the kidney than in the heart, beginning at day 1 and being maximal at day 3, when ten to twenty fold increases in expression have been shown (Milton et al, 1986a).

In the rat kidney both cortical and medullary tubules and arteriolar vascular endothelium have been shown to become strongly class II positive during rejection. The glomerulus, however, remains class II negative possibly because of the fewer infiltrating leucocytes and/or the washing away of lymphokines by the glomerular filtrate (Milton et al 1986). Widespread class II induction is also a feature of rejecting kidney grafts in humans (Hall, Duggin, Phillips et al, 1984) while in the mouse only the proximal convoluted tubules become positive (Benson et al, 1985). Surprisingly, however, it has been found that there is also a marked induction of class I and class II antigens in rat renal allografts in which graft rejection has been prevented by pre-operative donor-specific blood transfusion (Wood,Hopley,Dallman et al,1988).A recent report has confirmed this finding, but found that in passively enhanced animals, class II induction was delayed until 7 days after transplantation (Priestley and Fabre, 1989).

1.6.0. The rejection response

At the same time that early work on histocompatibility antigens was evolving, Dr. Peter Medawar was carrying out experiments which underlined the immunological nature of the rejection phenomenon. In 1943, Medawar along with Mr. Thomas Gibson, observed the fate of skin grafts in a burns patient in the Burns Unit of Glasgow Royal Infirmary (Gibson and Medawar, 1943). They found that a second graft from the same donor was rejected more rapidly than the first graft, the so called "second set phenomenon", which implied that there was some form of immunological memory. That rejection was a specific phenomenon was emphasised by further experiments in rabbits in which it was shown that accelerated rejection only occurred when the second set of skin came from the same donor as the first skin graft (Medawar, 1944, 1945). Despite many advances in our understanding of the rejection response since these early observations the mechanisms underlying rejection are still incompletely defined. The rejection response can for ease of description be divided into the <u>afferent limb</u> in which host sensitisation to graft antigens occurs and the <u>efferent limb</u> in which the effector cells mediate graft damage.

1.6.1. Host sensitisation

Donor dendritic cells, which constitutively express class I and class II antigens , are found as "passenger leucocytes" in all organ grafts . Dendritic cells are critically important in the afferent phase of graft rejection and are known to be potent stimulators of in vitro immune responses (Steinman and Witmer, 1978; Mason, Pugh and Webb, 1981). Their importance in host sensitisation is underlined by experiments in which removal of "passenger leucocytes" from thyroid or skin grafts by in vitro culture or irradiation results in prolonged graft survival (Lafferty,Bootes,Dart et al1976; Emma and Jacobs, 1981). Lechler and Batchelor (1982) have suggested that sensitisation may occur by two routes. One suggested route is direct presentation of donor antigen by donor cells. The second and possibly less efficient route is presentation of donor antigen by host antigen presenting cells. Evidence for the second route was recently provided by Sherwood ,Brent & Rayfield (1986) who found that adherent peritoneal cells isolated from allosensitised mice and depleted of T cells could cause accelerated rejection of skin grafts if injected into naive host strain mice. It should be noted that dendritic cells may be more important in sensitisation of the rodent than in man where the vascular endothelium expresses class II MHC and may therefore be able to present donor alloantigen.

Cultured human umbilical vein endothelial cells are able to stimulate unprimed lymphocytes in an MLR and can present soluble protein antigen to T cells (Hirschberg,Evenson,Henriksen et al, 1975;

Hirschberg, Berg and Thorsby, 1980). As described earlier, induction of class II antigens on the vascular endothelium is a feature of rejecting organ grafts. The finding by Pober ,Gimbrone,Cotran et al (1983) that co-culture with allogeneic T cells and gamma interferon induces Ia expression on vascular endothelium emphasises that vascular endothelium could present a potent allostimulus to the host in vivo.

The route of sensitisation may depend on the type of graft. In the case of a primary vascularised graft such as the kidney, induction of the response may occur within the graft (i.e. peripherally) while in a secondarily re-vascularised graft such as the skin it is generally thought to occur centrally in the lymphoid system. This conclusion was drawn on the grounds that infiltration of vascularised organ allografts by host lymphocytes occurs from the time of transplant, while in skin revascularisation takes several days and so sensitisation is more likely to be a result of donor dendritic cells (presumably Langerhans cells) travelling via the lymphatics to the central lymphoid organs. However Larsen,Morris & Austyn (1990), who followed the fate of donor dendritic cells from cardiac allografts in the mouse found they travelled to the recipient spleen where they were seen in the white pulp in close association with CD4 positive cells. This finding is in accordance with that of Forbes, Parfrey and Gomersall (1986) who showed that three to four days after cardiac transplant in the rat, localised dendritic cell-lymphoid cell aggregates could be seen in tissue sections and they proposed that

this was the counterpart of dendritic cell-lymphocyte clustering seen in vitro during an MLR (Austyn,Steinman,Weinstein et al, 1983; Steinman,Gutchinov, Witmer et al 1983; Green and Jotte, 1985).

1.6.2. Classification of rejection

In clinical organ transplantation, there are three main types of rejection response which have been classified on the basis of the tempo in which they occur and the histological appearance of the graft.

Hyperacute rejection occurs almost immediately following completion of the vascular anastamosis. It is due to preformed cytotoxic alloantibody in the recipient,directed either against ABO blood group antigens or donor histocompatibility antigens, the latter resulting from previous pregnancy,blood transfusions or renal transplant. In clinical practice hyperacute rejection is avoided by screening the patients' sera for cytotoxic antibody against a panel of lymphocytes of known HLA specificity.

Acute rejection occurs most frequently in the first two months after transplantation and often between two and ten days. Histologically there is widespread oedema and focal infiltration by leucocytes; endothelial swelling; fibrinoid necrosis of small arteries; platelet aggregates and fibrin thrombi in capillaries and foci of tubular necrosis. Acute rejection may be the result of a

humoral response, in which case, glomerular and arterial changes predominate or by a cellular mechanism where interstitial changes are most often found.

Chronic rejection usually occurs several months or later after transplantation and it is thought that antibody may play a role since deposits of immunoglobulin, complement and fibrinogen are found in varying patterns and quantities.

Before discussing specific immunosuppression and the mechanisms thought to mediate various forms of immunosuppression it is first necessary to look at the mechanisms responsible for graft damage during rejection.

1.6.3. The role of antibody in graft destruction

In 1966 Kissmeyer-Nielsen described the first cases of hyperacute rejection of renal allografts. This was found to be due to cytotoxic antibodies to donor histocompatibility antigens formed as a result of previous pregnancies, transfusion or transplants. In order to detect such antibodies in the sera of prospective transplant recipients, the lymphocytotoxicity test was developed (Terasaki, Mickey and Kreisler, 1971). Patient sera were reacted with a panel of leucocytes in the presence of complement and cell killing assessed by dye exclusion. Although lymphocytotoxic antibodies were initially said to be directed against HLA antigens on lymphocytes, it was found that some were in fact autoantibodies (Mottironi and Terasaki, 1970). These were mostly IgM antibodies and reacted more strongly in the cold or

at room temperature than at 37C, (Cicciarelli, Chia, Terasaki et al,1980) and bound more frequently to B cells than T cells (Park, Terasaki and Bernocco, 1977). The so called "cold antibodies" which react only at 5C are not indicators of sensitisation, while those which react at 37C (warm antibodies) are usually directed to HLA antigens and therefore harmful. A T cell crossmatch which is positive at the peak of sensitisation but which is negative at the time of transplant does not necessarily preclude transplantation. However, transplantation in the presence of a peak positive T cell crossmatch due to an anti-HLA antibody may only be successful if the antibody in the peak serum is of the IgG class(Chapman, Taylor, Ting et al, 1986). The precise role of antibody in acute or chronic rejection is not clear but graft damage could result from complement dependent cytotoxicity or antibody dependent cellular cytotoxicity (ADCC). In ADCC, cells bearing Fc receptors (K cells) interact with the Fc portion of IgG which is bound to the target cell and this results in the release of lysosomal enzymes or other toxins and ultimately cell death. K cells alone do not have lytic activity and the specificity of the response is a result of the specificity of the antibody (Perlmann and Holm, 1969). It has been difficult to properly evaluate the role of antibody in graft rejection since techniques of elution are fairly crude. However rejecting rat renal allografts show glomerular and tubular necrosis, gross oedema, fibrinoid necrosis of arterial walls, thrombosis and deposition of IgG and complement in arteries all of which could in principle be the result of antibody mediated damage (Abbas, Corson, Carpenter et al,1974a,b). Cells mediating ADCC have been isolated from rejecting heart

allografts in the rat (Tilney, Strom, McPherson et al 1975; Strom, Tilney,Paradysz et al, 1977). Complement dependent cytotoxic damage of allografts in the rat model is not readily demonstrable and a study by French (1969) found that hyperacute rejection of rat kidneys could only be demonstrated when hyperimmune serum was transferred along with guinea pig complement.

1.6.4. Delayed type hypersensitivity and specific cytotoxic T cells in rejection

T cells play an essential role in graft rejection and neither congenitally athymic nude or T cell depleted mice are able to reject allografts (Hall,Dorsch and Roser, 1978; Rolstad and Ford, 1974). However the precise mechanism(s) whereby T cells cause graft rejection remains a contentious issue since Brent, Brown and Medawar (1962) initially suggested a delayed type hypersensitivity reaction. Subsequently, Hayry and Defendi (1970) proposed that specific cytotoxic T cells were responsible for graft rejection. They showed that MHC incompatible lymphocytes proliferate in response to one another resulting in the generation of allospecific cytotoxic T cells and suggested that this was a complete in vitro model of allograft rejection. In this model optimal cytotoxicity was seen when responder and stimulator lymphocytes differed at both class I and class II regions of the MHC. Less cytotoxicity was generated in the response to an isolated class I and least cytotoxicity to an isolated class II disparity (Bach et al, 1976).

The studies of Mintz and Silvers (1970) of skin graft rejection in mice lent weight to the hypothesis that the specific cytotoxic T cell was the mediator of rejection. Using allophenic mice(a genetic mosaic formed by fusing early blastomeres of different MHC genotypes),they showed that when skin from an allophenic mouse containing H-2^k and H-2^b antigens was transplanted to either H-2^k or H-2^b recipient,there was focal destruction of melanocytes bearing the incompatible MHC antigens, while closely adjacent histocompatible melanocytes remained viable.

1.6.5. Studies examining the relative roles of CD4+ and CD8+ cells as effectors of rejection

The relative contribution of $CD4^+$ and $CD8^+$ T cells to the rejection response has been investigated using adoptive transfer of purified populations of T cells into T cell deficient hosts.

Naive $CD4^+$ cells alone are able to restore skin graft rejection in adult thymectomised bone marrow irradiated (ATXBM) hosts (Loveland, Hogarth, Ceredig et al,1981; Dallman, Mason & Webb,1982). Similarly $CD4^+$ cells alone restore heart allograft rejection in acutely irradiated hosts (Hall,de Saxe and Dorsch,1983) and renal allograft rejection in both acutely irradiated (Gurley, Lowry, and Forbes,1983) and nude hosts(Bolton, Gracie, Briggs et al,1989). Interpretation of these experiments is complicated, however, by the presence of host derived $CD8^+$ cells which have been found infiltrating grafts in ATXBM,acutely irradiated and nude hosts

respectively (Dallman and Mason,1982;Hall et al,1983 & Bolton et al,1989). Adoptive transfer of naive $CD8^+$ T cells to T cell deficient graft recipients is generally insufficient to initiate allograft rejection.

However,sensitised $CD8^+$ T cells have been found to mediate heart allograft rejection in acutely irradiated hosts (Lowry,Gurley & Forbes,1983; Herbert and Roser,1987),but not kidney graft rejection in nude hosts (Bolton et al,1989).

Recent studies in which the T cell requirements for grafts bearing isolated class I or class II MHC disparities suggest that both $CD4^+$ and $CD8^+$ T cells may contribute to rejection. Transfer of $CD4^+$cells alone causes rejection of class II disparate grafts and $CD8^+$ cells the rejection of class I disparate grafts.(Lowry and Gurley,1983; Sprent, Schaefer, Lo et al, 1986; Rosenberg, Mizuochi & Singer, 1986). Therefore the effector cell may depend on the nature of the MHC subregion disparity.

The demonstration that cloned $CD4^+$ cells can mediate cytotoxicity as well as $CD8^+$ cells (Krensky,Reiss,Mier et al, 1982; Spits,Borst,Terhorst et al, 1982; Strassman and Bach, 1984) underlines the fact that rejection associated with the transfer of $CD4^+$ cells does not necessarily imply a DTH mechanism. Kim,Rosenstein ,Weiland et al (1983) have demonstrated that skin graft rejection could be accelerated in sublethally irradiated mice by alloreactive, non-cytotoxic $CD4^+$ clones. However caution must be applied in interpreting the results of experiments using cloned cells as their in vitro and in vivo responses do not always correlate (Lynch,Weiland,Rosenberg et al, 1987).

1.6.6 Histologic and phenotypic analysis of the infiltrate

Early studies before the development of monoclonal antibodies to cell surface antigens showed that rejecting rat allografts were infiltrated by a heterogeneous population of mononuclear cells including T cells, B cells,and macrophages (Strom et al,1977; Hayry, von Willebrand; Soots et al,1979). More recent and detailed analysis has shown that most of the lymphocytes in rejecting rat renal allografts were T cells of the cytotoxic/suppressor phenotype (Renkonen,Soots, von Willebrand et al,1983; Christmas and McPherson,1982a;1982b). Interestingly, neither passive enhancement nor CyA treatment prevents the infiltration of rat renal allografts by leucocytes, but there is a predominance of $CD8^+$ cells in rejecting compared with passively enhanced rat kidney and heart allografts (Bradley,Mason & Morris,1985; Padberg,Lord, Di Stefano et al,1988). A preponderance of $CD8^+$ cells was also found in the rejecting grafts of rats which had received autologous blood transfusion compared with non-rejecting rats which had received donor-specific transfusion (Ruiz, Coffman & Howell,1988). Interestingly, neither passive enhancement nor cyclosporin treatment prevents the infiltration of rat renal allografts by leucocytes(Bradley et al,1985) . Similarly active and passive enhancement regimens did not alter the degree of cellular infiltration into cardiac allografts,though low dose cyclosporin given concomitantly reduces both the level and degree of activation of the infiltrate(Padberg et al,1988).

Studies on the nature of the cellular infiltrate in rejecting human renal allografts have produced variable results. Some have shown the infiltrate to be composed mainly of T cells (van Es, Meyer , Olijans et al 1984; Hall, Bishop ,Farnsworth et al(1984), while in others macrophages were the major cell type present (Hancock, Thomson and Atkins, 1983). The Oxford group has examined biopsies stained with monoclonal antibodies using morphometric analysis, to provide a quantitative measure of the area of the section infiltrated by cells of a particular phenotype (McWhinnie, Thompson, Taylor et al, 1985, 1986). T cells were found in greater numbers in rejection than during stable function, comprising 30-35% of the infiltrate, with monocytes and macrophages accounting for 60% and NK/K cells less than 10%. CD4:CD8 ratios were similar in both rejecting and stable grafts with CD8 cells always found in greater numbers than CD4 cells. This finding has been confirmed by some (Platt, LeBien and McMichael 1982; Hancock et al,1983) while others found greater numbers of $CD4^+$ cells (Tufveson, Forsum , Claesson et al, 1983; van Es et al, 1984). Such conflicting results could be due to the different monoclonal antibodies used, particularly those reacting with the $CD4^+$ cells, which react weakly in tissue section and label macrophages which may authentically express CD4.

It is difficult to determine the mechanism of rejection from such studies since one cannot correlate the presence of cells of a particular phenotype with functional activity in vivo. For this reason a number of groups have harvested the cells infiltrating rejecting grafts and assayed their functional activity in vitro.

1.6.7. Functional characteristics of cells infiltrating allografts

In a series of papers, Strom and Tilney's group, using mechanical and enzymatic disruption of human renal allografts and rat cardiac allografts, have identified cells mediating specific cytotoxicity and antibody dependent cell-mediated cytotoxicity(ADCC) (Tilney et al, 1975;Strom et al, 1977; Tilney,Notis-McConarty & Strom et al, 1978. Roberts and Hayry (1976), using a sponge matrix allograft model, identified anti-donor cytotoxic cells infiltrating the graft, a finding confirmed by Ascher's group using the same model (Ascher,Ferguson, Hoffman et al,1979). Nemlander,Saksela & Hayry,(1983) found high levels of both natural killer (NK) and specific cytotoxic cells in rejecting rat renal allografts.

Support for the role of the specific cytotoxic cell in graft rejection comes from the finding that rats receiving cyclosporin A or passively enhanced to prevent rejection do not have any demonstrable donor-specific cytotoxicity but do have high levels of NK activity(Mason & Morris,1984, Bradley et al,1985). Conversely it has been found that pre-transplant blood transfusion which results in prolonged survival of rat renal allografts does not prevent the appearance of cells in the grafts displaying donor-specific cytotoxicity(Dallman,Wood and Morris,1987; Ruiz et al,1988). However, one cannot conclude that cytotoxic cells are not important since their activity may be blocked in vivo by suppressor cells or antibody.

1.7.0. Transplantation tolerance

The first description of transplantation tolerance was made by Owen (1945) who observed that dizygotic twin cattle, which shared the same placental circulation at birth were tolerant to one another's red cells and other haemopoietic cells. These cattle twins were shown to accept skin grafts from each other but not from unrelated cattle. These findings led Burnet and Fenner (1949) to propose that lymphoid cells learned self tolerance during ontogeny and that antigens which reached the lymphoid tissues during the immunologically immature period would be regarded as 'self', thereby providing a means of distinguishing 'self' from 'non-self'. This prediction was confirmed by Billingham, Brent and Medawar (1953) when they induced specific tolerance to an allogeneic skin graft in adult mice by injecting the foetus with donor strain bone marrow cells. Such findings engendered hope that some form of antigen pretreatment could be devised which would lead to subsequent tolerance of a histoincompatible graft.

Two forms of specific immunosuppression have been extensively studied in animal models of transplantation;

a) Antigen induced suppression (ACTIVE ENHANCEMENT) where specific immunosuppression is induced by treatment of the recipients with preparations containing donor histocompatibility antigens.

b) Antibody induced suppression (PASSIVE ENHANCEMENT) where specific immunosuppression is induced by the administration to the recipient of antibodies directed against donor histocompatibility antigens.

Administration of donor blood is an example of the former and before describing this in detail I would first like to discuss briefly passive enhancement since part of this thesis discusses the possible role of transfusion-induced antibody in the mechanism of active enhancement and there may be parallels between the two models.

1.8.0. Passive Enhancement

1.8.1. Early observations

The term passive enhancement was coined by Kaliss and Molomut in 1952 when they showed that passively administered donor-specific alloantibody enhanced the growth of allogeneic tumours in the mouse. Interest in the phenomenon waned due to the inability to prolong skin graft survival by passive transfer of antibody. However with the advent of microsurgical techniques and the ability to perform vascularised organ allografts in inbred strains of rats, interest in passive enhancement was renewed. Stuart,Saitoh & Fitch (1968) were the first to report successful enhancement of renal allograft survival in inbred rats when they injected donor strain spleen cells intravenously 1 day before grafting followed by 1.0 ml of hyperimmune serum two hours before and one hour after grafting. French and Batchelor (1969) then demonstrated convincingly that the protective effect could be induced by alloantiserum alone. The advantage of this type of protocol over active enhancement,if it could be extrapolated to man,

would be that antiserum or combined antigen/antibody could be administered around the time of transplant, rather than several days before.

1.8.2. Factors influencing the induction of passive enhancement

In the rat renal allograft model,antiserum has a beneficial effect on graft survival, if given the day before transplant or up to two days after transplant(Morris, 1980). Dose response studies show that passive enhancement in the (DA x LEW)F1 to DA model can be achieved with as little as 10 ul of DA anti-Lewis antiserum while in the stronger (DA x LEW) F1 to LEW model 50ul may be necessary to prolong survival (Fabre & Morris,1972a;1973). Clearly there is a variation in effect due to strain combination and the ease of inducing enhancement is consistently greater in the heterozygous F1 to parental strains(Fabre & Morris, 1974). Different types of tissue allografts may also vary in their susceptibility to passive enhancement: the order of ease of enhancement of allografts being, kidney > cardiac > skin (Morris, 1980). The reasons for these differences are not fully understood but may partly reflect tissue antigen distribution,particularly class II antigens and whether or not the graft is primarily or secondarily revascularised.

1.8.3. Class and specificity of enhancing antibody

The donor specific antisera used to produce enhancement are usually generated by repeated immunisation of the recipient with donor lymphoid cells . Enhancing antibody is generally considered to be of the IgG class (Mullen, Raison & Hildemann,1977) although there is one report of enhancing activity associated with the IgA class (Voisin,Kinsky,Jansey et al,1969). Both non-complement fixing IgG1 and complement fixing IgG2 have been shown to mediate enhancement of rat renal allografts (Mullen et al, 1977). The Fc portion of IgG is important since preparations of $F(ab')_2$, are at least 100 fold less effective than whole IgG (Winnearls,Fabre,Millard et al, 1979).

Initially the bulk of evidence favoured anti-class II MHC antibody as the mediator of enhancement(Davies and Staines,1976). Davies and Alkins (1974) showed that enhancement of heterotopic cardiac allografts in the rat can be achieved by antisera absorbed with erythrocytes (which express only class I but not class II MHC antigens). These findings were substantiated by Staines ,Guy & Davies(1975) and McKenzie and Hemming (1977) using skin allografts between congenic mice and by Soulillou,Carpenter, d'Apice et al,(1975) in a rat renal allograft model. However there is contradictory evidence showing that anti-class I antibody may enhance skin graft survival in the mouse (Staines,Gray,Fish et al,1977) as well as cardiac and renal allograft survival in the rat (Jeekel,van Dongen,Majoor et al,1977; Gallico,Butcher & Howard,1979). Monoclonal antibodies to both class I and class II antigens have been found to

enhance survival of rat renal allografts (Stuart,McKearn & Fitch, 1979; Gallico et al 1979; Hart and Fabre, 1981c). Overall, however,the enhancing activity of hyperimmune serum is probably due to the anti-class II activity, since the immunisation protocol appears to favour the production of anti-class II antibody (Gallico and Mason, 1978; McKenzie,Fabre & Morris, 1980).

1.8.4 Mechanisms of passive enhancement

A number of alternative mechanisms have been proposed to explain passive enhancement namely: masking of graft antigens or opsonisation of graft dendritic cells by injected antibody; blocking of effector cells; clonal deletion; anti-idiotypic antibody; antigen-reactive cell opsonisation and suppressor cells. Similar mechanisms have also been proposed to explain active enhancement and there may be parallels between the two models.

The suggestion that injected alloantibody acts by masking graft alloantigens has been dismissed on the basis that the very small amounts of antiserum which induce enhancement would be insufficient to mask all of the graft alloantigens. In addition, it has been shown that ^{125}I-labelled anti-donor antibody initially binds to the graft,but then is lost within 48 hrs after transplantation(French and Batchelor, 1972). Another possibility is that small amounts of injected antibody label and then lead to opsonisation of donor class II positive dendritic cells in the graft. Dendritic cells are important for host sensitisation and long surviving kidney allografts obtained from passively enhanced recipients are not rejected when

retransplanted to unmodified recipients of the same genotype as the first recipient(Lechler and Batchelor,1982). Injection of recipients with donor dendritic cells restores rejection in this model. On the other hand,there is also evidence, that donor strain dendritic cells within a graft may be necessary for passive enhancement to be established (Hart & Fabre,1982).

Hutchinson (1980) proposed that when antigen reactive cells bind antigen in the presence of specific antibody reactive with other determinants, complexes are formed which are then removed by interaction of the Fc region of immunoglobulin with the Fc receptors on macrophages. This was termed Antigen-Reactive Cell Opsonisation (ARCO) and was based on the finding that when cells coated with antibody directed at a cell surface antigen are injected intravenously into a normal animal, the antibody coated cells are rapidly sequestered by Fc bearing macrophages in the reticulendothelial system (RES) (Hutchinson, 1980). This mechanism has been found to be operative in rats bearing passively enhanced kidney allografts (Hutchinson and Zola, 1977) and mice bearing enhanced tumour or skin allografts (Hutchinson and Brent, 1982).

The variable,antigen-combining regions of both antibody molecules and T cell receptors (TcR), can because of their unique conformation for specific antigen act as antigenic determinants (idiotypes). Antibody produced to the idiotypes of both antibody and TcR is known as anti-idiotype antibody and Jerne (1974) proposed that the immune system is regulated (both help and suppression) by idiotype-anti-idiotype interactions. After administration of spleen cells and alloantibody, Stuart, Scollard, McKearn et al (1976)

described an anti-idiotype response which peaked at day 10 after transplantation. They found that graft survival was enhanced if transplant was delayed until the peak of the anti-idiotype response. More recently Stuart, Fitch and McKearn (1982) showed some prolongation of renal allograft survival after immunising rats with an anti-idiotypic antiserum generated by immunising rats with an anti-class I monoclonal antibody. Presumably such anti-idiotype antibodies act by blocking antigen receptors on B and T cells for donor alloantigen, thereby preventing activation or by causing opsonisation of cells as envisaged in the ARCO mechanism.

Alternatively, passively administered antibody alone or in the form ofimmune complexes could block the effector arm of the immune response. There is little evidence for the presence of serum blocking factors since most groups have been unable to prolong survival in an unmodified host following the passive administration of serum from an enhanced rat (Morris, 1980). One exception to this rule is the finding by Tilney and Bell (1974) that passive transfer of serum from long surviving kidney allograft recipients to naive hosts prolonged kidney allograft survival. The reason for this discrepancy is not clear but prolonged survival may have been due to the absence of cytotoxic antibody at the time of transfer.

There is no convincing evidence that clonal deletion of alloreactive cells plays a role in passive enhancement. Animals with long term surviving grafts have a normal graft versus host (GvH) response in the popliteal lymph node assay and show cytotoxic

activity in vitro ,though this activity has been found to be delayed compared with rejecting rats (Biesecker, Fitch, Rowley et al,1973; Strom, Carpenter, Garaway,et al, 1975).

Suppressor cells have been found in passively enhanced rats by a number of groups. Batchelor, Phillips and Grennan (1984) looked for suppressor cells in the spleens of long term surviving rats bearing AS x AUG kidneys following passive enhancement. Previous attempts to adoptively transfer suppression from passively enhanced rats had failed (Batchelor, Brent and Kilshaw, 1977). It was therefore decided that an adoptive transfer system into rats bearing kidneys which were taken from an enhanced rat and were therefore depleted of passsenger cells would be more akin to the situation in the long term surviving rat. They found that spleen cells transferred significant suppression of rejection. Barber, Hutchinson and Morris (1984) used a similar protocol to transfer splenocytes or T cells from rats receiving a combined active and passive regimen into secondary hosts given passenger cell depleted kidney grafts. The latter were rejected in 15 days in the absence of "suppressor" cell transfer but survived for > 100 days if splenocytes or T cells from enhanced rats were administered using adoptive transfer into sublethally irradiated hosts. Hall (1985) demonstrated specific suppressor cells in the spleens of rats bearing passively enhanced rat cardiac allografts. Whole spleen cells were more effective that T cells alone at suppressing the ability of normal DA LNC to restore rejection, however, LNC and recirculating T cells from enhanced rats restored rejection.

A number of factors appear to be involved in the induction and maintenance of passive enhancement. However, attempts to passively enhance higher species such as dogs and monkeys have been largely unsuccessful, which has precluded the use of such a regimen in man, where the risk of hyperacute rejection is considered too great (Morris, 1980).

1.9.0. Active Enhancement

1.9.1 Early observations

I will review mainly the findings of experiments which have attempted to elucidate the mechanism of the blood transfusion effect in animal models with particular reference to the rat renal allograft model which is the subject of this thesis.

Earlier experiments on active enhancement were not necessarily concerned with blood components since the transfusion effect had not yet been described. One of the earliest descriptions was by Ockner, Guttman and Linquist (1970a) who showed that certain doses of bone marrow cells given at an optimum time prior to transplant could cause prolonged survival of rat renal allografts. The first group to show that donor blood given prior to transplantation could enhance survival of both renal and cardiac allografts in rats was Marquet,Heystek & Tinbergen(1971). This was confirmed by Jenkins and Woodruff (1971) in the rat cardiac model and by Fabre and Morris

(1972b) in the rat renal allograft model. As with passive enhancement there were a number of factors influencing the induction of active enhancement.

1.9.2. Factors influencing the induction of active enhancement

Fabre and Morris (1972b) found that several pre-operative injections of donor-specific blood led to long term survival of rat renal allografts. However, if only a single transfusion of whole blood was given the timing was critical. For example, blood given one day before grafting failed to produce active enhancement, while blood given one to two weeks before grafting led to prolonged survival.

As with passive enhancement the ability to induce active enhancement is strain dependent (Fabre and Morris, 1972b). The immune response to an allograft is under the influence of immune response (Ir) genes (Butcher and Howard, 1982) and since these control the strength of the rejection response it is likely that they are one of the important factors which influence the ease with which enhancement may be induced.

1.9.3 Blood components required for a "transfusion effect"

Several studies have attempted to elucidate which of the components of blood were responsible for the "transfusion effect", with much of the evidence leading to conflicting conclusions (Table A.1). Martin, Hewitt, Osborne et al (1982) found that while whole blood had the most potent enhancing activity, significant

enhancement could also be produced with purified populations of bone marrow mononuclear cells (BMM); thymocytes; red blood cells or platelets, but not with macrophages, plasma or blood lysate. El-Malik, Malik,Varghese et al (1984) found that red cells and B cells were more effective than whole blood in increasing graft survival in the LEW to DA rat renal allograft model. Jeekel, et al (1977) were able to produce enhancement of cardiac allograft survival in recipients by prior immunisation with donor erythrocytes (which carry class I but not class II MHC antigens). More recently Wood, Evins and Morris (1985) have convincingly shown that injection of highly purified donor erythrocytes can cause donor-specific prolongation of kidney graft survival. Furthermore, purified plasma membrane and endoplasmic reticulum expressing class I can also prolong survival of rat renal allografts (Foster, Wood and Morris , 1987). However pretreatment with water soluble class I MHC molecules , which do not elicit an antibody response,fail to prolong rat renal allograft survival (Priestley,Dalchau, Sawyer et al,1989). The majority of evidence seems to favour a role for erythrocytes in the transfusion effect but there are a few who have been unable to show this (Lauchart, Alkins and Davies, 1980; Hibberd and Scott, 1983). Platelets, which like erythrocytes only express class I, have also been shown by a number of groups to prolong graft survival in animal models (Batchelor, Welsh and Burgos, 1977; Hibberd and Scott 1983; Martin et al, 1982; El-Malik et al, 1984).

Both B and T cells have been found to prolong survival, however Lauchart et al (1980) was unable to produce an effect with T cells and El-Malik et al (1984) could do so only at low doses. Cranston,

Wood, Carter et al (1987) prepared purified populations of Lewis B and T cells by a rosette depletion method and found that both could enhance the survival of a Lewis kidney in a DA recipient. They extended these findings to show that $1x10^6$ B cells, T cells and T helper cells ($CD4^+$) prepared by flow cytometry could mediate enhancement, but the same number of $CD8^+$ (Tc/s) cells could not. Dose response studies were not carried out , so it is not clear whether a larger number of OX8 positive could cause enhancement.This study is more conclusive in implicating both B and T cells in the enhancing effect of blood since the methods used to prepare the sub-populations and to confirm their phenotype were more reliable than those used in earlier experiments. However, differences in dose and strain combinations may also have contributed to the contradictory findings. Donor dendritic cells which are strongly class I and class II positive do not seem to be good mediators of enhancement. El-Malik et al (1984) could find an effect only at very low doses and Peugh, Austyn, Carter et al (1987) using highly purified populations of dendritic cells were unable to enhance mouse cardiac allografts.

1.9.4 The role of class I and class II MHC antigens in the transfusion effect

The relative roles of class I and class II MHC products in the initiation of the blood transfusion effect have been examined using congenic rat strains. It has been shown in recipients of heart or kidney allografts pretreated with donor blood sharing class I,class II, or both with the graft donor, that class II antigens have

a strong immunosuppressive effect. While sharing of class I antigens between blood and graft donor led to prolonged graft survival, sharing of class I and class II together , had the greatest enhancing effect on both heart and kidney graft survival(Soulillou,Blandin,Gunter et al, 1984,Soulillou,Blandin & Gunter,1985; Hutchinson and Morris, 1986). However, an appropriate recombinant expressing only a class II gene product is not available to assess whether class II alone is enhancing. Furthermore, Hutchinson and Morris (1986) have found that they could enhance recipients against grafts bearing minor antigen differences by pretreatment with minor antigen plus third party class II products. The authors conclude that class II antigens alone induce unresponsiveness and are required to provide help to elicit unresponsiveness directed at class I and minor alloantigens. The role of class I and class II MHC antigens has been investigated further by pretreating murine recipients of cardiac allografts with mouse L cells transfected with class I and class II MHC genes of the donor (Superina,Wood & Morris, 1985;1987; Madsen, Superina, Wood et al,1988) They showed that pretreatment with either donor class I or class II antigens prolonged survival of cardiac allografts and was dependent on both the immunogenicity and the dose of MHC antigen.

1.9.5 Avoiding the sensitising effects of blood transfusion

In order to decrease the sensitising effect of donor antigen pretreatment, while still maintaining the enhancing effect, attempts have been made to alter the antigen or to administer antigen together with immunosuppressive drugs. A number of workers have

investigated the effect of CyA administered concomitantly with blood transfusion. It has been shown that a brief pre-transplant course of CyA combined with DST enhances the transfusion effect in a rat cardiac and renal allograft model (Miller, Martinelli, Racelis et al, 1982; Martinelli, Racilli, Giannone et al, 1983; Yasamura and Kahan, 1983). Attempts have been made to alter antigens by various treatments in order to decrease sensitisation while maintaining an enhancing effect. Cranston, Wood and Morris (1986) found that Lewis splenocytes which would normally enhance kidney graft survival in the Lewis to DA strain combination were ineffective after heat treatment or irradiation. Another group, using a different heat treatment protocol, found that administration of donor heat treated blood unlike fresh blood, did not induce a humoral cytotoxic response but still led to prolongation of cardiac allograft survival in the ACI to Lewis strain combination (Martinelli, Horowitz, Chiang et al, 1987). The beneficial effect of this protocol on graft survival was even greater when CyA was administered to recipients after transplantation.

Lenhard, Renner, Hansen et al (1985) looked at the effect of multiple Lewis blood transfusions on alloantibody levels in BN rats and found that cytotoxic antibody titres reached a maximum level after the second donor-specific blood transfusion. Therafter, administration of further blood transfusions resulted in a decline in antibody levels. This finding has important implications for donor-specific transfusion in the clinical setting where ideally one would like to maintain the maximum beneficial effect of transfusion but to minimise the degree of sensitisation. The influence of repeated transfusions alone or in combination with CyA on secondary alloantibody responses

was studied by Jones,Power,Cunningham et al,(1988). Lewis rats were first sensitised with DA splenocytes and then were given either no treatment,CyA alone,or weekly DA blood transfusions with or without CyA. CyA did not affect the spontaneous decline in alloantibody titers against class I antigens but did reduce significantly the response to class II antigens. It also prevented the maintenance of high anti-class I levels in rats repeatedly transfused. This suggests that transfusion given concomitantly with CyA could prevent the development of potentially harmful cytotoxic antibodies but it is not clear whether removal of these antibodies may diminish the beneficial effect of blood transfusion.

1.10.0. Mechanisms of the blood transfusion effect

Several different immunological mechanisms have been proposed to explain the beneficial effect of blood transfusion on allograft survival. These include clonal deletion, the development of serum blocking factors; Fc receptor blocking antibodies; anti-idiotypic antibodies and suppressor cells. The evidence favouring a particular mechanism is discussed in the next section.

1.10.1. Clonal deletion

Terasaki (1984),proposes that transfusion results in immunisation of the recipients such that upon transplantation, a vigorous anamnestic response occurs resulting in the rapid proliferation of alloreactive clones. The introduction of high dose

immunosuppression at the time of transplantation then kills or inactivates clones of reactive cells (clonal deletion). This model may play some role in the transfusion effect in the clinical situation but does not explain the prolonged survival in the rat model where exogenous immunosuppression is not required. Furthermore, Quigley, Wood and Morris (1988) have examined both lymphocyte proliferation and the generation of donor-specific cytotoxic cells in rats enhanced by donor-specific blood transfusion. Significant depression of the MLC response was found with LNC and thoracic duct lymphocytes (TDL) but not splenocytes harvested from enhanced rats. Specific cytotoxicity generated by in vitro MLC stimulation , however was augmented in the LNCs and TDLs, but not splenic lymphocytes compared with controls. Furthermore cytotoxic cells specific for donor alloantigen have been found in graft infiltrating cells harvested from rats receiving donor-specific transfusion (Dallman et al,1987;Ruiz et al,1988). The fact that there are cells capable of responding to donor alloantigen in transfused animals suggests that an active immunoregulatory mechanism is responsible for the absence of rejection in transfused animals and argues against the theory that alloantigen reactive cells are clonally deleted .

1.10.2 Serum blocking factors

In the early studies of active enhancement consideration was given to the possibility that serum factors were generated which blocked the effector response . However Jenkins and Woodruff (1971) failed to suppress cardiac allograft rejection by injecting serum

from rats given a donor-specific blood transfusion 14 days previously. Similarly, Hendry,Tilney, Baldwin, et al (1979) failed to prolong survival of cardiac allografts by the transfer of serum from rats which had received a combined protocol of active and passive enhancement at the time of cardiac transplant. Fabre and Morris,(1972c) were able to prolong survival of (DA X LEW)F1 to DA kidney by transfer of serum from (F1 to DA) actively enhanced long term surviving rats in 3 out of 4 animals,but in view of the small numbers of animals tested no firm conclusion could be drawn. Similarly, Nagata ,Ochiai,Asano et al(1984) found that serum but not spleen cells from rats transfused with donor blood 10 days previously prolonged cardiac allograft survival in sublethally irradiated hosts. They suggest that serum factors are important in the induction of the transfusion effect. The relatively small number of studies that have addressed this possibility do not rule out a role for serum factors in the enhancement of rat renal allografts.Furthermore, it is interesting that serum from long surviving recipients of orthotopic liver transplants (OLT serum) which are spontaneously accepted without pretreatment, can transfer enhancement to secondary hosts (Kamada Shinomiya, Tamaki et al, 1986). The same author has shown in an adoptive transfer assay with irradiated recipients that cells which would normally restore rejection are unable to if pretreated with OLT serum (Kamada, Sumimoto, Baguerizo, 1988).

1.10.3 Fc receptor blocking antibodies

In a series of studies MacLeod and her colleagues have shown that non-cytotoxic antibodies which inhibit EA rosetting with B lymphocytes (Fc blocking) develop following blood transfusion and correlate with graft survival(MacLeod, Mason, Stewart et al, 1982; MacLeod, Power, Mason et al, 1982). They have also found evidence that such antibodies are allo rather than auto-antibodies (MacLeod, Mason, Power et al, 1985; MacLeod, Stewart, Urbaniak et al, 1988). Forwell, Cocker, Peel et al (1987) also found Fc receptor(FcR) blocking antibodies in a proportion of sera obtained from transfused uraemic patients but could find no correlation with subsequent allograft survival. However, serum fractionation studies did reveal a striking correlation between graft survival and FcR blocking, mediated by a serum factor with a sedimentation coefficient of > 19S. It is not clear how such FcR blocking antibodies operate to induce a suppressive effect. If they do bind to, or near to, alloantigens then they could prevent allorecognition or could form complexes which are removed by macrophages by an ARCO mechanism.

1.10.4 Anti-idiotypic antibodies

Several groups have suggested that anti-idiotypic antibody may mediate enhancement by binding to the antigen binding site of T cells. Anti-idiotypic antibodies have been described both clinically and in animal models after blood transfusion. Fagnilli and Singal (1982) showed that sera from 17 multiply transfused uraemic

patients which had been depleted of lymphocytotoxic antibodies, bound to a higher percentage of T cells than untransfused controls as measured by indirect immunofluorescence. Singal, Joseph and Szewczuk (1982) found a correlation between graft survival and inhibition of MLC between donor and recipient. However, both studies involved very small numbers of patients and indirect evidence for anti-idiotypic interaction. Barkley, Sakai, Ettenger et al (1987) studied sera from post blood transfusion patients for the presence of anti-idiotypic antibodies to anti-HLA class I IgG. Such antibodies were assayed by their ability to inhibit an enzyme immunoassay for anti-HLA antibodies. Of the 16 patients studied, half showed inhibition which was specific for anti-HLA antibodies since no inhibition of other antigen antibody systems was found. It has been suggested that anti-idiotypic antibodies might modulate the appearance of anti-HLA antibodies following donor-specific blood transfusion and thus exert a favourable effect on graft outcome (Pohanka, Manfro, Oto et al,1989; Hillis, MacLeod ,Al-Muzairai et al, 1989).

Another study looked at the induction of anti-idiotypic antibodies following blood transfusion in mice (Singal, Ludwin, Joseph et al, 1986). Balb/c mice were given three blood transfusions from C3H mice. The Balb/c mice were bled a week later and the serum absorbed with C3H lymphocytes. IgG was prepared from this serum and normal mouse serum, and used to immunoprecipitate molecules on alloantigen activated (Balb/c anti-C3H) lymphocytes which had been labelled with 35S methionine. They were able to precipitate serum molecules from donor-specific lymphoblasts but not third party or syngeneic lymphocytes. The authors suggested that anti-idiotypic antibody was

binding and precipitating alloantigen-specific receptors on the recipient's T cells, but as they have not identified the molecules this is still speculative. Overall, therefore, the above studies provide good evidence that blood transfusion induces the development of anti-idiotypic antibodies but whether they play a role in enhancement has not been established.

1.10.5. Suppressor cells

Recent studies on the mechanism of enhancement have focussed on the possible role of suppressor T cells. Donor-specific suppressor cells have been found during both the induction and maintenance phase of active enhancement and are detected by adoptive transfer of lymphoid cells from enhanced recipients into naive hosts which are then challenged with an allograft. However, the naive hosts have to be lightly irradiated "to make space" for the adoptively transferred cells which makes interpretation of such experiments difficult. Tilney, Graves and Strom (1978) found suppressor cells in the thymus and spleen of rats conditioned by active and passive immunisation as early as one week after heart transplantation. The presence of an intact thymus is necessary for the induction of unresponsiveness since Hendry et al(1979) found that if rats bearing well functioning enhanced cardiac allografts were thymectomised between six and ten days post transplant this led to acute rejection. Spleen cells and thymocytes obtained from transfused rats bearing a cardiac allograft, are able, when adoptively transferred into sublethally irradiated

recipients bearing a heart graft, to prolong survival. This suppressive effect was not apparent when peripheral blood lymphocytes or lymph node cells were transferred (Marquet & Heystek,1981). Fractionation of suppressor spleen cells into T and B enriched populations and macrophages, showed that suppression was mediated by T cells. As discussed previously, alloantibody titres wane following successive donor-specific blood transfusions and this suppression can be adoptively transferred by splenocytes from multiply transfused rats(Lenhard et al,1985). Nagata et al (1984) who found a role for serum factors in the induction of the transfusion effect, conversely found that spleen cells but not serum harvested during the maintenance phase (30 days post transplant), were able to prolong survival of cardiac allografts. Similarly Singh,Marquet, de Bruin et al found that $OX8^+$ cells isolated from transfused rats 30 days after receiving a heart transplant could prolong survival of skin grafts when transferred to naive hosts. Hutchinson (1986) has found donor-specific suppressor cells in the spleens of long term surviving rat renal allografts following blood transfusion using an adoptive transfer assay. Furthermore he has shown that $CD8^+$ cells from long term surviving (LTS) kidney grafted rats prevent kidney but not heart graft rejection.

Recently, Quigley, Wood & Morris (1989a) examined the role of suppressor cells in the induction of the transfusion effect. Lymphoid cells were harvested from transfused and untreated rats and transferred either to lightly irradiated syngeneic hosts which were subsequently challenged with a kidney allograft or titrated as regulator cells into a naive unidirectional MLC. They found dose

dependent suppression of rejection by the adoptive transfer of thoracic duct lymph (TDL) or lymph done (LN) cells but not splenocytes from rats transfused 7 days previously. Similarly LN and TDL regulator cells transferred in vitro led to significant depression of a unidirectional donor/recipient MLC. These results correlated with the paper discussed earlier in which the same group showed that LN and TDL cells have reduced responsiveness to donor alloantigens in MLC, while splenocytes show increased proliferation (Quigley, Wood and Morris, 1988). Why the suppressive effect should be so dose dependent is not adequately explained, except to say that a fine balance must exist between suppressor and other effector cells. The same group found that the phenotype of these putative suppressor cells was $CD4^+$ and that they were found only transiently in the spleen and then in the TDL(Quigley,Wood & Morris,1989b,1989c). Shelby, Wakely and Corry (1984) also found suppressor cells in mice one week after donor specific blood transfusion, but they found them in the spleens. It is not clear from these studies why there is a discrepancy between the presence of suppressor cells in different lymphoid compartments in the various models. The phenotype of the suppressor T cell remains elusive but it appears that cells bearing either the CD4 or CD8 marker may be suppressive in different systems.

Recently,a hypothesis to explain both the specificity of the suppressive effect and the fact that both $CD4^+$ and $CD8^+$ cells can mediate suppression has been advanced (Batchelor,1989;Lechler, Lombardi,Batchelor et al (1990). It has been shown that rats with long surviving kidney allografts possess T cells which proliferate in response to autologous T cells specific for the kidney donor

alloantigen (Lancaster, Chui and Batchelor, 1985). Furthermore anti-idiotypic T cells can be generated in culture (Damle and Engelman, 1983; Mohagheghpour, Damle, Takada et al, 1986). These workers have grown $CD8^+$ T cell clones which proliferate specifically when stimulated with autologous $CD4^+$ cells harvested from specific but not third party MLRs and do not respond themselves to alloantigenic stimulator cells. Such $CD8^+$ cells are not demonstrably cytotoxic, but can inhibit $CD4^+$ T cells from proliferating in response to alloantigen. Recent evidence indicates that alloreactive T cells do not recognise allo MHC alone, but recognise allo MHC plus a peptide fragment (Marrack and Kappler, 1988). Lechler et al(1990) propose that $CD4^+$ and $CD8^+$ T cells bind to a complex of MHC plus peptide which would consist of an amino acid sequence derived from the idiotypic variable regions of the TCR of the alloreactive clones.

1.11 Aims of this study

It is clear from this survey of the literature that the precise mechanism of active enhancement has not been fully elucidated. It appears that both cellular and humoral factors may play a role in the beneficial effect of blood transfusion on renal allograft survival. The overall aim of this thesis is to examine the mechanism of prolonged allograft survival following donor specific blood transfusion using a rat renal allograft model. Much of the previous work has focussed on mechanisms maintaining the state of donor-specific unresponsiveness and so the intention in this study is to look more closely at possible mechanisms of induction of the enhanced state, both at the cellular and humoral level.

The rat model allows the immune response to defined histocompatibility differences to be examined, using inbred strains of rats. Furthermore the rat unlike the mouse has sufficiently large vessels such that anastamosis of the renal vessels may be performed with the aid of a dissecting microscope. While one must exercise caution in extrapolating the findings in an animal model, to the clinical situation one can hopefully gain information about, and a greater understanding of the "transfusion effect". This in turn may allow the development of strategies for inducing specific unresponsiveness in transplant recipients.

CHAPTER 2

MATERIALS AND METHODS

2.1 ANIMALS

Inbred male rats of the following strains were obtained from Harlan Olac Ltd, Bicester, Oxon: DA($RT1^a$), PVG($RT1^c$), Lewis(RT^l), Brown Norway ($RT1^n$), $(DAXPVG)_{F1}$ hybrids and PVG congenics $PVGRT1^{rl}$ ($RT1.A^aB^cC^cD^c$) and $PVG.RT1^{avl}$($RT1.A^aB^cC^cD^c$). Animals were kept in the animal facility of the University Department of Surgery, Western Infirmary Glasgow and maintained on standard rat diet and water. Rats were transplanted when 8-16 weeks old.

2.2 SURGICAL PROCEDURES

2.2.1 Renal transplantation

Kidney donors and recipients were anaesthetised with either a mixture of 5% Halothane in oxygen, or with diethylether, followed by an intraperitoneal injection of 0.7-1.2 ml of 7.5% chloral hydrate (BDH, Ltd). The dose of chloral hydrate required differed between rat strains. Renal transplantation was carried out essentially as described by Fabre and Morris [1975].

The donor rat was prepared first. The abdomen was opened through a midline incision and the intestines displaced and kept moist with a gauze swab soaked in saline. The left kidney, renal vessels and ureter

were then mobilised. This procedure was repeated in the recipient rat and after placing clips across the renal artery and vein, the renal vessels and ureter were cut to enable removal of the kidney.

The donor kidney was then transplanted into the left orthotopic site of the recipient rat with end-to-end anastamosis of the renal artery, vein and ureter using a 10/0 sterile polyamide interrupted suture (Ethicon Ltd). Cold ischaemia times were 25-35 minutes and during this period the kidney was kept cold by frequent application of small volumes of ice cold saline. Finally the intestines were replaced and the abdomen closed in two layers with a continuous 3/0 cat gut suture (Ethicon Ltd). Microsurgery was carried out with the aid of a Leitz Wild M650 microscope.

2.2.2 Contralateral nephrectomy

For graft survival experiments,contralateral nephrectomy was performed at the time of transplant by mobilising the right kidney, then positioning a 4/0 silk suture [Ethicon Ltd] around the right renal artery, vein and ureter followed by excision of the kidney.

2.3 Transfusion

Active enhancement of rat renal allografts was produced by the intravenous administration of 1.0ml of fresh heparinised donor strain blood, 7 days prior to transplantation.

2.4 HISTOLOGY

2.4.1 Antibodies

A range of mouse monoclonal antibodies [Mab] were used to label different rat leucocyte populations and MHC antigens as shown in Table 1.1. OX21, a monoclonal antibody against the human C3b inactivator (Hsiung, Brandon, Barclay et al,1982) was used as a negative control in all staining procedures. Peroxidase-conjugated rabbit anti-mouse Ig [Dako Ltd, High Wycombe] was used to detect primary monoclonal antibodies.

2.4.2 Preparation of cryostat sections

Tissue samples were embedded in OCT compound [Tissue-Tek, BDH Ltd], snap frozen in liquid nitrogen and 5μ sections cut with a Slee cryostat at -20^{o}C and transferred to multispot slides [C.A.Hendley, Essex] which had been gelatinised prior to use.

Table 1.1.

Mouse monoclonal antibodies to rat leucocyte antigens

Antibody	Specificity	Reference
MRC OX1	Common leucocyte antigen	Sunderland et al (1979)
MRC OX8	$CD8^+$ cytotoxic suppressor T cells, majority of NK Cells	Gilman et al (1982) Dallman et al (1982) Cantrell et al (1982)
W3/25	$CD4^+$ helper T cells, some macrophages	Brideau et al (1980) Barclay et al (1981)
MRC OX19	Peripheral T cells	Dallman et al (1982)
MRC OX39	IL-2 receptor	Patterson et al (1987)
W3/13	T cells, neutrophils and plasma cells	Williams et al (1977)
MRC OX18	MHC class I antigen	Fukomoto et al (1982)
MRC OX6	MHC class II I/A like antigen	McMaster et al (1979)
MN4-91-6	Polymorphic determinant of MHC class I. DA ($RT1^a$ positive), PVG ($RT1^c$ negative)	Milton & Fabre (1985)
F17-23-2	Polymorphic determinant of MHC class II, DA ($RT1^a$ positive), PVG ($RT1^c$ negative)	Hart & Fabre (1981) Milton & Fabre (1985)

2.4.3 Immunoperoxidase staining

Sections were fixed in acetone [BDH, Ltd] at room temperature [RT] for 10 min, wrapped in aluminium foil and stored at -70C until use. After rehydration in Dulbeccos A+B [DAB] for 5 mins. excess moisture was removed from around the section before adding 50ul of primary monoclonal antibody (appropriately diluted) to each section. Slides were incubated for 45 minutes in a humidified chamber and then washed three times in DAB. A 1:20 dilution of peroxidase-conjugated rabbit anti-mouse Ig [Dako Ltd], with 10% rat serum [to absorb out any cross-reacting activity], was added and the slides were incubated for a further 30 min at RT. After washing, the peroxidase substrate [0.6mg/ml 3,3-diaminobenzidine tetra hydrochloride, Sigma Chemical Co, plus 0.01% hydrogen peroxide] was added to each slide until there was a noticeable colour change. The substrate was then removed and the slides washed. Finally the slides were counterstained lightly with Harris's haematoxylin [BDH, Ltd] then dehydrated in alcohols and xylene before being mounted in DPX [BDH, Ltd].

2.4.4 Morphometric analysis of infiltrate

In order to assess the area of each immunoperoxidase-labelled tissue section infiltrated by cells of a particular phenotype, morphometric analysis was used employing the point counting technique (Aherne and Dunnill, 1982; McWhinnie et al, 1986). Sections were examined on a Leitz Dialux microscope at a magnification of 400 in the

presence of an eyepiece graticule, which had a square grid with either 121 intersections [1mm apart] or 745 intersections [0.5mm apart]. For each high power field, the number of positively stained cells superimposed by an intersection was counted and the area of the field occupied by cells of a particular phenotype was calculated as:

$$\% \text{ area of infiltrate} = \frac{\text{number of +ve grid intersections} \times 100}{\text{total number of grid intersections}}$$

The 745 point graticule was used for most sections except for those in which there was a heavy infiltrate in which case the 121 point graticule was utilised. Ten consecutive fields were counted for each section, so that for all sections the total number of points observed was well in excess of that required to maintain the accuracy of the point counting technique (Aherne and Dunnill, 1982). Slides were assessed independently by two observers (the author and Dr. E. Bolton) who had no prior knowledge of the time point or the group from which the kidney had been harvested. Each observer counted 10 adjacent high power fields of the kidney cortex and the results are expressed as the mean of the two observers.

2.5 MEDIA

2.5.1 Washing medium

Hanks buffered salt solution (HBSS) + 10mM Hepes + 5% heat inactivated fetal calf serum was used as a washing medium. Penicillin/Streptomycin were added during the preparation of sterile cell suspensions [Gibco,Ltd, Paisley, Scotland].

2.5.2 Complete medium

RPMI 1640 + sodium bicarbonate + 10mM Hepes + 10% FCS + 2mM glutamine + penicillin/streptomycin was used as a complete medium for cell culture [All from Gibco Ltd, Paisley, Scotland].

2.6 CELLS

2.6.1. Splenocytes

Spleens were placed in a petri dish with washing medium and gently teased apart with sterile plastic forceps. The cell suspension was then transferred to a plastic test tube and the debris allowed to settle out. The remaining cell suspension was transferred to another tube and washed at 200g for 10 minutes. The cell pellet was gently resuspended and the red cells lysed by addition of 3.0ml of distilled H_2O followed by 3.0ml of 2 X NaCl. Cells were washed twice, counted in trypan blue and resuspended at the appropriate cell concentration in complete medium.

2.6.2 Kidney graft infiltrating cells

In initial experiments freshly excised kidney grafts were placed in ice cold DAB/0.2% BSA and chopped up finely with a scalpel blade. The DAB was pipetted off and the kidney fragments transferred to another petri dish containing collagenase and hyaluronidase [Sigma Chemical Co, Poole, Dorset] at 1.0mg/ml in RPMI + 10mM Hepes. After 30 min incubation at 37^{o}C the pieces were passed gently through a stainless steel mesh in ice cold DAB/0.2% BSA. The resulting cell suspension was filtered through cotton gauze to remove debris, washed X 2 in ice cold DAB/0.2%BSA and finally resuspended in complete medium. Viability as assessed by trypan blue staining was usually 80% and the cell yield/kidney approximately 1- 3 x 10^{7}.

In later experiments an alternative method for preparing graft infiltrating cells was developed which gave better viability (>90%). After dicing the kidney, a cell suspension was produced by passage through a fine stainless steel mesh. This cell suspension was washed once and the mononuclear cells separated by Percoll centrifugation. Iso-osmolar 90% Percoll [Sigma Chemical Co.] stock solution was prepared in Ca^{2+} and Mg^{2+} free 10 X concentrated Hanks BSS containing 10mM Hepes buffer and this was further diluted with single strength HBSS/10mM Hepes to give 80% and 35% solutions of the stock 90% Percoll. The graft infiltrating cells were resuspended in 80% Percoll, layered underneath 35% Percoll and centrifuged at 500g for 30 min. The

mononuclear cells, free of dead cell debris and erythrocytes, were then retrieved from the interface and cell viability, assessed by trypan blue exclusion, was always >90%.

2.6.3 Concanavalin A stimulated lymphoblasts

Splenocytes from donor and third party rat strains were stimulated with concanavalin A (conA) to produce lymphoblasts for use as targets in ^{51} Chromium (^{51}Cr) release assays.

Splenocytes at a concentration of 2.0 X 10^6 cells/ml in complete medium containing 2 X 10^{-5} M 2-Mercaptoethanol were incubated with 5.0 mg/ml of con A [Sigma Chemical Co] in 25.0ml tissue culture flasks [Nunc] for 72 hours at 37^{o}C in a humidified CO_2 incubator.

2.6.4 Cell lines

The rat myeloma line Y3 (RT1^{u}: Galfre, Milstein and Wright, 1979) which is sensitive to natural killer cell mediated cytotoxicity was used as a target in cytotoxicity assays.

2.7 FUNCTIONAL ASSAYS

2.7.1 ^{51}Cr release assay

Graft infiltrating cells and splenocytes from transfused and unmodified PVG recipients were prepared as described previously and were tested for alloantigen-specific and non-specific cytotoxicity using a 6hr ^{51}Cr release assay. Donor alloantigen-specific cytotoxicity was assessed using ^{51}Cr-labelled DA con A blasts as targets, while LEW con A blasts were used as third party targets. Non-specific cytotoxicity was assessed using the rat myeloma Y3. Target cells were labelled with 5 Mega Becquerels (5MBQ) ^{51} Na-chromate activity and incubated at $37^{o}C$ with occasional agitation for 90 min. They were then washed X 5 and resuspended at 1.3 X 10^5 cells/ml in RPMI + 10mM Hepes + 5% FCS.

Graft infiltrating cells and splenocytes were prepared from experimental animals as described previously and resuspended at 1.3 X 10^7 cells/ml in RPMI + 10mM Hepes + 5%FCS. Doubling dilutions of effector cells were prepared to give effector : target ratios of 100 : 1; 50 : 1; 25 : 1; 12.5 : 1; 6 : 1 and 3 : 1 and each set up in triplicate or quadruplicate. Effector cells (75ul) were added to the wells of a 96 well V-bottomed plates [Sterilin] followed by 75ul of target cells.

Target cells were also incubated either with medium only (to measure spontaneous release) or Triton X [Sigma Chemical Co] to measure maximum release.

Plates were spun briefly, then incubated at 37°C in a humidified CO_2 incubator for 6 hours. After incubation, 75µl of supernatant was harvested from each well and released radioactivity determined with a Compugamma counter [LKB Pharmacia, Milton Keynes, U.K.]

The % specific ^{51}Cr release =

$$\frac{\text{Experimental Release - Spontaneous Release}}{\text{Maximum Release - Spontaneous Release}} \times 100$$

2.7.2 Popliteal lymph node assay

Graft infiltrating cells and lymph node cells prepared as described in sections 2.6.2 and 2.6.3 respectively were tested for their ability to mediate a regional graft versus host reaction (GvHR), using the Popliteal Lymph Node Assay of Ford, Burr & Simonsen (1970). Test cells were harvested and adjusted to $1x10^6$, $3X10^6$ and $9x10^6$ cells/ml in 0.1ml of DAB/2% FCS and the cell suspensions injected into the hind footpads of (DAXPVG)F1 rats. The animals were killed 7 days after injection and the popliteal lymph nodes weighed to an accuracy of 0.1 mg. The log median response of triplicate assays was then calculated for each dose level.

2.8 Fc RECEPTOR BLOCKING ASSAY

2.8.1 Preparation of antibody sensitised sheep erythrocytes (EA)

Rat anti-sheep erythrocyte serum was prepared by the method of Parish and Hayward (1974). Briefly, 5.0ml of sheep erythrocytes were washed 4 times in DAB, then 10^9 packed erythrocytes in 500ul of DAB were mixed with 500ul of Freunds Complete Adjuvant. 50ul of this suspension was then injected into the hind footpads of DA rats. Fourteen days later the animals were bled by aortic puncture and the collected serum was heat inactivated at 56C for 30 min, aliquoted and stored at -70^{o}C prior to use. A known volume of a 5% suspension of washed sheep erythrocytes was incubated with an equal volume of a 1 in 100 dilution of rat anti-sheep erythrocyte serum for 45 mins at 37C, then washed twice and adjusted to a 1.25% suspension in Hanks balanced salt solution (HBSS) plus 10mM Hepes.

2.8.2 Erythrocyte antibody rosette inhibition assay (EAI) assay

Lymphocytes were prepared from the spleens of normal DA (kidney donor strain) or Lewis (third party) rats in HBSS/Hepes plus 5% FCS. $1.25x10^6$ splenocytes were then resuspended in 125ul of test serum or serum fractions or in the appropriate controls (normal rat serum or PBS). Following incubation at 37^{o}C for 30min, cells were washed twice and resuspended in 175ul HBSS/Hepes and mixed with an equal volume of

1.25% sheep EA. Tubes were then centrifuged at 200g for 5 min to encourage rosette formation. Rosettes were gently resuspended and fixed in 250ul of 3% gluteraldehyde [BDH Ltd] for 20 min at room temperature (RT). Gluteraldehyde was then removed and the cells were finally resuspended in 125ul trypan blue and inspected under sealed coverslips. At least 400 lymphocytes per slide were counted (each slide being read blind on two occasions) and the percentage of rosetted lymphocytes determined. A rosette was defined as a mononuclear cell with 3 or more erythrocytes bound to it . EA rosette inhibition (EAI) was calculated as the percentage inhibition of rosette formation produced by test serum or IgG compared with controls. Typically, 20-30% of normal rat splenocytes formed EA rosettes in the absence of inhibitory factors.

A modification of the above procedure was developed in order to test small volumes of monoclonal antibodies and IgG preparations. Briefly, $2.5x10^5$ splenocytes in 25ul HBSS/Hepes were incubated in a microtitre plate at 37C for 30 min with 10ul test IgG at 1mg/ml. After washing twice, lymphocytes were rosetted with 50ul 2% sheep EA by centrifuging at 200g for 5min and fixed as before.

2.8.3 Preparation of Serum Fractions of graded molecular weight

Blood was obtained by aortic puncture and allowed to clot. Unfractionated sera were heat inactivated for 30 min at 56C and ultracentrifuged at 105,000g for 1hr to remove immune complexes prior to investigating their ability to inhibit EA rosette formation.

Serum fractions of graded molecular weight were prepared as described previously for human serum ,to yield six fractions of which fraction 2 contained the majority of serum IgM and fraction 4 contained the majority of serum IgG (Reid, Peel, Jarret et al, 1983). One ml of each of 4 sucrose solutions (20%, 27%, 33%, 40% sucrose weight/volume) was layered in a 0.5" X 2" polyallomer-tube. Onto this discontinuous sucrose gradient, 500ul of serum was layered and then ultracentrifuged at 105,000g for 16 hours at 4°C. This produced six serum fractions of graded molecular weight which were collected from the bottom of the gradient.

Fractions were screened by radial immunodiffusion to ascertain which contained IgM or IgG using a kit from Serotec, Oxford (Cat nos: AAR 08Z & AAR 02Z). Briefly 5ul of test serum was added to the wells of agar plates containing anti-IgG or anti-IgM . IgM was found in fraction 2 only, while IgG was found in fractions 3, 4, and 5, peaking in fraction 4. The fractions were also analysed by SDS polyacrylamide gel electrophoresis to confirm the position of other proteins within the fractions (Fig 1.4).

2.8.4 Purification of IgG by n-octanoic acid sedimentation

IgG was prepared according to the method of Steinbuch and Audran (1969). Approximately 6.0 mls of 0.06M sodium acetate buffer(BDH Ltd) pH 4.0 was added to 3.0 ml of serum. After gentle mixing, 225ul of n-octanoic acid [Sigma Chemical Co] was added to the buffer/serum mixture and stirred for 30 min at RT. The resulting precipitate was

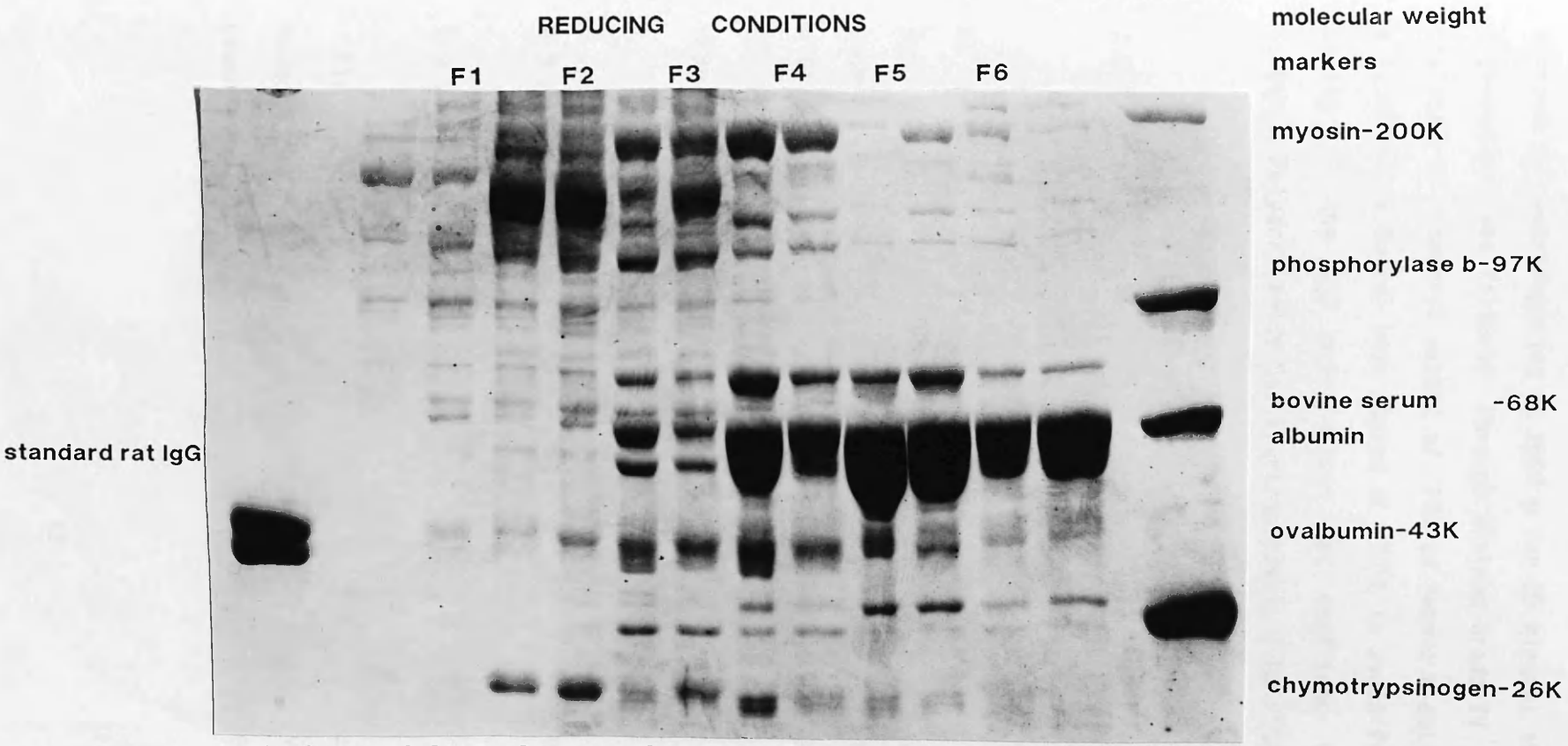


Fig 1.4 SDS-PAGE of serum fractions from an enhanced and rejecting rat prepared by discontinuous gradient centrifugation

Serum fractions 1 to 6 were prepared from an enhanced and rejecting rat by ultracentrifugation over a discontinuous sucrose gradient. Enhanced and rejecting serum fractions were separated by SDS-PAGE. The gel shows from left to right enhanced and rejecting serum fractions 1 to 6. Standard molecular weight markers were run alongside the serum fractions to determine the approximate molecular weight of components of the serum fractions.

removed by centrifugation at 2000 g for 20 minutes at RT. Finally, the supernatant was filtered through Whatman Grade IV filter paper and dialysed in several washes of PBS to remove n-octanoic acid. Small aliquots at 1.0mg/ml were stored at -70^{o}C to avoid freeze-thawing. The purity of the IgG preparations was confirmed by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis [SDS PAGE](Fig 1.5)

2.8.5 Absorption of IgG with erythrocytes and splenocytes

Fresh-Heparinised DA blood was washed 5 times in DAB with aspiration of the buffy coat and surface layer after each wash. Then, 200 ul of IgG (at 1.0mg/ml) was absorbed with 200ul of packed DA erythrocytes at room-temperature for one hour. Similarly 200 ul of IgG was absorbed with 10^{7} DA or PVG.RT1^{rl} splenocytes. After centrifugation at 1500g for 10 minutes, the supernatant was transferred to a second tube for a repeat absorption as before.

2.9 ANALYSIS OF SERUM FRACTIONS AND IgG BY GEL ELECTROPHORESIS

2.9.1 Running of gel

Electrophoresis in 8.5% Polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed essentially by the method of Laemmli (1970). The following stock solutions were prepared (See

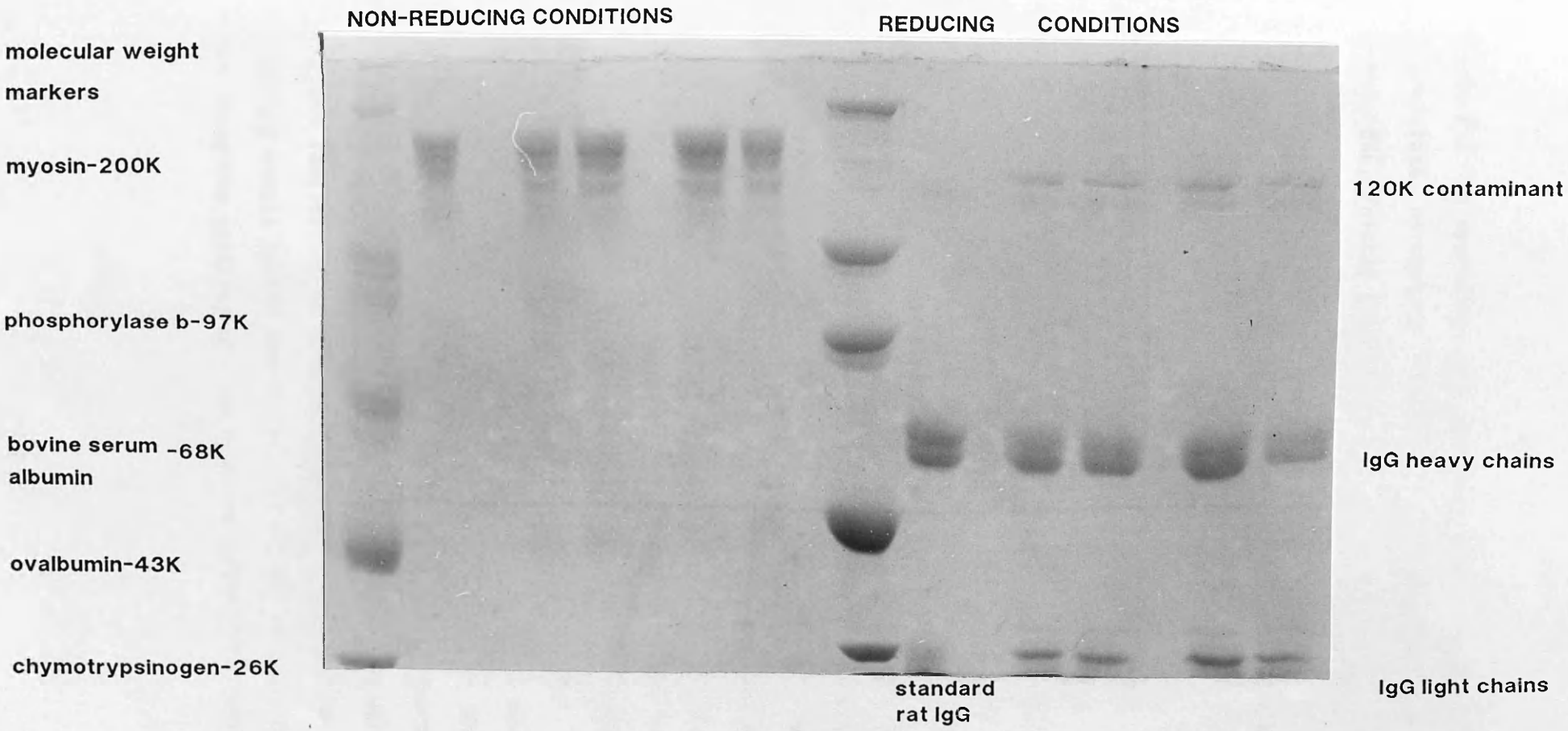


Fig 1.5 SDS-PAGE of IgG purified by n-octanoic acid sedimentation
IgG was purified by n-octanoic acid sedimentation and adjusted to 250ug/ml. 50ul of sample were added to both reducing and non-reducing buffers. Samples were reduced by boiling in a microcap tube for 3 minutes. Standard molecular weight markers and a commercial rat IgG standard were run alongside test samples.

table A.3 in appendix) and the quantities required to produce the appropriate percentage indicated. All reagents were purchased from Gibco, BRL, Paisley Scotland

Stock Solution	Running Gel (8.5%)	Stacking Gel (3%)
30% Acrylamide	12.0 (ml)	2.4 (ml)
Tris-HCl running buffer	12.0	-
Tris-HCl Stacking buffer	-	6.0
De-ionised water	24.0	15.6
10%ammonium persulphate	0.20	0.2
TEMED	0.02	0.01

Glass plates were washed thoroughly in decon, rinsed, then wiped with 70% alcohol before clamping together. The appropriate volumes of running buffer, acrylamide and deionised water were mixed together. Then, 2.0 ml of the resulting solution was removed and 200 ul of freshly prepared ammonium persulphate added followed by 20 ul of temed and this solution pipetted quickly between the plates to form a seal. Once the seal had set, the appropriate volume of ammonium persulphate and temed were added to the remaining gel solution and the gel poured immediately between glass plates separated by 1.5cm spacers. The gel was then overlaid with isobutanol to exclude air. When the gel had hardened the isobutanol was removed, stacking gel poured on top and a comb inserted between the plates to create sample wells. To reduce protein samples, 50 ul of each sample was mixed together with 10 ul of reducing sample buffer and the mixture boiled in a microcap tube for 3 min. Once the stacking gel had set, the combs were removed, the plates

clamped to the electrophoretic equipment and upper and lower tanks filled with their respective buffers. Protein samples were carefully layered onto the stacking gel and electrophoresis performed at a constant current of 40mA until the dye front was within 1 cm of the bottom of the gel. The gel was removed and stained as required.

2.9.2 Coomassie Blue Staining

Coomassie Blue stain was prepared by dissolving 0.3g of Brilliant Blue [Sigma Chemical Co] in 300 ml solution of H_2O, methanol and acetic acid (6:3:1). The resulting stain was filtered through grade 1 filter paper and stored at RT until required. Immediately after electrophoresis, the slab gel was placed in 200ml of Coomassie Blue and allowed to stain (1-16hrs). To destain, the dye was removed and replaced with approximately 200 ml of 10% acetic acid/10% methanol. The solution was replaced several times until the gel background was clear.

2.10 ALLOANTIBODY ASSAY

Antibodies against class I and class II ($RT1^a$) antigens were detected by a two-stage binding assay, essentially as described by Kamada (1986). Anti-class I antibodies were assayed using red cells from PVG.$RT1^a$ rats and anti-class II antibodies were assayed on PVG.$RT1^a$ lymph node cells.

2.10.1 Anti-class I assay

Round bottomed 96 well microtitre plates [Scotlab] were treated with 10% fetal calf serum (FCS) in DAB for 10 min (to prevent non-specific binding of rat Ig), washed with PBS and dried. Quadrupling dilutions of sera were prepared in DAB and dispensed into the microtitre wells (50ul per well). DAB was added to some of the wells to assess the background binding of the second antibody and hyperimmune serum was added to other wells as a standard control. Then 50ul of a 2.0% suspension of PVG.RT1^a red cells was added to each well and incubated for one hour at RT. Red cells were then washed four times and resuspended. Radio-iodinated, affinity purified sheep anti-rat Ig F(ab')$_2$ [Amersham International Ltd] was then dispensed into each well (100μl at a 1:320 dilution, giving 50 - 60,000 cpm per well), and incubated for a further hour. Finally the red cells were washed four times in the wells before transfer to clean RT25 tubes [Sterilin] for counting in a LKB Compugamma.

2.10.2 Anti-class II assay

Anti-class II antibodies were assayed by lymph node cell binding. Rat sera were first exhaustively absorbed with DA (RT1^a) red cells to remove anti-donor class I activity. This involved four successive absorptions of 200ul of packed DA red cells with 200ul of serum for

one hour at 4°C. Absorption was confirmed by testing of the serum in the class I assay for residual activity against $RT1^a$ red cells. The assay for anti-class II antibody followed the same procedure as the red cell binding assay except the targets were 50ul of lymph node cells (at 10^7/ml). All incubations were carried out at 4°C.

2.10.3 Complement dependent cytotoxicity assay

Serum samples were tested for cytotoxic antibody in a complement dependent cytotoxicity assay essentially as described by Winnearls,Fabre, Millard et al(1979). After heat inactivating at 56°C for 30min,sera were centrifuged briefly to remove protein aggregates, then serially diluted in RPMI/5%FCS/10mM Hepes.50ul of each dilution was then mixed with $5x10^4$ ^{51}Cr-labelled Con A lymphoblasts prepared from donor and third party strain splenocytes. Following incubation for 30 min at RT,100ul of freshly reconstituted guinea pig complement [Sera-Lab, Sussex] diluted 1:5 was added , and the cells were incubated for 1hr at RT. Finally 100ul of the supernatant was removed and counted in a gamma counter. Maximum and minimum release were measured as for the cellular cytotoxicity assay and the % cytotoxicity calculated as before.

2.10.4 Measurement of IgG and IgM component of the alloantibody response

Initially, 96-well round bottomed plates [Scotlab] were blocked with 2% BSA/DAB for 1hr at RT then washed in DAB and dried before use. Serum samples to be tested were diluted 1:2 in 1% BSA/DAB and 25ul added in triplicate to the plates. DAB controls were added to determine the background binding of the second antibodies. To measure the IgG and IgM component of the class I response unabsorbed sera were incubated with 50ul of 1% DA red cells for 1hr at RT. To measure the IgG and IgM component of the class II response ,DA red cell absorbed sera were incubated with 50ul of DA LNC at $1x10^7$/ml in 0.2% BSA/DAB at 4^oC. After washing three times with 1% BSA/DAB ,50ul of a 1:50 dilution of either biotinylated anti-rat IgG or anti-rat IgM were added and incubated for 1hr. Cells were washed three times and 50ul of a 1:50 dilution of ^{125}I-labelled streptavidin [Amersham International] added for 30 minutes. After four washes in 1% BSA/DAB cells were harvested into LP3 tubes and the cpm measured in a gamma counter. The cpm in 50ul of a 1:50 dilution of ^{125}I-streptavidin was measured and the % binding calculated as:

$$\frac{100 \times \text{Test} - \text{Background}}{\text{CPM Added} - \text{Background}}$$

2.11 MIXED LYMPHOCYTE REACTION

Allogeneic one-way mixed lymphocyte reactions (MLR) were set up to assess the inhibitory activity of serum or IgG from enhanced and rejecting rats. Initial experiments indicated that LNC responded better in MLR than splenocytes and therefore LNC were used as responders in all subsequent experiments. LNC were prepared and resuspended at a final concentration of 8.0 X 10^6/ml in complete medium containing 2 X 10^{-5}M ß-Mercaptoethanol and 5% normal rat serum. Splenocytes of either DA (donor-specific) or LEWIS (third party) rat strains were prepared as described previously and irradiated (2000R) before use as stimulator cells. The cells were washed after irradiation and resuspended at 8.0 X 10^6/ml in medium as before. In experiments where the inhibitory activity of serum was assessed, the concentration of normal rat serum added was adjusted so that the final concentration in each well was always 5%.

Doubling dilutions of either serum or IgG (50ul per well) were prepared in triplicate in 96 well sterile round-bottomed microtitre plates [NUNC] to which 4.0 X 10^5 responders and 4.0 X 10^5 stimulators per well were added to give a final volume of 200ul in each well. Control wells contained medium only or normal IgG. Plates were incubated at 37°C in 5% CO_2 for 4 days, pulsed with ^3H Thymidine (1uCi per well) for 16-18 hours, then harvested using a Dynatech Autowash automatic cell harvester. Finally the discs were placed in Ecosint scintillation fluid and counted in a Beta counter (LKB 1209).

2.12 STATISTICAL ANALYSIS

Statistical analysis of data was performed using the Mann-Whitney U test. A p value of 0.05 or less (two tailed) was considered significant.

CHAPTER 3

PHENOTYPE AND FUNCTION OF CELLS INFILTRATING REJECTING AND ACTIVELY ENHANCED RAT RENAL ALLOGRAFTS

3.1 Introduction

In the rat,renal or cardiac allografts undergoing unmodified rejection are characterised by a heterogeneous mononuclear cell infiltrate which is comprised of T cells, B cells, macrophages and NK cells (von Willebrand et al,1979; Renkonen et al,1983; and Strom et al, 1977). Associated with this infiltrate, is a striking increase of donor class I and class II MHC antigen expression (Milton & Fabre,1985; Milton et al, 1986a). Analysis of the functional activities of cells within rejecting renal and cardiac allografts has shown that cells displaying both specific and non-specific in vitro cytotoxicity can be isolated (Tilney et al,1975; Strom et al, 1977; Nemlander et al,1983).

Interestingly, although CyA treatment and passive enhancement, prolong allograft survival,they do not prevent leucocyte infiltration of renal allografts.However,they do affect the functional activities of the graft infiltrating cells, since cells isolated from these non-rejecting grafts do not display donor-specific cytotoxicity (Bradley et al,1985). Furthermore induction of class I and class II MHC antigens is reduced or absent in CyA treated rats and delayed in passively enhanced rats (Milton, Spencer, Fabre et al, 1986b; Priestley and Fabre,1989).

In view of these findings it was of interest to examine the phenotype and function of cells infiltrating rat renal allografts enhanced by preoperative donor blood transfusion and compare the

results with renal allografts undergoing unmodified rejection. The pattern and degree of class I and class II MHC expression in enhanced and rejecting rats was also studied using immunohistological techniques.Since there is no strict correlation between phenotype and function,the in vitro cytotoxicity of graft infiltrating cells harvested from enhanced and rejecting kidneys was also measured.

Results

3.2 Ability of preoperative blood transfusion to prevent allograft rejection

Preoperative blood transfusion is known to produce long term renal allograft survival in some but not all rat strain combinations (Fabre & Morris ,1972b). In most of the experiments in this thesis, the fully allogeneic DA into PVG strain combination was used. This strain combination was chosen because active enhancement of a renal allograft could be induced readily by preoperative blood transfusion and also because monoclonal antibodies were available which specifically label DA (donor) and not PVG (recipient) MHC thereby allowing unequivocal determination of donor MHC expression. Confirmation that the i.v. administration of DA donor blood 7 days prior to transplantation led to long term graft survival whereas unmodified PVG recipients rapidly reject their grafts is shown in

Table 1.2.Using this protocol,it was possible to make a comparison between unmodified (rejecting) and transfused (enhanced) rats in future experiments.

3.3 Kinetics of leucocyte infiltration into enhanced and rejecting rat renal allografts.

An analysis was made of the leucocyte infiltrates in rejecting DA kidneys from unmodified PVG recipients (rejecting) and non-rejecting kidneys from PVG recipients which had received a DA blood transfusion 7 days before transplantation (enhanced). Grafts were excised on days 1,3,5 and 7 post transplant(5 grafts/group/time point) and cryostat sections labelled with a range of mouse monoclonal antibodies to rat leucocyte antigens using the indirect immunoperoxidase technique . Sections were counted by the point counting technique in order to give a quantitative assessment of the area of each section that was occupied by cells of a particular phenotype.

The total leucocyte infiltration (determined by labelling with OX1,which binds to the common leucocyte antigen) is shown in Fig 2.1. Infiltration occured more rapidly in enhanced than rejecting grafts (at day 3, the % area infiltrate was 31+/-8 vs 11+/-3)($p<0.05$).(Fig 2.2a and 2.2b). By day 5 however there was a marked increase in the number of leucocytes infiltrating rejecting grafts, which was not significantly greater than that found in enhanced grafts,($p,0.05$). Day 7 rejecting grafts were too severely damaged to examine immunohistologically but levels of OX1 positive cells in day 7 enhanced grafts were similar to those at day 5. All the results shown

Table 1.2 Survival times for transfused and unmodified PVG recipients of DA kidneys

Donor-to-Recipient	Recipient pretreatment	No of animals	Survival (days)	MST[b] (days)
DA($RT1^a$) to PVG($RT1^c$)	None	8[a]	7,7,7,7,7,8,8,10	7
DA($RT1^a$) to PVG($RT1^c$)	1.0ml DA whole blood at day -7	5[a]	>100,>100,>100, >100,>100	>100

(a) Contralateral nephrectomy performed at day 7.

(b) Median survival time.

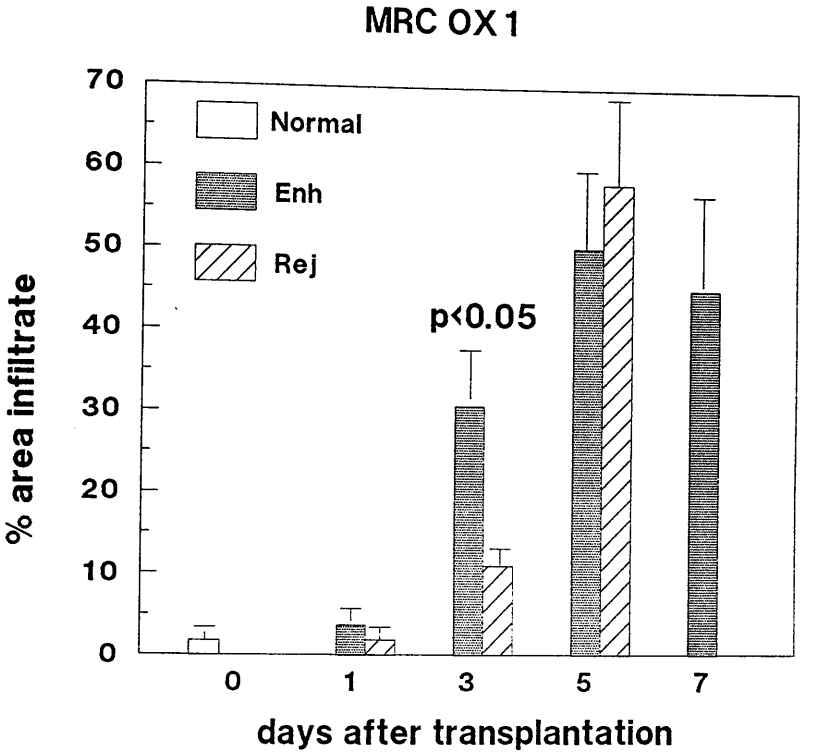


Fig 2.1 Morphometric analysis of OX1 infiltration into DA renal allografts in unmodified and transfused PVG recipients
DA grafts were removed on different days after transplantation from unmodified(n=5) and transfused (n=5) PVG recipients. Cryostat sections were stained by immunoperoxidase with MRC OX1(against the leucocyte common antigen), and the % area infiltrate of leucocytes was determined by morphometric analysis.Normal DA kidneys(n=4) were stained as a control.The results are expressed as the mean and standard deviation.

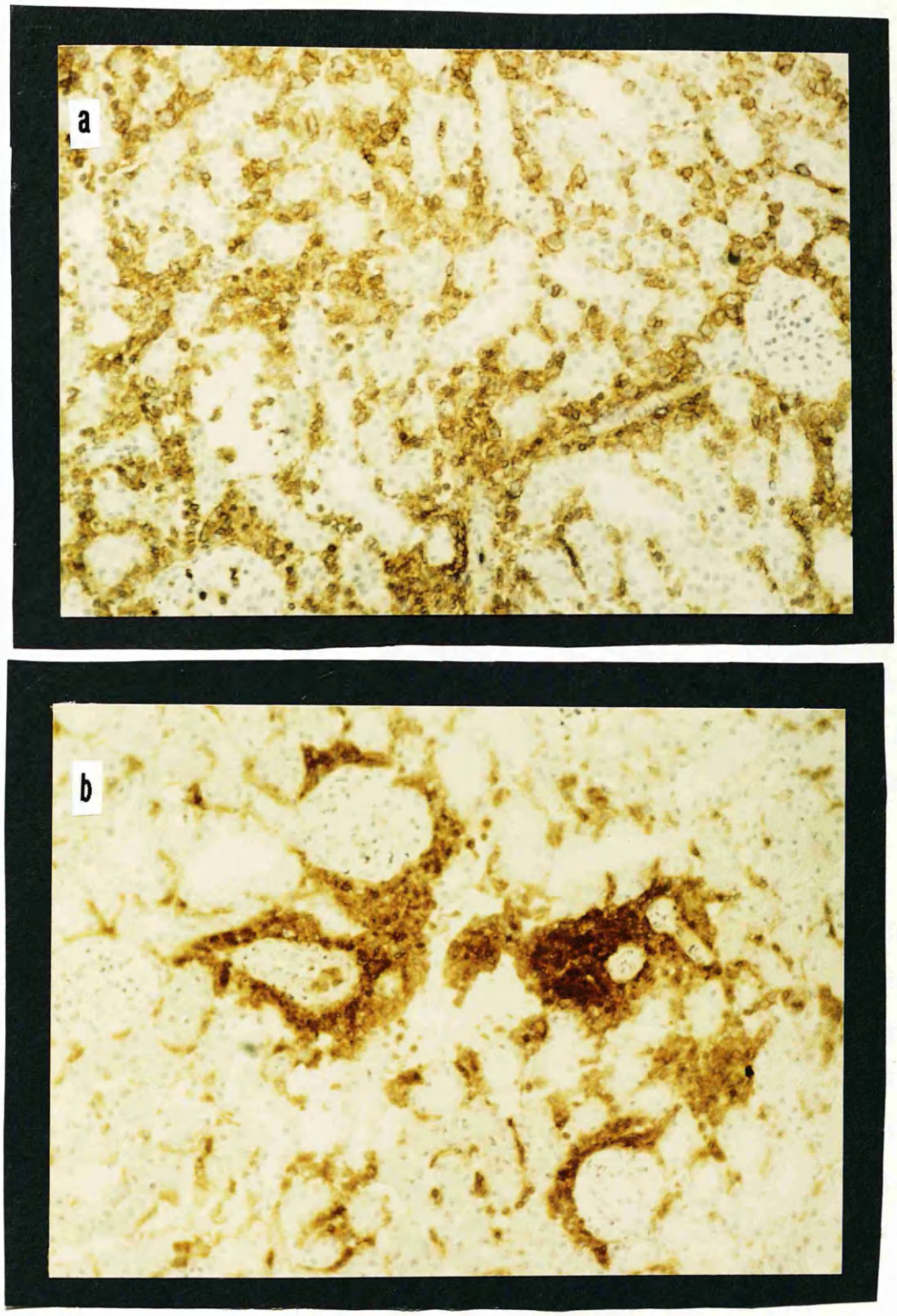


Fig 2.2. MRC OX1 labelling of enhanced and rejecting DA allografts in PVG recipients (day 3)

Cryostat sections of enhanced and rejecting DA allografts, 3 days after transplantation, were immunoperoxidase labelled with MRC OX1(common leucocyte antigen).
(a) Enhanced kidney labelled with OX1 (x200)
(b) Rejecting kidney labelled with OX1 (x200)

for the morphometric analysis are expressed as the mean of two observers (the author and Dr.E.Bolton). There was close agreement between the two observers for OX1 staining as shown by the high correlation coefficient (r=0.99) (Fig 2.3),and the low mean % difference between the two observers D = 6.7% . No attempt was made by the two observers to count the same area of the tissue section, therefore the close correlation between the observers indicated that the area counted was representative of the whole section. There was no apparent difference in the anatomical distribution of leucocytes within rejecting and enhanced grafts. Initially the infiltrate was focal and perivascular,however this progressed with time to become more diffuse with leucocytes clustering around the tubules and glomeruli , though never within the glomerulus itself. On day 3 after transplantation the rejecting infiltrate was focal but in the enhanced grafts had already become more diffuse(Fig 2.2a and 2.2b).

Fig 2.4 shows the results of staining with OX8 (which labels Tc and most NK cells). On day 3 post transplant there were significantly greater numbers of OX8 positive cells in the enhanced grafts compared with rejecting grafts(area infiltrate,6.5% +/- 2% vs 1.9% +/- 0.9%). Between days 3 and 5 there was a large influx of OX8 positive cells into rejecting grafts, so that by day 5 there were significantly greater numbers of $OX8^+$ than in enhanced grafts.($p<0.01$). The pattern of staining on day 5 post transplantation is shown in Fig 2.5a and 2.5b. Again there was close correlation between the two observers when counting OX8 positive cells (r=0.98,mean % difference D=6.7%).

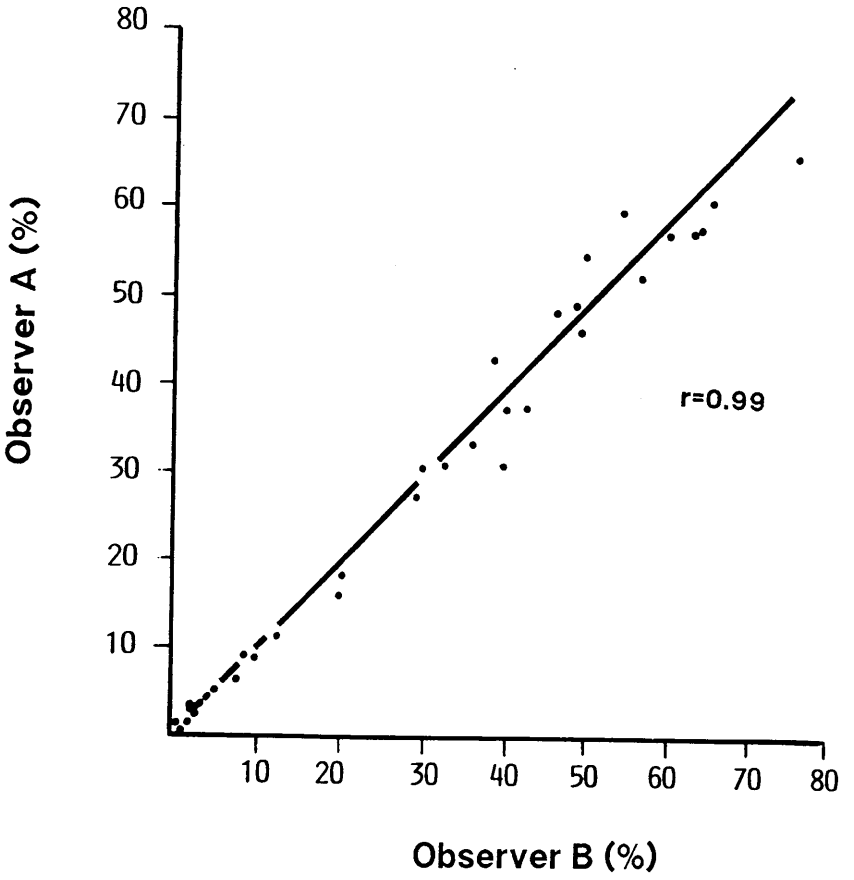


Fig 2.3 Correlation curve of observer A vs observer B for OX1
Counts of observer A were plotted against observer B for OX1 labelling and the correlation coefficent r was calculated.

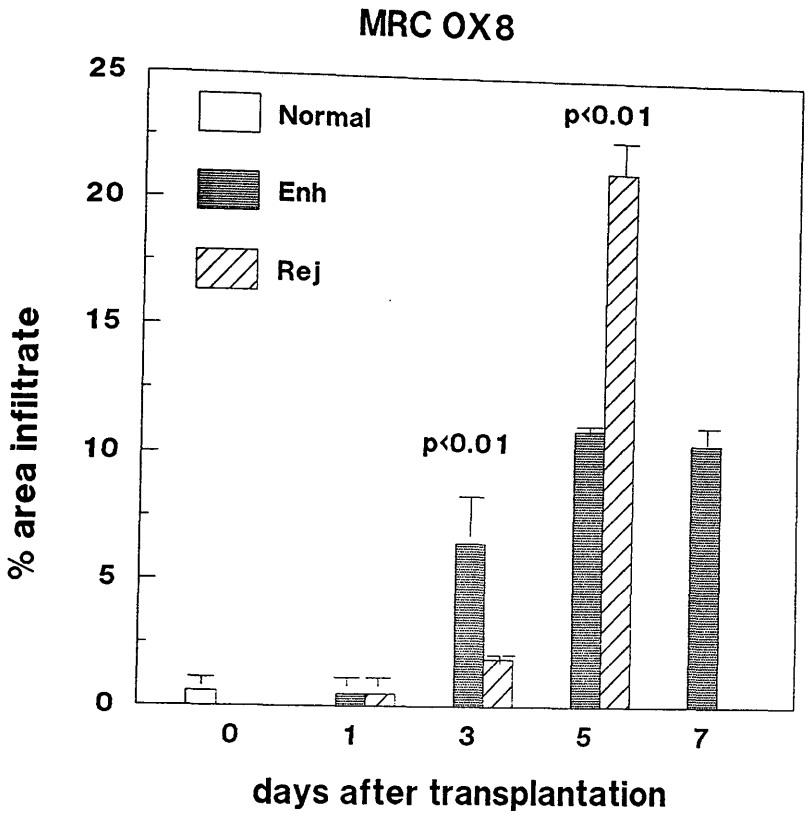


Fig 2.4 Morphometric analysis of OX8 staining of DA renal allografts in unmodified and transfused PVG recipients

DA grafts were removed on different days after transplantation from unmodified (n=5) and transfused (n=5) PVG recipients.Cryostat sections were stained by immunoperoxidase with MRC OX8 (against Tc and NK cells), and the % area infiltrate of labelled leucocytes was determined by morphometric analysis. Normal DA kidneys were stained as a control.The results are expressed as the mean and standard deviation.

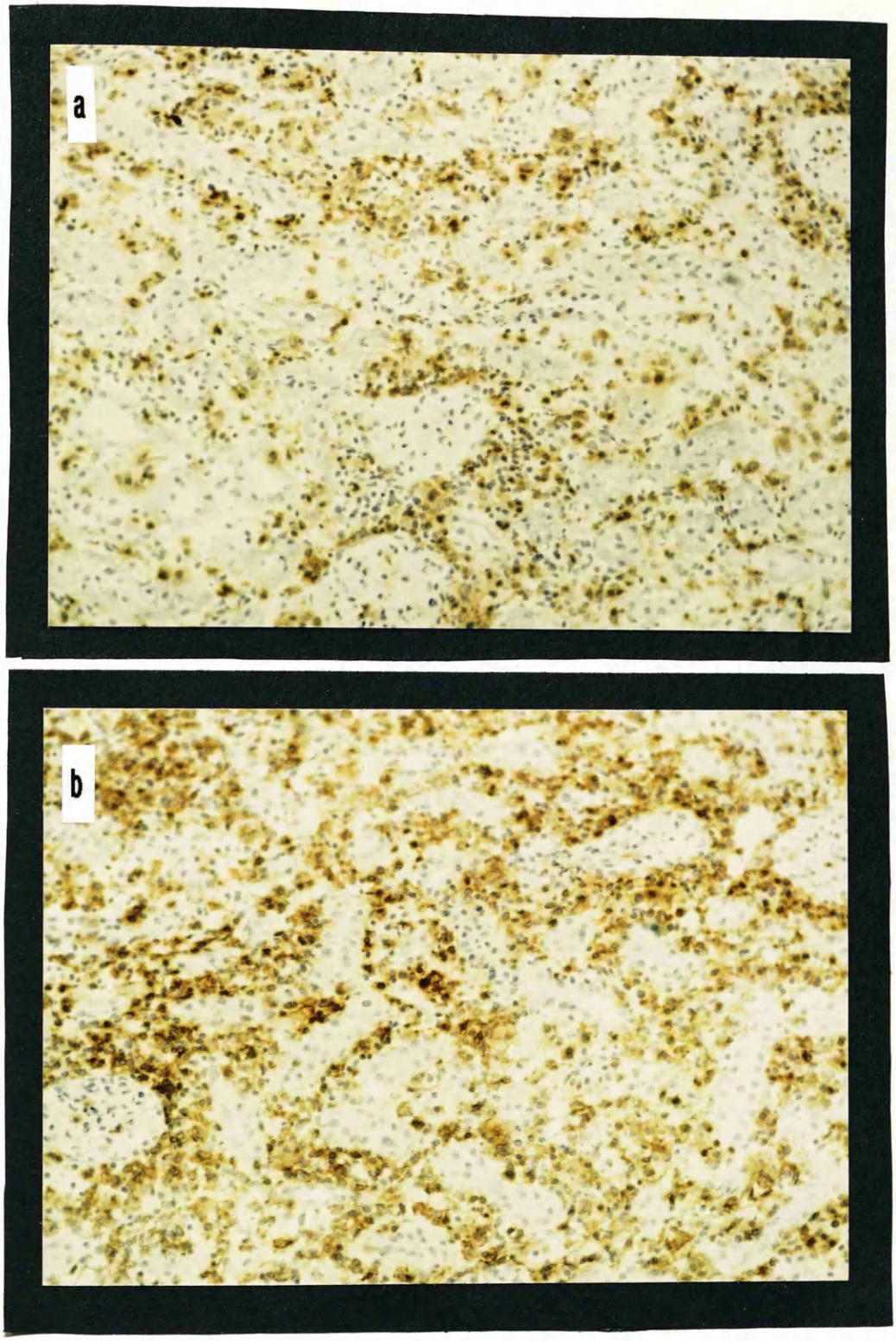


Fig 2.5 MRC OX8 labelling of enhanced and rejecting DA allografts in PVG recipients (day 5)

Cryostat sections of enhanced and rejecting DA allografts,5 days after transplantation were immunoperoxidase labelled with MRC OX8(labels cytotoxic T cells and NK cells)
(a) Enhanced kidney labelled with OX8 (x200)
(b) Rejecting kidney labelled with OX8 (x200)

There was a progressive infiltration of W3/25 positive cells (Th cells and macrophages) into both enhanced and rejecting grafts with no significant difference between the two groups at any of the days tested (Fig 2.6). The percentage area infiltrated by W3/25 positive cells was consistently less than that infiltrated by OX8 positive cells in both groups. Staining with W3/25 was always weaker and therefore more difficult to count than with OX8 ,reflected in the lower correlation coefficient (r=0.85) and mean % difference(D=16%) between the two observers.

To assess whether infiltrating cells simultaneously expressed the W3/25 and OX8 antigens , sections were labelled with a mixture of the two antibodies and counted as before. The results of the morphometric analysis are shown in Fig 2.7a. The percent area infiltrate determined in these sections was similar to that calculated as the sum of MRC OX8 positive and W3/25 positive cells for each section (Fig 2.7b), indicating that few of the infiltrating cells expressed both the W3/25 and MRC OX8 antigens simultaneously.

It can be seen in Fig 2.8 that the percentage area infiltrated by OX19 positive cells (pan T cell marker) in both rejecting and enhanced grafts is surprisingly low(maximum infiltration less than 6%) and is easily exceeded by the sum of W3/25 positive and OX8 positive cells . This may be due to labelling of NK cells which are $OX8^{+}OX19^{-}$ or macrophages $W3/25^{+}OX19^{-}$ or alternatively by weak labelling with OX19. Again, enhanced grafts were more quickly infiltrated than rejecting grafts (mean % area infiltrated by OX19 positive cells of 2.9% +/- 0.9% vs 1.6% +/- 0.9% at day 3,$p<0.05$, (Fig 2.9a and 2.9b). By day 5 the numbers of OX19 positive cells had

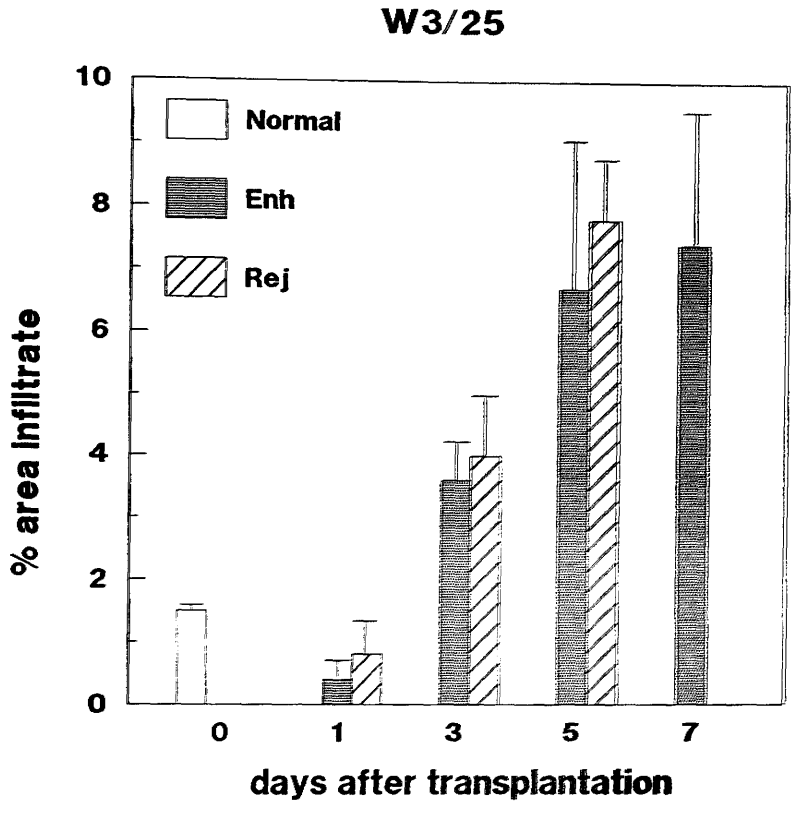


Fig 2.6 Morphometric analysis of W3/25 staining of DA renal allografts in unmodified and transfused PVG recipients
DA grafts were removed on different days after transplantation from unmodified (n=5) and transfused (n=5) PVG recipients. Cryostat sections were stained by immunoperoxidase with W3/25 (against Th and macrophages), and the % area infiltrate of labelled leucocytes was determined by morphometric analysis. Normal DA kidneys were stained as a control. The results are expressed as the mean and standard deviation.

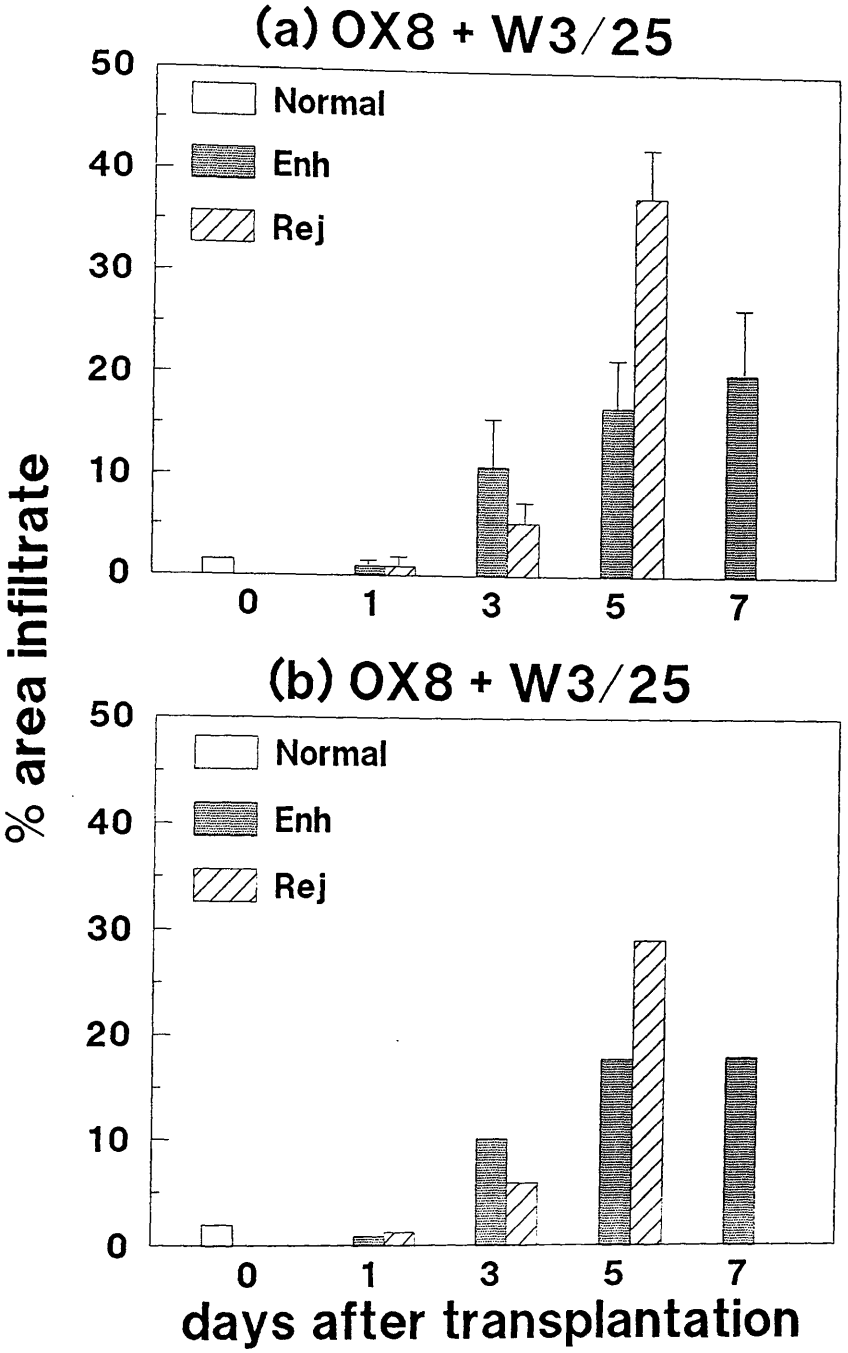


Fig 2.7 Comparison of % area infiltrate obtained when grafts were stained by a mixture of OX8 and W3/25 with that obtained by calculating the sum of OX8+W3/25
DA grafts were removed on different days after transplantation from unmodified (n=5) and transfused (n=5) PVG recipients. Cryostat sections were stained by immunoperoxidase with a mixture of MRC OX8 and W3/25 and the % area infiltrate of labelled leucocytes was determined by morphometric analysis. Results are expressed as the mean and standard deviation (a). Also shown is a graph of the % area infiltrate obtained when the sum of the observed values for OX8 and W3/25 was calculated(b).

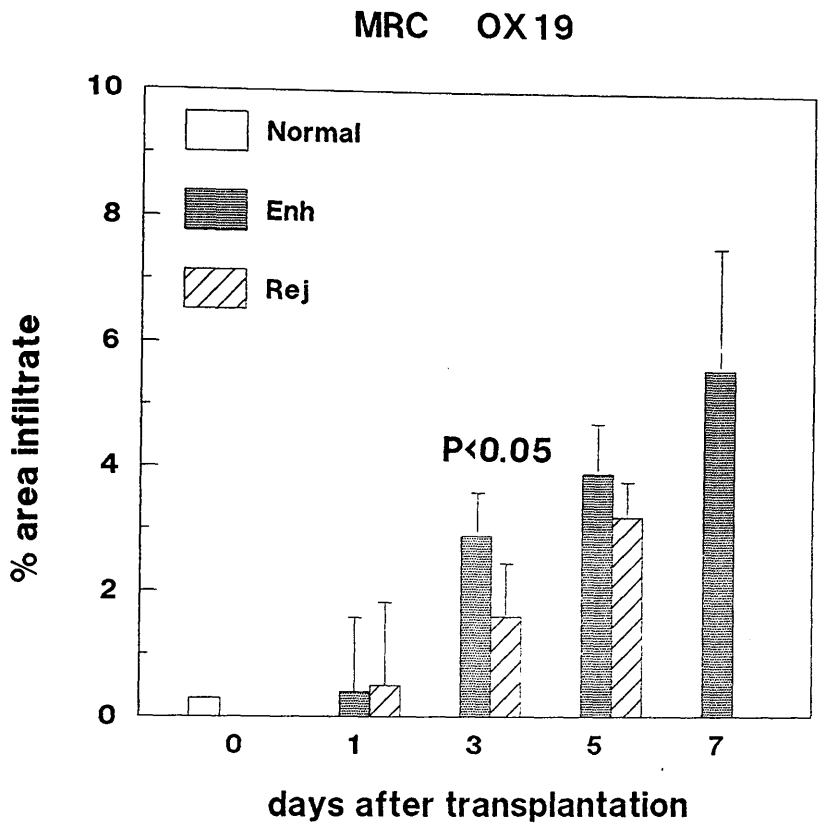


Fig 2.8 Morphometric analysis of OX19 staining of DA grafts in unmodified and transfused PVG recipients

DA grafts were removed on different days after transplantation from unmodified (n=5) and transfused (n=5) PVG recipients. Cryostat sections were stained by immunoperoxidase with MRC OX19 and the % area infiltrate of labelled leucocytes determined by morphometric analysis. Normal DA kidneys were stained as a control. Results are expressed as the mean and standard deviation.

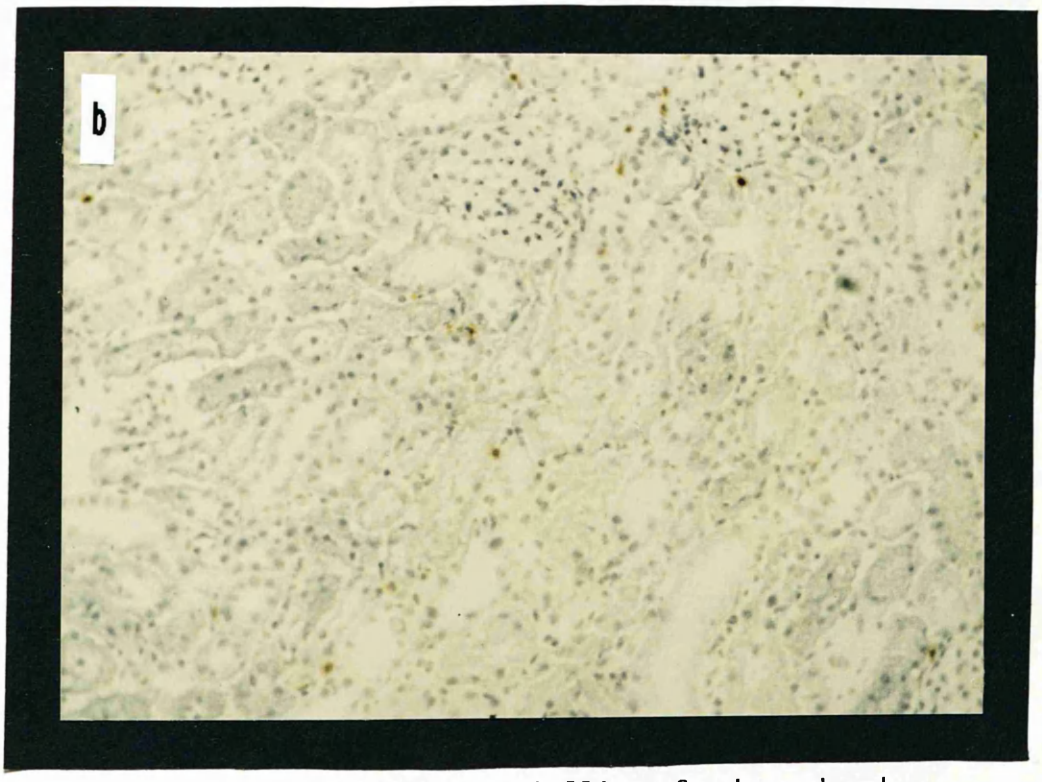


Fig 2.9 MRC OX19 labelling of enhanced and rejecting DA allografts in PVG recipients (day 3)
Cryostat sections of enhanced and rejecting DA allografts, 3 days after transplantation were immunoperoxidase labelled with MRC OX19(labels all T cells).
(a) Enhanced kidney labelled with OX19 (x200)
(b) Rejecting kidney labelled with OX19 (x200)

increased slightly but there was no significant difference between the groups. The kinetics of infiltration found when sections were stained with W3/13 (T cells, neutrophils and plasma cells) (Fig 2.10) was similar to that found with OX19, but the overall number of cells stained was slightly higher. There were no significant differences in levels of W3/13 positive cells between the two groups.

Interestingly, activated cells expressing the IL-2 receptor, as measured by labelling with OX39 (Fig 2.11) were found in greater numbers in enhanced than rejecting grafts at day 3, 2.6% +/- 0.7% vs 0.6% +/-0.5%, $p<0.01$. However by day 5 the numbers of OX39 positive cells had risen rapidly in rejecting grafts compared with enhanced grafts . The levels of OX39 positive cells remained low in enhanced grafts at day 7.

3.4. Cytotoxic function of cells infiltrating enhanced and rejecting grafts

Cells were harvested from enhanced and rejecting grafts at day 5 post transplant, and along with splenocytes were tested for specific and non-specific cytotoxicity in 6hr chromium release assays. Initially cells were harvested using an enzymatic technique, which produced a lot of cell debris. Experiments have subsequently been performed using cells harvested mechanically, then separated over Percoll, which led to a much cleaner cell preparation. A typical yield of cells was between $5\text{-}7x10^7$ cells per graft. The target cells used in the killing assay were lymphoblasts of either donor (DA) or third party (LEWIS)

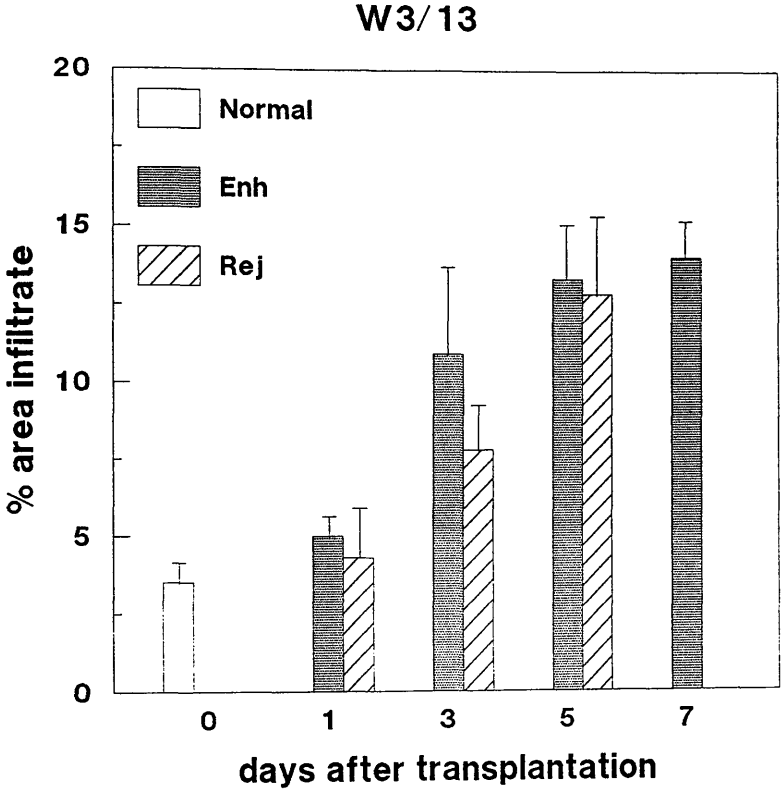


Fig 2.10 Morphometric analysis of W3/13 staining of DA grafts in unmodified and transfused PVG recipients
DA grafts were removed on different days after transplantation from unmodified (n=5) and transfused (n=5) PVG recipients. Cryostat sections were stained by immunoperoxidase with W3/13 and the % area infiltrate of labelled leucocytes determined by morphometric analysis. Normal DA kidneys were stained as a control. Results are expressed as the mean and standard deviation.

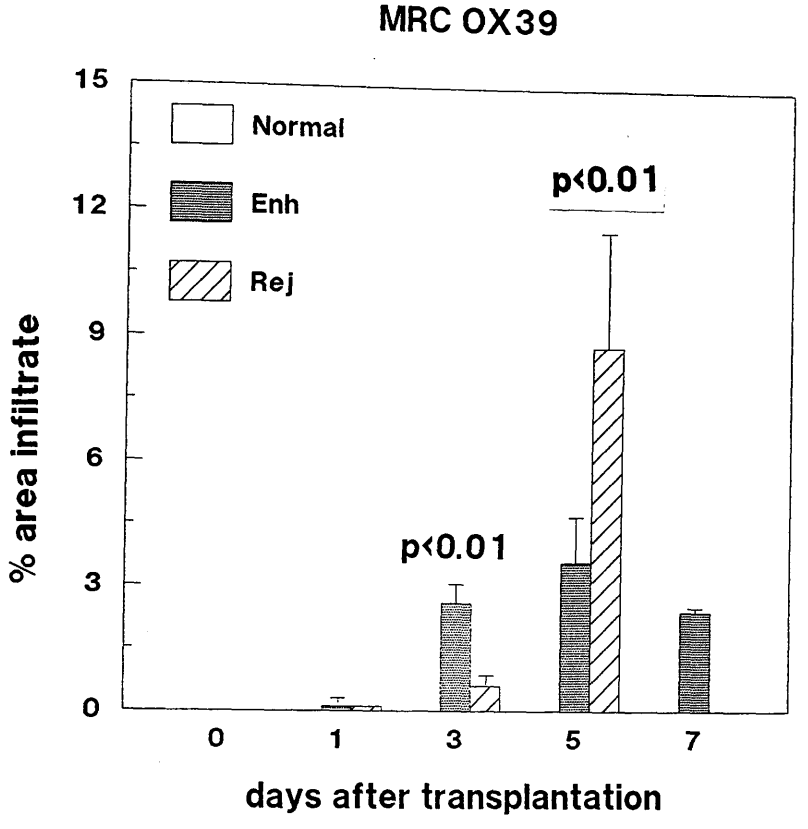


Fig 2.11 Morphometric analysis of OX39 staining of DA grafts in unmodified and transfused PVG recipients

DA grafts were removed on different days after transplantation from unmodified (n=5) and transfused (n=5) PVG recipients. Cryostat sections were stained by immunoperoxidase with OX39 and the % area infiltrate of labelled leucocytes determined by morphometric analysis. Normal DA kidneys were stained as a control. Results are expressed as the mean and standard deviation.

genotype. While these cells may not be appropriate in vitro correlates of the target cells in kidney tissue,they do bare appropriate MHC antigens which would be recognised by host effector cells. Before using these cells as targets, cytocentrifuge slide preparations of Con A blasts of various rat strains were stained with OX18 (anti-class I) and OX6 (anti-class II) monoclonal antibodies using the immunoperoxidase technique. In the rat strains tested all of the Con A blasts stained positively for class I antigens, and about one third stained strongly positive for class II antigens. The rat myeloma cell line Y3 was used as a non-specific target and was found to express class I antigens very weakly, but did not express class II antigens.

3.4.1 A comparison of the specific cytotoxicity found in enhanced and rejecting rat renal allografts

A feature of the assay in which the cells were harvested enzymatically, was the marked "prozone" effect at high effector to target ratios. The maximum level of release was found at an effector to target ratio of 50:1 and the results of 5 separate experiments are shown in Table 1.3. It can be seen that the levels of specific cytotoxicity produced by cells from the enhanced grafts were similar to or exceeded those found in rejecting grafts. Third party cytotoxic activity was assessed using Lewis Con A blasts as targets and was less than 6% in all experiments in which it was tested.(Table 1.3). When this experiment was repeated using graft infiltrating cells which had been harvested mechanically there was no prozone effect and cells from

Table 1.3

Specific cytotoxicity of graft infiltrating cells harvested from enhanced and rejecting rats 5 days after transplantation

% ^{51}chromium release at an effector:target ratio of 50:1

Experiment	Enhanced vs DA	Rejecting vs DA	Enhanced vs LEWIS	Rejecting vs LEWIS
1	15	22	ND	ND[a]
2	24	21	ND	ND
3	29	17	6.0	1.0
4	13	13	5.0	5.0
5	21	14	2.0	4.0

(a) ND = not done

Table 1.3 Graft infiltrating cells were harvested from enhanced and rejecting PVG recipients of a DA kidney, 5 days after transplantation. The cells were tested for cytotoxicity against donor-specific (DA) and third party (LEWIS) Con A blasts in a ^{51}Cr release assay. The results of 5 experiments at an effector:target ratio of 50:1 are shown.

the enhanced grafts showed considerably higher levels of specific cytotoxicity than the rejecting grafts(Fig 3.1). However this was not entirely specific for donor alloantigen,since there were high levels of third party activity(Fig 3.2). The levels of specific cytotoxicity found in the spleens of enhanced and rejecting rats in 5 separate experiments are shown in Table 1.4 and the results of a typical experiment are shown in Fig 3.3. Again there was no significant difference in the amount of cytotoxicity generated by each group. Levels of cytotoxicity were slightly higher in the spleens than in the kidneys but not all of this killing was directed towards donor alloantigen as at least half of this activity cross reacted with Lewis antigens (Table 1.4). In a number of experiments the spleens were depleted of macrophages but this did not consistently alter the levels of cytotoxicity (results not shown). This suggests that most of the cytotoxic activity measured in the grafts was mediated by lymphocytes. Therefore no consistent difference could be shown between the levels of specific cytotoxicity found in the grafts or spleens of enhanced and rejecting rats.

3.4.2 A comparison of the non-specific cytotoxicity found in enhanced and rejecting rats

Non-specific cytotoxicity was assessed by measuring cytotoxicity against the rat myeloma Y3. Levels of non-specific cytotoxicity found in the graft infiltrating cells harvested from enhanced and rejecting rats in five separate experiments are shown in Table 1.5. Surprisingly there was no prozone effect, and this may have reflected subtle

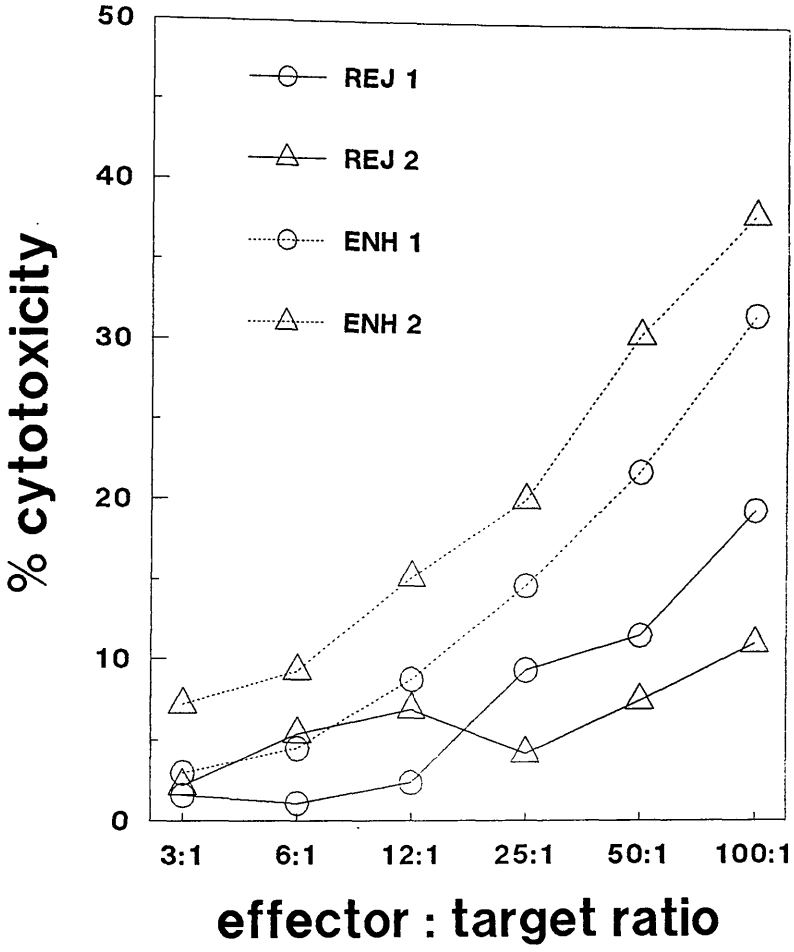


Fig 3.1 Cytotoxic activity of graft infiltrating cells harvested from enhanced and rejecting grafts against DA con A blasts
Graft infiltrating cells were harvested from enhanced (n=2) and rejecting(n=2) PVG recipients 5 days after transplantation with a DA kidney. They were then tested in vitro for their ability to lyse chromium-labelled DA con A blasts.

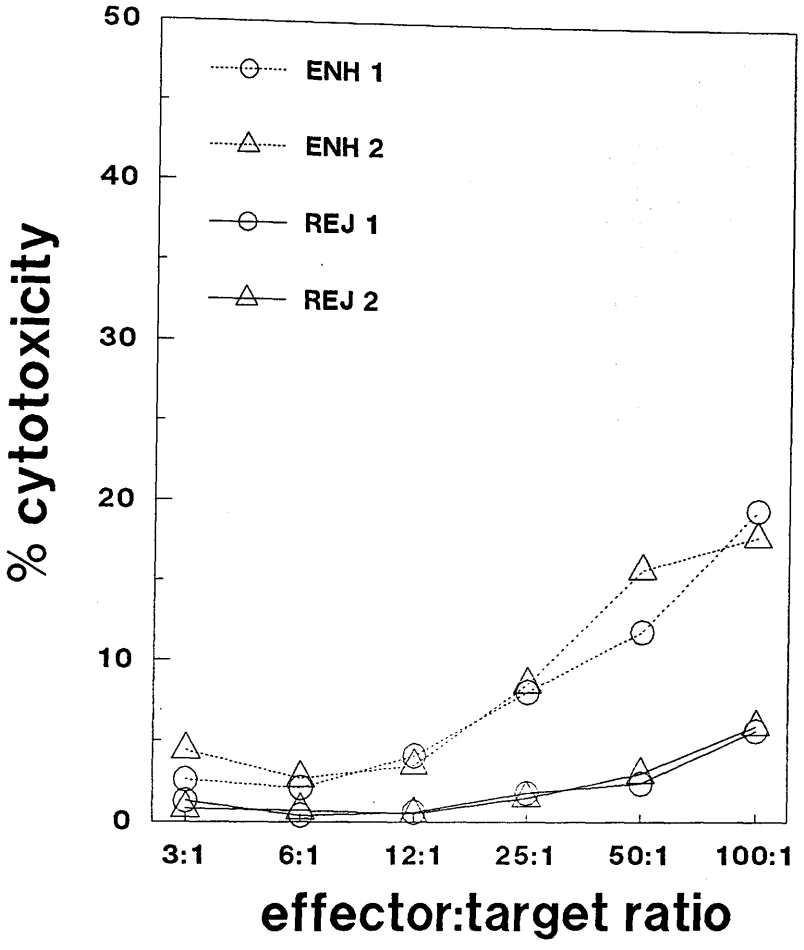


Fig 3.2 Cytotoxic activity of graft infiltrating cells harvested from enhanced and rejecting grafts against LEWIS con A blasts

Graft infiltrating cells were harvested from enhanced (n=2) and rejecting (n=2) PVG recipients 5 days after transplantation with a DA kidney. They were then tested in vitro for their ability to lyse chromium-labelled LEWIS con A blasts.

Table 1.4

Specific cytotoxicity of splenocytes harvested from enhanced and rejecting rats 5 days after transplantation

% ^{51}Cr release at an effector:target ratio of 100:1

Experiment	Enhanced vs DA	Rejecting vs DA	Enhanced vs LEWIS	Rejecting vs LEWIS
1	22	20	ND	ND[a]
2	16	18	ND	ND
3	26	30	17	16
4	22	19	14	10
5	26	25	18	10

(a) ND = not done

Table 1.4 Splenocytes were prepared from enhanced and rejecting PVG recipients of a DA kidney, 5 days after transplantation. The cells were tested for cytotoxicity against donor-specific (DA) and third party (LEWIS) Con A blasts in a ^{51}Cr release assay. The results of 5 experiments at an effector:target ratio of 100:1 are shown.

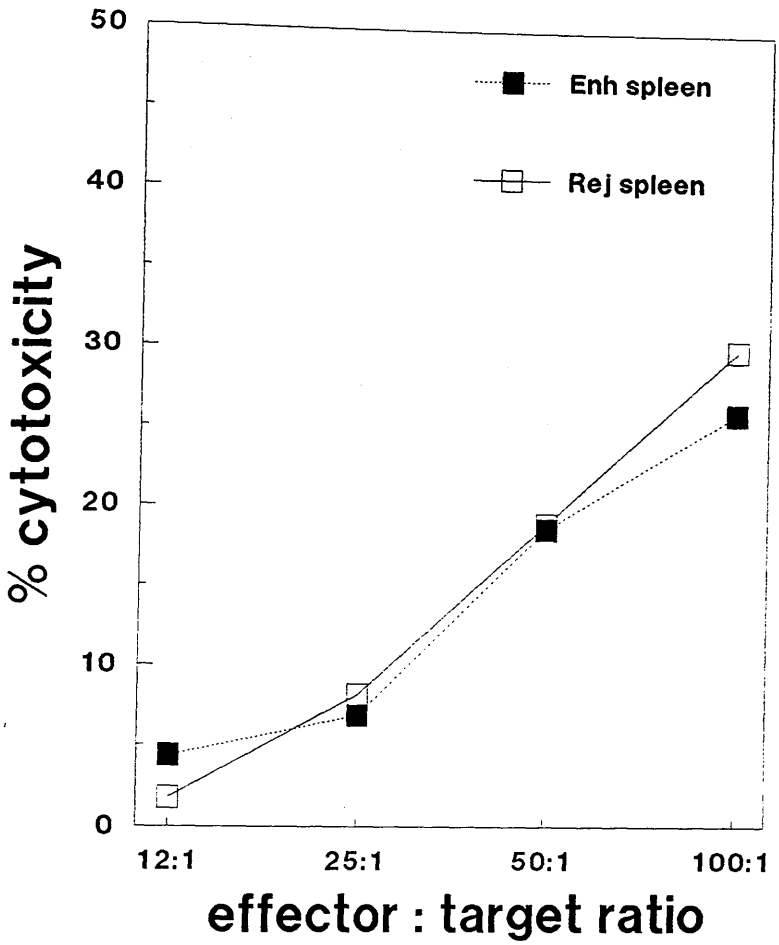


Fig 3.3 Cytotoxic activity found in the spleens of enhanced and rejecting PVG rats against DA con A blasts
Splenocytes were prepared from an enhanced and rejecting PVG rat 5 days after transplantation with a DA kidney . They were then tested in vitro for their ability to lyse chromium-labelled DA con A blasts.

Table 1.5

Non-specific cytotoxicity of graft infiltrating cells harvested from enhanced and rejecting rats 5 days after transplantation

% ^{51}Cr release at an effector:target ratio of 100:1

Experiment	Enhanced vs Y3	Rejecting vs Y3
1	15	22
2	29	17
3	13	13
4	21	14
5	24	21

Table 1.5 Graft infiltrating cells were harvested from enhanced and rejecting PVG recipients of a DA kidney, 5 days after transplantation. The cells were tested for non-specific cytotoxicity against the NK sensitive target Y3 in a ^{51}Cr release assay. The results of 5 experiments at an effector:target ratio of 100:1 are shown.

differences in the interaction between the effector and target cells. The results of a typical experiment are shown in Fig 3.4. Levels of non-specific killing found were slightly higher in the enhanced than in the rejecting group: the median and range for 5 separate experiments was 21%(13-29%) for the enhanced group and 17%(13-22%) for the rejecting group. High levels of non-specific cytotoxicity were found in the splenocytes of enhanced and rejecting rats and again levels were slightly higher in the enhanced group (Table 1.6) The results of a typical experiment are shown in Fig 3.5. The medians and ranges for the enhanced and rejecting groups in 5 separate experiments were 40%(35-74%) and 32%(29-68%).

3.5 Ability of lymph node cells from enhanced and rejecting rats to mediate GvH activity in the popliteal lymph node assay

In order to assess whether cells from a rat actively enhanced by blood transfusion could mount a GvH reaction, LNCs were harvested from a transfused and an unmodified rat five days post transplant. Three graded doses of cells were prepared from each group , then injected into the left and right hind footpads of 3(DAXPVG) F1 rats/ cell dose. After 7 days the popliteal lymph node was removed and weighed. The dose response curve is shown in Fig 3.6 It can be seen that cells from the enhanced animal responded as well as cells from the rejecting animal there being no significant difference between the two groups $p< 0.05$.

3.6 MHC antigen expression in rejecting and enhanced renal allografts

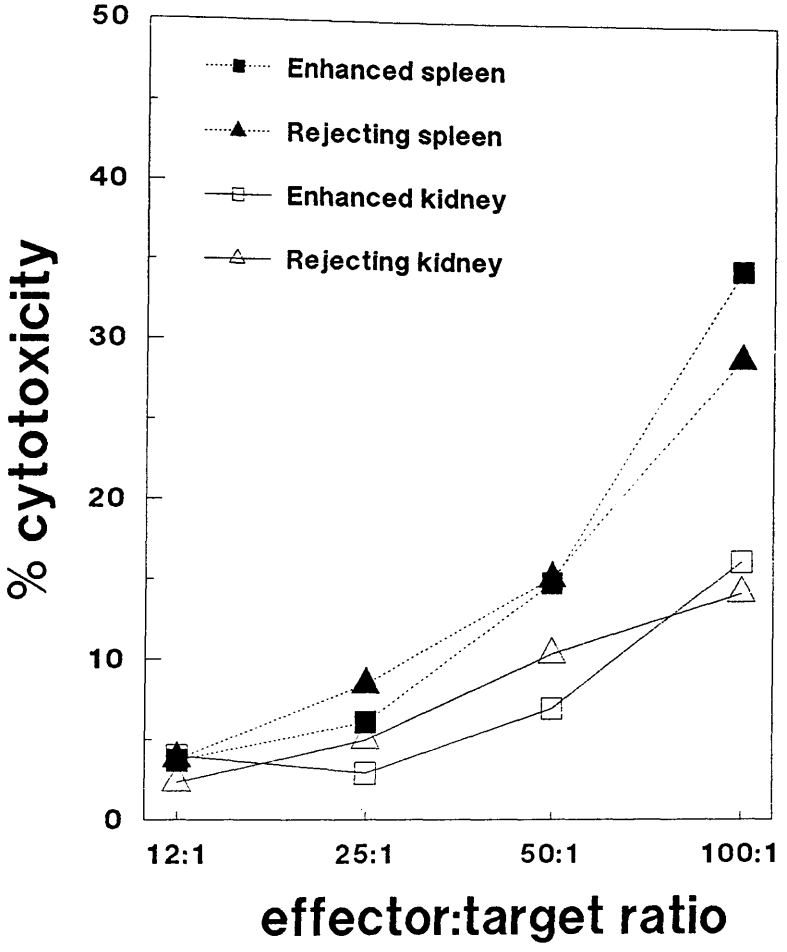


Fig 3.4 Non-specific cytotoxicity found in enhanced and rejecting spleens and kidneys against Y3 targets
Graft infiltrating cells and spleens were prepared from a transfused and unmodified PVG recipient 5 days after transplantation with a DA kidney. Cells were then tested in vitro for their ability to lyse chromium labelled Y3 targets.

Table 1.6

Non-specific cytotoxicity of splenocytes harvested from enhanced and rejecting rats 5 days after transplantation

% ^{51}Cr release at an effector:target ratio of 100:1

Experiment	Enhanced vs Y3	Rejecting vs Y3
1	35	29
2	40	32
3	45	47
4	39	30
5	74	68

Table 1.6 Splenocytes were harvested from enhanced and rejecting PVG recipients of a DA kidney, 5 days after transplantation. The cells were tested for non-specific cytotoxicity against the NK sensitive target Y3 in a ^{51}Cr release assay. The results of 5 experiments at an effector:target ratio of 100:1 are shown.

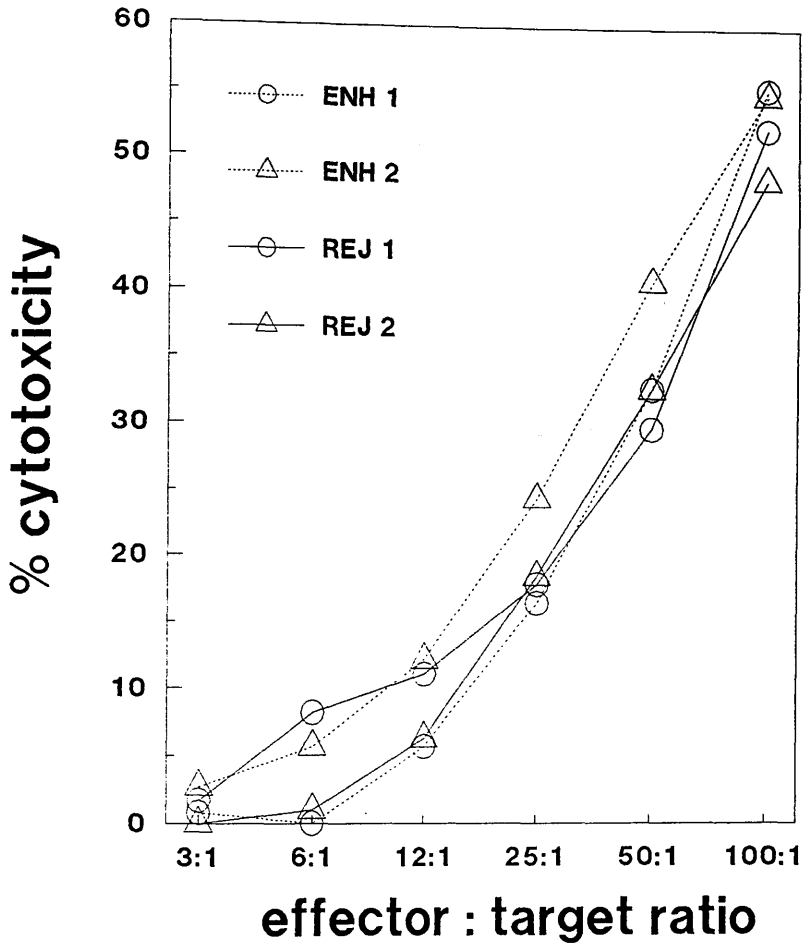


Fig 3.5 Cytotoxic activity of graft infiltrating cells prepared from enhanced and rejecting grafts against Y3 targets
Graft infiltrating cells were prepared from enhanced (n=2) and rejecting (n=2) PVG recipients 5 days after transplantation with a DA kidney. Cells were tested in vitro for their ability to lyse chromium-labelled Y3 targets.

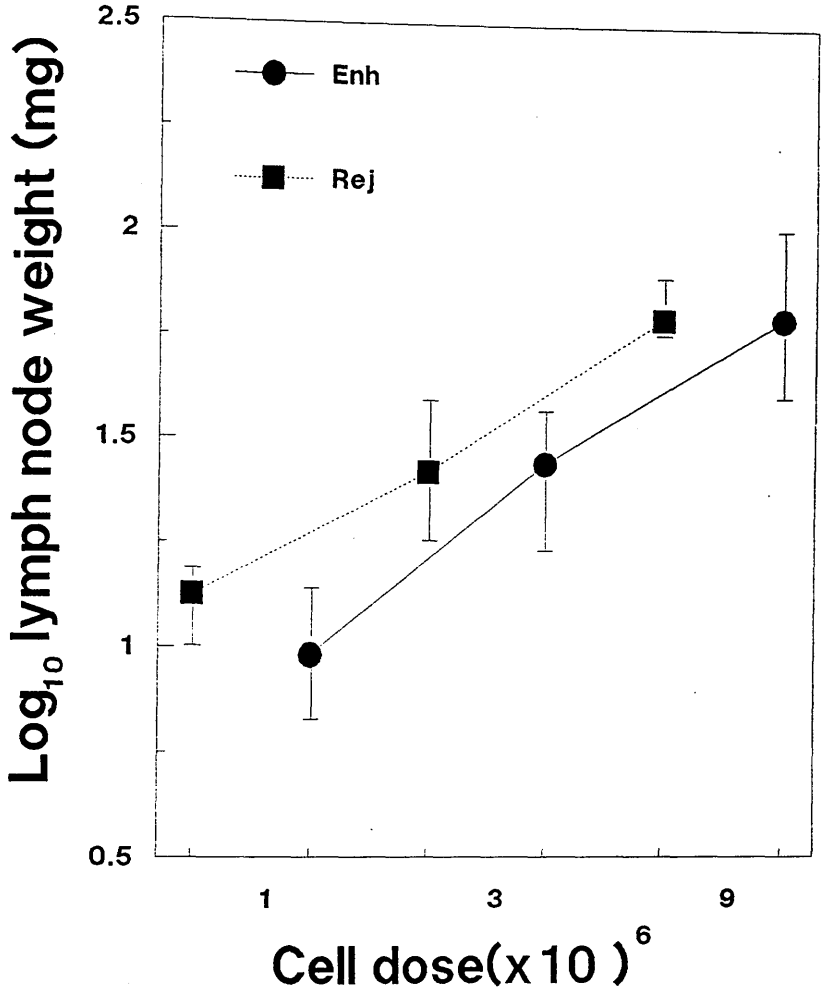


Fig 3.6 Ability of LNCs from enhanced and rejecting rats to respond in the popliteal lymph node assay

LNCs were prepared from an enhanced and a rejecting PVG rat 5 days after transplantation with a DA kidney. Three doses of cells (1,3 and 9 x 10^6) for each group were injected into the left and right hind footpads of 3(DAxPVG)F1 rats. After 7 days the popliteal lymph node was removed and weighed.

Serial cryostat sections of enhanced and rejecting renal allografts were stained on days 1,3,5,and 7 after transplant (5 rats/group/time point),with monoclonal antibodies to monomorphic and polymorphic determinants of class I and class II MHC antigens respectively. The monoclonal antibodies MN4-91-6 and F17-23-2 recognise polymorphic determinants of class I and class II respectively, which are expressed by DA but not PVG rat strains. (Milton et al,1986). This allowed the unequivocal detection of changes in donor MHC expression on the donor kidney tissue without the labelling of host infiltrating cells.

3.6.1 Immunohistological study of class I expression in rejecting and enhanced renal allografts

To gain a baseline of MHC expression, cryostat sections of normal DA kidneys (n=4) and DA isografts (n=3) were labelled with the anti-class I antibodies MRC OX18 and MN4-91-6.

Fig 3.7 shows the distribution of class I MHC antigens in normal DA kidney (indicated by labelling with MN4-91-6). There was strong staining of the interstitial areas(which includes dendritic type cells) and the arteriolar and venous endothelium, but only moderate staining of glomeruli, weak staining of the cortical tubules and occasionally some staining of medullary tubules. Similar staining was observed with OX18. DA isografts examined at days 3,5 and 7 occasionally showed slight induction on tubular cells but this was

Fig 3.7 Immunoperoxidase labelling of normal DA kidney with MN4-91-6
Cryostat sections of normal DA kidney were immunoperoxidase labelled with MN4-91-6 which labels a polymorphic determinant of DA but not PVG class I(x320)

much less than found with enhanced and rejecting allografts and was not a consistent finding. When DA renal allografts from both unmodified and transfused PVG recipients were examined, widespread induction was found in both groups. There was an increase in tubular staining as early as day 1 and by day 3 both rejecting Fig 3.8a and enhanced Fig 3.8b grafts showed strong staining of all tubular cells and glomeruli. Similar staining was found in day 5 rejecting, day 5 enhanced and day 7 enhanced allografts. Kidneys from three long surviving enhanced allografts were also labelled with MN4-91-6 and class I expression was found to have reverted to normal except for an absence of donor interstitial staining . When the same sections were labelled with MRC OX18, a similar pattern of expression was found except there were greater numbers of positively staining infiltrating cells which were presumably recipient dendritic cells (MN4-91-6 negative, MRC OX18 positive).

3.6.2 Immunohistological study of class II expression in rejecting and enhanced renal allografts

Class II expression was detected by staining with F17-23-2 which allowed a precise determination of donor class II expression and also with MRC OX6 which labelled a monomorphic determinant of class II. The distribution of class II MHC antigen in normal DA kidney is shown in Fig 3.9 The notable features of this staining were strongly positive dendritic-type cells scattered throughout the interstitial areas of the kidney. There was weak class II expression by the renal tubules

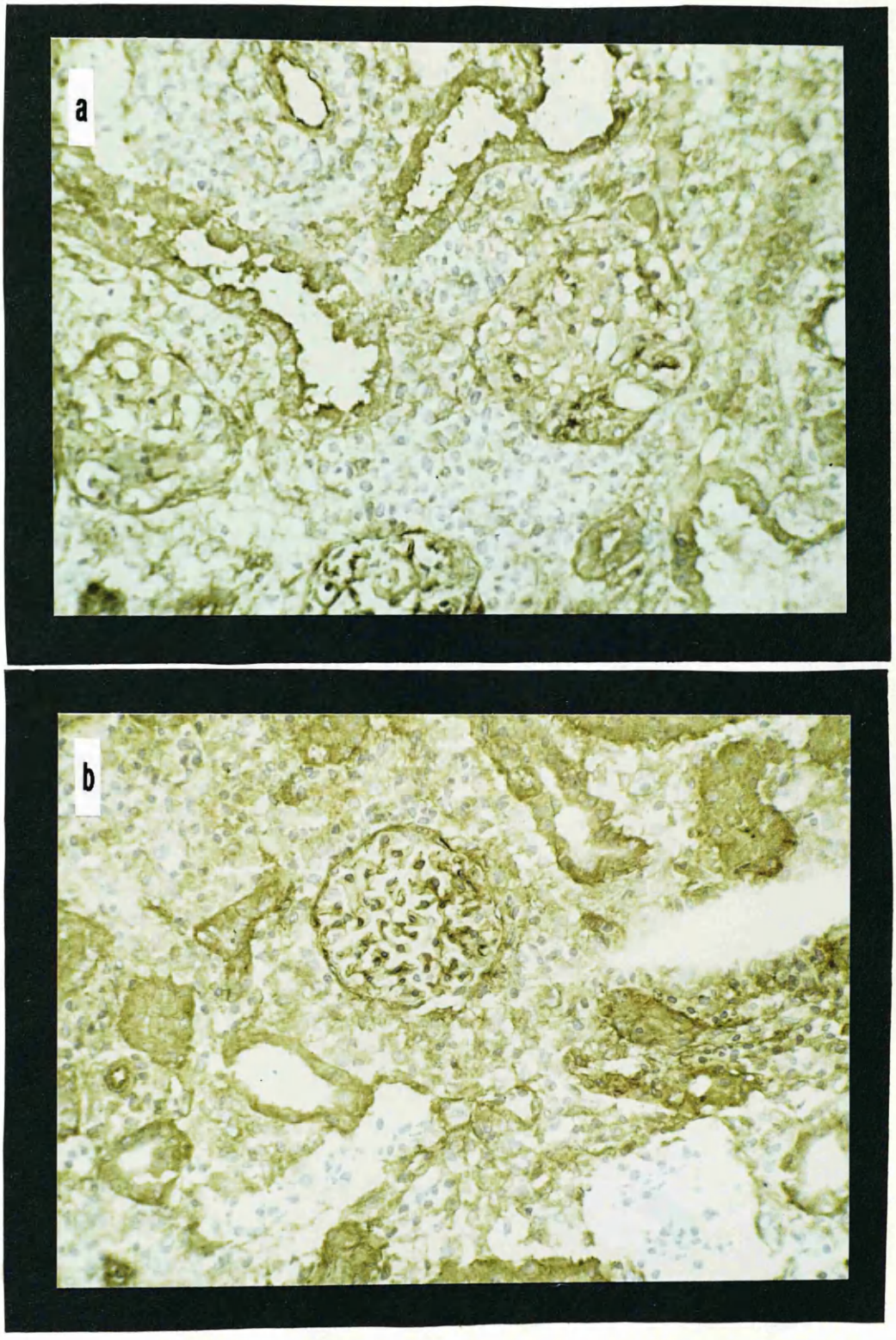
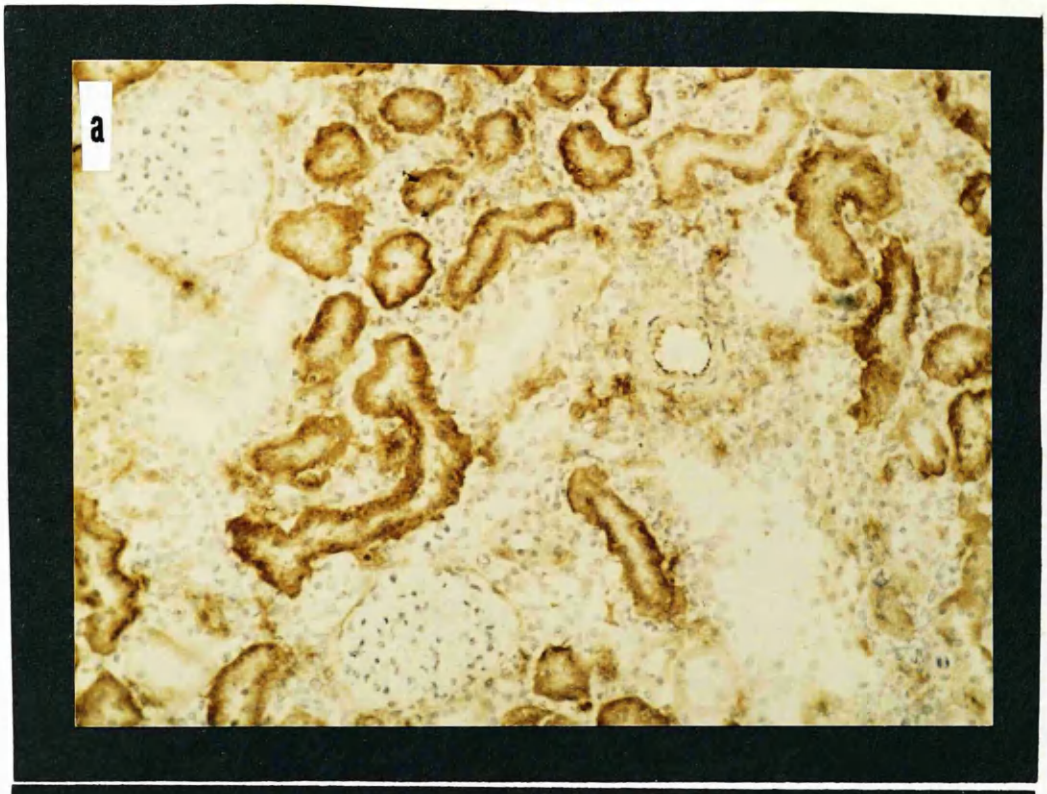


Fig 3.8 Immunoperoxidase labelling of enhanced and rejecting DA allografts in PVG recipients with MN4-91-6(day 3)
Cryostat sections of enhanced and rejecting DA allografts, 3 days after transplantation, were immunoperoxidase labelled with MN4-91-6 which labels a polymorphic determinant of DA but not PVG class I.
(a) Rejecting kidney labelled with MN4-91-6 (x200)
(b) Enhanced kidney labelled with MN4-91-6 (x200)

Fig 3.9 Immunoperoxidase labelling of normal DA kidney with F17-23-2
Cryostat sections of normal DA kidney were immunoperoxidase labelled with F17-23-2 which labels a polymorphic determinant of DA class II(x200).

(mainly the basal half of the proximal tubules), however there was no evidence of class II expression by vascular endothelium nor by glomeruli and the surrounding Bowman's capsule.

Day 1 enhanced allografts showed a slight focal increase in donor class II antigen expression on proximal tubules which was not found in rejecting grafts. Both unmodified and transfused recipients showed a slight decrease in the frequency of class II positive interstitial dendritic cells at day 1. By day 3, both enhanced (Fig 3.10a) and rejecting (Fig 3.10b) allografts had focal areas of increased class II expression , which appeared to be greater in the enhanced grafts than in the rejecting grafts. Furthermore, only enhanced grafts showed induction of class II antigen on arteriolar endothelium and on Bowman's capsules as well as a marked reduction in the number of interstitial dendritic cells. By day 5 the staining pattern was similar in both groups (Fig 3.11a and 3.11b). Grafts stained strongly for donor class II antigen on all tubular cells, arteriolar endothelium and Bowmman's capsules, however the glomeruli remained negative. There was virtually no F17-23-2 positive interstitial dendritic cells and these did not appear to have been replaced by recipient dendritic cells (MRC OX6 positive). A similar picture was seen in day 7 enhanced grafts. In long surviving enhanced grafts (day 100), there was still some class II expression on renal tubules and occasional expression on arteriolar vascular endothelium. These grafts contained a normal complement of recipient type dendritic cells (F17-23-2 negative, MRC OX6 positive)(Figs 3.12a and 3.12b). None of the DA isografts examined at days 3,5,or 7 showed any increase in class II expression after transplantation.



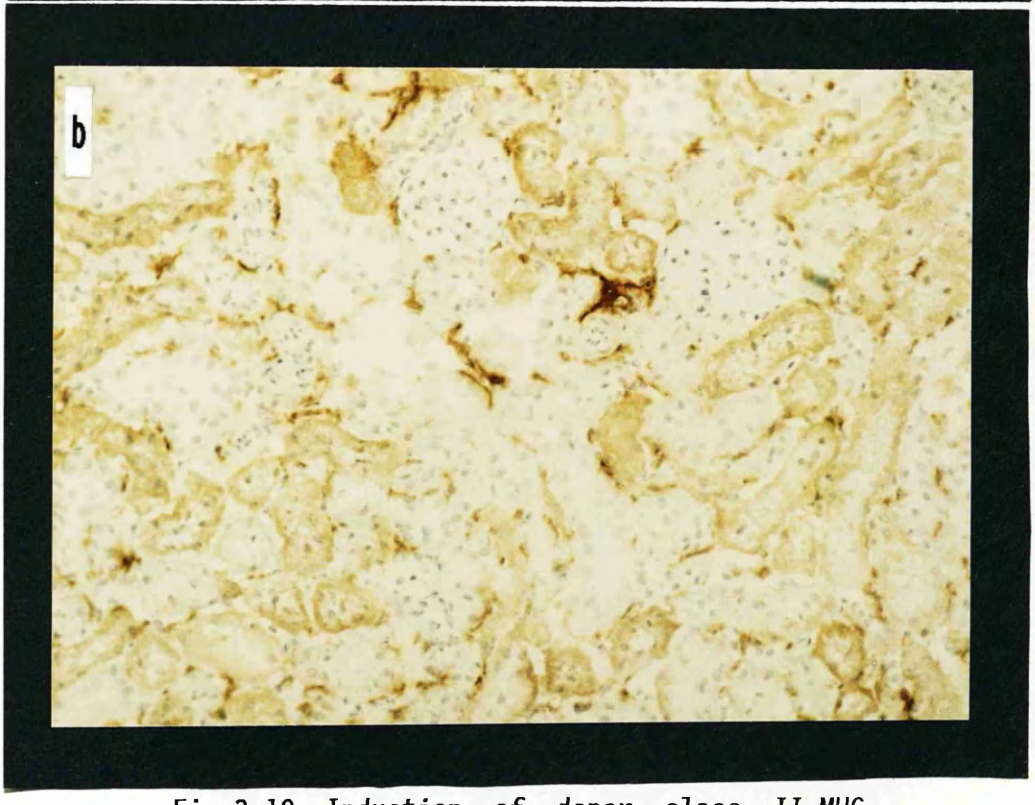


Fig 3.10 Induction of donor class II MHC antigens in enhanced and rejecting DA allografts in PVG recipients (day 3)
Cryostat sections of enhanced and rejecting DA allografts, 3 days after transplantation were immunoperoxidase labelled with F17-23-2 which labels a polymorphic determinant of DA (donor) but not PVG (recipient) class II.
(a) Enhanced kidney labelled with F17-23-2 (x200)
(b) Rejecting kidney labelled with F17-23-2 (x200)

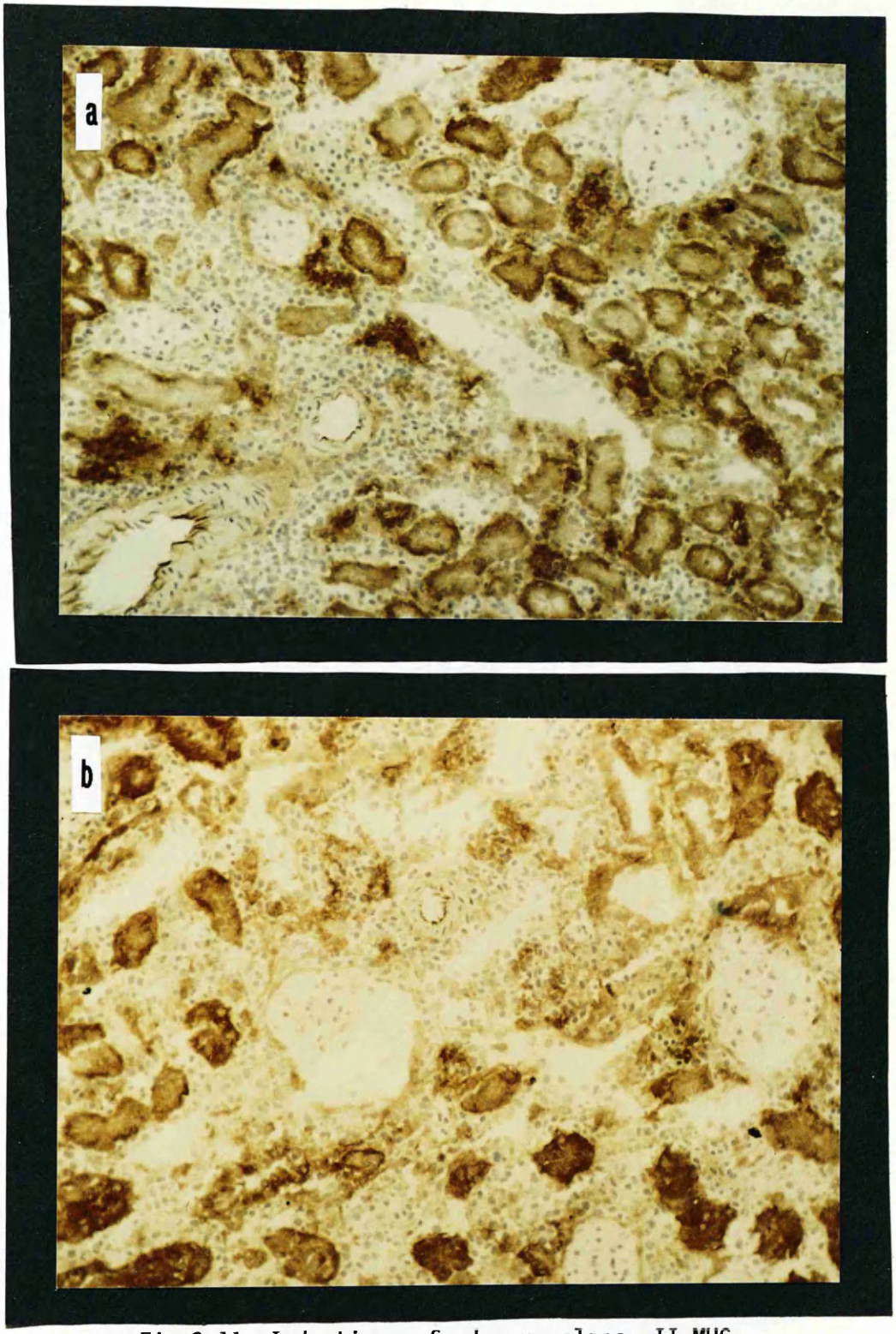
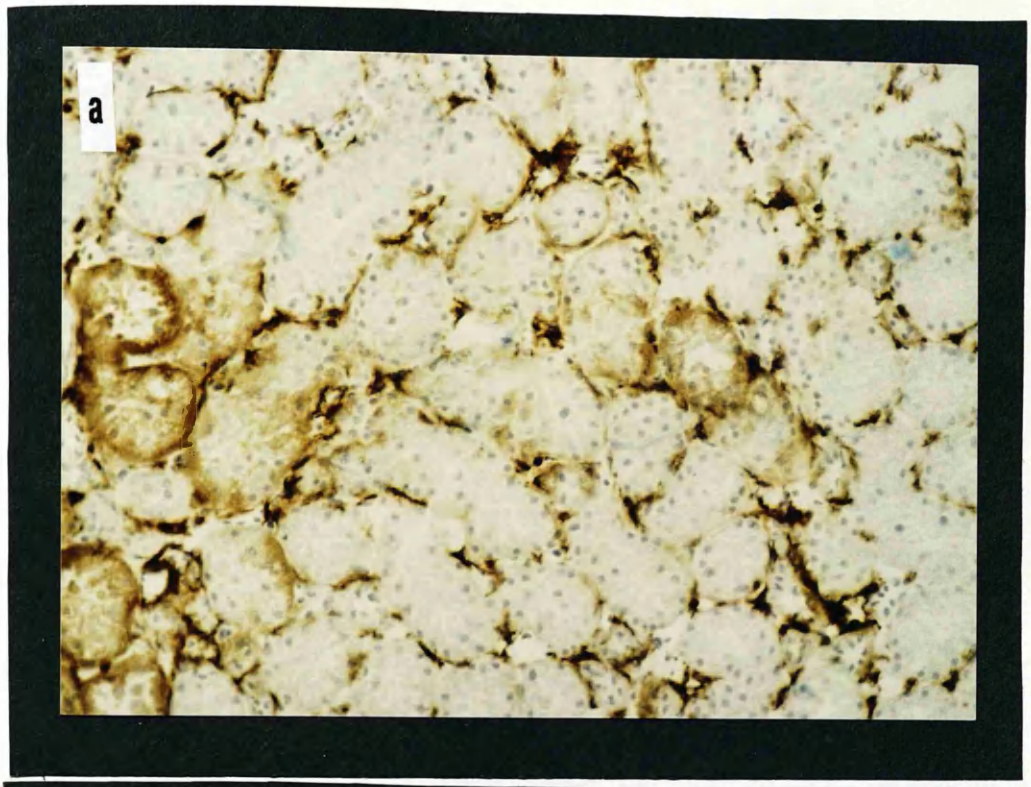


Fig 3.11 Induction of donor class II MHC antigens in enhanced and rejecting DA allografts in PVG recipients (day 5)
Cryostat sections of enhanced and rejecting DA allografts in PVG recipients, 5 days after transplantation were immunoperoxidase labelled with F17-23-2 which labels a polymorphic determinant of DA but not PVG class II.
(a) Enhanced kidney labelled with F17-23-2(x200)
(b) Rejecting kidney labelled with F17-23-2(x200)



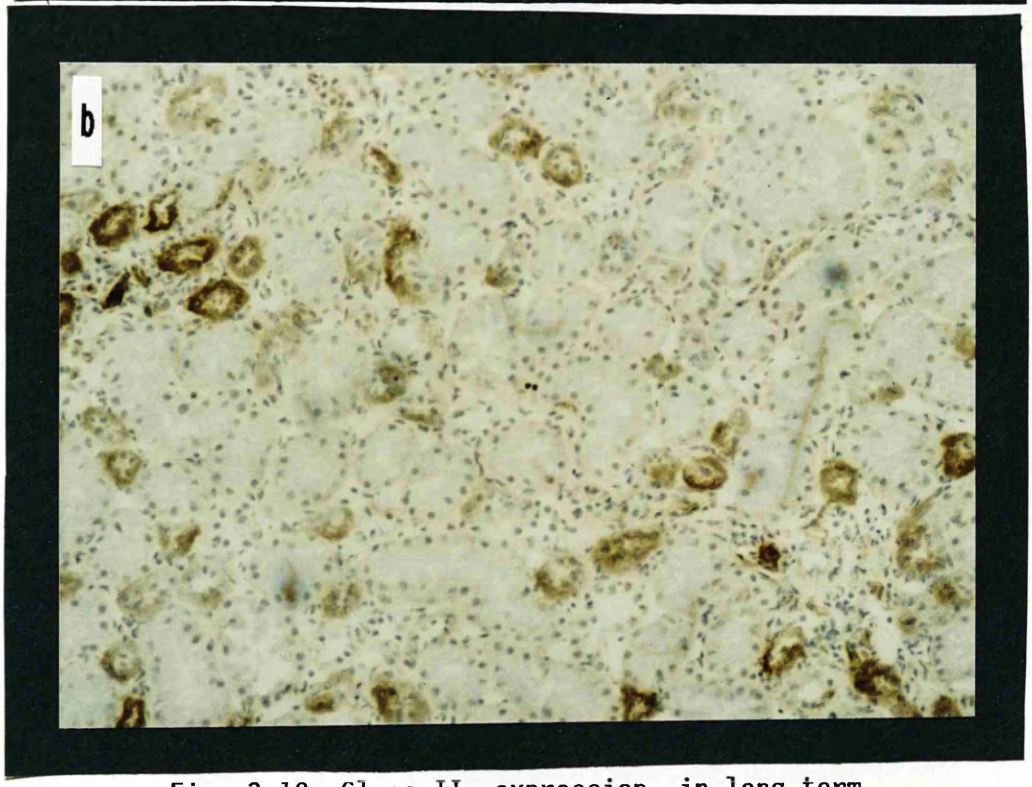


Fig 3.12 Class II expression in long term surviving enhanced PVG recipients of a DA renal allograft

Cryostat sections of long term surviving(day 100 post transplantation) DA kidneys in enhanced PVG recipients were immunoperoxidase labelled with OX6 (labels host and donor class II antigens) and F17-23-2 (labels donor class II antigens.

(a) Day 100 enhanced DA kidney labelled with OX6 (X200)

(b) Day 100 rejecting DA kidney labelled with F17-23-2 (X200)

3.7 Discussion

It has been shown in this chapter that although a single preoperative blood transfusion markedly prolongs allograft survival, there is an accelerated immunological response to the allograft. This was characterised by a rapid and substantial leucocyte infiltration ,early loss of graft dendritic cells, both alloantigen-specific and non-specific cytotoxicity and an accelerated and marked increase of donor strain class I and class II MHC expression within the graft. Such findings raise interesting questions both about the immunological process of renal allograft rejection and about the mechanism of induction of active enhancement.

The observation that passenger leucocytes disappeared more rapidly from the enhanced grafts was interesting in the light of their postulated role as mediators of host sensitisation (Lechler and Batchelor, 1982). If these cells travelled to a site of sensitisation at an earlier time point then this could explain the rapid appearance of host infiltrating cells in the enhanced grafts. An alternative explanation is that the host effector cells entering the graft at an early stage after transplantation have killed the dendritic cells in situ, hence removing an important alloantigenic stimulus.

The magnitude of the leucocyte infiltrate reached similar levels in both groups by day 5, however there were phenotypic differences which may influence the outcome of the rejection response. There were significantly higher numbers of OX8 positive cells in the rejecting

rats compared to the enhanced rats at day 5 post transplant. It is not clear from these results whether this reflects important functional differences between the two groups. It would be interesting to look at both the proliferative and cytotoxic responses of purified OX8 positive cells harvested from the grafts to examine this possibility. Recently, Ruiz et al,(1988) using FACS analysis of isolated graft infiltrating cells confirmed that the predominant infiltrating cell in rats receiving prior donor blood transfusion carried the $CD8^{+}$phenotype.

It was interesting that despite the greater numbers of OX8 positive cells in the rejecting grafts at day 5 there was no significant difference found between the two groups in the levels of specific cytotoxicity. This suggests that perhaps cytotoxicity was not mediated by OX8 positive cells or that the quantitative difference was not great enough to exert any effect on in vitro levels of cytotoxicity. The fact that few of the cells infiltrating either the enhanced or rejecting grafts were OX19 positive suggests that much of the graft infiltrate may be made up of non-specific effectors such as NK cells and macrophages . Bradley,Mason & Morris,1985 found that OX8 positive cells were the predominant phenotype of graft infiltrating cells in untreated LEW recipients of BN kidneys . This was in comparison with passively enhanced and CsA treated rats where there were fewer OX8 positive cells and W3/25 was the predominant phenotype. Overall these results imply that the relative preponderance of OX8 positive cells in acutely rejecting grafts may play a role in the rejection response.

Another important finding was that OX39 (anti-IL2R) positive cells in the enhanced grafts were higher than in the rejecting grafts at day 3 post transplant. This indicates that their were more activated cells present early in the response. However by day 5 there were twice as many activated cells in the rejecting rats , suggesting that the enhanced rats were deficient in either their ability to make or respond to IL-2. This observation has been confirmed by Dallman, Wood and Morris(1989), who analysed single cell suspensions of graft infiltrates by flow cytometry. They also found higher numbers of IL-2 receptor positive cells in the transfused rats compared with unmodified recipients at day 3. By day 5 the numbers in the rejecting group had risen considerably and were generally higher than the transfused group though the response was variable. Furthermore it was found that cells from the transfused rats had a lower proliferative response to IL-2 and that enhancement of graft survival could be abrogated by the administration of high doses of IL-2 for the first 5 days post transplant. The former finding suggests that there may be a defect in IL-2 responsiveness in the transfused animals, but it is debatable whether such high doses of IL-2 are physiological. This finding would appear to contradict the fact that cytotoxic cells which require IL-2 have been found in both enhanced and rejecting grafts in this and other studies(Dallman et al,1987 Ruiz et al,1988). However Dallman suggests that IL-4 ,which can also expand precursor cytotoxic cells , may be present and lead to maturation of the precursor cells. Further study at the molecular level to establish whether there is a switch off of the genes for IL-2 is required to clarify this finding.

If the infiltrate is largely non-specific as suggested by the phenotypic analysis then this would explain why the levels of specific cytotoxicity found in graft infiltrating cells harvested from both unmodified and transfused rats was not very high. The low levels of cytotoxicity may be partly due to the fact that PVG is a low responder to DA (Butcher & Howard, 1982). It has been shown that $CD4^+$ but not $CD8^+$ T cells are required to restore rejection of a DA kidney in a nude PVG recipient(Bolton et al,1989), perhaps indicating that a DTH response may be more important in this strain combination (Bolton et al,1989).

Alternatively the in vitro assay may not be a true correlate of cytotoxic activity in vivo where the targets are clearly different. The precise targets of the rejection response are not known but it is thought that vascular endothelium may be one of the primary sites of damage. It might therefore be more appropriate to use such cells as targets in cytotoxicity assays. One other possibility is that there may be qualitative differences in the cytotoxic cells present in transfused and untreated recipients. If specific cytotoxic T cells do play a role in the rejection response then they must be blocked in situ, either by blocking antibodies or specific suppressor cells.

An in vivo method of assessing the ability of cells from enhanced and rejecting rats to respond to donor alloantigen is the popliteal lymph node assay(Ford,1970). When graded doses of splenocytes were injected into the footpads of rats the local popliteal lymph node became enlarged. Furthermore the log dose / log mean lymph node weight plot was approximately linear throughout a range of doses.

The ability of cells from the transfused animals to respond to donor alloantigen in the PLN assay did not appear to be impaired though clearly this would have to be repeated to confirm this finding.

The induction of class I and class II antigens within rejecting grafts was in agreement with previous studies (Dallman & Mason,1983; Steiniger et al,1985; Milton et al 1985,1986a)However the substantial increase in class I and class II antigens in enhanced grafts was less expected, particularly in view of the fact that non-rejecting rat renal allografts in cyclosporin treated recipients have been shown to have diminished induction of class I and no induction of class II (Milton,Spencer & Fabre,1986b). Induction of class I and class II antigens in non-rejecting grafts in transfused recipients has been found by two other groups (Wood et al,1988; Priestley and Fabre, 1989). This clearly indicates that in the actively enhanced rats there must be activated T cells present which have differentiated to the stage of secreting lymphokines such as gamma-interferon. The increased expression of class I and class II MHC antigens could contribute to the rejection response by activating host effectors and making targets more susceptible to killing. In particular,specific cytotoxic T cells directed at class I targets may be augmented by increased expression of class I MHC antigens ,whereas DTH responses mediated by Th cells , directed primarily at class II targets may be augmented by increased expression of class II MHC antigens. However it appears that there is no correlation between increased MHC expression and rejection. Priestley and Fabre (1989), suggest that blood transfusion results in heightened effector and suppressor responses, but do not suggest how the latter dominates the former. In the

actively enhanced animals which initially show an accelerated response both in terms of MHC induction and cellular infiltration, it may be that lymphokines released within the graft are having opposing effects. The release of gamma interferon is normally associated with cellular activation (Spiegel,1988)and lymphocyte recruitment in vivo (Issekutz,Stalz and Meide,1988). However it has also been shown that gamma interferon augments suppressor activity,(Holda,Maier & Claman,1988), suppresses IL-1 production(Ghezzi and Dinarello,1988) and protects endothelial cells from lymphokine activated killer activity.(Renkonen,Ristimaki and Hayry,1988). The mechanism of this protective effect remains unclear, but it does appear that such a phenomenon may also occur in vivo.

It appears that enhanced grafts are able to survive despite increased MHC expression, a considerable cellular infiltrate and the presence of donor-specific cytotoxic cells. This suggests that the effector response to the graft is being blocked in vivo.

CHAPTER 4

Fc RECEPTOR BLOCKING ACTIVITY IN THE SERUM OF ACTIVELY ENHANCED RAT RENAL ALLOGRAFT RECIPIENTS

4.1 Introduction

The findings of the previous chapter suggest that the state of enhancement present in blood transfused rats is quite different from transplantation tolerance, where animals are unable to respond normally to alloantigens. Rather it appears that actively enhanced animals have a normal repertoire of alloantigenic responses, but these are held in check by some form of immunosuppression.

Of the several possible mechanisms which may be responsible for active enhancement, the generation of suppressor cells has received most attention and convincing evidence exists that such cells may be of importance in preventing graft rejection after blood transfusion (Hutchinson, 1986; Quigley, Wood and Morris, 1989a,b,c). However there is also a body of evidence from clinical studies which suggests that alloantibodies, induced by blood transfusion may be important (MacLeod et al, 1982;1985;1988). The mode of action of such alloantibodies might be analagous to passive enhancement whereby pre-treatment of the recipient with hyperimmune serum leads to long term graft survival (Morris, 1980).

The hypothesis that transfusion induced alloantibody plays a role in in the beneficial effect of pre-transplant blood transfusion is consistent with the clinical observation that transfusion is associated with the generation of non-cytotoxic IgG FcR blocking antibodies. These are detected by their ability to inhibit the formation of EA rosettes by donor lymphocytes and their presence may

correlate with subsequent allograft survival (MacLeod et al, 1982). Furthermore they appear to be alloantibodies rather then autoantibodies, (MacLeod et al 1985; 1988). Such Fc blocking antibodies have been found after platelet transfusion in man (Petranyi et al, 1988) and in patients who are long term survivors of renal allografts (Shohat,Cytron,Boner et al 1988). However a recent study of transfused renal dialysis patients, employing the EA rosette inhibition assay showed that graft survival correlated not with FcR blocking in the IgG serum fraction but instead with blocking activity in the high molecular weight >19S serum fraction (Forwell et al 1987).

The aim of this chapter is to further elucidate the nature of Fc blocking antibodies and whether such antibodies could be demonstrated in the serum of transfused rats which had received a renal allograft.

4.2 Development of the EARI assay

A study of Fc blocking activity in the serum of transfused kidney transplant patients in our own renal unit had used the method of MacLean, Goudie, MacSween et al(1984). The EA indicator cells in this assay were chick erythrocytes which had a rabbit anti-chick erythrocyte antibody bound. However the levels of rosetting between rat lymphocytes and these indicator cells were generally rather low (10-12%) and it was thought that this could be due to a weak interaction of the Fc region of rabbit IgG with the Fc R on rat

splenocytes, or that the chick red cells were too large to allow rosette formation. In order to overcome this problem a rat anti-sheep erythrocyte serum was raised and sheep cells used as indicator cells.

4.2.1 Preparation of a rat anti-sheep erythrocyte serum

Rat anti-sheep erythrocyte serum was prepared as described in section 2.8.1. 125ul of doubling dilutions of antiserum harvested from 2 DA rats were prepared and added to 125ul of a 5% solution of sheep red cells and incubated at $37^{o}C$ for 30 mins. The sheep cells were washed, resuspended and rosetted with $2X10^{6}$ DA splenocytes, by centrifuging at 1000rpm for 5 mins. The fixed cells were counted and the % of Fc rosetting cells calculated. The titration curves of the two antisera are shown in Figs 4.1a and 4.1b. At high concentrations of antisera, (neat-1:32) the red cells agglutinated, making the slides impossible to count. Maximum levels of rosetting were found at a dilution of between 1:64 and 1:256 in serum A and between 1:32 and 1:128 in serum B. Therefore the two antisera were pooled and used in subsequent experiments at a 1:100 dilution. The antiserum was tested by indirect haemagglutination to see whether it contained IgG or IgM. Briefly, two 5% solutions of sheep red cells were prepared one of which was sensitised as before. Doubling dilutions of sheep anti-rat IgG and sheep anti-rat IgM were made from 1:10 to 1:50,000 in a 96 well plate to which 100ul of 2% sensitised or unsensitised sheep cells were added. The plates were incubated at room temperature for 1 hr then tapped lightly to pellet the cells. Anti-IgG gave agglutination

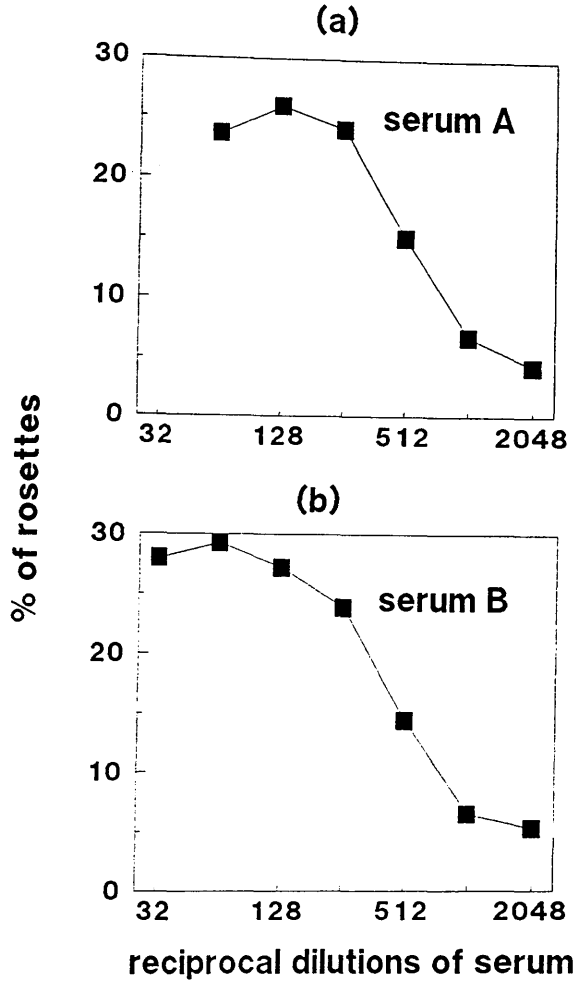


Fig 4.1 Titration of a rat anti-sheep erythrocyte serum
DA rats (n=2) were injected with 10^9 sheep red cells in Freund's complete adjuvant into the hind footpads of two Da rats. The rats were then bled 14 days after injection and the serum samples (a) and (b) titrated in the rosetting assay to determine the optimal dilution for sensitising the red cells

up to a 1:640 dilution while anti-IgM gave agglutination up to a 1:320 dilution.Therefore the antiserum appears to contain both IgG and IgM.

4.2.2 Conditions for erythrocyte antibody rosette inhibition assay

In order to assess the best conditions for the EARI assay a number of parameters were varied (the number of lymphocytes, the % of EA and the volume of EA). The results in Table 1.7 show that levels of between 20% and 30% were obtained but the optimum level of rosetting(30%) was obtained with $1x10^6$ splenocytes 1.25%, EA in 175uls of Hanks/Hepes. These conditions were then employed in all subsequent assays.

4.3 FcR blocking activity in serum from enhanced and rejecting rat renal allograft recipients

There were two main experimental groups namely;

1) Unmodified PVG($RT1^c$) recipients which were transplanted with a fully allogeneic DA($RT1^a$) kidney.

2) Actively enhanced PVG($RT1^c$) recipients which received 1.0 ml of heparinised donor strain whole blood i.v. 7 days prior to transplantation with a DA ($RT1^a$) kidney.

Table 1.7

Determination of the conditions for maximum rosetting in the EAR1 assay

No. of DA lymphocytes	% of EA	Volume of EA (ul)	% of rosetting lymphocytes
1×10^6	1.25	125	22
1×10^6	**1.25**	**175**	**31**
1×10^6	1.25	250	28
1×10^6	2.5	125	27
1×10^6	2.5	175	22
1×10^6	2.5	250	not counted[a]
2.5×10^6	1.25	125	18
2.5×10^6	1.25	175	24
2.5×10^6	1.25	250	23
2.5×10^6	2.5	125	23
2.5×10^6	2.5	175	25.0
2.5×10^6	2.5	250	not counted[a]

Table 1.7 The number of DA lymphocytes, % of EA and volume of EA were varied in order to ascertain the conditions required for maximum rosetting. Lymphocytes and EAs were centrifuged at 200g for 5 mins to encourage rosette formation. Rosettes were then fixed , stained, counted and the % of rosettes calculated.

(a) These slides were too crowded with red cells and lymphocytes to allow counting.

Serum was obtained at day 5 post transplant from 4 unmodified PVG recipients bearing a rejecting DA kidney and from 6 transfused PVG recipients bearing an actively enhanced graft and tested for the presence of FcR blocking activity against donor (DA) or third party (LEWIS) splenocytes. To remove the possibility of non-specific inhibition by aggregates the serum was ultracentrifuged, then added to the splenocytes. Serum from both groups showed specific inhibitory activity but inhibition was markedly higher in the enhanced sera (individual values for EAI were 80%, 81%, 94%, 94%, 91% and 82% vs 22%, 21%, 54%, and 13%). This inhibitory effect was donor specific since EAI was consistently <10% when tested against third party LEWIS splenocytes.

To determine which component of enhanced serum was responsible for donor specific EA rosette inhibition, 6 fractions of graded molecular weights were prepared from serum obtained from enhanced (n=4)and rejecting animals(n=4) by discontinuous gradient centrifugation. The ability of these fractionsto inhibit EA rosette formation with donor specific (DA) and third party (Lewis) lymphocytes is shown in Figs 4.2a and 4.2b respectively. The inhibitory activity in enhanced sera was found predominantly in fractions 3-5, all of which showed high levels of inhibition (median: 66%,70%,63% respectively) when compared with the the same fractions of rejecting sera (median:0%,10%,12% respectively). Again this activity was donor specific since inhibition against third party Lewis lymphocytes was minimal. The serum fractions were screened by radial immunodiffusion to determine which fractions contained IgG. The results in Table 1.8 show that IgG was

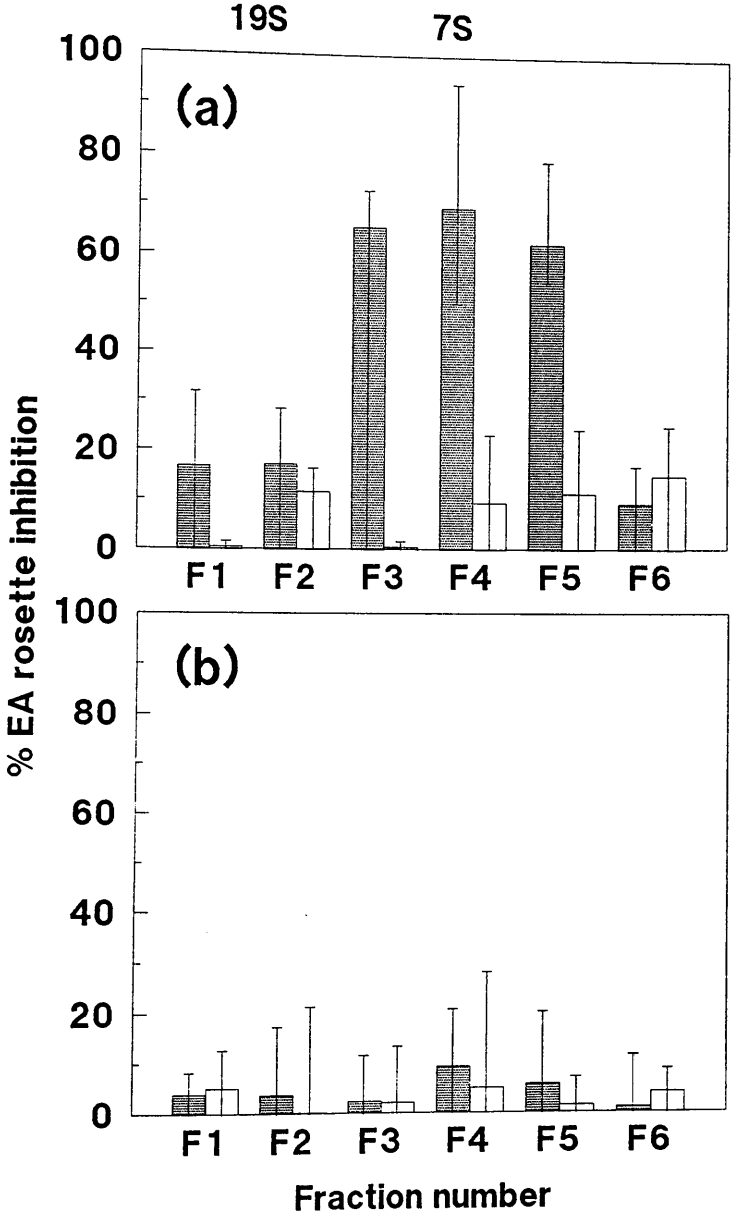


Fig 4.2 Ability of serum fractions from enhanced and rejecting rats to mediate Fc blocking activity against donor (DA) and third party (LEWIS) lymphocytes
Serum was collected from enhanced (n=4) and rejecting (n=4) PVG recipients 5 days after transplantation with a DA kidney. After fractionation over a discontinuous sucrose density gradient, enhanced(solid bars) and rejecting (open bars) serum fractions were tested for rosette inhibition against DA (a) and LEWIS (b) splenocytes. Results shown are the medians and ranges for each fraction tested.

Table 1.8

Determination of the concentrations of IgG and IgM found in enhanced and rejecting serum fractions by radial immunodiffusion

	Serum fraction number					
	1	2	3	4	5	6
Enhanced						
[IgG] mg/L	0	0	5360	11900	1810	0
[IgM] mg/L	0	424	0	0	0	0
Rejecting						
[IgG] mg/L	0	0	6900	21200	5360	0
[IgM] mg/L	0	97.8	0	0	0	0

Table 1.8 5 ul of serum from an enhanced and rejecting PVG recipient of a DA kidney, 5 days post transplant were added to agar plates containing anti-IgG and anti-IgM (Seralab). Precipitin rings were measured with the aid of an eyepiece graticule and the concentration calculated from a standard curve supplied in the kit.

present only in fractions 3-5 and was maximal in fraction 4. It seemed likely that donor specific Fc blocking was mediated by an IgG alloantibody present in enhanced but not rejecting serum.

4.4 Ability of serum from rats receiving single or double transfusion to mediate Fc blocking activity

In order to assess whether Fc blocking was a result of the initial blood transfusion given 12 days previously, serum fractions were prepared from rats 12 days after blood transfusion. As can be seen in Fig 4.3 relatively low levels of inhibition were found in all of the fractions in two of the rats though higher levels were found in fraction 5 of one of the rats(53%). However it was not necessary for the second allogeneic stimulus to be a renal allograft since a second transfusion given at the normal time of transplant also produced a marked increase in EA rosette inhibition in serum fractions 3-5 (median:43%,53% and 37%)(Fig 4.3). Similarly rats given a transfusion and then an intraperitoneal injection of kidney homogenate 7 days later also had high levels of Fc blocking activity in serum fractions 3-5 5 days after the injection of homogenate (Table 1.9). These experiments suggest that an IgG alloantibody produced after a secondary allogeneic stimulus may be responsible for the Fc blocking activity.

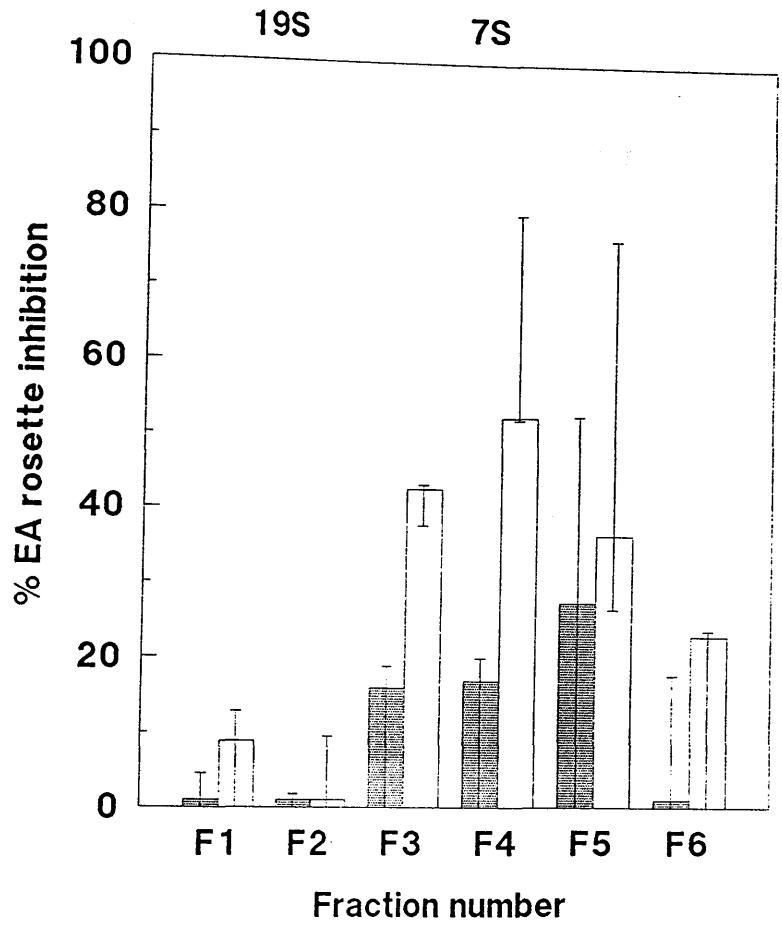


Fig 4.3 Fc blocking activity in PVG recipients following single or double transfusion

PVG rats received a single transfusion on day -7 (n=6) of which 3 also received a second transfusion on day 0 . Serum was collected on day 5 and fractionated over a discontinuous sucrose density gradient. Serum fractions from rats receiving single(solid bars) or double (open bars) transfusion were tested for inhibition of rosette formation with DA lymphocytes. Results shown are the medians and ranges for each fraction

Table 1.9

Fc blocking activity in the serum of PVG rats given a DA blood transfusion followed by an intraperitoneal injection of DA kidney homogenate

	% EA rosette inhibition vs DA splenocytes	
Fraction number	Rat no. 1	Rat no. 2
1	0	0
2	0	0
3	60.3	0
4	73.6	51.6
5	50.9	67.9
6	61.8	24.9

Table 1.9 DA rats (n=2) were given an i.v. injection of 1.0ml of DA blood on day -7 then an i.p. injection of homogenised DA kidney on day 0. Serum was collected on day 5 after the second injection and the serum fractionated over a sucrose density gradient into 6 fractions of graded molecular weight. Serum fractions were tested for Fc blocking activity in an EARI assay using DA splenocytes as targets.

4.5 Fc blocking activity of purified IgG from enhanced serum

4.5.1 Purification of IgG

IgG was prepared from enhanced serum and tested for EARI activity in order to confirm that the serum inhibition was due to IgG alloantibody. It was necessary to find a method of purification which required only small volumes of serum and gave good purity and yield. A number of methods were tried including DEAE Cellulose chromatography, Sepharose protein A absorption, and HPLC but a simple and effective method was found to be purification by n-octanoic acid sedimentation. Using this method a typical yield of protein from 2.5mls of starting serum was around 10mgs. IgG prepared by this method was tested for purity by running the samples on an 8.5% SDS polyacrylamide gel under both reducing and non-reducing conditions against a commercial rat IgG standard and molecular weight standards (Fig 1.5). The IgG was found to be relatively pure with a slight contaminant at 120K.

4.5.2 Conditions for EARI assay performed in microtitre plates

The original EARI assay required large volumes of test sample so a new assay was set up in 96 well plates in order to test the small volumes of IgG preparations. The number of lymphocytes, the % of EA, the volume of EA and the incubation temperature were varied in order to find the best conditions for rosetting. The results shown in Table

1.10 showed that higher levels of rosetting were obtained when lymphocytes were incubated at 37°C rather than 4°C, however there was little difference when the other parameters were varied. Maximum levels of rosetting (41%) were obtained when 25ul of lymphocytes at $1x10^7$ were incubated at 37°C with 50ul of 2% EA and these conditions were employed in subsequent assays.

4.5.3 Ability of IgG from enhanced serum to cause Fc blocking

IgG was prepared from 4 transfused PVG recipients on day 5 post transplant and adjusted to 1mg/ml in DAB. 10ul of doubling dilutions of test IgG or DAB were incubated with DA and LEWIS splenocytes to test for Fc blocking activity. As shown in Fig 4.4 all of the IgG preparations showed donor specific inhibition across a range of dilutions. There was no inhibitory activity against LEWIS splenocytes.

4.6 Ability of antibodies to rat leucocyte membrane antigens to cause Fc blocking

It had been shown previously that monoclonal antibodies to rat class I and class II MHC antigens were able to cause Fc blocking in an EARI assay (Power, Cunningham, Catto et al, 1987). In order to assess whether such antibodies had a similar inhibitory effect in the EARI assay used in this study, DA splenocytes were incubated with various

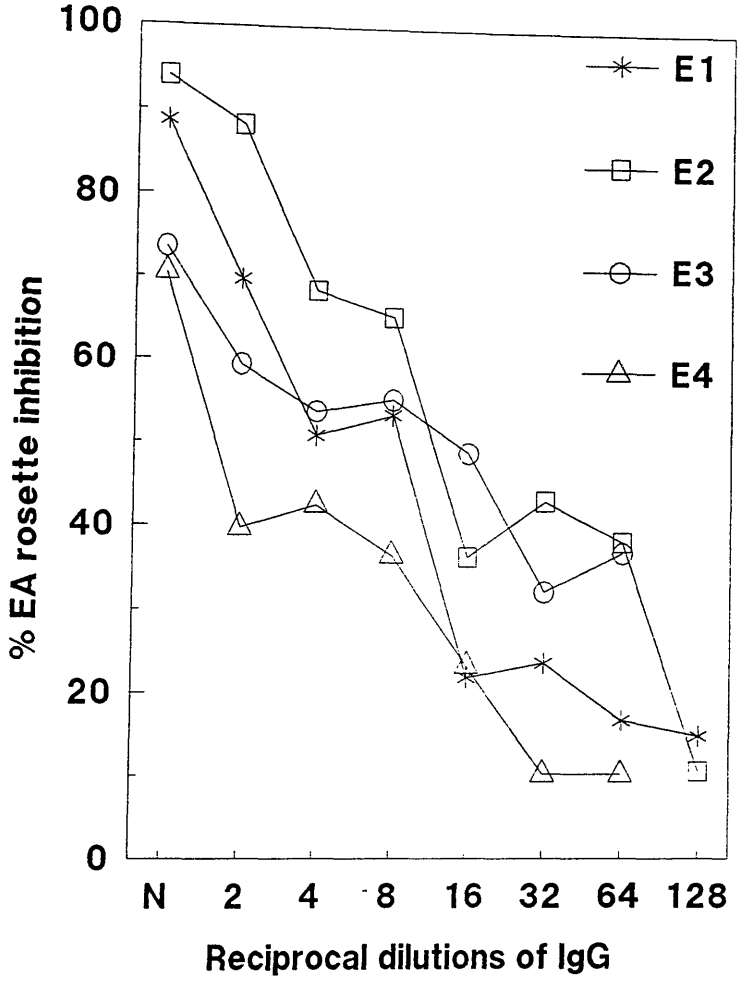


Fig 4.4 Ability of Enhanced IgG to inhibit rosette formation with DA lymphocytes
Serum was collected from enhanced PVG recipients (n=4) of a DA kidney on day 5 after transplantation. IgG was purified from the serum samples by n-octanoic acid sedimentation and reciprocal dilutions of IgG (from 10ug/well) were tested for their ability to block rosette formation with DA lymphocytes.

Table 1.10

Determination of the conditions required for maximum rosetting in the microtitre EARI assay

No. of DA lymphocytes	% of EA	Volume of EA (ul)	% of rosetting at 4°C	at 37°C
2.5×10^5	1.0	10	23	28
2.5×10^5	1.0	25	16	32
2.5×10^5	1.0	50	19	29
2.5×10^5	2.0	10	18	30
2.5×10^5	2.0	25	23	35
2.5×10^5	**2.0**	**50**	**17**	**41**
5×10^5	1.0	10	22	23
5×10^5	1.0	25	15	24
5×10^5	1.0	50	16	30
5×10^5	2.0	10	20	33
5×10^5	2.0	25	24	26
5×10^5	2.0	50	16	22

Table 1.10 The number of DA lymphocytes, % of EA, volume of EA and incubation temperatures were varied in order to find the best conditions for rosetting in microtitre plates. Lymphocytes and EAs were centrifuged at 200g for 5 mins. Rosettes were fixed, stained , counted and the % of rosette forming cells calculated.

monoclonal antibodies against cell surface antigens. In the first experiment, 10ul of doubling dilutions (from 1:800-1:100,000) of the following monoclonal antibodies were tested MRC OX18 (anti-class I), MRC OX6 (anti-class II, I-A homologue), MRC OX17 (anti-class II, I-E homologue). All of the antibodies were at a concentration of approximately 4mg/ml. The results of this experiment are shown in Fig 4.5. It can be seen that OX6 and OX18 were both very inhibitory and OX17 slightly less so: at a 1:800 dilution the % of EARI were 89%,82% and 77% respectively). The highest levels of inhibition were obtained when OX6 and OX18 or OX6 and OX17 were incubated together (82% and 93% at a 1:1600 dilution). The experiment was repeated to test the OX6 and OX18 again along with other monoclonal antibodies to cell surface antigens; OX12 (labels Ig on B cells), and W3/13 (all T cells, some neutrophils). Tripling dilutions of antibody were prepared from neat-1:18,000 and the lymphocytes were washed four times instead of twice since it was thought that unbound antibody could be contributing to the high levels of inhibition found even at high dilutions. This appeared to be the case since the inhibitory effect titrated out at a 1:729 dilution. However no inhibition was found with either OX12 or W3/13(Fig 4.6). It seems from these experiments that antibodies which bind to cell surface antigens near to the FcR on lymphocytes can cause Fc blocking and confirms that the assay could be detecting either anti-class I or anti-class II alloantibodies in the serum.

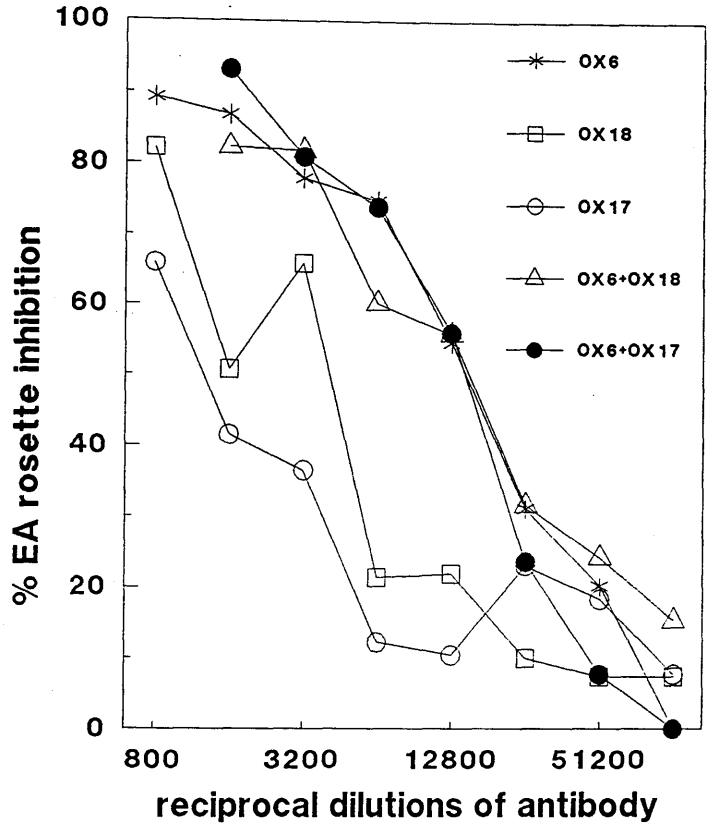


Fig 4.5 Ability of monoclonal antibodies to MHC antigens to cause Fc blocking
Monoclonal antibodies OX6,OX18, OX17,OX6+OX18 and OX6+OX17, were tested for their ability to block rosette formation with DA lymphocytes. All antibodies were at a concentration of 4-5mg/ml and 10ul of doubling dilutions from 1:800 to 1:51,200 were tested in the EARI assay.

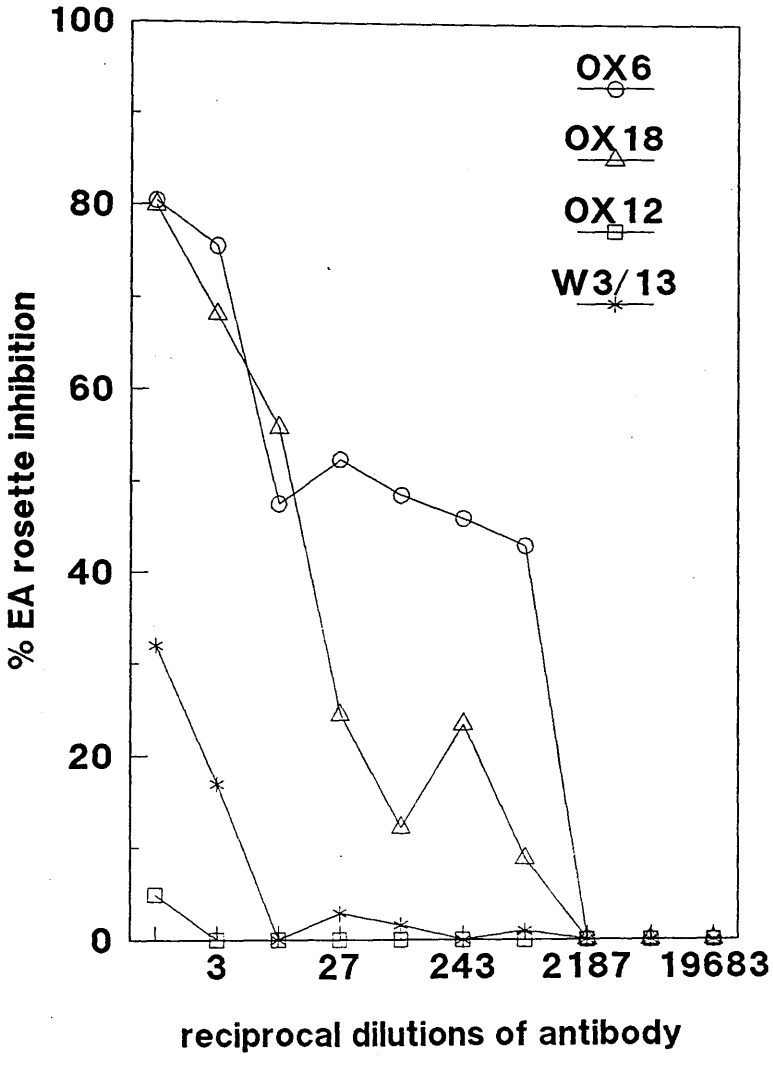


Fig 4.6 Ability of monoclonal antibodies to rat cell surface antigens to cause Fc blocking

Monoclonal antibodies OX6,OX18,OX12 and W3/13 were tested for their ability to inhibit rosette formation with DA lymphocytes. All antibodies were at 4-5mg/ml and 10ul of tripling dilutions of each from 1:3 to 1:19,683 were tested.

4.7 MHC specificity of FcR blocking alloantibody in IgG from enhanced serum

To determine the class of donor MHC antigen recognised by FcR blocking antibodies present in the purified IgG preparations from enhanced graft recipients, a series of absorption experiments was performed. Initially IgG from 3 enhanced rats was absorbed with DA (donor) and LEWIS (third party) erythrocytes (which in the rat only express class I) and tested for EARI with DA and LEWIS splenocytes. As can be seen in Table 1.11 absorption with both DA and LEWIS erythrocytes failed to remove any of the Fc blocking activity, against DA splenocytes . There was no third party Fc blocking (results not shown). Since it was not clear whether the absorption procedure had been effective the ability of the same 3 IgG samples before and after absorption to cause Fc blocking with splenocytes from the congenic rat strain PVG $RT1^{r}1$ was tested. The latter rat strain is haploidentical with DA class I A-locus ($RT1A^{a}B^{c}C^{c}D^{c}$), but otherwise syngeneic with recipient PVG MHC. Table 1.12 shows that there was minimal inhibition of Fc blocking in the absence of $RT1B^{a}$ and $RT1D^{a}$ class II MHC antigens. These results suggest that inhibition was predominantly mediated by anti-class II alloantibody. Since the appropriate recombinant rat strain expressing $RT1B^{a}$ and $RT1D^{a}$ class II MHC antigens was not available it was not possible to test directly whether absorption of DA class II MHC antigens alone eliminated Fc blocking in these experiments. The conclusion that antibodies to $RT1A^{a}$ were not contributing substantially to EA rosette inhibition was supported in a further experiment where IgG samples from two more enhanced rats were tested for inhibitory activity against DA,

Table 1.11

The effect of absorbing IgG with DA(donor) and LEWIS (third party) erythrocytes

IgG preparation	% of EAR1 in IgG samples		
	non-absorbed	absorbed with: DA rbc	LEWIS rbc
1	79	87	78
2	93	87	86
3	84	87	87

Table 1.11 Purified IgG preparations from transfused PVG recipients of a DA kidney 5 days after transplantation were absorbed with donor (DA) or third party (LEWIS) erythrocytes and tested for their ability to cause EA rosette inhibition with donor specific (DA) lymphocytes.

Table 1.12

Determination of MHC specificity of IgG alloantibody

IgG preparation		% of EARI in IgG preparations assayed with lymphocytes of: DA	PVG $RT1^{rl}$
1.	unabsorbed	95	12
	absorbed with DA rbc	92	0
2.	unabsorbed	86	0
	absorbed with DA rbc	88	3
3.	unabsorbed	96	8
	absorbed with DA rbc	97	0

Table 1.12 Unabsorbed and DA rbc absorbed IgG samples were prepared from 3 day 5 enhanced rats and tested for their ability to inhibit rosette formation with either DA or PVG-$RT1^{rl}$ (which shares class I with DA) splenocytes.

PVG,PVG-RT1^{rl} and PVG-RT1^{avl}(RT1A^{a}B^{a}C^{a}D^{a}) lymphocytes. Non-absorbed serum was again found to cause high levels of rosette inhibition against DA (94% and 98%) and PVG-RT1^{avl} (70% and 75%) lymphocytes, but not against PVG-RT1^{rl} lymphocytes (14% and 10%). Samples were absorbed with DA erythrocytes, and PVG-RT1^{rl} splenocytes to confirm that removal of anti-class I antibodies did not remove Fc blocking. Table 1.13 again shows that such absorptions did not significantly reduce inhibition against any of the targets. However absorption of the IgG samples with DA splenocytes significantly reduced inhibition in one animal (from 94% to 14%) and completely removed inhibition in the other (from 98% to 0%). Taken together these results strongly suggest that the Fc blocking alloantibody found in enhanced rats is an anti-class II IgG alloantibody.

4.8 Fc blocking activity in the DA to LEWIS strain combination

In the DA to LEWIS strain combination, rats normally reject their kidney grafts in 8 days, furthermore donor specific transfusion given 7 days before transplant does not usually cause prolongation of graft survival (Fabre and Morris, 1972b). Therefore it was interesting to see whether Fc blocking antibodies could be detected in LEWIS rats which had been transfused with DA blood and had received a DA kidney 7 days later. Serum was harvested 5 days post-transplant from 3 such rats and serum fractions and IgG were prepared . The serum fractions were tested for their ability to cause rosette inhibition with DA (donor) and Brown Norway(BN), (third party) lymphocytes. Despite the

Table 1.13

Determination of MHC specificity of IgG alloantibody

IgG preparations absorbed with:		% of EARI in IgG preparations assayed with lymphocytes of: DA	PVG-RT1^{rl}	PVG	PVG-RT1^{l}
1.	unabsorbed	94	16	0	70
	DA rbc	96	0	0	75
	PVG-RT1^{rl}	84	10	8	74
	DA splenocyte	14	ND	ND	ND
2.	unabsorbed	98	10	0	75
	DA rbc	84	10	8	74
	PVG-RT1^{rl}	87	0	10	84
	DA splenocyte	0	ND	ND	ND

Table 1.13 IgG samples which had been absorbed with DA rbc; PVG-RT1^{rl} splenocytes(which share class I antigens with DA) or DA splenocytes were tested for their ability to inhibit rosette formation with DA (RT1^{a}); PVG-RT1^{rl}(RT1A^{a}B^{c}C^{c}D^{c}); PVG (RT1^{c}) and PVG-RT1^{avl}(RT1A^{a}B^{a}C^{a}D^{a}) lymphocytes.

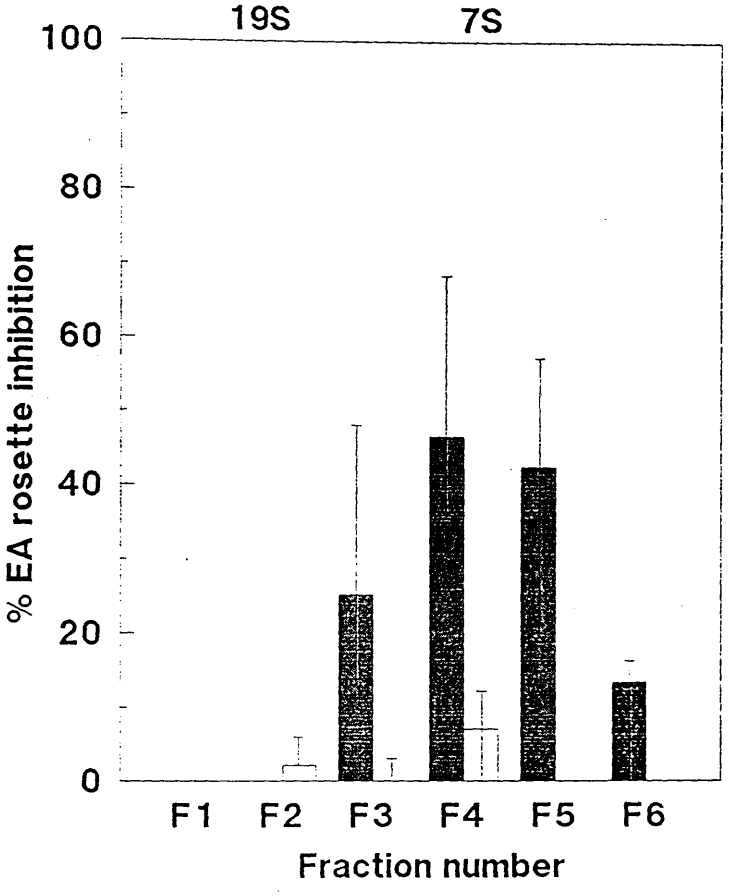


Fig 4.7 Fc blocking activity in serum fractions from transfused Lewis recipients of a DA kidney

Serum was collected from DA transfused LEWIS recipients (n=3), 5 days after transplantation with a DA kidney . The serum fractions obtained by discontinuous gradient centrifugation were then tested for their ability to inhibit rosette formation with donor DA (solid bars) or third party BN (open bars) lymphocytes. Results are expressed as the median and range for each fraction tested.

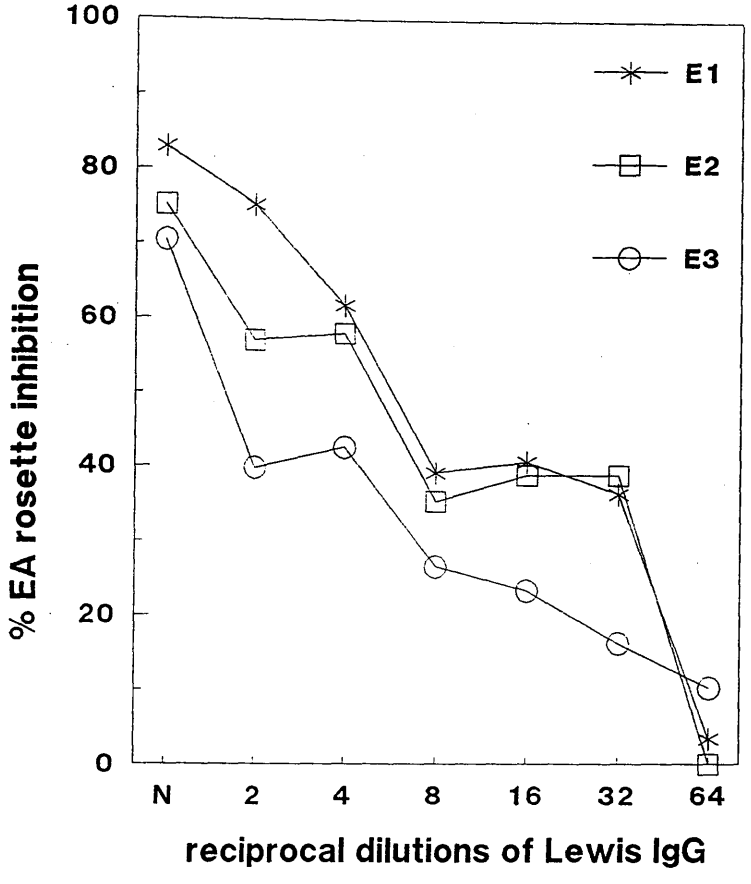


Fig 4.8 Ability of IgG from transfused Lewis recipients of a DA kidney to mediate rosette inhibition

Lewis rats (n=3) were given 1.0ml of DA blood on day -7, transplanted with a DA kidney on day 0 and serum was collected on day 5 after transplantation. Reciprocal dilutions of IgG(from 10ug/well) were tested for their ability to inhibit rosette formation with DA lymphocytes.

failure of transfusion to prevent rejection, donor specific rosette inhibition was found in fractions 3,4 and 5(median values:25%,47% and 42% respectively) (Fig 4.7), although these levels were lower than those found in the corresponding serum fractions from actively enhanced PVG recipients. IgG prepared from these rats similarly produced high levels of rosette inhibition(Fig 4.8). This finding does not rule out a role for anti-class II alloantibodies in the induction of enhancement since it has been shown that it is difficult to passively enhance survival in the DA to LEWIS strain combination (Fabre and Morris,1972a ;1973).

4.9 Discussion

Despite much debate, the mechanism responsible for the prolonged survival of MHC incompatible organ grafts after prior administration of donor antigens or anti-donor antiserum remains elusive. Some early studies addressed the possibility that alloantibodies were responsible for prolonged survival of both actively and passively enhanced rat renal allografts (Strom et al,1975; Soulillou et al,1976; Suthanthiran et al, 1979). The latter author found that alloantibody responses were profoundly depressed in passively enhanced animals and only appeared 7 days after transplant in animals actively enhanced with donor splenocytes. The lack of detectable anti-class I and anti-class II in passively enhanced animals confirmed the earlier finding of Strom that IgG mediated vasculitis and glomerulonephritis were abolished in passively enhanced animals. A more positive role for

antibody in the mechanism of enhancement was postulated by Soulillou et al who provided strong evidence that anti-class II antibodies alone were responsible for passive enhancement of rat renal allografts. He showed that hyperimmune serum which had been absorbed with red cells or platelets and which had no detectable cytotoxic or haemagglutinating activity , was as effective as unabsorbed serum in prolonging graft survival. Furthermore he showed that such sera could mediate donor-specific Fc blocking in a rosette inhibition assay. However there was contradictory evidence that the presence of antibodies which blocked the Fc receptor on lymphocytes was associated with graft loss (Suthanthiran, Gailunas, St.Louis et al, 1977b; Suthanthiran, Gailunas, Fagan et al,1978)

In this study the enhancing effect of blood transfusion on renal allograft survival was found to correlate with the development of high levels of donor-specific FcR blocking activity in the post-transplant serum. When whole serum from enhanced animals was separated into fractions of graded molecular weight and assayed for FcR blocking activity, inhibition resided in fractions 3-5 which contained most of the IgG. The suggestion that anti-donor IgG was the predominant FcR blocking factor in ultracentrifuged whole serum was supported by the observation that monomeric IgG isolated from enhanced serum by n-octanoic acid extraction showed high levels of donor-specific Fc blocking activity. This is in support of the clinical finding that non-cytotoxic Fc blocking antibodies develop during a course of elective blood transfusions prior to renal transplantation and that the presence of such antibodies correlated with improved cadaver donor renal transplant survival(MacLeod et

al,1982). However, in another clinical study of patients given blood transfusions prior to renal transplantation (Forwell et al, 1987), subsequent graft survival correlated not with Fc blocking by the IgG serum fraction (7S) but instead with the level of FcR blocking mediated by a serum factor of high molecular weight (19S). In the present study, little blocking activity could be detected in the (19S) fraction. The discrepancies in the clinical findings may reflect differences in the assay procedures, but the precise nature and specificity of such blocking factors remains unclear.

Early reports on the apparent close association between B lymphocyte Fc receptors and class II antigens (Dickler & Sachs,1974: Suthanthiran, Garavoy, Fagan et al,1977) and the pertinent suggestion that EA rosette inhibition was mediated entirely by anti-class II alloantibodies led several groups to use the EA rosette inhibition assay as a means of measuring anti-class II activity (Soulillou et al 1976; Suthanthiran et al 1979). On the other hand there was conflicting evidence in clinical studies about the relationship between Fc blocking and anti-HLA antibodies. Solheim, Thorsby & Moller (1976),found that anti-HLA antibodies could cause Fc blocking while Soulillou & Peyrat (1979) were unable to find Fc blocking activity against DRw determinants on B lymphocytes. However the previous studies with monoclonal antibodies to class I and II MHC antigens (Power et al,1987) together with the results of this study suggest that inthe rat antibodies directed against class I antigens may also inhibit rosette formation. This may possibly reflect differences in affinity for MHC antigens between monoclonal antibodies and antibodies produced by alloimmunisation. It is possible that

anti-class I antibodies can bind and cause blocking of the FcR in the absence of anti-class II antibodies which may be of higher affinity and compete for binding.

Nevertheless the finding that absorption of anti-donor class I antibody from enhanced IgG failed to abrogate its FcR blocking activity, together with the inability of the IgG from enhanced animals to cause EA rosette inhibition when tested against lymphocytes expressing only the donor $RT1^a$ haplotype both indicated, indirectly that FcR blocking activity of IgG in enhanced rats was due to anti-class II alloantibody.

It was interesting that high levels of EA rosette inhibition could be induced not only by a renal allograft, but also by a second blood transfusion. Since a second antigenic stimulus would be expected to result in an IgG response and a single challenge to result in an IgM response, the lack of FcR blocking activity in rejecting compared with enhanced serum, might reflect a lower level of circulating IgG. This confirms the earlier observation by MacLeod et al (1982) of increased levels of FcR blocking activity after multiple transfusions. Similarly, Power et al(1987) found that FcR blocking activity developed in the serum of rats as a result of multiple pregnancies, but in this case they appeared to be directed against class I antigens alone. Alternatively the lack of Fc blocking in the rejecting serum may be due to a lack of anti-class II antibodies. In the transfused animals such antibodies could cause opsonisation of donor dendritic cells or masking of antigenic determinants on vascular endothelium. In the previous chapter it was shown that MHC class II antigens are induced on the endothelium of both rejecting and actively

enhanced kidneys which could increase their susceptibility to attack. Furthermore it was noted that donor dendritic cells disappeared more quickly from the enhanced kidneys.

This study has demonstrated that the ability of donor strain blood transfusion to prevent renal allograft rejection in the DA to PVG rat strain combination correlates with the appearance in the post-transplant serum of high levels of donor specific FcR blocking activity, most likely as a consequence of IgG anti-donor class II antibody. It is not clear whether the generation of such alloantibody plays a role in prolonging graft survival in this model, or whether the alloantibody response is an epiphenomenon with no direct bearing on graft survival. It is notable that blood transfusion in the DA to Lewis strain also resulted in high levels of EA rosette inhibition in the post-transplant serum despite no prolongation of graft survival. However this is consistent with the finding that it is extremely difficult to passively enhance the DA to Lewis strain by administration of anti-donor alloantibody (Fabre & Morris, 1972a, 1973). Further evidence for the immunosuppressive effects of transplantation induced anti-class II alloantibodies, was recently provided by Kamada et al, (1986,1988), who showed that serum from rats which spontaneously accept liver allografts was able, after passive transfer to induce specific unresponsiveness.

In conclusion, the results reported here provide evidence that transfusion induced survival of rat renal allografts is associated with the generation of Fc-blocking antibodies which appear to be anti-class II alloantibodies. Although such antibodies appear to play a role in passive enhancement of rat renal allografts, and

spontaneous acceptance of rat liver allografts, further investigation is required to determine whether these antibodies might be responsible, in part, for the beneficial effect of blood transfusion on renal allograft survival.

CHAPTER 5

ALLOANTIBODY RESPONSES IN ENHANCED AND REJECTING RAT RENAL ALLOGRAFT RECIPIENTS

5.1 Introduction

In the previous chapter it was shown that blood transfused rats bearing a non-rejecting renal allograft had high levels of FcR blocking activity in their serum 5 days after transplantation. This FcR blocking activity was attributable mainly to anti-class II alloantibody, suggesting that there are important differences in the nature and timing of the alloantibody responses to a renal allograft in transfused and non-transfused recipients.

There are no detailed comparisons of the alloantibody response to a kidney allograft in blood transfused and unmodified rats, although there are a number of such studies in passively enhanced rats (Strom et al,1973; Suthanthiran et al,1979). Strom et al found that the lymphocytotoxic antibody response was similar in both unmodified and passively enhanced renal allograft recipients. However, Suthanthiran reported that passively enhanced rats showed a minimal anti-class II (measured by Fc blocking) and a weak anti-class I response (measured by haemagglutination and lymphocytoxicity). Interestingly, this study also showed that rats in which active enhancement was induced by preoperative injection with donor spleen cells, did not develop a detectable alloantibody response until 7 days after transplantation. Other studies in both humans and rats have suggested that repeated transfusions , may lead to a reduction in alloantibody levels.(Opelz et al,1981;Fabre and Morris,1972b ; Lenhard et al,1985).

Since transfusion-induced alterations in the nature of the alloantibody response to a renal allograft may be of relevance to enhancement it was decided to undertake a detailed analysis of the alloantibody response of unmodified and transfused PVG rats to a renal allograft.

5.2 Kinetics of antibody response to class I antigens in enhanced and rejecting rats

The kinetics of the antibody response to DA class I antigens was measured in unmodified (rejecting) and transfused (enhanced) PVG recipients of DA allografts. Enhanced recipients were transfused with 1.0ml of DA blood on day -7 and along with unmodified recipients were transplanted with a DA kidney on day 0. Rats were bled serially on days 1,3,5,7 and 10 from the tail vein. Sera were heat-inactivated at $56^{o}C$ for 30 mins , aliquoted and frozen at $-70^{o}C$ before use.

Quadrupling dilutions of the antisera were incubated in duplicate with PVG.RTI^{a} red cells which express only class I but not class II antigens of the donor. Furthermore they express the same minor antigens as the recipient so all of the antibody activity is directed against class I MHC. The amount of antibody bound was then detected by the addition of a ^{125}I labelled sheep anti-rat Ig antibody. Background binding of the second antibody was measured by incubating red cells with DAB. Results are expressed either in cpm with background adjusted or as a percentage of a hyperimmune serum control which was present in all assays .

The kinetics of the antibody response to class I MHC antigens can be seen in Fig 5.1 and 5.2. In Fig 5.1 the mean cpm is expressed as a percentage of the hyperimmune serum control, since test sera were not all assayed on the same day. In unmodified rats an anti-class I response was detectable on day 5 after transplantation and increased thereafter to 50% of the hyperimmune serum on day 10 after transplantation . In contrast, transfused rats bearing an enhanced kidney failed to show a detectable anti-class I alloantibody response during the first 10 days after transplantation. In Fig 5.2 individual curves are shown for enhanced and rejecting rats 5 days after transplantation and results are expressed as cpm since all the test sera were assayed simultaneously.

5.3 Kinetics of antibody response to class I in rats following single or double transfusion

In order to determine whether the lack of anti-class I in the serum of enhanced animals was due to failure to generate an antibody response, or due to absorption of antibody by the graft, the levels of anti-class I following single or double transfusion were measured. Seven PVG rats were given 1.0ml of DA blood i.v. on day 0, three of which were given a second transfusion on day 7. Rats in both groups were bled serially on days 7,8,10,12 14 and 17 after the first transfusion. The kinetics of the anti-class I response are shown in Fig 5.3. The levels of response in both groups were similar on day 7. The anti-class I activity reached a peak in the single transfusion

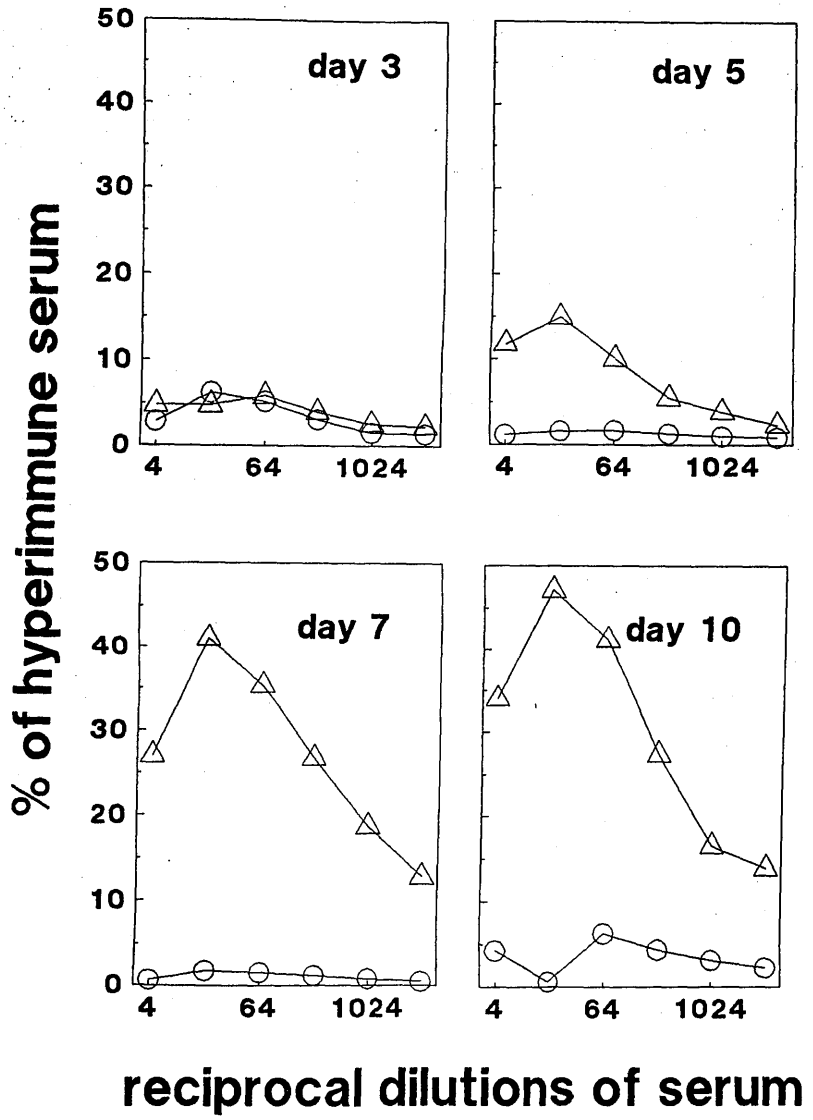


Fig 5.1 Kinetics of antibody response to class I MHC antigens in the serum of enhanced and rejecting PVG recipients of a DA kidney. Serum was collected from enhanced (n=3) and rejecting (n=3) PVG rats on days 0,3,5,7 and 10 after transplantation. Quadrupling dilutions of enhanced (open circles) and rejecting (open triangles) sera were tested for anti-class I activity . The mean cpm at each dilution is expressed as a percentage of the hyperimmune serum control.

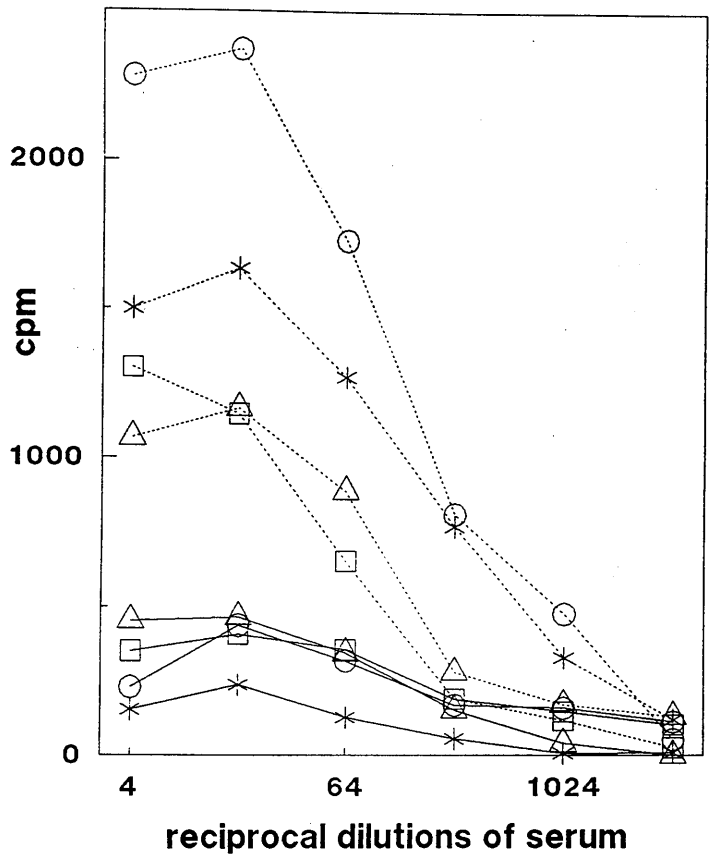


Fig 5.2 Antibody response to class I MHC antigens in the serum of enhanced and rejecting rats 5 days after transplantation
Serum was harvested from four enhanced (solid lines) and four rejecting (dashed lines) PVG rats 5 days after transplantation with a DA kidney, and tested for anti-class I activity.

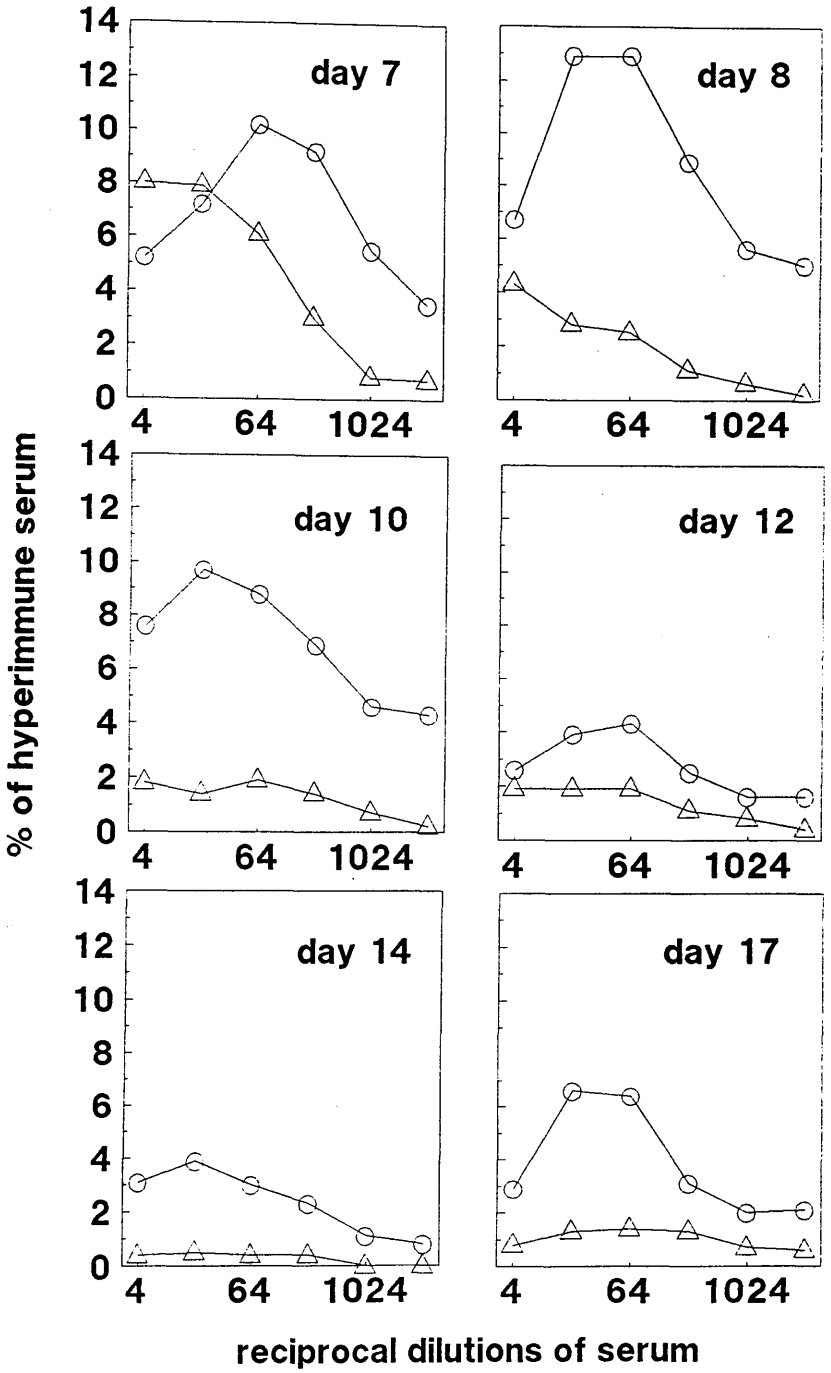


Fig 5.3 Kinetics of antibody response to class I MHC antigens in PVG rats following single or double transfusion

PVG rats (n=6) were given 1.0ml of DA blood on day 0, and three were given a second transfusion on day 7 . Both groups of rats were bled on days 7,8,10,12,14 & 17 after the first transfusion and the antibody response to class I MHC measured. The mean peak response is expressed as a % of the hyperimmune serum control for the single transfusion group (open circles) and the double transfusion group (open triangles).

group on day 8 after transfusion (13% of the HI serum) while levels in the double transfusion group fell to 4% of the HI serum control. The anti-class I activity in the single transfusion group declined rapidly between days 8 and 17 to 2% of the HI control and in the DT group fell to 1% of the control. The second transfusion does not appear to provoke a secondary response and the primary response produces only low levels of antibody compared to those found in the rejecting rats.

5.4 Absorption of serum with erythrocytes to remove anti-class I activity

Since there is no cell type in the rat which expresses only class II MHC antigens and there are no congenic strains which have the appropriate haplotype at the class II loci ($RT1B^a,D^a$), anti-class II antibody levels could not be assayed directly. Anti-class I activity was therefore removed by absorbing with DA red cells and the resulting sera were then tested for antibodies to $RT1B^aD^a$ by measuring binding to $RT1^a$ LNC. Preliminary studies were carried out to find out how many absorption steps were required to remove the anti-class I activity from the day 10 samples which had been found to contain the most activity. Two successive absorptions for 1 hr at 4^oC with equal volumes of packed DA erythrocytes at 4^oC were found to remove only 50% of the anti-class I activity, but the remaining activity was eliminated by a further 2 absorption steps (Figs 5.4a and 5.4b). To confirm that absorption with DA red cells was not removing any of the anti-class II antibody (by binding to contaminating leucocytes), two day 10 sera were absorbed with either DA ($RT1^a$)or PVG.$RT1^{rl}$ red

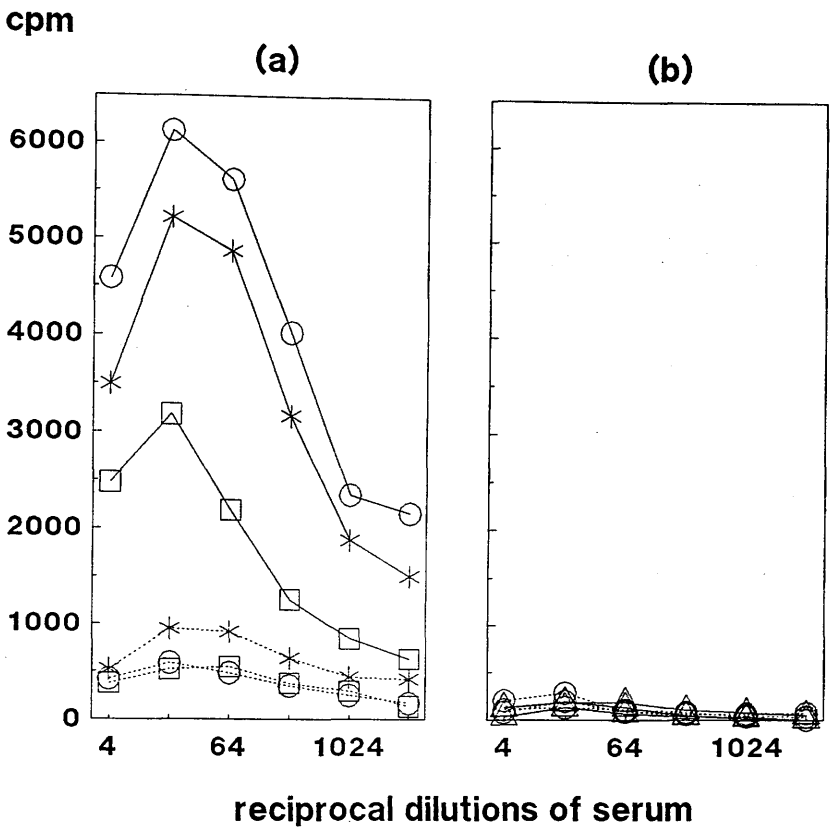


Fig 5.4 Antibody response to class I MHC antigens in serum before (a) and after (b) absorption with DA erythrocytes.
Serum was harvested from enhanced (n=3) and rejecting (n=3) rats 10 days after transplantation. Enhanced (broken lines) and rejecting (solid lines) sera were tested for antibody to class I MHC antigens before(a) and after(b) absorption with DA erythrocytes.

cells. Any contaminating leucocytes have the $RT1A^aB^cC^cD^c$ haplotype and therefore will not bind antibody to $RT1B^aD^a$. Absorption decreased activity slightly, probably as a result of diluting the sample out (the final volume was generally twice that of the starting volume). However there was little difference between the binding of DA red cell absorbed and the PVG.$RT1^{rl}$ red cell absorbed sera to PVG.$RT1^a$ LNC,(Figs 5.5a and 5.5b),so absorption is not removing notable amounts of anti-class II antibody.

5.5 Kinetics of antibody response to class II MHC antigens in enhanced and rejecting rats

The antibody response to class II MHC antigens was measured in 3 enhanced and 3 rejecting rats on days 1,3,5,7 and 10 after transplantation. All test sera were assayed simultaneously and the results are expressed as cpm after adjustment for background counts. The levels of background counts were high (1000-2000 cpm),due to the binding of the second antibody to immunoglobulin on B cells . The mean cpm for each dilution in both groups was calculated . Fig 5.6 shows the binding curves for days 1,3,5 and 7 after transplantation. It can be seen that on day 1 after transplantation levels of anti-class II Ab were slightly higher in the enhanced than in the rejecting group . By day 3,levels in the enhanced serum had risen from 1700cpm to 3000cpm and there was three times the amount of anti-class II Ab compared with the rejecting serum.The level of anti-class II Ab stayed around the same in the enhanced group on days 5 and 7 while in the rejecting

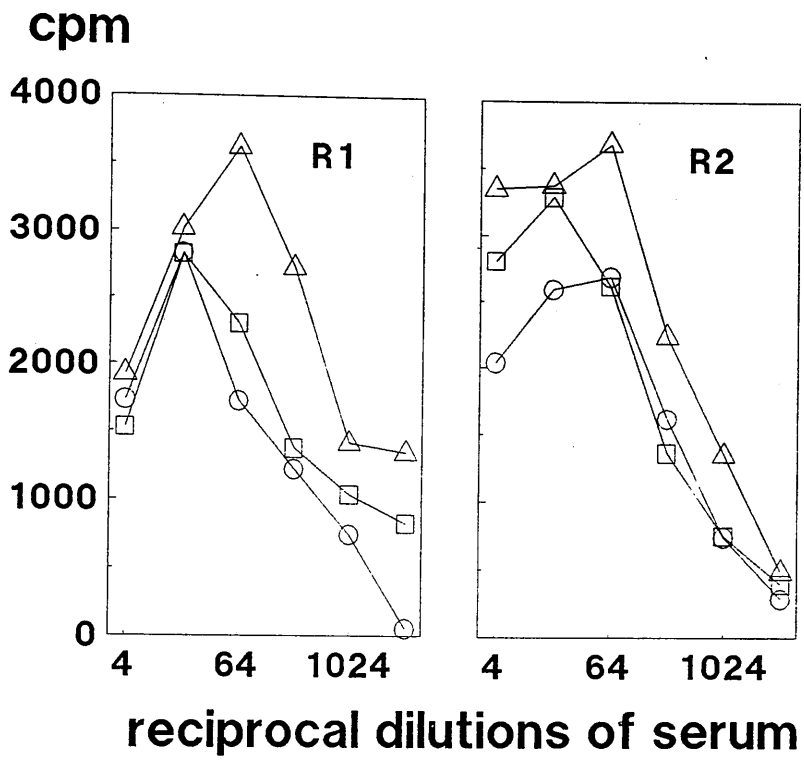


Fig 5.5 Comparison of the antibody response to class II MHC antigens in the serum of rats after absorption with DA or PVG.R1 red cells
Serum was collected from two PVG rats R1 (a) and R2 (b), 10 days after transplantation with a DA kidney. Unabsorbed (open triangles), DA red cell absorbed (open squares) and PVG.R1 red cell absorbed (open circles) sera was tested for anti-class II antibody activity.

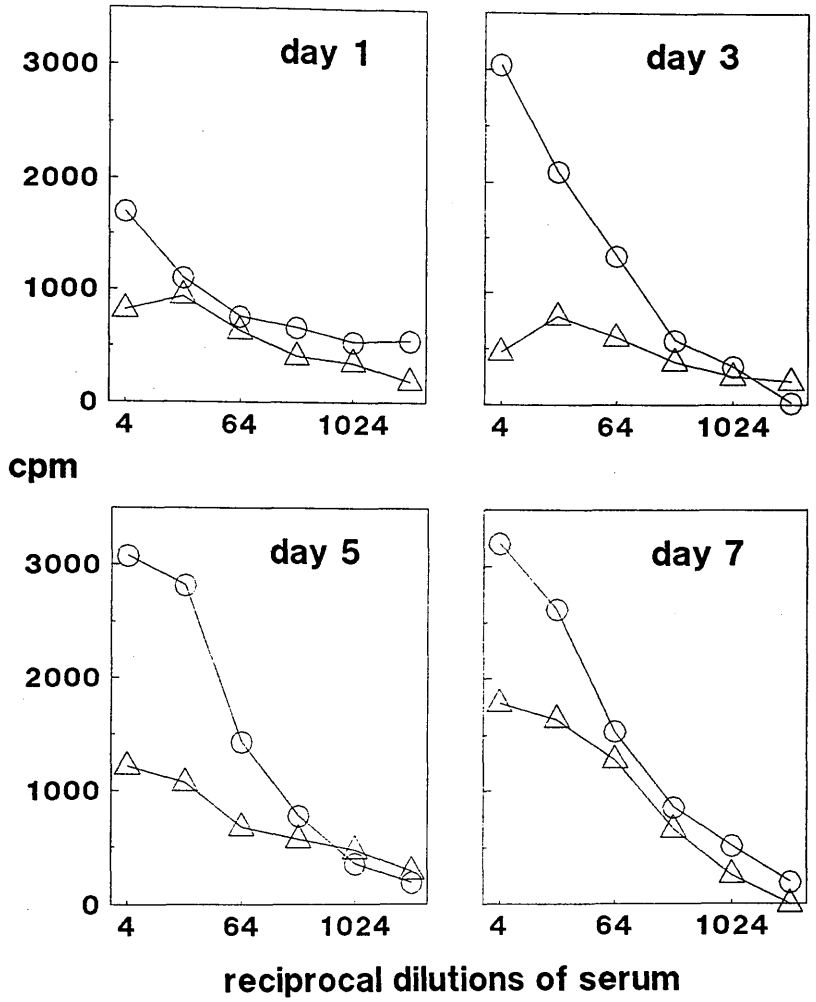


Fig 5.6 Kinetics of antibody response to class II MHC antigens in enhanced and rejecting PVG recipients of a DA kidney
Serum was harvested from enhanced (n=3) and rejecting (n=3) PVG rats on days 1,3,5 and 7 following transplantation with a DA kidney. Enhanced (open circles) and rejecting (open triangles) sera were then tested for anti-class II antibody activity.

group the levels steadily rose. By day 10 the levels of anti-class II Ab were still rising in both groups,with levels similar in each group: the mean peak level in the enhanced group was 3825 cpm compared with 3828 cpm in the rejecting group. The day 5 sera were assayed again with four sera in each group which confirmed that levels of anti-class II antibodies were considerably higher in the enhanced group compared with the rejecting group (Fig 5.7). The specificity of the antibody response was confirmed by testing serum from 3 enhanced and 3 rejecting rats 10 days after transplantation against third party (Lewis) LNCs.There was no net binding above background of day 10 sera (results not shown). IgG samples prepared by n-octanoic acid precipitation from enhanced (n=4) and rejecting (n=4) PVG rats 5 days after transplantation with a DA kidney were tested for anti-class II Ab activity. The IgG samples at 1mg/ml were absorbed with DA red cells as before and 50ul of doubling dilutions assayed as usual. It can be seen in Fig 5.8 that IgG from enhanced rats had twice as much anti-class II as IgG from rejecting rats.

5.6 Kinetics of anti-class II response following single or double transfusion

The levels of anti-class II antibody present in the single and double transfusion group described previously were also measured on days 8,10 12 14 and 17 after the first transfusion. As with the anti-class I response it appeared that the second transfusion did not evoke an immediate secondary response, though the levels of anti-class II Ab

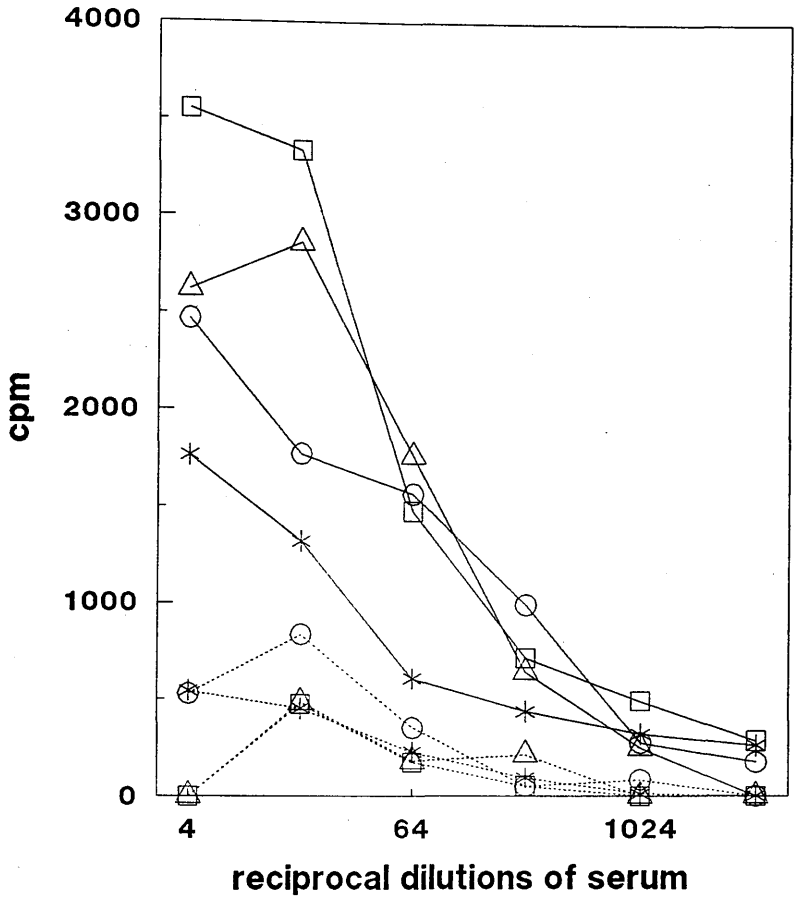


Fig 5.7 Antibody response to class II MHC antigens in the serum of enhanced and rejecting rats 5 days after transplantation
Serum was harvested from four enhanced (solid lines) and four rejecting (dashed lines) rats 5 days after transplantation and tested for anti-class antibody activity.

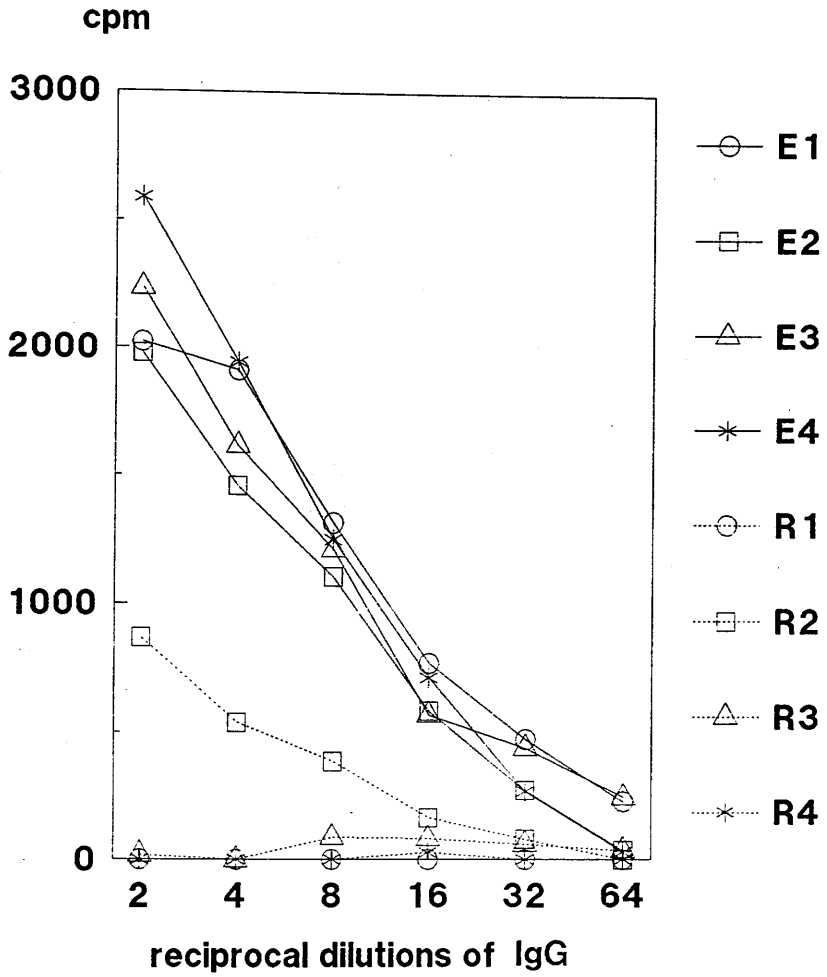


Fig 5.8 Antibody response to class II MHC antigens in IgG samples from enhanced and rejecting rats

IgG was prepared by n-octanoic acid precipitation from serum obtained from enhanced (n=4) and rejecting (n=4) PVG rats 5 days after transplantation with a DA kidney. Doubling dilutions of enhanced (solid lines) and rejecting (dotted lines) IgG(from 50ug/well) were then tested for anti-class II antibody activity.

were rising 8 days after the second transfusion (Fig 5.9). The anti-class II response in the double transfusion group was barely detectable immediately after the second transfusion, possibly due to absorption of antibody by the transfused blood. Levels of anti-class II were maximal in the single transfusion group 14 days after the first transfusion (33% of the HI serum control) while in the double transfusion group they were maximal on day 17 after the first transfusion (12% of the HI serum control).

5.7 Cytotoxic antibody response in enhanced and rejecting rats

Serum was collected from enhanced(n=3) and rejecting(n=3) rats on days 1,3,5,7 and 10 as described for the alloantibody assays.Doubling dilutions of antisera were tested for complement-dependent cytotoxicity against DA con A blasts in the presence of guinea-pig serum. The kinetics of cytotoxic antibody production are shown in Fig 5.10. On day 1 after transplantation there was no detectable cytotoxic antibody in the rejecting sera while in the enhanced sera there was a peak response of 28% killing. By day 3 levels of cytotoxicity had risen to 28% in the rejecting rats while in the enhanced rats levels had fallen to 18%. There was no change in cytotoxic antibody levels in the enhanced rats on day 5 ,however in the rejecting rats the % cytotoxicity had risen to 50%. There was little change on day 7 and and 10 in the levels of killing in the enhanced group, but levels continued to rise until day 10 in the rejecting group(not shown).

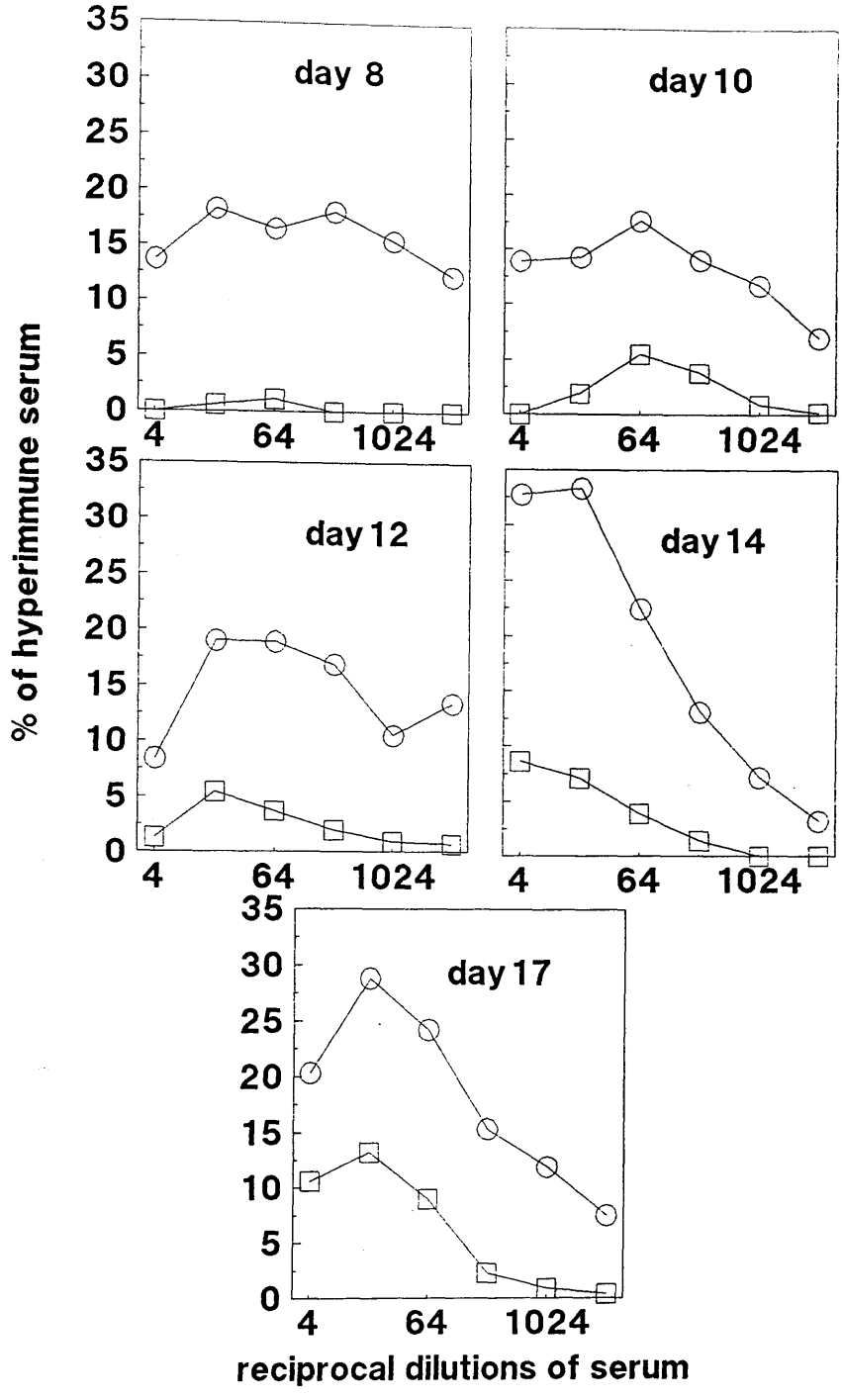


Fig 5.9 Kinetics of antibody response to class II MHC antigens in rats following single or double transfusion

PVG rats (n=6) were given 1.0ml of DA blood on day 0 , 3 of which were given a second transfusion on day 7. The single(open circles) and double (open squares) transfusion groups were bled on days 8,10,12,14 and 17 and antibody to class II MHC antigens was measured. The mean peak response is expressed as a % of the hyperimmune serum control.

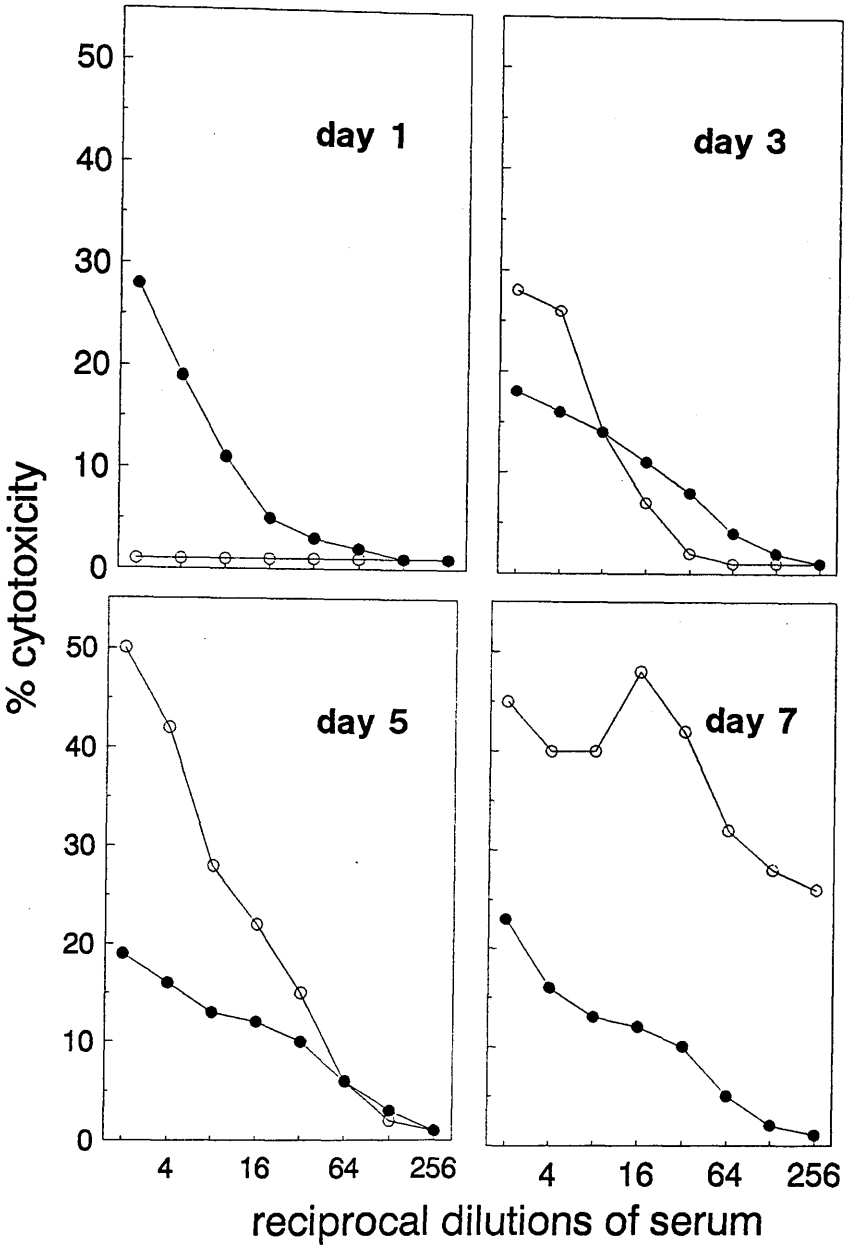


Fig 5.10 Kinetics of complement dependent cytotoxicity response in enhanced and rejecting PVG recipients of a DA kidney
Serum was collected from enhanced(n=3) and rejecting (n=3) PVG rats ,5 days after transplantation with a DA kidney. Doubling dilutions of enhanced (solid circles) and rejecting (open circles)sera were tested for cytotoxic antibody against DA blasts in the presence of guinea pig complement. Results are expressed as the % cytotoxicity.

There is some evidence that the complement system in the rat is not as effective as in other species (French and Batchelor,1972), so it was important to establish whether the sera were also cytotoxic in the presence of recipient (PVG) serum. Serum was collected from enhanced (n=3) and rejecting (n=3) rats 5 days after transplantation. The assay was performed essentially as before, however 100ul of a 1:4 dilution of fresh PVG serum was added instead of guinea pig complement. The specificity of the cytotoxic antibody response was also examined by using PVG.RT1^{rl} con A blasts as targets along with DA blasts. In Fig 5.11 it can be seen that levels of cytotoxicity against DA con A blasts in the presence of guinea pig serum were higher in the 3 rejecting rats than the 3 enhanced rats as was shown before. However if fresh PVG serum was added there were only low levels of cytotoxic antibody detectable in each group,the most dramatic reduction being in the rejecting group (Fig 5.12). Interestingly, when the sera were assayed for complement dependent cytotoxicity in the presence of guinea pig complement, against PVG.RT1^{rl}blasts(which share class I but not class II with DA), levels of cytotoxicity in the rejecting group were the same as against DA blasts. However in the enhanced group there was no cytotoxicity against PVG.RT1^{rl} blasts, suggesting that any cytotoxicity in this group is directed at class II MHC antigens(Fig 5.13).

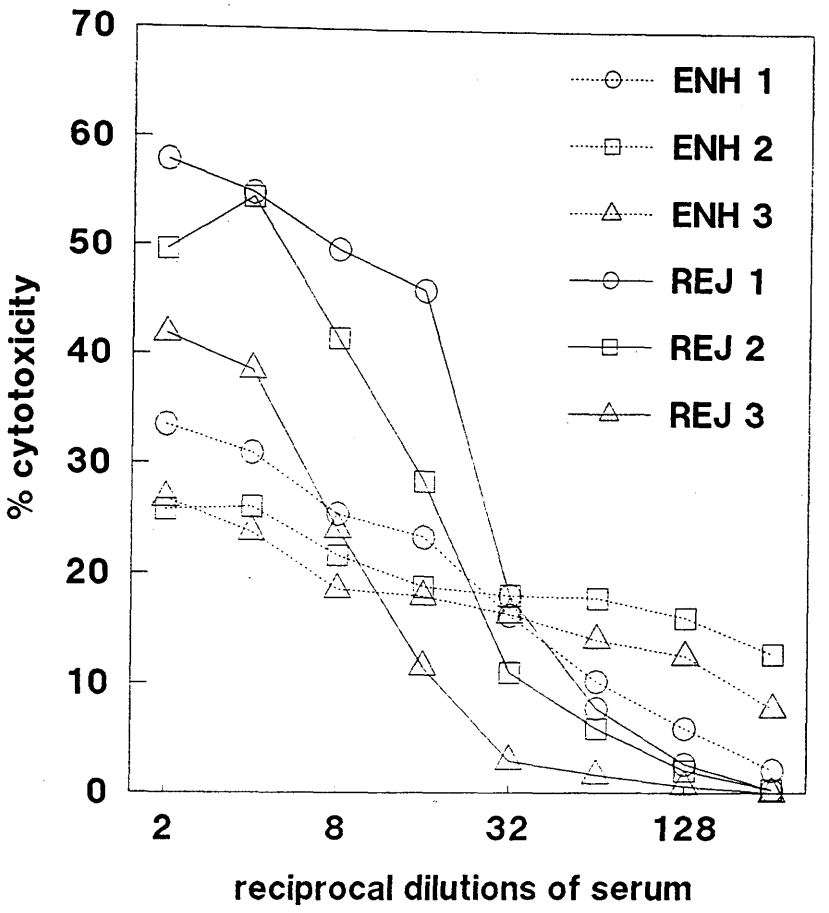


Fig 5.11 Ability of serum from enhanced and rejecting rats to mediate complement dependent cytotoxicity
Serum was collected from enhanced (n=3) and rejecting(n=3) PVG rats 5 days after transplantation with a DA kidney. Doubling dilutions of sera were tested for complement dependent cytotoxicity against DA con A blasts in the presence of guinea pig complement.

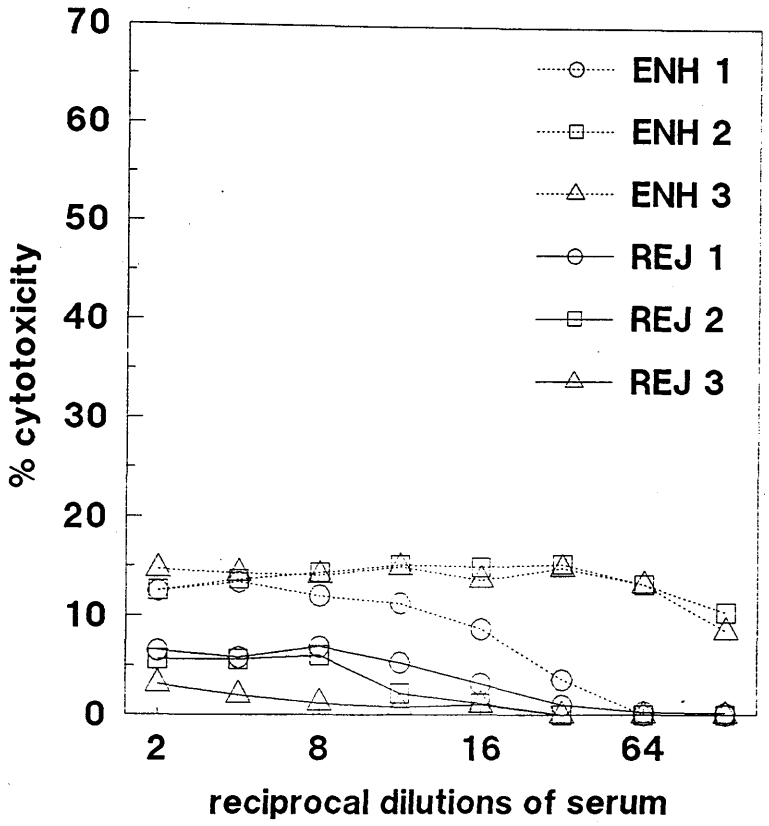


Fig 5.12 Ability of serum from enhanced and rejecting rats to mediate complement dependent cytotoxicity in the presence of PVG serum

Serum was collected from enhanced (n=3) and rejecting (n=3) PVG rats 5 days after transplantation with a DA kidney . Doubling dilutions of sera were tested for complement dependent cytotoxicity against DA con A blasts in the presence of PVG serum.

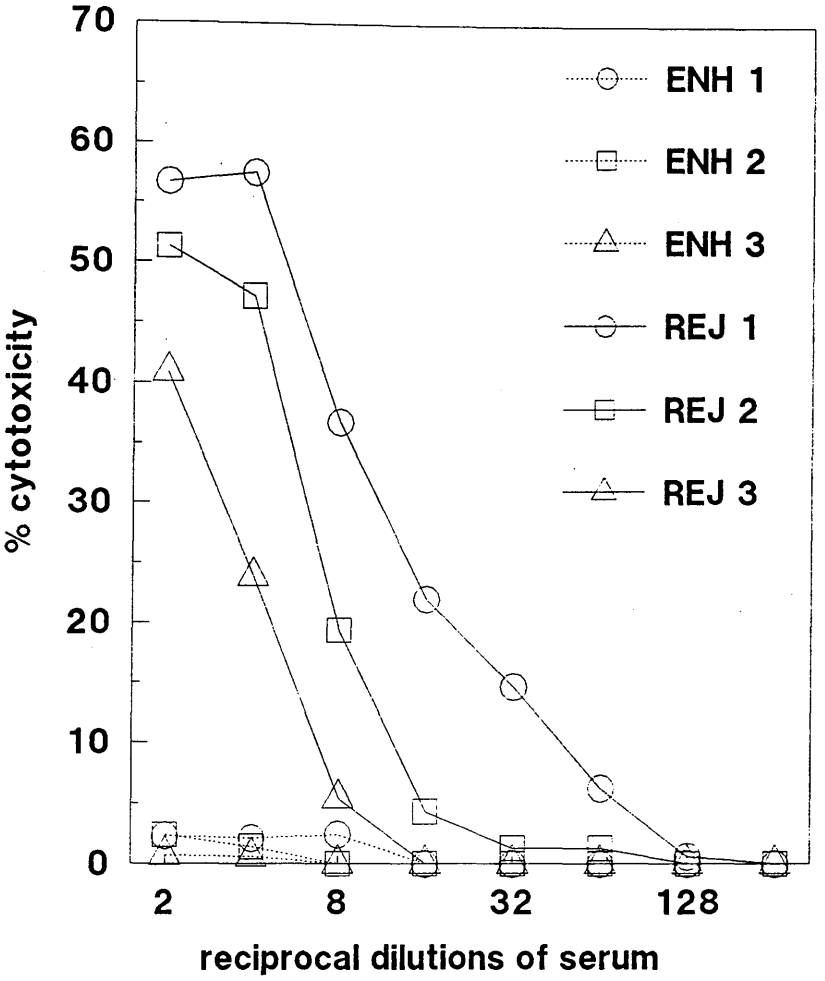


Fig 5.13 Ability of serum from enhanced and rejecting rats to mediate complement dependent cytotoxicity against PVG.R1 blasts
Serum was collected from enhanced (n=3) and rejecting (n=3) PVG rats 5 days after transplantation with a DA kidney. Doubling dilutions of sera were tested for complement dependent cytotoxicity against PVG.R1 con A blasts in the presence of guinea pig serum

5.8 Measurement of IgG and IgM in alloantibody response

A three-layer binding assay was used to detect the antibody class produced in response to either class I or class II MHC antigens. Briefly,equal volumes of serum samples from enhanced (n=3) and rejecting (n=3)rats on various days following transplantation were pooled. These were diluted 1:2 and were incubated in triplicate with either DA red cells or LNC as for the alloantibody assay. Then a biotinylated anti-IgG or anti-IgM antibody was added at a pre-determined optimum dilution(1:50). The amount of second antibody bound was then detected by the addition of ^{125}I-labelled streptavidin. The % binding was then calculated for each serum sample.

Clearly the % binding is also a reflection of the amount of anti-class I or anti-class II antibody binding in the first step. However a comparison can be made about the amount of IgG relative to the amount of IgM at a particular time point. Fig 5.14 shows the IgG and IgM components of the anti-class I response in enhanced and rejecting rats. The % binding was higher in the rejecting rats than in the enhanced rats as would be expected from the previous alloantibody results. The main component of the anti-class I response in the enhanced rats was IgG which was highest on day 3 post transplant. There was a drop in serum IgG and IgM on day 5 post transplant possibly as a result of absorption by the kidney which is expressing large amounts of class I at this time. Thereafter the levels of both IgG and IgM rose steadily until day 10 post-transplant. In the

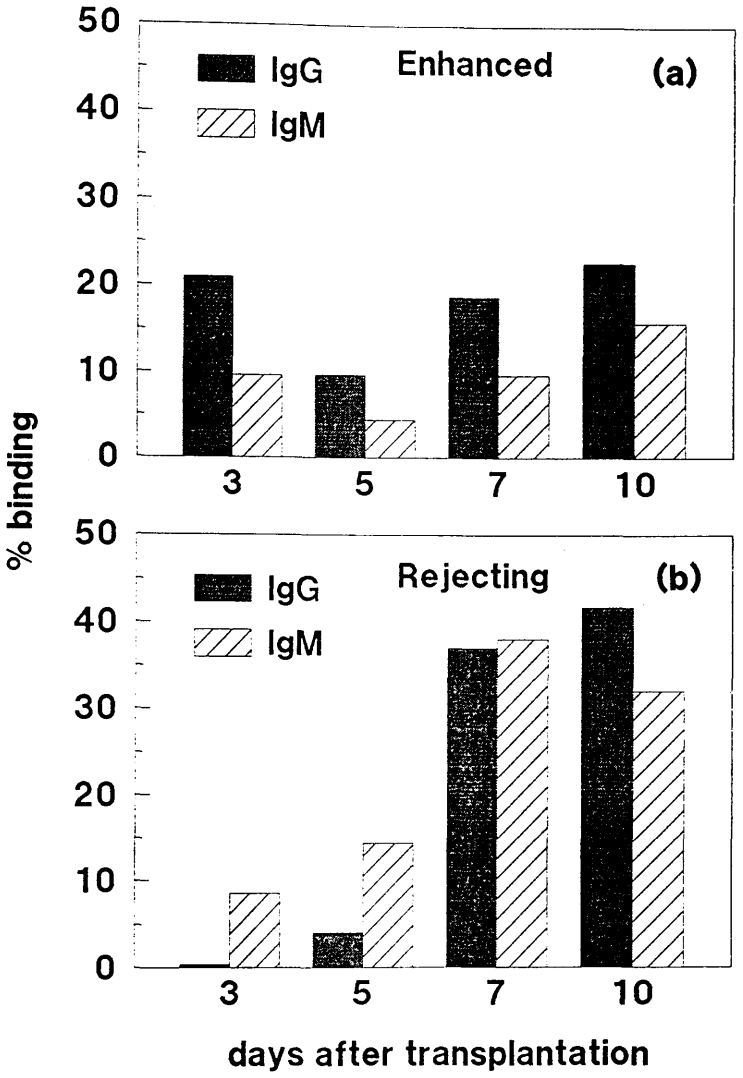


Fig 5.14 IgG and IgM component of the antibody response to class I MHC antigens in enhanced (a) and rejecting (b) rats
Sera were collected from enhanced (n=3) and rejecting (n=3) PVG rats on days 3,5,7,and 10 after transplantation. These were pooled to give one sample for each group on each day. After incubation with DA red cells biotinylated anti-IgG or anti-IgM was added followed by ^{125}I-labelled streptavidin. Results are expressed as % binding.

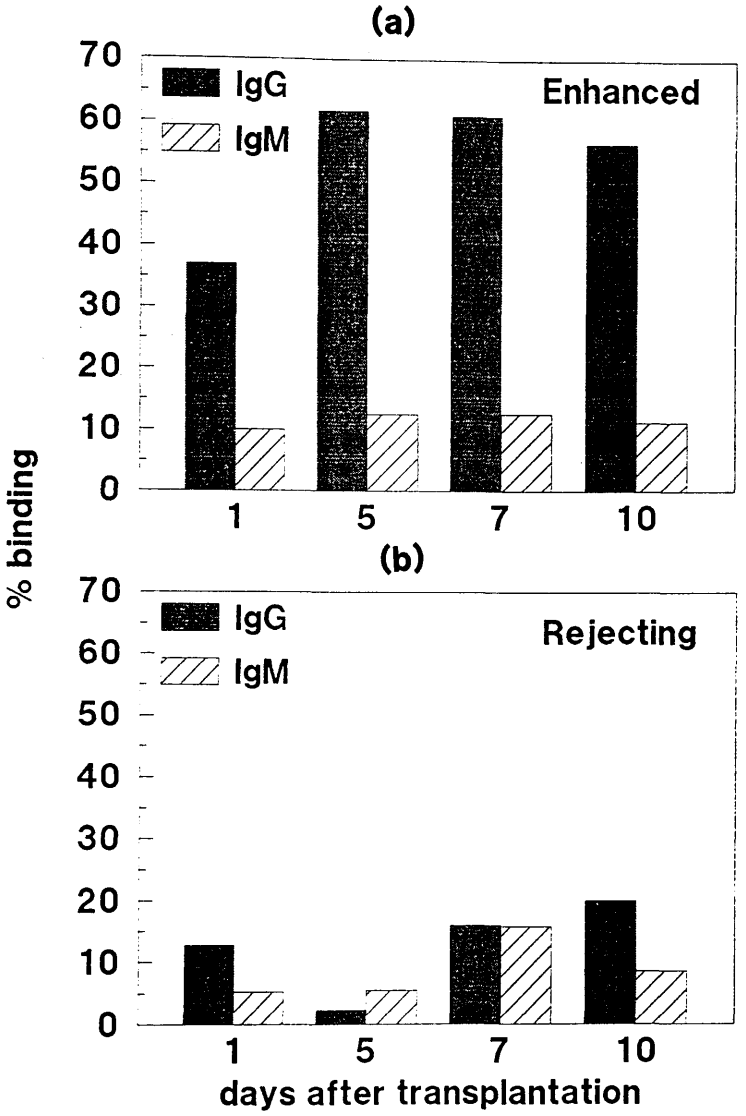


Fig 5.15 IgG and IgM component of the antibody response to class II MHC antigens in enhanced (a) and rejecting (b) rats
Sera were collected from enhanced (n=3) and rejecting (n=3) PVG rats on days 1,5,7 and 10 after transplantation. These were pooled to give one sample for each group on each day. After incubation with DA red cells , biotinylated anti-IgG or anti-IgM was added followed by ^{125}I-labelled streptavidin. Results are expressed as % binding.

rejecting rats on day 3 after transplantation only IgM was detected, the levels of which reached a peak on day 7 post-transplant. IgG was detected on day 5 post-transplant but by day 7 levels had increased greatly and were equal to the levels of IgM .

The IgG and IgM components of the anti-class II response of enhanced and rejecting rats can be seen in Fig 5.15 respectively. Despite high levels of background binding of anti-rat IgG (to Ig on B cells) there was still net binding of IgG. The % binding to enhanced and rejecting animals was again indicative of the levels of anti-class II originally present. The majority of the antibody present in the enhanced animals was of the IgG class . On days 1,5,7 and 10 after transplantation the % of IgG binding compared with IgM in the enhanced group was 35% vs 10%; 61% vs 12%; 60% vs 11% and 56% vs 10%, respectively. In the rejecting animals IgG was detected as early as day 3 after transplantation but levels dropped on day 5 and rose again on days 7 and 10. Levels of IgM were either equal or slightly less than IgG on all days tested. The % binding of both IgG and IgM on days 7 and 10 was not as high as would have been expected from the earlier Class II results .

5.9 Discussion

Previous studies on the blood transfusion effect have not made a detailed study of the alloantibody response in transfused and unmodified recipients following transplantation. Antibody has not been thought to be important in the mechanism of active enhancement,

with the demonstration of suppressor cells by a number of groups, (Hutchinson ,1986 ; Quigley et al, 1989a,b,c). Attempts to transfer suppression with serum have had varying degrees of success (Tilney & Bell,1974) . Since IgG antibodies directed either at class I or class II are able to transfer passive enhancement, it seemed reasonable that active enhancement could be due to the presence of IgG antibodies induced by blood transfusion and therefore present at the time of grafting. As discussed in the previous chapter such antibodies could either interfere with host sensitisation by removal of dendritic cells from the graft or by binding to endothelium thereby blocking allorecognition and preventing vascular damage by any alloactivated host cells.

Both anti-class I and anti-class II antibodies were measured serially following transplantation in enhanced and rejecting rats. In the DA to PVG strain combination enhanced animals had low levels of anti-class I compared with rejecting animals. It was thought that this might be due to absorption of antibodies by the graft but unfortunately animals were not bled before transplant in the transfused alone group. However the finding that there were high levels of anti-class I antibody in the rejecting rats was interesting since PVG has been shown to be genetically a low responder to RT1A (Butcher, Corralan, Licence et al, 1982). The latter authors showed that when splenocytes from PVG.RT1^{rl} rats(bearing an isolated class I disparity) were injected into PVG rats there was a poor cytotoxic antibody response compared with PVG rats which had received splenocytes from a DA rat (full MHC mismatch). Further experiments suggested that this defect in

responsiveness to class I was due to an Ir gene effect in the responder which could be overcome by help in the response to class II. Therefore the lack of response to class I in enhanced animals could result from the removal of the class II stimulus necessary to provide help for anti-class I antibody synthesis. A simpler explanation might be that the high amounts of class I MHC antigen presented by the blood result in high-zone tolerance either at the B or T cell level. However there are a number of reports which suggest that the antibody response to MHC antigens (induced by splenocyte injection) in other rat strain combinations is also directed mainly at class II MHC antigens (Gallico & Mason,1978; McKenzie,Fabre & Morris, 1980).

Single transfusion with donor blood also produced a poor anti-class I response, again suggesting that there may be some form of high-zone tolerance. A second transfusion led to further reduction in the anti-class I response possibly as a result of absorption . However antibody levels did not pick up and it could be that existing antibody formed immune complexes with newly injected red cells thereby preventing antigen from binding to memory cells and triggering a secondary response. Alternatively it has been suggested that the reduction in antibody levels found after repeated blood transfusions is mediated by suppressor cells (Lenhard et al,1985).

The anti-class II response found in the DA to PVG strain combination confirms the results of the previous chapter where it was postulated that Fc blocking antibodies found in enhanced rats were in fact anti-class II antibodies. High levels of anti-class II antibodies were detected in the enhanced group compared with the rejecting group on

day 5 after transplantation,though levels in the rejecting group equalled those in the enhanced group by day 10 . Most of the activity in the enhanced rats was of the IgG class while in the rejecting rats there was little IgG as shown in binding studies. It should be noted , that if such antibodies are involved in the induction of the enhanced state then the animals in the unmodified group are already rejecting their grafts by the time the levels have risen to those found in the serum of enhanced rats. When IgG samples prepared from enhanced and rejecting rats 5 days after transplantation were tested for anti-class II activity a similar pattern was found . Only one of four rejecting IgG samples had detectable antibody to class II MHC antigen while all four enhanced IgG samples had high levels (2000cpm) of activity at the optimum dilution. Double transfusion did not evoke such a good antibody response but actually led to a reduction in anti-class II levels after the second transfusion. Such a reduction in alloantibody after multiple blood transfusion has been observed in humans (Opelz et al,1981) and in rats (Fabre & Morris, 1972; Lenhard et al 1985).

The appearance of cytotoxic antibody in the rejecting and enhanced groups seemed to correlate with the appearance of antibody to class I and class II MHC antigens respectively. This was confirmed by measuring cytotoxic antibody levels against PVG.RT1^{rl} targets bearing an isolated class I disparity, which showed high levels of killing (comparable to those obtained against DA targets), while enhanced sera failed to kill PVG.RT1^{rl} blasts. This suggests that the high levels of cytotoxic antibody seen in the rejecting group are mainly

anti-class I. However it seems unlikely that the presence of such antibodies in the rejecting rats, but not in the enhanced rats is the cause of rejection. When normal PVG serum was used as a source of complement no killing of donor blasts was seen. This confirms the earlier observation by French and Batchelor,1969 that rats treated with hyperimmune serum, which would normally cause enhancement, rejected their grafts if injected with guinea pig complement. However this finding does not rule out the possibility of such antibody mediating damage in vivo via an ADCC type of mechanism.

CHAPTER 6

ABILITY OF SERUM FROM ENHANCED AND REJECTING RAT RENAL ALLOGRAFT RECIPIENTS TO INHIBIT THE DONOR-RECIPIENT MLR

6.1 Introduction

The mixed lymphocyte reaction (MLR) in which T cells are stimulated to proliferate in response to allogeneic cells, provides a model of cellular alloreactivity which may, to some extent, be taken as an in vitro correlate of graft rejection (Hayry and Defendi,1970). In general the rat MLR is the result of $CD4^+$T cells responding to allogeneic class II(Green & Jotte,1985), though there is increasing evidence that $CD8^+$ can be activated to alloantigen in vitro(Mason & Simmonds,1988). Previous studies in the rat have shown that lymphoid cells from blood-transfused animals may, depending on the lymphoid compartment and time after transfusion,show reduced proliferation in response to donor allogeneic cells(Quigley et al,1988; 1989a,b,c). Moreover the addition of lymphoid cells as "regulator" cells to a normal allogeneic MLR may cause a decrease in proliferation indicating that blood transfused animals contain cells with "in vitro" suppressor activity.

It is well known that alloantibody may in some circumstances inhibit the MLR,for example monoclonal antibody to class II antigens have been shown to inhibit the allogeneic MLR (Green & Jotte,1985). In the previous chapter it was demonstrated that blood transfused PVG animals developed a more rapid alloantibody response to kidney donor (DA) class II MHC when compared to non-transfused graft recipients . It was therefore of interest to examine whether this difference would be reflected in the ability of serum from transfused and unmodified rats to inhibit the allogeneic MLR.

6.2 Determination of optimal conditions in the DA to PVG MLR

Initial experiments were performed to determine the optimal conditions for the DA to PVG MLR. Normal PVG splenocytes and LNC at a concentration of $4x10^5$, were cultured with $4x10^5$ irradiated (2000R) DA (DA*) spleen cells over a period of 5 days in order to determine which responder population gave the greatest proliferative response. In this particular experiment the cells were cultured in 10% FCS. A consistent finding was that PVG LNC underwent a greater and more sustained proliferative response than PVG spleen cells (Fig 6.1).

The kinetics of the response were quite different for spleen and LNC responders. The maximum proliferation of splenocytes occured at 48hrs and decreased thereafter while peak proliferation of the LNC occured after 96hrs in culture. The reason for the reduced and early response of spleen cell responders is not clear. On the basis of these experiments PVG lymph node cells were used as responder cells in all subsequent experiments.

To determine the optimal concentrations of responder and stimulator cells different cell numbers and ratios were added to the wells. Plates were pulsed with 1uCi of ^{3}H-TdR at 24,48,72,96,120 and 144 hrs after initiation of culture. Fig 6.2 (a-d) shows the proliferative response with time when LNC responders ($0.5\text{-}4 \times 10^5$) responders were added to the cultures. It can be seen that there is a steady increase in proliferation with increasing numbers of both responders and stimulators. Maximum proliferation was achieved when

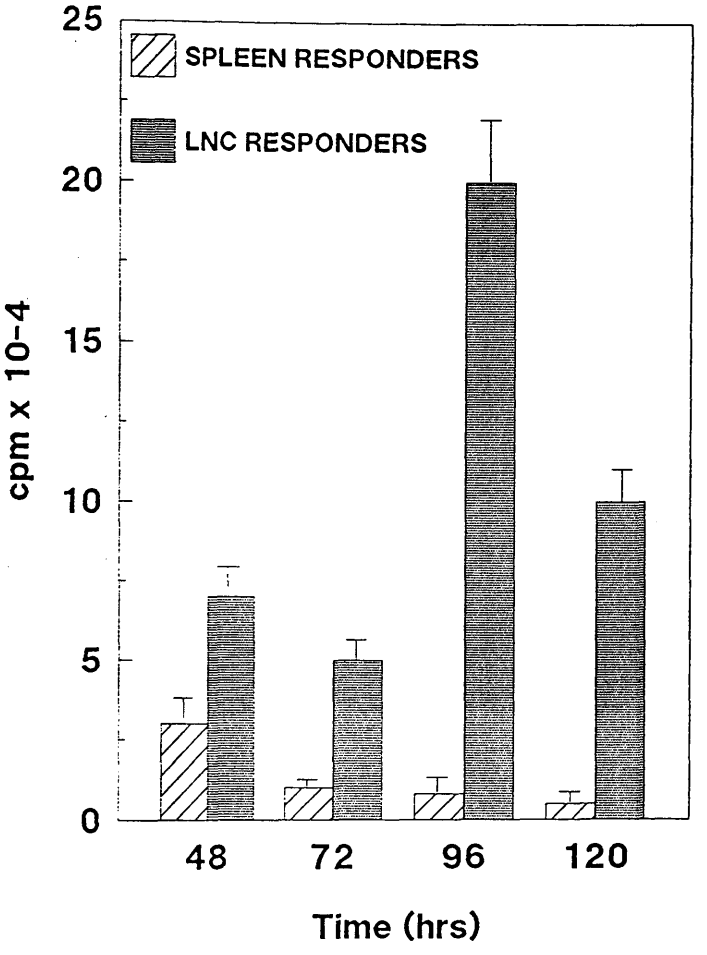


Fig 6.1 Comparison of PVG splenocytes and lymph node cells as responder cells in the DA* x PVG MLR
PVG splenocytes or lymph node cells (at $4x10^5$/well) were cultured with $4x10^5$ irradiated DA spleen stimulator cells/well. Cultures were pulsed for 18hrs with ^{3}H-TDR(1uCi/well) at 48,72,96 and 120 hrs after initiation of the culture. Results are expressed as cpm.

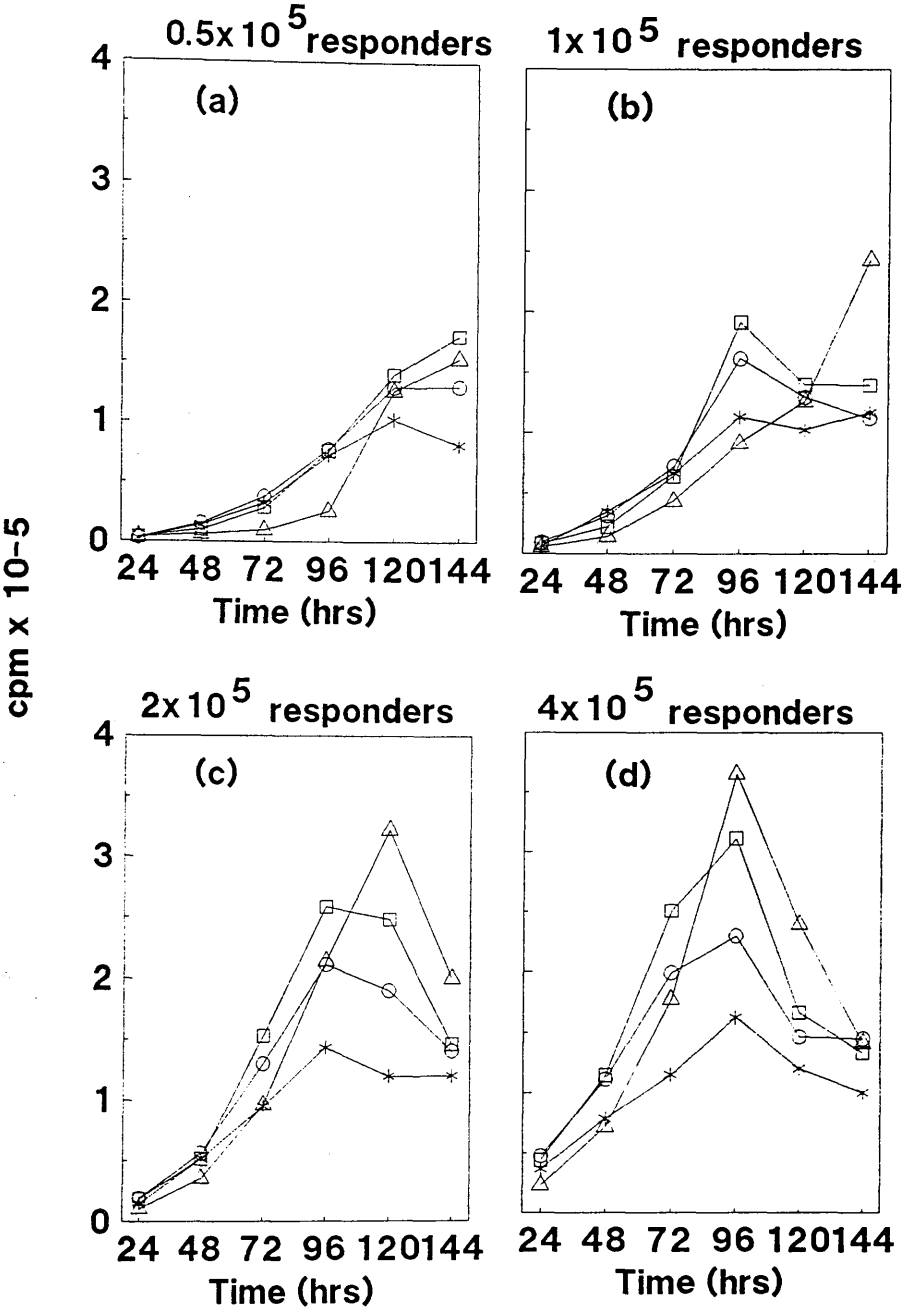


Fig 6.2 Kinetics of proliferation in the DA* x PVG MLR
Various concentrations of PVG responder LNC were cultured with 0.5(asterisk),1(open circle), 2(open square), or 4(open triangle)x10^5 irradiated DA stimulator cells. Cultures were pulsed for 18hrs with ^{3}H-TDR (1uCi/well) at 24,48,72,96,120 and 144 hrs after the initiation of the culture. Results are expressed as cpm.

$4x10^5$ responders and $4x10^5$ stimulators were cultured together (R:S ratio of 1:1). The peak proliferative response was again found to be 96hrs after the initiation of the culture.(Fig 6.2d)

It was felt that it might be more physiological if MLR experiments were established in homologous rat serum rather than FCS.A DA to PVG MLR was set up as before in the presence of either 5% heat inactivated FCS or 5% heat inactivated normal PVG serum. Cultures were pulsed 72,96 and 120 hrs after the initiation of culture. Again, maximum proliferation occured when $4x10^5$ responders and stimulators (R:S,1:1) were cultured for 96 hours. However there was a striking difference in the levels of proliferation between responders cultured in foetal calf serum(FCS)(Fig 6.3a) and those cultured in normal rat serum (Fig 6.3b).At the peak of the response counts obtained in FCS ($188x10^3$cpm) were three times higher than obtained in normal rat serum ($60x10^3$pm). However the levels of background counts obtained when responders were cultured alone were decreased from 18,000cpm in FCS to 35cpm in normal rat serum. This implies either that the FCS was mitogenic or that there was some inhibitory factor in the normal rat serum or possibly a combination of these effects. Since the levels of background obtained were lower with rat serum and levels of proliferation were still good , it was decided to carry out all future experiments in 5% homologous rat serum.

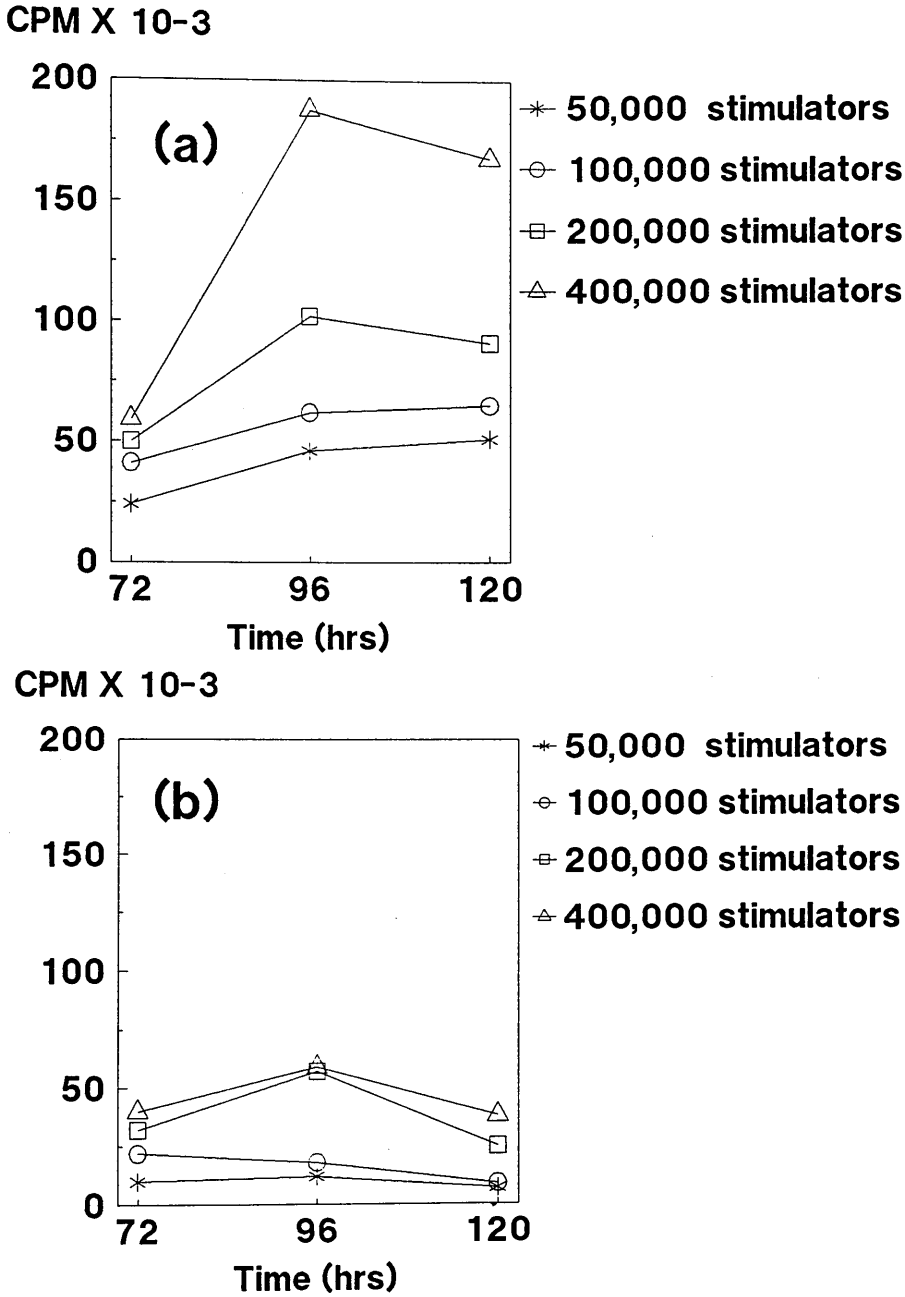


Fig 6.3 Comparison of foetal calf serum(FCS) and pormal rat serum (NRS) in the DA*xPVG MLR $4x10^5$ PVG responder/well were cultured with 0.5,1,2,or $4x10^5$ irradiated DA stimulator cells in medium containing either 5% FCS (a) or 5% NRS (b). Wells were pulsed for 18hrs with ^{3}H-TDR(1uCi/well) at 72,96 and 120 hrs after the initiation of the culture.

6.3 Ability of serum from enhanced and rejecting rats to inhibit the donor-specific proliferative response

Serum was obtained from unmodified and transfused PVG recipients 5 days after transplantation with a DA kidney. The test serum was heat-inactivated, sterile-filtered, and then frozen down in small aliquots at -70°C . Doubling dilutions of each test serum sample, starting at 5% (v/v) were then added to a DA* x PVG MLR. In order to compensate for those cultures containing less than 5% test serum appropriate amounts of normal rat serum were added such that all cultures contained a total of 5% rat serum. Control MLRs were set up in 5% PVG serum. Cell concentrations of $4x10^5$ cells per well of both responders and stimulators were used and cells harvested at 72 and 96hrs after the initiation of the culture. The results of one such titration are shown in Fig 6.4. Both enhanced serum samples displayed substantial inhibitory activity while the rejecting serum samples showed less inhibition. When this experiment was repeated with two further serum samples in each group levels of inhibition obtained with enhanced serum were significantly higher than those obtained with rejecting serum Fig 6.5 ($p<0.05$).

Following the demonstration in the previous set of experiments that enhanced, and to a lesser extent rejecting sera can appreciably inhibit the proliferative response of the DA*xPVG MLR, the specificity of this inhibitory effect was investigated. Serum samples 3 and 4 were added to the DA stimulated and Lewis-stimulated PVG MLR. The MLRs were performed simultaneously under optimal conditions and the plates

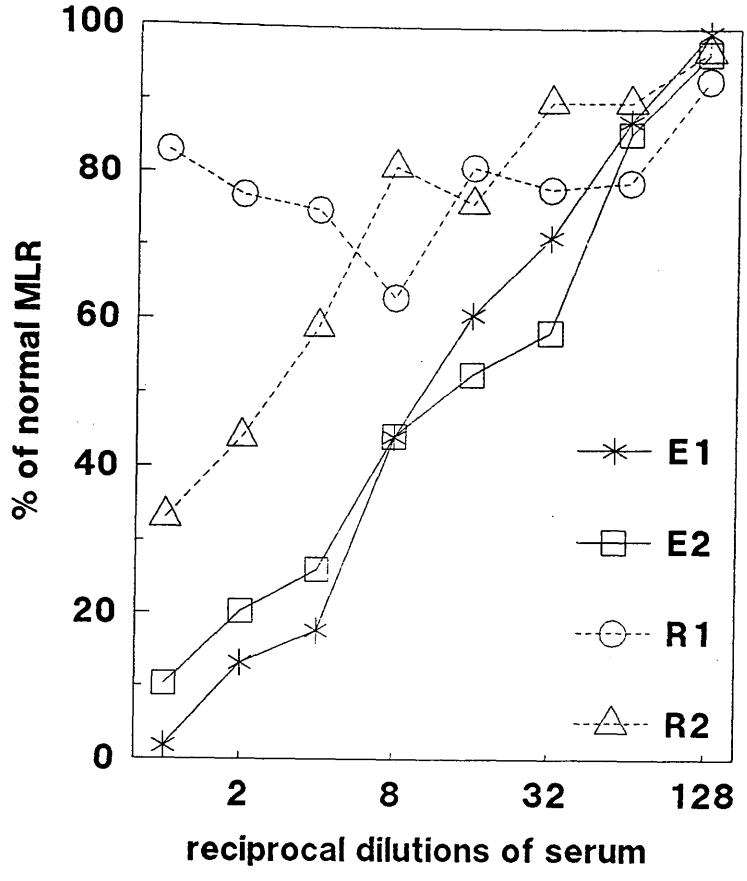


Fig 6.4 Effect of titrating enhanced and rejecting serum into the DA*x PVG MLR
Doubling dilutions of enhanced (E1 and E2) and rejecting (R1 and R2) serum samples(at an initial concentration of 5% /well)were added to the DA*xPVG MLR under optimal conditions. Normal rat serum at varying concentrations was added to the wells to maintain a final concentration of 5% serum/well.Cultures were incubated for 96hrs then pulsed with 1uCi/well of ^{3}H-TdR for 18hrs then harvested. Results are expressed as a % of the normal MLR .

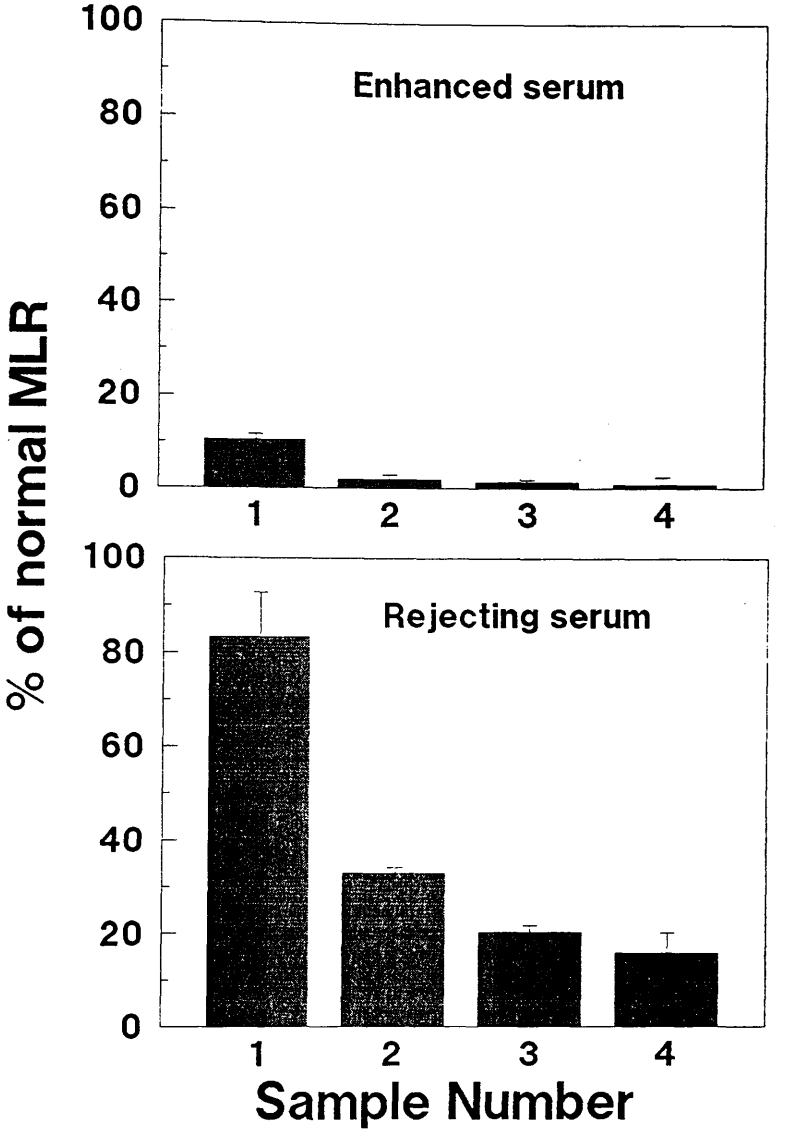


Fig 6.5 Ability of serum from enhanced and rejecting rats to inhibit proliferation in the DA*xPVG MLR
Serum was collected from enhanced (n=4) and rejecting (n=4) PVG recipients of a DA kidney, 5 days after transplantation and added to the DA*xPVG MLR to give a concentration of 5% serum/well. The results are expressed as a percentage of the control MLR which contained 5% normal PVG serum.Error bars denote the standard error of the mean.

pulsed after 96 hrs. The thymidine uptake for a normal LEWIS*xPVG MLR was found to be lower than that of the DA*xPVG MLR by approximately 50%. The results(table 1.14) are therefore expressed as a percentage of the control MLR for each strain combination. Both enhanced and rejecting sera inhibited the donor-specific and the third party MLR. The enhanced sera were 10 fold more potent in both their specific and non-specific inhibitory activity than rejecting sera. However there did not appear to be any difference in the level of donor-specific and third party inhibition in either the enhanced or rejecting group.

6.4 Ability of monoclonal antibodies to class I and class II MHC antigens to block proliferation in the MLR

Monoclonal antibodies directed to both monomorphic (OX18 and OX6) and polymorphic (MN4 and F17) determinants of rat class I and class II MHC antigens were added to determine whether they were able to inhibit the MLR. Cultures were set up under optimum conditions as before and doubling dilutions of antibody added. It can be seen from Fig 6.6 that at a concentration of 2ug/ml all of these antibodies are potent inhibitors of the MLR . The anti-class II monoclonal antibodies OX6 and F17 inhibited the response to a greater extent than the anti-class I antibodies OX18 and MN4.

Table 1.14

Serum	Stimulators	CPM ± SEM	% of normal MLR
NRS	DA	23636 ± 2198	-
	LEW	11002 ± 1538	-
ENH 3	DA	311 ± 32	1.33 ± 0.14
	LEW	656 ± 122	5.96 ± 1.11
ENH 4	DA	168 ± 1.5	0.72 ± 0.006
	LEW	132 ± 3.5	1.2 ± 0.03
REJ 3	DA	4767 ± 518	20.4 ± 2.22
	LEW	2637 ± 425	23.97 ± 3.86
REJ 4	DA	3724 ± 339	15.94 ± 1.45
	LEW	2191 ± 442	19.91 ± 4.02

Table 1.14 Ability of enhanced and rejecting serum samples to inhibit a donor-specific and third party MLR

Enhanced (n=2) and rejecting (n=2) sera were collected from transfused and unmodified PVG recipients, 5 days after transplantation with a DA kidney. Sera were added at a final concentration of 5% / well to a donor-specific DA*xPVG MLR or a third party LEW*xPVG MLR under optimal conditions.Control MLRs were set-up in 5% normal PVG rat serum. Cultures were pulsed 96 hrs later with ^{3}H-TdR (1uCi/well)for 18hrs,harvested and counted. Results are expressed as cpm and as a % of the normal MLR.

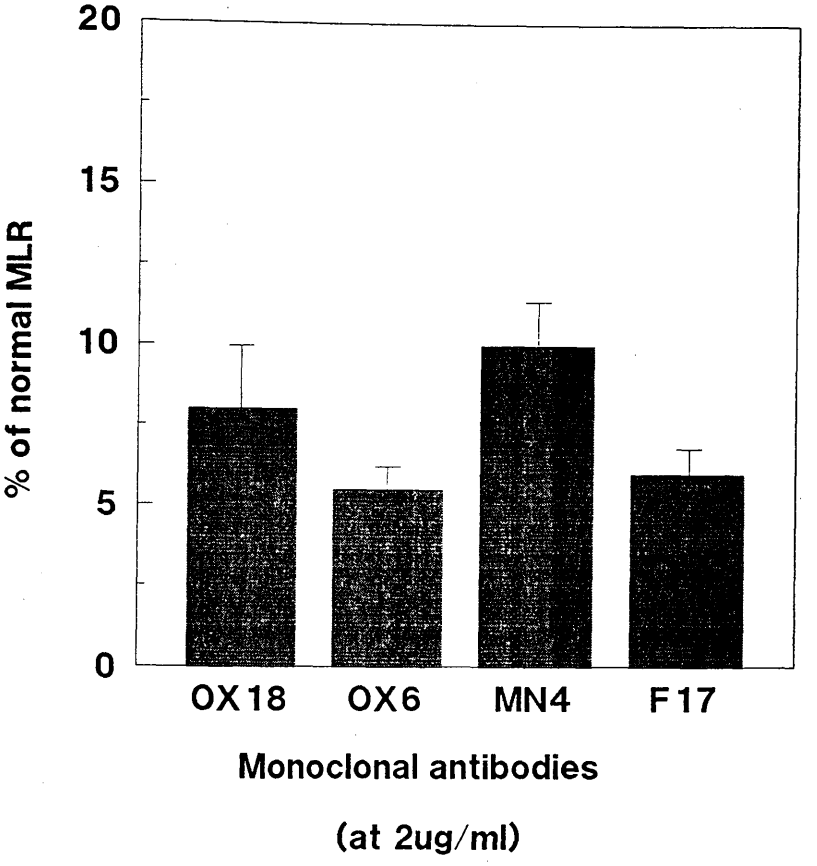


Fig 6.6 Inhibitory activity of monoclonal antibodies to class I and class II MHC antigens in the DA*xPVG MLR.
Monoclonal antibodies to monomorphic (OX18 and OX6) and polymorphic (MN4 and F17) determinants of class I and class II MHC antigens were added (at 2ug/well) in triplicate to the DA*xPVG MLR under optimal conditions.Cultures were pulsed as before 96hrs after the initiation of the culture. Results are expressed as a % of the control MLR carried out in normal PVG rat serum.Error bars denote the standard error of the mean.

6.5 Ability of enhanced and rejecting IgG to inhibit proliferation in the MLR

IgG samples were prepared by n-octanoic acid sedimentation from enhanced (n=4) and rejecting (n=4) PVG rats, 5 days after transplantation with a DA kidney. Purity of the preparations was assessed by SDS PAGE and found to be good apart from a small band at around 120K (Fig 1.5). Doubling dilutions of IgG samples from a starting concentration of 250ug/ml were added to the wells and each MLR was performed in triplicate. It can be seen in Fig 6.7 that the enhanced IgG samples showed greater inhibition than the rejecting IgG samples ($p<0.05$). The % of normal MLR obtained with 4 enhanced IgG samples (at 250 ug/ml) were 60%,40%,38% and 8% compared with 83%,77%,77% and 60% for the rejecting IgG samples. Levels of inhibition were considerably lower than achieved with whole serum and this may be due in part to the removal of non-specific inhibitory factors during the IgG preparation. In addition the concentration of IgG used in these experiments was only about 5% of that found in serum samples (assuming a serum IgG concentration of 16mg/ml).

The specificity of the inhibitory effect was investigated by adding IgG samples to both donor-specific and third party MLRs. Levels of donor-specific inhibition were not as high in this experiment since the final concentration of IgG added to the MLR was lower(125ug/ml). It can be seen from Fig 6.8 that generally there was greater specificity in the inhibition mediated by enhanced IgG samples

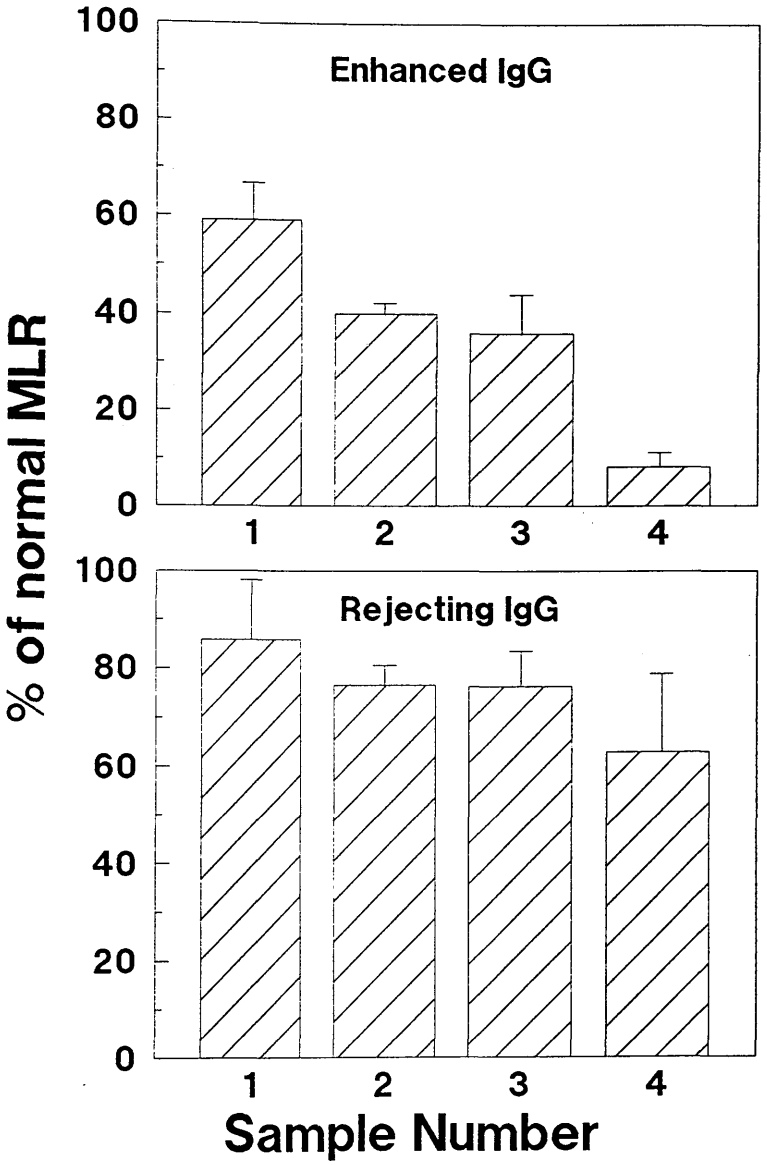


Fig 6.7 Inhibitory activity of enhanced and rejecting IgG in the DA*XPVG MLR
IgG was prepared from enhanced(n=4) and rejecting (n=4) PVG sera 5 days after transplantation with a DA kidney. This IgG(at 250ug/ml) was tested in triplicate for inhibitory activity in the DA*xPVG MLR under optimal conditions and pulsed as before . The mean of each triplicate is expressed as a % of the control MLR which was set up in 5% normal PVG serum. Error bars denote the standard error of the mean.

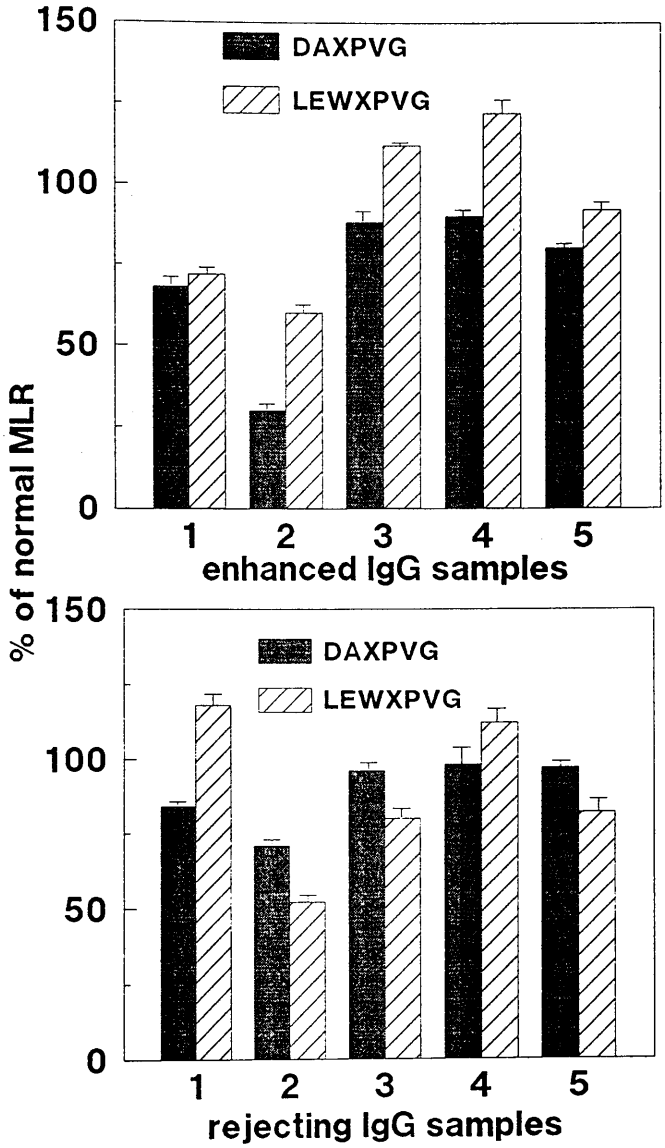


Fig 6.8 Ability of enhanced and rejecting IgG to inhibit the donor-specific and third party MLR

IgG was prepared from enhanced (n=5) and rejecting (n=5) PVG sera 5 days after transplantation with a DA kidney. This IgG (at 125ug/ml) was tested in triplicate for inhibitory activity in the DA*xPVG MLR under optimal conditions and pulsed as before. The mean of each triplicate is expressed as a % of the control MLR carried out in 5% PVG serum.Error bars denote the standard error of the mean

compared to the rejecting IgG samples however there was no significant difference between the two groups when tested in either the specific or the third party MLR.

These results do not accord with the previous experiment and suggest that the concentrations of IgG used were too low to detect inhibitory activity.

6.6 Investigation of the capacity of enhanced and rejecting serum fractions to inhibit the MLR

Sera from enhanced (n=3) and rejecting (n=3) PVG rats 5 days after transplantation with a DA kidney, were fractionated over a discontinuous sucrose density gradient as described previously. This yielded six fractions which were dialysed into RPMI 1640 medium and filter sterilised. Doubling dilutions of each fraction were added in triplicate (50ul/well) to $4x10^5$ PVG responders and $4x10^5$ DA stimulators in medium containing 5% normal rat serum. Cultures were pulsed with 1uCi/well of ^{3}H-TdR after 96 hrs and harvested after 18hrs. Results have been expressed as a % of the normal MLR occuring in rat serum alone.

The results of titrating fractions from one of the enhanced and one of the rejecting serum samples are shown in Figs 6.9 and 6.10 respectively. When fractions were added neat to the MLR it was found that all of the enhanced serum fractions produced some degree of inhibition but the greatest inhibition was in fractions 3,4 and 5(% inhibition 30%,1% and 1% respectively). The pattern was similar in the serum samples from the rejecting animal with most of the

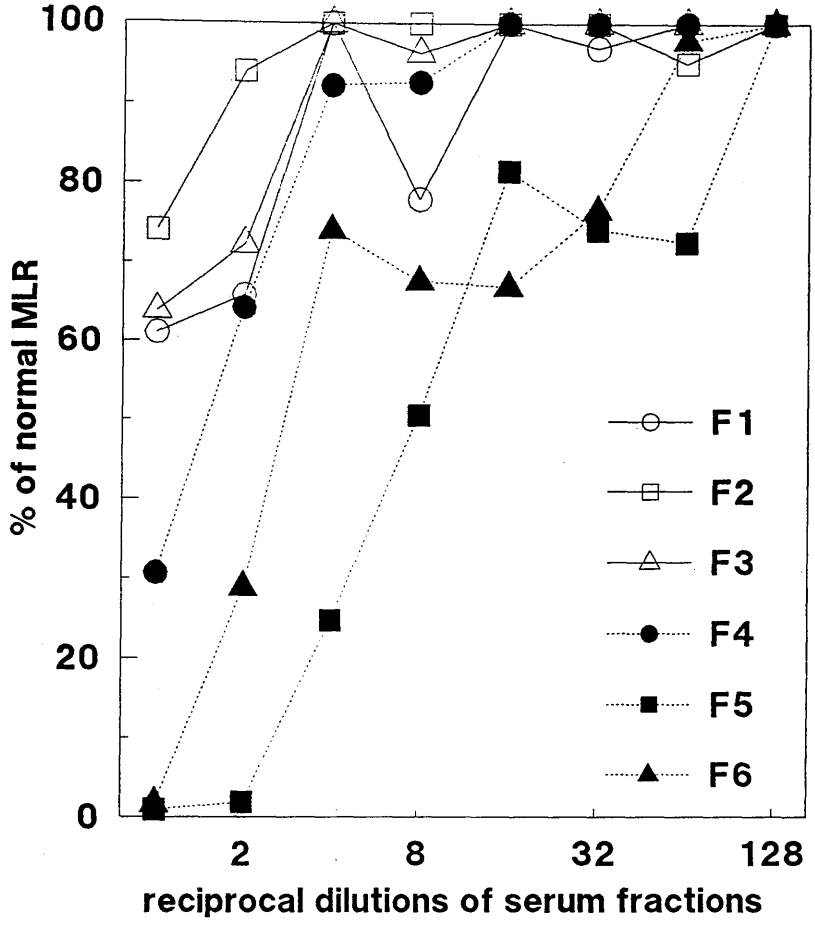


Fig 6.9 Ability of serum fractions from enhanced rats to inhibit the DA*xPVG MLR
Serum was collected from an enhanced PVG rat 5 days after transplantation with a DA kidney. Six serum fractions of graded molecular weight were prepared by discontinuous gradient centrifugation. Doubling dilutions of each fraction were added in triplicate (50ul/well) to the DA*xPVG MLR under optimal conditions and pulsed as before. The mean of each triplicate is expressed as a % of the normal MLR which was set up in 5% normal PVG serum.

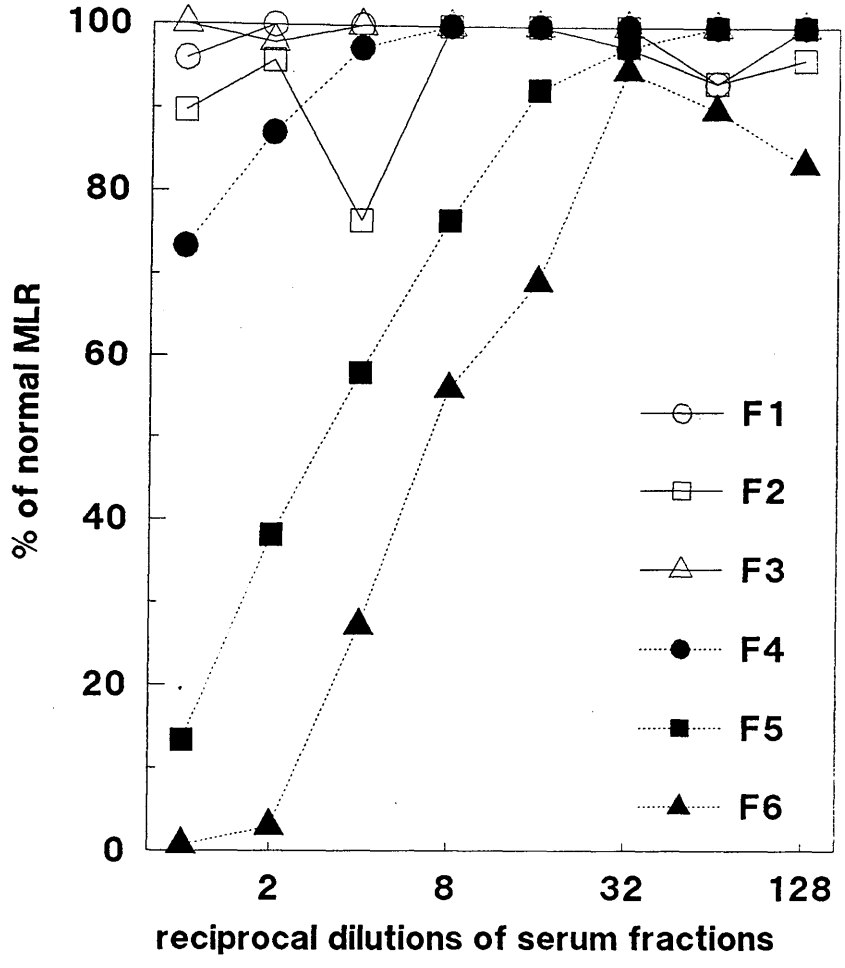


Fig 6.10 Ability of rejecting serum fractions to inhibit the DA*xPVG MLR
Serum was collected from an enhanced PVG rat 5 days after transplantation with a DA kidney. Six serum fractions of graded molecular weight were prepared by discontinuous gradient centrifugation . Doubling dilutions of each fraction were added in triplicate (50ul/well) to the DA*xPVG MLR under optimal conditions and pulsed as before. The mean of each triplicate is expressed as a % of the normal MLR which was set up in 5% normal PVG serum.

inhibition in fractions 5 and 6 (% inhibition 12% and 1% respectively) and some inhibition in fraction 4 (73%). A similar pattern of inhibition was found in the serum fractions of the other two enhanced and rejecting serum samples tested. The results are summarised in Fig 6.11 . The least inhibition (though this still represented about 50% of the normal MLR) was found in the high molecular weight fractions (F1 to F3),in both the enhanced and rejecting rats. The greatest inhibition was found in fraction 4,5 and 6 for the enhanced rats and in fractions 5 and 6 for the rejecting rats.

As was shown in chapter 4 , fractions 4,5 and 6 contain the IgG peak particularly fraction 4. It was therefore interesting that the level of inhibition in fraction 4 of the rejecting rats (median=52%) was far less than the enhanced rats(8%) confirming the findings with the purified IgG . It should be noted that the concentration of IgG in fraction 4 was higher than the starting concentration used in the IgG experiments(see Table 1.8). It was interesting however that the greatest inhibition was in fractions 5 and 6 in both groups. When an SDS PAGE gel of the fractions was analysed it could be seen that in fractions 4,5 and 6 apart from the IgG band there was also a very large band at around 68K , which corresponds to albumin (Fig 1.4). One possibility is that the non-specific inhibition found in the serum could be due to the high levels of albumin in the cultures, but this was not tested directly.

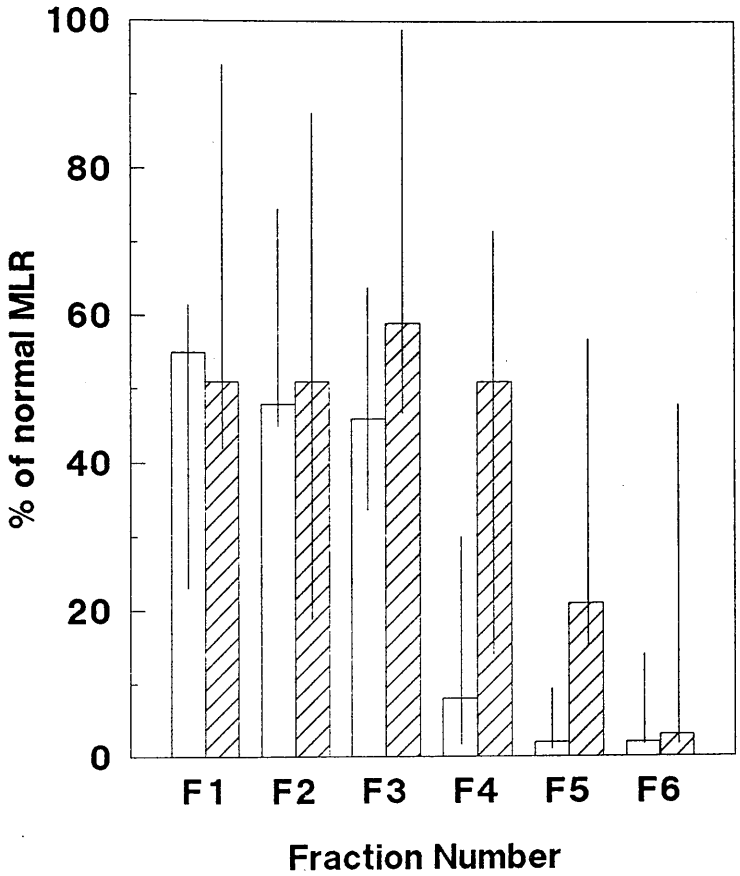


Fig 6.11 Ability of enhanced and rejecting serum fractions to inhibit the DA*xPVG MLR
Serum was obtained from enhanced (n=3) and rejecting (n=3)PVG rats 5 days after transplantation with a DA kidney. Sera were fractionated into six serum fractions of graded molecular weight(F1toF6). Each fraction (50ul/well) was added to the DA*xPVG MLR under optimal conditions and pulsed as before. Results shown are the medians and ranges.Enhanced = open bars, Rejecting = hatched bars.

6.7 Discussion

In this chapter the optimal conditions for PVG lymphoid cells responding in an MLR to irradiated DA stimulator cells were established. It was found that the proliferative response of LNCs was greater than splenocytes and this may have been due to suppressor macrophages within the spleen cell population which have previously been shown to inhibit the MLR(Oehler,Heberman, Campbell et al,1977; Weiss and Fitch, 1977). Maximum proliferationoccurred at day 4,which is consistent with the results of previous workers studying proliferation in rat MLR systems (Quigley et al,1988; Mason and Simmonds, 1988). MLR cultures were carried out in the presence of normal rat serum rather than FCS. Although levels of proliferation were considerably lower than in FCS, it was thought that this might be more physiological. It was not clear whether this was due to mitogenic activity in the FCS or inhibitory activity in the rat serum. However,previous reports have indicated that mouse sera can inhibit the proliferative response in an MLR by preventing T cell activation and this may be due to an IL2 antagonist (Lelchuk and Playfair, 1985).

Having established the optimal conditions for the MLR the effect of adding serum collected from unmodified and transfused PVG recipients, 5 days after transplantation with a DA kidney , was tested. There was significantly greater inhibition in enhanced serum samples compared with rejecting serum samples. Only two of the samples in each

group were tested for third-party inhibitory activity and this was found to be substantial in both groups. It cannot be completely excluded from the present study that the non-specific MLR inhibition may be due to antibody cross-reactive with third party alloantigens. However, this seems unlikely since no cross-reactivity was found when the serum was assayed against LEWIS alloantigens in the alloantibody assay. Furthermore, a DA blood transfusion does not enhance survival of a LEWIS kidney in a PVG recipient so if cross-reactivity is the explanation , then an assay of the inhibitory activity of enhanced and rejecting sera in the MLR is not a good correlate of graft survival. It seems more likely that the inhibition is due to some other serum protein and this will be discussed in relation to the findings of serum fractionation.

To eliminate the problem of contaminating proteins, enhanced and rejecting IgG samples were tested for inhibition of the donor and third party MLR .Levels of inhibition were far lower than found in serum. This may be due to a concentration effect, since levels of IgG were around 5% of that found in the serum. Enhanced IgG samples caused significantly greater inhibition of the MLR than rejecting IgG samples, at a concentration of 250ug/ml. This implies that an IgG alloantibody present in greater amounts in enhanced than in rejecting animals may be responsible for the MLR inhibition. It was shown in the previous chapter that levels of anti-class II antibodies are considerably higher in enhanced than in unmodified allograft recipients,5 days after transplantation. However both enhanced and rejecting IgG samples caused high levels of inhibition in a third party MLR and this inhibition cannot be explained by specific

alloantibody unless there is some degree of cross-reactivity with Lewis antigens. One possibility is that the contaminating protein seen on the SDS gel or trace amounts of caprylic acid which have not been removed by dialysis, cause some non-specific inhibition of the MLR, and that specific alloantibody is responsible for the difference found between enhanced and rejecting rats. Experiments in which an alternative purification procedure to obtain IgG eg. by HPLC may clarify these possibilities.

Mouse monoclonal antibodies to rat class I and class II MHC antigens were able to inhibit the rat MLR. This finding was slightly surprising since proliferation in the MLR is generally regarded to be a helper response driven by class II allo- antigens (Green & Jotte,1985). However the anti-class I monoclonal antibody also markedly blocks the response which is contradictory to the findings of Green and Jotte. Further experiments are required to elucidate whether this inhibition is a specific phenomenon or non-specific due to steric inhibition. This could be clarified by preincubating either responder or stimulator cell populations with the anti-class I and anti-class II antibodies. If anti-class I antibody is able to block the MLR then rejecting sera which have high levels of anti-class I at day 5 after transplantation would also be expected to block the MLR . It is perhaps not surprising then that both enhanced and rejecting sera are able to block the MLR. The varying degrees of blocking may reflect the greater ability of anti-class II compared with anti-class I antibody to block the MLR.

When enhanced and rejecting sera were fractionated by discontinuous gradient centrifugation into serum fractions of graded molecular weight it was found that fractions 5 and 6 from both groups caused the greatest inhibition of the MLR. Fraction 4 of the enhanced but not the rejecting group also caused a high level of inhibition and it is possible that this is responsible for the specific inhibition found in the serum and IgG from the enhanced rats and that the non-specific inhibition is due to fractions 5 and 6. SDS gel analysis of the serum fractions showed that in fractions 5 and 6, and to a lesser degree fraction 4, there was a large band migrating to the same level as albumin in the molecular weight markers. The third-party activity of the serum fractions has not yet been investigated but this may establish whether there is donor-specific inhibitory activity in fraction 4.

Addition of rat albumin at concentrations equivalent to those found in fractions 5 and 6 would indicate whether this was responsible for non-specific inhibition in the MLR. Attempts were also made to remove alloantibody activity from the test sera by absorption of sera with splenocytes(results not shown). However when the sera were re-tested in the alloantibody assay, absorption was incomplete and there was considerable residual alloantibody. It was not possible, therefore, to look at the effect of alloantibody-absorbed serum on the MLR.

If there is a correlation between in vitro proliferation and proliferation of alloreactive cells in vivo then it may be that the greater ability of enhanced serum compared with rejecting serum to inhibit the in vitro proliferative response to donor alloantigen may

also occur in vivo. Further studies are required to elucidate whether passively transferred alloantibody from enhanced animals is able to prevent rejection by blocking proliferation in vivo.

CHAPTER 7

FINAL DISCUSSION

7.0 Final Discussion

The beneficial effect of pre-operative blood transfusion in clinical renal transplantation was a striking discovery although its consequences have become less obvious as improved immunosuppressive drugs have been introduced. Terasaki (1988) reported that in 1987 one year survival for untransfused patients was 70% compared with 77% for patients who had received transfusion . Moreover, in some animal models preoperative donor-specific transfusion leads to long term graft survival even in the absence of any additional immunosuppressive drugs. Clearly, therefore, a greater understanding of the mechanisms underlying the blood transfusion effect are important and may, in the long term, lead to strategies of inducing specific unresponsiveness towards an organ graft and thereby reduce or eliminate the side effects of non-specific immunosuppression.

Despite intensive study of the blood transfusion effect in both humans and animal models, there is as yet no unifying hypothesis to explain why blood transfusion prolongs renal allograft survival. This may be a reflection of the complexity of the blood transfusion response and the wide ranging effects it produces on many aspects of both the cellular and humoral arm of the immune response to a subsequent allograft.

This study has utilised the rat renal allograft model to study the donor-specific blood transfusion effect. The DA to PVG rat strain combination was chosen because the PVG rat is a low responder to DA alloantigens (Butcher and Howard, 1982) and was therefore likely to be

readily susceptible to the induction of active enhancement. Furthermore antibodies were available which labelled class I and class II determinants of DA but not PVG, thereby allowing unequivocal evaluation of donor MHC expression. Therefore this study has the obvious limitation that the findings obtained may not necessarily be applicable to other rat strain combinations. Caution is also necessary in extrapolating the findings in the rat model to human renal transplantation because there are important differences in the immune systems of rat and man. In particular renal allografts in the rat appear less susceptible to antibody mediated damage, possibly as a result of a defect in the complement system (French and Batchelor, 1972).

The experiments reported herein, revealed several interesting findings which contribute to an understanding of the blood transfusion effect. The immunohistological and functional analysis of graft infiltrating cells reported in Chapter 3 revealed that the heavy mononuclear cell infiltration which occurs in rejecting renal allografts was not prevented by donor-specific blood transfusion. Instead, transfusion appeared to "sensitise" the recipient since the influx of mononuclear cells into the non-rejecting grafts was accelerated . It was also notable that at day 3 after transplantation, enhanced grafts in blood-transfused rats showed a greater number of "activated" cells bearing the IL-2 receptor. Although infiltration of mononuclear cells into rejecting grafts occurred more slowly, by day 5 there was a substantial infiltrate compared to enhanced grafts and there was a marked preponderance of cells bearing the CD8 antigen. Many of these $CD8^+$ cells appeared to have a phenotype ($OX8^+OX19^-$)

consistent with NK cells rather than $CD8^+T$ cells although further analysis using double labelling techniques are necessary to confirm this.

Previous studies have shown that treatment of rat renal allograft recipients with CyA or with anti-donor alloantibody(passive enhancement) prevents the infiltration of the graft by cells which display in vitro cytotoxic activity against donor strain lymphoblasts (Mason & Morris, 1984; Bradley et al, 1985). However, Dallman et al (1987) have recently shown that non-rejecting kidneys in blood transfused recipients contain infiltrating cells which display in vitro specific cytotoxicity similar to or in excess of that seen in rejecting grafts. This finding was confirmed in this study and raises questions about the role of specific cytotoxic T cells in graft rejection. There is convincing evidence that specific cytotoxic T cells contribute to graft rejection (reviewed in chapter 1). It may be that in actively enhanced rats the cytotoxic effectors are "blocked" from mediating their effector function, possibly by the presence of alloantibody. Alternatively it may be that assessment of cytotoxic function using donor lymphoblast targets is not relevant to the in vivo function of cytotoxic cells in graft rejection. The precise cellular target in renal allograft rejection is not known with certainty, but it seems likely that the vascular endothelium is a prime target of the rejection response. Future studies in which the ability of graft infiltrating cells from rejecting and actively enhanced renal allografts are tested for their ability to damage donor vascular endothelium in vitro may help to clarify this.

The ability of blood transfusion to prevent graft rejection could not be explained by a lack of target molecules on the donor cells within the kidney. The immunohistological analysis of donor class I and class II MHC expression demonstrated convincingly that marked induction of both class I and class II MHC antigens occurred in enhanced kidneys and was more rapid than the induction which occured in rejecting kidneys. This is in keeping with the accelerated graft infiltrate in enhanced kidneys because the induction of MHC antigens may be brought about by the release of lymphokines from the graft infiltrating cells. Other groups have subsequently confirmed the accelerated induction of MHC antigens in renal allografts in blood transfused rats (Wood et al,1988); Priestley & Fabre,1989). The apparent lack of any major differences in the cellular response to a renal allograft in unmodified and transfused recipients led to an examination of the effect of blood transfusion on the alloantibody response to a renal allograft as this could contribute to the transfusion effect.

The possibility that Fc blocking antibodies might contribute to graft survival induced by blood transfusion was investigated. Considerably higher levels of Fc blocking activity were found in serum of enhanced rats compared with rejecting rats on day 5 after transplantation. This activity was also found in serum fractions which corresponded to the 7S IgG peak and in purified IgG prepared from day 5 post transplant serum. Further experiments in which serum was absorbed with red cells to remove anti-class I activity suggested that this Fc blocking activity was mediated by anti-class II alloantibodies and this was substantiated by the demonstration that such antibodies could not block Fc receptors on congenic targets which share class II with PVG.

The finding that anti-class II antibodies were responsible for Fc blocking was in agreement with previous studies (Dickler & Sachs, 1974); Soulillou et al, 1975; Suthanthiran et al, 1977). However this may have been a fortuitous finding in the light of the demonstration that anti-class I monoclonal antibodies also mediate Fc blocking but that the predominant antibody induced by blood transfusion was an IgG anti-class II antibody.

As mentioned in the introduction IgG anti-class II antibodies are potent inducers of passive enhancement. It was therefore interesting that IgG anti-class II antibodies were present in higher amounts in the serum of enhanced rats than the rejecting rats early after transplantation. One mechanism which has been proposed to explain passive enhancement is that the presence of IgG anti-class II antibody at the time of transplantation results in the removal of graft dendritic cells by opsonisation. This could alter the course of normal host sensitisation leading to the induction of suppressor cells. It would therefore be interesting to follow the fate of dendritic cells in the enhanced grafts to see whether they appear in the local lymph node or spleen. It was noted in chapter 3 that dendritic cells disappeared more quickly from the enhanced grafts than the rejecting grafts. Alternatively anti-class II antibodies could prevent recognition of class II antigen on donor endothelium which has been shown to be capable of presenting donor antigen in vivo (Ferry,Halttunen, Leszczynski et al,1987). Since class II antigens are the main stimulators of the proliferative response it is unlikely that anti-class I antibody present in higher amounts in the rejecting animals would prevent sensitisation.

Clearly some degree of sensitisation has taken place as there are specific cytotoxic cells within the grafts though they may have been induced by blood transfusion and levels of cytotoxicity were low. However the paucity of cells expressing the Il-2R does suggest some down-regulation of the activation process. Anti-class II antibodies could also bind to shed donor antigen and prevent uptake of shed donor-class II antigen by host antigen presenting cells, thereby diminishing sensitisation.

Whether the dichotomy in the antibody response to the graft is true of other strain combinations remains to be elucidated, but previous studies have shown that in the DA to Lewis strain combination the alloantibody response induced by splenocyte injection was directed mainly at class II antigens (Gallico & Mason, 1978).

The greater inhibition of the MLR by enhanced serum and IgG could also be attributed to the higher levels of anti-class II antibodies present in the enhanced serum on day 5 after transplantation. Further experiments are required to confirm whether the anti-class II antibodies are cross-reactive with Lewis antigens. Alternatively, inhibition in vivo and in vitro (as seen in the MLR) of the response to DA may be mediated by anti-idiotypic antibody which is able to block the receptors for antigen on T cells. As mentioned in the introduction anti-idiotypic antibodies capable of inhibiting the MLR have been described in a number of studies on the blood transfusion effect. This is an area which was not investigated in the present study but which could be pursued both in relation to both blocking of the MLR and blocking of in vitro cytotoxicity.

There are arguments against anti-class II antibodies playing a major role in transfusion-induced suppression of graft rejection. The fact that purified erythrocytes can enhance survival of rat renal allografts (Wood et al, 1985) and mouse L-cells bearing only transfected class I can enhance survival of mouse cardiac allografts (Superina et al, 1985;1987; Madsen et al,1988) could be taken as evidence that antibody is not important. However IgG anti-class I antibody has also been found to be capable of passively enhancing kidneys, so presumably if IgG anti-class I is induced by erythrocytes this might have some effects similar to IgG anti-class II. Alternatively the immunosuppressive mechanisms induced by pre-treatment with class I alone may be entirely different from those evoked by whole blood containing a full MHC mismatch.

In conclusion, the mechanisms underlying the transfusion effect are undoubtedly complex, and are complicated by the fact that both the rejection response and the induction of enhancement are influenced by strain combination effects. A possible role for transfusion-induced IgG antibody has been highlighted and attempts to passively enhance naive PVG rats with day 5 enhanced sera may elucidate whether they are involved in graft survival. However, there are difficulties in this approach since, as mentioned in the introduction, the dosage and timing of antibody administration are critical in passive enhancement and therefore interpretation of such experiments is difficult. Moreover, most of the studies claiming a role for suppressor cells utilise adoptive hosts which have been lightly irradiated, and this might be required in order to demonstrate the suppressive effect of serum.

The relevance of the findings reported here to the transfusion effect in the clinical setting are still unclear. There is conflicting evidence from human studies about the enhancing effect of anti-class II antibodies. It may be that in humans the beneficial effect of such antibodies is masked by the fact that antibody may also be cytotoxic and therefore detrimental to graft survival. If so, the future for antigen pretreatment in the clinical setting will depend on finding ways to induce non-cytotoxic enhancing antibody.

APPENDIX

Table A.1.

Effect of Pretreatment with various donor cells on graft survival

Donor cells used for pretreatment	Allograft Model	Strain Combination	Graft Survival	Ref
Erythrocytes	Renal	BN--Wag	++	1
	Renal	LEW--BN	++	2
	Renal	DA--LEW	++	3
	Renal	BN--LEW	++	4
	Renal	DA--LEW	++	5
	Renal	LEW--DA	++	5
	Renal	$(DAxLEW)_{F1}$--DA	++	5
	Renal	DA--$(DAxHS)_{F1}$	-	6
	Cardiac	(LEWxBN)F1--LEW	-	7
Platelets	Renal	$(DAxLEW)_{F1}$--DA	++	8
	Renal	DA--$(DAxHS)_{F1}$	+	6
	Renal	BN--LEW	++	4
	Renal	DA--LEW	+	3
B cells	Renal	DA--LEW	++	3
	Renal	LEW--DA	++	9
	Cardiac	(LEWxBN)F1--LEW	++	7
T cells ($CD4^+$ but not $CD8^+$)	Renal	DA--LEW	+	3
	Renal	LEW--DA	++	9
	Cardiac	(LEWxBN)F1--LEW	-	7
Dendritic cells	Renal	DA--LEW	+	3
	Cardiac (mouse)	C57BL/10--DBA/2	-	10

++ = prolonged survival
+ = slightly prolonged survival
- = no effect on survival

Table A.2.

References used in table A.1.

Ref No.	Author and Journal
1	Jeekel J, van Dongen J, Majoor G et al (1977) Transplantation Proceedings **9**: 969-972
2	Majoor GD, van de Gaar M-JWH, Vlek LFM et al (1981) Transplantation **31**: 369-375
3	El-Malik, F., Malik, S.T.A. Varghese, Z. et al (1984) Transplantation **38**: 213-216
4	Martin DC, Hewitt CF, Osborne JG et al (1982 Transplantation Proceedings **14**: 407-409
5	Wood KJ, Evins J & Morris PJ (1985) Transplantation **45**: 759-767
6	Hibberd AD & Scott LJ (1983) Transplantation **35**: 622-624
7	Lauchart W, Alkins BJ & Davies DAL (1980) Transplantation **29**: 259-261
8	Batchelor JR, Welsh K & Burgos (1977) Transplantation Proceedings **9**: 931-936
9	Cranston D, Wood KJ & Morris PJ (1986) Transplantation **42**: 302-306
10	Peugh WN, Austyn JM, Carter NP et al (1987) Transplantation **44**: 706-710

Table A.3

Buffers for SDS-PAGE

Upper Tank Buffer:

Tris	- 31.6 g	
Glycrine	- 20.0 g	Make up to 1 litre with distilled H_2O
SDS	- 5.0 g	

Lower Tank Buffer:

Tris	- 60.5 g	Add 800 mls of distilled H_2O
SDS	- 5.0 g	Adjust pH to 8.1
		Make up to 1 litre with distilled H_2O

30% Acrylamide:

Acrylamide	- 28.5 g	Dissolve in 100 mls of distilled H_2O
Bis-Acrylamide	- 1.5 g	

Running Gel Buffer:

Tris	- 18.5 g	Add 50.0 mls of distilled H_2O
SDS	- 0.4 g	Adjust pH to 8.9
		Make up to 100 mls with distilled H_2O

Stacking Gel Buffer:

Tris	- 5.9 g	Add 50.0 mls of distilled H_2O
SDS	- 0.4 g	Adjust pH to 6.7
		Make up to 100 mls with distilled H_2O

Sample Buffer:

Reducing conditions		Non-reducing conditions	
Stacking Gel Buffer	- 2.5 mls	Stacking Gel Buffer	-5.0mls
2-Mercaptoethanol	- 2.5mls	SDS	-1.0g
SDS	- 1.0g		

Warm until SDS dissolves then add 1.0ml of glycerol and 4.0mls of 1% bromophenol blue.

All reagents from Gibco, Paisley except 2-Mercaptoethanol (Sigma Chemical Co., Poole, Dorset).

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