CELLULAR REQUIREMENTS FOR RENAL ALLOGRAFT REJECTION

by

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DECLARATION

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The investigations of the cellular events of unmodified allograft rejection (chapter 3) and the results of the irradiation studies (chapter 4) have been published, in part, as follows:

Armstrong,H.E., Bolton,E.M., McMillan,I.,Spencer,S.C. & Bradley,J.A. (1987) Prolonged survival of actively enhanced rat renal allografts despite accelerated cellular infiltration and rapid induction of both class I and class II MHC antigens. Journal of Experimental Medicine, **164**, 891-907.

Bolton,E.M., Armstrong,H.E., Briggs,J.D. & Bradley,J.A. (1987) Cellular requirements for first-set renal allograft rejection. Transplantation Proceedings, **19**, 321-323.

Part of the results of the renal graft versus host study (chapter 5), and of the studies of the athymic rats (chapter 6), were presented to the Meeting of the British Transplantation Society, Spring, 1988.

SUMMARY

Graft rejection remains a major problem in clinical renal transplantation despite recent improvements in immunosuppressive therapy. While it is accepted that T lymphocytes play an essential role in acute rejection, the relative contributions of the different effector pathways have not been established. The aim of this thesis was to examine the cellular effector mechanisms of renal allograft rejection in the rat.

In initial experiments, the characteristic features of unmodified rejection were observed in a serial immunohistological study of rejecting DA allografts transplanted into PVG recipients. The progressive mononuclear cell infiltration of the grafts initially comprised predominantly CD4 lymphocytes and subsequently CD8 cells. In addition to T cells, many of the infiltrating cells were of a phenotype consistent with NK cells and macrophages. This was associated with a striking increase in the expression, within the graft, of donor MHC class I and II antigens, together with the early disappearance from the graft of class II positive donor interstitial cells.

Mononuclear cells harvested from the grafts and spleens of these animals displayed moderate levels of *in vitro* allospecific cytotoxicity against donor strain ConA blasts as well as high levels of nonspecific cytotoxicity against the NK susceptible Y3 target.

Following on from these basic observations, subsequent experiments investigated the ability of CD4 or CD8 T lymphocyte subpopulations (prepared by negative selection) to mediate allogeneic kidney damage in different experimental models.

One approach was to examine the ability of adoptively transferred lymphocyte subpopulations to cause renal allograft rejection in rats rendered lymphocyte deficient by a sublethal dose of whole body gamma irradiation. Acutely irradiated (8.5 Gy) Lewis recipients were unable to reject DA renal allografts unless reconstituted with syngeneic lymph node cells (LNC). Whereas transfer of $5x10^6$ naive Lewis T lymphocytes rapidly restored graft rejection, similar numbers of either CD4 or CD8 lymphocytes were relatively ineffective.

Immunohistological examination of day 7 allografts in reconstituted recipients revealed, in all groups, a moderate leukocytic infiltrate of similar phenotypic composition, irrespective of the phenotype of the reconstituting cells, and with broad similarity to the infiltrate observed in unmodified rejection.

When harvested infiltrating cells and splenocytes were tested in functional assays, only effector cells from CD4+CD8 T cell reconstituted animals, and not those from animals receiving either separated subpopulation, demonstrated allospecific cytotoxicity. Interestingly, splenocytes from all animals (including unreconstituted rats) showed nonspecific cytotoxic activity against Y3, but graft infiltrating cells from the same groups showed none. Collectively, these experiments suggested that both T cell subpopulations were necessary for optimal graft rejection, and that in this particular strain and model, graft rejection correlated with specific cytotoxic T cell lysis rather than nonspecific cytotoxic activity.

The second approach examined the ability of T cell subpopulations to mediate allogeneic tissue damage in the renal graft versus host reaction (GVHR). Renal subcapsular injection of parental CD4 lymphocytes in F1 recipients was sufficient to produce, by day 7, a grossly observable renal GVHR, while CD8 lymphocytes (even if specifically sensitised) were ineffective. CD4 lymphocytes required the essential participation of radiosensitive F1 host, bone marrow derived cells to cause tissue damage. This occurred in the absence of demonstrable specific T cell lysis and appeared to be a DTH reaction. Experiments with PVG recombinant rats showed that an isolated MHC class II, but not a class I incompatibility was sufficient to provoke a response.

The final group of experiments examined the ability of adoptively transferred lymphocyte subpopulations to restore renal allograft rejection in the congenitally athymic PVG-rnu/rnu rat. CD4 lymphocytes alone were able to restore the first-set rejection response, while CD8 cells alone (naive or specifically sensitised) were ineffective, although the addition of CD8 cells to the inoculum had a synergistic effect on the ability of CD4 cells to restore rejection. Immunohistological studies revealed moderate cellular infiltration of non-rejecting grafts and increased cellular infiltration of the rejecting grafts, with a significant increase in the number of MRC OX8 positive cells.

An interesting finding was the presence, in unmodified PVG-rnu/rnu rats, of extrathymically derived cells with wide alloreactivity as detected by in vitro cytotoxicity assays against a range of allogeneic ConA blasts and NK susceptible targets. Treatment of the effector cells with anti-asialo GM1, and cold target inhibition assays together suggested the presence of populations of atypical, widely reactive NK cells, with the additional ability to preferentially recognise a specific target. These cells were also present in the rejecting grafts of CD4 reconstituted recipients.

Overall therefore, the transfer of CD8 lymphocytes alone was insufficient to cause tissue damage in allogeneic kidneys in any of the experimental models

studied. In contrast, CD4 lymphocytes alone were able to cause extensive parenchymal damage in the renal GVHR, and were sufficient to initiate allograft rejection in athymic recipients, but required the additional presence of CD8 cells to restore rejection in acutely irradiated animals.

ABBREVIATIONS

ATXBM	Adult thymectomised, lethally irradiated, bone marrow reconstituted
BSA	Bovine serum albumin
ConA	Concanavalin A
DAB	Dulbecco's A + B medium
DTH	Delayed type hypersensitivity
FCS	Foetal calf serum
GVHR	Graft versus host reaction
Ig	Immunoglobulin
IgG	Immunoglobulin G
IL2	Interleukin 2
IFN-ϑ	Interferon gamma
LAK cell	Lymphokine-activated killer cell
LNC	Lymph node cells
MAb	Monoclonal antibody
MHC	Major histocompatibility complex
mH	Minor histocompatibility
MLR	Mixed lymphocyte reaction
MST	Median survival time
NK cell	Natural killer cell
PBS	Phosphate buffered saline
RT	Room temperature
SD	Standard deviation
TDL	Thoracic duct lymphocyte
Th	Helper T lymphocyte
Tc	Cytotoxic T lymphocyte

CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Current status of clinical renal transplantation

Renal transplantation is now the treatment of choice for the majority of patients with end-stage chronic renal failure. Successful transplantation leads to improved quality of life and is also cost effective when compared with the alternative treatment of haemodialysis. Over the last decade, the results of cadaveric renal transplantation have steadily improved due to a variety of factors, including increased emphasis on tissue matching, the discovery of the beneficial effects of multiple pre-operative blood transfusions, and the use of the new and highly effective immunosuppressive drug, Cyclosporine (CyA). Nevertheless, immunological rejection of the graft remains the major unsolved problem in organ transplantation. This thesis is concerned with the immunological mechanisms which underly the rejection response, in the hope that a better understanding of these events will allow the development of a rational basis on which to plan strategies aimed at preventing rejection and reducing the inevitable morbidity associated with current methods of immunosuppression.

1.2 Historical perspective of renal transplantation

The first human cadaveric renal transplant was performed by Voronoy in 1933 in an attempt to treat a patient with acute renal failure secondary to mercury poisoning. Although this attempt was "technically successful" it did not prevent the early death of the patient (Voronoy, 1936).

The modern era of clinical transplantation began in Boston with the performance of renal transplants between identical twins. Initially, these were the only conditions in which clinical transplantation was acceptable since the otherwise insurmountable problem of rejection could not be avoided (Hume, Merrill, Miller et al, 1955). The discovery in the 1960s that treating allograft recipients with Azathioprine and steroids reduced graft rejection to "acceptable" levels meant that this form of treatment for endstage chronic renal failure was available to a much larger number of patients (Calne, Alexandre & Murray, 1962).

In the 1970s, the apparently immunosuppressive effects of pre-operative blood transfusion in renal transplant recipients were recognised (Opelz, Sengar, Mickey et al, 1973) and the institution of deliberate pre-operative transfusion policies resulted in a further improvement in graft survival.

Finally, the discovery and widespread use of CyA in the 1980s have meant that renal transplantation is now highly successful, and have allowed the successful transplantation of other vascularised organs, namely the heart and liver.

In spite of these clinical achievements, the immunological mechanisms of graft rejection are still unclear. Detailed observations of the responses of transplanted patients to their grafts led to the recognition of different clinical patterns of graft rejection which appeared to reflect different effector pathways. These have been described as follows (Williams, 1984):

a) Hyperacute rejection is antibody mediated and occurs within 24 hours of transplantation of an organ bearing the same histocompatibility antigens, or MHC antigens, to which the recipient has been previously exposed.

b) Accelerated rejection occurs within 5 days of transplantation and may be cell and/or antibody mediated. Its rapid onset suggests it is the equivalent of the "second set" response in animal experiments.

c) Acute rejection occurs within the first 3 months and is probably cell and antibody mediated. Its timing suggests the equivalent of a first set response to antigen.

d) Chronic rejection occurs insidiously after the first 3 months and appears to represent a continuing, but modified, response to the antigen.

There are several obvious difficulties in attempting to dissect the mechanisms of graft rejection by observing the responses of human recipients, since by necessity, the immune response in the patient is always modified by various types of immunosuppressive therapy. Most of our current knowledge of the rejection response is therefore based on animal models of transplantation, in which it is possible to examine the events comprising unmodified rejection of tissue transplanted between different, but usually inbred, strains of animals.

1.3 Early experimental transplantation

The current status of transplant immunology has its origins in the pioneering work of the late Sir Peter Medawar. As a postgraduate student at the Sir William Dunn School of Pathology, Oxford, Medawar had developed an interest in the treatment of severe burn injuries after observing the fate of a young, badly burned airman. Although it was appreciated that an obvious way of treating such injuries was with skin grafts from voluntary donors, it was also well known that, unless the two individuals were identical twins, any such skin

grafts would be rapidly destroyed by the recipient. However, there was little understanding as to how or why the grafts were rejected. In pursuit of his interest in burns, Medawar was seconded to the Burns Unit at Glasgow Royal Infirmary. Here, working with Tom Gibson, a young Glasgow surgeon, Medawar provided evidence that skin homografts (allografts) were rejected by an immunological process that resembled a specific adaptive response of the type which leads to protection against infecting organisms.

Gibson and Medawar treated a badly burned female patient with numerous small skin grafts taken from the non-burned areas of the patient's own skin, and from her brother. Several days later she received a second set of grafts from her brother. They observed that the grafts from the brother became infiltrated with mononuclear cells and did not "take", and that the second set of the brother's skin grafts were rejected in an accelerated fashion. This was the first demonstration of the phenomenon of "second set graft rejection", and supported an earlier proposal by Loeb (1930) that if "immune bodies" played a significant part in resistance to "homoplastic grafting", then a second graft transplanted when the reaction to a first was at its height should be attacked in an accelerated fashion. Gibson and Medawar described the second set response as occurring "in the absence of a demonstrable cellular mechanism", and suggested that the

"mechanism conforms to the general pattern of an antigen-antibody reaction". They also addressed the problem of histocompatibility. At that time there was a lack of consensus about whether blood incompatibility or tissue incompatibility was the more important cause of transplant rejection. However, since it was only then possible to match for blood groups and since in their case study the blood groups of donor and recipient were identical, they therefore concluded that "blood compatibility is not sufficient, and is not known to be necessary, to ensure compatibility of the skin".

These observations led Medawar to undertake a detailed series of studies of skin graft rejection in rabbits (Medawar, 1944, 1945). In these, he showed that the immune response to a skin graft was a systemic, not a local response, by virtue of the fact that neither the site nor the origin of the graft had any influence on the time taken for rejection; he also confirmed that the second set rejection response was donor specific, and hence was not obtained with a second graft taken from a different donor to that of the first. In addition he was able to postulate the existence of at least seven tissue antigens capable of provoking a rejection response in rabbits, and suggested that the survival time of the skin graft was an expression of the antigenic relationship between the donor and recipient.

These early findings form the basis of contemporary immunological dogma relating to graft rejection, namely: (a) that the graft rejection response is specific for the donor; (b) that it has memory and responds in an accelerated fashion to a second graft from the same donor; (c) that the first set response is primarily a cell mediated response; and (d) that second set rejection involves an antibody mediated response.

Following on from the basic observations of Medawar, a series of major advances over the next two decades provided the basis for our current understanding of graft rejection. These included the following major contributions:

1) The demonstration of a key role for recirculating lymphocytes in mediating both antibody responses to antigens, and cell-mediated responses to skin allografts (Gowans, McGregor, Cowan et al, 1962).

2) The proposal that the genetically determined histocompatibility antigens were responsible for the rejection of mismatched tissues (Snell, 1957).

3) The demonstration (Miller, 1962) of the importance of thymically-processed T cells in a range of immunological responses, including the ability to reject skin grafts, which, with the earlier observations of Bruce Glick in 1954 concerning the importance of Bursa-processed cells in antibody responses but not skin graft responses, revealed the dichotomy in the functions of lymphocytes.

4) The clonal deletion theory of self tolerance was proposed to explain the inability to generate self-directed antibody responses. This was further developed by Billingham, Brent & Medawar (1956) with their demonstration of the induction of neonatal tolerance.

1.4 Immunogenetics of transplantation

Graft rejection occurs because of genetic disparities between individuals, expressed as antigenic differences in cell surface histocompatibility antigens. The importance of these histocompatibility antigens in graft rejection was first proposed by Gorer et al (Gorer, Lymans & Snell, 1948) although it had long been recognised that variability in the rejection response to transplanted tissue was genetically controlled. For example, Little & Tyzzer (1916) had attributed the fate of transplanted tumours in Japanese waltzing mice to the influence of between 12 and 14 independent dominant genes acting simultaneously, which are now known to code for some of the MHC antigens.

Incompatibility between the graft donor and recipient for antigens encoded by genes of the Major Histocompatibility Complex (MHC) is the most important cause of rapid graft rejection. Minor histocompatibility

(mH) antigens encoded by genes outwith the MHC are undoubtedly weaker transplantation antigens than MHC antigens, but nevertheless, incompatibility for mH antigens alone may also cause graft rejection, albeit usually with a markedly reduced tempo.

MHC antigens During the early trials of clinical transplantation, attempts were made to "tissue match" the donor and recipient in order to reduce the risk of graft rejection. Serological methods were used to type the MHC gene products expressed on peripheral blood leukocytes (Dausset, 1958). These human leukocyte antigens (HLA) are now known to occur not just on leukocytes but on nearly all types of nucleated cells, and are known as class I MHC antigens. The existence of another group of MHC antigens became apparent when it was found (initially in mice and later in humans) that cells from different individuals which were apparently identical on serological typing still responded strongly to each other when tested in mixed lymphocyte culture. This additional group of MHC antigens has a more limited cellular distribution and constitutes class II MHC antigens.

A better understanding of the relationship between the complicated system of genetically determined MHC antigens and transplantation responses became possible with the development of highly inbred strains of animals. Both the mouse and to a lesser extent the rat have been extensively used for this purpose.

Mouse histocompatibility studies have had a long history and the genetically different strains are well classified. However, the data concerning the immunogenetics of transplantation in mice are almost exclusively derived from skin grafting experiments rather than transplantation of vascularised organ grafts, principally because of the technical constraints imposed by their small size. The development of appropriate inbred and genetically classified rat strains followed the pioneering immunogenetic work of Stark et al (Stark, Kren & Frenzl, 1967). This allowed the confirmation by Bildsoe (1972) that the rejection of vascularised heart and renal allografts (as well as skin grafts) in the rat was controlled by the RT1 locus genes.

Immunogenetic studies of transplantation in rodents have yielded invaluable information of relevance to clinical transplantation. Nevertheless, there is a need for caution when attempting to extrapolate the results obtained from rodent models. For example, organ transplantation is seldom performed across an ABO (erythrocyte antigen) incompatibility because of the presence of these antigens on all nucleated cells, and the presence of naturally occurring antibodies to foreign ABO types. Mice, however, have only a single red blood cell antigen. Rats have two red cell antigens but do not mount either a cellular or a humoral cytotoxic response against the incompatible type (Katz, Liebert, Gill et al,

1983) although they are able to make haemagglutinating antibodies. In order to understand the limitations of animal experiments with respect to the immunogenetics of transplantation it is necessary to consider first the components of the MHC and secondly, the differences between species and strains.

1.4.1 The Major Histocompatibility Complex

In man, the genes coding for the HLA system are situated on chromosome six, whereas the mouse MHC is encoded by genes located on chromosome 17 and in the rat, the chromosome(s) bearing the entire RT1 system have not been fully identified, nor has the position of the RT1 complex relative to the centromere been determined. The only loci providing a reference point between the species are the genetic markers glyoxalase 1 (glo1), located near to the MHC region in all cases, and the T/t locus (for tailless) in mice which confers growth and differentiation characteristics and appears to be analogous with the Grc (growth and reproduction complex) locus in rats, though the latter are located at opposite ends of the MHC region in these two species. However, enough information concerning the RT1 region itself has been obtained to allow a valid comparison between these species. Figure 1.1 shows a comparison of the MHC in man, mouse and rat.

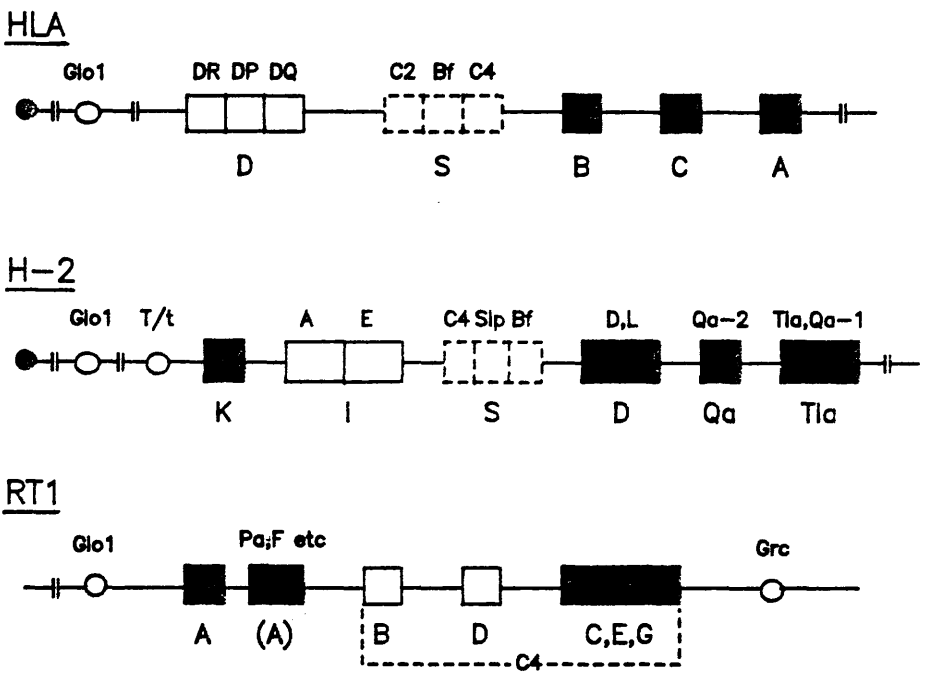


Fig. 1.1 Comparison of the MHC of man, mouse and rat. Shaded boxes represent loci encoding class I MHC antigens. Open boxes represent loci encoding class II MHC antigens. Broken lines represent loci encoding class III serum proteins.

1.4.1.1 The class I MHC region

Class I molecules in man are encoded by the HLA-A, HLA-B and HLA-C locus genes. In mice the serologically defined class I MHC antigens are encoded by the H-2D and H-2K regions, with a possible third class I antigen, H-2L being encoded by a locus situated in the D region (Hansen & Levy, 1978). These class I antigens are highly polymorphic, with at least 50 alleles present at both the K locus and at the D locus, which differ from each other in nucleotide sequence by up to 20% (Klein, 1978). Another group of class I genes located within the H-2 region are the "thymic-leukaemia" genes which are encoded by the Qa-1, Qa-2 and Tla genes. These non-transplantation antigens have a restricted distribution, being expressed preferentially on lymphocytes, thymocytes and certain leukaemic cells, and are much less polymorphic.

RT1A class I gene products in the rat are also highly polymorphic, although probably less so than mice. Gunther and Stark (1979) were able to describe more than 17 RT1 haplotypes in inbred rats alone, although these haplotypes often occurred in wild populations also. RT1C and RT1E antigens have a more restricted polymorphism and tissue distribution, and RT1C antigens are thought to be somewhat analogous to the mouse Qa and TL class I antigens (Stock & Gunther, 1982). Certainly they appear to have a less important role in transplantation

responses in that it was difficult to generate *in vitro* cytotoxic T lymphocyte responses against RT1C differences (Stock & Gunther, 1982).

1.4.1.2 The class II MHC region

In man, the class II genes are located in the D region of the HLA system and are organised into three families of genes, DR, DQ and DP, some of which are serologically detectable. Each family has one or two alpha chain genes and two or three beta chain genes, but the chains tend to associate with other chains within the same family so that class II molecules consisting of, say, a DR alpha chain and a DP beta chain are not expressed on the cell surface. It is not known which alpha and beta chains give rise to serologically detectable class II molecules, nor what determines the expression of a particular gene.

Class II molecules in the mouse have been most extensively studied and are encoded by H-2 I region genes, denoting immune responsiveness. This term was coined to identify genes responsible for the ability of the animal to mount a high or a low response to injected antigens or grafted allogeneic skin, and it was not clear initially whether or not they were class I genes. Observations based on the responses obtained in mixed lymphocyte cultures in mice suggested that the control of the response was independent of the serologically determined class I antigen genes, and hence a range of

genes determining MLR responses were described and denoted Ia for immune or I region associated genes (Davies, 1984). The H-2 class II I region genes include the IA, IE and IJ loci, and have been found to have direct counterparts in that human DR genes are analogous to IE while DQ are analogous to IA genes. IJ in mice has been found to confer a suppressor function on T lymphocytes (Smith, Steinmetz & Hood, 1986).

RT1 class II antigens are encoded by genes in the RT1B and RT1D regions which, by the repetition of experiments performed in mice and with the use of the rat recombinant strain r12, have been shown to be analogous to, though less well understood than, the IA and IE regions respectively (Blankenhorn, Cecka, Frelinger et al, 1980; Lobel & Cramer, 1981; Blankenhorn, Symington & Cramer, 1983). Their tissue distribution is restricted but variable, both on particular tissues and between strains, and this may be a manifestation of the responder status of the rat strain, determined by its class II haplotype.

1.4.1.3 The class III MHC region

Class III MHC genes encode soluble protein in all three species. Their products include the complement components C2 and C4, and factor B, and the serum-borne sex-linked protein in mice. In marked contrast to class

I and class II gene products, those of class III genes do not have a major role to play in initiating a rejection response to transplanted tissues.

1.4.2 Structure of MHC antigens

1.4.2.1 Class I MHC antigens

The basic structure of class I molecules was first determined in the mouse, but shows strong conservation between species and consists of two noncovalently linked glycoprotein chains which, in man, have approximate molecular weights of 45 Kilodaltons (Kd) and 12 Kd.

The smaller glycoprotein chain, known as β2-microglobulin, is not membrane bound and is easily dissociated, but is thought to be necessary for transport of the class I molecule to the cell surface. It has altered amino acid sequences between but not within species and is encoded by a gene on a separate chromosome from that bearing the MHC genes.

The larger chain is a transmembrane protein bearing three external domains, the third of which adjoins the transmembrane region and finishes with a short intracellular hydrophilic portion. The two external domains show high intraspecies polymorphism. It was previously thought that the alloantigenic specificity of class I molecules was conferred, at least in part, by glycosylation, but more recently it was shown that cells grown in conditions which did not permit insertion of

carbohydrate chains still exhibited alloantigenic specificity and it became apparent that the polymorphism resided in amino acid substitutions (Parham, Alpert, Orr et al, 1977; Ploegh, Orr & Strominger, 1980).

The degree of intraspecies polymorphism also shows considerable variation. For example in the mouse, K and D region products differ by up to 20% (Smith et al, 1986) whereas a class I region in the rat has recently been shown to encode for approximately six class I gene products which apparently differ by as little as a single amino acid (Gill, Kunz, Misra et al, 1987).

1.4.2.2 Class II MHC antigens

Class II MHC antigens differ structurally, functionally and in tissue distribution from class I molecules although, like class I molecules, they retain a similar basic structure between species. They consist of two non-covalently bound polypeptide chains (of approximate molecular weight 33 and 28 Kd) both traversing the cell membrane. Both chains are glycosylated and have two extracellular domains, one transmembrane domain and a hydrophilic intracellular domain. It is thought that the shorter chain is the more polymorphic, providing allo-antigenic specificity which resides in the polypeptide rather than the carbohydrate moeity (Cullen, Freed, Atkinson et al, 1975). The class II MHC gene products are encoded by the DR, DP and DQ loci in man, the IA, IE

and IJ region genes in the mouse, and the B and D locus genes in the rat. Their tissue distribution is much more restricted than that of the ubiquitous class I molecules in that they are expressed only on B lymphocytes, macrophages and monocytes, and in high density on dendritic cells. They are also present on activated T lymphocytes, and on some epithelial and endothelial cells where their expression is variable and inducible.

1.4.3 Role of the MHC in transplantation

The biological role of MHC molecules is to act as "restriction elements" in providing a means for rapidly distinguishing "self" from "non-self" or "altered self". Thus, MHC molecules function to direct T lymphocyte interactions with foreign antigens which are recognised as part of an MHC-antigen complex. The two major classes of MHC gene products preferentially direct the two main functional classes of T lymphocytes, in that cytotoxic T cells generally recognise antigens associated with class I MHC molecules whereas helper T cells predominantly recognise antigens associated with class II MHC molecules (Bach, Bach & Sondel, 1976; Miller, Vadas, Whitelaw et al, 1976).

In the context of transplantation, the MHC gene products are of critical importance because T cells also respond to allogeneic forms of MHC molecules in the absence of added antigen. Indeed, the frequency of T

cells which respond to foreign MHC molecules is very much greater than the frequency of T cells responding to conventional antigen and self MHC (Wilson, Howard & Nowell, 1972) although the reasons for this are not completely understood (Marrack & Kappler, 1988).

In general, allografts differing at the entire MHC will be rapidly rejected if transplanted into unmodified recipients although, as will be seen, there may be differences in the tempo of the rejection response depending on the nature of the tissue transplanted (Warren, Lofgreen & Steinmuller, 1973) and the particular strain combination examined. Both class I and class II MHC gene products are able to provoke strong transplant responses and lead to rapid graft rejection. This is clearly demonstrated by experiments in which grafts between inbred animals differing at isolated class I MHC or class II MHC antigens only have been shown to undergo rapid rejection in unmodified recipients (Katz et al, 1983; Burdick & Clow, 1986).

1.4.4 Role of non-MHC antigens in transplantation

The identification of non-MHC antigens as "minor" transplantation antigens is a misnomer, since congenic mice may reject skin grafts which differ only for mH antigens at a rate comparable with that seen in MHC disparate grafts (Schultz, Beals & Petraitis, 1976). In clinical transplantation, grafts performed between

siblings identical at the major histocompatibility loci still require immunosuppressive treatment to prevent rejection due to non-MHC incompatibilities. An example of non-MHC antigens is Epa-1 which is a tissue specific antigen, the expression of which is restricted to epidermal cells in mice, rats and man (Steinmuller & Tyler, 1983). Epa-1 incompatibility results in skin graft rejection and the generation of Epa specific cytotoxic T lymphocytes. The work of Tyler et al (Tyler, Galli, Snider et al, 1984) provided an example of how minor histocompatibility (mH) antigens are recognised in the context of self MHC, and also that reactivity to mH antigens is MHC restricted. They raised clones of Epa specific cytotoxic T cells which caused skin lesions when injected intradermally into Epa incompatible mice only if their MHC was identical to that in which the clone was raised.

Although less well defined, non-MHC antigens in rats are equally important in transplantation. One example whereby non-MHC incompatibility may lead to graft rejection in rats was described by Paul & Carpenter (1983). They presensitised MHC compatible recipients to an endothelial antigen, and subsequently were able to demonstrate acute rejection of an endothelial antigen incompatible kidney graft. In summary, therefore, the so-called minor histocompatibility antigens may play a

significant role in graft rejection and although they are probably less important in this respect than MHC antigens, they cannot be ignored.

1.4.5 Role of immune response genes in transplantation

Immune response (Ir) genes control the magnitude and specificity of immune responses to particular antigens, and may be MHC linked or non-MHC linked. McDevitt and colleagues first observed the influence of Ir genes in the strain-associated variability of antibody responses in mice challenged with a range of synthetic antigens (T,G)-A-L, (H,G)-A-L and (Phe,G)-A-L. They were able to demonstrate the highly specific nature of Ir gene control since H-2^b mice were high responders to (T,G)-A-L and low responders to (H,G)-A-L, while H-2^k mice showed reciprocal responses, and both strains were high responders to (Phe,G)-A-L (McDevitt, Deak, Schreffler et al, 1972). The ability of different strains to respond to these antigens mapped not to the serologically detectable regions of the MHC but to the H-2 I region.

In addition to Ir genes associated with the MHC, there are also Ir genes which are not inherited in association with MHC genes, but which can determine the resistance of animals to infectious organisms, often by coding for mechanisms such as the ability of macrophage enzymes to inhibit intracellular replication of organisms. Thus two

different strains of mice sharing the same H-2 haplotype may differ in their resistance to a particular organism (Krco & David, 1981).

In the context of tranplantation, the MHC linked immune response genes may determine not only the magnitude and specificity of the response to foreign antigens, but also the type of response, and it may be hypothesised that an animal which is unable to mount an observable cytotoxic cell mediated response can instead mount a potent alloantibody response and effect rejection in conjunction with nonspecific effector cells via an antibody-dependent cell-mediated cytotoxicity response.

There are several examples in animal experiments in which apparently inconsistent responses to transplanted tissues can be attributed to the influence of Ir genes. In some strain combinations, fully allogeneic rat liver grafts are spontaneously accepted with no graft rejection (Zimmerman, Butcher, Davies et al, 1979). The tempo of allograft rejection also appears to be determined by the immune response genes. In the rat, for example, the $RT1^{c}$ haplotype rejects MHC "a" haplotype skin grafts slowly, while the $RT1^{u}$ haplotype mounts a vigorous rejection response (Butcher & Howard, 1982). It might be concluded that the class II locus is more important in rejection since grafts between recombinants differing only at a class I locus antigen, but sharing class II and minor histocompatibility antigens are rejected slowly or

not at all, (Lowry, Forbes, Blackburn et al, 1985). However, it appears that the rejection of grafts differing at a class I locus alone may be determined overall by the immune response genes because isolated class I incompatible grafts on a "low responder" background such as $RT1^c$ do not reject, while class I incompatible grafts on a high responder background ($RT1^{c/u}$) are rejected rapidly (Butcher, Corvalan, License et al, 1982).

Like MHC antigens, responses to non MHC antigens also appear to be under Ir gene control, and this has been studied most extensively by observing responses to the male restricted non-MHC antigen H-Y (Fierz, Brenan, Mullbacher et al, 1982). Interestingly, $H\text{-}2^b$ mice respond to H-Y antigens in that they reject H-Y incompatible skin grafts although they do not mount a T cytotoxic cell response (detectable in in vitro studies). Conversely, mouse strains of H-2 haplotypes k, d and s do not reject H-Y incompatible skin grafts, although they can to some extent raise cytotoxic T cell responses. It has been suggested that the H-Y Ir genes operating here act at the induction of the immune response, and are both H-2 and non-H-2 Ir genes (Loveland & Simpson, 1986).

1.4.6 Tissue distribution of MHC antigens

Since MHC antigens are the major stimulus of the graft rejection response, the density and pattern of MHC expression by the different cellular components of an allograft is of obvious importance. In most species, MHC class I antigens are expressed strongly on interstitial dendritic and vascular endothelial cells, and on most other cells of the body. The cellular expression of constitutive class II MHC antigens, however, shows a much greater restriction and there are potentially important intraspecies differences. In the human kidney, class II MHC antigens are expressed strongly on the vascular endothelial cells of all vessels and on interstitial dendritic cells (Hart, Fuggle, Williams et al, 1981). They are also expressed by proximal renal tubular cells but are absent from distal renal tubular cells (Fuggle, Errasti, Daar et al, 1983).

In contrast, within rat kidneys the vascular endothelium does not constitutively express detectable class II MHC antigens, although these are strongly expressed by interstitial dendritic cells and are also present on the proximal renal tubules of most strains studied (Hart & Fabre, 1981a, 1981b).

The difference in class II MHC antigen expression by vascular endothelium between human and rat kidneys has potential implications when comparing renal allograft rejection in these species. For example, it has been

suggested as an explanation for the relative ease with which rat kidney allografts, in some strain combinations, can be prevented from undergoing rejection by a single injection of donor strain alloantibody (passive enhancement) whereas, by comparison, the expression of class II MHC antigens by human kidney vascular endothelium may prevent permanent acceptance of the graft unless immunosuppressive therapy is continued indefinitely (Fabre, 1982).

Not all investigators, however, agree that rat vascular endothelial cells do not express detectable class II MHC antigens. In particular, Paul (Paul & Carpenter, 1983), using rat alloantisera directed against class II IA-like antigens, reported demonstrable staining of rat vascular endothelium, suggesting that class II MHC antigens were indeed present. An explanation offered for this apparent anomaly was that class II antigen expression in vivo might be variable and subject to regulating mechanisms. Further discrepancies in the large amount of class II antigens detected in homogenates of rat kidneys by absorption studies (Hart & Fabre, 1979) compared with the small amount detected by absorption studies of intact viable kidney cells (Mason & Gallico, 1978) highlighted an important point concerning the distribution of class II antigens. Hayry et al, using a sensitive Staph. aureus binding assay, demonstrated that only a proportion of detectable class II antigens were expressed

on the surface of cells, and therefore the intracellular class II antigens, such as those of the proximal tubules, were not available for presentation to the host (Hayry, von Willebrand & Andersson, 1980; Lautenschlager, Nyman, Vaananen et al, 1983). These numerous apparent discrepancies in the literature concerning the expression of MHC antigens on various tissues in different species resulted in a critical reassessment of previous experiments, and the initiation of further careful studies from which a pattern began to emerge.

1.4.7 Induction of MHC antigens in rejecting allografts

The results of immunohistological studies over the last several years have now clearly shown that the expression of class II, and more recently class I, MHC antigens within a tissue is not predetermined and that under certain conditions MHC expression may be markedly increased. An early report (Lampert, Suitters & Chisholm, 1981) showed that during graft-versus-host reactions in rats, epidermal cells which do not constitutively express class II MHC antigens became strongly class II positive. This observation was followed by reports in both mice and rats that endothelial cells and keratinocytes in skin allografts undergoing rejection were induced to express class II MHC antigens (de Waal, Bogman, Maass et al, 1983; Dallman &

Mason, 1983). In clinical organ transplantation, increased HLA-DR expression on renal tubular cells was demonstrated in biopsies of renal transplants taken during rejection episodes (Hall, Duggin, Philips et al, 1984).

Experimental models of vascularised allografts undergoing unmodified rejection in rodents have all shown marked induction of class II MHC antigens within the rejecting tissue. Benson et al described the induction of large amounts of newly induced donor type Ia antigen in tubular and glomerular cells in transplanted allogeneic mouse kidneys (Benson, Colvin & Russell, 1985). This occurred in the absence of recipient class II antigen induction in the recipient's remaining native kidney, demonstrating the very localised induction of antigen associated with the rejection response.

Milton & Fabre have recently described in detail the induction of MHC antigens initially in rejecting rat heart (Milton & Fabre, 1985) and subsequently in rejecting rat kidney allografts (Milton, Spencer & Fabre, 1986a). With the use of monoclonal antibodies directed against polymorphic determinants of both class I and class II MHC antigens it was possible to demonstrate widespread increased expression of MHC antigens on donor tissues alone, since infiltrating host leukocytes were not labelled by these antibodies. They showed that myocardial cells which normally express little or no

class I MHC antigens and no class II MHC antigens became strongly positive for both antigens as rejection progressed. In addition, vascular endothelial cells in both hearts and kidneys became strongly class II positive. These findings were confirmed by quantitative absorption analysis of the MHC antigen expression in rejecting allografts which showed several fold increases in the amounts of both class I and class II MHC antigens in rejecting hearts and kidneys. It cannot be assumed, however, that allograft rejection is invariably associated with ubiquitous MHC antigen induction, since a recent paper by Forbes and colleagues described the rejection of ACI cardiac allografts by BN rats in the absence of donor MHC class II expression on either vascular endothelium or cardiac muscle cells (Forbes, Parfrey, Gomersall et al, 1986).

In addition to heart and kidney allografts, other vascularised organ allografts also show MHC antigen induction during rejection although the different graft components differ in their response. For example rejecting rat pancreas allografts show widespread class II antigen induction on acinar cells, pancreatic duct epithelium and vascular endothelium, although the islets of Langerhans remain class II negative (Steiniger, Klempnauer & Wonigeit, 1985). Similarly, rejecting human and rat liver allografts show strong induction of class

II MHC antigens on the bile duct epithelium (Takacs, Szende, Rot et al, 1985) although hepatocytes remain class II negative.

The induction of MHC antigens on the cellular components of rejecting allografts is almost certainly due to soluble mediators released from graft infiltrating leukocytes. It is well known that soluble mediators, such as alpha and beta interferons produced by a wide variety of cell types, have the capacity to induce class I expression on cells in vitro, while gamma interferon (IFN-ϑ), produced by activated T lymphocytes, causes the induction in vitro and in vivo of both class I and class II MHC antigens (Fellous, Nir, Wallach et al, 1982; Wong, Clark-Lewis, Harris et al, 1984; Basham, Smith, Lanier et al, 1984). It has been suggested that an important role of IFN-ϑ production is to enhance the function of immunological effector cells by improving the interaction between the antigen presenting target cells and the effector T lymphocytes, in which the expression of class II MHC antigen is essential (Farrar, Johnson & Farrar, 1981; Arenzana-Seisdedos, Virelizier & Fiers, 1985; Kelley, Fiers & Strom, 1984). This class II antigen expression may be constitutive, but is particularly susceptible to induction by IFN-ϑ especially on macrophages/monocytes, fibroblasts and endothelial cells

(Steeg, Moore, Johnson et al, 1982; Pober, Gimbrone, Cotran et al, 1983; Pober, Collins, Gimbrone et al, 1983).

Furthermore, it might be suggested that lymphokine production during the rejection response increases donor MHC antigen expression which, in turn, renders the transplanted tissue more susceptible to the rejection response. This suggestion is supported by the results of experiments in which the effect of altering MHC expression by, for example, IFN-ɣ or steroid treatment, has been examined.

1.4.8 Reduction of MHC antigen expression

Following their demonstration of widespread MHC antigen induction in rejecting rat heart and kidney allografts, Milton & Fabre subsequently showed that prevention of rejection by administration of CyA was associated with only slight induction of class I MHC antigens and absence of class II antigen induction (Milton, Spencer & Fabre, 1986b). This followed a suggestion by Groenewegan et al that constitutive MHC class II antigen expression by vascular endothelium *in vivo* was dependent on IFN-ɣ, since they were able to demonstrate that class II expression disappeared from kidney endothelial cells of dogs in which lymphokine production was inhibited by treatment with Cyclosporin A (Groenewegan, Buurmnan & van der Linden, 1985).

Leszczynski et al found that a single injection of IFN-ϑ caused a marked increase in class II expression by rat renal endothelial cells which was entirely abolished by three daily doses of methylprednisolone (Leszczynski, Ferry, Schellekens et al, 1986); while the continuous local infusion of steroids also prevented MHC antigen induction in rat renal allografts (Ruers, Buurman, van Boxtel et al, 1987).

These findings were contradicted, however, by the demonstration that in vivo administration of IFN-ϑ by continuous infusion (Ijzermans, Bouwman, de Bruin et al, 1987) did not result in increased class II expression in allografted rat heart tissues nor did it alter graft survival time.

1.4.9 Susceptibility of different tissues to rejection

MHC antigen expression is not the only factor that influences the susceptibility of a particular tissue to the rejection response. It is well known that within a particular strain combination different tissues reject at different rates (for example, Warren et al, 1973) and there may be a number of explanations for this. A primarily vascularised organ allograft may be more instantly immunogenic to the recipient since there will be immediate contact between donor antigens and circulating host immune cells. A skin graft, by contrast, is not immediately vascularised, but eventually

develops a blood supply provided by the growth of host and not donor vessels, so that the majority of vascular endothelial cells will express syngeneic and not allogeneic MHC antigens.

Heart allografts sometimes appear to be less susceptible to rejection than kidney allografts, and this may be attributed to the fact that they express lower levels of class I and class II MHC antigens (Hart & Fabre, 1979). However, liver allografts which express large amounts of class I antigens but little class II, are also relatively less susceptible than kidneys to allograft rejection, and in some fully allogeneic strain combinations are accepted indefinitely (Kamada, 1985). One explanation may be that liver allografts are accepted partly because of their large size, and the very large amounts of alloantigen they contain simply overwhelm the recipient's immune system.

1.5 Cellular pathways of graft rejection

The remainder of this introduction reviews the cellular mechanisms which underly the rejection of an allograft. The data discussed are derived, for the most part, from rodent models of graft rejection and, wherever possible, the experiments described relate specifically to renal allograft rejection.

It is convenient and conventional to consider the process whereby a vascularised allograft undergoes rejection as comprising three phases (Mason & Morris, 1986). During the first or afferent phase of graft rejection, graft alloantigens are presented by either donor or host strain antigen presenting cells and induce an immune response by the host's antigen reactive lymphocytes. Immune induction is followed by the second or central phase of rejection. This phase encompasses the lymphocyte-lymphocyte interactions, proliferation and clonal expansion which occur in both the circulating and sessile lymphocyte populations within the lymphoid tissue of the recipient. During the third, or efferent phase of graft rejection, effector cells (and alloantibody) are released into the efferent lymph and thereafter reach the graft via the systemic circulation. Here the effector mechanisms cause the tissue destruction which characterises graft rejection. These three phases will be considered in detail.

1.5.1 Afferent phase of graft rejection

It is generally accepted that the immune response to conventional protein antigens usually (but not always) involves the processing of the antigen by antigen presenting cells prior to the induction of an immune response. Hence, although some small antigenic peptides may be recognised directly by T cells (Kovac & Schwartz,

1985), most antigenic proteins are internalised and re-expressed on the surface of an antigen presenting cell in close association with a self MHC molecule, and this antigen-MHC complex acts as the ligand for the alpha-beta receptor of the T cell (Shimonkevitz, Kappler, Marrack et al, 1983). In the context of transplantation, recipient T cells respond to allogeneic MHC molecules in the absence of foreign antigen.

When the antigen consists of MHC alloantigens expressed on donor strain antigen presenting cells, these may directly activate host antigen reactive cells either within the graft or, conceivably, when the antigen presenting cells have migrated to the recipient lymphoid tissue. Alternatively, antigenic material shed by the graft may be processed and presented by recipient antigen presenting cells, thereby initiating graft rejection.

With vascularised allografts, there is no consensus regarding whether sensitisation to donor MHC antigens occurrs predominantly within the graft or within the draining lymph nodes. With regard to the route of sensitisation, a distinction can be made between skin and vascularised allografts. Skin allografts are transplanted as free, non-vascularised grafts, and while the lymphatic drainage of the graft is rapidly restored, it takes several days before the vascular supply is re-established. The importance of the lymphatic drainage to the afferent phase of skin graft rejection is

illustrated by experiments in which skin allografts, which have a vascular supply but are prevented from re-establishing lymphatic connections (by supporting the skin on an impenetrable pedicle), show prolonged survival (Barker & Billingham, 1968). This observation contrasts with similar experiments in vascularised organ allografts where diversion of the lymphatic connections away from the graft does not prolong graft survival (Pedersen & Morris, 1970).

Regardless of the mechanism by which the host recognises the graft as foreign, the presence within the graft of parenchymal cells which express MHC antigens may not, in itself, be sufficient to initiate graft rejection. Lafferty et al proposed a "two signal" model for the initiation of graft rejection (Lafferty, Prowse, Simeonovic et al, 1983). In this scheme, recognition of foreign MHC antigens on parenchymal cells alone does not provoke a rejection response. Instead, presentation of foreign MHC antigens by a cell that can also deliver "co-stimulatory activity" is necessary to initiate rejection. The evidence supporting this two signal theory is based largely on in vitro experiments demonstrating, for example, that metabolically active cells are required to stimulate alloantigen reactive T cell responses. Killed cells, although they express MHC antigens, are unable to do so (for example, Lindahl-Kiessling & Safwenberg, 1971). Further

supportive evidence is provided by the observation that cells such as erythrocytes, platelets and fibroblasts are unable to stimulate allogeneic lymphocyte responses despite expressing MHC antigens on their cell membranes.

1.5.2 Role of donor strain dendritic cells in the afferent phase

There is little doubt that the major immunogenic component of a vascularised allograft resides in the bone-marrow derived interstitial dendritic cells which are widely distributed throughout the tissue. These cells are present in large numbers scattered throughout the parenchyma of all commonly transplanted organs . They have a striking branched morphology, express large amounts of both class I and class II MHC antigens, and are potent antigen presenting cells as well as stimulators of the in vitro mixed lymphocyte reaction (Steinman & Witmer, 1978; Mason, Pugh & Webb, 1981).

It is not a new concept that bone-marrow derived cells of donor origin occurring within the grafted tissue provide the major immunogenic stimulus (Snell, Dausset & Nathenson, 1976). However, in earlier studies it was generally believed that this immunogenicity resided in blood leukocytes remaining within the vasculature and tissue spaces of the donor organ after harvesting and that these leukocytes were carried over into the recipient of the graft - hence the term "passenger

leukocyte". An important role for passenger leukocytes in graft rejection was demonstrated by Guttman, Lindquist & Ockner, (1969) using a rat renal allograft model in which radiation bone marrow chimaeric rats were used as kidney donors so that the bone marrow derived cells were syngeneic with the graft recipient. They showed convincingly that whereas the unaltered donor kidney was rapidly rejected by the host, the chimaeric kidneys in which the passenger leukocytes were syngeneic with the host had good renal function on day 7 and little histological evidence of rejection. Unfortunately, no survival data were presented, and no attempts were made to determine the degree of chimaerism of the donor kidneys.

Similar experiments by Stuart (Stuart, Bastien, Holter et al, 1971) also demonstrated a convincing role for passenger leukocytes in graft rejection. In addition to using radiation chimaeras, they also transplanted donor kidneys into immunosuppressed intermediate hosts which were syngeneic with the eventual recipient animals. During its residence in the intermediate host it was expected that passenger leukocytes would be eliminated from the graft prior to its retransplantation. Again it was demonstrated that reduction or elimination of passenger leukocytes significantly prolonged graft survival although permanent survival was by no means always obtained.

More recently, immunohistological studies demonstrated that strongly class II MHC antigen positive, bone marrow derived, interstitial dendritic cells were widespread throughout all tissues, with the exception of the nervous system (Hart & Fabre, 1981c; Daar, Fuggle, Fabre et al, 1984). Simultaneously, it was realised that the presence of these cells within the graft accounted for the immunogenic properties previously attributed more broadly to passenger leukocytes. The importance of interstitial dendritic cells in graft rejection was shown by Lechler & Batchelor (1982) in an elegant series of experiments. They transplanted donor rat kidneys to an intermediate host syngeneic with the eventual recipient and prevented graft rejection by pretreatment of the intermediate host with donor alloantibody (passive enhancement). Several weeks later, by which time the donor strain dendritic cells were judged to have been replaced by those of recipient origin, the transplanted kidney was retransplanted to a fresh unmodified allogeneic recipient. Although the second recipient was unable to reject the renal allograft, the simultaneous injection of a small number (10^4) of donor strain dendritic cells was sufficient to restore graft rejection.

A further demonstration that interstitial dendritic cells play a key role in graft rejection is provided by experiments in which allogeneic tissue (most often

isolated pancreatic islets or thyroid fragments) is cultured under *in vitro* conditions aimed at selectively eliminating passenger leukocytes. Many such studies have shown that this treatment reduces graft immunogenicity, although few have attempted to critically examine the effectiveness of the protocol in eliminating passenger leukocytes. Early experiments showed that culture of allogeneic mouse thyroid tissue in an atmosphere of high oxygen content prior to transplantation to the renal subcapsular site of allogeneic hosts was able to abrogate rejection (Lafferty, Cooley, Woolnough et al, 1975; Lafferty, Bootes, Kilby et al, 1976). Attempts to repeat these findings with isolated pancreatic islets were initially unsuccessful because of the susceptibility of islets to oxygen toxicity. This problem was overcome by forming aggregates of mouse pancreatic islets which could then be cultured and successfully transplanted into allogeneic recipients where they were shown to reverse streptozotocin-induced diabetes (Bowen & Lafferty, 1980; Bowen, Prowse & Lafferty, 1981; Donohoe, Andrus, Bowen et al, 1983). Similar results were reported for rat islets (Lacy & Davie, 1984).

However, in contrast to these findings, it has also been shown that culture of pancreatic islet tissue, and hence putative elimination of interstitial dendritic cells, prior to transplantation does not invariably prevent graft rejection (Simeonovic, Bowen, Kotlarski et

al, 1980). Furthermore, in other experiments confirming that pretransplant culture of rodent islets leads to long term graft survival, it has been suggested that the decrease in immunogenicity is not due solely to the depletion of Ia^+ dendritic cells (Gores, Sutherland, Platt et al,1986).

1.5.3 Alternative routes of sensitisation

Although it is apparent that donor strain dendritic cells play a major role in initiating graft rejection, they do not represent the sole immunogen. On the basis of their retransplantation experiments, Lechler & Batchelor (1982) postulated the existence of two routes whereby graft recipients became sensitised to alloantigen. The first route involved the direct recognition of MHC antigen on donor dendritic cells. The second, and possibly less efficient, route involved recognition and processing of alloantigen present in the graft by recipient strain dendritic cells or macrophages. The second route of sensitisation has received support from the recent experiments of Sherwood et al (1986). Mice were sensitised to allogeneic cells, and their sensitised spleen or peritoneal cells were then transferred to naive syngeneic secondary hosts. They showed that when the secondary host was given a skin graft from the strain of the sensitising animal, it was rejected in an accelerated fashion, implying the ability

of the transferred cells from the primary host to present alloantigen. These cells, which appeared to be dendritic cells, were surprisingly effective even after inactivation by paraformaldehyde , thus contradicting Lafferty's observations on the "two signal" mechanism of host sensitisation which required the presence of metabolically active cells.

1.5.4 Ability of other graft components to initiate rejection

It must not be assumed that the interstitial dendritic cells are the only cells capable of initiating the rejection response, although it cannot be doubted that, by virtue of their expression of large amounts of class II MHC antigens, they are highly efficient in this respect.

The relatively recent demonstrations of the inducible nature of class II MHC antigens on a number of tissues raised the possibility that other cell types might have the capacity to present antigen, in addition to those cells such as dendritic cells that constitutively express class II antigens.

It seems likely that it is the expression of class II MHC antigens that confers on a cell type the ability to present antigens to class II restricted responder cells. This was confirmed by experiments in which mouse L-cell fibroblasts which do not normally express class II MHC

antigens under any circumstances, were transfected with genes encoding mouse class II I-A molecules of the "k" haplotype, and subsequently expressed this molecule on their cell surface. These transfected L-cells were then shown to be able to present protein antigens such as KLH to KLH-specific, $I\text{-}A^k$ restricted T cell hybridomas which, on specific activation, were induced to secrete IL2 (Malissen, Peele Price, Goverman et al, 1984).

With more direct relevance to transplantation, experiments by Pober et al have shown that cultured human umbilical vein endothelial cells that have been induced to express class II antigens by the addition of recombinant IFN-ϑ develop the added capacity to present class II antigen to cloned allospecific CTL and, ipso facto, to act as targets (Pober, Collins, Gimbrone et al, 1983). This observation was further developed by Ferry et al, who successfully cultured rat heart endothelial cells in vitro and showed that they expressed increased amounts of MHC class II antigen when grown in the presence of recombinant rat IFN-ϑ. These cultured endothelial cells were highly immunogenic in vivo since when they were injected into allogeneic rats they were able to specifically sensitise the animals so that subsequent organ allografts were rejected in accelerated fashion (Ferry, Halttunen, Leszczynski et al, 1987). Although the expression of class II MHC antigens may confer upon a cell the ability to function in an antigen

presenting capacity it remains to be seen whether this route is sufficiently effective to be of biological significance. Further experiments along these lines should lead to an increased understanding of the initiation and direction of alloimmune responses in allograft rejection.

1.6 Central phase of graft rejection

The central phase of graft rejection encompasses the lymphocyte interactions which lead to the generation of immunological effector mechanisms directed against the graft. Optimal development of the immune response against an allograft is ensured by the process of lymphocyte migration and recirculation which results in the activation of relevant antigen reactive cells (Ford, 1975). Transplantation of either skin or vascularised organ allografts leads to a rapid and vigorous proliferative response by host lymphocytes, particularly those in the regional lymph nodes and spleen (Anderson, Anderson & Wyllie, 1975; Baldwin, Hendry, Birinyi et al, 1979). Furthermore, in the case of vascularised allografts, there is a marked proliferation and clonal expansion of lymphocytes within the tissues of the transplanted organ (Ascher, Chen, Hoffman et al, 1983).

During this phase of the graft rejection response, lymphocytes with T helper function are thought to play a central role (Mason & Morris, 1986). These cells, principally through lymphokine release, are able to facilitate the proliferation and differentiation of different potential effector pathways. Interleukin 2 is the most well known lymphokine produced by activated T helper cells and results in the differentiation of cytotoxic T cells (Farrar, Benjamin, Hilfiker et al, 1982) as well as activating and potentiating the function of natural killer cells and antibody dependent cellular cytotoxicity (Kawase, Brooks, Kuribyashi et al, 1983). In addition, activated T cells may release several other lymphokines which influence the generation of the anti-graft response, notably IFN-ϑ, as well as other interleukins which are involved in B cell differentiation and proliferation.

1.7 Effector mechanisms in allograft rejection

As a consequence of the initiation of the process of graft rejection, a potent immunological response is mounted against the transplant which, in the unmodified recipient, results in the complete destruction of the transplant, usually within several days. The immunological effector mechanisms by which the recipient rejects an allograft are a source of considerable debate. Since a major part of this thesis concerns this area, the experimental evidence concerning the effector mechanisms of allograft rejection is reviewed in detail in this section.

1.7.1 Target antigens of the immune response

The rejection of a vascularised allograft, as already described, is due principally to MHC antigenic differences between the recipient and the donor. It follows, therefore, that the cellular distribution and density of MHC class I and class II target antigens within an allograft may influence its vulnerability to immunological effector mechanisms. Furthermore, the widespread induction of MHC antigen expression that occurs within a rejecting allograft might be expected to further increase its susceptibility to effector cell damage.

Surprisingly, the precise cellular targets of graft rejection are not known although it seems likely, in vascularised allografts such as the kidney, that donor vascular endothelial cells represent a major target and that damage to the microvasculature leads to ischaemia and necrosis of the graft (eg. Cramer, 1987). In contrast, in skin allografts, where revascularisation by host strain vascular endothelium occurs, the epidermal cells are thought to be the major targets of the immune response. This latter suggestion is suported by the observation that in rejecting skin grafts, host leukocytes have been observed to be in close contact with epidermal cells.

In addition to their role as potent initiators of graft rejection, it has also been suggested that passenger leukocytes represent an important target within renal allografts. Stuart et al (1971) transplanted donor rat kidneys from radiation chimaeras in which the passenger leukocytes were allogeneic with the graft recipient, but the kidney parenchyma was syngeneic with the recipient. Controversially, they reported that such kidneys underwent graft rejection with a tempo comparable to that seen with fully allogeneic grafts. Similar experiments by Fabre & Morris (1973) did not support this finding, although it was acknowledged that this may have been due to the different rat strain combination used.

The *in vitro* activity of effector cells in the rejection response is usually assessed by examining their ability to lyse allogeneic donor strain radiolabelled lymphocyte target cells. Clearly, however, these lymphocyte target cells, which are usually mitogen activated lymphoblasts prepared from donor strain spleen or lymph node cells, may not be the most appropriate targets in the rejection of a vascularised organ graft. Ideally, purified preparations of more appropriate targets, such as isolated vascular endothelial cells, would be used. However, with only occasional exceptions (MacPherson & Christmas, 1984), technical difficulties in isolating sufficient numbers of pure cells have usually precluded their use. Methods describing the isolation of purified mouse kidney and human vascular endothelial cells are now reported and future information based on the use of such cells may be of use for the better understanding of the processes involved in the initiation of graft rejection (Ramos & Cox, 1987; Auerbach, Alby, Grieves et al, 1982).

1.7.2 The role of alloantibodies

A major controversy has existed over the last two decades as to which of the possible immune effector mechanisms is responsible for the destruction of an allograft. It is accepted that the uncommon clinical problem of hyperacute rejection is due to the presence of

preexisting cytotoxic antibodies against the donor MHC antigens (Kissmeyer-Nielsen, Olsen, Petersen et al, 1961). The dramatic and irreversible consequences of hyperacute rejection are the results of the cytotoxic alloantibodies binding to the vascular endothelium of the graft followed by complement fixation, platelet aggregation, fibrin deposition and destruction of the graft (Busch, Reynolds, Galvanek et al, 1971). Fortunately, in clinical practise, hyperacute rejection is rare since kidney recipients are screened for the presence of cytotoxic antibodies against their prospective allograft, prior to transplantation.

Alloantibodies may also play a role in chronic renal allograft rejection. However, although this is a relatively common clinical problem, it has not been well studied. A satisfactory animal model with which to examine the problem of chronic graft rejection has not been devised, but it does appear from patient studies that the humoral immune response is involved to a greater extent than the cellular immune response (Cramer, 1987).

Although alloantibody is responsible for hyperacute graft rejection and possible chronic rejection in immunosuppressed patients, it is generally assumed that antibody does not play an essential role in acute graft rejection but, rather, that cellular mechanisms are involved. Nevertheless, alloantibody could, in principle, contribute to acute graft rejection. For

example, specific alloantibody could bind to the graft and allow nonspecific effector cells, such as natural killer cells and macrophages, to mediate specific lysis via the recognition of the Fc portion of the alloantibody. In this process of "antibody dependent cellular cytotoxicity" (ADCC), the specificity of the response depends on the attachment of alloantibody to the target cells and hence there is no bystander damage to adjacent cells which have not bound alloantibody (Perlmann & Perlmann, 1970).

It has been suggested that antibody dependent cellular cytotoxicity is the mechanism by which passive transfer of alloantibodies is able to contribute to allograft rejection in some experimental models (Carpenter, d'Apice & Abbas, 1976), but the precise role of antibody in acute graft rejection remains unclear. Indeed, in some animal models of renal or cardiac transplantation, the administration of donor specific alloantibodies may have a protective rather than a destructive effect on the graft, leading, in some strain combinations, to long term graft survival (passive enhancement) (Fabre & Morris, 1972).

1.7.3 The essential role of T lymphocytes in acute rejection

In contrast to the uncertain role of alloantibody in acute graft rejection, it was established at an early stage (Miller, 1962) that thymus-derived T lymphocytes played an essential part in the rejection process when it was shown that animals which had either undergone neonatal thymectomy or were congenitally athymic were unable to reject a skin allograft. Strong support for the necessary role of T lymphocytes but the apparently non-essential role of B lymphocytes (and hence alloantibody) was provided by the detailed adoptive transfer experiments of Dorsch & Roser (1974) and Hall et al (Hall, Dorsch & Roser, 1978a). They showed that administration of a sublethal dose of whole body irradiation (9.0 Gy) to adult rats was sufficient to ablate the immune system (destroying >99.9% of circulating small lymphocytes) and rendered the animals unable to reject a skin allograft. Irradiated animals which were reconstituted with a sufficient number of syngeneic T lymphocytes (TDL or LNC) promptly rejected their skin grafts whereas those reconstituted with B lymphocytes alone were not capable of rejecting their grafts. Furthermore, B lymphocytes did not contribute any synergistic effect towards graft rejection when they were given along with T lymphocytes.

Consequently, the role of B cells and antibody in experimental models of graft rejection have subsequently been somewhat neglected and attention has focussed instead on the role of cellular effector mechanisms in causing rejection and, in particular, the relative roles played by DTH and specific cytotoxic T cells in graft rejection. A summary of the cellular events which characterise DTH and specific cytotoxic T cell lysis will be given, followed by a description of the available evidence suggesting a role for one or other of these mechanisms in allograft rejection. In addition, a table of adoptive transfer experiments providing evidence for either mechanism in allograft rejection is included for reference, in Appendix A.

1.7.4 DTH and nonspecific effectors

A DTH response implies that a small number of T helper (CD4) lymphocytes recognise class II MHC antigens and are able, by releasing lymhpokines, to recruit nonspecific effector cells, including macrophages and NK cells (McKenzie, 1983). Although the specificity of the DTH response is mediated through T helper lymphocytes, tissue destruction is effected by nonspecific cells and soluble mediators (North, 1978). Therefore, although a small number of Th cells are thus able to indirectly mediate

the destruction of a large number of target cells, the efficiency of the response may be counterproductive if adjacent self tissue suffers from bystander damage.

As previously noted, both macrophages and NK cells are found in large numbers within rejecting allografts and, in principle, could mediate graft damage as part of a DTH response. Activated macrophages are capable of a large variety of cell functions and in particular the release of many inflammatory mediators including lysozyme, proteases, tumour necrosis factor (TNF) and reactive oxygen intermediates. NK cells, or large granular lymphocytes (LGL), which are defined by their ability to spontaneously lyse susceptible target cells, are able to recognise target cells by an, as yet, unidentified receptor (not the T cell receptor) and thereafter cause target cell lysis by a mechanism which appears to be similar to that of cytotoxic T cell mediated lysis.

Little is known about the precise role, if any, which these two nonspecific cell types play in allograft rejection. Attempts have been made to specifically deplete experimental animals of macrophages or NK cells with a view to investigating this in adoptive transfer experiments. However, both of these cell types are relatively radioresistant, and although alternative approaches have been investigated, these have met with little success. For example, MacPherson & Christmas (1984) found that the intravenous administration of a

potent antimacrophage serum to rats failed to significantly reduce the numbers of circulating macrophages and did not prevent macrophages from accumulating in cardiac allografts in these animals.

Although it is difficult to assess the individual roles of macrophages and NK cells in allograft rejection, they appear unlikely to have a significant role in the absence of T cells. Neither congenitally athymic animals nor ATXBM animals are able to reject an allograft despite the presence of large numbers of functionally active macrophages and NK cells and therefore, by definition, these cell types must play a secondary role if they contribute to graft rejection. This would seem most likely to be a DTH response although it should not be overlooked that both activated macrophages and NK cells possess Fc receptors and may therefore be capable of causing specific target cell damage by an ADCC mechanism.

1.7.5 Specific cytotoxic T cells

Cytotoxic T lymphocytes mediate their destructive effects on target cells by direct cell contact via specific MHC surface antigens (Cerottini & Brunner, 1974), resulting in specific cytolysis of the target cell (Berke, 1983) without a requirement for either lymphokine release or recruitment of nonspecific cells. Cytotoxic T cells (of the CD8 phenotype) are class I restricted (Bevan, 1975) and cannot be generated against cells with

isolated class II disparities in mixed lymphocyte reactions (MLRs). However, their proliferation is markedly enhanced in fully MHC incompatible MLRs by the addition of T helper cells, which, in response to the class II incompatibilities, provide help for the differentiation of T cytotoxic precursors (Bach et al, 1976).

Target cell lysis by cytotoxic T cells is a divalent cation-dependent process requiring intimate cell-cell contact, mediated through T cell receptor interaction with specific ligands on the target cell, or nonspecifically by adhesion mediated through lectins or mitogenic antibodies such as those directed against the CD3 and CD2 molecules. Following a successful Mg^{++} dependent adhesion phase, the Ca^{++} dependent cytolytic phase is then triggered, involving the release of cytoplasmic granule contents at the junction of the two cells. Pore forming proteins, or perforins, released from the cytotoxic cell granules bind to the target cell membrane, polymerise in the presence of Ca^{++}, and form lesions in a manner similar to that of complement mediated cell lysis, while the effector cell remains unharmed and able to move on to fresh targets (Podack, 1985; Pasternack, Verret, Liu et al, 1986). Death of the target cell soon follows, observed initially by the loss of ions and small molecules from the damaged cell membrane, but followed rapidly by extensive breakdown of

the nuclear DNA into numerous 200-base pair fragments. A recent report (Ucker, 1987) has suggested that this DNA fragmentation occurs as a result of the triggering of a glucocorticoid-mediated "self destruct" process, rather than as a direct result of membrane damage. Ucker was able to demonstrate resistance to both synthetic glucocorticoid mediated and to CTL mediated DNA fragmentation in a mouse thymoma mutant in spite of demonstrably normal CTL:target cell contact. A single spontaneous reversion event occurring in cells derived from the subcloned mutant rendered those cells subsequently equally susceptible to both glucocorticoid and CTL mediated killing, suggesting that the cell played a part in its own destruction, rather than being entirely at the mercy of the action of perforins. This raises the question of whether the pore forming proteins are essential in specific CTL mediated killing, or whether their role is more in the nonspecific killing mediated by NK cells and in ADCC mechanisms.

1.7.6 The roles of Tc and Th lymphocytes in graft rejection - early observations

The experimental evidence for DTH and Tc in contributing to graft rejection is considered below, although as will be seen in the light of current knowledge, it may be that the type of cellular effector response is largely determined by the nature of the MHC

subregion disparity between recipient and allograft. Thus, Th (CD4) lymphocytes predominantly recognise and respond to a class II MHC disparity while Tc (CD8) lymphocytes recognise and respond predominantly to a class I disparity.

In the early descriptive studies by Brent, Brown & Medawar (1958) the histological appearances of rejecting grafts were noted to resemble the features characteristic of a classical DTH response. The large number of graft infiltrating lymphocytes, macrophages and nonspecific inflammatory cells led them to suggest therefore that DTH might be the major effector mechanism in acute graft rejection.

The alternative view, that cytotoxic lymphocytes might be responsible for graft rejection, followed the first demonstration by Govaerts (1960) of lymphoid cells which showed cytotoxic activity. During experiments on the mechanism of kidney allograft rejection in dogs, he noted that TDLs from animals which had previously rejected an allograft had the ability to specifically lyse donor kidney epithelial cells in vitro. These observations were soon confirmed and extended by numerous other studies such as that of Cerottini's in 1976, in which he demonstrated the presence of lymphocytes with allospecific cytotoxic activity in spleens from mice which had rejected allogeneic cells (Cerottini, Engers, Fitch et al, 1976). The view became widely held that

graft rejection was due to cytotoxic T lymphocytes rather than DTH. An elegant experiment by Mintz and Silvers (1970) provided convincing evidence in favour of a specific cytotoxic T cell mechanism in graft rejection. They grafted skin from allophenic mice, which had been created by fusing blastomeres of two different H-2 strains, to recipients of one of the parental strains. It was found that whereas the melanocytes and hair follicles of the foreign parental strain were destroyed, the immediately adjacent cells of the graft which were syngeneic with the recipient remained undamaged. This convincing demonstration of the exquisite specificity of the graft rejection response in the complete absence of bystander damage strongly supported a role for cytotoxic T cells rather than DTH in graft rejection.

1.7.7 Ability of adoptively transferred lymphocyte subpopulations to mediate graft rejection in immunocompromised recipients

The view that specific cytotoxic T cells played an essential role in graft rejection was strongly challenged by the adoptive transfer experiments of Loveland & McKenzie (Loveland, Hogarth, Ceredig et al, 1981). They were able to show that the ability of ATXBM mice to reject a skin allograft was restored by the injection of specifically sensitised Lyt1+ lymphocytes and they interpreted their experiments as providing evidence of

DTH in mediating graft rejection. Similar experimental results were reported by Dallman, Mason & Webb (1982) when they showed that the ability of ATXBM rats to reject a skin graft was completely restored by thoracic duct lymphocytes that had been depleted of cells bearing the cytotoxic precursor phenotype (MRC OX8+). However, unlike the former study, Dallman emphasised that this did not exclude a role for specific cytotoxic cells in graft rejection. On the contrary, immunohistological analysis of rejecting grafts in ATXBM rats reconstituted with W3/25+ T helper cells alone still contained many MRC OX8+ (CD8) T lymphocytes, suggesting that the ATXBM animal contained cytotoxic precursors which were activated in the presence of W3/25+ (CD4) cells. In subsequent experiments, Dallman administered a further dose of irradiation to ATXBM animals after they had been skin grafted but before reconstitution. Using this more stringent experimental protocol, the adoptive transfer of CD4 cells was found to restore rejection in the absence of demonstrable MRC OX8+ cells within the skin graft. Nevertheless, the criticisms of the conclusions drawn by Loveland & McKenzie remain, since it cannot be excluded that ATXBM animals possess some cytotoxic precursor cells.

Following these experiments on the ability of Th cells to mediate skin allograft rejection in ATXBM mice and rats, reports appeared on the ability of adoptively

transferred lymphocyte subpopulations to mediate rejection of vascularised organ (almost exclusively heart) allografts in the rat. Because of the criticisms of the ATXBM model, these later reports were based on an acute irradiation model in which rats received a sublethal dose of whole body irradiation immediately prior to organ grafting and lymphocyte reconstitution. Using this model, Lowry and colleagues demonstrated that the transfer of specifically sensitised CD4 lymphocytes was both necessary and sufficient to restore rapid graft rejection (Lowry, Gurley & Forbes, 1983; Lowry & Gurley, 1983; Gurley, Lowry & Forbes, 1983). Furthermore, they showed the apparent absence of specific cytotoxic activity by the splenocytes of irradiated allograft recipients reconstituted with CD4 cells alone. In contrast, they found that reconstitution of graft recipients with purified, specifically sensitised CD8 cells could restore rejection, and resulted in the presence of splenocytes which could mediate specific cytotoxic activity when tested in vitro. A complicating factor in Lowry's experimental protocol was that the animal from which the donor organ was obtained was also irradiated (presumably to prevent the possibility of GVH) and there is evidence that such treatment may affect the immunogenicity of the donor organ (Leszczynski, Renkonen & Hayry, 1985).

Using a similar acute irradiation model of rat cardiac transplantation, Hall, de Saxe & Dorsch (1983) showed that the adoptive transfer of unsensitised CD4 cells alone was also able to restore graft rejection, and they concluded that helper T cells play a pivotal role in allograft rejection. However, since the reconstituted animals were shown to contain cells of the CD8 phenotype, they were careful to qualify their conclusions by stating that the main action of the T helper cells in this model was the augmentation of a cytotoxic response by the radioresistant or rapidly regenerating cytotoxic cells derived from the irradiated host.

The adoptive transfer experiments in both ATXBM and acutely irradiated allograft recipients are therefore in general agreement since the transfer of CD4 T cells alone is sufficient to initiate the rejection of a fully MHC disparate graft. However, there remains doubt as to whether or not cytotoxic effector mechanisms contribute significantly to graft destruction in these animals. Cytotoxic effector cells may derive from residual CD8 cytotoxic precursors in the host and/or result from the presence of class II restricted CD4 cytotoxic cells derived from the reconstituting inoculum.

The ability of adoptively transferred lymphocyte subsets to mediate the rejection of allografts differing from the recipient at isolated class I or class II MHC antigens has also been addressed. Lowry et al (1983)

demonstrated that the adoptive transfer of CD4 cells to acutely irradiated rats bearing a cardiac allograft differing only at the class II locus was sufficient to cause rejection. Unexpectedly, they also demonstrated the presence of donor directed CD8 cytotoxic T cells in the irradiated animals. In fact, it has now become apparent that the donor and recipient strains used, WF(RT1.A^uB^u) and a recombinant, RF(RT1.A^uB^l) inherit their class I and background genes from separate inbred lines, and although their RT1.A loci appear on serological typing to be identical, their RT1.C and RT1.E loci (also class I) have not been fully immunochemically classified and they have sufficient differences to elicit a class I restricted cytotoxic response. For this reason, the results obtained are inconclusive.

Using a similar adoptive transfer model, Lowry et al (1985) went on to show that CD8 cells were important for the rejection of class I disparate cardiac allografts. They and others had previously demonstrated that PVG recipient rats were unable to reject heart allografts from class I-disparate PVGr1 (recombinant) donors (Lowry & Blackburn, 1984) and they attributed this lack of response to the apparently low responder status of the recipient towards the donor haplotype (Rozing, Bonthius, Joling et al, 1983). Instead, when F1 hybrids were created between the high responder WF strain and the PVG

recombinants, heart allografts performed between the two F1 strains bearing a single class I disparity were rapidly rejected, and furthermore, the presence of adoptively transferred sensitised CD8 cells in the inoculum was essential to restore the rejection response to sublethally irradiated recipients.

Finally, an important study on the effects of single class I MHC incompatabilities on skin graft rejection was published by Rosenberg et al (Rosenberg, Mizuochi & Singer, 1986). They made use of a range of Kbm mutant mice which differed from the wild type B6 mice only at an isolated class I antigen locus. The mutants all had biochemically distinct antigenic differences of the Kb molecule and their skin, when grafted onto B6 recipients, was rejected at different rates. The rate of rejection was found to correlate closely with the number of Kbm-reactive, Lyt2+, IL2-secreting T cell precursors elicited in vitro in mixed Kbm x B6 lymphocyte cultures; in other words, the more Kbm reactive T cell precursors generated, the faster the rejection of the particular Kbm skin graft. The apparently necessary role for Lyt2+ cells in the rejection of class I incompatible grafts was supported by adoptive transfer experiments in which B6 nude mice were grafted with bm1 mutant skin, and reconstituted with B6 lymphocyte subsets. It was found that isolated Lyt2+ T cells were both necessary and

sufficient to reject bm1 skin grafts, thus supporting a role for cytotoxic T cells in skin graft rejection of class I incompatible grafts.

1.7.8 Effect of *in vivo* depletion of lymphocytes on allograft rejection

Another approach to the investigation of the role of lymphocyte subsets in allograft rejection has been the *in vivo* administration of anti-T cell monoclonal antibodies to selectively deplete allograft recipients of T cell subsets and thereby gain information on the requirement of the depleted subset for the rejection process. Using this technique, Cobbold et al showed that the depletion of either Lyt2+ (CD8) or L3T4+ (CD4) T cells from mice impaired their ability to reject a skin allograft (Cobbold, Jayasuriya, Nash et al, 1984). Whereas treatment with anti-Thy1 + anti-Lyt1 antibodies resulted in long term survival of allogeneic skin grafts, treatment with anti-L3T4 resulted in moderate prolongation of graft survival. In contrast, treatment with anti-Lyt2 antibody did not significantly prolong graft survival. In further experiments (Cobbold, Martin, Qin et al, 1986), it was shown that the delayed graft rejection seen in CD4-depleted animals was due to CD8 cells, indicating the ability of this cell type alone to cause skin graft rejection, albeit in a delayed fashion.

A similar experimental model was also used by Wheelahan & McKenzie (1987). They recently reported a detailed study of the requirements for CD4 and CD8 T cell subsets in the rejection response towards mouse skin grafts which were incompatible with the recipient at class I, class II or non-H-2 loci. They demonstrated that *in vivo* treatment of mice with a combination of anti-CD8 and anti-CD4 antibodies prevented them from rejecting a class II incompatible skin graft within 80 days, although class I incompatible grafts were rejected by 50 days, which was the same time taken for rejection of class I incompatible grafts in mice depleted of CD8 cells. To further summarise their findings, depletion of CD8 cells alone resulted in prolonged survival of class I incompatible grafts (which were not prolonged by depletion of CD4 cells), and slight prolongation of class II incompatible grafts. Depletion of CD4 T cells prolonged the survival of class II incompatible grafts, whereas survival of fully MHC incompatible grafts was not prolonged by any of the monoclonal antibodies used.

Overall, the results of these experiments suggest that CD8 cytotoxic cells alone are sufficient to cause rejection of grafts with an isolated class I disparity.

1.7.9 Demonstration that cloned cytotoxic cells can mediate *in vivo* allospecific damage

The development of techniques for cloning cytotoxic T cells has allowed the direct investigation of their ability to cause allospecific tissue damage. In an elegant and important study, Tyler et al (1984) clearly showed that cloned CD8 cells were capable of producing local tissue necrosis when injected into the skin of mice. In these experiments, a CD8 CTL clone was raised which was specifically active against the non-H-2 Epa-1 epidermal cell antigen presented on an H-2K^k MHC background. These cytotoxic cells, when injected intradermally into H-2K^k mice, were able to cause local tissue damage and necrosis. Although the lesion was associated with an inflammatory infiltrate of host mononuclear cells and macrophages, the tissue damage was not dependent on the participation of host derived lymphocytes since it still occurred in irradiated hosts. These findings suggested a paradox whereby CD8 cells were able to cause direct tissue damage, presumably by a cytolytic T cell mechanism, but were also able to elicit a local inflammatory response reminiscent of the DTH reaction in the absence of T helper cells of either donor or host origin. Interestingly, Tyler's CTL clone had a requirement for exogenous IL2 to maintain its viability in *in vitro* experiments but not for its *in vivo* ability to lyse mouse epidermal cells.

The results of Tyler's experiments were consistent with the earlier work of Engers et al who had demonstrated that the intravenous injection of cloned CD8, H-2^d restricted CTL into mice bearing H-2^d tumour cells in their peritoneal cavity was able to mediate destruction of the tumour cells (Engers, Glasebrook & Sorenson, 1982). Unlike the CTL clone used by Tyler, that employed by Engers was capable of producing its own IL2.

Together, these two reports clearly demonstrate the ability of cytotoxic T cells alone to cause alloreactive tissue damage in an MHC class I restricted fashion, although they do not, of course, provide direct evidence that this mechanism operates in the rejection of an organ allograft.

1.7.10 Comparison of cellular effectors in rejecting and nonrejecting allografts

A further approach to the investigation of the cellular mechanisms of graft rejection has been the comparison of the cellular events occurring in rejecting grafts from unmodified recipients with those seen in nonrejecting allografts in either immunosuppressed or enhanced recipients. Rat renal allografts undergoing unmodified graft rejection are rapidly infiltrated by a heterogeneous population of mononuclear cells, including T lymphocytes of both the CD4 and CD8 phenotypes, B

cells, NK cells and macrophages (Hayry, von Willebrand & Soots, 1979; Bradley, Mason & Morris, 1985). Mason & Morris (1984) showed that infiltrating leukocytes harvested from rejecting rat renal allografts displayed both specific and nonspecific cytotoxic activity when tested in vitro. In contrast, cells obtained from nonrejecting grafts in cyclosporine treated animals showed minimal specific cytotoxicity but levels of nonspecific cytotoxicity similar to those in rejecting allografts. Similar results were obtained in rejecting rat renal allografts and nonrejecting allografts in passively enhanced recipients (Bradley et al, 1985). In these experiments, the correlation of graft rejection with the presence of cytotoxic T cells in the graft is consistent with, although not proof of, cytotoxic cells playing an important role in allograft rejection. Nevertheless, the presence of cytotoxic T cells in an allograft does not necessarily imply acute rejection, as demonstrated by recent reports of the presence of moderate levels of specific cytotoxic T cell activity in actively enhanced rat renal allografts (Dallman, Wood & Morris, 1987).

1.7.11 Rejection of mH incompatible grafts

The cellular requirements for the rejection of MHC identical but mH incompatible grafts have been less extensively studied, but it appears that a DTH mechanism predominates over a cytotoxic T cell mediated mechanism.

There is a requirement for in vivo immunisation of the responder strain lymphocyte donor prior to their incorporation in mixed lymphocyte cultures for the purpose of raising mH antigen specific cytotoxic T cells. This probably reflects not only the relatively low precursor frequency of T cells capable of recognising minor H, compared with MHC, antigens, but also the relative lack of importance of a Tc effector mechanism in the rejection of an mH incompatible graft. Indeed, it appears from experimental evidence that the generation of mH specific CTL and the rejection of mH incompatible grafts are under the control of separate Ir genes in mice, since mouse strains exist that can reject H-Y incompatible skin grafts in the absence of H-Y specific CTL, and conversely, that are able to generate H-Y specific CTL but are unable to reject H-Y incompatible skin grafts (Hurme, Chandler, Hetherington et al, 1978). However, it is difficult to entirely exclude a role for CTL, since it has been described convincingly that while rejection of mH incompatible skin by irradiated rats was restored by the transfer of primed T helper cells alone,

the addition of either primed or unprimed Tc precursors to the inoculum dramatically reduced the rejection time (Mason, Dallman, Arthur et al, 1984).

1.7.12 Summary and current views on effector mechanisms of allograft rejection

From the foregoing account, it is apparent that there is strong evidence, from different types of experiments, that T lymphocytes of both the Th (CD4) and Tc (CD8) phenotypes may play a role in the graft rejection process. Although the data arising from adoptive transfer experiments appear to support a role for DTH in graft rejection, the possibility of a contribution from host cytotoxic cells, particularly in the ATXBM animals, must be considered. Furthermore, as pointed out by Mason et al (1984), it is possible that transferred CD4 T cells may interact with class II positive cells in the allograft (e.g. interstitial dendritic cells) resulting in lymphokine production and thereby induction of class II MHC antigens on vascular endothelium and other target cells of the graft. These class II positive cells may then be susceptible to class II restricted cytotoxic T cells of the T helper (CD4) phenotype. Caution is therefore required in equating function with phenotype of T cells, and consideration must also be given to the concept that the lymphocyte surface antigens may not necessarily be stable markers.

If both cytotoxic T cells and DTH mechanisms contribute to graft rejection, the balance between these may well depend on the genetically determined responder status of the recipient with respect to the donor strain, and its effect on the degree of dominance of the class I or class II restricted response. Therefore it is important to take into account the donor and recipient strain combination in the interpretation of data on mechanisms of graft rejection.

A further important factor determining the mechanism of rejection may be the nature of the allograft. There may well be crucial differences between the predominant mechanism of skin allograft rejection and that responsible for rejection of a vascularised graft, as well as differences between the different types of vascularised grafts. Of particular relevance to the present work is the fact that in only one reported adoptive transfer study was a renal allograft model used.

Lastly, although T cells play an essential role in allograft rejection, it may be that the possible contribution of antibody to acute graft rejection has not received sufficient attention. Alloantibody production by B cells is a T cell dependent process and antibody might contribute to graft rejection by ADCC involving NK cells or macrophages, both of which are found in abundance in rejecting allografts.

1.8 Aims

The overall aim of this thesis was to examine the cellular effector mechanisms of renal allograft rejection in the rat. This was approached initially by observing the cellular events occurring in rat renal allografts undergoing unmodified rejection. Subsequent experiments were performed on rats which had been rendered lymphocyte deficient, either by irradiation or by inheritance (congenitally athymic animals), such that they were unable to reject an allogeneic kidney. The ability of T lymphocytes to cause renal allograft damage was investigated by adoptively transferring T cell subpopulations, prepared by negative selection, to lymphocyte depleted allograft recipients. In addition, this thesis studied the cellular mechanisms by which the subcapsular injection of T lymphocyte subpopulations was able to mediate a local renal graft versus host reaction in F1 hosts.

It was anticipated that the results of such experiments might provide a rational basis for the development of strategies to prevent graft rejection.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Animals

Inbred male rats of the following strains were obtained from Harlan Olac Ltd.: PVG(RT1c), DA(RT1a), LEW(RT1l), AO(RT1u), PVGr1(RT1.Aav1,Bc,Cc,Dc); F1 hybrid rats of (LEWxDA), (PVGxDA), and (PVGr1xPVG) strains; and PVG-rnu/rnu(RT1c). The rnu gene was backcrossed from an outbred population onto the inbred PVG line in 1977, which has since under gone 12 backcrosses and 12 intercrosses altogether, and is now firmly established (A.G.Peters, Harlan Olac Ltd., personal communication).

PVGr19(RT1.Aav1,Bav1,Cc,Dav1) strain rats were kindly provided by Dr Geoff Butcher (AFRC Institute of Animal Physiology, Babraham, Cambridge). F1 hybrids of (PVGr1xPVGr19) strains were bred in the animal facility at the University Department of Surgery, Western Infirmary, Glasgow. All animals were housed in the animal facility and maintained on standard rat diet and water. PVG-rnu/rnu rats and irradiated rats were housed in sterile cages within laminar flow isolators, and were provided with sterile water and bedding. Irradiated animals received oxytetracycline hydrochloride (5 g/L) in their drinking water.

2.2 Irradiation

Whole body gamma irradiation was administered from a ^{60}Cobalt source at a rate of 1-1.2 Gy per minute. Animals were irradiated at a distance of one metre to the mid-plane of the body and the irradiation time, which was electronically controlled, was calculated with a program correcting for isotope decay. During irradiation the rats, which were not anaesthetised, were restrained in a plastic container.

2.3 Surgical procedures

2.3.1 Renal transplantation

Kidney donor and recipient rats were anaesthetised with 5% halothane in oxygen, or with ether, until they lost consciousness, and were then given an intraperitoneal injection of 0.7-1.2 mls of 7.5% chloral hydrate in saline. The precise dose of chloral hydrate depended on the rat strain used since strains differed in their susceptibility. Anaesthetised animals were shaved and swabbed with chlorhexidine in 70% alcohol. Renal transplantation was performed essentially as described by Fabre & Morris (1975).

The donor rat was opened with a midline abdominal incision, the intestines were displaced and covered with a gauze swab moistened with sterile saline, and the left kidney, renal vessels and ureter were mobilised. This procedure was repeated in the recipient rat, and after placing occlusive clips across the renal artery and vein, the renal vessels and ureter were divided and the kidney removed. Next, the donor rat was heparinised (100 units per rat), the donor kidney was excised and immediately transferred to a Petri dish containing ice-cold saline. The excised kidney was flushed of excess blood by applying gentle external pressure.

The donor kidney was then transplanted into the left orthotopic site of the recipient rat with end-to-end anastomosis of the renal artery and renal vein using a 10/0 interrupted suture (Ethicon Ltd.). The vascular clips were removed, thereby re-establishing the blood flow to the transplanted kidney. The donor ureter was then anastomosed end-to-end to the recipient ureter with a 10/0 interrupted suture. Ischaemia times of the kidney during transplantation were approximately 25 min (maximum 35 min) and during the ischaemic period, the graft was cooled by frequent application of cold saline to minimise ischaemic damage. After completion of the anastomosis, the intestines were replaced, and the abdomen was closed in two layers with a continuous 3/0 cat gut suture (Ethicon Ltd.).

2.3.2 Contralateral nephrectomy

Depending on the experiment, contralateral nephrectomy was performed either at the time of transplantation (immediate nephrectomy) or seven days later (delayed nephrectomy). Immediate nephrectomy was performed by mobilising the right kidney and placing a single 3/0 silk suture (Ethicon Ltd.) around the right renal artery, renal vein and ureter, then excising the kidney. Delayed nephrectomy was performed in a similar fashion, but usually through an incision in the right flank.

2.3.3 Thymectomy

The thymus was removed from young adult rats through a cervical incision as described by Rolstad & Ford (1974).

2.3.4 Thoracic duct cannulation

The thoracic duct of adult rats was cannulated by the method described by Ford (1978). Animals were then placed in restraining cages and received an intravenous infusion of sterile Dulbecco's A & B medium (DAB, Oxoid Ltd.) containing one unit per ml heparin, via the tail vein.

2.3.5 Skin grafting (for sensitisation)

Rats received two sequential full thickness skin grafts to the dorsal side, two weeks apart, as described by Roser & Ford (1972). Sensitised rats were used in experiments 1-2 months after rejection of the second skin graft.

2.4 Cells and tissues

All procedures with cells were performed in DAB containing either 2% foetal calf serum (DAB/FCS) or in DAB containing 0.2% bovine serum albumin (DAB/BSA).

2.4.1 Lymph node cells

Cervical and mesenteric lymph nodes were removed from rats that had been sacrificed by cervical dislocation. The excised lymph nodes were pooled, cut with a scalpel blade and then pushed through a fine stainless steel mesh with a 5ml syringe plunger, in DAB/FCS or DAB/BSA. Lymph node cells were washed two or three times and aggregates of dead cells removed with a pasteur pipette.

2.4.2 Spleen cells

Spleens were removed from sacrificed rats, and teased apart with disposable plastic forceps in DAB/FCS or DAB/BSA. Cells were washed once and aggregated dead cells were removed. Erythrocytes were removed from the

cell pellet by hypotonic lysis (5mls distilled water followed by 5mls double strength saline). Splenocytes were washed three more times before use.

2.4.3 Sensitised lymphocytes

Specifically sensitised lymphocytes were prepared from the pooled mesenteric and cervical lymph nodes of animals that had rejected two sequential skin allografts (Hall, Dorsch & Roser, 1978).

2.4.4 Thoracic duct lymphocytes

Lymph was collected on ice from the thoracic duct cannula draining into a sterile flask containing 5 ml DAB plus 100 units heparin. Thoracic duct lymphocytes (TDL) were washed once in DAB/FCS or DAB/BSA before use.

2.4.5 Bone marrow cells

Bone marrow was collected from the femurs and tibia removed aseptically from sacrificed rats, by flushing through with Hanks balanced salt solution (HBSS, Gibco Ltd.) containing 10 u/ml heparin and 2% FCS. The bone marrow was aspirated several times through a 21 gauge needle to disperse the cells, then filtered through a loose cotton wool plug. Cells were washed once in HBSS/FCS and counted in a haemocytometer, before injecting intravenously into the recipient.

2.4.6 Foetal liver cells

Foeti were removed from pregnant rats sacrificed on days 17-19 of gestation to provide foetal liver cells, as described by Hunt & Fowler (1981). Foetal livers were dissected and placed in cold DAB/FCS. Livers were pushed through a fine stainless steel mesh, then the resulting cell suspension was washed twice in DAB/FCS and the aggregated dead cells were removed. Cells were counted in a haemocytometer then injected intravenously in a volume of 1ml into the recipient rat.

2.4.7 Cell lines

The rat myeloma line Y3 ($RT1^u$; Galfre, Milstein & Wright, 1979) and the mouse lymphoma line YAC-1 (Sjogren & Hellstrom, 1965), both of which are susceptible to NK cell mediated lysis, and the mouse mastocytoma P815 (Dunn & Potter, 1957) which is reported to be NK resistant but "activated NK-susceptible" were used as target cells in in vitro cytotoxicity assays. They were maintained by frequent subculture in tissue culture medium comprising RPMI 1640, 10% foetal calf serum, 2 mM glutamine, and 100 U/ml penicillin + 100 ug/ml streptomycin (all from Gibco Ltd.).

2.4.8 Concanavalin A-stimulated lymphoblasts

^{51}Chromium (^{51}Cr) labelled Concanavalin A (ConA)-stimulated lymphoblasts were used as target cells in lymphocyte mediated cytotoxicity assays.

Lymphocytes were prepared from rat spleens as described above, and adjusted to a concentration of 2-2.5 x 10^6 cells/ml in RPMI containing 10% FCS, glutamine, penicillin,streptomycin and 2 x 10^{-5}M 2-mercapto-ethanol (BDH Ltd.). 10 mls cell suspension were incubated in tissue culture flasks with 5 ug/ml Concanavalin A (Sigma Chemical Co.) for 72 hours at 37C in 5% CO2 in air. Lymphoblasts were washed once and counted before use.

2.5 Cell subpopulations

Lymphocyte subpopulations were prepared by negative selection using a modification of the indirect rosette depletion method described by Mason (1981). The purity of the subpopulations was checked by cell sorter analysis or by immunoperoxidase labelling of cytocentrifuged slide preparations.

2.5.1 Indirect rosette depletion

LNC were depleted of lymphocyte subpopulations according to their reactivity with the mouse anti-rat monoclonal antibodies MRC OX12, MRC OX8, W3/25 and MRC

OX22 (see 2.6 Antibodies). LNC were incubated with saturating concentrations of the appropriate antibodies for one hour, on ice. After washing the cells three times in DAB/FCS, they were mixed with sheep erythrocytes which had been coated with rabbit anti-mouse Ig (Dako Ltd.) using 0.1% chromic chloride. This resulted in rosette formation between the sheep cells and the antibody-coated lymphocytes. To improve the separation and purity of the subpopulations, large lymphocyte-erythrocyte aggregates were formed by mixing the cells with sheep erythrocytes coated with mouse IgG (Dako Ltd.). The rosette aggregates were then separated from the unlabelled lymphocytes by layering over a mixture of Ficoll (BDH Ltd.) and sodium metrizoate (Nyegaard Ltd.) with a specific gravity of 1.088, and centrifuging at 1200g for 30 min at room temperature (RT). The depleted lymphocyte subpopulation was harvested from the interface, and washed three times in DAB/FCS before use.

2.5.2 Analysis of lymphocyte subpopulations

The purity of the cells obtained was determined on every occasion and found to be contaminated with less than 0.5% unwanted cell types.

2.5.2.1 EPICS cell fluorescence analysis

Aliquots of 1-2 x 10^6 cells were incubated with relevant or control monoclonal antibodies for one hour on ice, then washed twice in DAB/BSA. They were then incubated for one hour at 4C with FITC-conjugated goat anti-mouse antibodies (Sigma Chemical Co.) containing 20% normal rat serum to prevent cross reaction of the antibody with cell surface bound rat Ig. After washing once in DAB/BSA, the purity of the cells was determined by counting 10,000 cells using an EPICS fluorescence activated cell analyser (Coulter Electronics Ltd.).

2.5.2.2 Immunoperoxidase analysis

Aliquots of 10^4 cells were centrifuged onto microscope slides in a Cytospin 2 cytocentrifuge (Shandon Ltd.). Cytopreps were fixed for 30 seconds at 4C in cold buffered formalin/acetone (Yam, Li & Crosby, 1971) then labelled for 45 minutes with the relevant or control monoclonal antibodies, in a humidified perspex staining chamber at room temperature. After washing three times in DAB, peroxidase-conjugated rabbit anti-mouse antibodies (Dako Ltd.) containing 10% normal rat serum were added to the slides, for 30 min at RT. Slides were washed again, then incubated with the peroxidase substrate, 0.6 mg/ml 3,3'-diaminobenzidine tetra-hydrochloride (Sigma Chemical Co.) plus 0.01% hydrogen peroxide, for 5 min. Slides were washed, counterstained

lightly with Harris's haematoxylin (BDH Ltd.), dried and mounted in DPX (BDH Ltd.), for microscopy. 400 cells per slide were counted, with the aid of a squared eyepiece graticule.

2.6 Antibodies

Monoclonal mouse anti-rat antibodies (MAbs) used for identifying rat leukocyte populations are described in Table 2.1. A range of mouse MAbs were used both to characterise and to quantify MHC antigen expression, and they are described in Table 2.2. MHC OX21 is a mouse MAb against human C3b inactivator (Hsiung, Barclay, Brandon et al, 1982) and was used as a negative control.

Secondary polyclonal antisera were used to detect the presence of the primary MAbs by a range of techniques, for which the antiserum had been appropriately conjugated. Peroxidase-conjugated rabbit anti-mouse Ig was purchased from Dako Ltd., (High Wycombe). FITC-conjugated goat anti-mouse Ig was obtained from Sigma Chemical Co (Dorset), and ^{125}I-conjugated sheep anti-mouse F(ab')2 was from Amersham International, Aylesbury.

Table 2.1 Monoclonal mouse antibodies detecting rat leukocytes

ANTIBODY	SPECIFICITY	REFERENCE
MRC OX1	Leukocyte common antigen	Sunderland et al (1979)
MRC OX8	CD8+ cytotoxic/suppressor T cells, majority of natural killer cells	Gilman et al (1982) Dallman et al (1982) Cantrell et al (1982)
W3/25	CD4+ helper T lymphocytes, some macrophages	Brideau et al (1980) Barclay et al (1981)
MRC OX19	Peripheral T lymphocytes	Dallman et al (1982)
MRC OX12	Ig kappa chain on B lymphocytes	Hunt & Fowler (1981)
MRC OX39	IL-2 receptor	Jefferies et al
W3/13	T cells, neutrophils & plasma cells	Williams et al (1977)
MRC OX22	B lymphocytes, MRC OX8+ and 67% of W3/25+ peripheral T cells, NK cells	Spickett et al (1983)
ED1	Macrophages, dendritic cells	Dijkstra et al (1985)

Table 2.2 Monoclonal mouse antibodies detecting rat MHC antigens

ANTIBODY	SPECIFICITY	REFERENCE
MRC OX18	MHC class I antigen	Fukumoto et al (1982)
MRC OX6	MHC class II I/A-like antigen	McMaster et al (1979)
MRC OX17	MHC class II I/E-like antigen	Fukumoto et al (1982)
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	Milton & Fabre (1985) Hart & Fabre (1981)

2.7 Histology

2.7.1 Preparation of cryostat sections

Tissue samples were embedded in OCT compound (Tissue-Tek, BDH Ltd), snap frozen in liquid nitrogen and 5μ cryostat sections were cut at -20C onto gelatinised multispot slides (C.A.Hendley, Essex).

2.7.2 Immunoperoxidase staining

Sections were fixed in acetone at room temperature for 10 min, then washed in DAB. Excess moisture was dried from around each section, then 50μl of primary monoclonal antibody, appropriately diluted, were applied to each section. Slides were incubated for 45 min at RT in a humidified chamber, then washed three times in DAB. The secondary antibody was applied, consisting of peroxidase-conjugated rabbit anti-mouse Ig containing 10% normal rat serum to absorb out any crossreacting activity. After 30 min incubation at RT, the slides were washed three times and the substrate was added, as for cytopreps (section 2.5.2.2). After counterstaining lightly in Harris's haematoxylin, the sections were dehydrated and cleared in alcohols and xylene, then mounted in DPX (BDH Ltd).

2.7.3 May-Grunwald and Giemsa staining

For morphological identification of different cell types, cytocentrifuge slide preparations were flooded with May-Grunwald solution (BDH Ltd.) for 3 min, washed with distilled water, flooded again with Giemsa (BDH Ltd.) diluted 1:30 for 12 min, washed again with distilled water, air dried and mounted in DPX.

2.7.4 Morphometric analysis of cellular infiltrate

The area of each immunoperoxidase-labelled tissue section infiltrated by leukocytes of a particular phenotype was determined by morphometric analysis using the point counting technique (Aherne & Dunnill, 1982; McWhinnie, Thompson, Taylor et al, 1986). Sections were examined at a magnification of 400 in the presence of a microscope eyepiece graticule engraved with a squared grid bearing 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:

$$\% \text{ area of infiltrate} = \frac{\text{no. +ve grid intersections}}{\text{total no. grid intersections}} \times 100$$

Most sections were counted with the 745 point graticule but if there was a very heavy infiltrate the section was counted with the 121 point graticule. 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 & Dunnill, 1982).

This technique has been evaluated in our own laboratory and has been shown to produce a reliable estimate of cellular infiltration in kidney allografts, since two independent observers counting different areas of the same section obtained results that correlated very closely (Armstrong, Bolton, McMillan et al, 1987).

2.8 ATXBM rats

Adult thymectomised, irradiated, bone marrow reconstituted (ATXBM) rats were investigated as potentially suitable kidney allograft recipients in adoptive transfer experiments. Adult rats were thymectomised and allowed to recover for 3-4 weeks before being lethally irradiated with 100 Gy. They then received an intravenous inoculum of approximately 10^7 bone marrow cells from a syngeneic donor that had undergone thoracic duct drainage for 48-72 hours to deplete mature T lymphocytes from the bone marrow (Howard & Scott, 1972). ATXBM rats were housed in an isolator

and received 5 g/l oxytetracycline hydrochloride in their drinking water, until they were used not less than two weeks later.

2.9 Cell mediated cytotoxicity

2.9.1 Preparation of effector cells

Spleen cells or graft infiltrating cells were used as effectors in cell mediated cytotoxicity assays. Spleen cells were prepared as described in section 2.4.2. On occasions, macrophages were removed by either plastic adherence (cells were incubated in plastic petri dishes in the absence of serum, at 37C for one hour, and the effector cells retrieved) or by carbonyl iron (Sigma) treatment (cells were incubated with 10 mg/ml carbonyl iron for one hour at 37C, then adherent macrophages and iron particles removed with a strong magnet). Splenocytes were resuspended in RPMI/5%FCS/20mM Hepes for use in the cytotoxicity assay.

Graft infiltrating cells were prepared by mincing the excised kidney allograft with a scalpel blade. In earlier experiments, effector cells were extracted by enzyme digestion with hyaluronidase and collagenase, as described by Bradley, Mason & Morris (1985). However, it was difficult to get consistently good cell viability using this technique and in subsequent experiments a Percoll separation method was employed as follows. The

kidney pieces were pushed through a fine stainless steel mesh, then washed once in RPMI/5%FCS and effector cells were separated by Percoll centrifugation (Sigma). 90% stock Percoll was prepared with calcium- and magnesium-free 10x HBSS plus 10mM Hepes buffer. This was further diluted with single strength HBSS/10mM Hepes to give an 80% and a 35% solution of the stock 90% Percoll. Graft infiltrating cells were resuspended in the 80% Percoll which was layered underneath 35% Percoll in a centifuge tube. Cells were centrifuged at 500g for 30 min at RT, during which time the erythrocytes and most macrophages and granulocytes pelleted, and dead cell debris formed a layer on top of the 35% Percoll. Mononuclear effector cells retrieved from the interface were washed once in RPMI/5%FCS/20mM Hepes and resuspended in the same medium, to be used in the 51Cr-release assay. Viability was always >90%, assessed by trypan blue exclusion.

2.9.2 ^{51}Chromium release assay

A standard ^{51}Cr-release assay based on the experiments by Brunner et al (1968) was used, and the method was essentially as described by Bradley et al (1985). Target cells in RPMI/FCS were labelled by incubating with 5 MBq ^{51}Cr-sodium chromate for 90 min at 37C with frequent mixing. Cells were washed five times then resuspended in medium. Effector cells were

prepared as described above, and serial twofold dilutions were made, to give effector:target cell ratios of 100:1, 50:1, 25:1, 12.5:1, 6:1 and 3:1.

Equal volumes (75μl) of effector and target cells were mixed in wells of 96 V-well microtitre plates (Sterilin, Teddington) so that each well contained $5x10^3$ or 10^4 target cells, and each cell combination was set up in triplicate or quadruplicate. Spontaneous release from targets was determined using medium instead of effector cells, and maximum release obtained by adding 10% Triton X (BDH) instead of effectors. The plates were centrifuged briefly then incubated for 6 hours at 37C in 5% CO2 in air. From each well, 75μl supernatant was harvested and the amount of released isotope determined using a Compugamma counter (LKB, London). The following formula was used to calculate effector cell mediated ^{51}Cr-release from the targets:

$$\% \text{ cytotoxicity} = \frac{\text{(experimental-spontaneous release)}}{\text{(maximum-spontaneous release)}} \times 100$$

2.10 Quantitative analysis of MHC antigens

The increased expression of MHC class I and class II antigens observed in immunoperoxidase stained cryostat seections of rejecting kidney allografts was measured quantitatively by preparing tissue homogenates of the rejecting grafts and performing MHC absorption analyses

on those tissues using antibodies that detected only donor strain MHC antigens and not MHC expressed on infiltrating host leukocytes.

2.10.1 Preparation of tissue homogenates

Kidney grafts were excised at various time points after transplantation, halved and frozen in liquid nitrogen, and crushed with a cooled pestle and mortar. Three or five half kidneys were pooled for each time point and stored at -70C until homogenates were prepared following the method of Milton & Fabre (1985). The pooled tissues were homogenised mechanically with a Teflon pestle at 4C in the presence of Tris-buffered saline containing 5mM EDTA, 2mM phenylmethylsulphonyl fluoride and 2.5mM iodoacetamide to inhibit proteolysis. The washed homogenates were frozen at -70C until use.

2.10.2 Quantitative analysis of donor class I MHC antigens

80µl of tripling dilutions of each kidney homogenate were incubated with 80µl of a predetermined dilution of donor-specific anti-class I antibody MN4-91-6 representing conditions of target antigen excess in the assay system. After incubation for one hour at 4C the supernatants were assayed for the presence of unbound antibody using an indirect radioactive binding assay (Morris & Williams, 1975). Duplicate 25µl samples of

supernatant were incubated with 25µl of DA erythrocytes at 10^9 cells/ml (class I target) for one hour at 4C, which were then washed twice and incubated with 100µl ^{125}I-labelled sheep anti-mouse F(ab')2 at saturating concentrations for one hour at 4C. The cells were then washed and the cell-bound radioactivity measured in a gamma counter.

2.10.3 Quantitative analysis of donor class II MHC antigens

The donor-specific anti-class II antibody F17-23-2 was unsuitable for absorption analysis because, although it worked well for immunoperoxidase labelling, its affinity did not appear to be sufficient to prevent high free antibody concentrations in the analysis. Instead, an assay using purified DA class II (I-A-like) MHC antigen as the specific target was developed and performed by Dr Sarah Spencer in the laboratory of Professor John Fabre, Blond McIndoe Centre, East Grinstead, Sussex. Briefly, tripling dilutions of kidney homogenates were incubated with a predetermined dilution of a polyspecific PVG anti-DA antisreum. After incubation for one hour at 4C, the supernatants were assayed for the presence of unbound anti-DA class II antibody using a radioactive binding assay. 25µl samples of supernatant were placed in 96-well PVC plates (Dynatech, Sussex) which had been previously coated with the purified DA class II antigen.

After incubation for one hour the wells were washed and incubated for a further hour with 50µl of ^{125}I-labelled F(ab')2 anti-mouse F(ab')2 which is highly crossreactive (>80%) with rat F(ab')2 and has been described elsewhere (Milton et al, 1986a). The wells were again washed, then cut out individually and the bound radioactivity measured in a gamma counter.

2.11 Induction of renal graft versus host reaction

A local graft versus host reaction (GVHR) was produced in F1 rats by injecting parental cells under the kidney capsule as described by Elkins (1964). Under ether anaesthesia, F1 rats were opened by a midline incision and the left kidney was exposed. An inoculum of parental lymph node cells in a volume of 0.1 mls DAB/FCS was then injected under the left kidney capsule with a 30 guage stainless steel needle. Seven days later, animals were sacrificed and both kidneys were removed and weighed. The ratio of the weights of the injected to the noninjected contralateral kidney (Ki/Kc) was calculated to provide a means of comparing different experimental groups (Elkins, 1966). In addition, injected kidneys were graded for the presence of a local GVHR by assessment of grossly observable lesions and by histological examination of haematoxylin and eosin stained paraffin sections.

2.12 Popliteal lymph node assay

The ability of graft infiltrating cells to mediate a regional GVHR was assessed by extracting the cells as described in section 2.9.1 and injecting them into the footpads of F1 rats, according to the method of Ford (1978). Three graded doses of graft infiltrating cells were compared with similar doses of splenocytes from the same rat for their ability to cause enlargement of the popliteal lymph nodes by day 7 after injection. The nodes were weighed to an accuracy of 0.1 mg and the log median response of triplicate assays was calculated for each dose level.

2.13 Radiation chimaeras

Chimaeric rats were produced by whole body irradiation and immediate reconstitution with haemopoietic tissue. The resulting chimaeras were used as kidney donors so that the grafts had bone marrow-derived passenger leukocytes that were syngeneic with the graft recipient but parenchymal cells and vascular endothelium that were allogeneic. Chimaeric rats were also used as recipients of injected parental cells in the investigations of the renal graft versus host reaction. F1 rats were irradiated with 100 Gy from a ^{60}Cobalt source of gamma

radiation and were reconstituted the same day with approximately 10^8 haemopoietic cells prepared from parental strain foetal livers as described in section 2.4.6 (Hunt & Fowler, 1981). The chimaeric status of all animals was tested on lymphocytes prepared from a single excised cervical lymph node. Lymphocytes were incubated with cytotoxic alloantisera (raised against each parental strain) and complement, and the subsequent viability of the treated cells was assessed by staining them with fluorescein diacetate and ethidium bromide, and counting the cells using fluorescence microscopy. In truly chimaeric animals, cells were lysed by alloantisera raised against the same parental strain as the foetal liver donor but not by antisera directed against the other parental strain. Only animals that were fully chimaeric by the above criteria were used in experiments.

2.14 Analysis of serum antibody titres

The titre of circulating antibodies directed against donor strain rats was determined from a sample of blood taken from the renal allograft recipient at different time points after transplantation. Serum samples were decomplemented at 56C for 30m, centrifuged briefly to remove protein aggregates, serially diluted in RPMI/5%FCS/20mM Hepes and mixed with ^{51}Cr-labelled splenocytes prepared from relevant and third party strain

rats. After incubating for 30m at RT, freshly reconstituted guinea pig complement (Sera-Lab, Sussex) diluted 1 in 5 was added, and the cells were incubated for one hour at 37C. The ^{51}Cr released into the supernatant was counted in a gamma counter, and titration curves of % ^{51}Cr release against serum dilution were constructed for each serum sample.

2.15 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 THREE

CELLULAR EVENTS OCCURRING IN RAT RENAL ALLOGRAFTS UNDERGOING UNMODIFIED REJECTION

3.1 Introduction

The cellular events which precede the destruction of an allograft have been extensively studied in a variety of animal models. A well recognised feature occurring in all types of allograft undergoing unmodified rejection is the rapid and progressive accumulation of a heterogeneous population of mononuclear cells within the graft (Hayry, von Willebrand, Parthenais et al, 1984). The functional repertoire of these cells, when harvested and tested in vitro, has typically included allospecific cytotoxicity against the donor which, as already described, gave rise to the notion that cytotoxic T cells may cause graft rejection. More recently, it has become apparent that cellular infiltration of rejecting allografts may be associated with an increase in the expression of class I and class II MHC antigens by the tissues within the graft - presumably as a consequence of lymphokines released from graft infiltrating cells (Fabre, Milton, Spencer et al, 1987). This phenomenon might be important since increased MHC expression may, in principle, contribute to graft rejection both by initiating the rejection response and by making the graft more susceptible to effector mechanisms.

This thesis is concerned with the rat renal allograft model and in this chapter a detailed analysis of the cellular events occurring during unmodified rat renal

allograft rejection was undertaken. Immunohistological observations were made to determine both the magnitude and phenotype of cellular infiltration of the graft and the pattern of associated induction of donor strain MHC antigen expression. A functional analysis of harvested graft infiltrating cells was carried out and, in addition, experiments were performed to assess the contribution of donor strain dendritic cells, as well as isolated MHC subregion disparities to graft rejection. The results of these experiments provided a comprehensive description of the cellular events occurring in this particular model and enabled the construction of a paradigm from which it was possible to interpret the results of experiments reported in subsequent chapters.

In the following experiments, fully allogeneic DA ($RT1^a$) strain rat kidneys were transplanted into the orthotopic site of unmodified PVG ($RT1^c$) recipients. Renal allografts in this strain combination consistently undergo rapid rejection. This was confirmed in 8 recipient animals which received a DA renal allograft and underwent simultaneous contralateral nephrectomy. Survival times for these animals were 7,7,7,7,7,8,8 and 10 days (median survival time [MST] 7 days) and blood urea levels were markedly elevated at day 5 (mean 44 mM/L; range 32-54 mM/L), increasing thereafter until

death. As expected, five PVG control animals receiving a syngeneic kidney transplant all survived indefinitely (>100 days) with a normal blood urea (<15 mM/L) throughout.

3.2 Magnitude and phenotype of leukocyte infiltration in rejecting renal allografts

3.2.1 Total leukocyte infiltration

Recipients of renal transplants were sacrificed on days 1, 3 and 5 following transplantation and serial cryostat sections were labelled with a range of mouse monoclonal antibodies against rat leukocyte membrane antigens, using the indirect immunoperoxidase staining technique. The area of each tissue section that was occupied by infiltrating leukocytes of a particular phenotype was assessed by morphometric analysis using a point counting technique with a microscope eyepiece graticule. Five allografts and three DA syngeneic grafts were examined at each time point.

The results of morphometric analysis of cryostat sections stained with MRC OX1 (which labels the leukocyte common antigen) are shown in Fig 3.1. DA allografts showed a rapidly progressive infiltration of the rejecting graft which was minimal at day 1 (<5%), rising to a mean percent area infiltrate of 11% (s.d.3%) at day

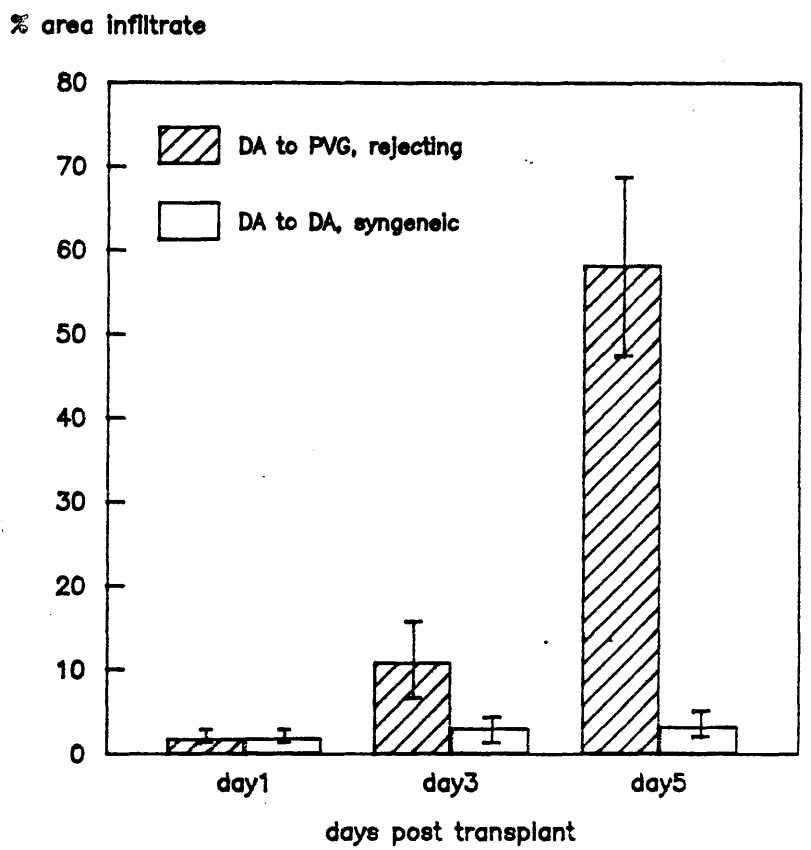


Fig. 3.1 Morphometric analysis of cellular infiltrate in DA renal allografts transplanted into unmodified PVG recipients, showing infiltration by leukocytes.
Grafts were removed on different days after transplantation, cryostat sections were stained by immunoperoxidase with MRC OX1 (against the leukocyte common antigen), and the % area infltrate of leukocytes was determined by morphometric analysis and compared with the infiltrate in DA kidneys transplanted to syngeneic hosts, removed on the same days. The results are expressed as the mean and standard deviation of five allografts and three syngeneic grafts examined at each time point.

3 and increased to the observed maximum of 58% (s.d.12%) by day 5. This infiltrate was initially focal in distribution, with distinct perivascular and periglomerular accumulations of cells, but as rejection progressed the infiltrate became heavier and more diffuse, showing an intertubular distribution. Grafts were excised on day 7 after transplantation but were found to be too severely damaged by rejection to allow immunohistological analysis.

In contrast to allografts, DA syngeneic grafts showed only a sparse cellular infiltrate which did not exceed 5% in any of the grafts examined. The cellular infiltrate in rejecting grafts was significantly greater than that of syngeneic grafts on days 3 and 5 after transplantation ($p < 0.05$, Mann-Whitney U test).

Normal non-transplanted DA kidneys were also stained and found to contain occasional and widely distributed MRC OX1 positive cells (<2% area infiltrate).

3.2.2 Infiltration by leukocyte subpopulations

The results of the phenotypic analysis of leukocyte subpopulations infiltrating rejecting renal allografts are shown in Fig 3.2. In day 3 allografts there was a predominance of W3/25 positive cells (Th and some marophages) over MRC OX8 positive cells (Tc and NK cells) ($p < 0.05$, Mann-Whitney U test). However, by day 5 the

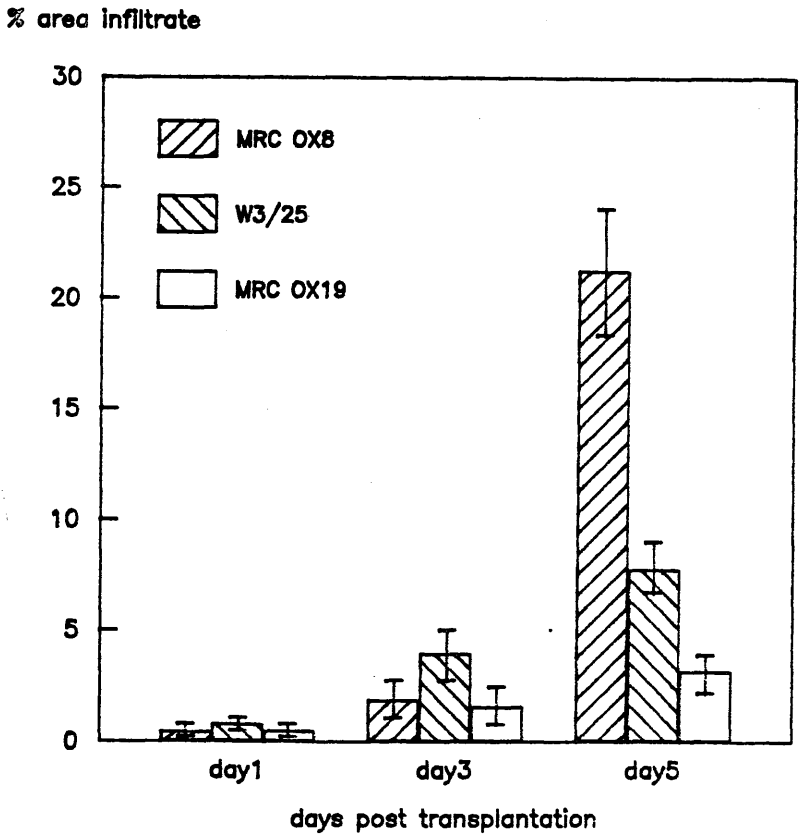


Fig. 3.2 Phenotypic analysis of the cellular infiltrate in DA renal allografts transplanted into unmodified PVG recipients, stained with MRC OX8, W3/25 and MRC OX19.
Cryostat sections (five grafts at each time point) were labelled with MAbs directed against lymphocyte subpopulations: MRC OX8 labels T cytotoxic/suppressor cells and NK cells; W3/25 labels T helper cells and some macrophages; MRC OX19 labels T cells. Results are expressed as the mean and standard deviation of the % area infiltrates.

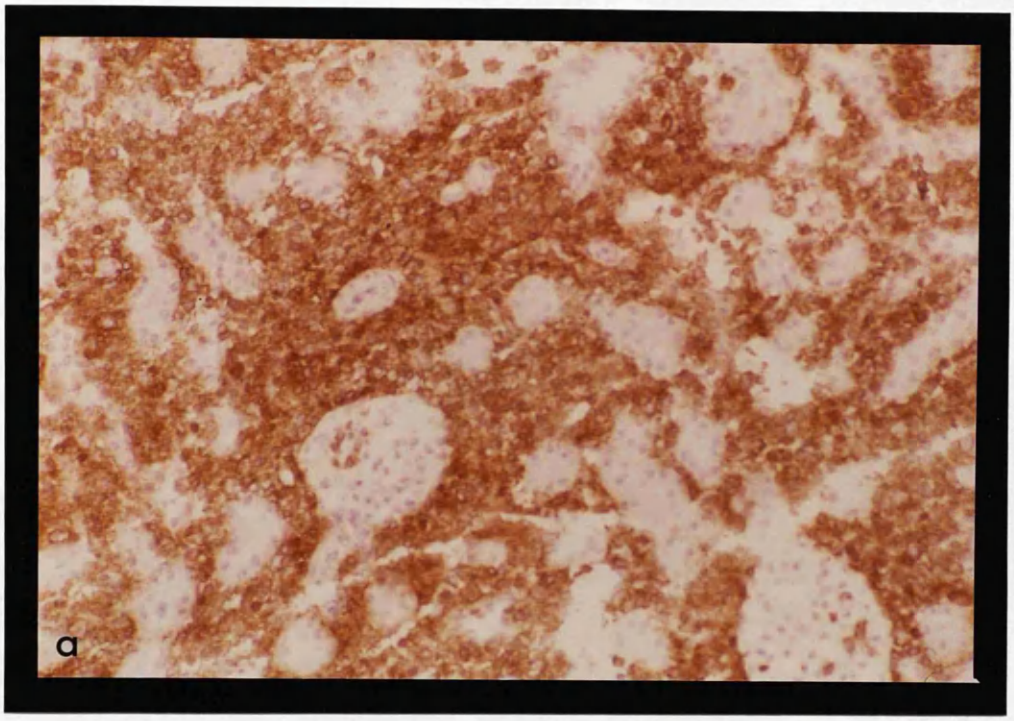
MRC OX8 positive infiltrate had increased markedly (mean % area infiltrate 21%, s.d.3%) and exceeded that labelled by W3/25 (8%, s.d.1%) ($p < 0.01$, Mann-Whitney U test).

Cryostat sections were also labelled with a mixture of MRC OX8 and W3/25 MAbs and the % area infiltrate was found to be very similar to that calculated as the sum of MRC OX8 and W3/25 positive cells for each section, indicating that few of the cells expressed detectable W3/25 and MRC OX8 antigens simultaneously.

Allografts showed a small but progressive infiltration by MRC OX19 positive cells (all T cells) but the % area infiltrate was surprisingly low (3% at day 5) and was far exceeded by the sum of values for MRC OX8 and W3/25 labelling. The low counts obtained with the MRC OX19 antibody may be due, in part, to the presence of weakly labelled cells which were not readily detected with the immunoperoxidase technique. Alternatively, and perhaps more likely, a large component of the cellular infiltrate at day 5 after transplantation may consist of secondarily recruited NK cells (MRC OX8 positive, MRC OX19 negative) and activated macrophages (W3/25 positive, MRC OX19 negative).

The typical patterns of leukocyte infiltration in rejecting and in syngeneic grafts may be seen in Figs 3.3 & 3.4, where there is clearly more cellular infiltration in the rejecting grafts.

Fig. 3.3 Pattern of cellular infiltration in rejecting DA renal allografts in PVG recipients (day 5).
Cryostat sections of the grafts were labelled, by immunoperoxidase, with mouse MAbs to rat leukocyte subpopulations:
a) labelled with MRC OX1 (leukocytes), x160.
b) labelled with MRC OX19 (T cells), x160.
c) labelled with MRC OX8 (Tc & NK cells), x160.
d) labelled with W3/25 (Th & macrophages), x160.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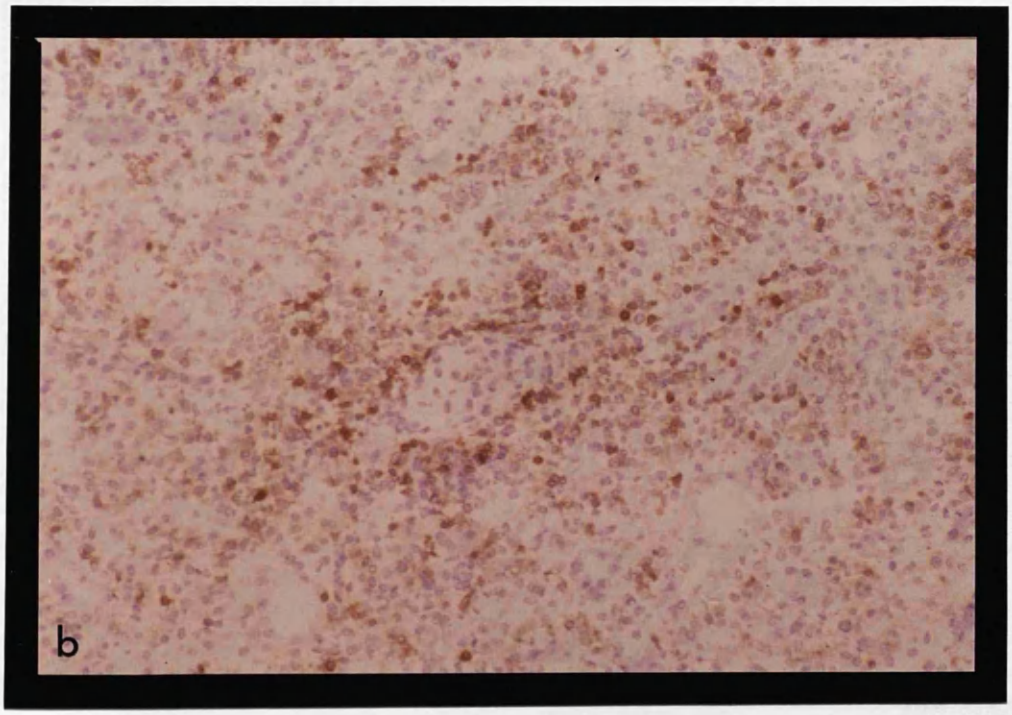
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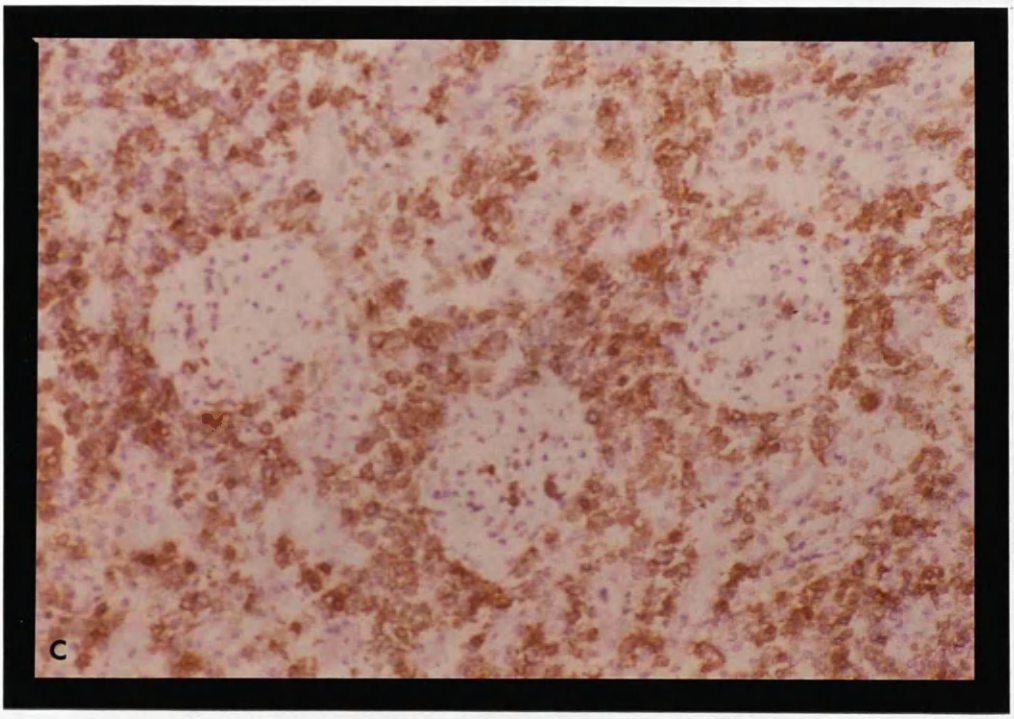
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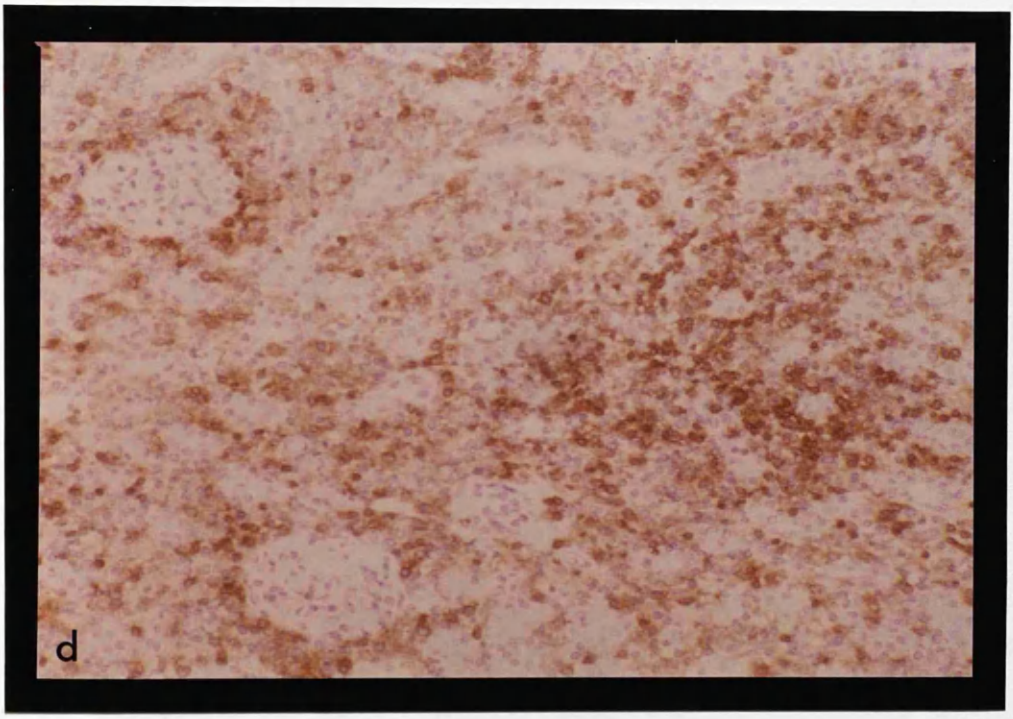
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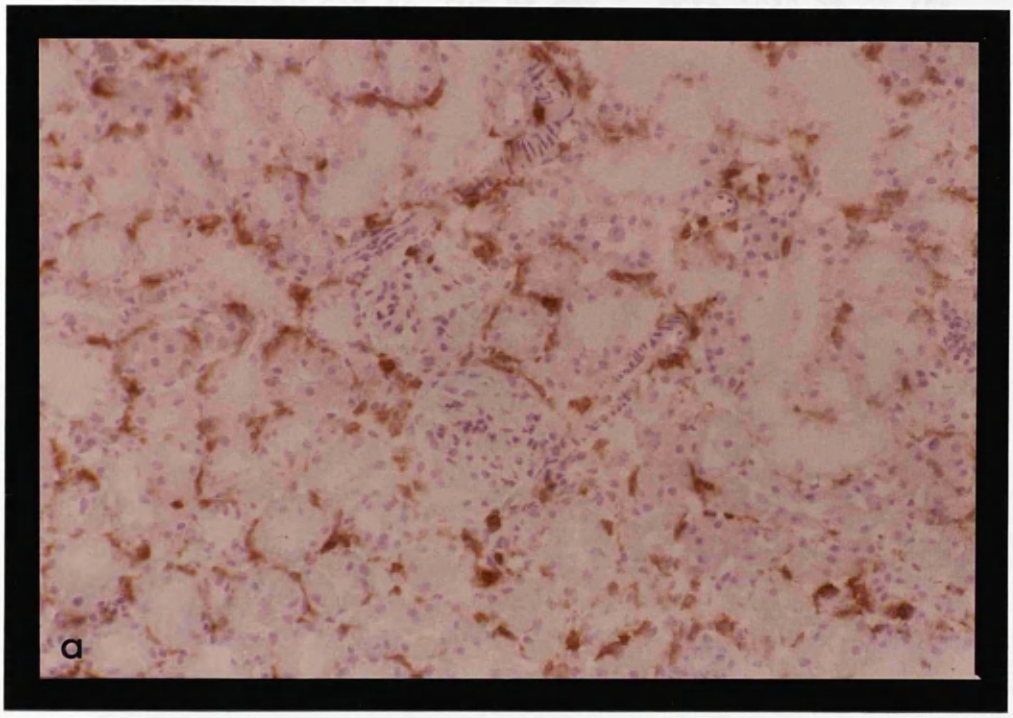
Fig. 3.4 Pattern of cellular infiltration in syngeneic, non-rejecting DA kidneys transplanted into DA recipients (day 5).
Cryostat sections of the grafts were labelled, by immunoperoxidase, with mouse MAbs to rat leukocyte subpopulations:
a) labelled with MRC OX1 (leukocytes), x160.
b) labelled with MRC OX19 (T cells), x160.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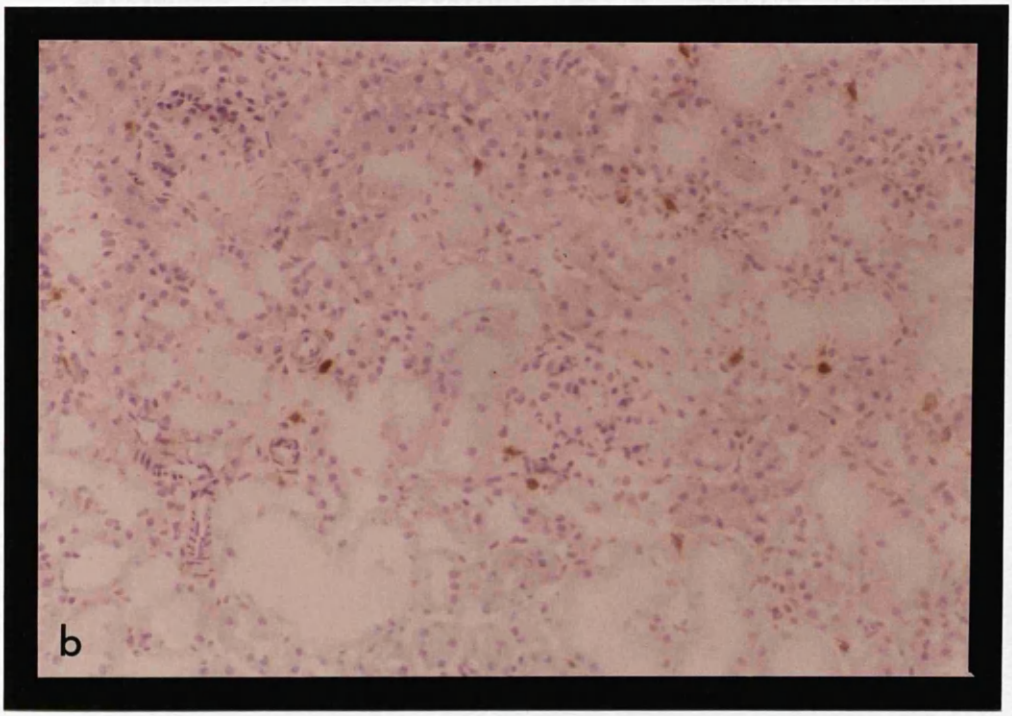
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Tissue sections labelled with an antibody to the interleukin 2 receptor, MRC OX39, showed less than 1% infiltration by day 3, but by day 5 more than 8% of the tissue was occupied by cells expressing this activation marker.

Sections were also labelled with MRC OX12 (anti-kappa light chain on B cells) in an attempt to enumerate graft infiltration by B lymphocytes. However, staining with this antibody revealed the presence of widespread tissue bound immunoglobulin throughout the graft which was presumably allospecific antibody, and which precluded analysis of the infiltrate.

In all immunohistology experiments, sections were stained with the irrelevant MAb, MRC OX21 (mouse anti-human C3b inactivator) as a negative control; positive labelling was never detected.

3.3 Functional cells in rejecting renal allograft recipients

PVG recipients of DA renal allografts were sacrificed on day 5 after transplantation and the cytotoxic repertoire of graft infiltrating cells and splenocytes was examined in <u>in vitro</u> ^{51}Cr-release assays.

In all *in vitro* cytotoxicity assays reported in this thesis, different strains of rat ConA blasts have been used as target cells. Although they may bear little resemblance to the actual target cell of the rejection response in allograft recipients, the important criterion for their use as targets *in vitro* is that they express either relevant or irrelevant MHC antigens. Cytocentrifuge slide preparations of ConA blasts were stained, using the immunoperoxidase technique, with monoclonal antibodies to rat class I and class II MHC antigens. It was found that in all strains investigated, all of the ConA blasts stained moderately strongly for class I antigens, while one third of the cells stained strongly for MHC class II antigens and the remainder were negative. The rat myeloma Y3 cells stained very weakly for class I antigens and not at all for class II antigens. Thus ConA blasts should be appropriate targets for demonstrating the presence of cytotoxicity directed at either, or both class I and class II alloantigens.

In these experiments, graft infiltrating cells were released from the kidney by enzyme digestion, and their capacity to lyse a range of target cells was compared with that of splenocytes from the same recipients. Yields of graft infiltrating cells were typically 5-10 x 10^7 cells per graft.

Both infiltrating cells and splenocytes from rejecting grafts demonstrated moderate levels of cytotoxic activity against ^{51}Cr-labelled donor strain ConA blasts, with a marked prozone effect seen in graft infiltrating effector cells (Fig 3.5). There was minimal activity against third party Lewis strain ConA blasts, indicating that lysis of donor strain targets was mediated by alloantigen specific cytotoxic cells rather than nonspecific effector cells.

Cytocentrifuge slide preparations of graft infiltrating cells stained with May-Grunwald/Giemsa revealed the presence of large amounts of cellular debris, and it was felt that this might, at higher effector : target ratios, have had an inhibitory effect on the lysis of donor specific targets, thus accounting for the prozone effect. In subsequent experiments, the use of enzymes was discontinued, and cell debris was removed by Percoll centrifugation.

Nonspecific activity of graft infiltrating cells and splenocytes was investigated using the rat myeloma Y3 as the NK susceptible target. Moderately high levels of lytic activity were found in both populations of effector cells (Fig 3.6). These experiments were performed on five occasions with similar results.

On two occasions, the effector cells were depleted of macrophages by incubating the cells with carbonyl iron powder and then using a strong magnet to remove adherent

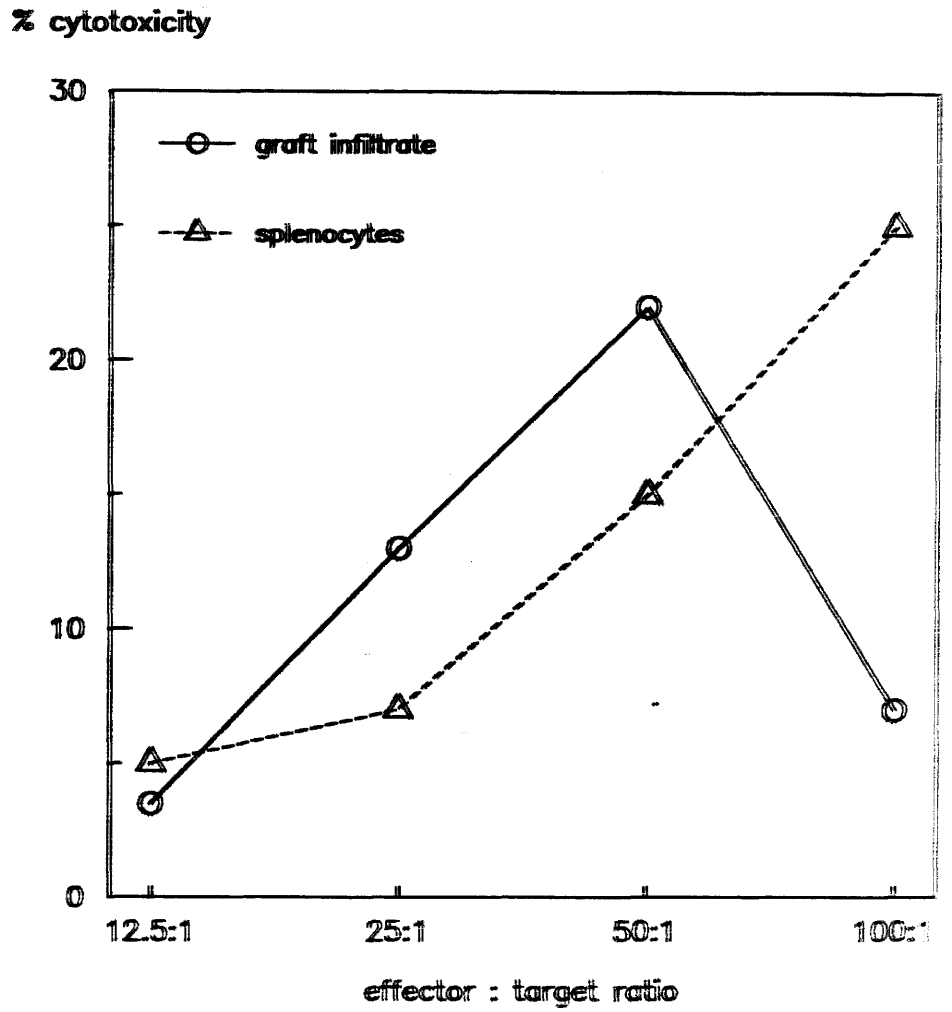


Fig. 3.5 Cytotoxic activity of graft infiltrating cells and splenocytes harvested on day 5 from rejecting DA renal allografts in PVG recipients, against donor specific DA ConA blasts.
Graft infiltrating cells were harvested by enzymic digestion of the graft. Splenocytes were macrophage depleted. Effector cells were tested in a 6-hour chromium release assay against DA ConA blasts.

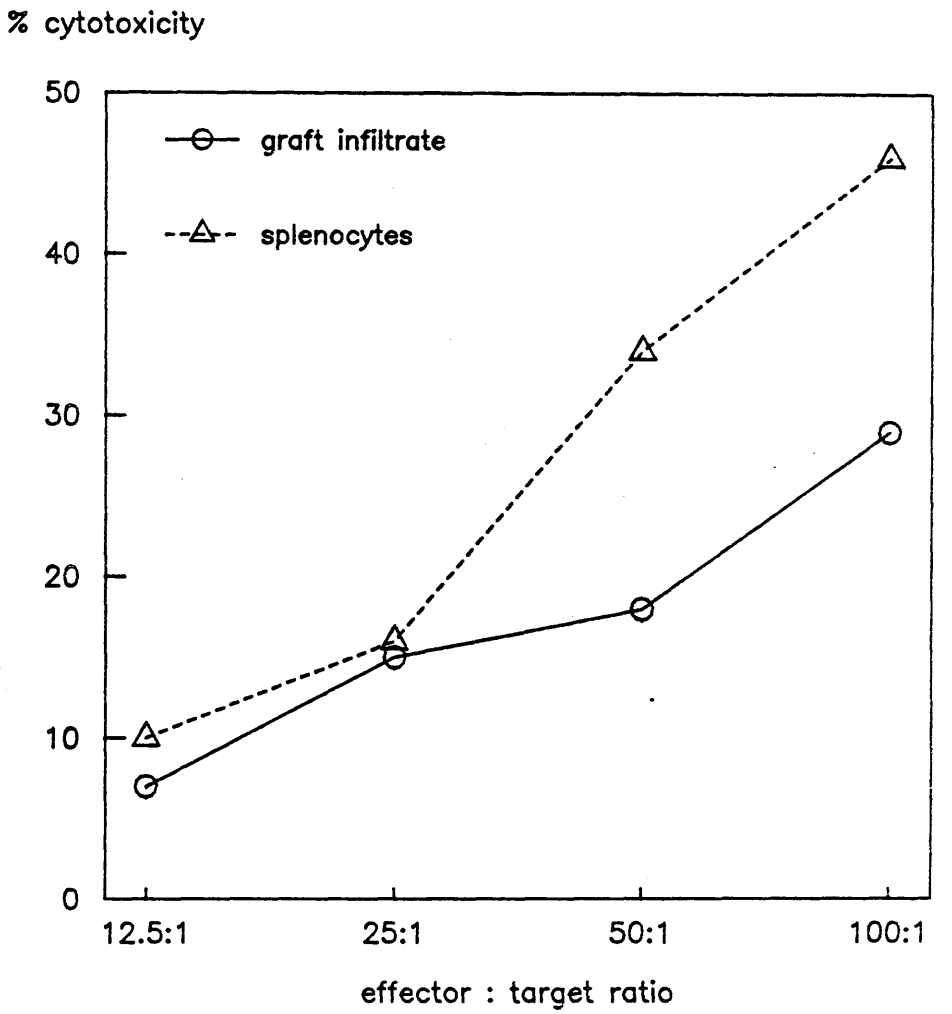


Fig. 3.6 Cytotoxic activity of graft infiltrating cells and splenocytes harvested on day 5 from rejecting DA renal allografts in unmodified PVG recipients, against NK susceptible Y3 targets.
Graft infiltrating cells were harvested by enzymic digestion of the graft. Splenocytes were macrophage depleted. Effector cells were tested in a 6-hour chromium release assay against Y3 targets.

cells. This treatment failed to deplete either the donor specific or the nonspecific activity, indicating that target lysis was not mediated by macrophages.

3.4 MHC antigen expression in rejecting renal allografts

The kinetics of MHC antigen induction in rejecting grafts were examined using monoclonal antibodies directed against both monomorphic and polymorphic determinants of class I and class II molecules. The MAbs MRC OX18 and MRC OX6 recognise monomorphic determinants of class I and class II MHC antigens respectively, and label MHC antigens of both donor and recipient strain.

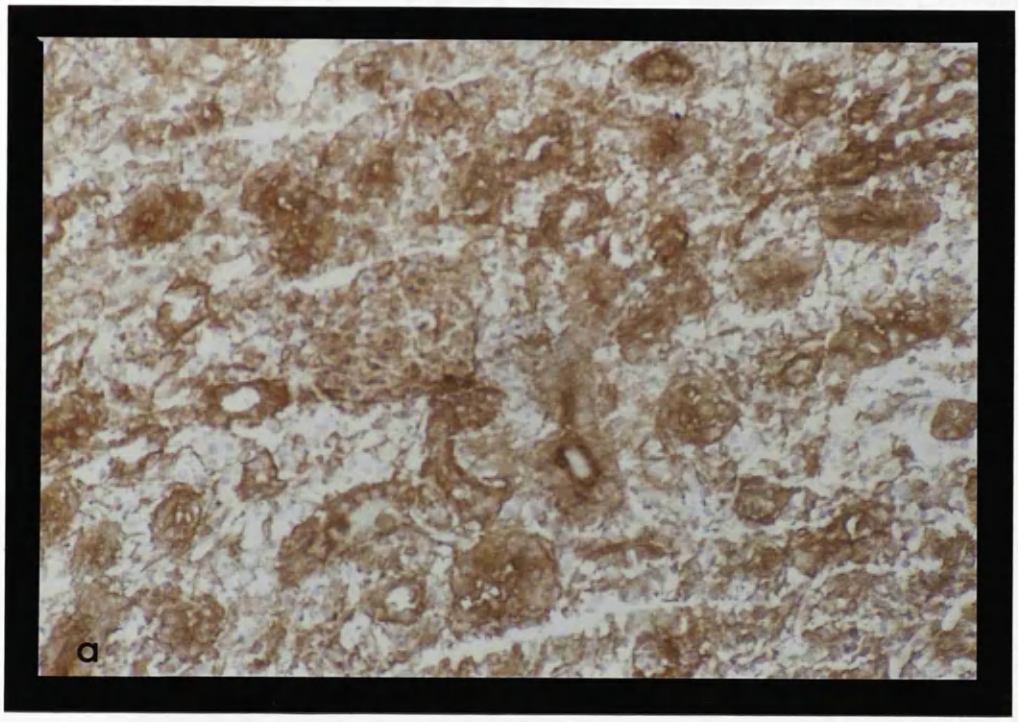
MN4-91-6 labels a polymorphic determinant of MHC class I present in DA but not PVG rat strains, and F17-23-2 labels a polymorphic determinant of MHC class II (I/A-like) antigen present in DA but not PVG rats. The use of these two antibodies was critical for enabling the detection of changes in donor MHC antigen expression on renal parenchymal structures, in the absence of additional staining of infiltrating cells, and their suitability for this investigation has previously been conclusively demonstrated in Fabre's laboratories (Milton et al, 1986a).

A description of the distribution of MHC antigens was made by examining cryostat sections of the grafts stained with the relevant MAbs, while quantitative absorption analysis of the same grafts provided a more objective means of assessing changes with time in the expression of MHC antigens in rejecting grafts.

3.4.1 Class I MHC antigen expression: Immunohistology

Cryostat sections of normal DA kidneys labelled with the anti-class I antibodies MRC OX18 and MN4-91-6 revealed strong staining of interstitial dendritic cells and venous and arteriolar endothelium, moderate staining of glomeruli and weak staining of cortical tubules and some medullary tubules. Rejecting grafts labelled with MN4-91-6 showed rapid induction of class I antigens with increased tubular staining apparent as early as day 1 after grafting. Strong staining of all tubular cells and glomeruli was detected by day 3, and by day 5 there was widespread class I induction apparent on all structures throughout the graft. DA isografts showed a slight increase in class I MHC antigen expression on tubular cells, but this was less intense and more variable than that seen in rejecting grafts. Fig 3.7 demonstrates the pattern of increased MHC class I expression in day 5 rejecting grafts compared with normal DA kidneys, as detected by labelling with MN4-91-6.

Fig. 3.7 Induction of MHC class I antigen expression in rejecting DA renal allografts in PVG recipients (a), compared with class I expression in normal DA kidneys (b).
Cryostat sections of day 5 grafts were labelled, by immunoperoxidase, with MN4-91-6 which detects donor (DA) but not recipient (PVG) class I antigens. x100.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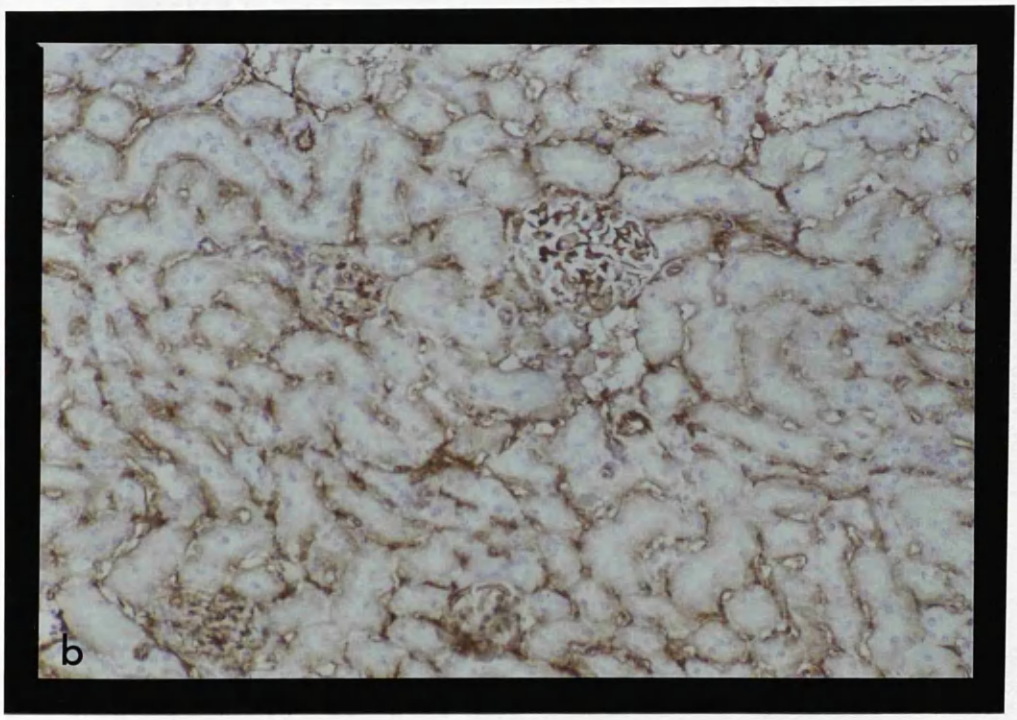
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3.4.2 Class I MHC antigen expression: Quantitative absorption analysis

Induction of class I MHC antigens in rejecting grafts was assessed quantitatively by performing absorption analyses with MN4-91-6 on homogenates of five pooled grafts at each time point after transplantation. The principle of this assay depended on the incubation of each pooled kidney homogenate, representing a different time point, with a predetermined quantity of ^{125}I-labelled, anti-class I monoclonal MN4-91-6. During the incubation period, different amounts of the MAb were absorbed by the tissues depending on the amounts of class I antigen they were expressing. The amount of labelled MN4-91-6 remaining in the supernatant was then quantified by binding it to an excess of class I specific targets (DA erythrocytes). Thus the more MHC antigen present in the homogenates, the lower the counts in the supernatant bound to the DA red blood cells.

The rapid and substantial increase in class I MHC antigen expression confirmed the immunohistologic findings and is depicted in Fig 3.8, where a shift of the curve to the right represents an increase in the amount of class I MHC antigens expressed by that tissue. By day 3 there was already a twofold increase in class I antigens which increased to a maximum ninefold (approximately) by day 5, compared with the normal level.

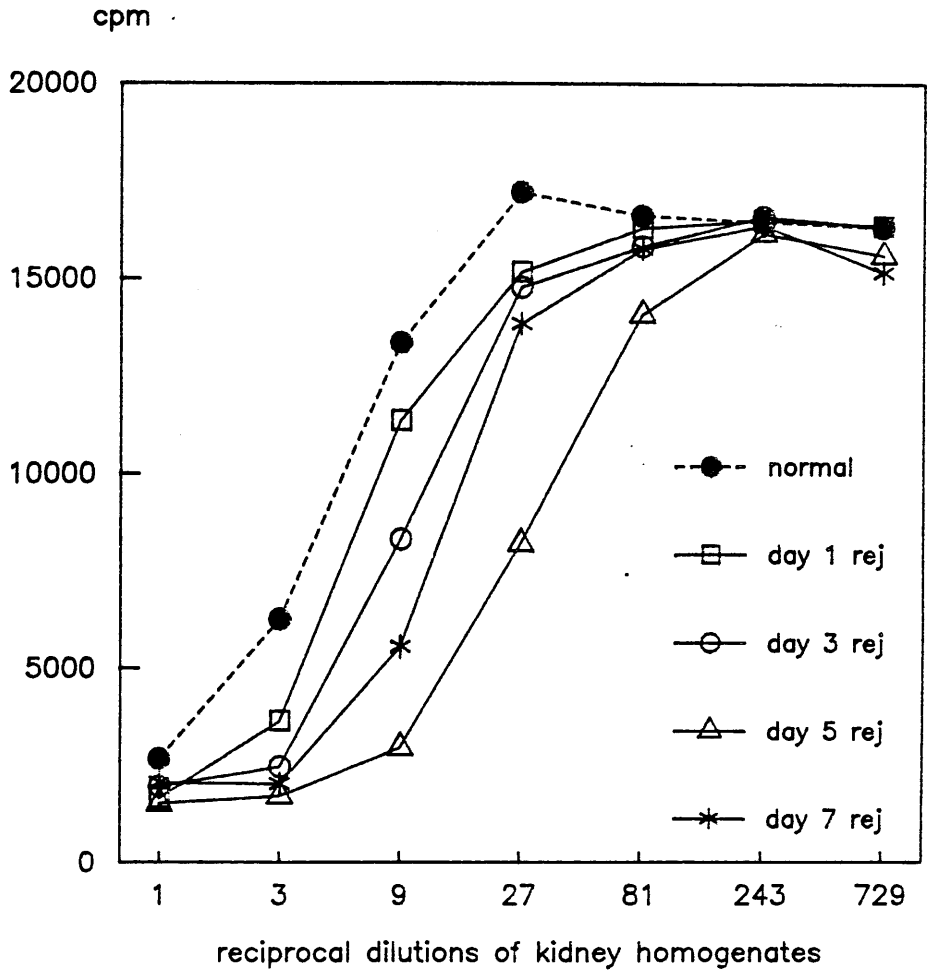


Fig. 3.8 Kinetics of donor class I MHC antigen induction in rejecting DA renal allografts in unmodified PVG recipients.
Quantitative absorption analysis was performed with the MAb MN4-91-6 which was absorbed with tripling dilutions of kidney homogenates prepared from five pooled grafts for each time point. The residual antibody was assayed by binding to DA erythrocytes. A shift of the curve to the right indicates increased class I MHC antigen expression.

3.4.3 Class II MHC antigen expression: Immunohistology

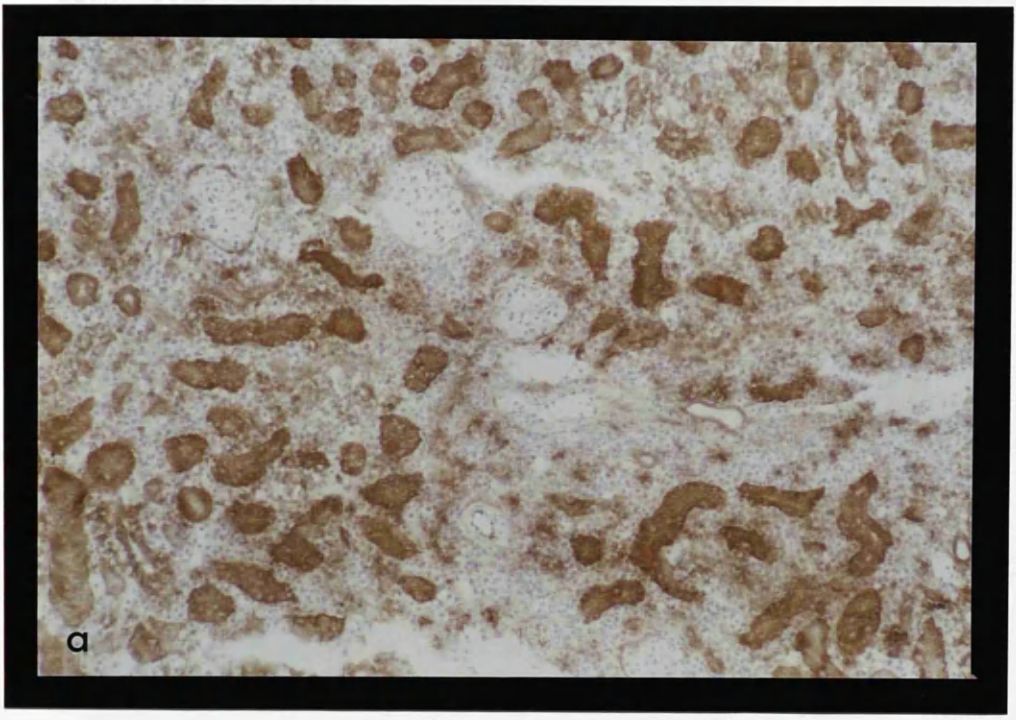
Normal DA kidneys stained with MRC OX6 and F17-23-2 showed strong class II antigen expression on interstitial dendritic cells and weak expression on proximal renal tubules, but none on vascular endothelial tissues nor in the glomeruli.

Class II expression in rejecting grafts was detected by staining with F17-23-2, which revealed, at day 1, an apparent slight decrease in the frequency of interstitial dendritic cells, but no other changes. By day 3, foci of increased donor class II antigen expression were apparent on some proximal cortical tubules. By day 5, all tubular cells were strongly stained and, in addition, donor class II antigen was induced on arteriolar endothelium and on Bowman's capsule, although not on the glomerular epithelial cells or capillary endothelium. Donor strain interstitial dendritic cells had virtually disappeared, and staining with the monomorphic anti-class II antibody MRC OX6 indicated that they had not yet been replaced with host dendritic cells.

DA isografts showed no increase in class II MHC antigen expression at any time after transplantation.

Fig 3.9 demonstrates the pattern of increased MHC class II expression in day 5 rejecting grafts compared with normal DA kidneys, as detected by labelling with F17-23-2.

Fig. 3.9 Induction of MHC class II antigen expression in rejecting DA renal allografts in PVG recipients (a), compared with class II expression in normal DA kidneys (b).
Cryostat sections of day 5 grafts were labelled, by immunoperoxidase, with F17-23-2 which detects donor (DA) but not recipient (PVG) class II antigens. x100.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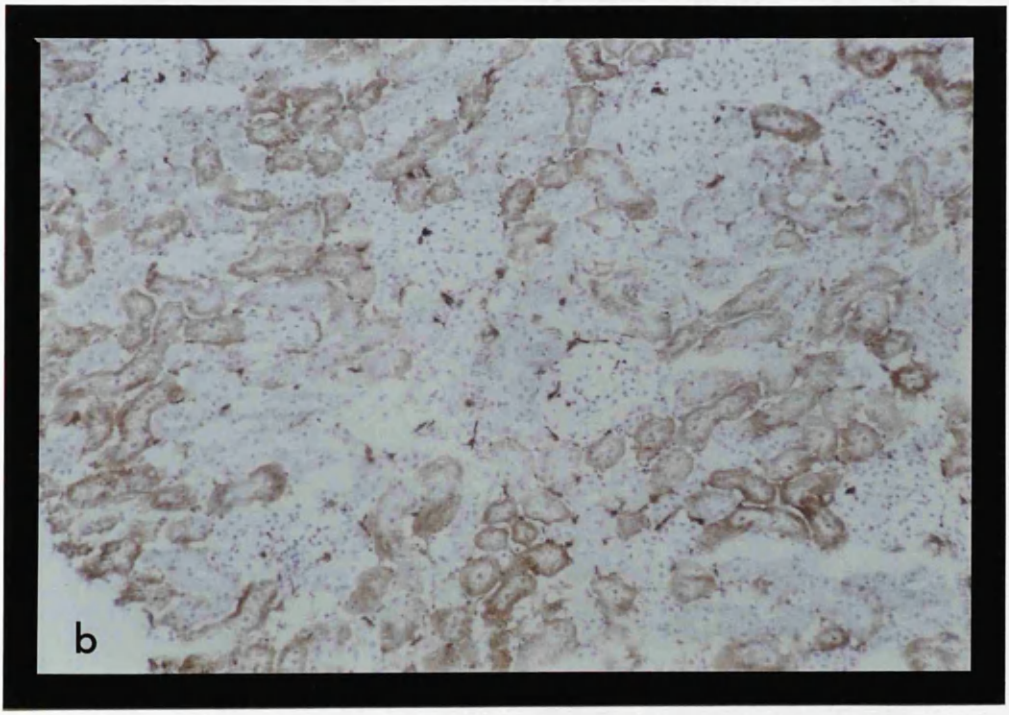
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3.4.4 Class II MHC antigen expression: Quantitative absorption analysis

Quantitative absorption analysis of class II MHC antigens was performed on aliquots of the same homogenates analysed for class I antigen content.

The antibody F17-23-2 was unsuitable for absorption analysis because its low affinity resulted in high free antibody concentrations at the higher dilutions, even in conditions of antigen excess in the absorption (Milton et al, 1986a). Instead, an assay was performed along the same lines as the class I quantitation assay, but using an alloantiserum in place of the anti-class II MAb, and the specificty of the asssay derived from the use of purified donor class II MHC antigens as the target for detecting the remaining activity in the supernatants. The class II assay was carried out by a collaborator, Dr Sarah Spencer, who had developed the procedure for the preparation of purified DA class II MHC antigen, and the results are reported herein with her permission.

These results (Fig 3.10) confirmed the histological findings, with an initial slight decrease in class II expression at day 1 reflecting the disappearance of donor dendritic cells. This was rapidly followed by an induction of class II MHC antigens which brought the

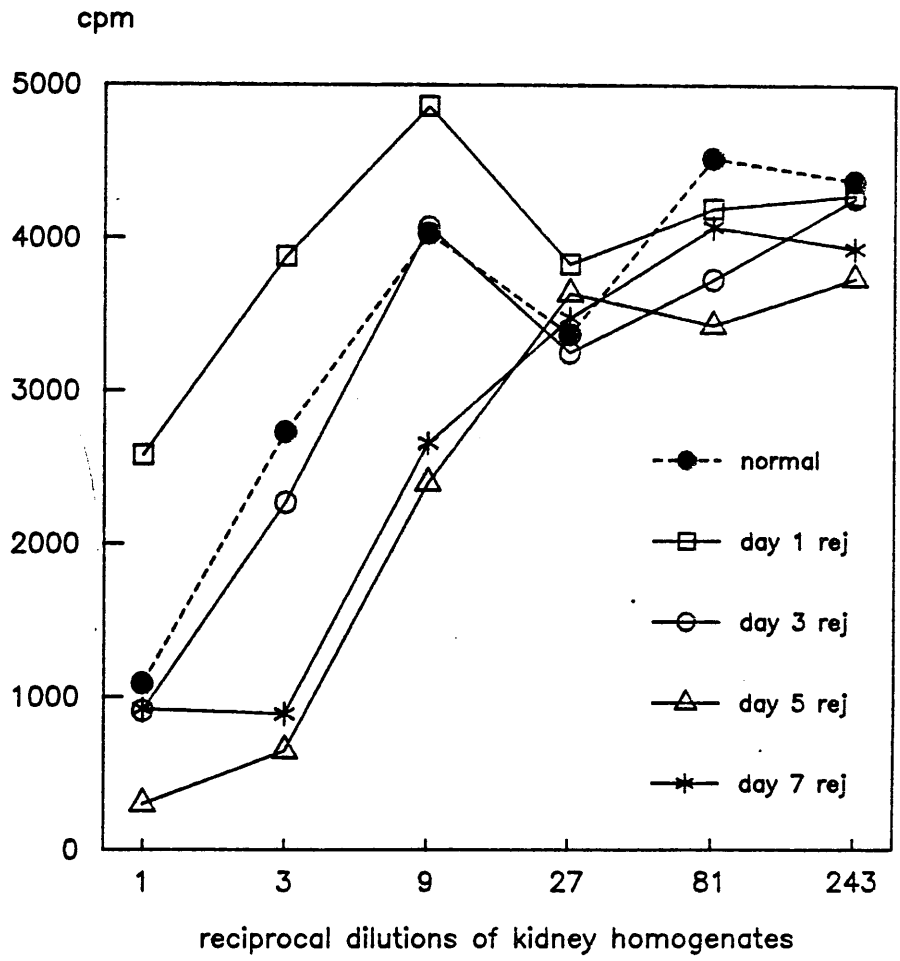


Fig. 3.10 Kinetics of donor class II MHC antigen induction in rejecting DA renal allografts in unmodified PVG recipients.
Quantitative absorption analysis was performed with PVG anti-DA serum which was absorbed with tripling dilutions of kidney homogenates prepared from five pooled grafts for each time point. Residual antibody was assayed by binding to microtitre plates coated with pure DA class II antigen. A shift of the curve to the right indicates increased class II MHC antigen expression.

levels to just above normal by day 3 and which continued to rise to a maximum fivefold increase (approximately) by day 5 after transplantation.

3.5 Role of dendritic cells in graft rejection

During this series of experiments investigating unmodified renal allograft rejection, the earliest cellular event observed was the apparent disappearance of donor strain dendritic cells. The role of dendritic cells in graft rejection was investigated by creating chimaeric rats to be used as kidney donors, so that their interstitial dendritic cells were syngeneic with the eventual graft recipients. The use of the polymorphic MAb F17-23-2 enabled an assessment of the extent of chimaerism in the donor kidneys.

Preliminary experiments were performed in an attempt to create fully allogeneic chimaeras, but on testing, chimaerism was found to be incomplete, presumably because a proportion of the reconstituting haemopoietic tissue had been destroyed by natural cytotoxicity. To avoid this situation, a semi-allogeneic strain combination was used instead.

Male (DAxPVG)F1 rats were irradiated (10.0 Gy) and immediately reconstituted with an intravenous inoculum of approximately $8x10^7$ haemopoietic cells prepared from

18 days gestation PVG foetal livers. Two months later, chimaerism was assessed as described in Chapter 2, and fully chimaeric animals were used as kidney donors for PVG recipients. Contralateral nephrectomy was performed on day 7 after transplantation and the survival of recipients was compared with the survival time of PVG recipients of normal (DAxPVG)F1 kidneys.

Two kidneys obtained from separate chimaeric animals, instead of being transplanted, were sectioned and stained with monoclonal antibodies detecting MHC class II positive interstitial dendritic cells, and the numbers of cells labelled with each antibody were counted and compared with those in normal (DAxPVG)F1 kidneys.

Fig 3.11 shows that chimaeric kidneys survived significantly longer than normal (DAxPVG)F1 kidneys in PVG recipients. Five recipients of F1 kidneys died on days 9,10,10,10 & 10 after transplantation, while the six recipients of chimaeric kidneys died on days 11,14,15,15 and two of the animals survived for longer than 100 days ($p < 0.005$).

Immunohistological examination of normal (DAxPVG)F1 kidneys revealed the presence of numerous class II positive dendritic cells staining with both antibodies, although there were fewer F17-23-2$^+$ cells, as expected, since this antibody is of relatively low affinity. Examination of the chimaeric kidneys showed (Table 3.1) that approximately 90% of the original

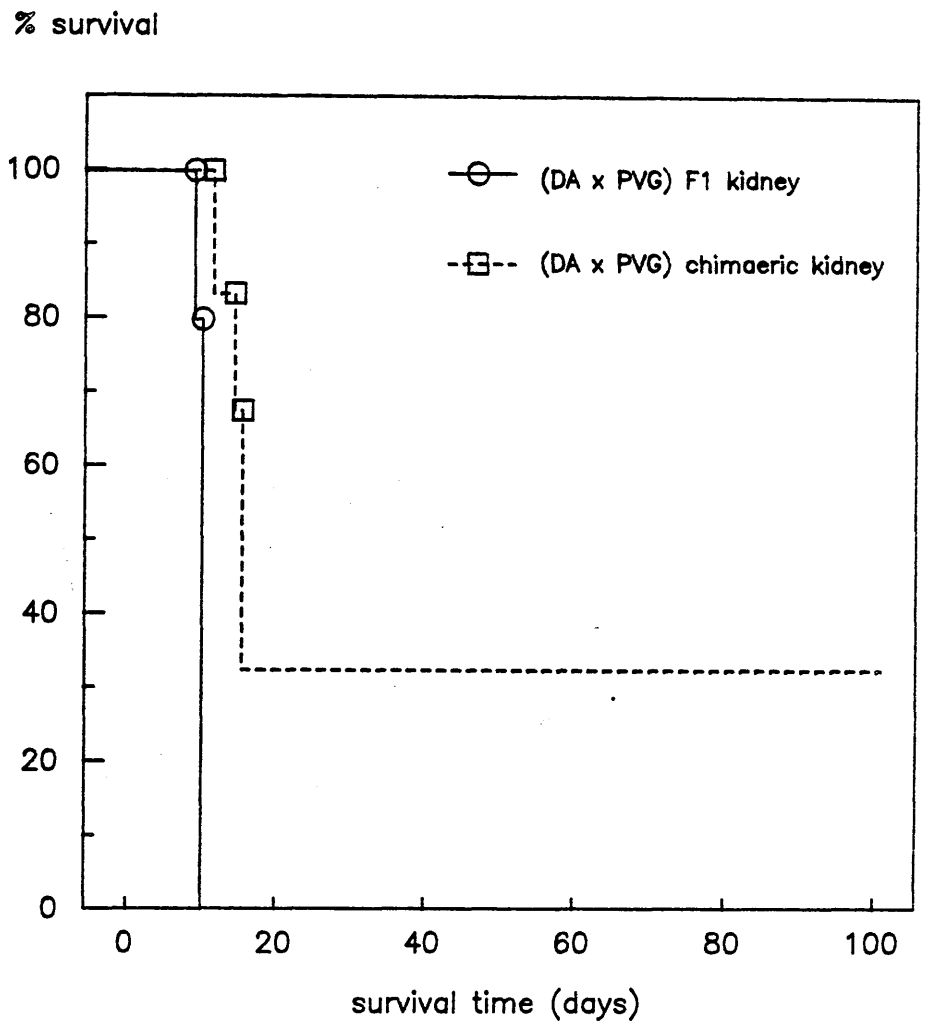


Fig. 3.11 Prolonged survival of chimaeric PVG(DAxPVG) kidneys in unmodified PVG recipients, compared with (DAxPVG)F1 kidneys.
Radiation chimaeric kidney donors were prepared with bone marrow derived cells, of PVG strain, syngeneic with the graft recipient. The survival of six chimaeric kidneys in PVG recipients was significantly prolonged in comparison with the survival of five F1 kidneys. ($p < 0.005$, Mann-Whitney U test).

Table 3.1 Replacement of (DAxPVG)F1 class II positive interstitial cells by PVG cells in PVG(DAxPVG) chimaeric kidneys

ANTI-CLASS II ANTIBODY	NO. OF LABELLED CELLS PER FIELD IN KIDNEYS (DAxPVG)F1	PVG(DAxPVG)	PVG(DAxPVG)
MRC OX6	91 (+/-15)	64 (+/-10)	70 (+/-19)
F17-23-2	48 (+/-13)	5 (+/-2)	4 (+/-2)

Table 3.1 Cryostat sections of one (DAxPVG)F1 kidney and two PVG(DAxPVG) chimaeric kidneys were stained by immunoperoxidase with MRC OX6 which detects class II molecules of all strains, and F17-23-2 which labels DA but not PVG class II molecules. The number of labelled cells in ten consecutive high power fields was counted in each kidney and the results are expressed as the mean and s.d. of the counts.

dendritic cells had been replaced by foetal liver-derived PVG strain dendritic cells which stained with MRC OX6 but not with F17-23-2. The photographs in Fig 3.12 illustrate this finding. However, in both kidneys up to 10% of the original dendritic cells remained.

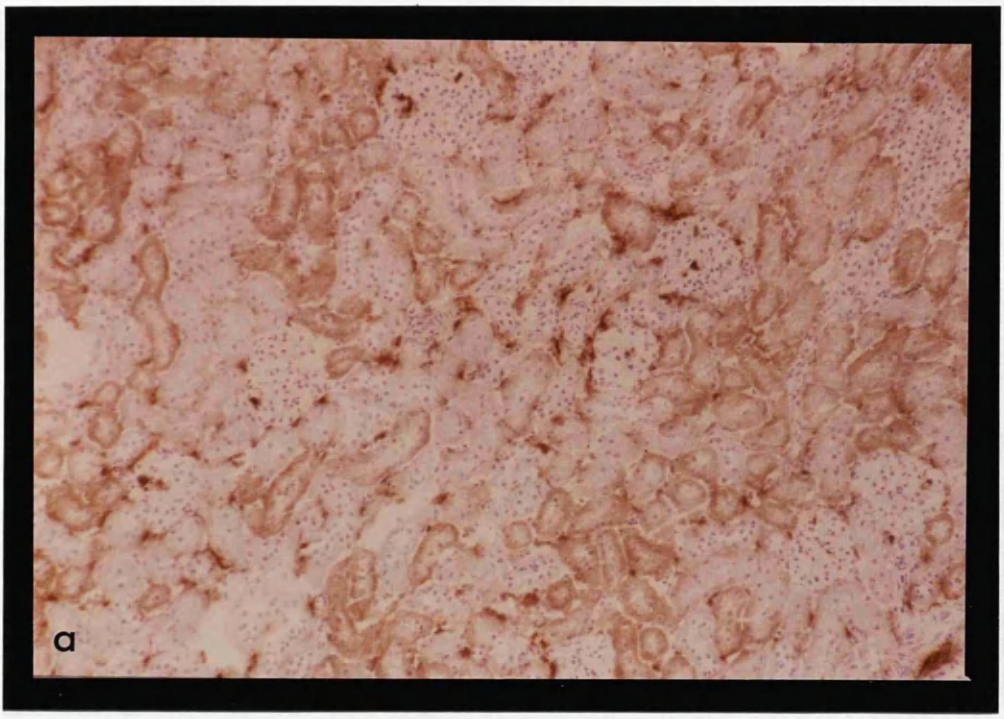
The results of these experiments suggested that donor dendritic cells are important for initiating graft rejection since when they were replaced by cells syngeneic with the kidney recipient, graft survival was prolonged. However, the wide range of survival times may reflect differences in the numbers of residual donor strain bone marrow derived interstitial cells in the donor kidney prior to transplantation.

3.6 The influence of MHC subregions on graft rejection

The recent development of recombinant series of inbred rats has enabled the design and interpretation of experiments to investigate the role of MHC subregion genes in influencing the outcome of transplants performed between individuals differing from each other at isolated MHC class I or class II region genes.

It is possible to select pairs of rats from the PVG recombinant series, such that the kidney donor strain differs from the recipient either at the RT1A class I locus, or at the RT1B and RT1D class II loci.

Fig. 3.12 Expression of MHC class II antigens on interstitial dendritic cells in kidneys from (DAxPVG)F1 animals and from PVG(DAxPVG) chimaeric animals.
Cryostat sections of F1 and chimaeric kidneys were labelled, by immunoperoxidase, with MRC OX6 detecting both DA and PVG class II antigens, and with F17-23-2 detecting DA but not PVG class II antigens.
a) (DAxPVG)F1 kidney labelled with MRC OX6. x100.
b) (DAxPVG)F1 kidney labelled with F17-23-2. x100.
c) Chimaeric kidney labelled with MRC OX6. x100.
d) Chimaeric kidney labelled with F17-23-2. x100.
Note the absence of F17-23-2-positive dendritic cells in the chimaeric kidney, suggesting that the dendritic cells derive from the PVG haemopoietic tissue.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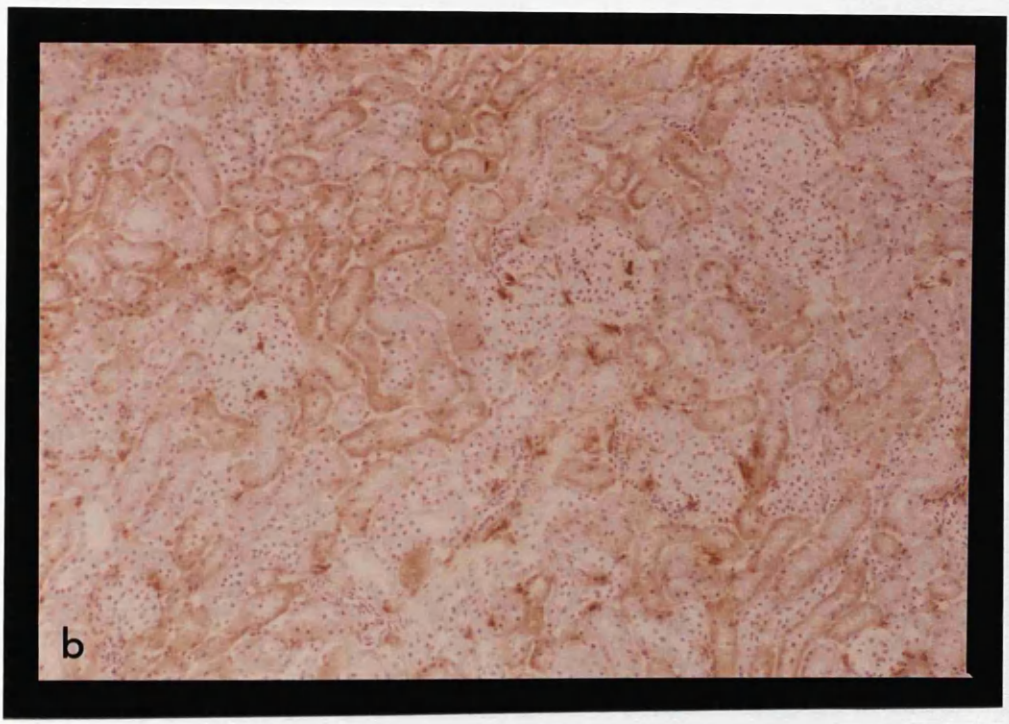
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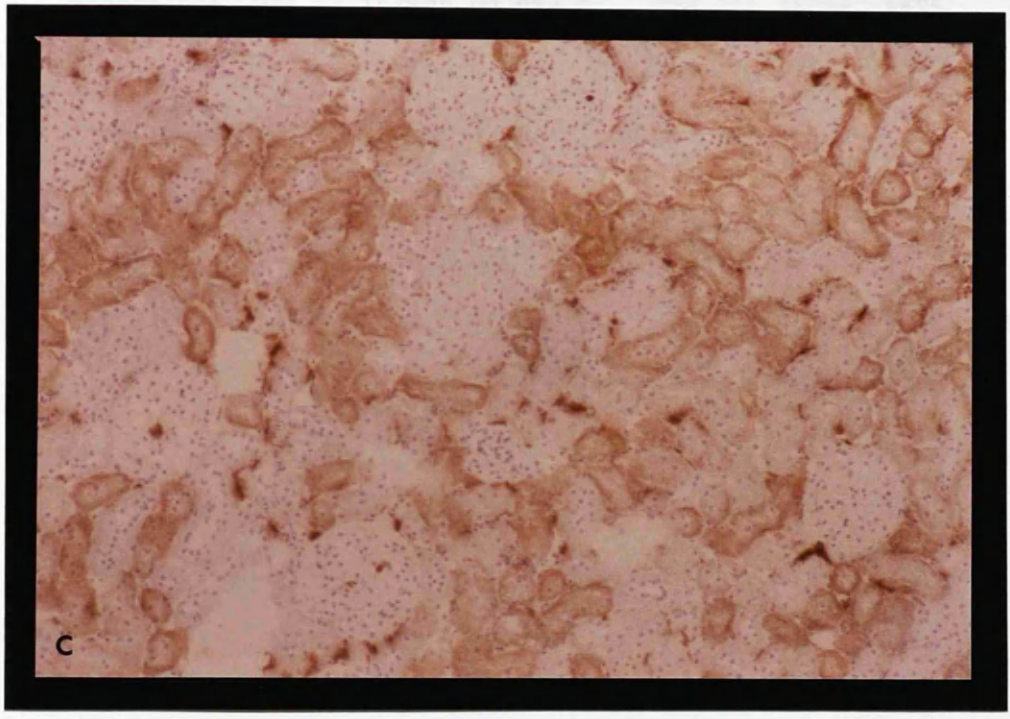
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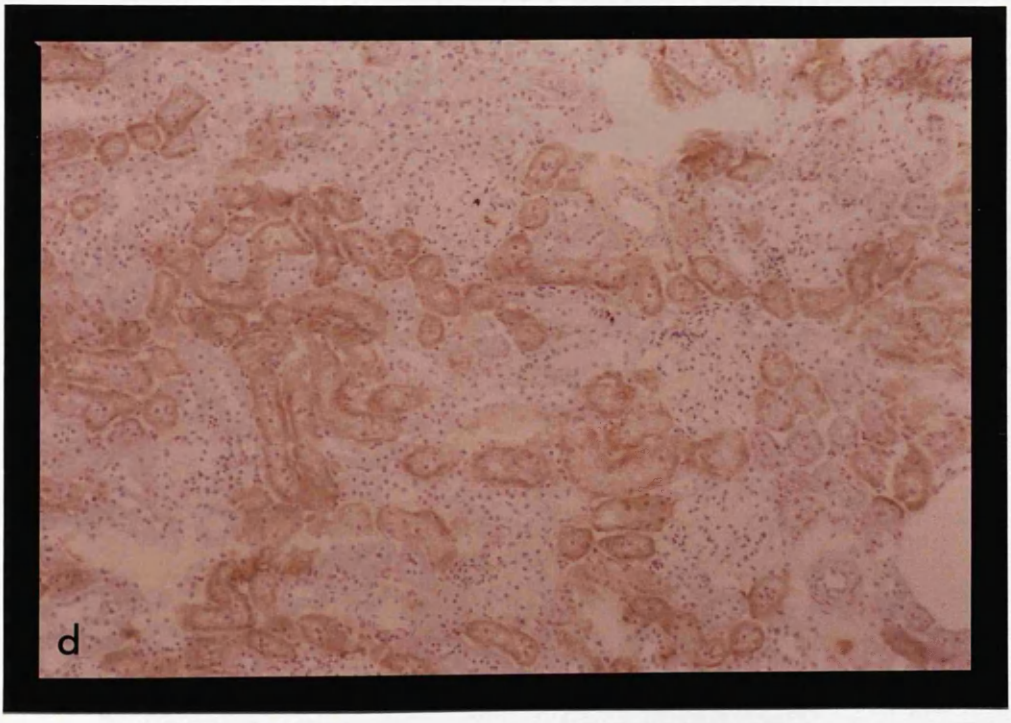

d

Three PVG rats were transplanted with kidneys from PVG.R1 rats whose RT1A class I region genes were of the "a", rather than the "c" haplotype. Contralateral nephrectomy was performed on day 7, and all three rats survived for longer than 100 days, with no significant rise in serum urea and creatinine levels.

To investigate the influence of class II region genes, three PVG.R1 rats were transplanted with kidneys from PVG.R19 donors whose RT1B and RT1D region genes were of the "a", rather than the "c" haplotype. Following contralateral nephrectomy on day 7, serum urea and creatinine levels in all three animals rose rapidly until their deaths on days 16, 17 and 19 after transplantation.

Although the numbers are small, these survival data suggest that, in this particular strain combination, an isolated class I disparity is insufficient to provoke a rejection response, and is not a necessary adjunct to a class II disparate graft which is rejected in the absence of class I incompatibility.

3.7 Discussion

The experiments described in this chapter provided information on several aspects of the cellular events occurring in rat renal allografts undergoing unmodified rejection.

Detailed morphometric analysis of the graft infiltrate showed that by the third day after transplantation the infiltrating cells were predominantly MRC OX19 positive, W3/25 positive cells of the T helper phenotype. However, by day 5 there was a predominance of cells with a phenotype consistent with that of NK cells (MRC OX19 negative, MRC OX8 positive) and, to a lesser extent, macrophages (MRC OX19 negative, W3/25 positive). This pattern would be consistent with an early infiltration of the graft by specific T helper cells followed by lymphokine release and the consequent recruitment of nonspecific effector cells by day 5.

Analysis of the functional repertoire of these infiltrating cells revealed that they possessed moderate levels of donor specific cytotoxic activity in spite of the relative dearth of T cells, and high levels of nonspecific, presumably NK cell mediated cytotoxicity. The demonstration of specific cytotoxicity suggests that this mechanism is involved in the rejection response. This is supported by the findings of Bradley et al (1985) and of Mason & Morris (1984) that the levels of specific, but not nonspecific, cytotoxicity were reduced in nonrejecting grafts from passively enhanced or CyA-treated animals. However it is contradicted by evidence from Dallman et al (1987) and from our own laboratory (Armstrong et al, 1987) that specific

cytotoxic activity was also found in graft infiltrating cells from actively enhanced rats that retained their grafts indefinitely.

The functional activity of NK cells, however, may not be necessary for graft rejection. This was proposed by Heidecke, Araujo, Kupiec-Weglinski et al (1985) who treated heart allografted rats with anti-asialo GM1 to remove NK cells, and showed that the nonspecific cytotoxic activity of infiltrating cells was abrogated by this treatment, but graft rejection was unaffected, or prolonged by up to two days following repeated administration of anti-AGM1. They were unable to rule out the possibility that the slight prolongation of survival was due to the depletion of other cells in addition to NK cells (for example, Tc lymphocytes), but from their evidence it appears that the presence of NK cells in rejecting grafts is an epiphenomenon.

In parallel with the influx of infiltrating cells was the widespread induction of MHC antigens throughout the rejecting graft, and notably the induction of class II MHC antigens on the previously class II-negative vascular endothelium. It was difficult to discern an increase with time in the expression of class I MHC antigens, but this data was provided by quantitative absorption analysis which revealed a more rapid induction of class I than class II antigens. These results agree with Fabre's findings but contrast with data for MHC antigen

induction in heart allografts, where class I and class II induction is delayed, possibly because there is less initial expression of MHC antigens in normal hearts than in normal kidneys (Milton et al, 1986a). The induction of MHC antigens is most probably mediated by the release of IFN-ϑ from infiltrating, activated leukocytes (Wong, Clark-Lewis, Harris et al, 1984) and serves to increase possible target antigen density on graft tissues, thus rendering the graft more susceptible to both class I restricted and class II restricted alloimmune responses. It must be remembered, however, that induction of MHC antigens in allografted tissues is not invariably associated with destruction of the graft (Armstrong et al, 1987), and that rejection of allogeneic tissues is not invariably associated with MHC antigen induction (Forbes et al, 1986).

A further notable finding was the apparent disappearance, by day 5, of interstitial dendritic cells from the grafted tissue. The use of monoclonal antibodies directed against polymorphic determinants of class I and class II MHC antigens enabled this observation to be made unequivocally, since the dendritic cells were of characteristic morphology, strongly stained and were the only interstitial cells labelled with these antibodies. On day 1 after transplantation, there was no increased MHC antigen expression on kidney parenchymal cells, and so the reduction in the numbers of

interstitial dendritic cells compared with normal DA kidneys was quite striking. When consecutive tissue sections were stained with MRC OX6 (directed against a monomorphic determinant of class II MHC antigens) it was clear that these cells had not been replaced by dendritic cells of recipient origin. These highly immunogeneic cells of donor origin are generally considered to be important in the initiation of the rejection response (Lechler & Batchelor, 1982; McKenzie, Beard & Hart, 1984) but the route by which this occurs is unknown. It was not possible to determine whether the dendritic cells migrated to recipient lymphoid tissue, or whether they were destroyed in situ by infiltrating host cells, although it should be noted that the host infiltrate in the graft was negligible at day 1.

Experiments using chimaeric kidney donors addressed the question of whether rejection would occur in the absence of donor strain dendritic cells. As discussed in section 1.5, Guttman et al (1969) investigated this problem by creating radiation chimaeras and using them as kidney donors, so that the donor kidneys had allogeneic parenchymal tissues but the bone marrow derived passenger leukocytes were syngeneic with the graft recipient. They were able to demonstrate good renal function in the grafts on day 7 after transplantation but they failed to report survival data or to determine the degree of chimaerism in the donors. Fabre & Morris (1973) were

unable to substantiate these findings and suggested that this phenomenon might be highly strain dependent. The findings reported in this chapter confirmed the importance of donor strain dendritic cells in initiating allograft rejection, since in situations where the numbers of donor dendritic cells were depleted by 90%, survival of renal allograft recipients was significantly prolonged. It could not be ascertained whether or not rejection of chimaeric kidneys was due to residual dendritic cells, or to an alternative route of recipient sensitisation proposed by Sherwood et al (1986) in which host antigen presenting cells are able to present donor class II molecules to antigen reactive cells.

The limited number of experiments investigating the influence of isolated class I and class II disparities on allograft rejection suggested that a class I incompatibility in this strain combination did not provide a significant allogeneic stimulus to the recipient. However, the MHC disparities could not be considered to be the only influence, since it has previously been shown that whereas a vascularised PVGr1 heart graft was not rejected by PVG recipients, skin grafts in the same strain combination usually were rejected (Stewart, Butcher, Herbert et al, 1985). The survival of class I- but the rejection of class II-disparate grafts reported herein are consistent with the observation of only moderate levels of <u>in vitro</u> donor

specific cytotoxicity in PVG cells infiltrating DA grafts, and suggests that in this strain combination rejection is initiated by class II restricted cells and mediated by a DTH type of reaction.

This investigation has revealed the presence of a number of cellular events that appear to be associated with graft rejection, such as the presence of specific cytotoxic activity and MHC antigen induction, but they cannot be considered to be sufficient for rejection since these events are also observed in allogeneic grafts that are retained indefinitely. It has not therefore been possible to define the effector mechanisms of graft rejection.

CHAPTER FOUR

THE CELLULAR REQUIREMENTS FOR RENAL ALLOGRAFT REJECTION IN LYMPHOCYTE DEPLETED RATS

4.1 Introduction

The relative contributions of specific cytotoxic T cells and DTH mechanisms in graft rejection was critically reviewed in chapter 1 and the necessity to define the roles of the different potential effector mechanisms in terms of species, strain combination and type of allograft was emphasised. Much useful information has been provided by adoptive transfer experiments in which the ability of lymphocyte subpopulations to restore graft rejection in immunocompromised hosts has been studied. However, there is a need for caution in interpreting the results because of the difficulty in preparing truly T cell deficient animals and because T cell subpopulations defined according to their phenotype may have overlapping functions.

With a single known exception (Gurley, Lowry & Forbes, 1983), there are no previous adoptive transfer studies which have examined the cellular requirements for renal allograft rejection. The majority of previous studies have been concerned with skin or heart allograft rejection and many have used only sensitised cells for adoptive transfer.

The work reported in this chapter describes attempts to establish the T cell requirements for rat renal allograft rejection in acutely irradiated, lymphocyte depleted

recipients. The ability of adoptively transferred CD4 and CD8 lymphocytes to restore rejection was assessed and the phenotype of graft infiltrating cells was examined. In addition, the in vitro cytotoxic activity of splenocytes and graft infiltrating cells from irradiated, reconstituted allograft recipients was defined.

4.2 Investigation of the ATXBM rat as a model for determining the cellular requirements for renal allograft rejection

In preliminary experiments, consideration was given to the use of the ATXBM rat model as a T cell deficient host in which to investigate the cellular requirements for restoring first-set renal allograft rejection. Before examining the role of T cell subpopulations in rejection, it was necessary to establish firstly, that nonreconstituted ATXBM allograft recipients were unable to reject a renal allograft and secondly, that reconstituting ATXBM graft recipients with unsensitised syngeneic lymphocytes would restore rejection in, or near, first-set tempo. For these experiments, the fully allogeneic DA ($RT1^a$) into Lewis ($RT1^l$) rat strain combination was chosen, since it is generally recognised to provoke a strong rejection response, and

this is corroborated by the inability to prevent graft rejection in this strain combination by active enhancement (Fabre & Morris, 1972).

Lewis ATXBM rats were prepared by lethal irradiation (10.0 Gy) of thymectomised animals followed by immediate reconstitution with bone marrow obtained from a syngeneic donor rat depleted of T cells by chronic thoracic duct drainage. ATXBM animals were used as allograft recipients approximately six weeks after preparation. Graft rejection was judged by observing the survival of allograft recipients which had undergone contralateral nephrectomy at the time of renal transplantation, together with monitoring serum urea levels in serial blood samples.

The results are summarised in Table 4.1. Unmodified Lewis recipients all rapidly rejected DA renal allografts, with a median survival time of only 8 days and markedly elevated day 7 blood urea levels. As expected, ATXBM Lewis rats did not reject DA renal allografts and all three animals survived until sacrifice at day 50 or later, with normal blood urea levels throughout. Histological examination of the kidney allografts showed no evidence of rejection. Unexpectedly, immediate reconstitution of ATXBM allograft recipients with an intravenous inoculum of $5x10^7$ syngeneic LNC failed to restore graft rejection, and despite a mild elevation in serum urea levels, three out

Table 4.1 Survival of DA renal allografts in unmodified and ATXBM Lewis recipients.

HOST	LNC DOSE	SURVIVAL (days)	MST (days)	UREA LEVELS (mM/L; med & range) d7	d14
normal	none	6,7,7,7,8,8,8,8	8	124 (101-163)	-
ATXBM	none	>50,>50,>50	>50	13 (8-26)	8 (8-10)
ATXBM	$5x10^7$	17,>50,>50,>50	>50	22 (16-45)	18 (12-61)
ATXBM	$1.5x10^8$	13,>50	32	25 (21-28)	60[a]

Table 4.1 Unmodified and ATXBM Lewis recipient rats recieved a DA renal allograft, and underwent immediate contralateral nephrectomy. Graft rejection was monitored by observing the survival time of the recipient, and measuring serum urea levels in serial blood samples.

a) One rat died before day 14.

of four animals survived for longer than 50 days. In two additional animals, an inoculum of $1.5x10^8$ LNC also failed to restore graft rejection in a normal tempo.

The inability of relatively large numbers of syngeneic lymphocytes to restore rapid graft rejection in ATXBM rats precluded further experiments to examine the role of T cell subpopulations in rejection because of the technical difficulty in preparing the large numbers of purified lymphocytes that would obviously be required. From these results, the possibility existed that the inability of adoptively transferred lymphocytes to restore graft rejection was due to the presence, in ATXBM recipients, of suppressor mechanisms. Although of potential interest, this possibility was not pursued since the primary object was to examine the role of T cell subpopulations in graft rejection.

4.3 Establishing the acute irradiation model

As an alternative to the ATXBM model, the decision was taken to examine the cellular requirements for renal allograft rejection in sublethally irradiated recipients.

The experimental design for adoptive transfer experiments required that the potential allograft recipients be sublethally irradiated, transplanted with a kidney from a normal allogeneic donor rat and

reconstituted with an intravenous inoculum of lymphocytes prepared from the lymph nodes of a normal rat syngeneic with the kidney recipient, all within 24 hours of irradiation. The contralateral kidney would be removed at an appropriate time and the integrity of the transplanted kidney would then be monitored at frequent intervals by determining the blood serum urea and creatinine levels, and by recording the survival of the graft recipient.

Preliminary experiments were performed to discover the lethality of different doses of irradiation and their effects on the levels of blood cellular components, in particular, lymphocytes and platelets.

4.3.1 The effect of "sublethal" irradiation on rat haematological profiles

From previous studies, it is evident that in order to consistantly produce long term skin or vascularised heart allograft survival, it is necessary to administer a near lethal dose (typically 7.0-9.0 Gy) of gamma irradiation. Preliminary experiments were carried out in which normal adult rats were given a single "sublethal" dose of gamma irradiation (7.5, 8.5 or 9.25 Gy). All irradiated animals (3-6 per group) survived until sacrifice (at >50 days). Serial measurements of peripheral blood leukocyte, lymphocyte and platelet counts were made and Figs 4.1, 4.2 and 4.3 show the results for three Lewis

rats treated with 8.5 Gy. The profiles thus obtained were indistinguishable from haematological profiles in rats treated with either 7.5 or 9.25 Gy.

During the first two days after irradiation, total leukocyte counts showed a profound fall (Fig 4.1), followed over the next few days by a transient rise before falling back to the nadir. This "abortive rise" is a well recognised phenomenon in radiation biology and is due to replenishment of circulating cells from leukocyte precursors, until after one or two cell divisions they succumb to the effects of radiation induced chromosome abnormalities. The cells constituting the abortive rise do not have normal function (Bond, Fliedner & Archambeau, 1965). From about 14 days, there was a steady recovery in leukocyte numbers, frequently with a period of overcompensation before normal levels were regained.

The profile for circulating lymphocyte counts (Fig 4.2) followed a similar general pattern to that seen for total leukocytes. Lymphocytes were particularly radiosensitive (circulating levels were reduced to less than 2% of normal by 48 hours) but nevertheless showed an early "abortive rise" and eventual recovery from day 14 onwards.

Platelet counts (Fig 4.3) fell less rapidly, but reached very low levels by day 7, before rising to normal from day 14 onwards.

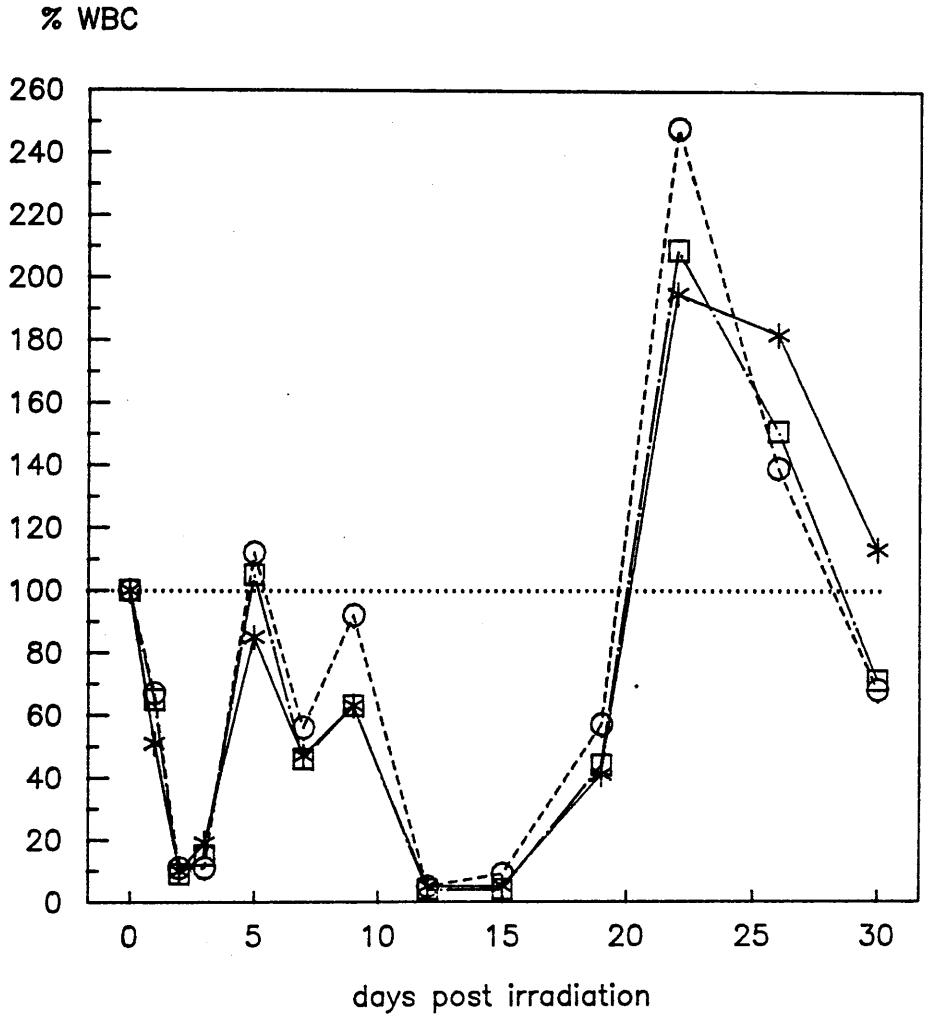


Fig. 4.1 Effect of irradiation (8.5 Gy) on circulating leukocyte numbers in three Lewis rats.
Results are expressed as a percentage of normal leukocyte numbers in non-irradiated rats.

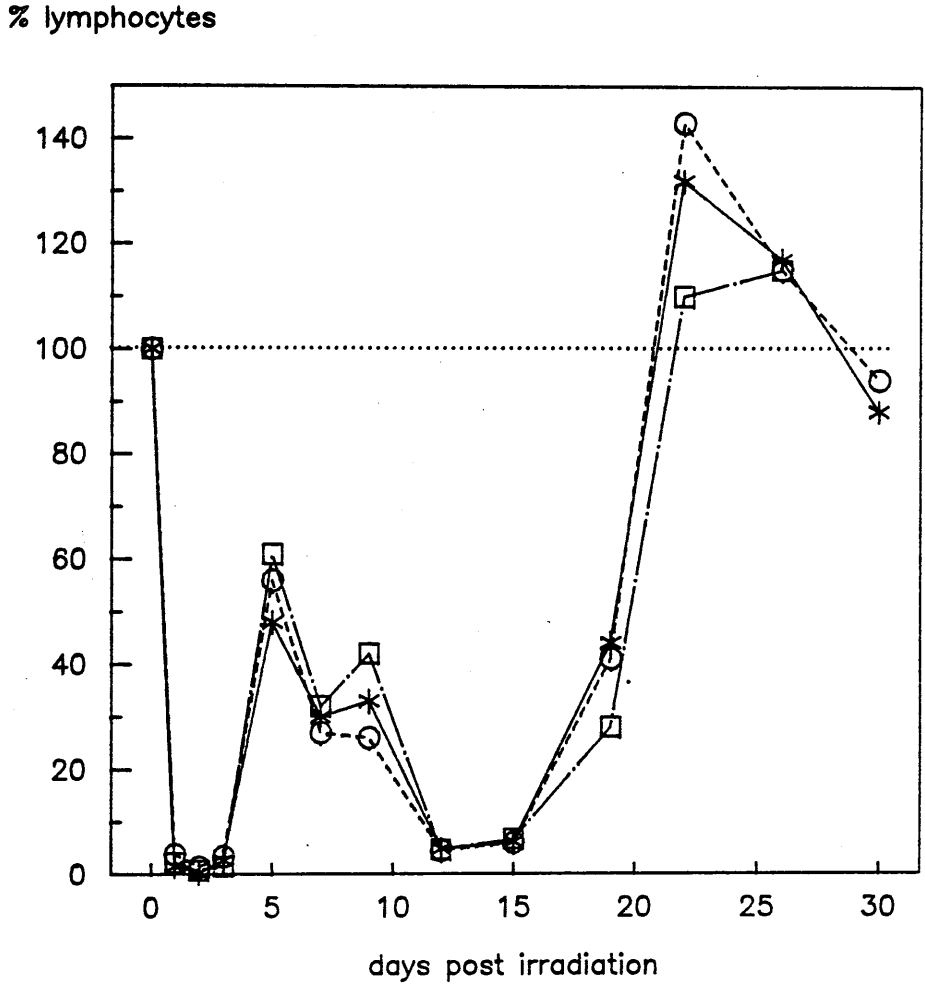


Fig. 4.2 Effect of irradiation (8.5 Gy) on circulating lymphocyte numbers in three Lewis rats.
Results are expressed as a percentage of normal lymphocyte numbers in non-irradiated rats.

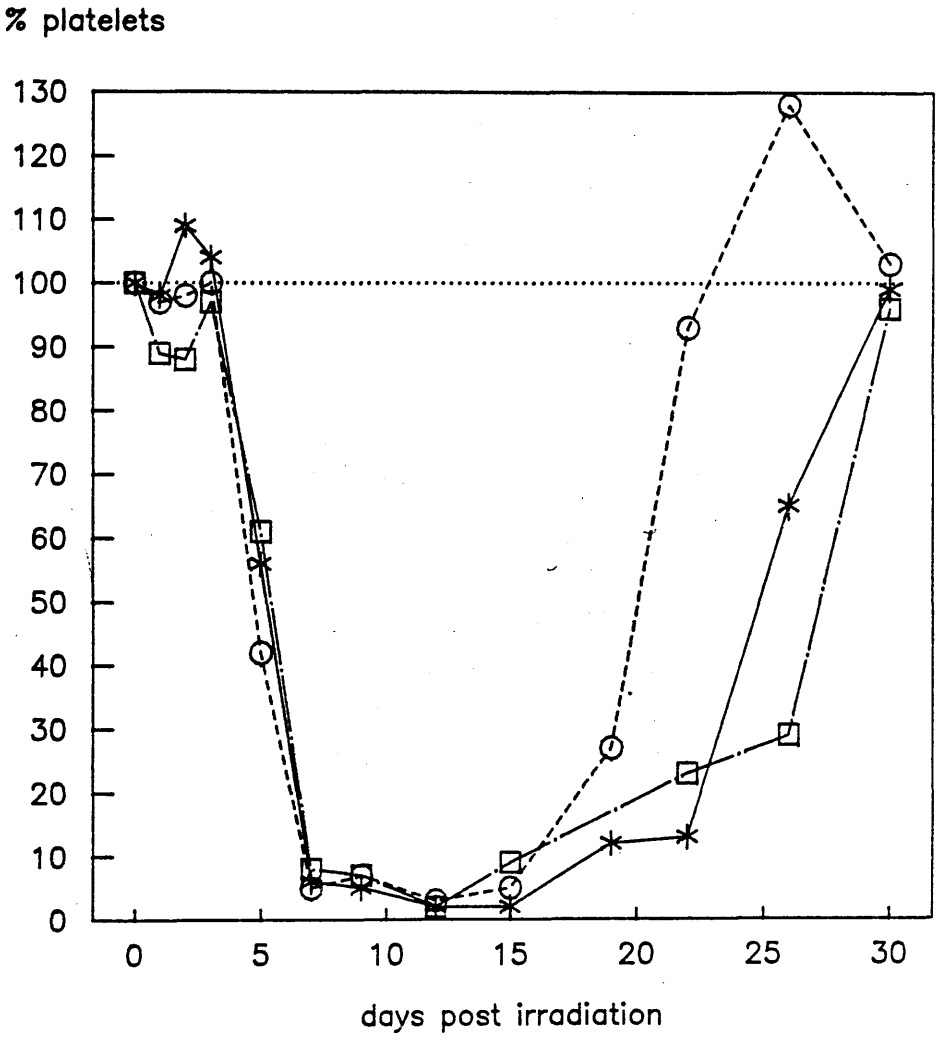


Fig. 4.3 Effect of irradiation (8.5 Gy) on circulating platelet numbers in three Lewis rats.
Results are expressed as a percentage of normal platelet numbers in non-irradiated rats.

The survival and haematological tests in the above experiments with Lewis rats were repeated with identical results in irradiated DA strain rats.

4.4 Ability of acute irradiation to prevent renal allograft rejection

Lewis rats were irradiated with either 7.5 or 8.5 Gy and immediately transplanted with a DA strain kidney. Contralateral nephrectomy was performed at the time of transplantation, and recipient survival times and serum urea levels were monitored.

The results are summarised in Table 4.2. As previously noted, unmodified Lewis recipients rapidly rejected their grafts (MST 7 days) and died with a markedly elevated blood urea level. In contrast, irradiation of recipients with either 7.5 or 8.5 Gy significantly prolonged graft survival (MST >50 days) One rat given 7.5 Gy died on day 4 because of a ureteric leak and three rats given 8.5 Gy died (or were sacrificed) on days 11, 15 and 21 because of radiation induced hind leg paralysis and severe diarrhoea.

As shown in Table 4.2, allograft recipients given 7.5 Gy had a significantly higher day 7 serum urea than those receiving 8.5 Gy ($p<0.02$), although by day 14 this had fallen and serum urea levels were similar in both groups.

Table 4.2 Survival and serum urea levels of irradiated Lewis recipients of DA renal allografts.

IRRADIATION (Gy)	N	SURVIVAL (days)	MST (days)	UREA LEVELS (mM/L; median & range) d7	d14	d21
none	8	6,7,7,7, 8,8,8,8	8	124 (101-163)	-	-
7.5	6	4[a], >50, >50,>50, >50,>50	>50	37 (31-49)	17 (10-21)	7 (7-9)
8.5	9	11,15,21,[b] 35[c],>50, >50,>50,>50	>50	16 (8-38)	15 (7-39)	8 (6-33)

Table 4.2 Irradiated (7.5 or 8.5 Gy) Lewis recipients received a DA renal allograft with immediate contralateral nephrectomy. Graft rejection was monitored by observing recipient survival time and by measuring serum urea levels in serial blood samples.

Serum urea levels on day 7 in rats receiving 7.5 Gy irradiation were significantly higher than in rats treated with 8.5 Gy ($p<0.02$, Mann-Whitney U test).

a) Death due to ureteric leak.

b) The three rats dying on days 11,15 & 21 had normal urea levels.

c) Anaesthetic death, normal urea.

Kidney allografts were excised from irradiated rats, after sacrifice at 50 days or later, for routine histology. None of the grafts were swollen or showed any of the features of acute graft rejection (irrespective of whether they had received 7.5 or 8.5 Gy). However, it was thought likely that the high day 7 serum urea in the 7.5 Gy group might have been an indication of transient rejection due to incomplete ablation of the immune response. It was decided, therefore, to use the higher dose of 8.5 Gy for subsequent adoptive transfer studies, despite the increase in radiation associated morbidity.

4.5 Ability of adoptively transferred LNC to restore renal allograft rejection in acutely irradiated Lewis recipients

Irradiated (8.5 Gy) Lewis rats were transplanted with a DA strain kidney and then given an intravenous inoculum of syngeneic lymphocytes prepared from the pooled cervical and mesenteric lymph nodes of normal Lewis rats. Contralateral nephrectomy was performed at the time of transplant. Table 4.3 shows the ability of different numbers of LNC to restore first set graft rejection. The adoptive transfer of $1x10^6$ LNC failed to fully restore rapid graft rejection (MST 14 days) although the day 7 urea levels were moderately elevated. However,

Table 4.3 The ability of transferred LNC to restore DA renal allograft rejection in acutely irradiated Lewis rats

LNC TRANSFERRED	N	SURVIVAL(days)	MST (days)	DAY 7 UREA (mM/L) (median & range)
5×10^7	4	7,7,8,9	8	137 (101-191)
1×10^7	5	7,8,8,9,9	8	102 (63-127)
1×10^6	3	12,14,15	14	46 (23-49)

Table 4.3 Irradiated (8.5 Gy) Lewis recipients received a DA renal allograft with immediate contralateral nephrectomy, and were reconstituted with an intravenous inoculum of syngeneic, unseparated LNC. Graft rejection was monitored by observing recipient survival time and by measuring serum urea levels in serial blood samples.

increasing the number of transferred cells to $1x10^7$ or more restored rejection to the tempo seen in unmodified animals (MST 8 days) with markedly elevated day 7 serum urea levels. Allografts excised after death for histological examination all showed signs of severe graft rejection.

4.6 Ability of negatively selected LNC subpopulations to restore renal allograft rejection in irradiated Lewis rats

Further adoptive transfer experiments were performed in which the ability of negatively selected LNC subpopulations to restore first set allograft rejection was examined. The purity of depleted T cell subpopulations was checked on each occasion, either by FACS analysis or by immunoperoxidase staining of cytocentrifuged slide preparations of the depleted cells, counting at least 400 cells on each slide. Typically, normal Lewis LNCs before depletion comprised approximately 63% T cells (MRC OX19+), 33% B cells (MRC OX12+), 21% CD8 cells (MRC OX8+) and 48% CD4 cells (W3/25+). Following rosette depletion, approximately 10% of the selected cells failed to label with any of the antibodies used and were regarded as "null" cells phenotypically, while the remaining 90% were labelled

with both MRC OX19 and either MRC OX8 or W3/25. On every occasion, contaminating cells of the unwanted phenotype amounted to less than 0.5%.

Based on the finding that $1x10^7$ unseparated LNC effectively restored graft rejection, graft recipients were injected with $5x10^6$ T lymphocytes alone (LNC depleted of B cells by rosetting with MRC OX12). As shown in Table 4.4, allograft recipients rapidly rejected their grafts (MST 8 days) indicating that T cells were as effective as unseparated LNC in restoring graft rejection.

In contrast, reconstitution of irradiated recipients with negatively selected T cell subpopulations was relatively ineffective at restoring first set graft rejection (Table 4.4). An inoculum of $5x10^6$ CD4 lymphocytes (depleted of MRC OX12+ and MRC OX8+ cells) resulted in a median survival time of 24 days (range 10-38 days) with a median day 7 urea of 40 mM/L, while the same number of CD8 lymphocytes (depleted of B cells and W3/25+ cells) gave a median survival time of 20 days (range 8->50 days) with a median day 7 urea of 34 mM/L. Increasing the number of CD4 cells transferred from $5x10^6$ to $2\text{-}5x10^7$ still failed to restore optimal first set rejection (MST 28 days). Although purified T cell subsets were clearly unable to restore graft rejection to normal tempo, it could not be determined with certainty that they were unable to cause

Table 4.4 Ability of depleted LNC subpopulations to restore DA renal allograft rejection in acutely irradiated Lewis rats

CELLS TRANSFERRED (No. & phenotype)	N	SURVIVAL (days)	MST (days)	DAY 7 UREA (mM/L) (median & range)
5×10^6 T cells	5	8,8,8,9,9	8	53 (39-109)
5×10^6 CD4	5	10,13,24, 34,38	24[a]	40 (13-50)
$2\text{-}5 \times 10^7$ CD4	5	17,19,28, 30,>50	28[a]	33 (16-46)
5×10^6 CD8	7	8,9,14,20, >50,>50,>50	20[a]	34 (15-42)

Table 4.4 Irradiated (8.5 Gy) Lewis recipients received a DA renal allograft with immediate contralateral nephrectomy, and were reconstituted with an intravenous inoculum of syngeneic LNC subpopulations. LNC subpopulations were negatively selected by labelling the unwanted cell types with MAbs and rosetting them with sensitised sheep erythrocytes. Unlabelled lymphocytes were separated from rosettes by density gradient centrifugation, washed and resuspended for injection into the recipient. Graft rejection was monitored by observing recipient survival time and by measuring serum urea levels in serial blood samples.

a) Graft survival was significantly prolonged in CD4 ($p<0.01$) or CD8 ($p<0.05$), compared with T cell, reconstituted rats; (Mann-Whitney U test).

delayed graft rejection in some animals. Nevertheless, the survival data in animals reconstituted with T cell subpopulations did not show a statistically significant difference from the survival of irradiated, non-reconstituted recipients. It was therefore thought that the range of survival times and the moderately raised serum urea levels probably reflected the general ill health of the allograft recipients following acute irradiation, rather than the possibility that the dose of cells administered was a "borderline" dose, and that the scatter of survival times were simply biological variation in the response to the inocula.

Overall, these results therefore suggest that neither T cell subpopulation alone can effectively restore first set rejection to irradiated Lewis recipients of DA renal allografts.

4.7 Examination of the cellular requirements for first set renal allograft rejection in the reciprocal Lewis into DA strain combination

Experiments were also performed with a view to investigating the cellular requirements for first-set renal allograft rejection in the reciprocal Lewis into DA strain combination. This strain combination is known to be "weaker" than the DA into Lewis combination as

illustrated, for example, by its relative susceptibility to enhancement protocols. Thus, passive or active enhancement may readily produce long term survival of Lewis renal allografts in DA recipients but not vice versa (Fabre & Morris, 1972).

In an attempt to demonstrate that acute irradiation (8.5 Gy) of DA recipients prevented them from rejecting a renal allograft, six irradiated animals were transplanted with a Lewis kidney. As in the previous experiments, contralateral nephrectomy was performed at the time of transplantation. However, only one of the six animals survived for longer than 50 days and the individual survival times for the remainder were 3,3,7,8 and 12 days. Death appeared to be due to radiation induced side effects (particularly gastrointestinal syndrome) rather than graft rejection, since none of the animals developed a high (>20 mM/L) blood urea level, and histological examination of their kidney grafts after death showed no convincing evidence of graft rejection. It was concluded that irradiated DA strain rats were less able than Lewis rats to tolerate renal transplantation and immediate contralateral nephrectomy. Consequently, in a further five irradiated animals, renal transplantation was performed but contralateral nephrectomy was delayed until day 7 (although this meant performing nephrectomy on animals with a grossly reduced platelet count). As shown in Table 4.5, all animals

Table 4.5 Survival of Lewis renal allografts in acutely irradiated DA recipients

RECIP. RADS	RATS (N)	SURVIVAL (days)	MST	UREA LEVELS (mM/L, median & range) d9	d14	d28
none	6	10,10,10 10,10,10	10	89 (85-104)	-	-
8.5 Gy	5	>50,>50 >50,>50,>50	>50	nd	6 (4-19)	6 (5-12)

Table 4.5 Irradiated (8.5 Gy) and unmodified DA recipients received a Lewis renal allograft and contralateral nephrectomy was performed on day 7 after transplantation. Graft rejection was monitored by observing recipient survival time and by measuring serum urea levels in serial blood samples.

survived for longer than 50 days with no apparent rise in blood urea levels. This contrasted with six non-irradiated DA rats which all rapidly rejected their Lewis kidney grafts and died (MST 10 days) with a markedly elevated blood urea level.

Surprisingly, it was very difficult to restore the rejection response by transferring lymphocytes prepared from the lymph nodes of normal, syngeneic DA rats. Varying numbers of B cell-depleted lymphocytes were transferred without causing graft loss, although there was often a marked, but temporary rise in serum urea levels on the second day after contralateral nephrectomy (Table 4.6). This rejection episode eventually resolved spontaneously and the recipients survived for longer than 50 days when they were sacrificed, often with normal urea levels. Even the transfer of very large numbers of unseparated LNC failed to cause irreversible graft rejection, in spite of extremely high serum urea and creatinine levels. Similarly, the transfer of specifically sensitised cells did not cause graft loss except in one animal, which died on day 9 after transplantation with a moderately high urea level (Table 4.6). Clearly, there was little to be gained from studying the effects of transferring lymphocyte subpopulations if first set graft rejection could not be

Table 4.6 Inability of transferred lymphocytes to restore Lewis renal allograft rejection in acutely irradiated DA recipients

RATS (N)	CELLS TRANSFERRED (N. & cell type)	SURVIVAL (days)	MST (days)	UREA LEVELS (mM/L, med & range) d9	d14	d28
3	$1x10^7$ T cells	>50,>50 >50	>50	33 (14-40)	13 (8-24)	10 (6-10)
4	$3-5x10^7$ T cells	11, 48, >50,>50	49	56 (24-68)	17 (15-25)	12 (11-24)
3	$5-13x10^7$ LNC	>50,>50, >50	>50	59 (45-96)	23 (23-33)	17 (15-32)
4	$1-2x10^7$ sens. T cells[a]	9, >50, >50,>50	>50	52 (30-68)	36 (22-44)	26 (22-27)

Table 4.6 Irradiated (8.5 Gy) DA recipients received a Lewis renal allograft and were immediately reconstituted with an intravenous inoculum of syngeneic LNC, or T cells depleted of MRC OX12 positive cells by rosette depletion. Contralateral nephrectomy was performed on day 7 after transplantation. Graft rejection was monitored by observing recipient survival time and by measuring serum urea levels in serial blood samples.

a) Sensitised cells were prepared from DA rats that had rejected two Lewis skin grafts.

restored with relatively large numbers of unseparated naive LNC and the decision was taken to discontinue the adoptive transfer studies in this strain combination.

All subsequent experiments described in this chapter therefore refer to investigations performed in Lewis recipients of DA renal allografts.

4.8 Immunohistology of DA renal allografts in irradiated, reconstituted Lewis recipients

Cryostat sections of DA renal allografts removed from Lewis recipients on day 7 after transplantation were stained, using an indirect immunoperoxidase technique, with a range of monoclonal antibodies to rat leukocytes and to MHC class I and class II antigens. The extent of leukocyte infiltration of the grafts was determined by morphometric analysis, and the salient features are described below.

DA renal allografts undergoing unmodified rejection in non-irradiated Lewis rats showed a progressive and heavy mononuclear cell infiltrate, and associated induction of MHC class I and class II antigen expression in the graft (as determined by labelling with the MAbs MRC OX18 and MRC OX6). The phenotype of the infiltrate and pattern of

MHC induction appeared broadly similar to that already described in detail for rejecting DA kidneys in PVG recipients (chapter 3).

Non-rejecting DA kidneys obtained at days 5-7 after transplantation into irradiated (8.5 Gy) Lewis rats contained, by day 5, occasional small foci of infiltrating leukocytes scattered throughout the renal cortex which became heavier and more widespread by day 7 (20.5% area infiltrate when assessed by morphometric analysis). Although a moderate number of cells stained with MRC OX8 and/or W3/25, there was little staining with MRC OX19, suggesting that the cellular infiltrate comprised radioresistant or rapidly regenerating NK cells or macrophages. There was evidence of slight class I and class II MHC antigen induction within the grafts.

In contrast, rejecting grafts obtained from irradiated rats reconstituted with unseparated LNC contained a heavy mononuclear cell infiltrate, indistinguishable in magnitude and phenotype from grafts in non-irradiated hosts, together with a similar pattern of MHC antigen induction.

Day 7 allografts from irradiated recipients reconstituted with either CD4, CD8 or unseparated T cells showed a moderately heavy and diffuse cellular infiltrate as detected by staining with MRC OX1. Irrespective of the phenotype of the reconstituting inoculum, all kidneys contained many cells that were either MRC OX8+ or W3/25+,

but relatively few which were of the MRC OX19 phenotype, suggesting again that much of the inflammatory graft infiltrate in irradiated, reconstituted animals derived from radioresistant host NK cells and macrophages rather than from the inoculum. It is apparent from Fig 4.4 that there was very little difference in either the magnitude or the phenotype of the cellular infiltrate in these three groups although, paradoxically, there were slightly fewer MRC OX8 positive cells in the grafts of animals reconstituted with CD8 cells ($p < 0.05$). Interestingly, grafts in both groups demonstrated moderate induction of both class I and class II MHC antigens in spite of the absence of a first-set rejection response, although this MHC induction was less marked and more variable than that seen in animals restored with unseparated LNCs.

4.9 Functional repertoire of spleen cells and graft infiltrating cells from irradiated Lewis recipients of DA renal allografts

In view of the observation that reconstitution of irradiated Lewis renal allograft recipients with T lymphocyte subpopulations failed to restore first set allograft rejection in spite of the presence within the graft of a moderate cellular infiltrate, it seemed

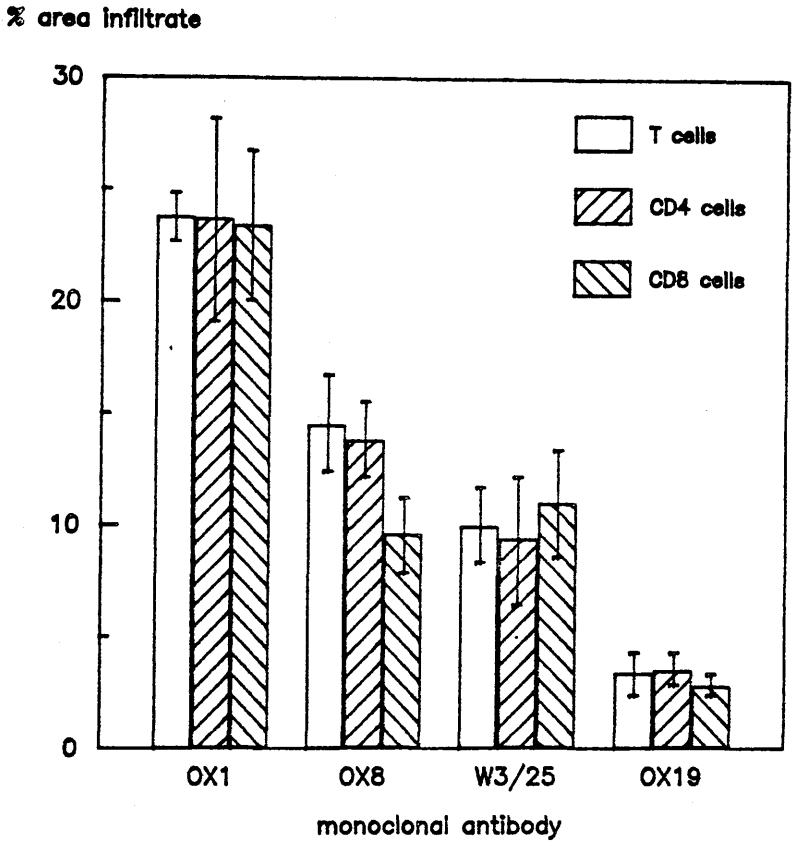


Fig. 4.4 Phenotypic analysis of the cellular infiltrate in DA renal allografts transplanted into irradiated Lewis recipients reconstituted with either CD4, CD8 or unseparated T cells.
Cryostat sections (four grafts at day 7) were labelled with MRC OX1 (all leukocytes), MRC OX8 (Tc and NK cells), W3/25 (Th and macrophages), and MRC OX19 (T cells). The % area infiltrate was determined by morphometric analysis; results are expressed as the median and range.

appropriate to investigate the functional capacity of recipient lymphoid cells to lyse donor specific target cells *in vitro*.

In the initial experiments, the spleen was used as the source of effector cells. Graft infiltrating cells were the preferred source of effector cells since the aim of the experiments was to investigate events occurring within the graft, and although in earlier experiments the grafts were used for immunohistological examination of infiltrating cells, in the later experiments, graft infiltrating cells were harvested (by enzyme digestion) and also tested for cytotoxic activity.

Cytotoxicity assays were performed as described in sections 2.9.1 and 2.9.2, using ^{51}Cr-labelled donor strain (DA) ConA blasts as the allospecific target and PVG ConA blasts as third party controls. The NK susceptible Y3 cell line was used as a nonspecific target.

4.9.1 *In vitro* cytotoxicity of splenocytes from irradiated Lewis recipients of a DA renal allograft

In an initial experiment, splenocytes obtained, at day 7, from an acutely irradiated (8.5 Gy) Lewis recipient of a non-rejecting DA renal allograft were compared with splenocytes obtained from a non-irradiated renal

allograft recipient and splenocytes from an irradiated but LNC (10^7) reconstituted graft recipient (both of which would be expected to reject their grafts).

As depicted in Fig 4.5, spleen cells from the irradiated graft recipient displayed no ability to lyse DA ConA blasts. However, the irradiated animal which had been reconstituted with 10^7 LNC showed levels of allospecific lysis comparable to those found in splenocytes from the non-irradiated graft recipient. The absence of specific cytotoxicity in splenocytes from the irradiated graft recipient was in marked contrast to the high levels of nonspecific cytotoxicity which they displayed. Fig 4.6 reveals that the levels of cytotoxicity against Y3 in irradiated rat splenocytes exceeded those shown by speen cells from either the non-irradiated graft recipient or the irradiated but LNC reconstituted recipient. This experiment was repeated with almost identical results.

The possibility that the presence of macrophages in the effector cell population was contributing to the observed specific or nonspecific cytotoxicity was investigated. On two occasions, an aliquot of the effector splenocytes from irradiated but LNC reconstituted graft recipients was treated (by incubation with carbonyl iron powder) to remove macrophages, and then compared with untreated effector cells for their ability to lyse donor specific or NK susceptible targets.

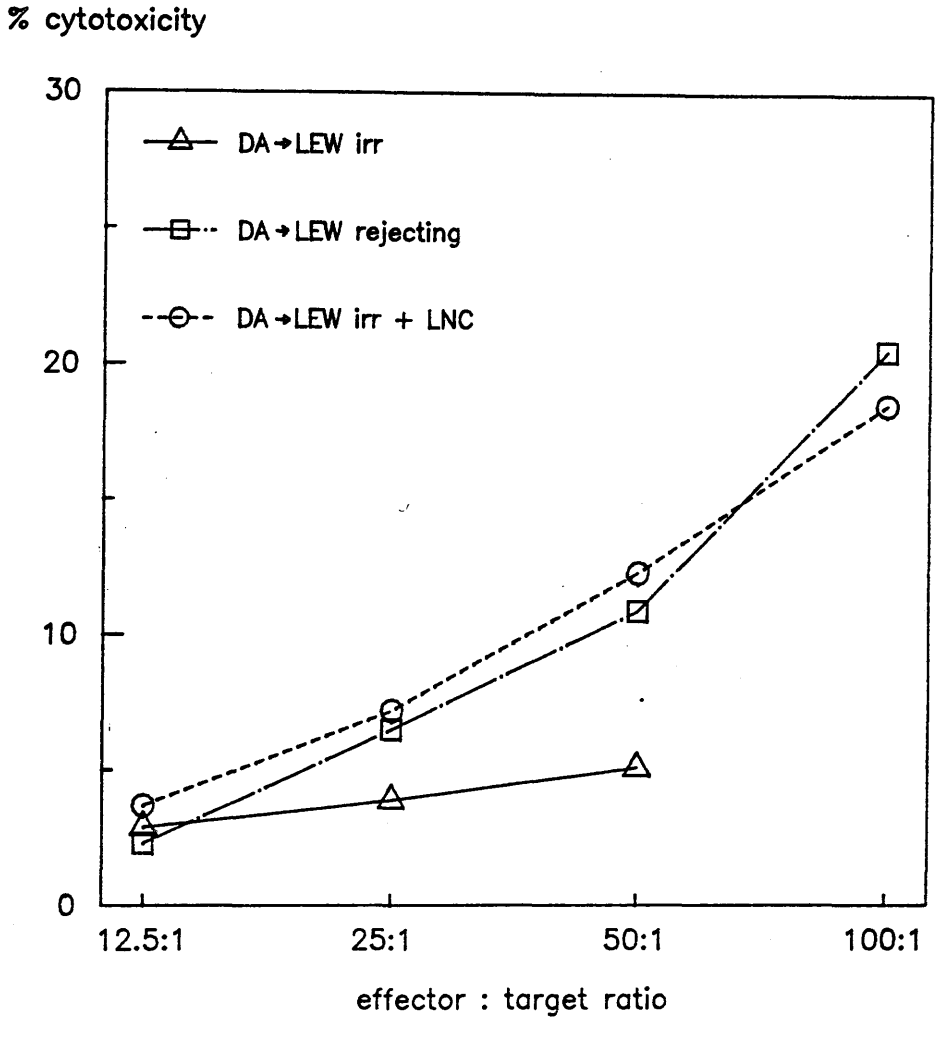


Fig. 4.5 Cytotoxic activity of splenocytes harvested on day 7 from Lewis recipients of DA renal allografts, against donor specific DA ConA blasts.
Splenocyte effector cells were prepared, with macrophage depletion, from Lewis recipients which were either unmodified (□), irradiated (Δ), or irradiated and reconstituted with 10^7 LNC (○). Insufficient cells ($<10^7$) were obtained from irradiated, unreconstituted animals to perform assays at the 100:1 effector : target ratio.

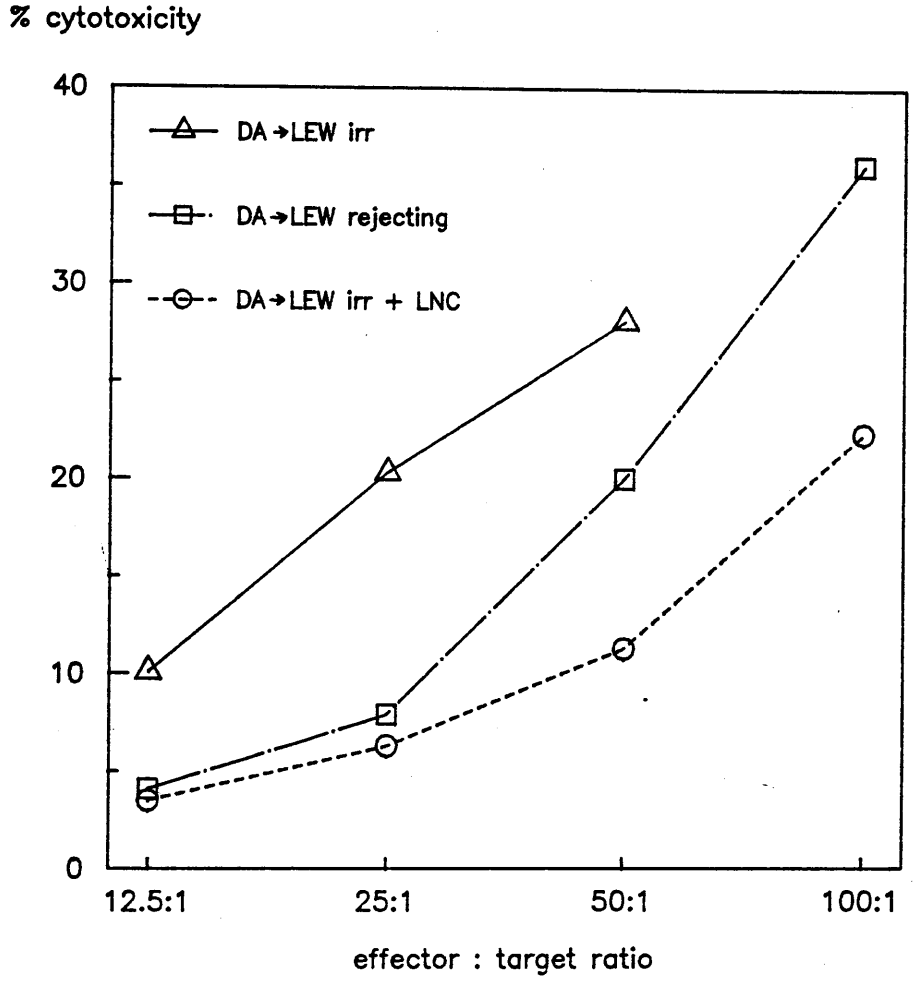


Fig. 4.6 Cytotoxic activity of splenocytes harvested on day 7 from Lewis recipients of DA renal allografts, against NK susceptible Y3 targets.
Splenocyte effector cells were prepared, with macrophage depletion, from Lewis recipients which were either unmodified (□), irradiated (△), or irradiated and reconstituted with 10^7 LNC (○). Insufficient cells ($<10^7$) were obtained from irradiated, unreconstituted animals to perform assays at the 100:1 effector : target ratio.

On neither occasion were the levels of cytotoxicity reduced, and in one experiment they were slightly raised; in subsequent experiments, effector splenocytes were routinely macrophage depleted.

4.9.2 Cytotoxic activity of splenocytes obtained from irradiated allograft recipients reconstituted with LNC subpopulations

On a number of occasions, experiments were performed in which three Lewis rats were simultaneously irradiated (8.5 Gy) then transplanted with a DA kidney. In each experiment, one of the transplanted animals was then reconstituted with $1x10^7$ unseparated LNC (as a positive control) and the other two were reconstituted with $1x10^7$ cells of purified lymphocyte subpopulations, either T cells or CD4 cells or CD8 cells. On day 7, the spleen cells of each animal were tested for alloantigen specific and nonspecific cytotoxicity.

4.9.2.1 Reconstitution with T cells

Purified T lymphocytes were prepared by rosette depletion of LNC using MRC OX12. The ability of spleen cells, from the three reconstituted graft recipients, to lyse DA ConA blasts is shown in Fig 4.7. The two T cell reconstituted animals demonstrated high levels of cytotoxicity which were comparable to those seen in the LNC reconstituted animal. This cytotoxicity was

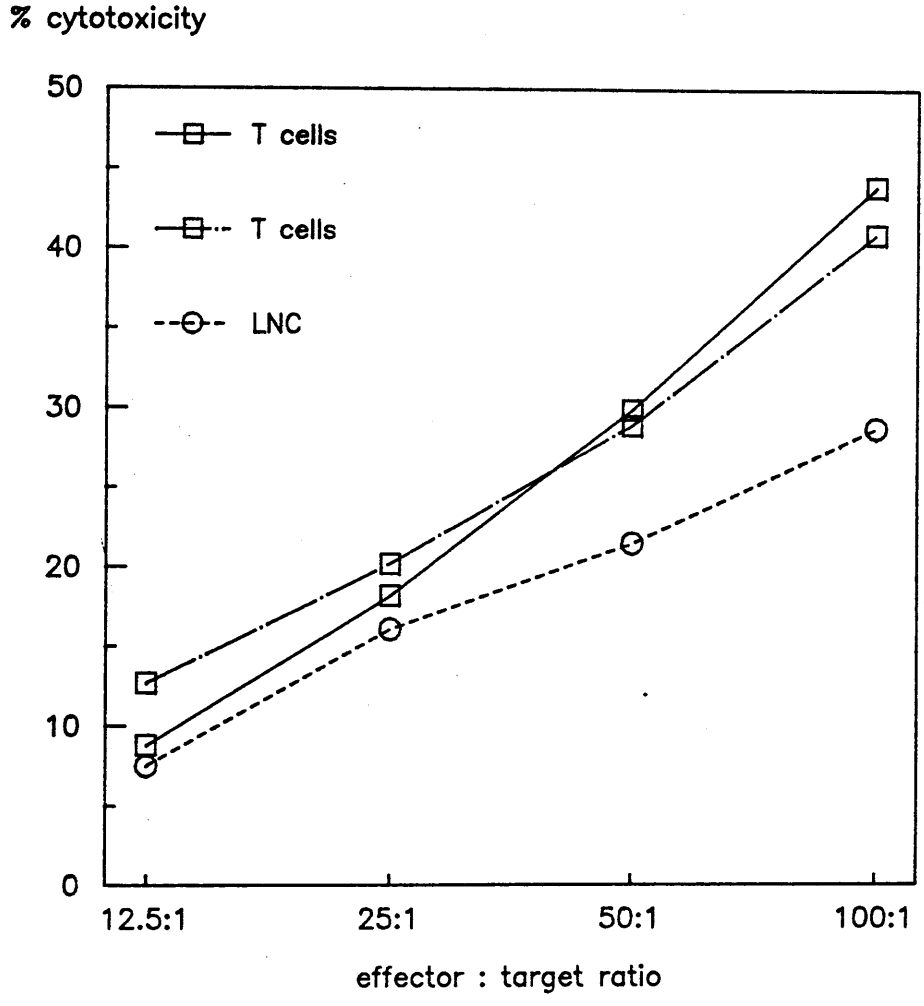


Fig. 4.7 Cytotoxic activity of splenocytes harvested on day 7 from irradiated, reconstituted Lewis recipients of DA renal allografts, against donor specific DA ConA blast targets.
Splenocyte effector cells were prepared, with macrophage depletion, from Lewis recipients reconstituted with 10^7 cells, either T cells (□) or unseparated LNC (○).

allospecific, since there was no detectable lysis of third party PVG ConA blasts (results not shown). Spleen cells from all three animals also showed similarly high levels of nonspecific cytotoxicity against Y3 targets (Fig 4.8). This experiment was repeated with the same results.

4.9.2.2 Reconstitution with CD4 cells

In this experiment, two of the irradiated, allografted Lewis recipients were reconstituted with CD4 LNC depleted of both B cells and CD8 cells, and one animal received unseparated LNC. Spleen cells obtained at day 7 from the two CD4 reconstituted animals showed minimal levels of specific lysis of DA ConA blasts when compared with spleen cells from the third animal (Fig 4.9). In contrast, splenocytes from all animals demonstrated high levels of nonspecific cytotoxicity against the Y3 target. This experiment was repeated with identical results.

4.9.2.3 Reconstitution with CD8 cells

In two further experiments, one irradiated graft recipient was reconstituted with LNC and the other two received CD8 cells prepared from LNC depleted of B cells and CD4 cells. Spleen cells from the two CD8 reconstituted animals showed only modest levels of lysis of DA ConA blasts when compared with splenocytes from

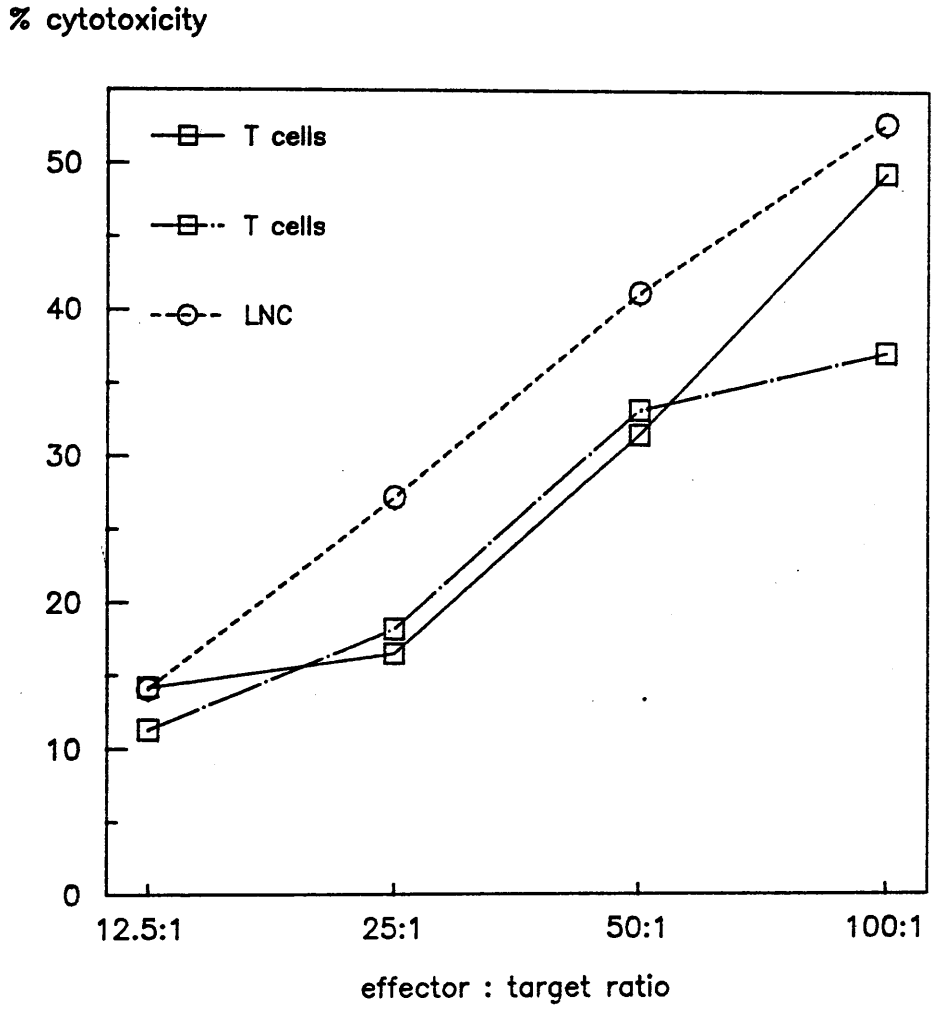


Fig. 4.8 Cytotoxic activity of splenocytes harvested on day 7 from irradiated, reconstituted Lewis recipients of DA renal allografts, against NK susceptible Y3 targets.
Splenocyte effector cells were prepared, with macrophage depletion, from Lewis recipients reconstituted with 10^7 cells, either T cells (□) or unseparated LNC (○).

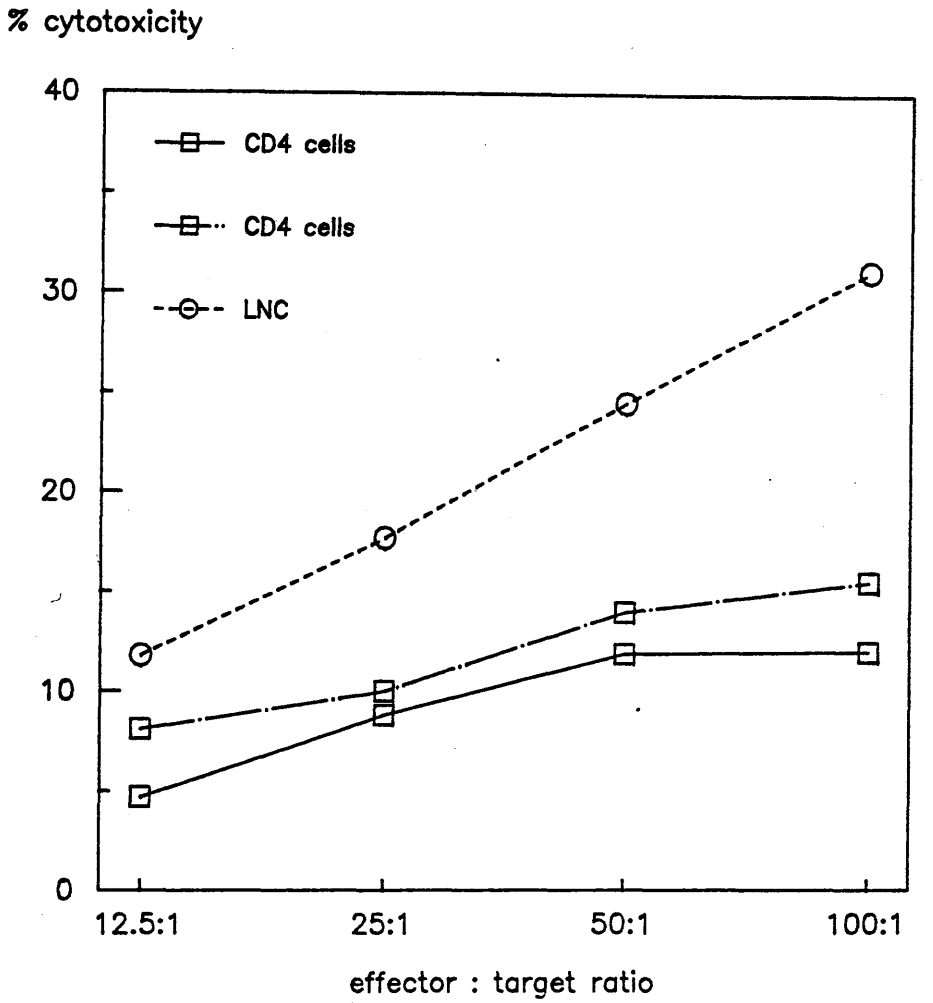


Fig. 4.9 Cytotoxic activity of splenocytes harvested on day 7 from irradiated, reconstituted Lewis recipients of DA renal allografts, against donor specific DA ConA blast targets.
Splenocyte effector cells were prepared, with macrophage depletion, from Lewis recipients reconstituted with 10^7 cells, either CD4 cells (□) or unseparated LNC (○).

the LNC reconstituted animals (Fig 4.10). As before, nonspecific cytotoxicity against Y3 was the same in all animals, and the two experiments gave the same results.

4.9.3 Cytotoxic activity of graft infiltrating cells and splenocytes from irradiated allograft recipients reconstituted with LNC subpopulations

The following experiments were performed in order to demonstrate that any observed differences in specific cytotoxicity in splenocytes from reconstituted animals were entirely due to true functional differences between the injected cell subpopulations and not artifacts of the methods used for cell separation. Two aliquots of LNCs were simultaneously depleted of B cells and either CD4 cells or CD8 cells. In two identical experiments, three irradiated Lewis recipients of DA renal allografts were reconstituted with $1x10^7$ cells which were either CD4 cells alone, CD8 cells alone, or a mixture of equal numbers of each. Animals were sacrificed on day 7 and both splenocytes and graft infiltrating cells were tested for in vitro cytotoxicity. The results of a representative experiment are shown in Figs 4.11 and 4.12, and summaries of a total of three separate experiments are shown in Tables 4.7 and 4.8.

As predicted from the previous experiments, spleen cells from animals reconstituted with a mixture of CD4 and CD8 lymphocytes showed high levels of specific

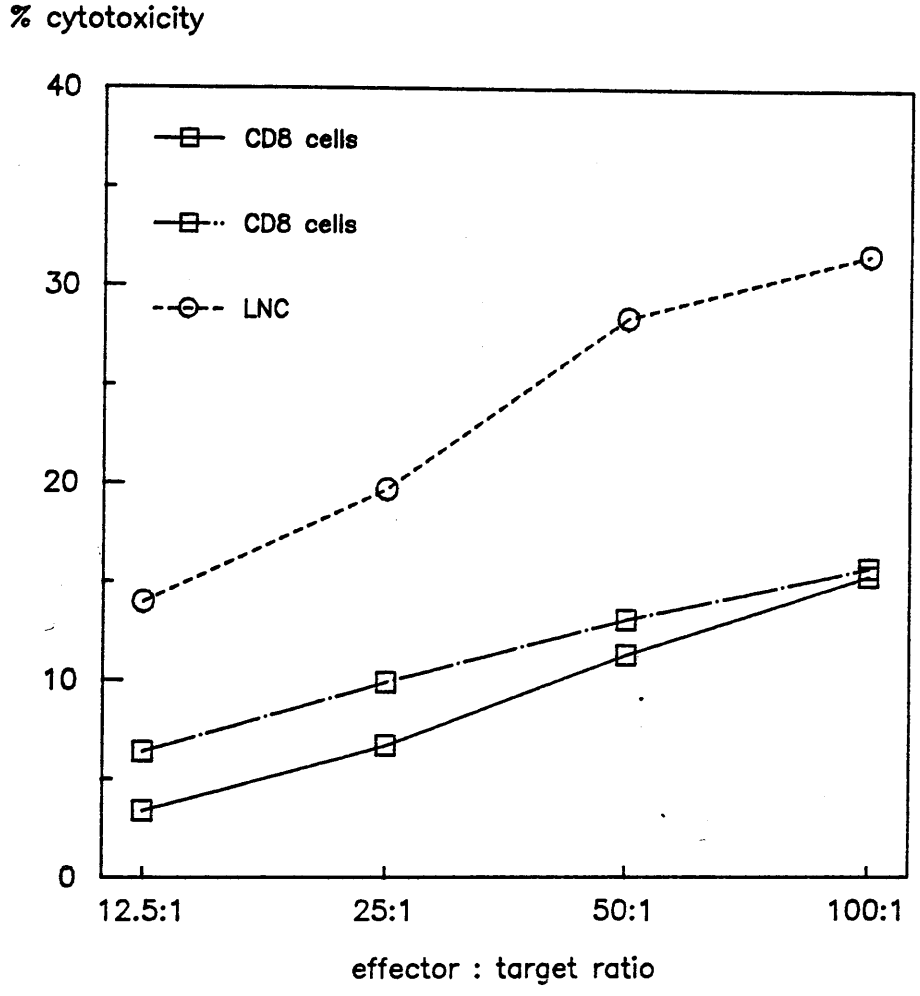


Fig. 4.10 Cytotoxic activity of splenocytes harvested on day 7 from irradiated, reconstituted Lewis recipients of DA renal allografts, against donor specific DA ConA blast targets.
Splenocyte effector cells were prepared, with macrophage depletion, from Lewis recipients reconstituted with 10^7 cells, either CD8 cells (□) or unseparated LNC (○).

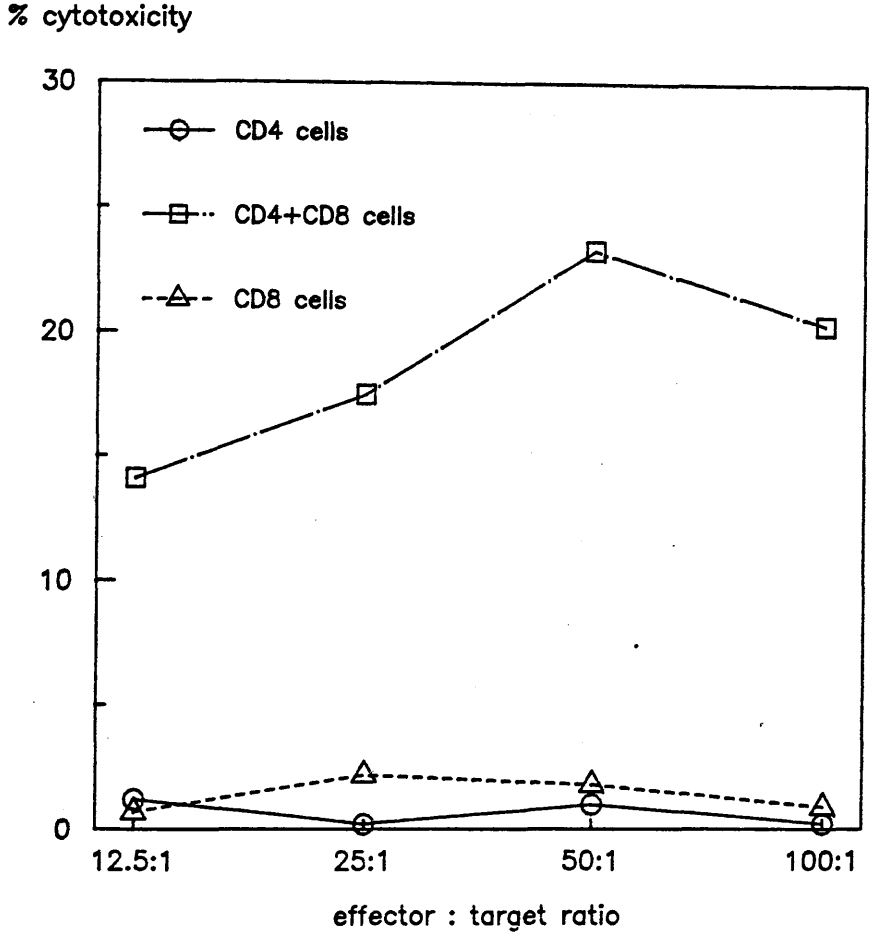


Fig. 4.11 Cytotoxic activity of splenocytes harvested on day 7 from irradiated, reconstituted Lewis recipients of DA renal allografts, against donor specific DA ConA blast targets.
Splenocyte effector cells were prepared, with macrophage depletion, from Lewis recipients reconstituted with 10^7 cells, either CD4 cells (O) or CD8 cells (Δ) or an equal mixture of CD4+CD8 cells(□).

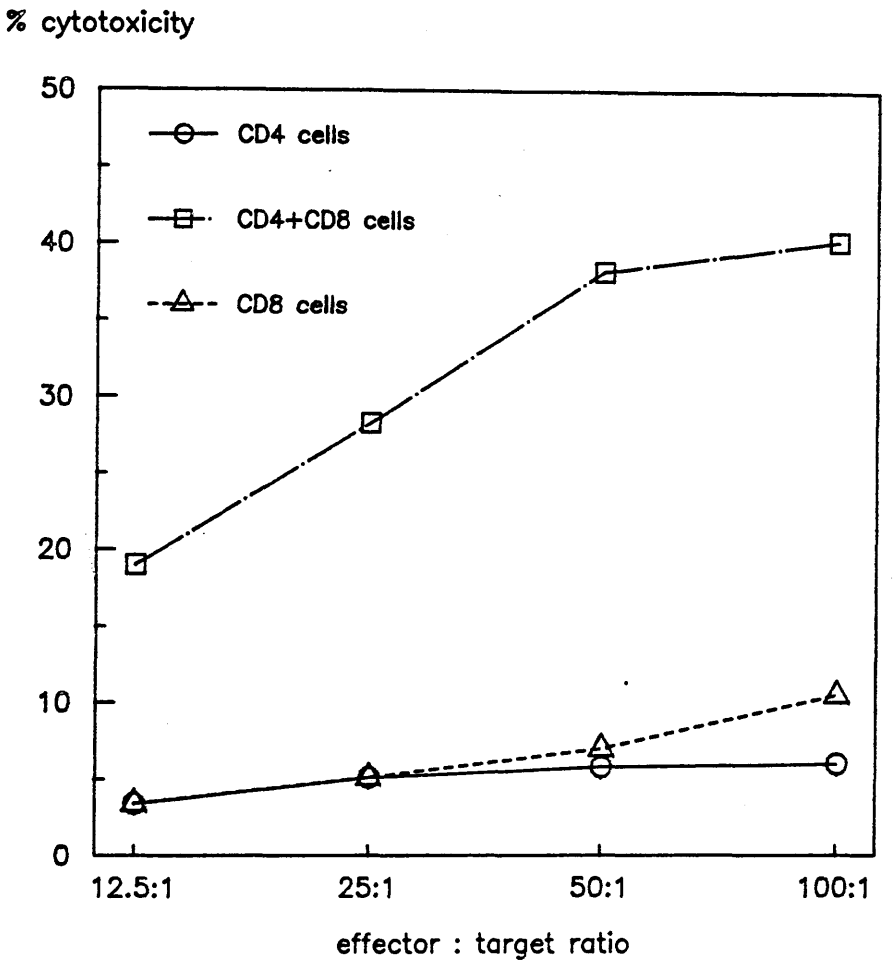


Fig. 4.12 Cytotoxic activity of graft infiltrating cells harvested on day 7 from irradiated, reconstituted Lewis recipients of DA renal allografts, against donor specific DA ConA blast targets.
Graft infiltrating effector cells were extracted, by enzyme digestion of the graft, from Lewis recipients reconstituted with 10^7 cells, either CD4 cells (○) or CD8 cells (△) or an equal mixture of CD4+CD8 cells(□)

Table 4.7 Specific and nonspecific cytotoxic activity of spleen cells obtained from irradiated, reconstituted Lewis recipients of DA renal allografts

RECONSTITUTING CELLS	% CYTOTOXICITY (E:T RATIO 50:1) AGAINST VARIOUS TARGETS		
	DA ConA blasts	PVG ConA blasts	Y3 cell line
$5x10^6$ CD4 + $5x10^6$ CD8 LNC	44% (39%,38%,56%)	<1% (0%,2%,0%)	22% (12%,28%,27%)
$1x10^7$ CD8 LNC	13% (11%,7%,22%)	<1% (0%,0%,0%)	33% (13%,29%,57%)
$1x10^7$ CD4 LNC	5% (1%,6%,7%)	<1% (0%,2%,0%)	29% (13%,32%,42%)

Table 4.7 Spleen cells were harvested on day 7 after transplantation and tested for their ability to lyse chromium-labelled target cells as described in chapter 2. Results are expressed as the mean and individual values for cytotoxicity levels at E:T ratio 50:1 obtained from three separate experiments.

Table 4.8 Specific and nonspecific cytotoxic activity of graft infiltrating cells from irradiated, reconstituted Lewis recipients of DA renal allografts

RECONSTITUTING CELLS	% CYTOTOXICITY (E:T RATIO 50:1) AGAINST VARIOUS TARGETS		
	DA ConA blasts	PVG ConA blasts	Y3 cell line
$5x10^6$ CD4 + $5x10^6$ CD8 LNC	21% (23%,24%,15%)	<1% (0%,0%,0%)	3% (4%,4%,1%)
$1x10^7$ CD8 LNC	3% (0%,2%,7%)	<1% (0%,0%,0%)	3% (0%,4%,6%)
$1x10^7$ CD4 LNC	2% (0%,1%,5%)	<1% (0%,0%,0%)	4% (1%,4%,8%)

Table 4.8 Graft infiltrating cells were harvested (using enzymes) on day 7 after transplantation and tested for their ability to lyse chromium-labelled target cells as described in chapter 2.
Results are expressed as the mean and individual values for cytotoxicity levels at E:T ratio 50:1 obtained from three separate experiments.

cytotoxicity against DA ConA blasts but no ability to lyse PVG ConA blasts, indicating the allospecific nature of the cytotoxicity (Fig 4.11 and Table 4.7). In contrast, splenocytes from animals reconstituted with either subpopulation alone showed low levels of allospecific cytotoxicity. As before, splenocytes from all irradiated graft recipients showed high levels of nonspecific cytotoxicity against Y3 (Fig 4.13).

Mononuclear cells harvested from rejecting DA allografts obtained from irradiated recipients reconstituted with a mixture of CD4 and CD8 lymphocytes demonstrated moderate levels of cytotoxic activity against DA ConA blasts and no lysis of PVG ConA blasts (Fig 4.12 and Table 4.8). In contrast, graft infiltrating cells obtained from animals reconstituted with either CD4 or CD8 cells alone showed no ability to lyse DA ConA blasts.

When graft infiltrating cells were tested against the nonspecific target Y3, the findings were notably different from those observed with spleen cells from the same animals. None of the graft infiltrating cells, including those from the rejecting grafts in (CD4+CD8) reconstituted animals, showed any detectable lysis.

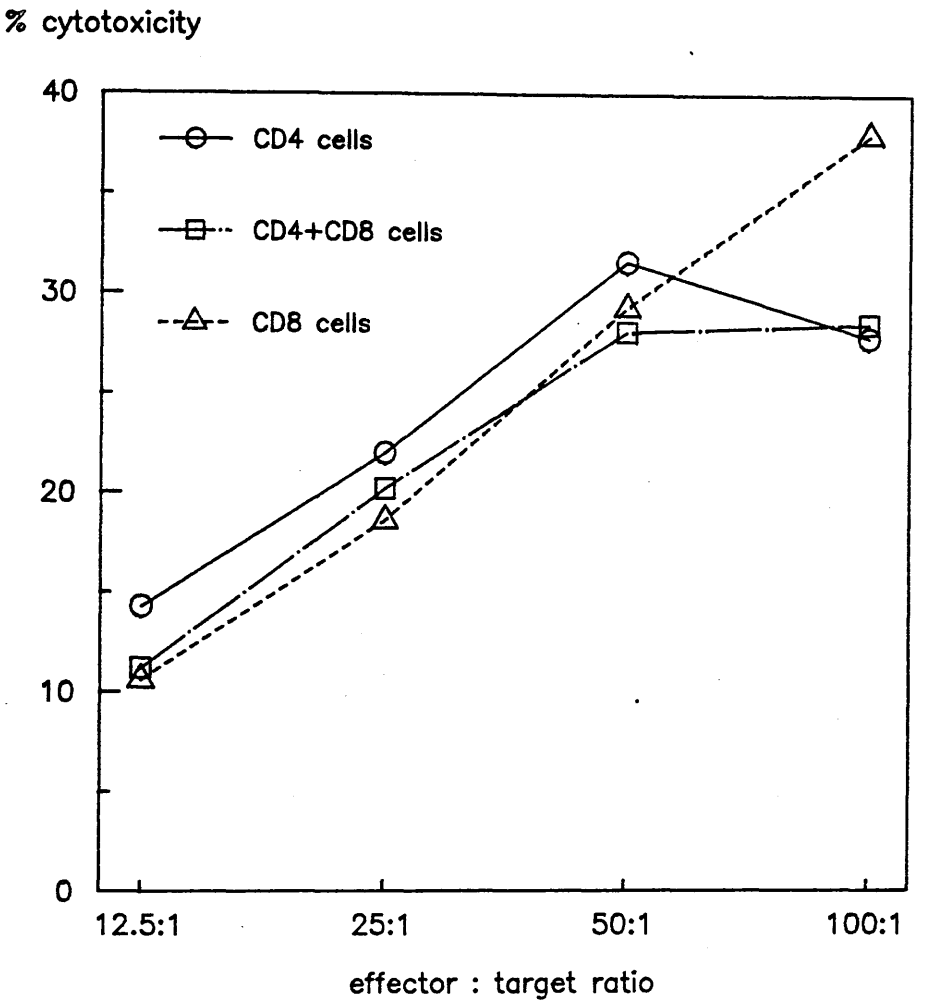


Fig. 4.13 Cytotoxic activity of splenocytes harvested on day 7 from irradiated, reconstituted Lewis recipients of DA renal allografts, against NK susceptible Y3 targets.
Splenocyte effector cells were prepared, with macrophage depletion, from Lewis recipients reconstituted with 10^7 cells, either CD4 cells (o) or CD8 cells (Δ) or an equal mixture of CD4+CD8 cells (□).

4.10 Discussion

The experiments described in this chapter sought to determine the ability of non-sensitised CD4 and CD8 lymphocyte subpopulations to restore renal allograft rejection when adoptively transferred to T cell depleted recipients. It was initially planned to use the ATXBM rat as the T cell deficient host, on the basis that T lymphocytes are essential for graft rejection and ATXBM rats are T cell deficient, although they have a normal complement of B lymphocytes. Because they also have relatively normal levels of platelets and other bone marrow derived leukocytes, ATXBM rats are generally healthier than sublethally irradiated (but not bone marrow reconstituted) animals. Hence they would be expected to tolerate the surgical procedure of renal transplantation well and their subsequent survival and blood urea levels would give a good indication of graft rejection. The inability of ATXBM animals to reject a renal allograft was confirmed, as expected, but it became apparent that very large numbers of lymphocytes ($>10^8$ LNC) would be necessary to consistently restore first set rejection. It has been reported that as few as $2x10^5$ thoracic duct lymphocytes caused rejection of, or necrotic changes in, skin grafts in ATXBM rats, and $5x10^7$ TDL consistently restored the skin graft rejection response to normal (Dallman, Mason &

Webb, 1982). This may be an indication of the particular susceptibility of skin allografts to rejection, since other investigators have reported that $1x10^8$ specifically sensitised lymphocytes are necessary to restore heart allograft rejection in ATXBM recipient rats (Clason, Duarte, Kupiec-Weglinski et al, 1982; Lear, Heidecke, Kupiec-Weglinski et al, 1983). The discrepancy observed in the cell numbers required to restore fist set organ allograft rejection in ATXBM recipients compared with acutely irradiated recipients in both the present experiments and previous reports suggests the induction, in ATXBM recipients, of suppressor pathways, although no attempt was made to confirm this suggestion in this thesis. Clason et al (1982) used only 7.5 Gy to prepare ATXBM rats following thymectomy, thus there would undoubtedly have been extensive host bone marrow regeneration by the time the animals were grafted, although the lymphocytes would not, of course, be thymically processed. These authors reported that they had been unable to detect the presence, in nonrejecting ATXBM allograft recipients, of either suppressor cells, blocking antibodies, or of reduced graft immunogenicity. Instead, they suggested that the requirement for adoptive transfer of large numbers of cells to restore graft rejection might be accounted for by altered migration patterns of injected lymphocytes in ATXBM rats. This interesting suggestion

may be worthy of further investigations.

As an alternative to ATXBM animals, it was decided to use rats which had received a heavy but sublethal dose of whole body gamma irradiation as T cell deficient hosts. This model has been used by several other groups (see Appendix A) with reproducible results. Preliminary experiments in non-transplanted animals confirmed the rapid and dramatic reduction of circulating lymphocyte levels (>98% reduction) after irradiation, although by two weeks there was evidence of bone marrow recovery and replenishment of circulating leukocyte levels - indicating the long-term limitations of this particular model. Inevitably, acutely irradiated animals were less healthy than ATXBM animals, since a near lethal dose of irradiation was necessary to completely ablate any detectable graft rejection response (8.5 Gy in this study). Consequently, monitoring renal allograft rejection in acutely irradiated rats on the basis of recipient survival is less satisfactory since it is sometimes difficult, even with serial blood urea measurements, to ascertain whether early deaths after transplantation are due predominantly to rejection and are not radiation induced.

Nevertheless, within these constraints it was possible, using the DA into Lewis acute irradiation model, to clearly demonstrate that relatively small numbers of syngeneic unsensitised lymphocytes ($0.5\text{-}1x10^7$ LNC)

were sufficient to consistently restore rejection in a tempo comparable with that seen in non-irradiated hosts. Conversely, the inability of even large numbers of adoptively transferred lymphocytes in the reciprocal Lewis into DA strain combination provided convincing evidence of the relatively weak response of DA ($RT1^a$) recipients to Lewis ($RT1^l$) alloantigens, presumably as a result of Ir gene control. This example emphasises the importance of interpreting the results of adoptive transfer experiments of this kind, in the context of the particular strain combination of animals used.

The results of adoptive transfer experiments in irradiated Lewis recipients of DA renal allografts confirmed that LNC which had been depleted of B lymphocytes were as effective as unseparated LNC in restoring first-set rejection - a finding which was in agreement with previous reports for first-set heart allograft rejection (Hall et al, 1983). However, when equivalent numbers of purified CD4 or CD8 lymphocytes were tested for their ability to restore rejection, neither subpopulation tested was able, at the cell numbers used, to restore rejection in first-set tempo. This was only achieved when both CD4 and CD8 cells were transferred simultaneously, suggesting that cellular cooperation between the CD4 and CD8 subsets occurred in order to cause rejection - a mechanism which is

apparently similar to the cellular cooperation required for the efficient generation in vitro of specific cytotoxic cells in fully allogeneic MLRs (Bach et al, 1976).

Interestingly, increasing the number of adoptively transferred CD4 cells by 4- to 10-fold over the number of unsparated T cells that caused rapid rejection still failed to initiate first-set graft rejection. This observation contrasts with that of Hall et al (1983) who found that purified unsensitised CD4 cells alone restored first-set rejection of heart allografts in acutely irradiated recipients.

The immunohistological studies of allografts in irradiated recipients provided evidence that radioresistant or rapidly regenerating host cells are found in allografts and may be involved in the rejection response. Although non-rejecting grafts in irradiated hosts showed only a sparse cellular infiltrate, adoptive transfer of either CD4 or CD8 cells alone increased the magnitude of the infiltrate and irrespective of which population had been used for reconstitution, there were many cells of both MRC OX8+ and W3/25+ phenotypes, which appeared to be predominantly MRC OX8 positive NK cells and W3/25 positive macrophages. Rejecting grafts from animals reconstituted with both T cell subsets showed a heavy mononuclear cell infiltrate, which was similar in magnitude and phenotype to that observed in allografts

undergoing unmodified rejection (chapter 3) and it was considered likely that the majority of these cells were secondarily recruited host cells, although their degree of involvement is difficult to assess.

As shown by the *in vitro* cytotoxicity results, acute irradiation of unreconstituted allograft recipients effectively eliminated cells with allospecific cytotoxicity from the spleen, although nonspecific cytotoxic activity appeared to be radioresistant. However, the principle observation from the functional analysis was that graft rejection, in T cell reconstituted rats, correlated with the presence, in both the spleen and the graft, of cells which displayed allospecific cytotoxicity. This contrasted with absent or only low levels (in the CD8 and CD4 reconstituted animals respectively) of allospecific cytotoxicity in the spleens and non-rejecting grafts of animals reconstituted with T cell subsets.

The presence of cells in rejecting grafts of T cell reconstituted recipients which showed allospecific cytotoxicity contrasted sharply with the virtual absence of graft infiltrating cells showing nonspecific cytotoxicity. Collectively, these findings are consistant with the suggestion that specific cytotoxic T cells are involved in renal allograft rejection but that generation of an optimal allospecific cytotoxic response in this model requires T-T cell collaboration. The presence of

non-specific cytotoxic cells (presumably NK cells) within the rejecting graft does not appear to be an essential requirement for graft rejection - a finding which is consistent with the observation of Heidecke et al, that the elimination of NK cells by in vivo treatment with anti-asialo GM1 does not abrogate the ability of rats to reject a heart allograft (Heidecke et al, 1985).

As already noted, the absence of cells showing nonspecific cytotoxicity in the rejecting grafts of T cell reconstituted animals occurred despite the presence within the grafts of numerous cells with a phenotype (MRC OX8 positive, MRC OX19 negative) consistent with that of NK cells. This suggests that these cells, which are presumably host derived, do not have normal function despite their ability to migrate to the graft in the presence of rejection.

CHAPTER FIVE

EFFECTOR MECHANISMS IN THE PATHOGENESIS OF THE LOCAL RENAL GRAFT VERSUS HOST REACTION

5.1 Introduction

A striking demonstration of the ability of alloreactive lymphocytes to mediate in vivo renal parenchymal damage is provided by the unidirectional renal graft-versus-host reaction (GVHR) in the rat, as originally described by Elkins (1964, 1966). When parental strain lymphocytes are injected into the renal subcapsular site of F1 recipients, they do not readily migrate from the site of inoculation, and hence mediate their effects on the subjacent renal parenchyma. By day 7, they produce an easily observable invasive destructive lesion of the renal parenchyma, with mononuclear cell infiltration and local tissue necrosis. The histopathology of this response is broadly similar to that of acute renal allograft rejection which, as already shown, is characterised by heavy mononuclear cell infiltration and destruction by alloreactive lymphocytes.

A particular advantage of the renal GVH model is that it provides a method whereby allogeneic lymphocyte subpopulations can be tested for the ability to cause in vivo tissue damage in the presumed absence of any contributory specific effector response from the host, since the F1 recipient would not be expected to mount a response against either the injected parental cells or its own kidney. The experiments described in this chapter were performed in order to investigate further

the cellular basis of the local renal GVHR because of its possible relevance to the effector mechanisms of graft rejection.

5.2 Ability of parental LNC to mediate a potent renal GVHR in F1 recipients.

For the purposes of the experiments described in this chapter, parental LNC were prepared from pooled cervical and mesenteric lymph nodes and 1-5x10^7 lymphocytes in a volume of 0.1 ml DAB/FCS were injected under the left renal capsule of F1 recipients, as described in Chapter 2. Seven days later, the recipient was sacrificed and the presence or absence of a renal GVHR was determined by both gross and histological evaluation of the injected kidney. In addition, the weights of the injected and the noninjected contralateral kidneys were expressed as a ratio (Ki/Kc). The results were invariably unequivocal in that, depending on the particular experiment, the injected cells either produced no histological evidence of a GVHR, and the Ki/Kc ratio was 1.0, or there was an easily recognisable renal GVHR, with an associated increase in Ki/Kc.

Injection of 10^7 Lewis cells under the kidney capsule of (LEWxDA)F1 recipients resulted, by day 7, in the development of a large GVHR lesion in all animals.

Fig 5.1 shows a typical renal GVHR lesion involving a large part of the injected kidney, with the contralateral, uninjected kidney for comparison. Histological examination revealed extensive cellular infiltration and renal tubular destruction, and this was associated with a significant increase in the weight of the injected kidney (Table 5.1). This experiment was repeated with PVG LNC injected into (DAxPVG)F1 recipients and this again resulted in a grossly observable renal GVHR in all animals (Table 5.1).

When F1 recipients were given a heavy dose of whole body irradiation (10Gy) prior to renal subcapsular injection of LNC, the renal GVH response was entirely abrogated. This confirmed the observations of Elkins (1966), and indicated the essential participation of host radiosensitive cells which were, presumably, bone-marrow derived (Table 5.1).

5.3 Ability of lymphocyte subpopulations to mediate the renal GVHR

The ability of different parental lymphocyte subpopulations to mediate a renal GVHR in F1 recipients was investigated by preparing highly purified lymphocyte subpopulations from LNC by rosette depletion, as described in Chapter 2. The results are shown in Table

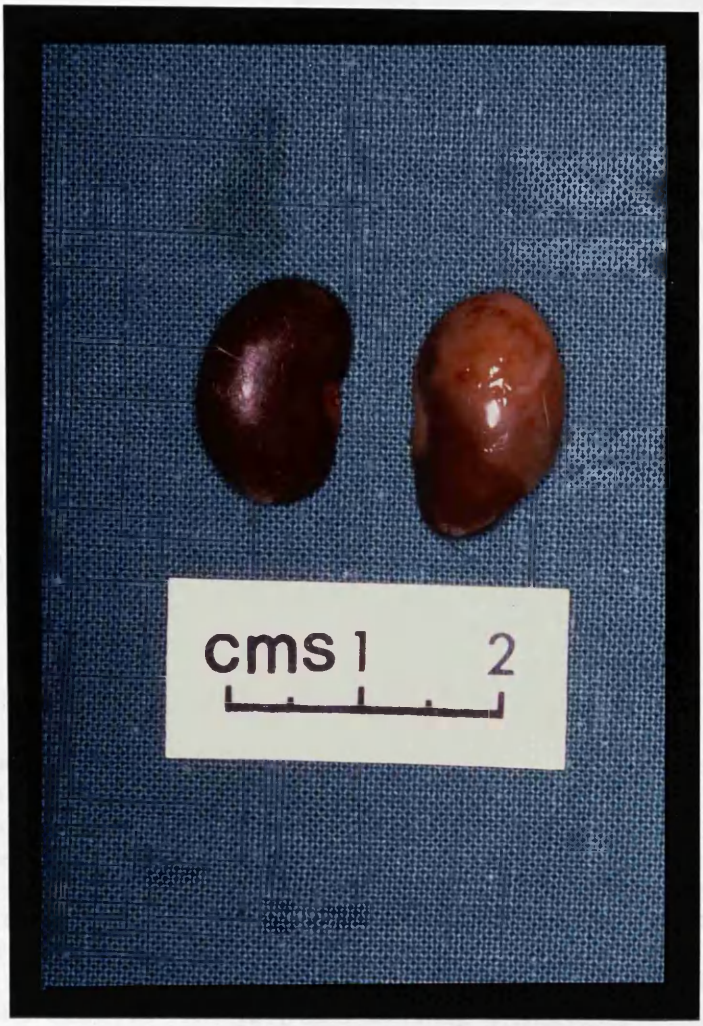


Fig. 5.1 Demonstration of renal GVHR lesion at day 7 in F1 kidney injected with parental lymphocytes, compared with non-injected contralateral kidney.
A typical renal GVHR lesion is grossly observable, and results in a significant increase in the weight of the injected kidney.

Table 5.1. Ability of parental LNC to mediate a local renal GVHR in F1 recipients

DONOR LNC (10^7 cells)	RECIPIENT STRAIN	IRRADIATION OF HOST	GVH LESIONS/ ANIMALS	Ki/Kc[a]
LEWIS	(LEWxDA)F1	none	4/4	1.32 (1.2-1.43)[b]
PVG	(DAxPVG)F1	none	8/8	1.30 (1.21-1.57)[c]
LEWIS	(LEWxDA)F1	10 Gy	0/3	1.03 (1.0-1.11)

Table 5.1 $1x10^7$ parental strain LNC were injected into the renal subcapsular site of F1 recipient rats. On day 7, evidence of a renal GVHR was sought by gross and histological examination of the injected kidney. In addition, the weights of the injected (Ki) and contralateral (Kc) were determined and expressed as a ratio Ki/Kc. The Ki/Kc ratio in experimental groups was compared with the Ki/Kc in control F1 animals injected with $1x10^7$ syngeneic F1 LNC (median Ki/Kc = 1.0, range 0.99-1.03). P values are shown for groups with a significant increase in Ki/Kc (Mann-Whitney U test).

a) Ki/Kc ratio expressed as median and range.

b) $p<0.02$

c) $p<0.005$

5.2. As expected, Lewis LNC depleted of B cells were as effective as unseparated LNC in causing a renal GVHR when injected into (LEWxDA)F1 recipients. When the T cell subpopulations were tested, it was found that Lewis CD4 cells were also effective in mediating a renal GVHR. However, injection of Lewis CD8 cells into the renal subcapsular site of (LEWxDA)F1 recipients failed to produce a detectable renal GVHR (Fig 5.2). Specifically sensitised Lewis CD8 cells (prepared from lymph node donors that had previously rejected two full thickness (LEWxDA)F1 skin grafts) were also tested and found to be ineffective at provoking a renal GVHR. These experiments were repeated by testing PVG lymphocyte subpopulations in (DAxPVG)F1 recipients and the same pattern of results was obtained (Table 5.2).

It was apparent from these experiments that the CD4 lymphocyte subpopulation alone was responsible for causing the renal GVHR, and it therefore seemed likely that cells in the inoculum were responding to class II alloantigens expressed by renal parenchymal cells and/or bone-marrow derived cells within the recipient kidney. Two possible mechanisms whereby CD4 cells might cause parenchymal damage are considered. Firstly, damage may be the result of a classical DTH type reaction in which activated class II restricted CD4 cells release lymphokines and tissue damage is due to nonspecific inflammatory cells. Secondly, parenchymal damage may be

Table 5.2. Ability of LNC subpopulations to mediate a local renal GVHR (10^7 cells injected)

P DONOR STRAIN	LNC SUBGROUP	F1 RECIPIENT STRAIN	GVH LESIONS/ ANIMALS	Ki/Kc[a]
LEWIS	T cells	(LEWxDA)F1	4/4	1.32 (1.21-1.42)[b]
LEWIS	CD4	(LEWxDA)F1	6/6	1.23 (1.07-1.34)[c]
LEWIS	CD8	(LEWxDA)F1	0/6	1.00 (0.98-1.0)
LEWIS	CD8[d] (sensitised)	(LEWxDA)F1	0/3	0.94 (0.94-1.04)
PVG	CD4	(DAxPVG)F1	4/4	1.19 (1.14-1.3)[b]
PVG	CD8	(DAxPVG)F1	0/3	1.02 (1.0-1.03)

Table 5.2 $1x10^7$ parental strain lymphocytes (negatively selected by rosette depletion) were injected into the renal subcapsular site of F1 recipient rats. On day 7, evidence of a renal GVHR was sought by gross and histological examination of the injected kidney. The Ki/Kc ratio in experimental groups was compared with the Ki/Kc in control F1 animals injected with syngeneic F1 LNC. P values are shown for groups with a significant increase in Ki/Kc (Mann-Whitney U test).

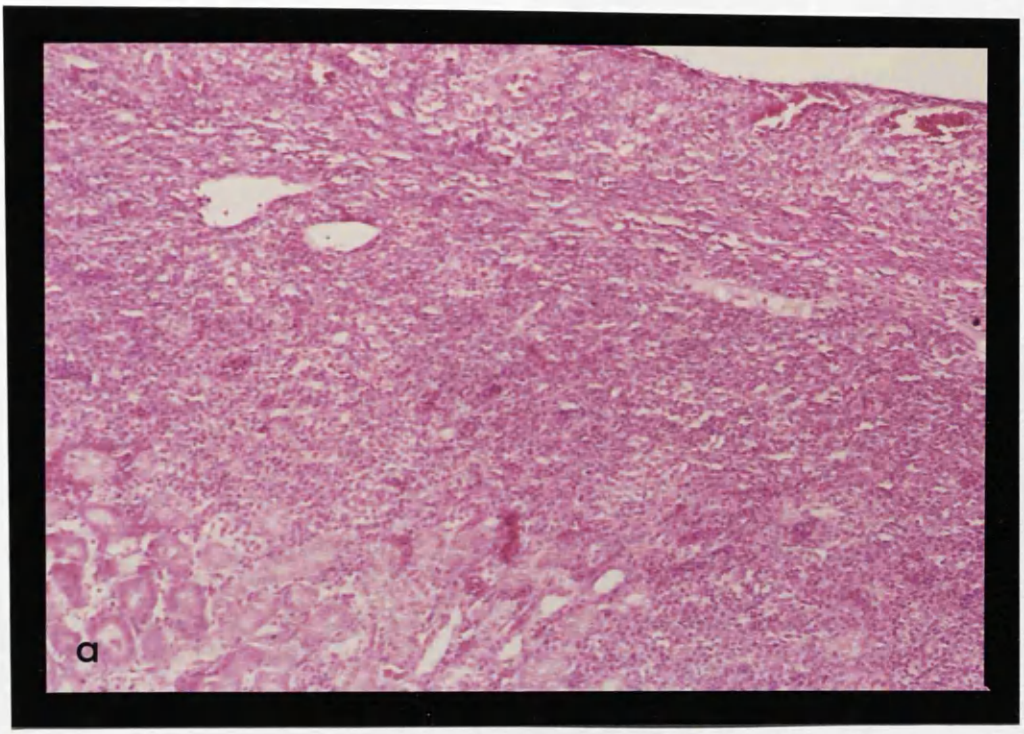
a) Ki/Kc ratio expressed as median and range.

b) $p<0.02$

c) $p<0.005$

d) Lewis donors sensitised by two DA skin grafts.

Fig. 5.2 Histological comparison of F1 kidneys injected with parental CD4 cells (a & c) or with CD8 cells (b).
a) Section through a renal GVHR lesion induced by the subcapsular injection of CD4 cells, showing extensive parenchymal destruction. (Day 7, H&E, x100).
b) Section through the site of renal subcapsular injection of CD8 cells, with the injected cells clearly visible in a layer beneath the capsule, but no evidence of parenchymal damage. (Day 7, H&E, x100).
c) High power view of the lesion produced by CD4 cells, showing damaged renal parenchyma but relatively well preserved glomeruli. (H&E, x160).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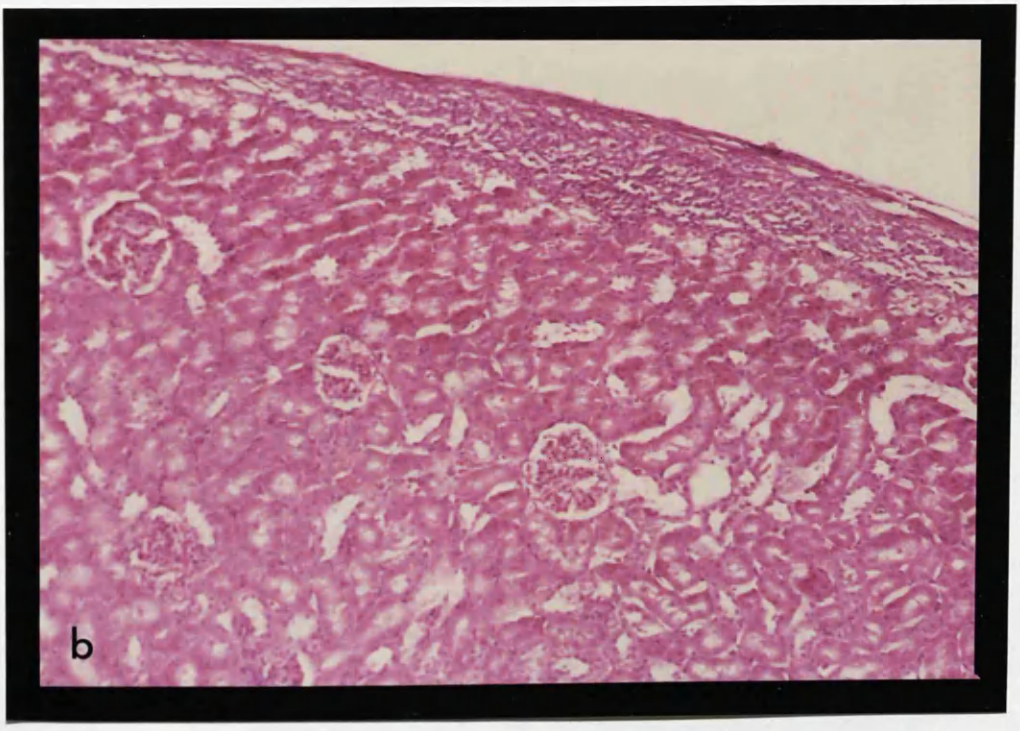
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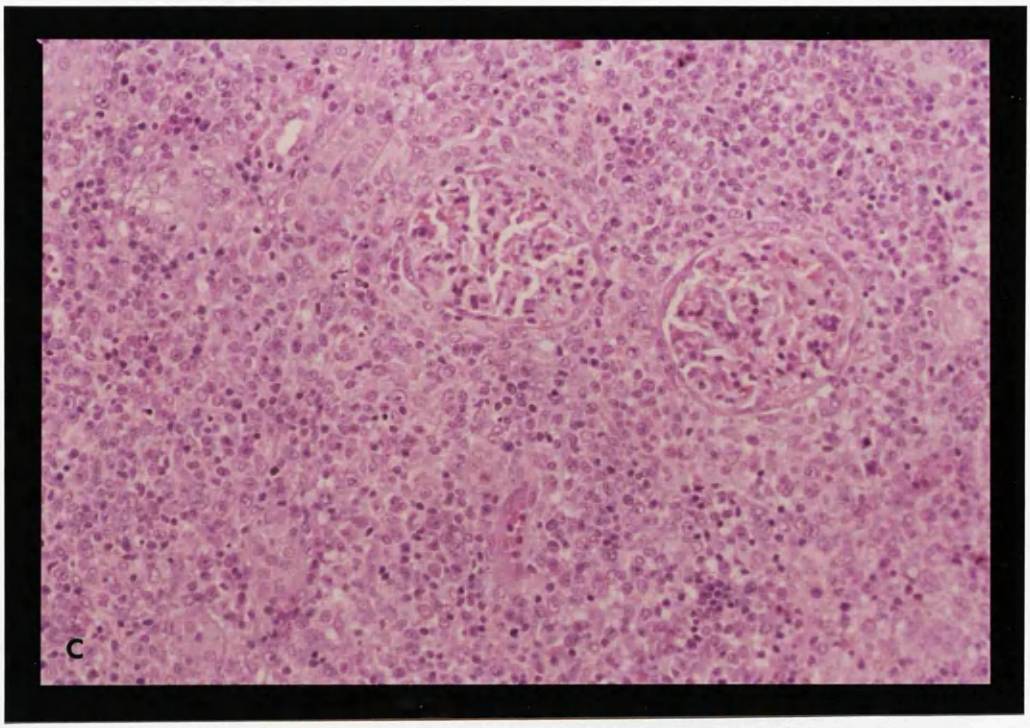

C

mediated directly either by CD4 lymphocytes releasing cytotoxic lymphokines, or by CD4 cell mediated cytotoxicity.

5.4 *In vitro* cytotoxic activity of mononuclear cells from the renal GVHR lesion

While the majority of proliferating cells in the renal GVHR appear to be of donor origin (Elkins, 1970), there is, in addition, a substantial "nonspecific" host contribution in the renal infiltrate.

In the following experiments, evidence for a role of cytotoxic CD4 lymphocytes in the renal GVHR was sought by harvesting mononuclear cells from an established renal GVHR and testing them for *in vitro* allospecific cytotoxic activity, as well as nonspecific NK cell mediated cytotoxicity. Clearly, however, because of the host contribution in the GVH lesion, the cytotoxic activity of harvested infiltrating cell cannot be strictly attributed to donor cells.

Effector cells were prepared by mechanical disruption and Percoll centrifugation of renal GVH lesions induced in (DAxPVG)F1 recipients by the inoculation of either unseparated PVG LNC or PVG CD4 lymphocytes. These cells were tested in a standard 6-hour chromium release assay to detect both specific cytotoxic activity against

relevant and third party ConA blast targets, and nonspecific activity against the NK susceptible targets Y3 and YAC-1.

Fig 5.3 shows the results of a typical experiment in which effector cells were harvested on day 7 from a renal GVH lesion induced by an inoculum of unseparated LNC. There were high levels of nonspecific activity against both Y3 and YAC-1 targets, but only low levels of activity against DA ConA blasts expressing class I and class II antigens of donor phenotype. Activity directed against third party Lewis ConA blasts was minimal.

Fig 5.4 shows the cytotoxic activity of effector cells harvested from lesions induced by the injection of CD4 cells alone. Again there were high levels of nonspecific activity against Y3 and YAC-1 but in this experiment, specific activity against DA ConA blasts was barely detectable, as was activity against third party Lewis targets.

These experiments were repeated on two occasions with the same results. The findings failed to support a role for allospecific cytotoxicity in the renal parenchymal damage of the renal GVHR, especially in lesions induced by the inoculation of CD4 cells alone. The low levels of allospecific cytotoxic activity detected in renal GVH lesions induced by injection of unseparated LNC were probably caused by parental strain cytotoxic precursor

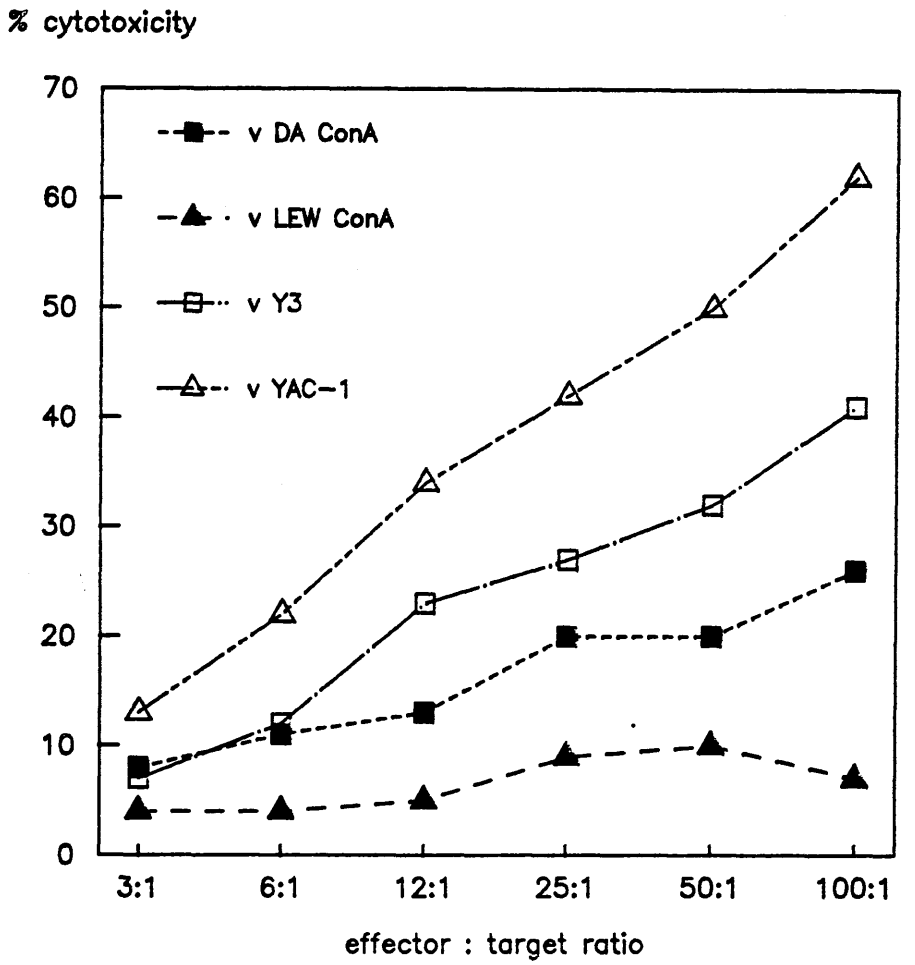


Fig. 5.3 Cytotoxic activity of renal GVHR cells, extracted on day 7 from LNC-induced lesions, against specific DA and third party Lewis ConA blasts, and against NK susceptible Y3 and YAC-1 targets.
Effector cells were prepared, by Percoll centrifugation, from four pooled lesions induced by injection of 10^7 PVG LNC into the renal subcapsular site of (DAxPVG) F1 recipients.

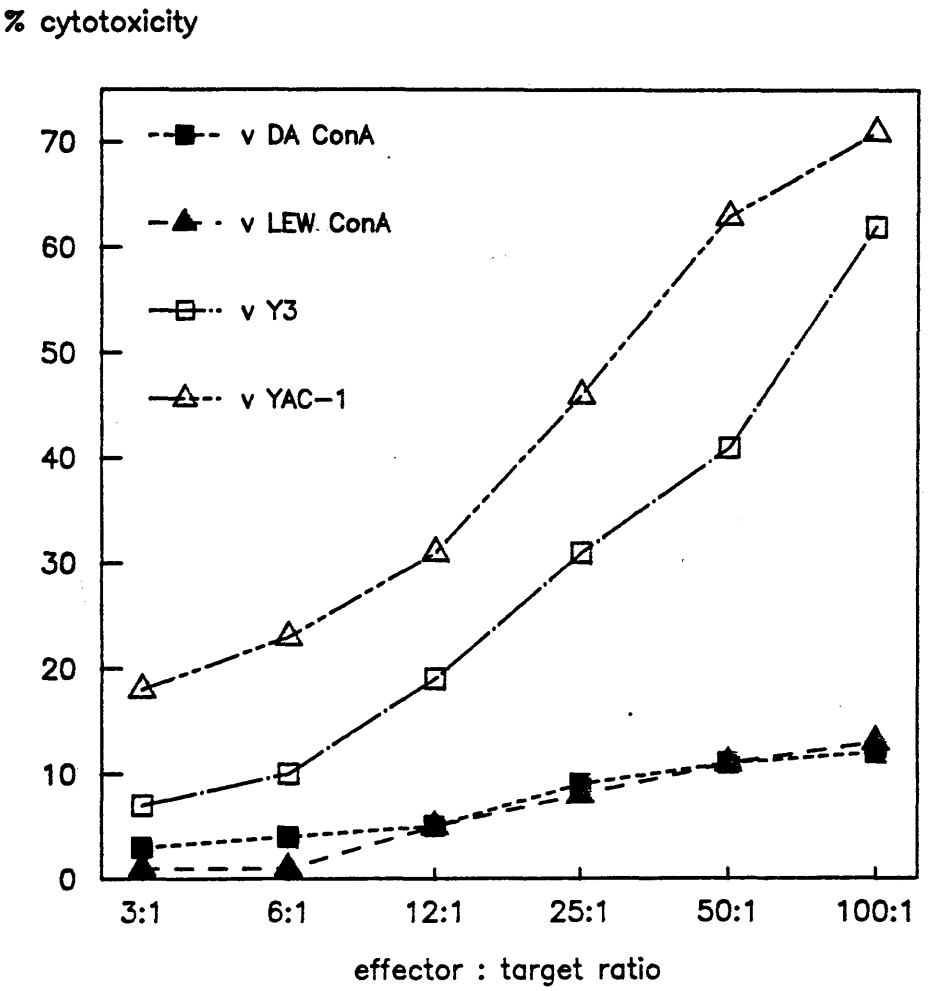


Fig. 5.4 Cytotoxic activity of renal GVHR cells, extracted on day 7 from CD4-induced lesions, against specific DA and third party Lewis ConA blasts, and against NK susceptible Y3 and YAC-1 targets.
Effector cells were prepared, by Percoll centrifugation, from four pooled lesions induced by injection of 10^7 PVG CD4 cells into the renal subcapsular site of (DAxPVG) F1 recipients.

cells of CD8 phenotype in the inoculum responding, with the help of parental CD4 cells, to recipient alloantigens and developing into activated cytotoxic T cells.

5.5 Role of MHC class disparities in the renal GVHR

The above experiments were performed in animals whose strain combinations represented MHC incompatibilities at both the class I and the class II loci (as well differences at the minor histocompatibility loci), and yet there was no evidence of a requirement for a class I restricted response by the injected cells in order to raise a renal GVH lesion.

In the following experiments, rats of the recombinant PVG series, whose members differ from each other at points within the MHC region, were used to study the influence of MHC subregion gene disparities on the subsequent development of the GVH response. The results of injecting parental strain LNC under the kidney capsule of F1 recipients differing at only class I or at class II MHC loci are shown in Table 5.3. As might have been predicted, parental cells were unable to respond to RT1A class I incompatible recipients differing at either the "a" haplotype (groups 1 & 2) or the "c" haplotype (groups 3 & 4). In contrast, an incompatibility at the class II locus alone was sufficient stimulus to provoke a renal

Table 5.3. Influence of MHC subregion disparities on the local renal GVHR

GROUP	P DONOR TO F1 RECIPIENT STRAIN COMBINATION	NO. LNC INJECTED	RT1 (MHC) DISPARITY	LESIONS/ ANIMALS	Ki/Kc[1]
1	PVG to (PVGr1xPVG)F1	$1x10^7$	A^a	0/4	1.02 (0.98-1.11)
2	PVG to (PVGr1xPVG)F1	$5x10^7$	A^a	0/4	1.03 (0.98-1.05)
3	PVGr1 to (PVGr1xPVG)F1	$1x10^7$	A^c	0/4	1.00 (0.98-1.02)
4	PVGr1 to (PVGr1xPVG)F1	$5x10^7$	A^c	0/4	0.99 (0.98-1.04)
5	PVGr1 to (PVGr1xPVGr19)F1	$1x10^7$	B^aD^a	4/4	1.16 (1.11-1.28)[2]
6	PVGr1 to (PVGr1xPVGr19)F1	$5x10^7$	B^aD^a	4/4	1.17 (1.12-1.27)[2]
7	PVGr19 to (PVGr1xPVGr19)F1	$1x10^7$	B^cD^c	4/4[3]	1.11 (0.98-1.16)
8	PVGr19 to (PVGr1xPVGr19)F1	$5x10^7$	B^cD^c	4/4	1.24 (1.16-1.29)[2]

Table 5.3 1x or $5x10^7$ parental strain lymphocytes were injected into the renal subcapsular site of F1 recipient rats. On day 7, evidence of a renal GVHR was sought by gross and histological examination of the injected kidney. The Ki/Kc ratio in experimental groups was compared with the Ki/Kc in control F1 animals injected with syngeneic F1 LNC. P values are shown for groups with a significant increase in Ki/Kc (Mann-Whitney U test).

1) Ki/Kc ratio expressed as median and range.

2) $p<0.02$.

3) Mild GVHR apparent only on histological examination.

GVHR. Parental cells responded to RT1B + RT1D class II incompatible recipients with either "a" haplotype (groups 5 & 6) or "c" haplotype (groups 7 & 8) disparities. In group 7, when 10^7 cells were injected, the renal GVHR response was not sufficient to significantly raise the Ki/Kc ratio, but it was clearly visible on histological examination.

From these experiments, it is apparent that a class I disparity is not necessary, and is not by itself sufficient, to provoke the renal GVHR.

5.6 Inability of the host kidney to provoke a renal GVHR

As demonstrated earlier (Table 5.1) whole body irradiation of F1 recipient animals prior to the subcapsular injection of parental LNC completely abrogated the local renal GVHR. Although the most striking effect of this dose of irradiation is on the circulating bone marrow derived cells of the recipient, it may also result in a decrease in the constitutive MHC class II antigen expression by both renal tubular cells and interstitial dendritic cells in the rat kidney (Leszczynski, Renkonen & Hayry, 1985), and observations in our own laboratory have confirmed these findings. In order to try and exclude the possibility that such a reduction in renal MHC expression may have contributed to

the abrogation of the renal GVHR, recombinant rat IFN-ɣ was administered intraperitoneally to F1 recipients daily for 6 days either before or after irradiation. This protocol has been evaluated in our laboratory and shown to produce a rapid and marked increase in the expression of class I and class II MHC antigens in many tissues of the body, including the renal parenchyma, as demonstrated by widespread and intense immunoperoxidase labelling of tissue cryostat sections with monoclonal antibodies directed against class I and class II MHC antigens. For example, by day 6, all renal tubules stained strongly with MRC OX18, and renal vascular endothelium, which does not constitutively express class II antigens, showed strong staining with MRC OX6.

However, as shown in Table 5.4, despite widespread MHC antigen induction, this treatment failed to restore the ability of parental T cells administered after irradiation to provoke a detectable renal GVHR. Thus there appears to be an absolute requirement for the participation of a radiosensitive host cell in the generation of the renal GVHR.

In a series of elegant experiments using bone marrow chimaeric animals, Elkins (1971) showed that the immunogenic stimulus which provoked a renal GVHR resided in the bone marrow derived cells rather than in the kidney parenchyma. The bone marrow derived cells provided both the stimulus (or target) for the response

Table 5.4 Ability of Lewis T lymphocytes[a] to mediate a renal GVHR in (LEWxDA)F1 recipients

GROUP	HOST IRRADIATION	IFN-δ TREATMENT	GVH LESIONS/ No. TESTED	Ki/Kc (median + range)
1)	none	none	4/4	1.32 (1.2-1.43)[b]
2)	10 Gy	none	0/5	1.01 (0.95-1.11)
3)	10 Gy	10^6U/Kg/ days -6 to 0	0/4	0.93 (0.89-1.10)
4)	10 Gy	10^6U/Kg/ days 0 to 6	0/4	1.03 (1.0-1.11)

Table 5.4 $1x10^7$ Lewis T lymphocytes were injected into the renal subcapsular site of (LewxDA)F1 recipient rats. On day 7, evidence of a renal GVHR was sought by gross and histological examination of the injected kidney. The response in this group (group 1) was compared with the response in irradiated recipients (group 2), and with irradiated recipients which had been treated with IFN-δ either before (group 3) or after (group 4) subcapsular injection.

a) 10^7 T cells (prepared from LNC by rosette depletion with MRC OX12) were injected into left renal subcapsular site.

b) Only group 1 showed a significant ($p<0.02$) increase in Ki/Kc.

c) Recombinant rat IFN-δ was given by daily intraperitoneal injection,resulting in a marked increase in class I and class II MHC expression.

as well as a population of effector cells. The possibility remained, however, that the specific allogeneic stimulus might be provided by the renal parenchyma alone if MHC antigens were expressed in sufficient density, while the effector population might be recruited from the bone marrow derived cells. To answer this question, radiation chimaeras were prepared, using another strain combination, in which only F1 renal parenchyma displayed allogeneic MHC antigens necessary to provoke a reaction, since the circulating bone marrow derived cells were syngeneic with the injected parental lymphocytes. As expected, none of three DA(Lewis x DA)F1 chimaeras given a renal subcapsular injection of 10^7 DA LNC showed histological evidence, at day 7, of a renal GVHR (Ki/Kc ratios 1.02, 1.04, and 1.07). Furthermore, three additional chimaeric animals treated with IFN-ϑ for 7 days after subcapsular LNC injection also failed to show evidence of a renal GVHR (Ki/Kc ratios 0.90, 0.95 and 1.04) despite marked induction of class II MHC antigens on the renal tubular cells and vascular endothelium. These experiments therefore indicated that the renal parenchymal tissue alone was incapable of provoking a detectable response by the injected DA lymphocytes.

5.7 The ability of graft infiltrating cells to mediate GVH reactions

It is clear that there are similarities between the cellular events occurring in the renal GVHR and those occurring in unmodified rat renal allograft rejection, both of which result in extensive renal parenchymal damage associated with mononuclear cell infiltration. In a final group of experiments to discover the extent of this relationship, lymph node cells and graft infiltrating mononuclear cells were harvested on day 5 from PVG recipients rejecting a DA renal allograft, and tested in vivo for their ability to cause GVH reactions both in the renal subcapsular site and in the popliteal lymph node enlargement assay.

Infiltrating PVG mononuclear cells were extracted by mechanical disruption of the kidney followed by Percoll centrifugation. 10^7 cells were injected under the kidney capsule of two (DAxPVG)F1 recipients, but they failed to induce renal GVH lesions that were detectable either macroscopically or on histological examination on day 7, and the Ki/Kc ratios were not raised (0.97 & 0.98).

Three graded doses of graft infiltrating cells were injected into the footpads of (DAxPVG)F1 recipients, and were compared with injected LNC from the same animals for their ability to cause popliteal lymph node enlargement

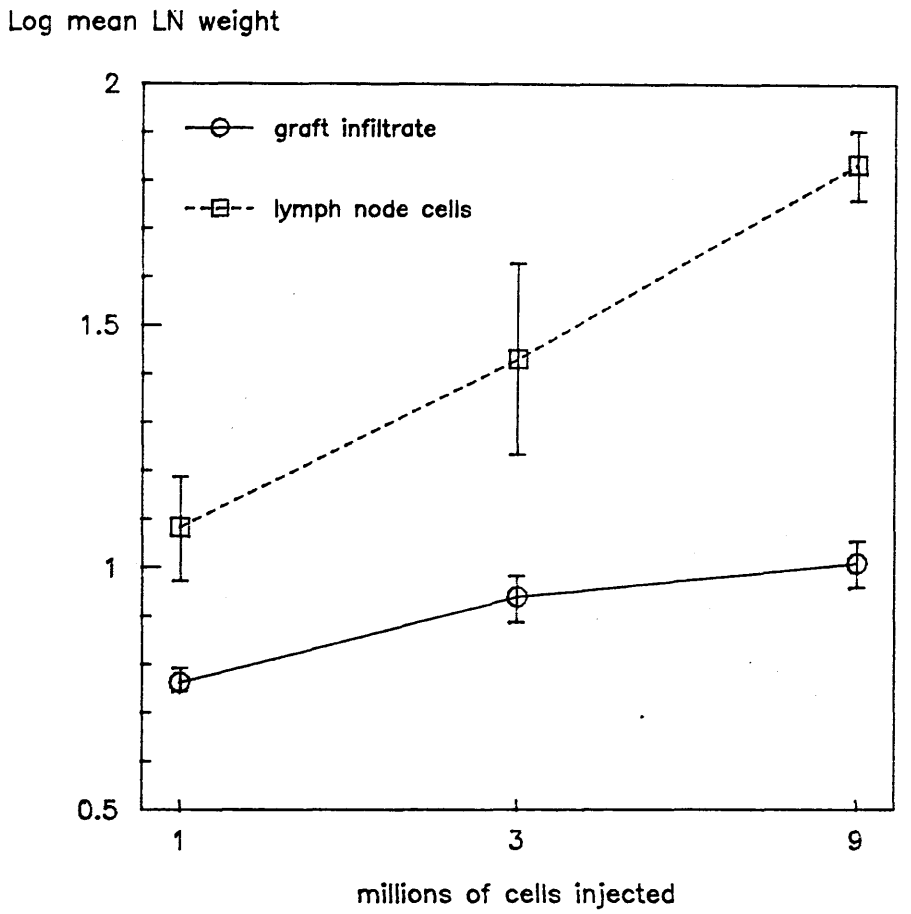


Fig. 5.5 Ability of graft infiltrating cells and LNC, harvested on day 5 from PVG recipients rejecting a DA renal allograft, to cause popliteal lymph node enlargement in (DAxPVG)F1 hosts.
F1 rats received footpad injections of graded doses of cells, and popliteal lymph node enlargement was assessed on day 7. Results are expressed as the log mean and s.d. of 3 or 4 lymph node weights.

by day 7 after injection. The results in Fig 5.5 show that while LNC were able to cause significant popliteal lymph node enlargement, indicating an efficient GVH response, graft infiltrating cells had no effect.

These findings suggest that there may be insufficient cells in 10^7 graft infiltrating cells to cause graft versus host rections when transferred to F1 recipients, presumably because only a minority of the cells are of the CD4 phenotype. This is in keeping with the results of the phenotypic study of graft infiltrating cells (chapter 3) which showed that only a small percentage of the cells were T lymphocytes. Alternatively, the graft infiltrating cells may be fully differentiated as effector cells and may be unable to respond in an appropriate manner to raise a GVH reaction in F1 recipients.

5.8 Discussion

The renal GVHR provides a clear demonstration of the ability of parental strainn lymphocytes to mediate extensive <u>in vivo</u> tissue damage in semiallogeneic kidneys, with pathological appearances that are broadly similar to those of rejecting renal allografts. In the renal GVHR, unlike in the irradiated graft recipients in the preceeding chapter, it could be assumed with

confidence that only the injected parental lymphocytes were able to cause direct allospecific damage and that any contribution from the host would comprise nonspecific cells only (since there would be no alloantigenic stimulus to activate host lymphocytes in the parental strain into the F1 combination). It was therefore of particular interest to investigate the ability of purified parental strain lymphocytes to cause renal damage in this model. The finding that lymphocytes of the CD4 phenotype alone were capable of producing extensive parenchymal damage in F1 recipients is in keeping with the findings of Mason et al (1984).

Support for the view that CD4 class II reactive cells alone were able to cause a renal GVHR was obtained from the experiments using recombinant rats differing at isolated MHC class II subregions. It was shown that an isolated class II MHC disparity between the parental lymphocytes and F1 recipient was sufficient to provoke a strong renal GVHR. This finding is consistent with the results obtained from other assays of GVH reactivity in the rat such as the popliteal lymph node enlargement assay which suggest that class II MHC antigens are the principal stimulating antigens in GVH reactions (Butcher, Licence & Roser, 1981).

The failure of CD4 lymphocytes to mediate a renal GVHR in heavily irradiated recipients, together with the chimaera experiments reported by Elkins (1971), suggests

that the renal GVHR is initiated by parental CD4 lymphocytes recognising and responding to class II MHC alloantigens expressed on host bone marrow derived cells. Interestingly, the stimulatory function of these bone marrow derived cells could not be replaced by upregulating class II MHC alloantigen expression by the vascular endothelium and renal tubular cells in the cortex of the kidney subjacent to the injected lymphocytes, in either the irradiated recipients or the chimaeric animals. Thus there is an absolute requirement for the allogeneic stimulus to be present on recipient bone marrow derived cells, and while the precise nature of this radiosensitive cell was not determined, a likely candidate would be the strongly class II positive interstitial dendritic cell. In this respect, a parallel can be drawn between the renal GVHR and renal allograft rejection, since the importance of interstitial dendritic cells in graft rejection, and the improved graft survival achieved by reducing their numbers was demonstrated in chapter 3.

The rat CD4 lymphocyte is known to be the T cell subpopulation which proliferates strongly in the mixed lymphocyte reaction (Mason, Arthur, Dallman et al, 1983) and which, in vivo, is responsible for causing a positive response in the popliteal lymph node enlargement assay (White, Mason, Williams et al, 1978) as well as causing lethal GVH disease when injected into

irradiated recipients (Mason, 1981). However, the ability of CD4 cells to cause kidney damage in the local renal GVHR (and other tests of GVH reactivity) contrasts with the inability of adoptively transferred CD4 cells to cause renal allograft rejection in acutely irradiated hosts in the preceeding chapter.

Although there are several possible explanations for this paradoxical finding, one possibility is that the CD4 lymphocytes mediate their destructive effects (in the absence of CD8 lymphocytes) through the presence of nonspecific effector cells which, in the irradiated allograft recipients, may have been affected by the 8.5 Gy irradiation dose. This explanation, however, is difficult to reconcile with other reports in the literature which have shown that CD4 cells alone can mediate allograft rejection in acutely irradiated hosts (see Appendix A).

There is convincing evidence from the results of <u>in vitro</u> tests described in this chapter that the CD4 subpopulation mediates tissue damage in the renal GVHR by lymphokine release and recruitment of nonspecific effectors such as macrophages and NK cells, rather than by class II restricted specific T cell lysis, since damage occurred in the absence of detectable specific T cell lysis by mononuclear cells harvested from CD4 induced renal GVH lesions.

Parental lymphocytes of the CD8 phenotype, even when obtained from specifically sensitised animals, were unable, by themselves, to cause a renal GVHR in F1 recipients. Since evidence for the presence of a renal GVHR was sought only on day 7 after induction, the possibility that CD8 cells are able to mediate the response, but more slowly, cannot be excluded. However, this is unlikely in view of the inability of CD8 cells from specifically sensitised donors to cause a renal GVHR.

Rat CD8 cells have been reported to be unable to produce a positive response in the popliteal lymph node enlargement assay (White et al, 1978) and yet they are able to cause lethal GVH disease in irradiated rats, albeit less effectively than CD4 lymphocytes (Mason, 1981). In addition, cloned, specifically sensitised cytotoxic CD8 lymphocytes are able to cause tissue damage when injected intradermally (Tyler, Galli, Snider et al, 1984) and are able to restore class I incompatible skin allograft rejection in mice (Rosenberg et al, 1986), while Sprent et al have demonstrated the ability of CD8 cells alone to mediate lethal GVH disease in class I disparate irradiated mice (Sprent, Schaefer, Gao et al, 1988). Thus there is evidence that CD8 cells alone are able to directly mediate tissue damage under certain conditions, although they are not, at least in the rat

strain combinations tested, able to cause a renal GVHR nor, as shown in chapter 4, are they able to restore renal allograft rejection in acutely irradiated rats.

The finding that an isolated class I MHC disparity was insufficient to provoke a renal GVHR is consistent with the suggestion that CD8 cells do not play a significant role in the renal GVHR. However, caution is required in interpreting the inability of an isolated class I disparity reported in this chapter to provoke a renal GVHR, since the PVG ($RT1^c$) rat is a "low responder" to the isolated A^a class I antigen of the PVGr1 rat and vice versa (Butcher et al, 1982). This was also highlighted in chapter 3 when it was shown that renal allografts with an isolated A^a disparity were not rejected.

In summary, this chapter has demonstrated that in an appropriate rat model, the renal GVHR, CD4 lymphocytes may cause extensive renal parenchymal damage by a DTH type reaction. Whether or not a similar type of effector mechanism operates to cause rejection of a renal allograft must remain largely speculative because of the obvious fundamental differences between the two models.

CHAPTER SIX

CELLULAR MECHANISMS OF RENAL ALLOGRAFT REJECTION IN THE ATHYMIC PVG-RNU/RNU RAT

6.1 Introduction

The problems associated with the use of acutely irradiated animals as allograft recipients in adoptive transfer studies have been outlined in chapter 4. In particular, the difficulty of completely eliminating T cells and precursors, and the incompletely understood effects of irradiation on platelets and other bone marrow derived cells involved in the rejection response, together emphasise the poorly defined nature of this experimental model.

In this chapter, the ability of adoptively transferred T lymphocyte subpopulations to mediate renal allograft rejection in congenitally athymic PVG-rnu/rnu rats was investigated. It has long been appreciated that mutant mice carrying the nu gene, resulting in congenital absence of the thymus gland and associated hairlessness, have absent T cell immunity. As such, they are unable to reject either allografts or xenografts. The development, by backcrossing, of inbred strains of athymic mice with defined MHC antigens enabled experiments to be performed in which the ability of adoptively transferred T cell subpopulations to restore skin graft rejection was examined. In contrast with acutely irradiated animals, athymic rodents have intact non-thymically processed bone marrow derived cells including platelets, macrophages, NK cells and B lymphocytes. However, it is essential to

appreciate that they also possess cells with the apparent identity of T cytotoxic precursor (Tcp) cells, as shown by several groups (Gillis, Union, Baker et al, 1979; Hunig & Bevan, 1980), although there is a lack of consensus regarding their functional identity with Tcp.

More recently, the Rnu gene in rats has been backcrossed onto inbred lines with defined MHC antigens. Adoptive transfer experiments in both mice and rats have demonstrated that skin allograft rejection may occur in athymic recipients which have received an intravenous inoculum of T cells prepared from syngeneic but euthymic donors (Rosenberg, Mizuochi, Sharrow et al, 1987; Bell, Sparshott, Drayson et al, 1987). The athymic rat, because of its size, now provides a model in which it is possible to investigate the T cell requirements for rejection of a primarily vascularised organ graft, such as the kidney, as described in this chapter.

Congenitally athymic animals tend to be healthier and physiologically more normal than irradiated animals. Furthermore, with intact bone marrow derived cells, it is likely that the transfer of T cells to restore allograft rejection will induce the recruitment of nonspecific inflammatory cells in a normal manner.

A further important advantage in the use of the PVG rnu/rnu rat as host in adoptive transfer experiments lies in the observation that nude rats have patterns of natural cytotoxicity or allogeneic lymphocyte

cytotoxicity (ALC) that are not dissimilar to those of normal rats, except that their ALC response is even more efficient than that of euthymic PVG rats (Rolstad & Ford, 1983). While the recirculation patterns of allogeneic and syngeneic lymphocytes in normal animals with a full complement of T cells cannot be truly compared with recirculation patterns seen in nude rats, it has been observed that syngeneic cells in nude rats appear to behave normally. By contrast, syngeneic cells injected into irradiated rats have very disturbed migration patterns (McNeilage & Heslop, 1983).

The results of the adoptive transfer experiments performed in this chapter will be discussed with reference to these characteristics of athymic animals.

6.2 Ability of adoptively transferred LNC to restore renal allograft rejection in athymic PVG-rnu/rnu rats

In the following experiments, PVG-rnu/rnu rats were transplanted with a fully allogeneic DA strain kidney and then immediately reconstituted with an intravenous inoculum of lymphocytes (or lymphocyte subpopulations) prepared from pooled cervical and mesenteric lymph nodes of syngeneic but euthymic PVG donors. Contralateral nephrectomy was performed on day 7 after transplantation, and the function of the graft was monitored by observing

recipient survival time and by measuring serum urea and creatinine levels in serial blood samples. All animals were housed in an laminar flow isolator to minimise the risk of life-threatening infections and this appeared to be very effective since deaths from infection were rare, while those that did occurr were almost exclusively in non-reconstituted animals (see below).

As expected, PVG-rnu/rnu rats were unable to reject DA renal allografts (Table 6.1). Two of the four non-reconstituted renal allograft recipients survived until sacrifice at more than 100 days, with normal serum urea and creatinine levels throughout. The other two animals developed severe wasting from approximately day 65 onwards and were sacrificed on days 75 and 79. Their urea and creatinine levels showed a modest rise during the few days before sacrifice but this was thought to be due to illness and dehydration rather than graft rejection. Grafts removed from all four animals at sacrifice appeared completely healthy and showed no histological evidence of rejection.

Athymic PVG rats were readily induced to reject their grafts by injection of PVG LNC (Table 6.1). Reconstitution of renal allograft recipients with an inoculum of $5x10^7$ unseparated LNC immediately after transplantation caused rapid graft rejection (median survival time 10 days; markedly elevated day 9 serum urea and creatinine). Kidneys removed from these animals

Table 6.1. Ability of adoptively transferred LNC to restore DA renal allograft rejection in PVG-rnu/rnu recipients

LNC INOCULUM	SURVIVAL (days)	MST (days)	SERUM UREA (mM/L; median + range) d9	d14
none	75, 79, >100,>100	92	11 (9-14)	9 (5-10)
$5x10^7$	9,10,15	10	80 (58-97)	61 (1 rat)

Table 6.1 PVG-rnu/rnu rats received a DA renal allograft and were immediately reconstituted with an intravenous inoculum of syngeneic PVG LNC. Contralateral nephrectomy was performed on day 7 after transplantation, and graft rejection was monitored by observing recipient survival time and by measuring serum urea levels in serial blood samples.

at the time of death were grossly swollen and discoloured, and on histological examination showed severe rejection. The tempo of graft rejection in PVG-rnu/rnu recipients reconstituted with this dose of LNC was comparable to that seen previously in unmodified PVG recipients of a DA renal allograft (Chapter 3).

6.3 Ability of depleted LNC subpopulations to restore renal allograft rejection in PVG-rnu/rnu recipients

In this series of experiments, PVG-rnu/rnu recipients of DA renal allografts were injected with an intravenous inoculum of separated LNC subpopulations to examine their ability to restore rejection. Lymphocyte subpopulations were negatively selected by rosette depletion in the same way that they were prepared for reconstituting acutely irradiated graft recipients (chapter 4). On each occasion, the purity of the selected population was checked by immunoperoxidase staining of cytocentrifuge slide preparations of the cells, and the level of contamination by unwanted cell types was always found to be under 0.5%.

The results of adoptively transferring different doses of either CD4 or CD8 lymphocytes to allograft recipients is shown in Table 6.2. Injection of 10^7 CD4 lymphocytes failed to consistently restore graft

Table 6.2. Ability of depleted LNC subpopulations to restore DA renal allograft rejection in PVG-rnu/rnu recipients

CELLS TRANSFERRED (N. & phenotype)	N	SURVIVAL (days)	MST (days)	DAY 9 UREA (mM/L) (median & range)
$1x10^7$ CD4	7	9,10,83,>100 >100,>100,>100	>100	16 (9-83)
$2.5x10^7$ CD4	3	9,10,13	10	68 (59-88)
$5x10^7$ CD4	5	9,10,10,11,21	10	25 (21-78)
$1x10^7$ CD8	4	60,76, >100,>100	99	8 (6-9)
$2.6x10^7$ CD8	2	>100,>100	>100	10 (8-11)
$7x10^6$ CD4 + $3x10^6$ CD8	7	9,9,9,9,12, 13,23	9	60 (18-106)

Table 6.2 PVG-rnu/rnu rats received a DA renal allograft and were immediately reconstituted with an intravenous inoculum of syngeneic PVG LNC subpopulations, which were negatively selected by rosette depletion. Contralateral nephrectomy was performed on day 7 after transplantation, and graft rejection was monitored by observing recipient survival time and by measuring serum urea levels in serial blood samples.

rejection (MST >100 days) but increasing the number of CD4 cells administered to $2.5x10^7$ or more caused rapid graft rejection in all animals (MST 10 days; markedly elevated day 9 serum urea).

Adoptive transfer of CD8 lymphocytes, however, failed to restore graft rejection. It can be seen from Table 6.2 that the number of CD8 cells transferred was not as great as the highest number of CD4 cells transferred. It was difficult to obtain very large numbers of highly purified CD8 lymphocytes because they represent a relatively smaller proportion of the LNC (<20%). However, because they represent a smaller proportion of LNC than the CD4 lymphocytes, it was considered appropriate to transfer an equivalent number of CD8 lymphocytes. Thus, since LNC comprise approximately 50% CD4 cells and 20% CD8 cells, transferring 10^7 CD8 cells was equivalent to transferring $2.5x10^7$ CD4 cells which, as described, was sufficient to cause renal allograft rejection.

The results of adoptive transfer experiments in acutely irradiated allograft recipients described in Chapter 4 raised the possibility that the transfer of CD4 and CD8 lymphocytes together was more effective than transfer of either subpopulation alone. Evidence for such an effect in the present experiments was sought by transferring a mixture of negatively selected lymphocyte subpopulations to PVG-rnu/rnu recipients of DA renal allografts.

Since 10^7 naive CD4 lymphocytes were unable to consistently restore graft rejection (Table 6.2), the same number of mixed negatively selected cells (comprising 70% CD4 and 30% CD8 cells) was tested. There did appear to be a synergistic effect when transferring the combined subpopulations, since recipient survival times were reduced. The individual survival times in seven animals were 9,9,9,9,12,13 and 23 days (MST 9 days) which is significantly less ($p<0.05$) than the survival of animals reconstituted with 10^7 CD4 cells alone.

6.4 Ability of specifically sensitised LNC subpopulations to restore renal allograft rejection in PVG-rnu/rnu rats

The following experiments examined the ability of adoptively transferred, specifically sensitised lymphocyte subpopulations to restore graft rejection. PVG lymph node donors were sensitised by two consecutive DA skin grafts, and were used approximately one month after the second graft had rejected. The results are shown in Table 6.3. Whereas 10^7 naive CD4 lymphocytes did not consistently cause rejection, the same number of specifically sensitised CD4 cells restored rejection with a first set tempo (median survival time 9 days and high day 9 serum urea levels). Furthermore, reducing the number of sensitised CD4 cells to $2x10^6$

Table 6.3. Ability of specifically sesitised lymphocyte subpopulations to restore renal allograft rejection in PVG-rnu/rnu recipients

CELLS TRANSFERRED (N. & phenotype)	N	SURVIVAL (days)	MST (days)	DAY 9 UREA (mM/L) (median & range)
$1x10^7$ sCD4	3	8,9,9	9	75 (72-77)[a]
$2x10^6$ sCD4	4	9,10,10,11	10	79 (77-87)[a]
$1.3x10^7$ sCD8	2	>100,>100[b]	>100	14 (11-17)
$1.4x10^7$ sCD8	2	>100,>100[b]	>100	14 (12-16)

Table 6.3 PVG-rnu/rnu rats received a DA renal allograft and were immediately reconstituted with an intravenous inoculum of specifically sensitised syngeneic PVG LNC subpopulations. Sensitised LNC donors had previously rejected two sequential DA skin grafts, and LNC subpopulations were negatively selected by rosette depletion. Contralateral nephrectomy was performed on day 7 after transplantation, and graft rejection was monitored by observing recipient survival time and by measuring serum urea levels in serial blood samples.

a) One rat died with no blood urea measurement.

b) Animals were sacrificed with normal graft function (median serum urea 9, range 6-20 mM/L at death).

still resulted in first set graft rejection (MST 10 days). In contrast, the transfer of relatively large numbers of specifically sensitised CD8 lymphocytes ($1.4x10^7$) was ineffective at restoring graft rejection.

6.5 Ability of MRC OX22 depleted CD4 cells to restore renal allograft rejection in PVG-rnu/rnu recipients

In a final series of adoptive transfer experiments, PVG-rnu/rnu recipients of DA renal allografts were reconstituted with an intravenous inoculum of CD4 lymphocytes that had been additionally depleted of cells bearing the MRC $OX22^+$ phenotype. MRC OX22 reacts with the high molecular weight form of the rat leukocyte-common antigen and labels B cells and CD8 lymphocytes in thoracic duct lymphocyte populations, but labels only two thirds of the CD4 lymphocyte subset (Spickett, Brandon, Mason et al, 1983). Functional studies have shown that MRC OX22 splits the CD4 lymphocyte subpopulation according to whether they are able to mediate GVH reactivity, as detected by the popliteal lymph node enlargement assay (positively labelled), or whether they provide T help for antibody responses by B cells (unlabelled by MRC OX22). Since it is widely accepted that alloantibodies are present in animals

rejecting renal allografts but are not generally thought to play a crucial role in rejection, it might be expected that the adoptive transfer of CD4 cells depleted of the population responsible for cellular responses such as GVH reactivity would be unable to restore graft rejection. However, the results showed that transferring $3x10^7$ MRC OX22 depleted CD4 lymphocytes restored the rejection response in three of the four animals tested. Three rats died on day 9 with a median day 9 serum urea of 77 mM/L (range 70-81 mM/L), whereas the remaining rat had a raised serum urea at day 9 (35 mM/L) which quickly returned to normal and the animal was sacrificed on day 123 with an apparently normal kidney.

6.6 Immunohistological examination of DA renal allografts in non-reconstituted and in reconstituted PVG-rnu/rnu recipients

Non-rejecting DA renal allografts were excised, on day 7, from non-reconstituted PVG-rnu/rnu recipients and were compared with rejecting renal allografts obtained, at day 7, from PVG-rnu/rnu recipients reconstituted with either $5x10^7$ LNC or $5x10^7$ CD4-positive PVG LNC.
A small portion of the graft was immediately embedded in Tissue-Tek and frozen in liquid nitrogen for immunohistological studies, while the remainder of the

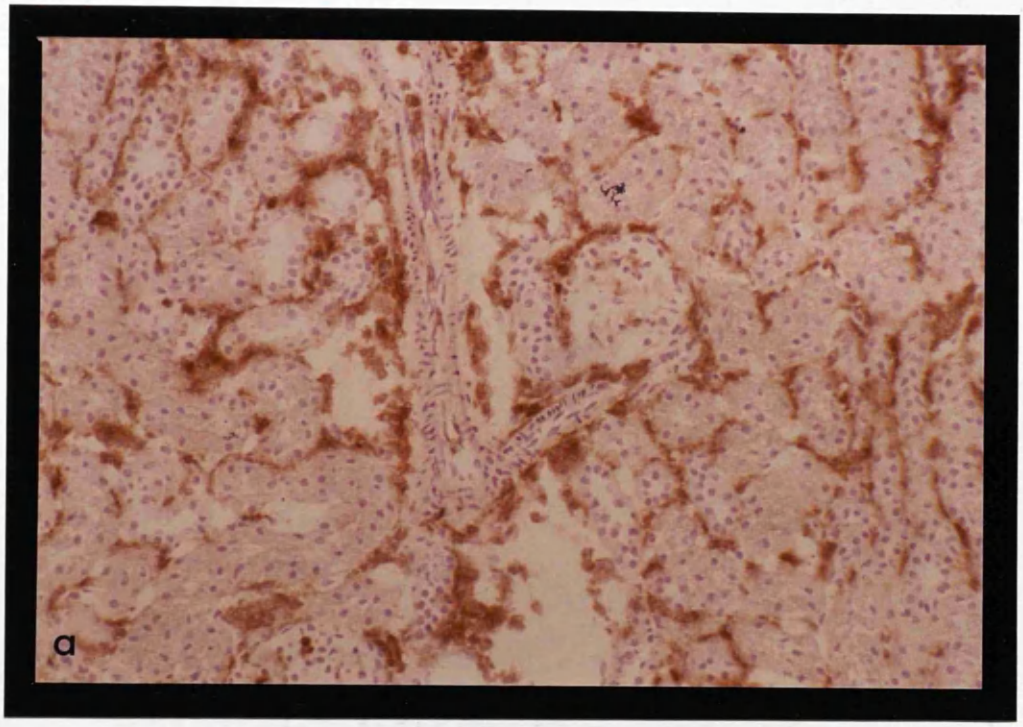
kidney (and the spleen) were used to investigate the ability of graft infiltrating cells and splenocytes to lyse a range of target cells in in vitro functional assays.

Cryostat sections (5 μ thick) were labelled with a range of mouse monoclonal antibodies to rat leukocyte subpopulations and MHC antigens using an indirect immunoperoxidase staining technique. With light microscopy, the percentage area of each section that was infiltrated by leukocytes of a particular phenotype was determined by morphometric analysis using a standard point counting technique.

6.6.1 Magnitude and phenotype of mononuclear cell infiltration

Non-rejecting renal allografts obtained, at day 7, from PVG-rnu/rnu recipients which had not been reconstituted with lymphocytes showed a mild cellular infiltrate (median area infiltrate 11%) when labelled with MRC OX1 (against the leukocyte common antigen)(Fig 6.1). In contrast, rejecting renal allografts from recipients reconstituted with unseparated LNC or purified CD4 lymphocytes showed a heavier cellular infiltrate (26% and 25% median area infiltrate respectively) when labelled with MRC OX1 (see Figs 6.1 & 6.2).

Fig. 6.1 Comparison of leukocyte infiltration of a DA renal allograft in a PVG-rnu/rnu recipient, either non-reconstituted (a) or reconstituted with PVG LNC (b), on day 7 after transplantation.
Cryostat sections of the grafts were stained, by immunoperoxidase, with MRC OX1 (rat leukocytes). (x160)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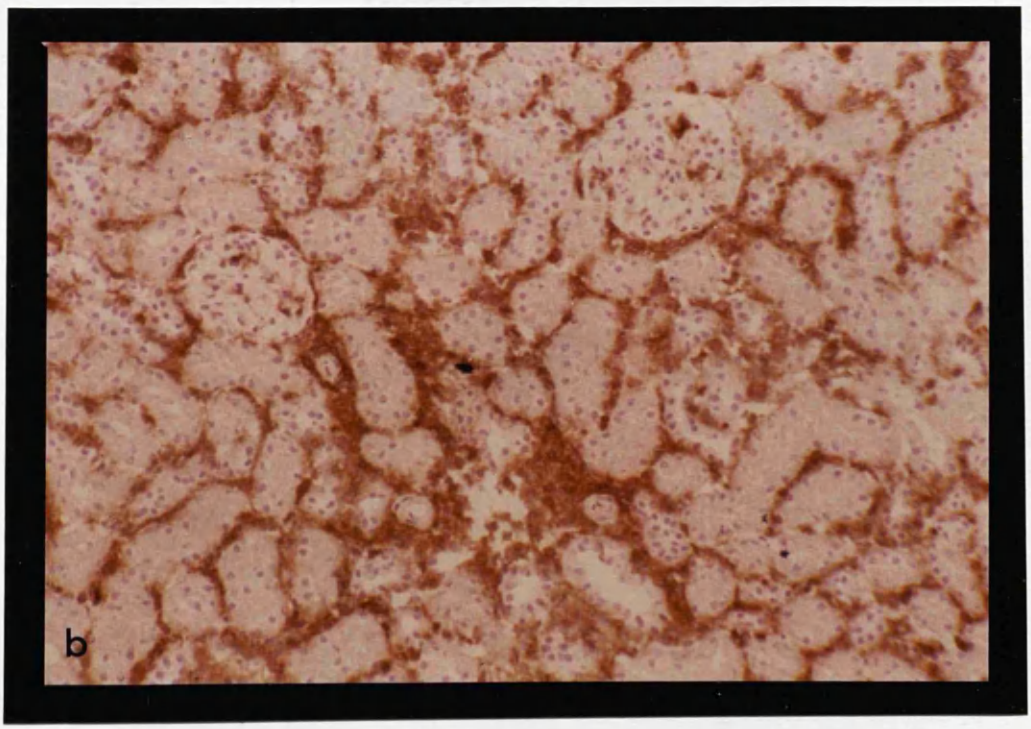
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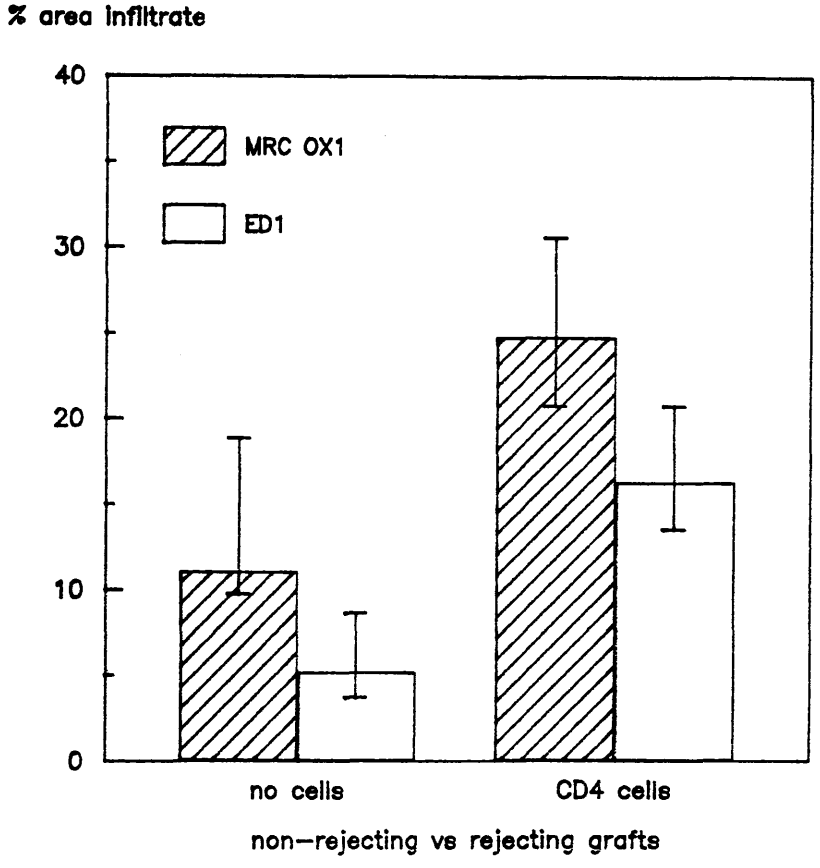


Fig. 6.2 Morphometric analysis of cellular infiltrate in DA renal allografts transplanted into unmodified or CD4-reconstituted PVG-rnu/rnu recipients.
Grafts were removed on day 7 after transplantation and cryostat sections were stained by immunoperoxidase with the leukocyte common antibody, MRC OX1 and a MAb detecting tissue macrophages, ED1. Results are expressed as the median % area infiltrate and range of four grafts in each group.

In nonrejecting grafts, the majority of infiltrating cells appeared to be macrophages since they were labelled with the antibodies W3/25 (which labels CD4 lymphocytes and activated macrophages) and ED1 (which labels most tissue macrophages and dendritic cells), but showed very little staining with the T cell antibody MRC OX19. Labelling with MRC OX8 showed that there were very few CD8 lymphocytes and NK cells present, and no activated cells as detected by the antibody MRC OX39 raised against the IL2 receptor. In contrast, rejecting grafts in LNC reconstituted and CD4 cell reconstituted animals showed almost identical patterns of cellular infiltration. There were large numbers of macrophages as shown by labelling with ED1 (approximately 15-17% area infiltrate, Fig 6.2). There were few MRC OX19 positive T cells (median area infiltrate 2%), but around 6-8% of the sectional area was occupied by MRC OX8-positive cells (presumably mostly NK cells) and around 10% by W3/25-positive cells which were presumably mostly macrophages (Fig 6.3). Infiltrating cells in rejecting grafts showed only a slight increase in the expression of detectable IL2 receptor (shown by MRC OX39 labelling).

Taken together these results suggest that in spite of the requirement for the adoptive transfer of immunocompetent lymphocytes to restore the graft rejection response in athymic animals, the majority of

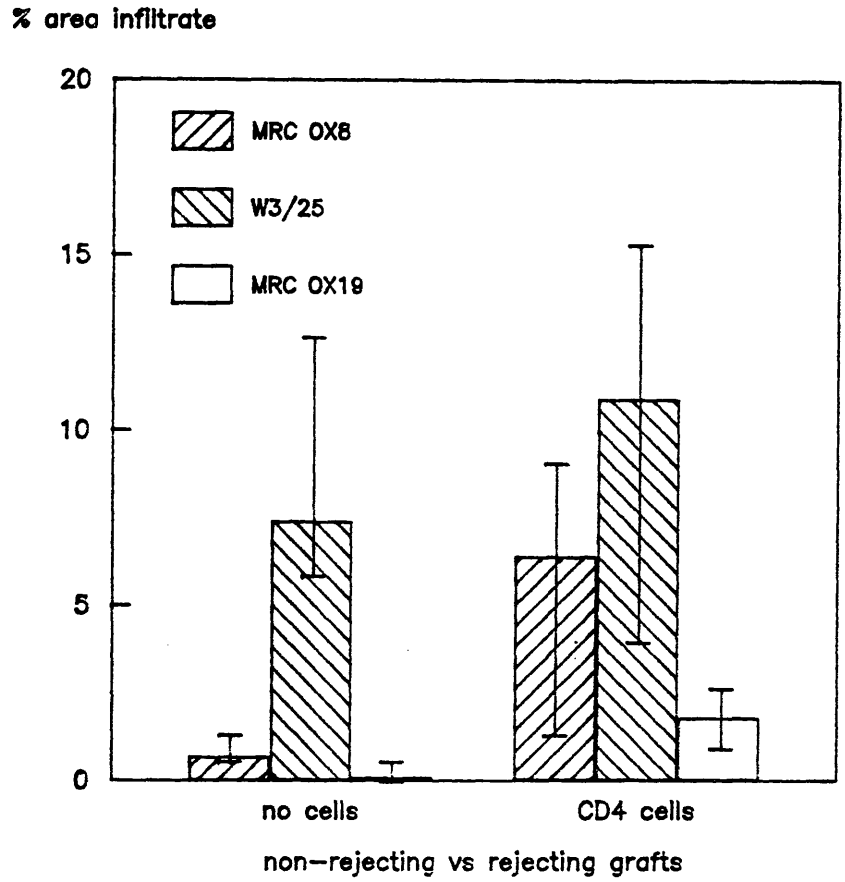


Fig. 6.3 Morphometric analysis of cellular infiltrate in DA renal allografts transplanted into unmodified or CD4-reconstituted PVG-rnu/rnu recipients.
Grafts were removed on day 7 after transplantation and cryostat sections were stained by immunoperoxidase with MAbs directed against leukocyte subpopulations. Results are expressed as the median % area infiltrate and range of four grafts in each group.

the cellular infiltrate is composed of nonspecific inflammatory cells such as macrophages and NK cells. Sections were also labelled with MRC OX12 (which labels Ig kappa chain on B cells) but in all grafts examined, labelling of tissue bound antibody masked the staining of B cells. The presence of large amounts of tissue bound antibody within the grafts of non-reconstituted animals was surprising since the animals would not be expected to be able to mount a specific alloantibody response in the absence of T help.

6.6.2 MHC antigen expression in DA renal allografts

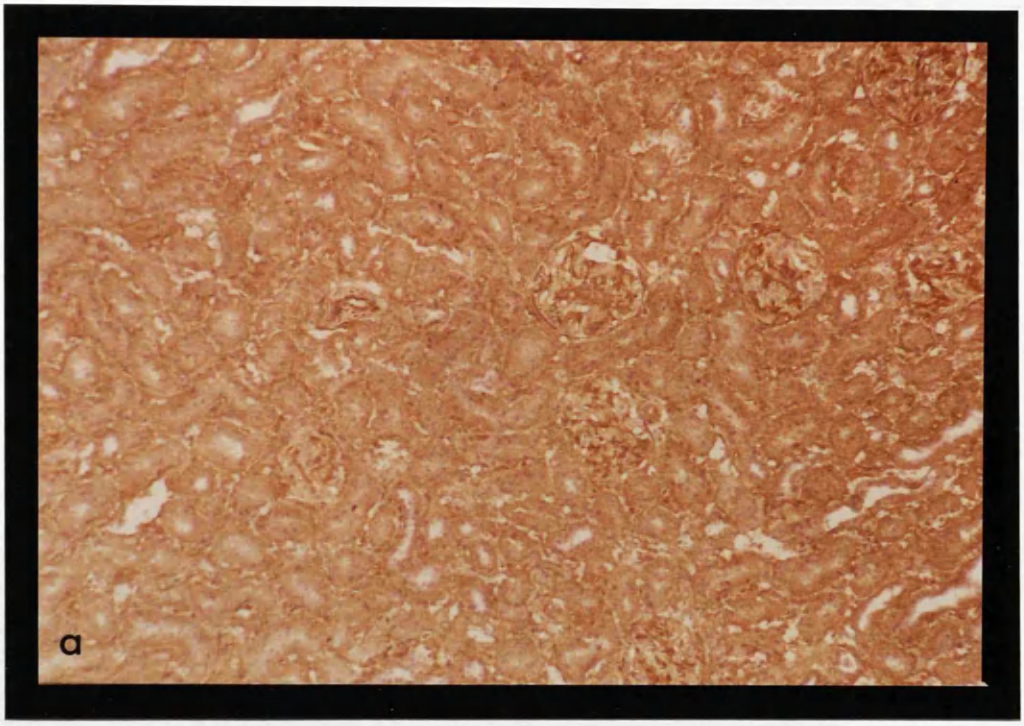
Cryostat sections were stained, by indirect immunoperoxidase, with antibodies directed against the monomorphic and polymorphic determinants of MHC class I antigens (MRC OX18 and MN4-91-6 respectively) and against the monomorphic and polymorphic determinants of MHC class II antigens (MRC OX6 and F17-23-2 respectively).

Non-rejecting renal allografts from unreconstituted animals showed moderate, but variable induction of class I and class II antigens on cortical tubules and occasional class II induction on renal vascular endothelium (Fig 6.4).

Rejecting grafts in animals restored with CD4 lymphocytes also demonstrated moderate induction of both class I and class II MHC antigens on cortical tubules and also occasional induction of class II MHC antigens on Bowman's capsule and on arteriolar vascular endothelium.

Rejecting grafts in animals restored with unseparated LNC demonstrated heavy induction of both class I and class II MHC antigens, often with strong class II expression on vascular endothelium and Bowman's capsule (Fig 6.4). Again, the cellular infiltrate comprised large numbers of MRC OX6 positive, polymorphic antibody negative host leukocytes, together with a few clusters of cells labelled with the donor specific antibodies. Thus much of the induction of MHC antigens in these animals was apparently closely associated with the cellular infiltration of the graft resulting from adoptive transfer of immunocompetent lymphocytes.

Fig. 6.4 Comparison of MHC antigen expression on DA renal allografts in PVG-rnu/rnu recipients which were either not reconstituted (a & b) or reconstituted with PVG LNC (c & d).
Cryostat sections of day 7 grafts were stained, by immunoperoxidase, with MN4-91-6 detecting DA but not PVG MHC class I antigens (a & c), and with F17-23-2 detecting DA but not PVG MHC class II antigens (b & d). (All x100).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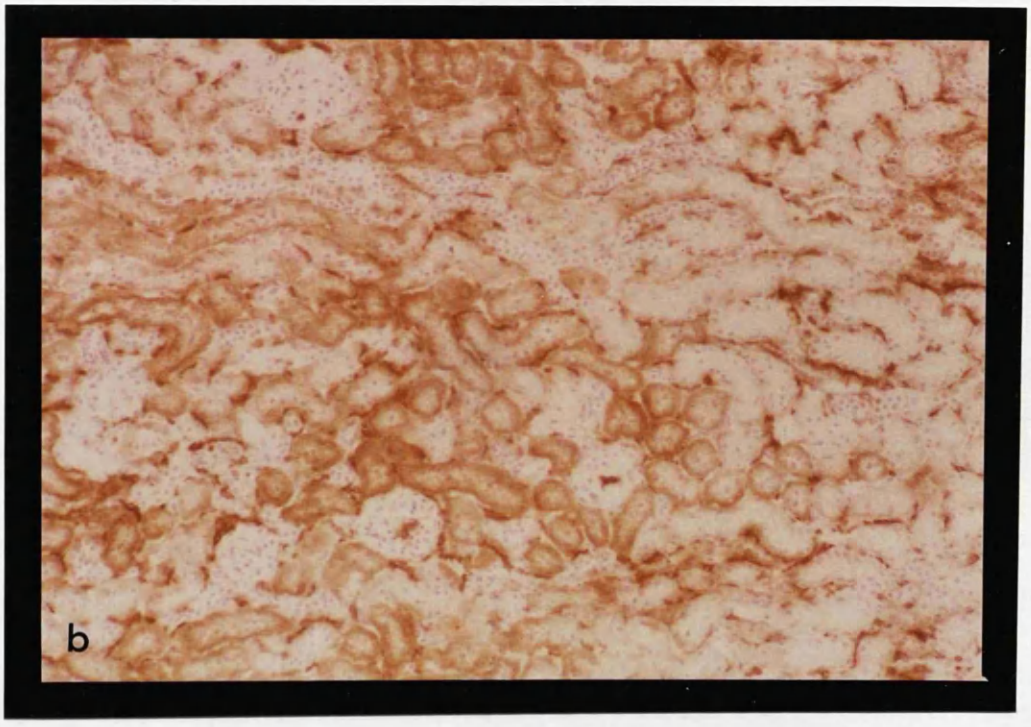
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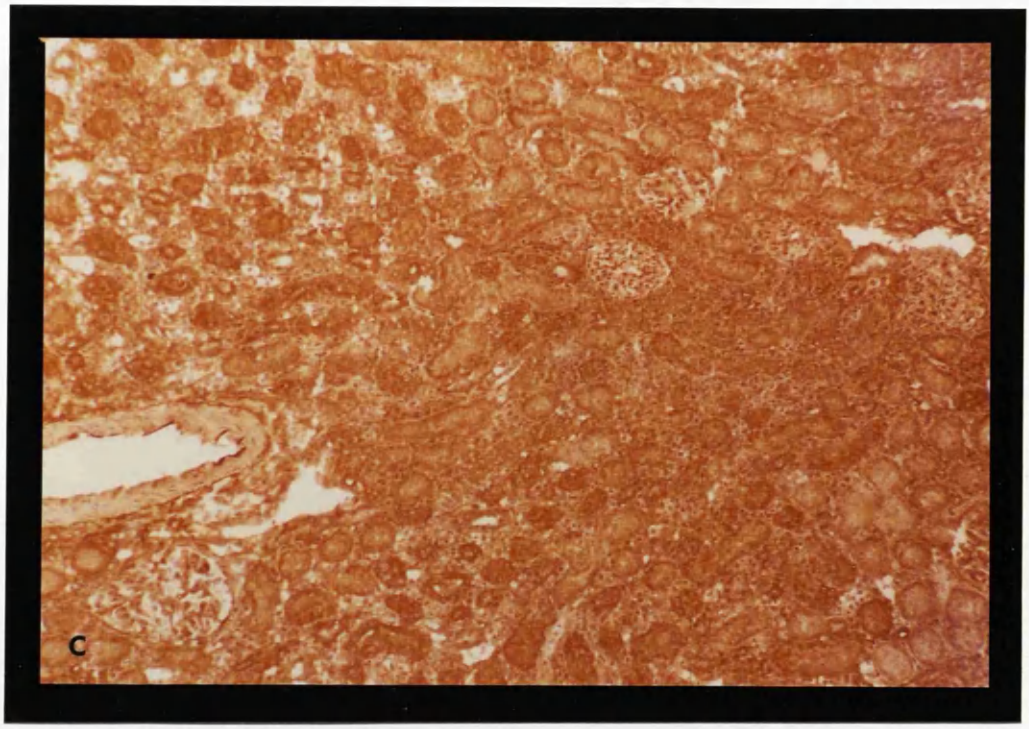
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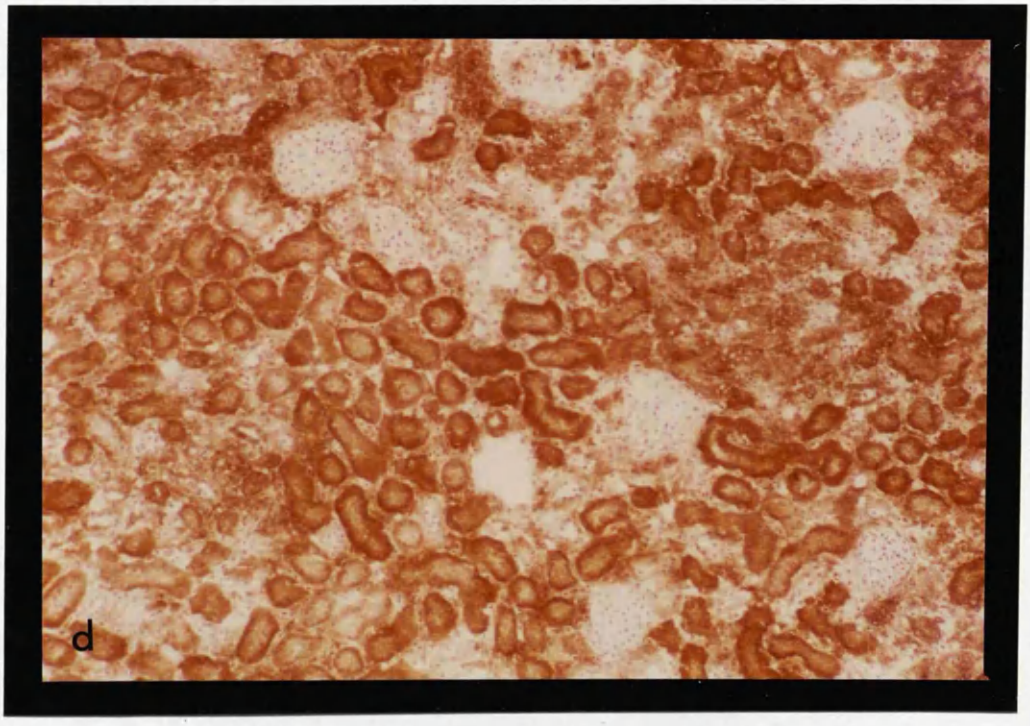
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6.7 *In vitro* cytotoxic activity of effector cells from PVG-rnu/rnu rats

6.7.1 *In vitro* cytotoxic activity of splenocytes from unmodified PVG-rnu/rnu rats

The demonstration that adoptively transferred CD4 lymphocytes restored the ability of athymic rats to reject a renal allograft promoted an investigation into the cytotoxic repertoire of spleen cells and graft infiltrating cells from these animals.

In this section, therefore, experiments were performed to assess the ability of such effector cells to lyse both allospecific and nonspecific targets in *in vitro* 6-hour ^{51}chromium release cytotoxicity assays. During the course of preliminary experiments, the cytotoxic activity of spleen cells from unmodified, i.e. non-transplanted, PVG-rnu/rnu rats was also tested. As predicted, spleen cells from unmodified athymic recipients showed high levels of nonspecific cytotoxic activity against the NK susceptible targets Y3 and YAC-1, which usually exceeded those of spleen cells obtained from normal PVG animals (Figs 6.5 & 6.6).

Interestingly, spleen cells from unmodified PVG-rnu/rnu animals also showed significant lysis of the mouse mastocytoma line, P815. This cell line is susceptible to lysis by lymphokine activated killer cells, or LAK cells, but is reported to be resistant to lysis by nonactivated

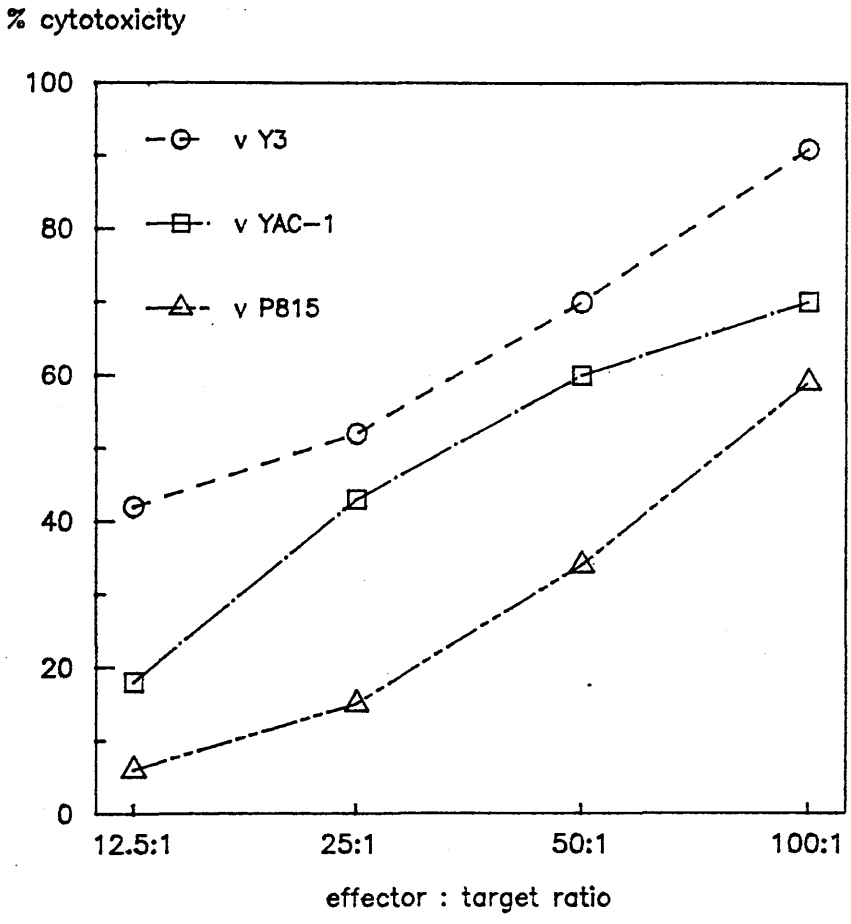

Fig. 6.5 Cytotoxic activity of splenocytes from unmodified PVG-rnu/rnu rats against NK susceptible targets Y3 and YAC-1.
Splenocyte effector cells were prepared, with macrophage depletion, from unmodified PVG-rnu/rnu rats and tested for their ability to lyse chromium-labelled target cells.

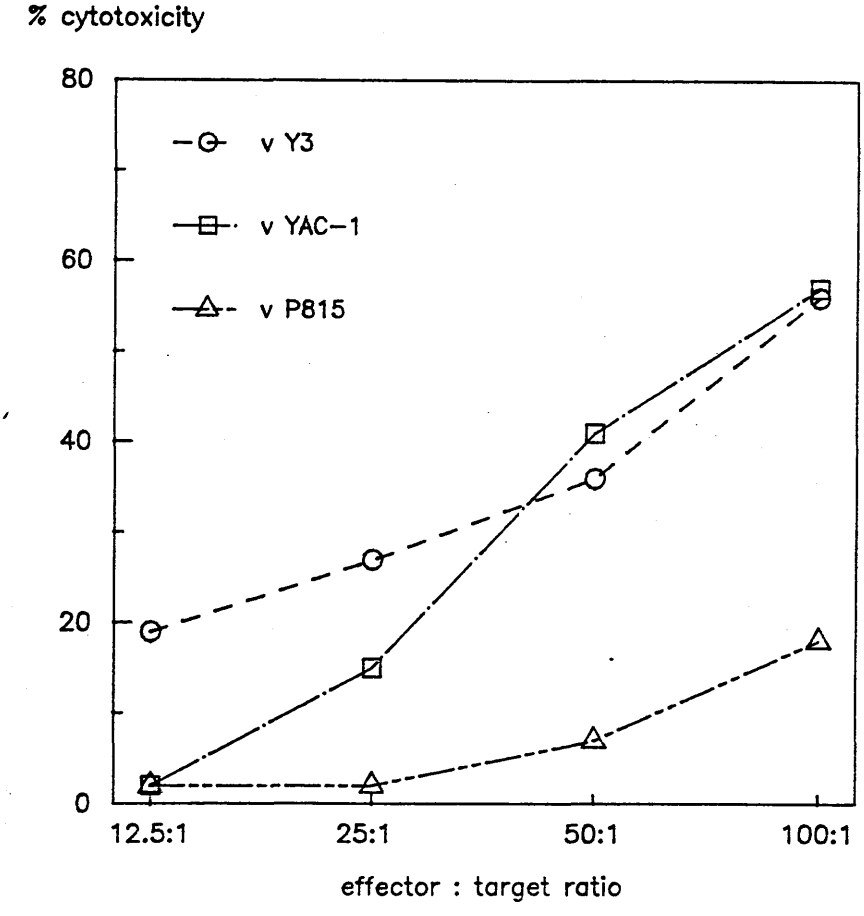

Fig. 6.6 Cytotoxic activity of splenocytes from normal PVG rats against NK susceptible targets Y3 and YAC-1. Splenocyte effector cells were prepared, with macrophage depletion, from normal PVG rats and tested for their ability to lyse chromium-labelled target cells.

lymphocytes and NK cells (Dunn & Potter, 1957; Vujanovic, Herberman & Hiserodt, 1988). Spleen cells from euthymic PVG rats showed minimal levels of cytotoxic activity against P815.

Unexpectedly, spleen cells from non-transplanted athymic PVG rats also showed high levels of cytotoxic activity against allogeneic (DA, AO and Lewis) ConA lymphoblasts, despite the absence of prior sensitisation, although they showed no ability to lyse syngeneic PVG lymphoblasts. This cytotoxicity against allogeneic ConA blasts contrasted markedly with the inability of spleen cells from normal PVG animals to lyse allogeneic ConA blast targets. The results of one such experiment are shown in Figs 6.7 and 6.8, and these are representative of similar experiments performed on six occasions.

The ability of spleen cells from PVG-rnu/rnu rats to lyse allogeneic ConA blasts was not lectin related since a similar pattern of reactivity was observed against freshly isolated, chromium-labelled allogeneic splenocyte targets, although the levels of spontaneous chromium release in such assays were high (e.g. 40%, results not shown).

The observation that spleen cells from unmodified athymic animals were cytotoxic against allogeneic ConA blasts was thought worthy of further investigation, as described below.

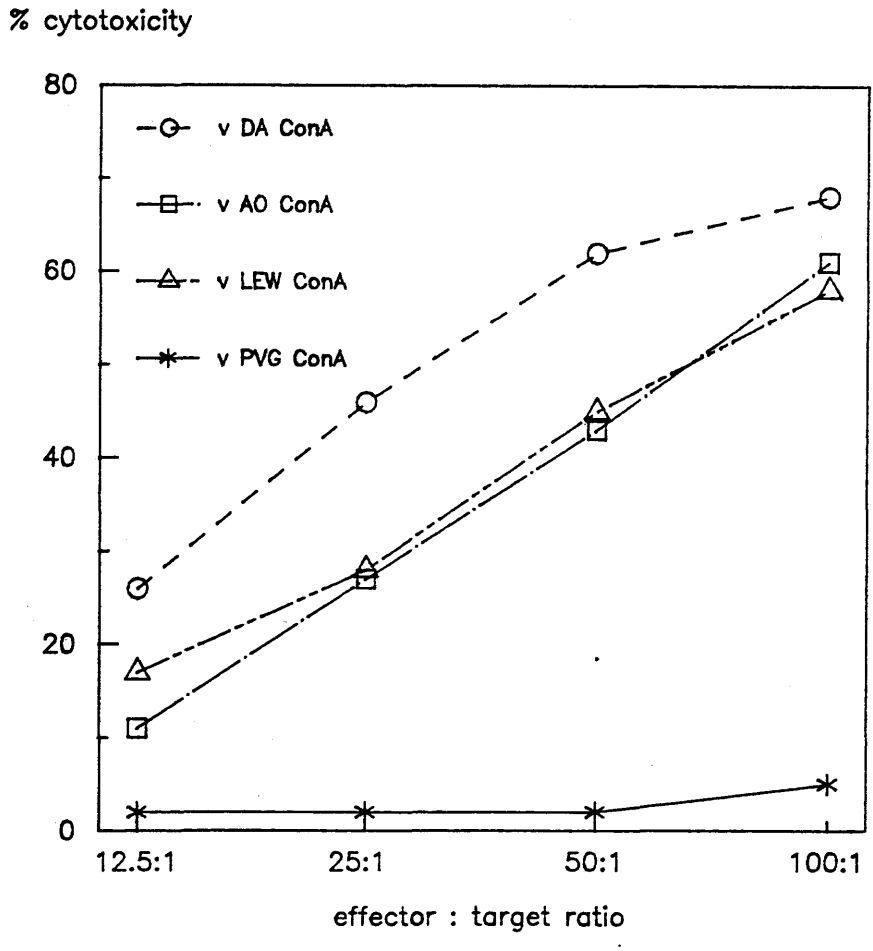

Fig. 6.7 Cytotoxic activity of splenocytes from unmodified PVG-rnu/rnu rats against a range of ConA blasts.
Splenocyte effector cells were prepared, with macrophage depletion, from unmodified PVG-rnu/rnu rats and tested for their ability to lyse chromium-labelled target cells.

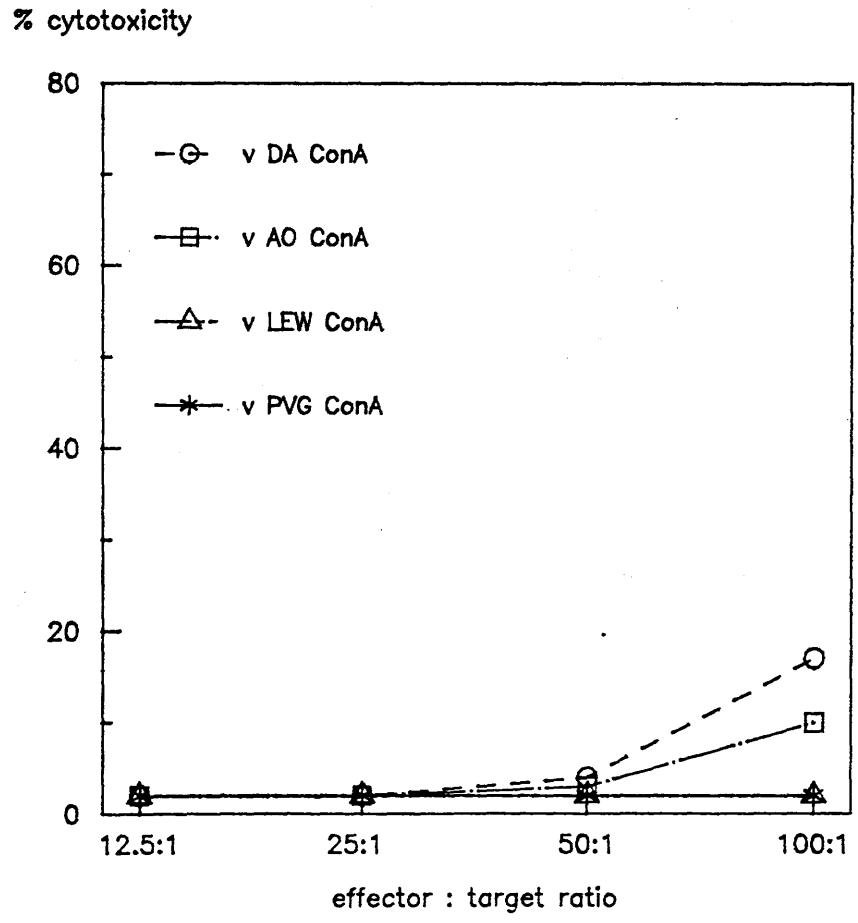

Fig. 6.8 Cytotoxic activity of splenocytes from normal PVG rats against a range of ConA blasts.
Splenocyte effector cells were prepared, with macrophage depletion, from normal PVG rats and tested for their ability to lyse chromium-labelled target cells.

6.7.2 Effect of anti-asialo GM1 treatment on the alloreactivity of PVG-rnu/rnu splenocytes

The results of the experiments described above suggested that the alloreactive cells in nude rats might be NK cells - atypical because NK cells are not normally able to lyse ConA blasts. Mouse and rat NK cells are reported to bear the cell surface glycolipid, GM1, which serves as a marker by which NK cells may be either identified, or eliminated with the use of antibody and complement (Kasai, Iwamori, Okomura et al, 1980; Reynolds, Sharrow, Ortaldo et al, 1981; Kasai, Yoneda, Habu et al, 1981). Attempts to positively identify NK cells in tissue sections and in cytocentrifuge slide preparations of PVG-rnu/rnu rat splenocytes using a rabbit anti-asialo GM1 (anti-AGM1) antiserum (Wako Chemicals, Dallas, Texas) with an immunoperoxidase staining technique (Fossum & Rolstad, 1986) were unsuccessful because of extensive background staining.

An alternative approach was to remove the putative NK cells from the effector cell populations by treating the cells with anti-AGM1 and complement to see if the ability of the cell population to lyse NK sensitive targets was reduced. Briefly, $2\text{-}3x10^7$ splenocytes per ml were incubated for 45 min at 4C with a 1:50 dilution of the anti-AGM1 antibody. After two washes,

the cells were incubated with a 1:5 dilution of freshly reconstituted guinea pig complement for 45 min at 37C, and then washed again prior to use.

Treated and untreated PVG-rnu/rnu splenocytes were compared with treated and untreated splenocytes from specifically sensitised (by DA skin grafting) PVG donors, for their ability to lyse a range of chromium labelled target cells in vitro. ("Untreated" splenocytes were incubated with DAB, in place of anti-AGM1, followed by guinea pig complement as for treated cells.)

The results of the experiment depicted in Figs 6.9, 6.10, 6.11 & 6.12, are representative of three experiments which gave similar results. As before, results from both euthymic and athymic PVG rats showed high levels of cytotoxic activity against the NK susceptible targets Y3 and YAC-1. This nonspecific activity was entirely abrogated by anti-AGM1 treatment (Figs 6.9 & 6.10).

PVG-rnu/rnu splenocytes also showed very high levels of cytotoxic activity against both DA and Lewis ConA blasts, which, again, were abrogated by treatment of the effector cells with anti-AGM1 (Figs 6.11 & 6.12).

Specifically sensitised PVG splenocytes demonstrated low levels of cytotoxic activity against third party Lewis ConA blasts, and moderate levels against the specific target DA ConA blasts. Unexpectedly, however, this activity was similarly diminished following

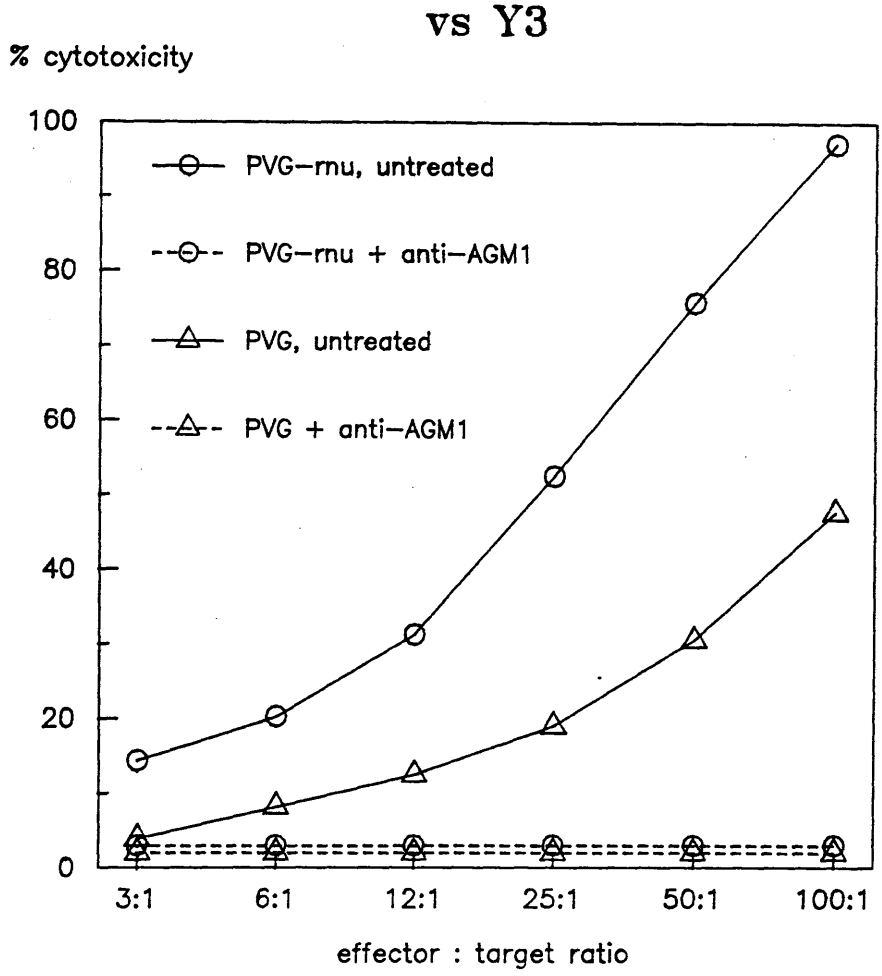

Fig. 6.9 Effect of anti-asialo GM1 treatment on cytotoxic activity of splenocytes from unmodified PVG-rnu/rnu and from specifically sensitised PVG rats, against Y3 targets.
Effector splenocytes were prepared from unmodified athymic rats and from PVG rats which had previously been specifically sensitised with two DA skin grafts. Effector cells were treated with either anti-AGM1 or with DAB (control) followed by guinea-pig complement, to remove NK activity, then tested for in vitro cytotoxic activity against a range of chromium-labelled targets.

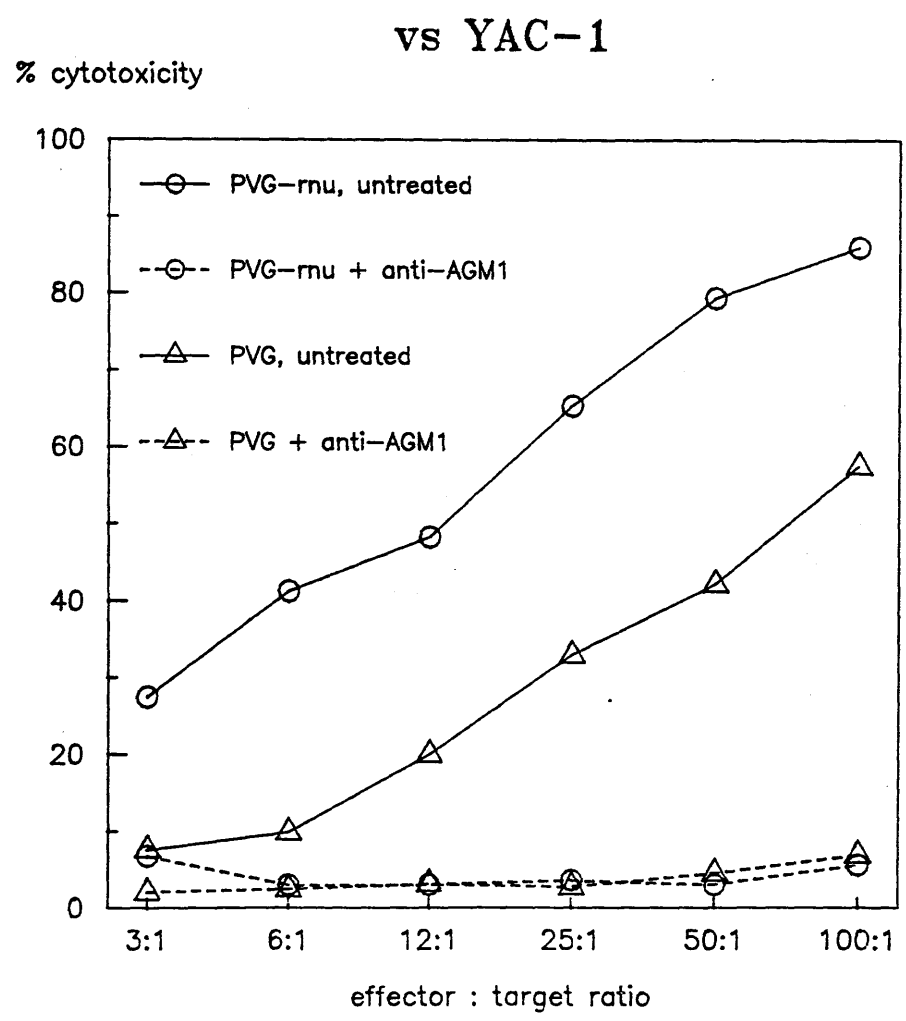

Fig. 6.10 Effect of anti-asialo GM1 treatment on cytotoxic activity of splenocytes from unmodified PVG-rnu/rnu and from specifically sensitised PVG rats, against YAC-1 targets.
Effector splenocytes were prepared from unmodified athymic rats and from PVG rats which had previously been specifically sensitised with two DA skin grafts. Effector cells were treated with either anti-AGM1 or with DAB (control) followed by guinea-pig complement, to remove NK activity, then tested for in vitro cytotoxic activity against a range of chromium-labelled targets.

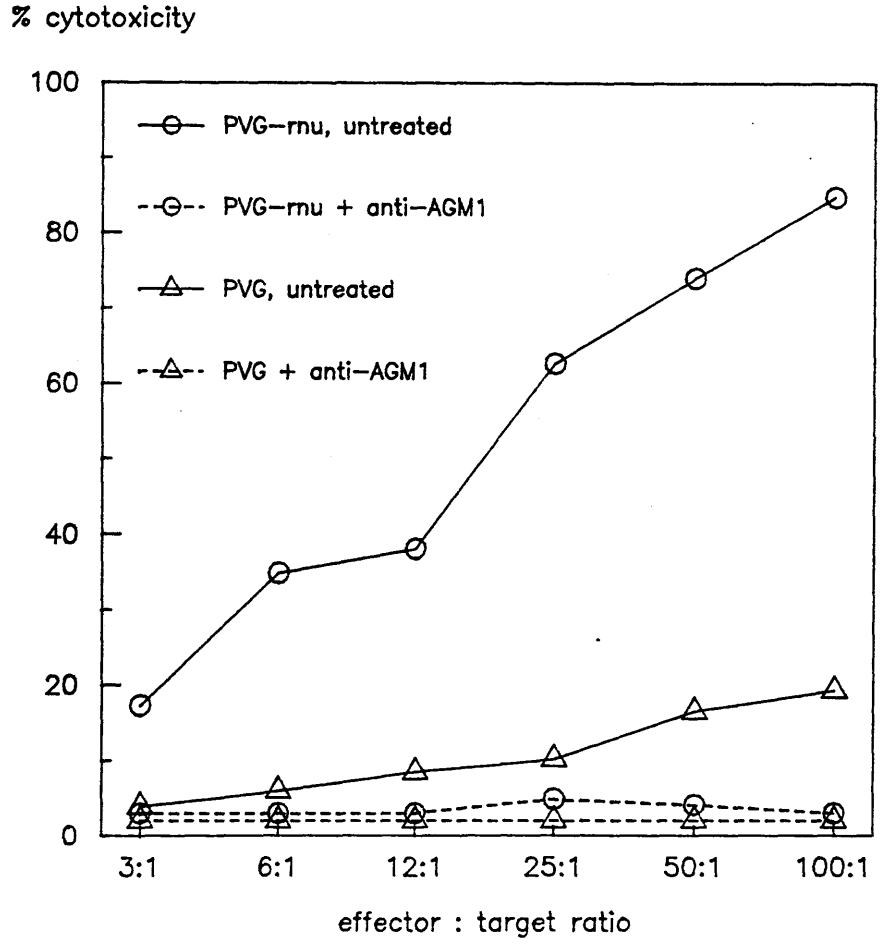

Fig. 6.11 Effect of anti-asialo GM1 treatment on cytotoxic activity of splenocytes from unmodified PVG-rnu/rnu and from specifically sensitised PVG rats, against DA ConA blast targets.
Effector splenocytes were prepared from unmodified athymic rats and from PVG rats which had previously been specifically sensitised with two DA skin grafts. Effector cells were treated with either anti-AGM1 or with DAB (control) followed by guinea-pig complement, to remove NK activity, then tested for in vitro cytotoxic activity against a range of chromium-labelled targets.

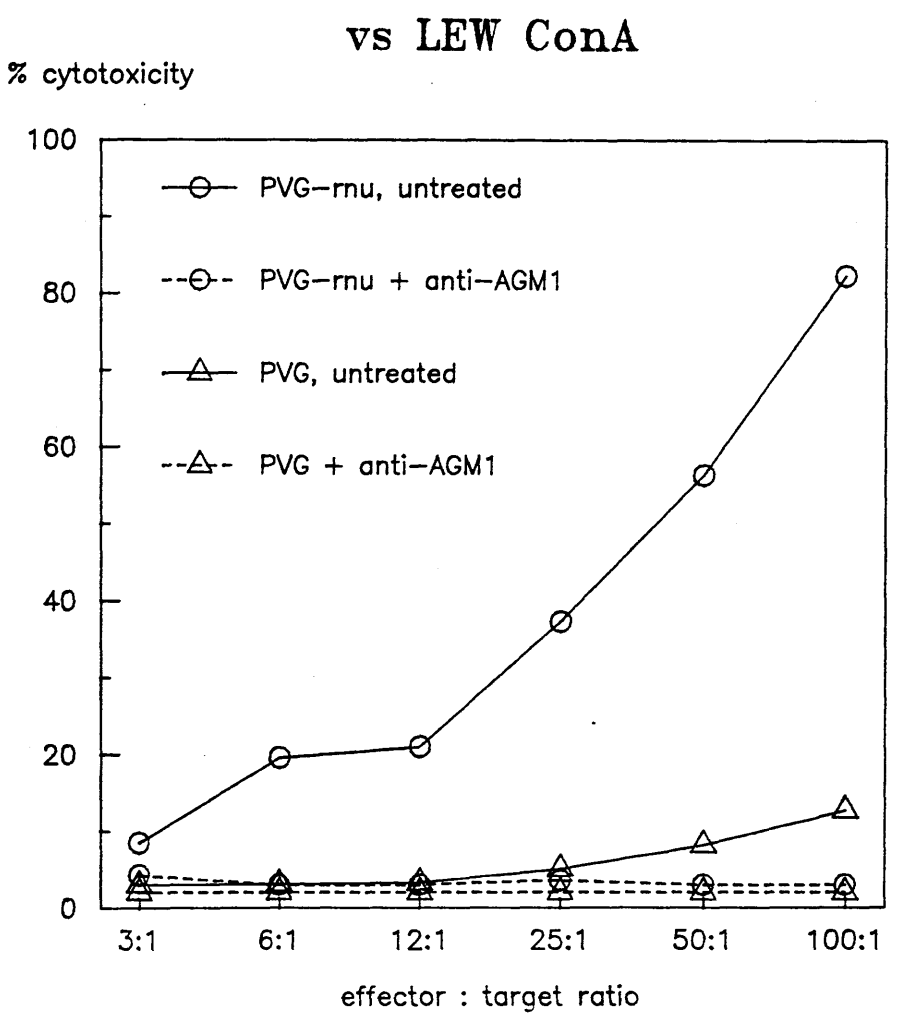

Fig. 6.12 Effect of anti-asialo GM1 treatment on cytotoxic activity of splenocytes from unmodified PVG-rnu/rnu and from specifically sensitised PVG rats, against Lewis ConA blast targets.
Effector splenocytes were prepared from unmodified athymic rats and from PVG rats which had previously been specifically sensitised with two DA skin grafts. Effector cells were treated with either anti-AGM1 or with DAB (control) followed by guinea-pig complement, to remove NK activity, then tested for in vitro cytotoxic activity against a range of chromium-labelled targets.

anti-AGM1 treatment (Fig 6.11), and it was therefore not possible to conclude that anti-AGM1 was effective only against NK cells.

Cytocentrifuge slide preparations of PVG-rnu/rnu effector cells prepared before and after anti-AGM1 treatment confirmed that the number of cells with the morphological appearance of large granular lymphocytes (i.e. cells containing azurophilic cytoplasmic granules) were dramatically reduced from 10-15% of effector cells before treatment to 2-3% of effector cells following treatment.

The results of these experiments appeared to support the hypothesis that the wide alloreactivity of PVG-rnu/rnu splenocytes was due to NK cells. This suggestion must be interpreted with caution, however, since it is likely that the GM1 glycolipid may be expressed on leukocytes other than NK cells, albeit less densely.

6.7.3 Inhibition of widely alloreactive cytotoxic cells by the addition of unlabelled targets

The demonstration that spleen cells from PVG-rnu/rnu recipients were able to lyse allogeneic ConA blasts raised the question as to whether different subpopulations of effector cells were specifically

killing targets of certain haplotypes or whether, as seemed more likely, effector cells were able to lyse all of the different targets in a nonspecific manner.

This question was investigated by performing cold target inhibition assays, in which addition of excess unlabelled targets to the assay was used to inhibit the killing of labelled targets. If target cell lysis were entirely nonspecific, then the addition of unlabelled targets of a different specificity should be as inhibitory as the addition of syngeneic but unlabelled targets. Conversely, if alloreactive lysis were due to the specific recognition of the target, then the addition of irrelevant, unlabelled targets should not affect chromium release from the specific target.

The results of two experiments are depicted in Table 6.4. As shown, at lower ratios of unlabelled (cold) to labelled (hot) targets (e.g. 20:1), there was marked heterogeneity of effector cell interaction with the various targets, since inhibition of cytotoxicity was generally substantially greater with syngeneic targets than with other cold targets. However, at higher cold:hot ratios, there was clear evidence of varying degrees of cross inhibition as well as homologous inhibition, indicating shared specificity of effector cells against the different targets.

Table 6.4 Inhibition of ^{51}Cr-release by the addition of unlabelled targets in cytotoxicity assays

LABELLED TARGET	UNLABELLED: LABELLED	% INHIBITION BY UNLABELLED TARGETS DA	LEW	Y3	YAC-1
Expt 1					
DA	20:1	68	16	nd	22
(62%)*	10:1	47	11	nd	17
	5:1	30	21	nd	19
YAC-1	20:1	0	3	nd	100
(39%)*	10:1	0	0	nd	100
	5:1	8	1	nd	100
Expt 2					
DA	80:1	93	71	31	nd
(55%)*	40:1	82	47	22	nd
	20:1	71	24	29	nd
LEW	80:1	71	86	75	nd
(65%)*	40:1	60	71	38	nd
	20:1	72	69	38	nd
Y3	80:1	52	75	88	nd
(48%)*	40:1	25	35	90	nd

Table 6.4 Cold target inhibition assays were performed in which unlabelled target cells, added to the wells in standard *in vitro* cytotoxicity assays, competed with the labelled targets for lysis by effector cells prepared from athymic PVG splenocytes. The ratio of effector cells to labelled target cells was 100:1 throughout.

Results (expressed as % inhibition of chromium release [mean of triplicate determinations]) are shown for two experiments. In both experiments, control wells containing unlabelled PVG splenocyte "filler" cells were included, and inhibition was less than 10% in all cases.

* % cytotoxicity at effector : target ratio of 100:1 in absence of unlabelled competitor targets.

There was little "crowding effect" in the wells of the assay, since the addition of unlabelled PVG splenocytes (instead of cold targets) at 80:1 and 40:1 ratios resulted in less than 10% inhibition.

6.7.4 The role of FCS in the *in vitro* cytotoxicity assay

Why are PVG-rnu/rnu rats unable to reject a renal allograft and yet they possess cells which have the capacity to lyse a large variety of allogeneic targets *in vitro*? Two possibilities related to the experimental protocol were investigated: firstly, it was possible, but unlikely, that PVG-rnu/rnu splenocytes were activated by the presence of FCS in the assay. If so, substitution of FCS by heat inactivated serum from nude rats might remove the wide alloreactivity of the splenocytes. Secondly, it was possible that nude rat serum is deficient in a factor, e.g. an adherence factor which was present in FCS. If so, effector cells may be able to lyse targets *in vitro* in the FCS environment, but be unable to do so *in vivo*.

In the following experiment, effector cells were prepared from two PVG-rnu/rnu spleens and compared with effectors from two normal PVG spleens for their ability to lyse a range of target cells in the presence of either FCS or PVG serum or PVG-rnu/rnu serum. The results presented in Table 6.5 show very little difference between the groups and therefore demonstrate that the

Table 6.5. Effect of FCS and rat serum on cytotoxic activity of effector splenocytes from nude and normal PVGs

SERUM SOURCE	EFFECTOR CELLS	E:T RATIO	% CHROMIUM RELEASE FROM TARGET CELLS					
			DA	AO	PVG	P815	Y3	YAC-1
FCS	PVG-rnu	100:1	68	61	5	59	91	70
		50:1	62	43	1	34	70	60
		25:1	46	27	0	15	52	43
		12:1	26	11	0	6	42	18
	PVG	100:1	17	10	1	18	56	57
		50:1	4	3	0	7	36	41
		25:1	2	0	0	1	27	15
		12:1	0	0	0	1	19	1
PVG	PVG-rnu	100:1	80	63	25	63	84	85
		50:1	57	37	24	31	58	57
		25:1	51	30	3	15	39	47
		12:1	25	20	9	20	19	31
	PVG	100:1	28	19	20	19	52	58
		50:1	18	11	16	8	35	34
		25:1	19	19	17	20	20	19
		12:1	14	6	13	16	18	9
PVG-rnu	PVG-rnu	100:1	67	44	8	33	80	76
		50:1	47	25	2	16	58	56
		25:1	31	9	0	5	43	33
		12:1	12	1	0	0	24	19
	PVG	100:1	16	7	2	7	44	43
		50:1	5	0	0	1	22	24
		25:1	0	0	4	1	18	12
		12:1	0	0	0	0	11	3

Table 6.5 To investigate the possibility that the presence of 5% FCS in the medium had a contributing effect on the observed wide alloreactivity of unmodified PVG-rnu/rnu splenocytes *in vitro*, cytotoxicity assays were performed simultaneously in the presence of either FCS, or heat-inactivated normal PVG serum, or heat-inactivated normal PVG-rnu/rnu serum, against a range of ^{51}chromium-labelled target cells.

wide alloreactivity of PVG-rnu/rnu splenocytes is not explained by a "serum effect". Interestingly, the presence of normal PVG serum resulted in moderate levels of autoreactivity by both normal and nude PVG effector cells.

6.7.5 Cytotoxic activity of splenocytes from PVG-rnu/rnu recipients of DA renal allografts

In each individual experiment, three PVG-rnu/rnu recipients of DA renal allografts were reconstituted, at the time of transplant, with $5x10^7$ LNC (one animal), or the same number of CD4 lymphocytes (one animal), or were not reconstituted (one animal). On day 7 after transplantation, the animals were sacrificed in order to study the capacity of their lymphoid cells to lyse a range of chromium labelled target cells in vitro. (It was not possible to obtain sufficient graft infiltrating cells or lymph node cells from individual animals [$<10^7$] to perform an assay.) Splenocytes were compared, after macrophage depletion, for their ability to lyse the different target cells. These included the NK susceptible Y3 and YAC-1 rat myeloma and mouse lymphoma lines, as well as a variety of ConA blasts.

Overall, the results of these experiments differed little from the results of the above experiments using effector splenocytes prepared from non-transplanted athymic rats. As before, it was found that all effector

cells showed the same high levels of cytotoxic activity against all targets tested, with the exception of PVG ConA blasts which were not lysed by any of the effector populations. This experiment was repeated on three occasions, with the same result, and the results of one experiment are shown in Figs 6.13, 6.14 & 6.15, where the targets were DA and Lewis ConA blasts and Y3 cells respectively.

Thus there was little evidence of allospecific activity even in the spleens of reconstituted animals rejecting a renal allograft. The levels of lysis of both DA and Lewis ConA blasts were similar, and the overall pattern of lysis against ConA blasts differed little from that already seen. Splenocytes prepared from normal euthymic PVG rats, by contrast, showed no activity against DA, PVG and Lewis ConA blasts, but showed moderately high levels of activity against Y3 targets (approximately 40% lysis at 100:1 effector : target ratio).

Cytocentrifuge slide preparations of effector splenocytes from two of the above experiments were stained with May-Grunwald & Giemsa for morphological examination, and with monoclonal antibodies and the immunoperoxidase technique for phenotypic examination. A differential count was performed on 500 cells in each effector population, and a total of 400 cells with the morphological appearance of lymphocytes was counted in

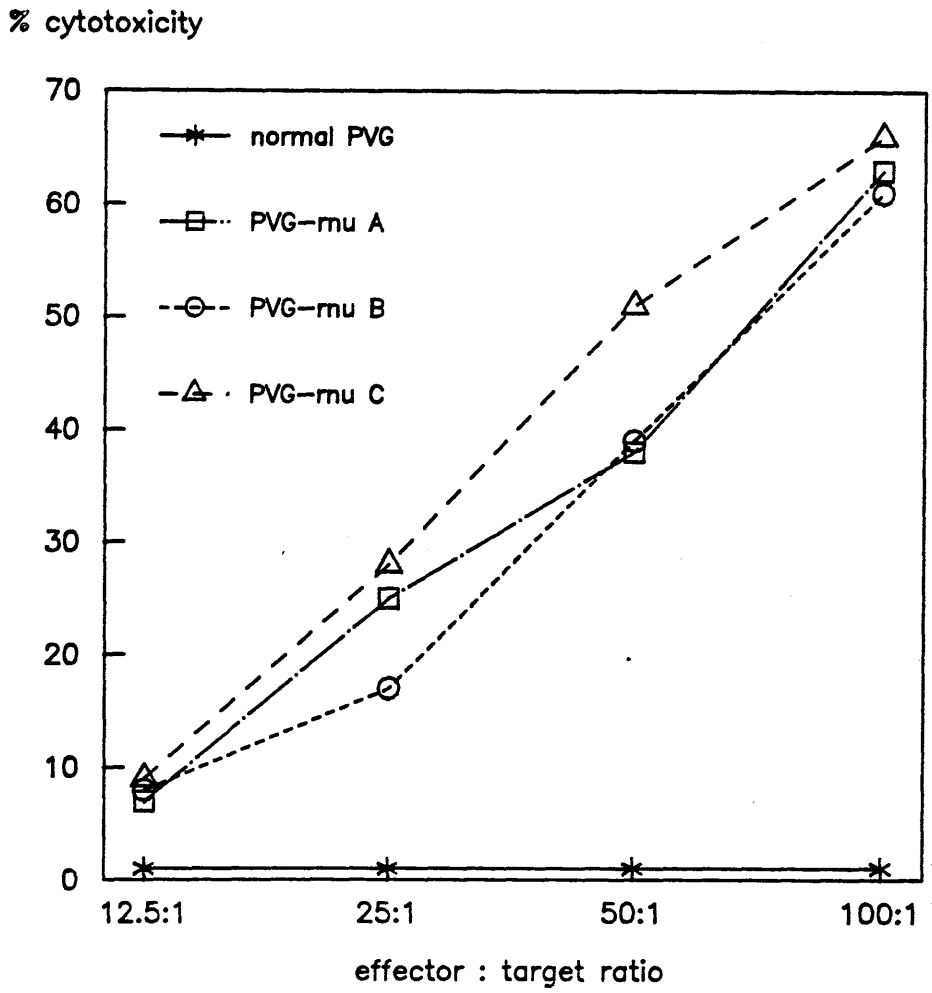

Fig. 6.13 Cytotoxic activity of splenocytes harvested on day 7 from PVG-rnu/rnu recipients of DA renal allografts, against DA ConA blast targets.
Splenocyte effector cells were prepared, with macrophage depletion, from PVG-rnu/rnu recipients which had received no cells (A), or which had been reconstituted with $5x10^7$ cells, either CD4 (B) or LNC (C). Normal PVG spleen effector cells were included for comparison.

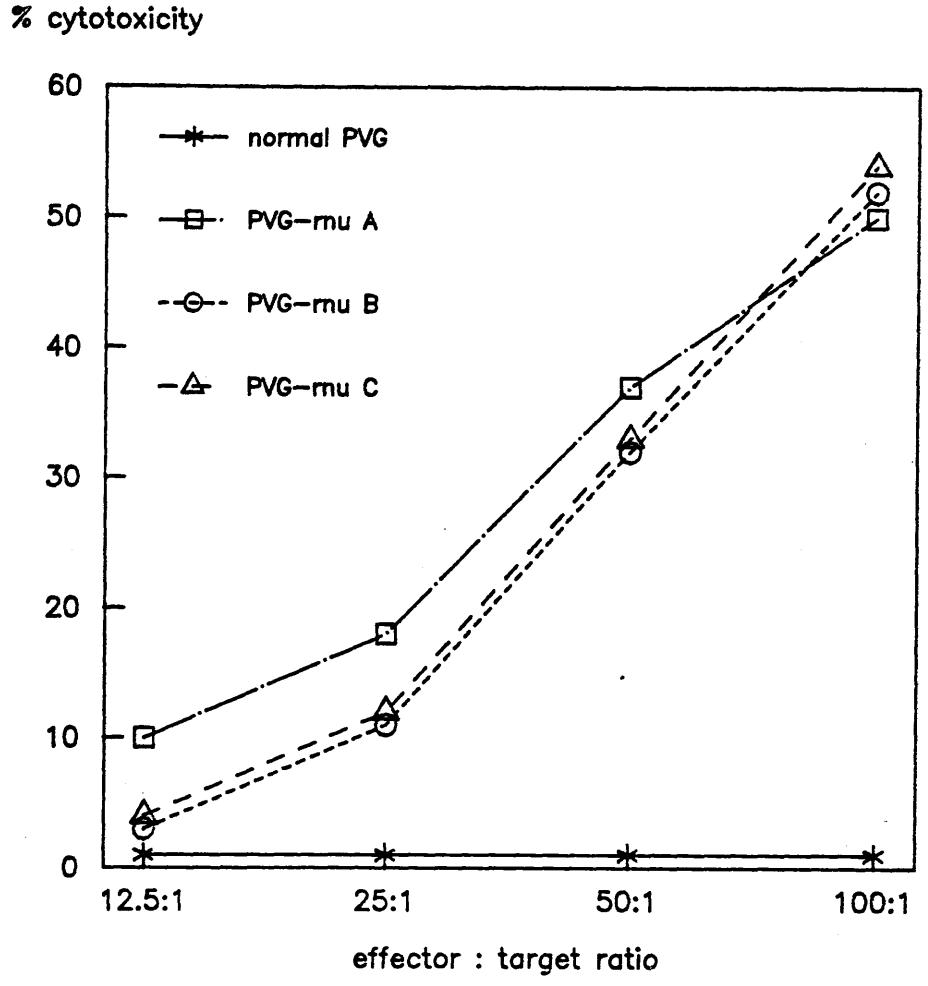

Fig. 6.14 Cytotoxic activity of splenocytes harvested on day 7 from PVG-rnu/rnu recipients of DA renal allografts, against irrelevant Lewis ConA blast targets.
Splenocyte effector cells were prepared, with macrophage depletion, from PVG-rnu/rnu recipients which had received no cells (A), or which had been reconstituted with $5x10^7$ cells, either CD4 (B) or LNC (C). Normal PVG spleen effector cells were included for comparison.

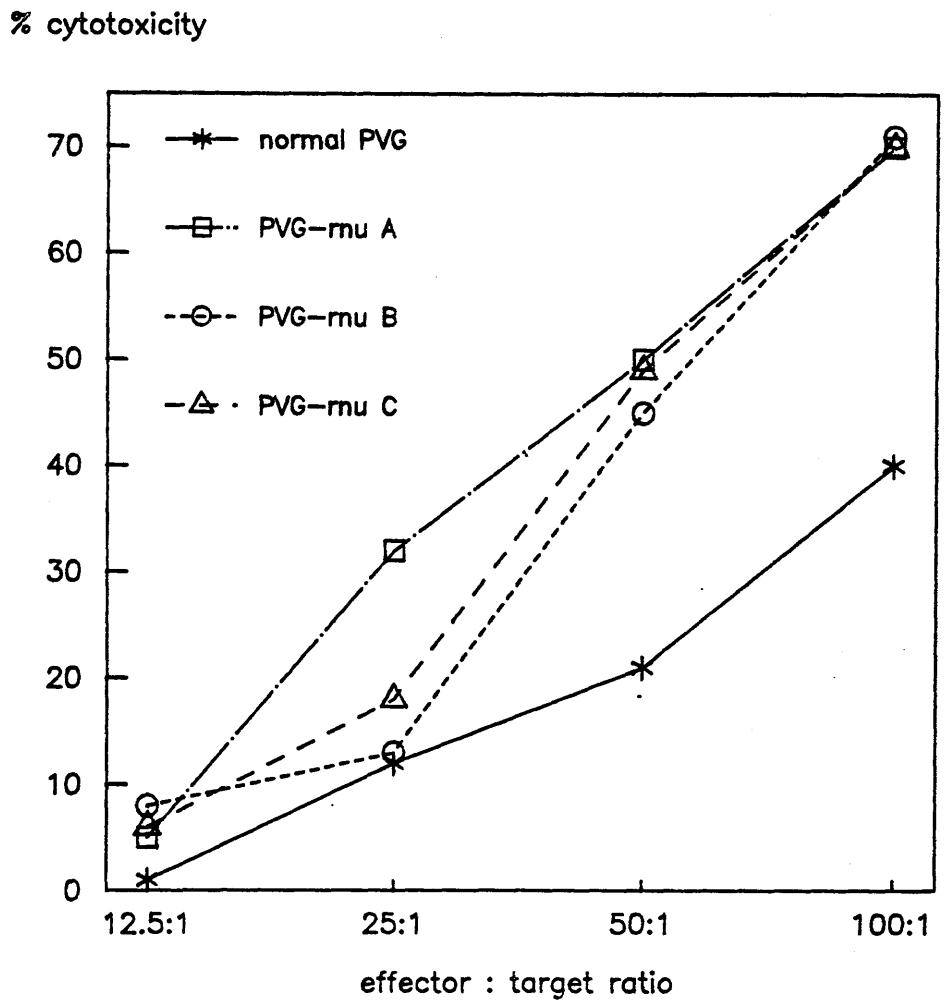

Fig. 6.15 Cytotoxic activity of splenocytes harvested on day 7 from PVG-rnu/rnu recipients of DA renal allografts, against Y3 targets.
Splenocyte effector cells were prepared, with macrophage depletion, from PVG-rnu/rnu recipients which had received no cells (A), or which had been reconstituted with $5x10^7$ cells, either CD4 (B) or LNC (C). Normal PVG spleen effector cells were included for comparison.

each slide labelled with a different monoclonal antibody. The results appear in Table 6.6, and show a similar pattern, irrespective of whether or not they derived from animals that had been reconstituted with syngeneic lymphocytes. Approximately 20% of the cells were neutrophils, about 70% had the morphological appearance of lymphocytes, and the remaining 10% comprised macrophages and eosinophils. Of the lymphocytes, most were labelled with MRC OX12 and were therefore B cells. A number appeared to be MRC $OX8^+$ cells, some of which were MRC $OX19^-$ and were probably NK cells, and a few appeared to be $W3/25^+$ lymphocytes. The main difference between the groups of effector cells was that those from unreconstituted animals appeared to contain fewer MRC $OX19^+$ T cells.

It appeared from the functional experiments described in this section that PNG-rnu/rnu rats possess cells which are morphologically similar to lymphocytes, and some of which are phenotypically similar to NK cells. These cells demonstrate wide <u>in vitro</u> alloreactivity and yet are unable to mediate allograft rejection in the absence of additional immunocompetent syngeneic lymphocytes.

Table 6.6 a) Morphological identity of effector splenocytes from PVG-rnu/rnu recipient of DA renal allograft

RAT NO.	CELLS TRANSFERRED	DIFFERENTIAL LEUKOCYTE COUNT OF EFFECTORS (%) PMNs	LYMPHs	MACROs	EOSINs
1	none	22	69	8	1
2	none	14	79	6	1
3	CD4	22	70	7	1
4	CD4	28	59	9	4
5	LNC	16	76	7	1
6	LNC	15	77	7	1

Table 6.6 b) Phenotypic identity of effector splenocytes from PVG-rnu/rnu recipients of DA renal allografts

RAT NO.	CELLS TRANSFERRED	% EFFECTOR SPLENOCYTES LABELLED WITH MAbs OX8	W3/25	OX19	OX39	OX12
1	none	19	5	11	1	nd*
2	none	10	9	1	3	80
3	CD4	19	31	47	3	nd
4	CD4	17	21	10	3	69
5	LNC	16	17	22	3	nd
6	LNC	12	13	8	3	73

* nd = not done

Table 6.6a Cytocentrifuge slide preparations of effector cells were stained with May-Grunwald/Giemsa and a differential leukocyte count was performed by counting a minimum of 400 cells on each slide.
Table 6.6b Cytocentrifuge slide preparations of effector cells were stained with MAbs to rat leukocytes, using an immunoperoxidase technique, and the percentage of lymphocytes (ie. cells with the morphological appearance of lymphocytes) labelled with each MAb was determined by counting a minimum of 400 cells on each slide.

6.7.6 Cytotoxic repertoire of graft infiltrating cells and LNC in PVG-rnu/rnu rats rejecting DA renal allografts

In order to obtain sufficient numbers of graft infiltrating cells and lymph node cells from reconstituted animals rejecting their grafts, it was necessary to pool the tissues of 2-3 animals.

Effector cells were therefore prepared on day 7 from renal allografts and from lymph nodes of two or three rats which had been simultaneously reconstituted with $5x10^7$ CD4 cells at the time of transplantation. Graft infiltrating cells were harvested by mechanical disruption of the kidney followed by Percoll centrifugation and tested for in vitro cytotoxic activity against a range of chromium labelled targets.

Targets included DA ConA blasts, syngeneic PVG ConA blasts and third party Lewis ConA blasts, together with the NK susceptible Y3 cell line, and also the mouse mastocytoma line, P815. This experiment was repeated on three occasions with the same result. Graft infiltrating cells showed modest levels of autoreactivity and high levels of activity against all other targets. The levels of cytotoxic activity shown by graft infiltrating cells (Figs 6.16 & 6.17) were not higher than those of either LNC (Fig 6.18 & 6.19) or of splenocytes (reported earlier). In one experiment, PVGr1 ConA blasts (which differ from PVG cells only at the RT1A "a" haplotype which is of DA type) were used as a

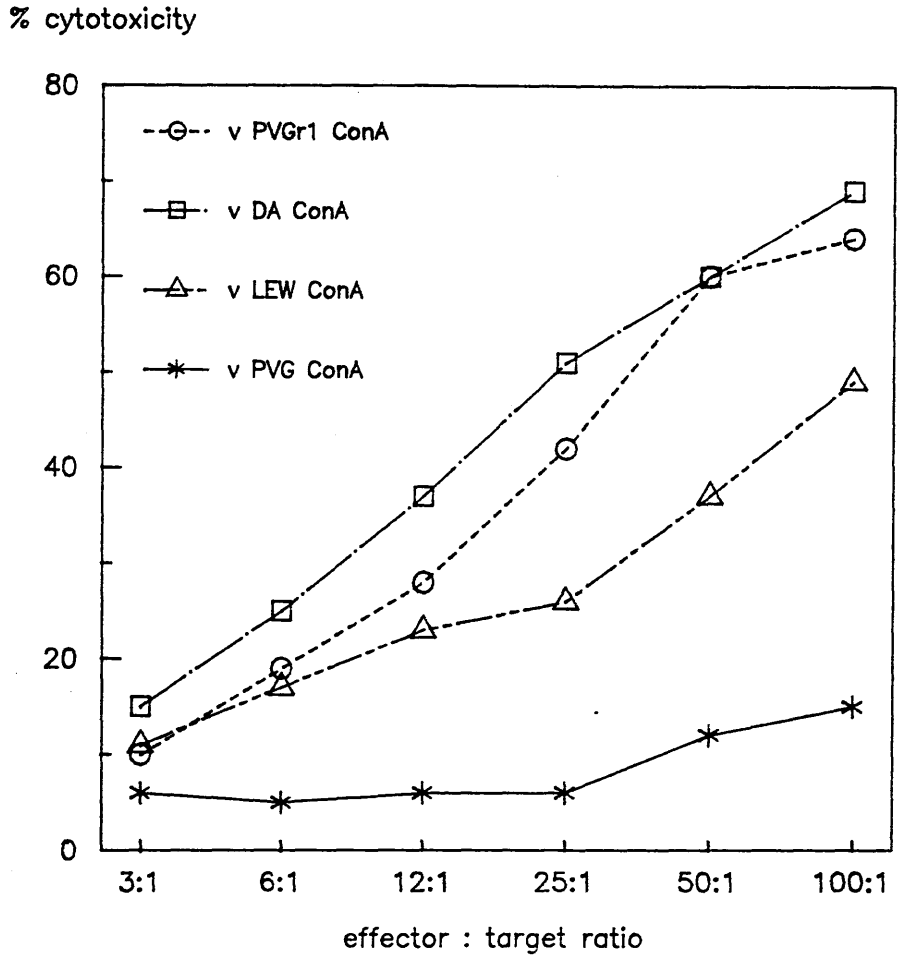

Fig. 6.16 Cytotoxic activity of graft infiltrating cells harvested on day 7 from CD4 cell reconstituted PVG-rnu/rnu recipients rejecting a DA renal allograft, against a range of ConA blast target cells.
Graft infiltrating cells were prepared, by Percoll centrifugation, from two pooled kidneys removed from animals reconstituted with $5x10^7$ CD4 cells. They were tested in vitro for their ability to lyse chromium-labelled ConA blast targets of PVGr1, DA, PVG and Lewis strains.

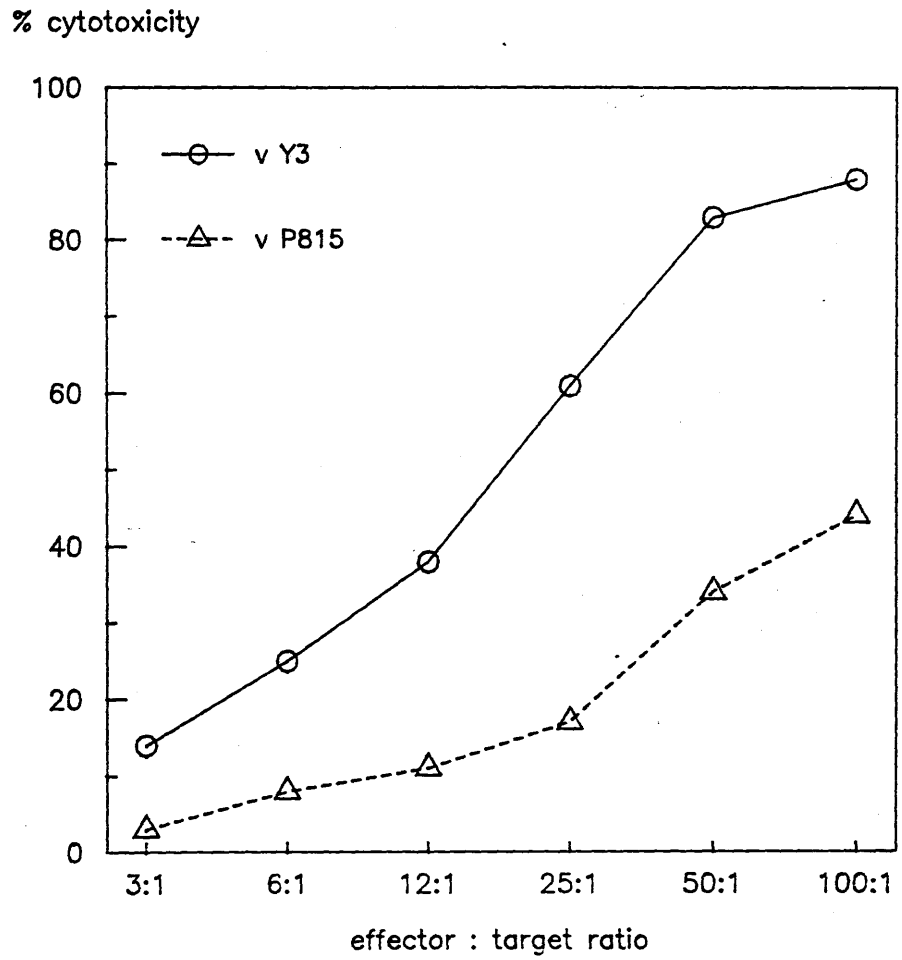

Fig. 6.17 Cytotoxic activity of graft infiltrating cells harvested on day 7 from CD4 cell reconstituted PVG-rnu/rnu recipients rejecting a DA renal allograft, against Y3 and P815 target cells.
Graft infiltrating cells were prepared, by Percoll centrifugation, from two pooled kidneys removed from animals reconstituted with $5x10^7$ CD4 cells. They were tested in vitro for their ability to lyse chromium-labelled Y3 and P815 targets.

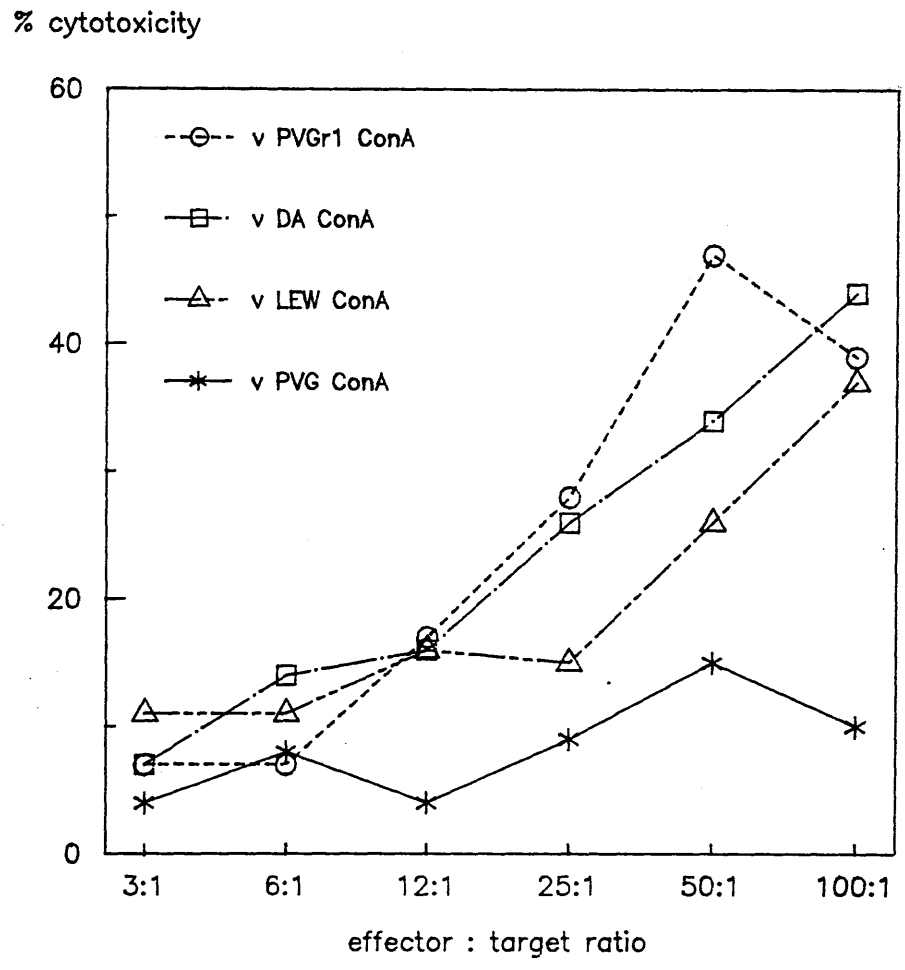

Fig. 6.18 Cytotoxic activity of lymph node cells harvested on day 7 from CD4 cell reconstituted PVG-rnu/rnu recipients rejecting a DA renal allograft, against a range of ConA blast targets.
LNC effector cells were prepared from mesenteric and cervical lymph nodes removed from animals reconstituted with $5x10^7$ CD4 cells. They were tested *in vitro* for their ability to lyse chromium-labelled ConA blast targets of PVGr1, DA, PVG and Lewis strains.

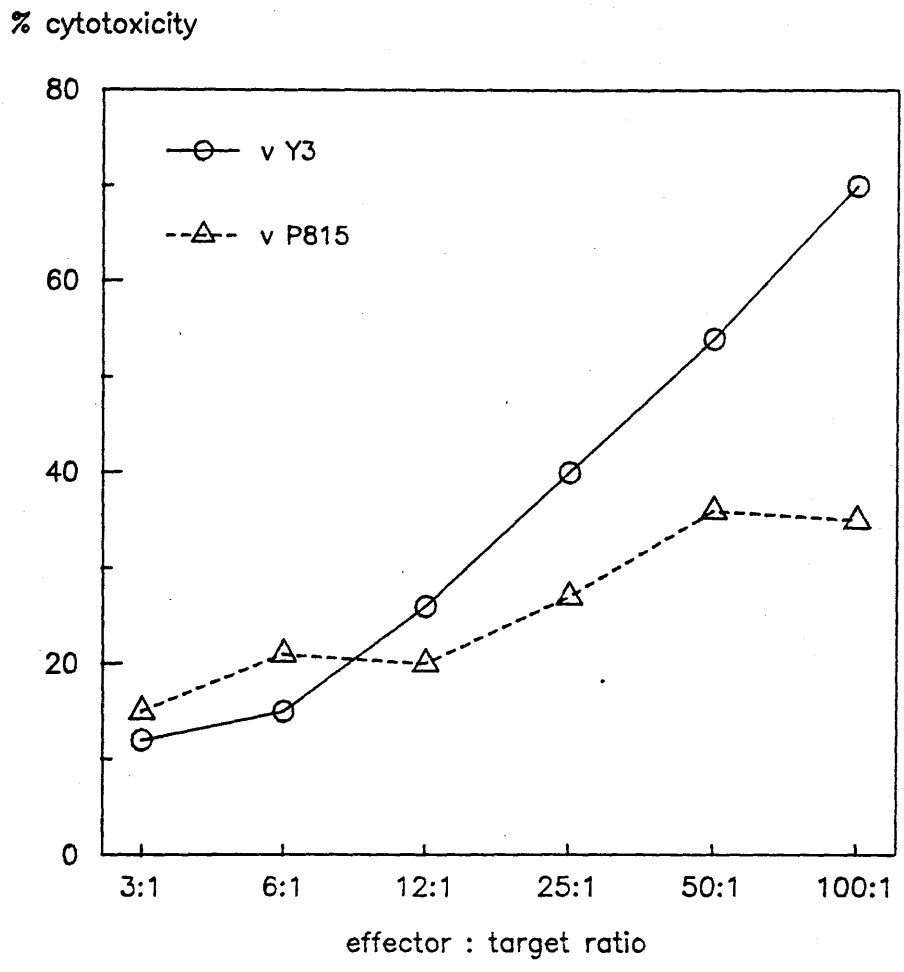

Fig. 6.19 Cytotoxic activity of lymph node cells harvested on day 7 from CD4 cell reconstituted PVG-rnu/rnu recipients rejecting a DA renal allograft, against Y3 and P815 targets.
LNC effector cells were prepared from mesenteric and cervical lymph nodes removed from animals reconstituted with $5x10^7$ CD4 cells. They were tested in vitro for their ability to lyse chromium-labelled Y3 and P815 targets.

target. Graft infiltrating cells showed considerably higher levels of cytotoxicity against PVGr1 targets than against PVG targets, and it was unexpected that cells from a CD4 reconstituted animal could specifically lyse a class I disparate target. Graft infiltrating cells also demonstrated high cytotoxic activity against P815 targets, which suggests the presence, within PVG-rnu/rnu rats, of activated leukocytes with characteristics more generally attributed to LAK cells.

Cytocentrifuge slide preparations of the harvested graft infiltrating cells were stained with monoclonal antibodies and the immunoperoxidase staining technique. The percentages of leukocytes of each subpopulation were determined by counting 400 cells on each slide, and the results are reported in Table 6.7. When compared with Table 6.6, it can be seen that there were fewer B cells present in graft infiltrating cells than in spleen or lymph node effector cells.

6.8 The role of soluble factors in mediating rejection of DA renal allografts in PVG-rnu/rnu recipients

In view of observations discussed above that PVG-rnu/rnu rats possess cells which display wide alloreactivity in vitro and yet the animals are unable to reject allografts in the absence of additional

Table 6.7 **Phenotype of mononuclear effector cells harvested from rejecting DA renal allografts and from lymph nodes in PVG-rnu/rnu recipients restored with CD4 lymphocytes**

EFFECTOR CELLS FROM	% EFFECTOR CELLS LABELLED WITH MAb			
	OX8	W3/25	OX19	OX12
Kidney	22 (17,23,25)	31 (29,32,32)	30 (24,28,40)	15 (13,13,19)
LNC	7 (5,7,9)	31 (27,30,37)	31 (30,31,33)	54 (51,53,57)

Table 6.7 Cytocentrifuge slide preparations of effector cells were stained with MAbs to rat leukocytes, using an immunoperoxidase technique, and the percentage of "lymphocytes" labelled with each MAb was determined by counting a minimum of 400 cells on each slide.
Results are mean (and individual values) from three rats.

immunocompetent cells, the possibility remained that these animals are deficient in some kind of soluble mediator. The roles of two soluble factors were investigated: firstly, DA renal allograft recipients were treated with exogenous IL2 to try to restore the rejection response. Secondly, it was possible that antibody-mediated cellular cytotoxicity was a mechanism involved in graft rejection in animals restored with CD4 cells, although the *in vitro* cytotoxicity assays showed high levels of activity in the absence of alloantibody.

6.8.1 Effect of administering recombinant IL2 *in vivo*

It was suggested by Gillis et al (1979) that "a major function of the thymus may be to effect the maturation of TCGF- (or IL2-) producing cells" and that the reason behind the T cell immunodeficiency of nude mice is their inability to produce IL2. In a single experiment, three PVG-rnu/rnu rats received a DA renal allograft and were treated twice daily for the next six days with an intraperitoneal injection of 100 units of recombinant human IL2. One rat died from intraperitoneal haemorrhage during injection. The two remaining rats underwent contralateral nephrectomy on day 7, and survived for longer than 70 days with no significant increase in serum urea or creatinine levels. It seems likely, in retrospect, that higher and more frequent doses would be necessary to provide a useful amount of IL2 in the

local environment of the renal allograft. This might be achieved with the use of implanted mini-osmotic infusion pumps, although it might be difficult to overcome the problem of the short half life of IL2 when administered in vivo.

6.8.2 The role of alloantibody in rejection of DA renal allografts in PVG-rnu/rnu recipients

It was possible that the function of transferred CD4 cells in restoring rejection in PVG-rnu/rnu recipients of DA renal allografts was to provide help for B cells in the production of alloantibodies. These antibodies might then be involved in an ADCC type of effector mechanism in graft rejection. If this were the case, then the administration of exogenous alloantibody might restore graft rejection in PVG-rnu/rnu rats in the absence of transferrred cells.

Three PVG-rnu/rnu recipients of DA renal allografts were treated with six daily intravenous injections of 0.5 ml serum harvested (on day 10) from normal PVG rats which were rejecting DA renal allografts. (This time was chosen as being the day on which circulating levels of alloantibody were likely to be at their highest.) Following contralateral nephrectomy on day 7, one PVG-rnu/rnu rat died on day 20 but showed no rise in serum urea and creatinine levels (at day 14). One rat survived for 63 days and the other for >100 days with no

evidence of rejection. To reduce the possibility that these animals had received insufficient alloantibody, three more recipients of DA renal allografts were treated with 0.5 ml hyperimmune serum (from DA-skin-grafted PVG rats that had received four fortnightly booster injections of DA splenocytes). All three rats survived with no evidence of rejection.

In all six animals treated with alloantibodies, free circulating cytotoxic alloantibodies were detectable in their serum 24 hours after the last injection, so it was improbable that the animals received insufficient amounts of alloantibody. Circulating cytotoxic antibodies were detected (see Methods) by a colleague, Dr Alastair Gracie, who was also able to demonstrate the presence of cytotoxic anti-DA alloantibodies in serum from PVG-rnu/rnu allograft recipients which had been reconstituted with CD4 cells or LNC, and including those restored with a synergistic dose of CD4 + CD8 cells, but not in unreconstituted rats.

6.9 Graft immunogenicity is reduced in "late reconstituted" PVG-rnu/rnu recipients of DA renal allografts

In this final group of experiments, the role of passenger leukocytes in DA renal allografts transplanted to PVG-rnu/rnu recipients was investigated. In the experiments described in this chapter, allograft rejection was achieved when recipients received transferred cells immediately after transplantation. The possibility existed that if reconstitution was delayed for several weeks, the immunogenicity of the graft might decrease, and it might become resistant to rejection even in the presence of transferred immunocompetent cells. Lechler & Batchelor (1982) desribed how the use of an intermediate, immunoincompetent host resulted in the prolonged survival of rat renal allografts when transferred to fully immunocompetent recipients. This type of strategy has formed the basis of many experiments designed to remove "passenger leukocytes" (probably interstitial dendritic cells) from allogeneic tissues in order to achieve prolonged survival of the graft in the final recipient (see chapter 1).

PVG-rnu/rnu recipients of DA renal allografts underwent contralateral nephrectomy on day 7 after transplantation. They received an intravenous inoculum of $5x10^7$ PVG CD4 cells, either naive or specifically sensitised, at

between four and eight weeks after transplantation, and graft function was monitored by measuring serum urea and creatinine levels in frequent tail blood samples. Animals reconstituted with naive cells showed prolonged survival, and died on days 16, 49 and later than day 50 after reconstitution with high serum urea levels which did not begin to rise until 12 days after reconstitution. Animals receiving sensitised cells rapidly rejected their grafts on days 11, 11 and 13 after recontitution, with associated high serum urea levels. The prolonged graft survival may be accounted for by the migration of passenger leukocytes out of the graft and their subsequent destruction, without the generation of immunological memory, by natural cytotoxicity. Thus, perhaps, the more efficient antigen presentation pathway was impaired (Lechler & Batchelor, 1982) but sufficient allogeneic stimulus remained in the graft to eventually provoke a response from the transferred cells.

6.10 Discussion

The observation, in this chapter, that congenitally athymic PVG-rnu/rnu rats were unable to reject a renal allograft was expected and is entirely consistent with the reported inability of such animals to reject skin allografts. There is, however, a single contrasting

report that aged athymic nude rats may, on occasions, be able to reject skin allografts, suggesting that extrathymic routes of lymphocyte development may allow alloreactive lymphocytes to develop (Hedrich, Wonigeit & Schwinzer, 1987). The possibility that these latter observations were a consequence of the particular rat colony used can only be speculative.

Although it is generally accepted that athymic mice and rats are unable to reject allografts, it is also clear that nude mice may develop lymphocytes with in vitro cytolytic activity as reported by several groups (Gillis et al, 1979). At least one group has shown that the development of this functional activity was largely dependent on the development, with age, of Thy-1 and Lyt-2 T cell markers (MacDonald, Lees, Sordat et al, 1981; Maryanski, MacDonald, Sordat et al, 1981). Similarly, several groups have reported the age-related development of T cell markers in nude rats, together with T functional activity in vitro (Vaessen, Broekhuizen, Rozing et al, 1986; Schwinzer, Hedrich & Wonigeit, 1987).

There is no doubt that nude rats (and mice) possess cells that bear T lymphocyte markers : 2%-4% of rat thoracic duct lymphocytes have been shown to label with the monoclonal antibodies W3/13, W3/25 and MRC OX8, and the density of lymphocytes bearing T cell markers in the lymph nodes is considerably higher (Fossum, 1983). There is less convincing evidence, however, that these cells

are able to perform the functions of T cells. A detailed study of the activity of cells bearing T lymphocyte markers in the nude mouse failed to demonstrate normal T cell activity (Chen, Scollay, Shortman et al, 1984), which was consistent with the apparent absence of any demonstrable T cell receptor gene rearrangement signifying thymic processing (Owen, Jenkinson, Williams et al, 1986). Nude rats lacking alloreactivity have been shown to possess as many as $5x10^7$ cells bearing T lymphocyte markers (Fossum, 1983), while repopulation of nude rats with one fifth of this number of T cells is sufficient to restore their ability to rapidly reject skin allografts (Bell, Sparshott, Drayson et al, 1987). Collectively, the evidence suggests that there is an absolute requirement for lymphocytes to be thymically processed before they can mediate such T cell dependent responses as graft rejection.

The experiments described in this chapter showed, as expected, that the adoptive transfer of syngeneic lymphocytes to PVG-rnu/rnu rats effectively restored their ability to reject a renal allograft. Transfer of purified CD4 cells alone, in appropriate numbers, restored rapid graft rejection, while specifically sensitised CD4 cells were several fold more potent in this respect. In contrast, both naive and specifically sensitised CD8 cells were totally ineffective at provoking renal allograft rejection. However, there was

a suggestion that the transfer of a mixture of CD4 and CD8 cells might be a more potent inducer of graft rejection than an equal number of CD4 cells alone. Such a synergistic effect would be consistent with the earlier observation that transfer of unseparated T cells to acutely irradiated rats restored graft rejection while similar numbers of CD4 cells alone were considerably less effective.

It was of interest that CD4 cells, which had been further depleted of those lymphocytes expressing the MRC OX22 antigen, were also able to mediate graft rejection. The MRC OX22 antibody distinguishes between CD4 cells which provide helper activity for B cells (W3/25 positive, MRC OX22 negative) and those which mediate graft-versus-host reactions (Spickett et al, 1983). It was a somewhat unexpected finding, from this preliminary experiment, that a CD4 positive but MRC OX22 negative T cell population could effectively restore graft rejection. Animals rejecting their grafts after reconstitution with this T cell population showed a cytotoxic alloantibody response. The possibility therefore existed that the alloantibody response generated may have an effector role in rejection (via ADCC) rather than being dismissed as an epiphenomenon. This point was not extensively pursued, but the serum transfer studies (section 6.8.2) failed to support the concept that the presence of alloantibody alone in

athymic graft recipients was sufficient to result in irreversible graft damage. The ability of CD4 positive, MRC OX22 positive cells to cause renal allograft rejection was not studied, but it was anticipated that they also would be effective.

When splenocytes from athymic rats were tested for *in vitro* cytotoxicity, it was found, unexpectedly, that even cells from unmodified, non-transplanted animals displayed cytotoxic activity against allogeneic target cells. This observation was pursued further, to investigate the possibility that this activity was of relevance to the mechanism whereby CD4 cells restore the rejection response, and for more general interest.

It has previously been described that nude rats, like nude mice, have increased numbers of circulating cells with the phenotype and function of NK cells (Reynolds, Timonen, Holden et al, 1982) and that these high levels arise as a protective mechanism because of the absence of functional T cells (Grzelak, Olszewski, Fossum et al, 1984). *In vitro* cytotoxicity experiments described in this chapter consistently showed higher levels of chromium release from the NK susceptible targets Y3 and YAC-1 by nude splenocytes when compared with normal PVG splenocytes. Moreover, PVG-rnu/rnu splenocytes also showed consistently high levels of cytotoxic activity against all strains of ConA blasts tested except syngeneic PVG ConA blasts. This was never observed with

normal PVG splenocytes, and has not been reported to occur in cultured nude mouse cytolytic cell lines, where third party lysis was never observed (Gillis, Union, Baker et al, 1979). Another group investigating the ability of cultured nude mouse splenocytes to kill a range of targets showed that while high levels of cytotoxicity were observed with all tumour lines tested, moderate levels of cytotoxicity against allogeneic ConA blasts were detected after an 18 hour incubation period, but not after 6 hours incubation (Kedar, Ikejiri, Sredni et al, 1982). This wide alloreactivity in PVG-rnu/rnu rat splenocytes has not, to my knowledge, been previously reported. It is interesting that despite possessing large numbers of these cells that are able to lyse allogeneic cells in vitro, PVG-rnu/rnu rats are unable to reject DA renal allografts.

The use of anti-AGM1 to ablate this wide alloreactivity in vitro suggested that these cells are NK cells with the unusual property of being able to lyse not only the NK susceptible Y3 and YAC-1 targets, but also the traditionally NK resistant P815 mouse mastocytoma (Dunn & Potter, 1957), and allogeneic ConA blasts. Furthermore, while cold target inhibition assays suggested that the wide alloreactivity of PVG-rnu/rnu splenocytes might be accounted for by their recognition of a common

determinant on target cells, they also provided evidence that these splenocytes are capable of additional specific recognition of allogeneic targets.

It is reasonable to suggest that these cells exist as a form of immune defence in the absence of thymically processed cells and it is possible that these are the pre-thymic T cells proposed by Jerne (1971) - there are more of them in nude rats because they never become thymically processed. NK cells have been implicated in responses to transplants, but they appear to be more important in destroying single cells, as seen in the resistance to bone marrow transplantation and the induction of GVH disease (Herberman, 1982), rather than the response to a solid organ graft. Heidecke et al have shown that in vivo treatment of normal rats with anti-AGM1 did not alter their response to heart allografts, and they concluded that NK cells do not play an active role in organ allograft rejection (Heidecke, Araujo, Kupiec-Weglinski et al, 1985). It is possible that the in vitro lysis of allogeneic targets reported in this chapter is more akin to in vivo ALC mediated lysis and NK mediated resistance to transplanted bone marrow than to any known mechanisms of organ allograft rejection.

In view of the widely alloreactive, cytotoxic nature of these nude rat splenocytes, an alternative suggestion is that these cells are LAK cells, or lymphokine-activated

killer cells. This term is normally used to imply an in vitro functional characteristic of lymphocytes (often large granular lymphocytes [LGL] which are sometimes considered synonymous with NK cells) which have been cultured in the presence of IL2. It appears that IL2-mediated activation of NK cells confers upon them the ability to lyse target cells in a non-MHC restricted fashion, such that they are able to lyse a wider range of different tumour lines.

Rat LAK cells bear the charateristics of activated NK cells, in that they are 98% LGL and express high levels of MRC OX8 reactivity and asialo GM1, few of them label with MRC OX19, and most of them express class II MHC antigens but not the IL2 receptor detected with MRC OX39 (Vujanovic, Herberman, Maghazachi et al, 1988). LAK cells have previously only been identified in cells cultured in vitro under certain conditions that include the presence of IL2, but the generation of mouse LAK cells in vivo has recently been described in mice colonised with the IL2 producing mouse T lymphoma EL4 (Nishimura, Uchiyama & Hashimoto, 1988). Similarly, LAK activity has been observed in peripheral blood lymphocytes of cancer patients treated with IL2 (Hank, Kohler, Weil-Hillman et al, 1988). The widely alloreactive nude rat splenocytes described in this chapter appear to behave similarly to these in vivo stimulated LAK cells but it is difficult to understand

how LAK cells might exist in PVG-rnu/rnu rats unless nude rats differ from nude mice in their inability to produce IL2 (Gillis et al, 1979). The activity of PVG-rnu/rnu splenocytes resembles that of LAK cells in that they show non-MHC restricted cytotoxicity and are able to lyse NK-resistant targets such as P815. Moreover, the results of the cold target inhibition study suggesting the presence of widely alloreactive effector cells with the additional ability to specifically recognise different targets are in agreement with the results of similar cold target inhibition assays performed on mouse LAK cells grown from cultured foetal thymus: there was only slight cross inhibition of YAC-1 cells by P815 and vice versa (Skinner & Marbrook, 1988).

While LAK cells may be able to control the growth of metastatic tumours in animal models (Rosenberg, 1986), widely alloreactive nude rat cells are incapable of destroying renal allografts, and are not present in the graft unless rejection is initiated by the transfer of syngeneic T lymphocytes. The absence of increased specific alloreactivity in cells harvested from rejecting grafts, when compared with the activity of splenocytes, was surprising, but in view of the high levels of nonspecific cytotoxicity recorded, specific activity would be difficult to detect. This lack of increased specific activity in graft infiltrating cells suggests that nonspecific, rather than specific, mechanisms are

involved in renal allograft rejection in PVG-rnu/rnu rats, and these may well be mediated by LAK cells and NK cells, but there is still an absolute requirement for T cells to initiate the response.

An important question that remains unsolved concerns the mechanism by which T cells are able to initiate a response in PVG-rnu/rnu rats culminating in the rejection of DA renal allografts, apparently by cellular mechanisms, but with minimal numbers of T cells actually present in the grafts, and no clear evidence of T cell mediated specific cytotoxic activity.

CHAPTER SEVEN

FINAL DISCUSSION

7.1 General discussion

The overall aim of this thesis was to investigate the cellular events which culminate in the rejection of an allogeneic kidney transplant. Experiments of this type are of importance since they should provide a better understanding of the graft rejection process and thereby a rational basis from which to develop strategies aimed at improving kidney allograft survival in clinical practise.

The rat renal allograft model was used throughout, since appropriate inbred strains are available and unlike in the mouse, kidney transplantation by microsurgical methods is feasible with a high level of success. It should be remembered, however, that there may be important differences in the rejection response when compared with humans. For example, human kidneys undergo hyperacute rejection in the presence of preformed cytotoxic alloantibodies whereas it is difficult to demonstrate a detrimental effect of alloantibody on rat kidney allografts - presumably because rat complement is, by comparison, less potent. Furthermore, there are important differences in the constitutive MHC expression between human and rat kidneys. Whereas human renal vascular endothelium expresses class II MHC antigens, rat renal vascular endothelium does not. However, as demonstrated in chapter 3, rat renal vascular endothelium

does become strongly class II positive during the course of acute rejection. Notwithstanding these considerations, it is reasonable to assume that the cellular mechanisms leading to the rejection of a rat renal allograft can, in broad terms, be extrapolated to apply to human kidney graft rejection.

Three main approaches were used in this thesis to investigate the cellular events causing tissue damage in allogeneic rat kidneys. First, observations were made in chapter 3 on the cellular events occurring in kidney allografts transplanted into unmodified recipients. Second, in chapters 4 and 6, the ability of T cell deprived hosts to reject a renal allograft following adoptive transfer of T cell subsets was examined. Third, the capacity of T cell subsets to mediate renal parenchymal damage in a local GVH model was investigated. Although the experiments described have already been discussed in their respective chapters, it is helpful to consider further the implications and interrelationships of the different experimental findings.

The observations reported in chapter 3 on the events occurring in rat kidney allografts after transplantation into unmodified hosts provided a detailed analysis of the pattern and phenotype of graft infiltrating mononuclear cells and the associated induction of donor MHC class I

and class II antigens, the results of which are in agreement with other similar reports. Also consistent with previous reports (Mason & Morris, 1984; Bradley et al, 1985) was the demonstration that mononuclear cells harvested from rejecting grafts displayed both donor specific as well as nonspecific cytotoxicity when tested in vitro. These functional results raise the question as to whether the observed specific and nonspecific cytotoxic activity are (a) an essential component of the rejection response, (b) whether they contribute to rejection or (c) whether they are simply epiphenomena. Assuming the in vitro cytotoxicity assay bears relevance to mechanisms operating in graft rejection, then the demonstration of cells within the graft which are able to lyse donor strain targets in vitro would suggest that such cells might, in vivo, be expected to cause tissue damage. It is interesting, however, that specific cytotoxic T cells are also found in the non-rejecting allogeneic kidneys obtained from actively enhanced rats. The explanation for this paradoxical finding is not clear but, as outlined by Dallman, it may be that there are qualitative differences in the cytotoxic cells present in transfused and untreated recipients (Dallman et al, 1987), or that the action of cytotoxic cells in transfused animals is blocked, possibly by enhancing antibodies.

Similarly, it is apparent that the presence of nonspecific cytotoxic cells within an allograft is not necessarily associated with rejection, since these are also found in non-rejecting rat kidney allografts in CyA-treated or passively enhanced hosts (Mason & Morris, 1984; Bradley et al, 1985). Furthermore, such nonspecific cytotoxic cells do not appear to be an essential component of rejection since, in the acute irradiation experiments described in chapter 4, rejecting grafts in T cell reconstituted animals contained cells with demonstrable specific, but not nonspecific, cytotoxic activity.

A major part of this thesis concerned the ability of immunologically compromised rats to reject a renal allograft following the adoptive transfer of different lymphocyte subpopulations. This approach has been widely used by many different investigators with the aim of determining the cellular requirements for graft rejection, and these studies are summarised in Tables A1-A4. As shown, these reports relate almost exclusively to rodent models of either skin or heart transplantation, and many were concerned with the adoptive transfer solely of specifically sensitised cells. Only one group has previously investigated the cellular requirements for restoring renal allograft rejection and they examined the ability of specifically sensitised cells to cause

rejection in acutely irradiated rats (Gurley et al, 1983). The conclusions of this latter study were based on isolated day 7 serum creatinine measurements and not graft survival data. In addition, the kidney donor, as well as the recipient, was irradiated and this may have altered the immunogenicity of the graft.

It is clear from the various adoptive transfer experiments summarised in Tables A1-A4, and those described in this thesis, that the ability to restore graft rejection to immunocompromised hosts depends on several factors. These include the nature of the immunocompromised host, the type of tissue allograft (eg, skin or vascularised organ), and the influence of Ir genes in the particular strain combination studied.

7.2 The function and phenotype of T cells

Throughout this thesis, the ability of T lymphocyte subpopulations to mediate allogeneic tissue damage has been examined. The purified T cell subpopulations were prepared by a negative selection procedure according to their reactivity with the non-overlapping monoclonal antibodies W3/25 and MRC OX8. These label the two phenotypically distinct T cell subpopulations designated CD4 and CD8 respectively. In this respect they are analogous to the MAbs commonly used for identifying human

CD4 and CD8 lymphocytes, for example, Leu3 and Leu2, or OKT4 and OKT8. The equivalent MAbs in the mouse, of which there are several, are not entirely non-overlapping, and for this reason caution should be exercised in the interpretation of results from similar experiments performed in mice.

The phenotypic characteristics of cells determined by their pattern of labelling with MAbs do not necessarily designate their function according to original definitions. CD4 lymphocytes have traditionally been synonymous with T helper cells and CD8 lymphocytes with T cytotoxic and T suppressor cells. It is now apparent that this dichotomy of function does not strictly correlate with phenotype. Instead the T cell phenotype may correlate more closely with MHC-restricted function so that, in the context of graft rejection, CD4 cells respond to class II MHC alloantigens whereas CD8 cells respond principally to class I MHC alloantigens (Swain, 1983; Zinkernagel & Doherty, 1979). Therefore, CD4 lymphocytes may, under certain circumstances, have a cytotoxic function towards target cells expressing class II MHC antigens; evidence for such a mechanism was provided by studies on the phenotype and specificity of human cytotoxic T cell clones (Spits, Borst, Terhorst et al, 1982). Similarly, in certain mutant mouse strains, CD8 cells may mediate class I restricted GVH disease and rejection of class I disparate skin grafts in the absence

of CD4-mediated T cell help, since these mutant mouse CD8 cells are themselves able to produce IL2 (Sprent, Schaefer, Gao et al, 1988; Rosenberg et al, 1987). Furthermore, it has been shown that GVH disease, which was classically believed to be a CD4 cell mediated disease, is indeed caused by CD4 cells when they are responding to an isolated MHC class II disparity in mice, but it can also be caused by CD8 cells in the presence of an isolated class I disparity (Sprent, Schaefer, Lo et al, 1986). Thus it is more convenient, in transplantation studies, to interpret the functions of transferred T lymphocyte subpopulations in terms of their observed, rather than their expected responses to MHC alloantigens.

7.3 Role of CD4 and CD8 T cells in allograft rejection

Tables A1-A4 represent a summary of experiments designed to investigate the ability of purified lymphocyte subpopulations to restore graft rejection, and provides evidence that either CD4 or CD8 cells are able to cause graft rejection, depending on the species, model, tissue, MHC disparity and status of the transferred cells. In other words, there is no consensus of opinion on this matter, and it is now apparent that the debate of the last decade about whether CD4 cell-

mediated DTH reactions or CD8 cell-mediated cytotoxic responses cause graft rejection has been superceded by a requirement for a more detailed explanation of mechanisms of rejection. As discussed above, the function of a particular subpopulation of T cells is not exclusively defined by its phenotype, so while it is possible to determine the phenotype of cells that are required to be adoptively transferred in order to cause graft rejection, the mechanism by which they achieve rejection can only be postulated by examining the functions of cells extracted from rejecting tissues. The rat renal allograft model and the renal GVH reaction are ideal for this type of approach because adoptively transferred cells cause rejection or tissue damage that is easily and objectively diagnosed, and relatively large numbers of cells may be extracted and tested in *in vitro* functional studies.

7.3.1 Role of CD8 T cells

CD8 T cells alone (ie, W3/25 negative lymphocytes) were unable to mediate allogeneic tissue damage in any of the experiments reported in this thesis. Thus, neither naive nor specifically sensitised CD8 lymphocytes were effective in restoring the ability of either acutely irradiated or congenitally athymic rats to reject a fully allogeneic renal allograft. Furthermore, they were also unable to cause renal parenchymal damage when tested in

the renal GVH assay. These observations correspond with the classical scheme whereby alloreactive cytotoxic CD8 cells are generated from cytotoxic precursors under the influence of inductive lymphokines (principally IL2) produced by CD4 cells. Nevertheless, there is now good evidence that CD8 cells alone are able to mediate graft rejection in the absence of cooperation from CD4 cells. However, these circumstances appear to be restricted to isolated class I disparities in certain mutant mouse strains, whose CD8 cells produce their own lymphokine signals necessary for their differentiation into mature cytotoxic cells. As already discussed, there is also clear evidence of the ability of specifically active cytotoxic T cell clones to cause tissue damage in vivo (Engers et al, 1982; Tyler et al, 1984).

Although the present work provided no evidence that CD8 cells alone were able to cause allogeneic tissue damage, there was evidence that they did contribute to rejection when transferred together with CD4 lymphocytes. This synergy was seen most clearly in the acutely irradiated renal allograft recipients where optimal first set rejection only occurred in rats reconstituted with unseparated lymphocytes and not with CD4 or CD8 cells alone. In the congenitally athymic renal allograft recipients there was also a trend suggesting that a mixture of CD4 and CD8 lymphocytes may have been more effective at restoring rejection than an equivalent

number of CD4 cells alone. Interestingly, this evidence for T-T cell cooperation is supported by other experiments. For example, Lowry reported that there was an absolute requirement for the adoptive transfer of both CD4 and CD8 cells in order to restore rejection of class I disparate heart allografts in acutely irradiated rats (Lowry et al, 1985). Hall also described a synergistic effect in the addition of a small number of CD4 cells to an inoculum of naive CD8 cells in adoptive transfer studies (Hall, 1987).

7.3.2 Role of CD4 T cells

The adoptive transfer of CD4 cells alone to ATXBM or acutely irradiated allograft recipients does, in several of the studies outlined in Tables A1-A4, lead to graft rejection. Experiments in which CD4 cells alone were sufficient to restore skin graft rejection in ATXBM animals prompted Loveland to suggest that this was mediated by a DTH mechanism (Loveland, Hogarth, Ceredig et al, 1981). This was contradicted by the demonstration that cytotoxic precursor cells were present in CD4 reconstituted, ATXBM recipients of skin allografts (Dallman & Mason, 1982) and thus the possible involvement of T cytotoxic cells in graft rejection could not be entirely eliminated. Nevertheless, additional experiments using acutely irradiated animals showed that the adoptive transfer of either specifically sensitised or naive CD4

lymphocytes were sufficient to restore heart allograft rejection (Lowry & Gurley, 1983; Lowry et al, 1985; Hall et al, 1983). Furthermore, the failure to detect cytotoxic T cells in the rejecting heart grafts appeared to favour a DTH response as the mechanism of graft damage.

In the acute irradiation experiments reported in this thesis, unsensitised CD4 cells alone were not sufficient to cause rapid renal allograft rejection in the particular rat strain combination used (DA to Lewis). By contrast, the adoptive transfer of CD4 cells alone to congenitally athymic rats restored their ability to rapidly reject a renal allograft. It is relevant here to recall the results of experiments described in chapter 3, showing that PVG rats are able to reject renal allografts bearing an isolated class II, but not class I, disparity of the DA haplotype, suggesting that a class II restricted (and CD4 mediated) rejection response predominates in this strain combination.

It was clearly shown that allograft rejection in CD4 reconstituted athymic rats occurred in the presence of numerous CD8 as well as CD4 cells within the grafts. Many of these cells appeared to be either CD8 positive NK cells or CD4 positive macrophages rather than T lymphocytes. It was originally anticipated that detailed functional analysis of these infiltrating cells harvested from rejecting grafts in CD4 reconstituted animals would

allow insight into the mechanism of rejection. This achievement was frustrated by the unexpected finding that unmodified athymic animals possessed mononuclear cells, particularly in the spleen, which in the absence of prior sensitisation, demonstrated high levels of lysis against allogeneic targets. This interesting observation was discussed more fully in chapter 6 and undoubtedly merits further study.

In view of the experiments discussed above, it would have been interesting to examine whether adoptive transfer of CD4 positive lymphocytes to athymic rats enabled them to reject a renal allograft bearing only an isolated class I disparity.

7.4 The role of MHC antigens and Ir gene control

It became clear, from investigating the ability of lymphocyte subpopulations to cause renal parenchymal damage, that the response depended not only on the functions of the transferred cells, but on the antigens to which they were responding, as well as the predisposition of the recipients to respond in a particular way to the donor strain. Thus the minimum requirements for a local renal GVHR were CD4 cells and an isolated class II disparity, together with the presentation of alloantigen on host bone marrow derived

cells. There was no response in the renal GVHR to an isolated class I disparity and no requirement for CD8 cells. This was consistent with the findings in chapter 3 that PVG rats of the recombinant series were unable to reject class I disparate renal allografts but rejected class II disparate grafts relatively rapidly. Further evidence for the ability of PVG CD4 cells to respond to DA alloantigens was provided by their efficiency at restoring graft rejection in PVG-rnu/rnu rats. Thus in a low responder strain combination (Butcher et al, 1982) renal parenchymal damage may be caused by CD4 cells recognising and responding to MHC class II alloantigens in the absence of a requirement for CD8 cells. However, there is evidence that in other strain combinations, CD8 cells are required in order to cause tissue damage by responding to a class I disparity, either in the presence or absence of CD4 cells (Lowry et al, 1983; Wheelahan & McKenzie, 1987; Sprent et al, 1988).

Similarly, adoptive transfer experiments performed in this laboratory in different strain combinations demonstrated very different cellular requirements for restoring graft rejection. Thus in one strain combination (DA to Lewis, normally regarded as a " strong combination" in which it is not possible to consistently achieve active enhancement), restoration of rapid graft rejection required the transfer of both CD4 and CD8 cells, whereas in the reciprocal combination (regarded as

"weak" and easy to enhance) even the presence of very large numbers of unseparated LNC failed to provoke a response to the allograft.

The predominance of a class I restricted , CD8 mediated response over a class II restricted, CD4 mediated response, or vice versa, is largely determined by the influence of Ir genes operating in the particular strain combination, and this must be a consideration when attempting to postulate mechanisms of graft rejection or GVH reactions.

7.5 Conclusion

The experiments performed in this thesis comprise a detailed investigation into the minimal requirements, in terms of lymphocyte subpopulations, for restoring first set renal allograft rejection in immunocompromised recipients, and an attempt to investigate the cellular interactions culminating in the cell mediated damage of renal parenchymal tissue characteristic of both allograft rejection and the renal GVHR. The different cellular requirements in the various models studied serve to emphasise the influence of Ir gene control and MHC restriction of T cell responses on the results of adoptive transfer studies, and highlight the complexity of these reactions. In order to understand more about the cellular mechanisms of graft rejection, it will be

necessary in the future to dissect the responses to MHC class I and II alloantigens in isolation in order to understand how they might interact in rejection responses. The use of functionally and phenotypically defined clones of cells might provide some useful information in adoptive transfer studies, although preliminary experiments in this laboratory have confirmed reports that it is difficult to maintain clones of alloreactive rat lymphocytes *in vitro*. Finally, the mechanisms of cell damage might be investigated with better effect if a more appropriate target cell than ConA lymphoblasts were available for *in vitro* studies. A suitable candidate might be monolayers of vascular endothelial cells, which have been implicated as targets of the rejection response *in vivo*, and which may be manipulated to express increased amounts of MHC class I and class II antigens, thereby mimicking their behaviour in graft rejection.

To date, the cellular requirements for causing tissue damage may be defined according to the conditions of the experiments, but the precise pathways of target cell damage by alloreactive effector cells remain undisclosed.

APPENDIX

Table A.1 Ability of transferred lymphocyte subpopulations to restore graft rejection in acutely irradiated recipients

TISSUE	INCOMPATIBILITY I	II	minor	ANIMAL	DOSE (Gy)	CELLS TRANSFERRED TO RESTORE REJECTION	CELLS N/S[a]	REF
Skin	+	+	+	Rat Wag → DA	7.5	Small recirculating cells	N	1
Heart	+	+	+	Rat DA → PVG	7.5	LNC, TDL. Potency relates to recirculation of cells	N	2
Heart	+	+	+	Rat DA → PVG	7.5	S cells more potent than N cells. Rejection slower in absence of minor incompatibilities	S N	3
	+	+	-	" congenics	"			
Heart	+	+	+	Rat WF → LEW	7.8 (also donor)	Both CD4 and CD8 cells. CD4 more potent. In vitro cytotoxicity only when CD4 cells also present.	S	4
Kidney	+	+	+	Rat WF → LEW	7.8 (also donor)	CD4 and T cells. CD8 not tested. Spleen cytotoxicity only with T cells. No data on survival (d7 creat only).	S	5
Heart	-	+/-	+/-	Rat WF, PVG, congenics	7.8	CD4 and T cells. CD8 not necessary in absence of class I incompatibility.	S	6
Heart	+	-	-	Rat PVGr1 → PVGxWF	7.8	Requirement for CD8 for rejection with only class I disparity.	S	7
Heart	+	+	+	Rat PVG → DA	9.0	CD4 cells alone sufficient for normal tempo rejection.	N	8
Neonatal	+	+	-	Rat PVGRT1a → PVG	7.5	OX22- CD4 cells restore rejection, CD8 necessary for memory.	N	9

a) Cells transferred were either naive (N) or specifically sensitised (S).

Table A.2 Ability of transferred lymphocyte subpopulations to restore graft rejection in ATXBM recipients

TISSUE	INCOMPATIBILITY I	II	minor	ANIMAL	CELLS TRANSFERRED TO RESTORE REJECTION	CELLS N/S[a]	REF
Skin	+	+	+	Mouse	CD4 cells alone sufficient	S	10
Tumour	+	+	+	Mouse	N - both CD4 + CD8 necessary. S - CD4 can, CD8 cannot.	N S	11
Skin	+	+	+	Mouse	CD4 cells alone sufficient	N	12
Skin	+	+	+	Rat DA→PVG	CD4 cells alone sufficient	N	13
Tumour	+	+	+	Mouse	Cloned CD8 CTL effective.	S	14
Heart	+	+	+	Rat LxBN→L	Require 10^8 S splenocytes plus IL2, plus adherent cells.	S	15
Heart	+	+	+	Rat LxBN→L	Require 10^8 S T cells +/- IL2	S	16
Heart	+	+	+	Rat LxBN→L	CD4 can slowly, CD8 cannot. T cells + IL2 necessary for rejection in normal time.	S	17
Skin	+	+	+	Mouse	Either CD4 or CD8 sufficient. NB regeneration of host CTL.	S	18

a) Cells transferred were either naive (N) or specifically sensitised (S).

Table A.3 Ability of purified lymphocyte subpopulations to mediate tissue damage in allogeneic (and immunocompromised) recipients

TISSUE	INCOMPATIBILITY I	II	minor	ANIMAL	MODEL	CELLS TRANSFERRED TO RESTORE REJECTION	CELLS N/S[a]	REF
Skin	-	-	+	Mouse	normal	Cloned Epa1-specific CTL caused intradermal lesion.	S	19
Skin	+	+	+	Mouse	MAb[b]	Either CD4 or CD8 depletion still allowed rejection.	N	20 21
Skin	+	-	-	Mouse	nu/nu	IL2-secreting CD8 cells alone caused rejection.	N	22
Skin	+/-	+/-	+/-	Mouse	MAb	CD8 alone only for class I disparity. Others require CD4.	N	23

a) Cells transferred were either naive (N) or specifically sensitised (S).

b) Animals were treated with monoclonal antibodies to selectively deplete them of T cell subpopulations.

Table A.4 References used in Tables A.1, A.2, & A.3

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