ALLOANTIBODY AND RENAL TRANSPLANTATION

BY

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A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF MEDICINE

FROM

THE UNIVERSITY DEPARTMENT OF SURGERY, THE LEICESTER GENERAL HOSPITAL

MAY 1988.

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ALLOANTIBODY AND RENAL TRANSPLANTATION - J.D. TAYLOR.

With the introduction of the immunosuppressive drug Cyclosporin A (CyA) into clinical practice, renal transplantation has become a safer and more successful therapy for end stage renal failure. Rejection by both humoral and cellular mechanisms remains the main cause of graft failure. The allosensitised patient is at greater risk from early rejection episodes that are difficult to treat. Patients become allosensitised through exposure to alloantigen by organ transplantation, pregnancy, and blood transfusion. The detection of alloantibody by a complement mediated lymphocytotoxicity test is widely used as a measure of allosensitisation.

The first part of this study is a detailed review of the Leicester experience with CyA and the management of the allosensitised patient. This confirms a relationship between allosensitisation as detected by alloantibody and renal allograft rejection resistant to treatment. Nevertheless, using current methods 50% of allosensitised patients were transplanted without early rejection episodes.

The second part of the study describes a new model of accelerated renal allograft rejection by allosensitised dogs, where recipients receive a kidney across a positive lymphocytotoxic crossmatch. CyA is of no benefit in delaying rejection by these allosensitised recipients, except when the lymphocytotoxic crossmatch test is negative. Two other strategies were unsuccessful in the face of a positive lymphocytotoxic crossmatch test: the use of cyclophosphamide and cytosine arabinoside to immunomodulate the allosensitised recipient prior to transplantation, and prostacyclin given in conjunction with CyA after renal transplantation. This experimental model may be of use in the further study of the clinical problem posed by the allosensitised patient.

John Desmond Taylor MD Thesis

Binding errors:

Page 37 is duplicated.

Page 257 is followed by Page 237-257 inclusive, and this duplicated section should be deleted.

Typographical errors:

Page 26 Para 5 line 1, 'Persiyn' should be 'Persijn'.

Page 175 Line 2 'of rejection' should be deleted.

Page 272 Incomplete reference under 'Doveren'. (Section and pagination omitted).

Page 272 line 9 'Obstectrics' mis-spelt.

Page 284 Incomplete reference under 'Reed' (year omitted).

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ACKNOWLEDGEMENTS

The work presented in this thesis has been made possible by the help I have received from many people, together with financial support from several sources, though primarily the University Department of Surgery.

The experimental canine work was performed in the Department of Biomedical Services, University of Leicester. Dr. David Morton gave invaluable practical advice in the design of the model, supervised the care and welfare of the dogs, and provided the anaesthesia service. Dr. Kenny Simpson helped with the anaesthesia. The operations and biopsies were performed in the operating theatres with the unceasing and patient help of Linda Reid. Mr. Peter Veitch helped with the renal transplant operations. The day to day care of the dogs was the responsibility of Carol Husken, Val Patrick, Trusha Patel, Lin Scrimshire, Barbara Gossage, Caron Jackson, Paula Read, Steve Blagden and Mark Bodycote. The excellent care that the dogs received was vital to the success of this project, facilitating the repeated handling of the dogs and their continuing good health. The haematology and biochemical services were provided by Val Patrick and Trusha Patel.

The laboratory work was largely carried out by Dr. Natu Mistry in the Transplantation Laboratory at the General Hospital. Natu was responsible for the enormous amount of work involved in screening the dog sera for alloantibody and the lymphocyte crossmatch tests. The ELISA assay for tetanus toxoid antibody was modified by Natu from that used for a clinical study, and he went on to screen the dog sera. The CyA levels were also measured by Natu.

Both Anne Simpson and Anne Bell helped considerably in the retrieval of the clinical data presented in this study .

The staff in the Department of Histopathology at the General Hospital prepared slides from over 180 biopsies. The needle biopsies were all reported by Dr. H. McKay and the nephrectomy biopsies by Dr. K. O'Reilly.

The photographs were taken in the Department of Biomedical Services and developed by the Medical Illustrations Department at the Leicester Royal Infirmary. The photomicrographs were taken with the assistance of Sally Brown.

The large quantity of CyA used in this study and by other workers was provided by the Pharmacy Department at the General Hospital who saved all partially used bottles of CyA returned from the wards. The equivalent of 200 bottles of CyA was provided in this way. Mr. Clive Haylott of Upjohn Ltd. made a valuable gift of cyclophosphamide. Dr. Williams of The Wellcome Foundation Ltd. donated the prostacyclin used. The loan of the two pumps from

Pharmacia Ltd. was arranged by Mr Colin McLean. Financial support was also received from Sandoz Pharmaceuticals.

This thesis was prepared on my Macintosh personal computer and printed on a laserprinter in the Department of Biomedical Services.

Finally I would like to acknowledge the help of three people. Mr. Peter Veitch, my supervisor, who remained undaunted despite all the problems encountered during this study and whose practical help contributed to the solution of these problems. Dr Terry Horsburgh for his help in designing the project, continuing advice and for permitting the laboratory work in the Transplantation Laboratory. Finally thankyou to Professor P.R.F. Bell for his continuous encouragement.

Summary

The clinical problem.

With the introduction of the immunosuppressive drug Cyclosporin A into clinical practice, renal transplantation has become a safe and successful therapy for end stage renal failure. However, rejection by both humoral and cellular mechanisms remains the main cause of graft failure. Patients with alloantibody, commonly directed against histocompatibility antigen, are at greater risk from rejection, particularly from very early rejection episodes which are more difficult to treat. Patients are tested for the presence of alloantibody, directed against lymphocytes from their prospective donor, immediately prior to transplantation by means of a complement dependent lymphocytotoxicity test - the crossmatch test. A positive result increases the risk of hyperacute rejection of the kidney (Kissmeyer-1966). Hyperacute rejection occurs when the recipient has humoral antibody directed against antigens in the transplanted kidney, and following antigen - antibody interaction a sequence of events is set in train which leads, within hours, to cessation of perfusion of the kidney. However, the crossmatch test also identifies recipient antibodies, also directed against donor lymphocytes, that need not necessarily preclude successful transplantation (Falk-1985).

In our experience of 156 renal cadaver transplants immunosuppressed with Cyclosporin A, we have observed one case of hyperacute rejection and seven instances of rejection occurring in the first two weeks after transplantation, only one of which was successfully treated. Furthermore we have observed an association between primary non function (PNF) of a kidney, i.e. dialysis has been needed in the first week post transplant, patients with alloantibody, and immunosuppression with Cyclosporin A. These patients are at an increased risk of graft failure. The mechanism of this association is unknown.

Patients with alloantibody also wait longer for a transplant, because they are more likely to have a positive crossmatch test, which is a contraindication for transplantation. These observations confirm that transplantion of patients with alloantibody presents a problem in clinical management.

The aims of this study

The first part of this study was an in depth analysis of the effects of the new immunosuppressive agent Cyclosporin A on the results of cadaver renal transplantation in Leicester, with special reference to the problem of the allosensitised patient. The main cause of patient allosensitisation was a previous graft, and the management of failed grafts was examined to determine whether allograft nephrectomy influenced the degree of allosensitisation associated with allograft failure.

The second part of this study was to devise an experimental model to examine potential strategies for managing allosensitised patients. This required a model of either hyperacute or accelerated rejection of renal allografts by allosensitised recipients. There is no good small animal model model for the investigation of allosensitisation. The vast majority of experimental transplantation has been in the rat and mouse. Neither of these species are capable of producing the accelerated, treatment resistant, graft rejection seen clinically. We chose a large animal, the dog, for the experimental study. Renal allografts to dogs have been extensively studied and parallels with clinical renal allografting have been shown. Examples of these are the failure to induce immunological tolerance, the beneficial association between azathioprine and blood transfusion, and the association between allosensitisation and early graft failure. There has been little work in the area of allosensitisation and CyA in the dog, and it was first necessary to study these aspects in the proposed model of canine accelerated renal allograft rejection. Various routes of allosensitisation, the natural history of alloantibody, and the outcome of transplantation with CyA were examined.

In the experimental model alloantibody was induced by either skin grafting, blood transfusion, or renal transplantation between unrelated recipients and donors, thus hopefully ensuring some difference in major and minor histocompatibility antigens, the usual clinical situation. Recipients were transplanted with a kidney from a donor to which they had cytotoxic alloantibody directed against donor lymphocytes, i.e. a positive crossmatch at the time of transplantation. In the clinical context this would be contra-indicated because of the risk of hyperacute rejection.

The kidney was placed in a subcutaneous pouch so as to facilitate examination and biopsy. The recipient kept its native kidneys so that alloantibody responses could be studied after graft failure. Alloantibody responses were assayed by a complement mediated cytotoxicity test (CDC) against donor peripheral blood lymphocytes. Progress of the renal transplant was assessed by daily examination of the recipient and the transplant. Frequent needle biopsies of the kidney were taken for histopathology, so as to monitor the time course and intensity of the rejection process. The ultimate test of success of a transplant was removal of the native kidneys. There were nine experimental groups. The first six groups in the study validated the model. Renal allografts were given to the following groups:

1) Unsensitised recipient, negative CDC test, and no immunosuppression.

2) Unsensitised recipient, negative CDC test, and immunosuppression with Cyclosporin A.

3) Recipient allosensitiseded by previous renal allograft, positive CDC test, and no immunosuppression.

4) Recipient allosensitised by skin grafting, positive CDC test, and no immunosuppression.

5) Recipient allosensitised by blood transfusion, positive CDC test, and no immunosuppression.

6) Allosensitised recipient, positive CDC test, and immunosuppression with Cyclosporin A.

Three strategies for transplanting the allosensitised recipients were examined. Firstly the avoidance of target antigen by finding a crossmatch negative donor. Secondly, attempts to reduce alloantibody production by the use of cyclophosphamide and cytosine arabinoside prior to renal allografting. Thirdly, attempts to modify the consequences of antibody interraction at the time of transplantation with prostacyclin. Renal allografts were given to the following groups:

7) Allosensitised recipient, but negative CDC test, and immunosuppression with Cyclosporin A.

8) Allosensitised recipient, positive CDC test, immunosuppression with Cyclosporin A and prostacyclin administered continuously into the transplant renal artery for six days, together with a small dose of aspirin by mouth.

9) Allosensitised recipient, positive CDC test, immunosuppression with Cyclosporin A and heparin administered continuously into the transplant renal artery for six days.

CHAPTER 1

ALLOSENSITISATION AND CLINICAL RENAL TRANSPLANTATION.

1.1 The problem - results of transplantation to the allosensitised patient.

The consequences of allosensitisation depend on the immunological memory, cellular and humoral, and they give rise to the second set response. Patients become allosensitised by exposure to antigen from another individual, as occurs with transplantation, pregnancy and blood transfusion. Humoral sensitisation can be measured by examining patient serum for alloantibody reactivity against allogeneic cells. Alloantibodies are directed against antigens not found in the host. Screening of sera by complement dependent lymphocytotoxicity is a simple technique compared to the tests required to demonstrate cellular sensitisation. Sera are more easily stored and transported than cells, and the screening of sera for alloantibody against panels of lymphocyte donors is a test of allosensitisation used by all transplantation laboratories.

Alloantibody can be detected by complement dependent lymphocytotoxicity against either the donor lymphocytes from a prospective donor, or lymphocytes from a panel of donors (Opelz-1972), and expressing the result as a percentage panel reactivity (%PRA). The panels are made up of peripheral blood lymphocytes from 20 to 100 donors. A serum with cytotoxic antibody to half of the panel, has a %PRA of 50%. If the panel of lymphocyte donors is carefully selected to include most alloantigens, then the %PRA provides some idea of the probabilities of obtaining a positive crossmatch test against a particular cadaveric donor. In Leicester, sera from patients on the renal transplant waiting list are collected monthly, both for alloantibody screening and for pretransplant testing against prospective donors. At the

time of the pretransplant lymphocyte crossmatch test these sera are described as historical in contrast to the acute or current serum obtained immediately prior to transplantation.

Screening for alloantibody tests one part of the humoral effector arm, the presence of preformed lymphocytotoxic antibody. The presence of B memory cells is not tested, although inferred by the presence of alloantibody, nor is antibody that may damage the graft without causing lymphocytotoxicity, such as anti-endothelial antibody (Cerilli-1985). Cellular sensitisation is not directly tested by alloantibody measurement. However, just as the presence of alloantibody can be correlated with graft destruction, it may be presumed that alloantibody as measured by %PRA reflects cellular sensitisation to some extent. Hence, though this thesis is concerned with allosensitisation and renal transplantation, the term alloantibody reflects the universal practice of measuring allosensitisation by the detection of alloantibody.

In patients receiving azathioprine (Aza) and prednisolone (Pred), allosensitisation reduced first cadaver graft survival by 5 -12% and second graft survival by 15% (Keown-1987). Opelz (1987a) for the Collaborative Transplant Study (CTS) observed a significant effect of %PRA on the survival of first cadaver grafts given CyA. Recipients were stratified by the pretransplant %PRA (0, 1-5, 6-10, 11-30, 31-50, 51-90, >90). The one year graft survival for the unsensitised patients was 79% and for the >90% PRA it was 63%. However, for recipients with a %PRA of less than 50% the graft survival was close to that for the unsensitised, and conversely for those between 51-90% the survival approximated to that for the >90% PRA. Thus, even with CyA, allosensitisation has an adverse effect on graft survival. In the CTS data %PRA was not correlated with patient survival. The CTS data indicated that the beneficial effect of HLA-matching was stronger in allosensitised patients than in unsensitised patients. With second transplants the effect of matching on graft outcome was even more striking in the allosensitised patient. Transplanted allosensitised patients also spend longer in hospital and have a higher incidence of primary non function (Iwaki-1985).

There is no agreement on what level of %PRA constitutes a highly allosensitised patient. Generally the highly allosensitised patient has been defined as having a %PRA of greater than 80%, but the CTS data above suggests that a level of current %PRA of greater than 50% may be more appropriate as the category at greater risk. Because %PRA changes with time, the prevalence of allosenstisation depends on whether the peak level of %PRA or the current level are considered, as illustrated by data from the United Kingdom Transplant Service (UKTS) - Table 1.1.

TABLE 1.1 - Allosensitisation by %PRA on the UKTS renal transplant waiting list, as presented to User's meeting in Bristol - June, 1987. Highest or peak %PRA in either historical or current sera and %PRA in current sera.

	Peak %PRA	Current %PRA
Negative	39.8%	65%
5-50%	28.6%	15%
51-80%	11.1%	9%
81-99%	20.5%	11%

Considering only the peak serum (serum with highest %PRA) will magnify the problem of the allosensitised patient, particularly so as it has been proposed that the outcome of the pretransplant crossmatch test with the acute serum is of greater relevance to the outcome of the graft than that with the historical sera.

Bradley (1987) for the Council of Europe study of high sensitisation in renal transplantation observed a significant extra risk of graft failure associated with high sensitisation. Graft survival was analysed for 638 highly sensitised patients (more than 80% PRA-peak or current not specified) and 653 control first grafts (≤10% PRA) together with 527 highly sensitised regrafts and 439 corresponding controls (control regrafts ≤30% PRA) transplanted during the period 1982-85. By one week after transplantation 14% of highly sensitised and 7% of control regrafts had failed; corresponding failure rates for first grafts at two weeks were 11% and 6%. The significant risk associated with allosensitisation as measured by %PRA continued up to three months, but was diminished thereafter.

Martin (1987a,b) has shown the post-transplant production of panel reactive antibody to be significantly associated with poor graft outcome. The antibodies were frequently directed against donor HLA antigens, particularly class 1 (HLA-A,B & C). Thus HLA matching not only holds out the prospect of improved graft survival but it also minimises the induction of anti-donor antibodies.

Allosensitisation, as measured by %PRA, decreases both the availability and success rates for renal transplantation. These adverse effects persist with the use of CyA.

1.2 Natural history of alloantibody.

As a consequence of pregnancy, blood transfusion, and renal allografting there has been a steady growth in the population of allosensitised patients with preformed alloantibodies on waiting lists for renal transplantation. The near-universal adoption of preoperative blood transfusion after the report of Opelz (1980) on the beneficial effects of blood transfusion in renal transplantation has caused a modest increase in allosensitisation. What is the extent of

the problem of allosensitisation? Bradley (1987) for the Council of Europe study of highly sensitised patients (HSP) reported that of 17,000 patients on the waiting list of seven European transplant registries 13% were highly sensitised as defined by a greater than 80% peak panel reactivity. The prevalence varied from a high of 26% to a low of 6%.

Sanfilippo (1982) analysed 2879 cadaveric donor transplants performed during the Southeastern Organ Procurement Foundation Prospective Study (SEOPF) from 1977 to 1981 and found that previous transplantation had the greatest quantitative effect on allosensitisation as measured by the %PRA. Pregnancy had an intermediate effect, while transfusion resulted in a low, but significant increase in %PRA levels.

Tongio (1985) has described cytotoxic antibodies in the absence of an obvious history of alloantigen exposure. "Natural antibodies" occur with a low incidence (0.1% to 1.0%) in both females and males, are generally of the IgM immunoglobulin class, of low affinity, and frequently monospecific. These natural antibodies can only be detected when B cells are used as targets. Approximately 40% of females develop alloantibodies by the fourth pregnancy (Terasaki-1970). These are frequently directed against the paternal haplotype, although they may be broadly reactive. It is frequently difficult to distinguish the impact of graft failure alone on allosensitisation, as blood transfusion is often required around the time of graft failure. Approximately two thirds of graft failures not accompanied by blood transfusion do not develop broad sensitisation. Other factors contributing to allosensitisation may include poor HLA matching and the withdrawal of immunosuppression after graft failure, which may then permit full expression of the immune response. The risk of sensitisation from blood transfusion approximates to 10% of patients developing 5% to 100% panel reactivity after five or more blood transfusions (Opelz-1981).

Patients may have a high %PRA by the production of antibodies with certain specificities, which gives a high %PRA by means of crossreactivity between MHC antigens (Oldfather-1983). Thus an antibody produced against a specific HLA antigen may crossreact with several HLA specificities and cause a higher panel reactivity.

Norman (1985) reported on the natural history of alloantibody, as measured by %PRA, in the renal failure population of a single centre, immunosuppressed with Aza and Pred between 1980 and 1985. Alloantibody prolonged the waiting time for a renal transplant. Alloantibody disappeared over time, especially in men but less so in multiparous women. Patients with a failed graft, in whom immunosuppression was withdrawn, had more alloantibody than those in whom the graft was removed before stopping immunosuppression, or those who continued on immunosuppression until they were regrafted. The numbers were small in the three groups (14, 24, and 10), but the observations have interesting implications for management, in that they suggest that the removal of a failed graft before stopping immunosuppression could reduce the degree of allosensitisation of the patient. Some

support for this comes from a review of the Leicester data on allograft failure (Taylor-1986), which found that allograft nephrectomy whilst on immunosuppression resulted in less allosensitisation compared to leaving the failed graft in-situ or removing the graft sometime after stopping immunosuppression.

A renal transplant is the most important cause of allosensitisation. The level of alloantibody as measured by %PRA changes with time, tending to fall in the absence of further exposure to alloantigen.

1.3 The crossmatch test.

The antibody dependent, complement mediated, lymphocytotoxicity test as described by Amos (1976) is always performed before renal transplantation. It is a measure of donor-specific presensitisation in the potential recipient. Briefly lymphocytes from a prospective donor are incubated with sera from a potential recipient, then rabbit complement contalning propidium iodide is introduced. The cell viability is assessed under green fluorescence light one hour after complement is added. Dead cells are stained orange-red by propidium iodide. The exact conditions of the test vary slightly from laboratory to laboratory, for example the duration and temperature of incubation.

In man the Major Histocompatibility Complex (MHC) is the Human Leucocyte Antigen (HLA) gene cluster located on chromosome 6. Since many of the MHC encoded proteins were first Identified by serological analysis they are frequently called MHC or HLA antigens. The MHC proteins are of three different generic types as determined by their structures and functions. Class 1 proteins consist of two polypeptides, the larger is encoded by the MHC and is non-covalently associated with the polypeptide β_2-microglobulin, which is encoded outside the MHC. Class 2 proteins consist of two non-covalently associated peptides referred to as å chains and ß chains, both of which are encoded by the MHC. Class 3 proteins are those complement components which are encoded by the MHC. In man the main regions of the MHC are D, B, C & A. There are many distinct antigenic specificities detectable at each HLA subregion, consequently the total number of possible genotypes is enormous, which makes for difficulties in matching for HLA specificities in organ transplantation. The D region contains genes for class 2 proteins, and the A, B, and C regions for class 1 proteins.

Essentially all nucleated cells carry the class 1 proteins of the A, B and C regions in varying amounts, but the class 2 proteins have a restricted distribution, namely to B lymphocytes, macrophages, monocytes, and some activated T lymphocytes. HLA-antibodies are directed against MHC specificities, and they are examples of alloantibodies.

It was originally thought that all the antibodies causing lymphocytotoxicity were reacting with HLA-A,B antigens present on T and B cells. However, there are other specificities

involved, some on T and B lymphocytes and others on B lymphocytes alone. It appears that the non-HLA antibodies are not associated with graft rejection, and therefore it is important to be able to distinguish clearly the non-HLA antibodies from the HLA antibodies (Ting-1985).

1.3.1 Historical

Kissmeyer-Nielsen (1966) and Williams (1968) both reported hyperacute rejection of renal allografts in the presence of pre-existing alloantibody directed against donor cells. The recognition that a renal transplant performed in the presence of a positive serological lymphocytotoxic crossmatch between donor and recipient usually resulted in hyperacute or accelerated rejection of the kidney was described by Ting (1983) as probably the most important contribution of the tissue typing laboratory to clinical renal transplantation. Patel (1969) reported 30 patients with a positive crossmatch: 24 failed immediately, though 6 functioned, two beyond three months. Of 27 patients with alloantibody and a negative crossmatch four failed immediately. Thus it was proposed that a positive crossmatch test should be considered an absolute contraindication to transplantation, as it was believed that the antibodies in the recipients serum directed at the donor's mismatched HLA-A,B antigens were the cause of hyperacute rejection. Terasaki (1971) observed a clear association between preformed lymphocytotoxic antibody and hyperacute rejection as shown by the 13 fold higher incidence of hyperacute rejection among patients with preformed alloantibody (26/197) in contrast to patients without antibodies (6/273). Despite a negative crossmatch, 7 out of 93 patients with alloantibody hyperacutely rejected a renal allograft, indicating that antibody specificities not detected by the standard crossmatch test might be involved in hyperacute rejection.

1.3.2 B cell positive crossmatch test

Until the mid 1970's transplants were performed only in the presence of a negative crossmatch result on all available sera, killing of as little as 10% of the target cells (peripheral blood lymphocytes, spleen lymphocytes and lymph node lymphocytes) above background was usually considered a positive result. In the mid-1970's a dramatic change occurred in interpreting the significance of a positive crossmatch when it was discovered that not all lymphocytotoxic antibodies were damaging to renal allografts, and that some transplants could be successful in the presence of a positive crossmatch. Amongst others, Beleil (1972) described 8 females whose allografts did not undergo hyperacute rejection despite a positive crossmatch. The results at one year were poor, only two having good function. There were 5 living related donors (LRD), 1 living unrelated (LURD), and two cadaver donors. Beleil identified these cases from a retrospective survey of pretransplant sera from 1450 renal transplant recipients in the United States. The crossmatch techniques were not described, but all pretransplant sera contained panel reactive antibody. Separate crossmatches on T and B lymphocytes were encouraged by these reports. This occurred

together with the discovery of the HLA-DR system, the antigens of which are present predominantly on B lymphocytes. It was also found that anti-Ia antibodies (Ia is the mouse equivalent of DR in man) in murine transplant models enhanced graft survival.

Ettenger (1976) reported the successful outcome of seven transplants performed with a positive B cell (but negative T cell) crossmatch, though the standard crossmatch was negative in all eight with peripheral blood lymphocytes (pbl). The recognition of a B cell crossmatch may depend on the source of target lymphocytes. Lobo (1977) observed that percentages of B lymphocytes were low in peripheral blood (10%), variable in lymph nodes (28.8%) and highest in spleen (41.4%). Crossmatch assays with sera with B cell specific antibody were invariably negative with pbl, but positive with lymph node or spleen preparations. Lobo observed a successful outcome in four cadaver allografts with a positive B cell crossmatch against lymph node or spleen, but negative with pbl. Morris (1977) reported 13 cadaver grafts with a positive crossmatch against donor B lymphocytes: 12 of these were positive against pbl, lymph node and spleen. There were 10 successes and two failures due to rejection. Myburgh (1977) reported that 13 of 14 patients with positive B cell crossmatches, but negative T lymphocyte crossmatches, had normal graft function at three months to three years. The crossmatches were negative with pbl and positive with lymph node lymphocytes.

There were simultaneous reports that a B cell positive crossmatch could be associated with early graft loss from allograft rejection. Dejelo (1977) reported hyperacute rejection of an HLA-A,B & C identical cadaver graft with a positive B cell crossmatch. Opelz (1987a) from the CTS data observed a significantly increased risk of graft loss in those patients with a positive B cell crossmatch with a current PRA of greater than 50%. Ting (1981) reported on 191 renal transplants in Oxford from 1976-1980. Sixty had a positive crossmatch on either historical or current sera, 45 a positive B cell crossmatch and 15 a positive T and B cell crossmatch. Overall the survival rate of positive crossmatches was not significantly lower than that for negative crossmatches. However, of the B cell positives, 25 had sera reactive with 85% of panel cells, and eight of these failed within one month, compared with three failures within one month in the 20 B cell matches with less than 85% reactivity. Of 18 grafts that never functioned, 13 had a positive crossmatch - Table 1.2.

TABLE 1.2 - Crossmatch results of 18 allografts in Oxford that never functioned - Ting 1981.

Crossmatch result	No	(%)	Current serum	
			Positive	Negative
Positive B	9	(20%)[1]	5	4
Positive T+B	4	(27%)	1	3
Negative	5	(4%)	-	-

1 - Grafts that never functioned as a percentage of all positive B cell crossmatches.

Bignon (1985) from Nantes made similar observations from a series of 322 grafts performed between 1978 and 1984. This series only contained 28 second grafts. %PRA was assessed against both T and B cell panels. Equivalent success rates were observed in B positive and negative crossmatches with first grafts, except for patients grafted with B positive crossmatches and reacting with at least 70% of the B cell panel, who had very poor results, only one out of eight functioning at a year. This is obviously a small subgroup, but once again the highly immunised patient with a positive B cell match did badly. Bradley (1987) for the Council of Europe Study reported that positive B-cell crossmatches were associated with a worse graft survival in allosensitised patients. Alarif (1987) reported 36 B cell positive crossmatches, there were seven graft losses, all but one occurring in the 11 patients with a %PRA ≥10% in current sera. Overall B positive crossmatches did not affect graft survival, but in the subgroup with ≥10 %PRA at transplantation, graft survival was 45%. This study suggested that a B cell positive crossmatch, with any degree of allosensitisation measured by %PRA, adversely affected graft survival, though the numbers were small.

This data suggests that there is heterogeneity of antibodies giving rise to B cell positive crossmatches, some of which will be harmless, and some damaging to the graft. The positive crossmatch patients appear to have more losses within one month, particularly those with high %PRA. This may reflect variables such as antibody specificity, titre, affinity, immunoglobulin class, and ability to fix complement. Whether B cell antibodies are directed against classical HLA-DR or another B cell antigen system remains to be resolved.

Controversy exists as to whether HLA-DR antibodies are damaging or not, mostly because it is difficult to show that the antibody is only anti-DR. Mohanakumar (1981) reported on two patients receiving a second cadaver graft, each with at least one identical DR mismatch to that with the first transplant. Alloantibody reactive to the mismatched DR specificity was demonstrated after failure of the first graft, and a positive B cell crossmatch was obtained with the second graft. Both second grafts were rejected in about a month, demonstrating that specific presensitisation to HLA-DR could result in acute graft failure of cadaveric renal grafts, though as to whether this specific sensitisation was causal of the rejection or an

epiphenomenon was impossible to say. Jeannet (1981) reported successful transplantation in 14 out of 16 patients with a positive B cell crossmatches, which were claimed to be due to anti-DR antibodies, because the antibodies were not removed by platelet absorption and the majority were not autoreactive.

Ahern (1982) reported two cases of hyperacute rejection of HLA-A,B identical (DR mismatched) grafts associated with high serum titres of donor specific B cell alloantibody. In both cases the level of pre-existing donor specific antibody was high, and this may have been important. The antibodies were proven to be reactive with donor endothelium (skin) in one case, and were almost completely absorbed in vivo within 24 hours of transplantation in case two. D'Apice (1980) thought that most B-cell positive crossmatches were not due to HLA-DR antibodies as the B cell positive sera, when tested in families, did not segregate with HLA-DR and specificity analysis of the sera showed that only 4 of 34 sera tested had DR antibodies against the specific antigen of the respective kidney donor.This suggested that B cell antibodies in different patients did not necessarily have the same specificity, although it was originally supposed that these antibodies were all directed at HLA-DR antigens.

If B cell reactive antibodies are not anti-HLA-DR, what specificities of these antibodies are responsible for a positive B cell crossmatch? Reekers (1977) described an antibody that reacted with autologous B lymphocytes in addition to allogeneic B lymphocytes, and that a kidney could be successfully transplanted in the presence of a positive B cell crossmatch resulting from these antibodies. These findings were supported by Ting (1977) and Ettenger (1983). Ting (1977) reported seven B cell positive crossmatches due to autoantibodies, and six were successful at three months. Ettenger (1983) observed autoantibodies in nine of 38 paediatric recipients, and observed a better graft outcome in those with autolymphocytotoxic antibodies. However only two B-cell crossmatches were positive, both at 5 deg C. The B cell autoantibodies are generally IgM, and the immunising antigen is not known. The appearance of the antibody does not seem to be related to previous pregnancies or blood transfusions and this type of antibody has been found in non transfused males. Ting (1978) observed that the autoantibody reacts with normal B lymphocytes, but it does not react with B lymphocytes from patients with chronic lymphatic leukaemia. Interestingly, the reported incidence of these antibodies in the chronic renal failure population varies enormously: Lobo (1981) reported a 42% incidence in dialysis patients whilst Major (1987) in Leicester has found an incidence of only 2%. However the finding of autolymphocytotoxic antibody has benefited a few highly sensitised patients, who have been shown to have B cell autoantibodies.

Weak HLA-A,B,C antibodies can also react exclusively with B cells probably because of a higher density of these antigens on B cells, as compared with that on T cells (Pellegrino-1978). As yet, positive B cell crossmatches due to these antibodies have not been documented.

Not all T and B cell antibodies are directed at HLA-A,B,C & DR antigens, and the non-HLA antibodies may not be damaging to the graft. Stastny (1976) described a patient who received a graft from an HLA-identical sibling, despite a positive T and B cell crossmatch at 22 deg C (negative at 37 deg C). Clearly the antibody was not directed at HLA-A,B,C or DR antigens.

1.3.3 T cell positive crossmatch test

Cross (1976) described nine successful transplants in patients with killing of donor T and B cells; this was based on killing of 30-80%, too great for B cells. The positive crossmatches were on historical sera. Falk (1985) described successful transplantation in patients who were crossmatch negative to donor T cells at the time of transplantation but donor crossmatch positive on at least one historical or non-current serum (NCS) sample. Of the 61 patients, 40 had received at least one previous transplant, and 22 of the 31 females had had one or more pregnancies. All patients had been transfused with an average of 14 units per patient. Forty-three of the 61 patients had a peak %PRA level greater than 70% . The reactivity at the time of transplantation had fallen to an average of 40%. Five patients received a transplant with a donor positive crossmatch on samples taken less than two months before transplantation. Ten received grafts from donors expressing HLA antigens shared with a previous donor; three of the 10 had demonstrated antibody with an allospecificity expressed by the donor. The study group was compared with 586 patients given a cadaver graft during the same period, but with a negative crossmatch on all sera. Of the 10 patients receiving grafts from NCS-positive donors sharing HLA antigens with a previous donor, six had functioning grafts at 12 to 70 months of follow up. Patients with reactivity to donor T cells on NCS, but negative at the time of transplantation, were not at higher risk of graft rejection when compared with patients with no reactivity to donor T cells in any sample tested. Of the 61 patients, 56 received prophylactic anti lymphocyte serum, nine of these being maintained on CyA and Pred. Three were given CyA and Pred only (Cardella-1985). In the positive crossmatch group 19 grafts were lost to rejection, 12 in the first six months. The histological pattern of rejection did not differ between the positive and negative crossmatch patients.

Falk (1986) also examined the outcome in retransplanted patients, comparing those whose previous graft had failed within 12 months of transplantation with those whose graft had either failed after 12 months or had had a technical failure. The outcome of a prior graft was predictive of outcome of a subsequent graft, in that those in the early loss group from rejection had a lower rate of success with a subsequent graft. When the groups were compared as to NCS positive or negative crossmatch, there was no significant difference in either the high risk or the low risk group in graft survival at one year. Falk (1987) found that in the high risk group (rejection of previous kidney within one year of transplantation) the %PRA at the time of retranspiantation was correlated with graft survival, and was worse

when the current %PRA was greater than 50%. In contrast the %PRA in the low risk group did not affect outcome of a further transplant.

These results have been supported by others. Matas (1984) used only Aza and Pred in five patients with positive historical sera: one graft underwent accelerated rejection at day three, and a second was removed at three months after repeated rejection episodes. Three others were functioning at three months. Norman (1985) observed a successful outcome in 6 patients highly sensitised by blood transfusion and with a positive historical crossmatch, who were immunosuppressed with Aza and Pred. Rejection episodes were treated with ATGam (anti-thymocyte globulin from Upjohn) or OKT3 (mouse monoclonal antibody against the T cell antigen T3). Sanfilippo (1984) examined the SEOPF Prospective Study data base and confirmed that crossmatch results with peak serum could be ignored and the interval between peak and current %PRA levels did not appear to significantly affect outcome. Rosenthal (1985) reported 12 cadaver grafts with positive historical crossmatches with sera from 3 to 24 months before transplantation, using CyA and Pred. There were two graft losses (one never functioned and one failed at four weeks), ten were functioning at one year. As in other studies the recipients were highly sensitised and poorly matched.

Chapman (1986) has examined the specificity and class of antibody causing positive T cell crossmatches. Dithiothreitol (DTT) reduction was used to determine whether the antibody was of the IgG or IgM class. By using a noncytotoxic monoclonal antibody (PA2.6), directed at a monomorphic determinant of HLA class I antigens, to inhibit the crossmatch test, it was possible to determine whether or not the target of the antibody was HLA class I. Of 14 positive historical T cell crossmatches, four out of seven due to IgM antibodies were successful compared to the seven transplants with IgG antibodies, which all failed. Thus transplantation in the presence of a peak positive T cell crossmatch due to an anti-HLA antibody might only be successful if the antibody in the peak serum was of the IgM class.

It can be seen that refinement of the standard crossmatch test has been successfully applied to the problem of finding acceptable donors for highly sensitised patients. There are conflicting results on the outcome of B cell positive crossmatches, but the CTS data does indicate that the highly sensitised patient with a positive B cell crossmatch is at increased risk of graft loss. Some of the conflicting evidence on B cell antibodies may reflect the variation between centres in detecting them. Schafer (1987) showed that T cell antibodies could be detected with a high degree of reliability in different laboratories, whereas the results of B cell antibodies were often discordant. In a prospective study of 1,176 transplants performed at 50 centres in the CTS group it appeared that matching for HLA-A,B was particularly important when the recipient possessed T cell antibodies, and matching for HLA-DR was important when B warm antibodies were present. The lack of agreement in interpretation of the crossmatch test could be related to variations in the method of the crossmatch test such as the incubation time, temperature, cell types, criteria of a positive

result, and the specificity of the antibodies detected.

Efforts have been made to increase the sensitivity of the T cell crossmatch to see if early graft loss can be related to false negative crossmatches. These have included the antiglobulin-augmented T cell crossmatch (Zachary-1987), and more recently the use of flow cytometry with a FACS machine (fluorescence activated cell sorter). Iwaki (1987) reported that 18 of 113 (16%) first cadaver grafts and 6 of 23 (26%) regrafts had a positive T cell crossmatch by flow cytometry crossmatching. Graft survival rates at three months were significantly better in the negative crossmatch patients, but the method still lacked specificity as 16 of 24 positive crossmatches were functioning at three months. Thus flow cytometry crossmatches have not yet been shown to be practically useful in selecting donor/recipient pairs. Lazda (1987) has shown a correlation between rejection episodes in the post transplant period and a positive flow cytometry crossmatch, suggesting a possible role in the prediction of early rejection episodes.

Most centres are now transplanting allosensitised patients with current positive B cell and historical T cell positive crossmatches. There are variations in crossmatch results: the UKTS SOS scheme (see 1.3.4) has documented crossmatches in highly sensitised patients using the same sera and targets as positive in one laboratory and negative in another. There is variation in clinical practice also, with centres differing in their willingness to expose patients to a repeat HLA mismatch and to the minimum time interval between a positive historical T cell crossmatch and transplantation. Opelz (1978) observed in a survey of 1900 retransplants that repeating the HLA-A,B incompatibilities did not worsen graft survival, with the exception of those who lost a first graft within three months from rejection. The latter had a significantly shorter second graft survival compared with those who lost a first graft after three months from rejection.

1.3.4 Donor selection for allosensitised patients.

For highly sensitised patients the crossmatch test is used, both to screen sera in order to define acceptable HLA mismatches prior to transplantation, and to screen the sera of several allosensitised patients against prospective donors as they become available. The standard crossmatch test may not always detect alloantibody harmful to the graft. Cerilli (1985) demonstrated that vascular endothelial cells and peripheral blood monocytes share a restricted non-HLA antigen system that can function as a potent immunogen in transplantation. Antibodies to this antigen system have been implicated in the hyperacute rejection of HLA identical transplants.

Persijn (1987) gave details of the Eurotransplant scheme for defining acceptable HLA-A,B mismatches. Highly sensitised patients are crossmatched against a panel of tissue typed blood donors for acceptable mismatches. They have a panel of 25,000 typed donors and clearly the

scheme is very labour intensive. Thirty-five transplants were reported, with 5 failures, and a follow up extending up to 2 years.

In the United Kingdom a national scheme for transplanting highly sensitised patients with %PRA greater than 85% was initiated in January 1984. The latest results of the UKTS SOS scheme were given by Klouda (1987). By the end of 1986 115 transpiants had been performed with a one year graft survival of 56%. Of the 54 failures 26 failed within the first 10 days post transplant. No effect of HLA-A and B matching was observed on graft survival, though there was some effect of HLA-DR matching.

Opelz (1987a) described the HIT (Highly Immunised Tray) scheme which involves 24 European centres and accepts patients with a %PRA of greater than 80%. Unlike the UKTS SOS scheme an effort is made to match donor and recipient, no more than one mismatch at one locus being recommended. There have been 113 patients transplanted with approximately a 70% one year survival, and a trend to improved survival with better matching.

1.4 Alloantibody and rejection.

Allosensitisation increases the risk of graft loss from immunological causes. The %PRA is used as a measure of alloantibody in the allosensitised patient, and a high %PRA is associated with an increased risk of graft failure within the first three months of transplantation (Bradley-1987). Both Opelz (1976) and Kocandrle (1985) have observed that in the face of a negative crossmatch with donor cells at the time of transplantation the duration of graft function was still correlated with levels of %PRA.

Is alloantibody responsible for graft loss or is it a marker of cellular sensitisation? There is no doubt that donor-reactive antibodies can cause hyperacute rejection. This rejection is immediate, and is usually the result of antibody-induced complement dependent endothelial cell damage leading to platelet aggregation and microvascular blockage (Forbes-1984). In contrast, the role of antibodies in acute rejection, starting some days after transplantation is unclear. This rejection type is more dependent upon cell mediated effector mechanisms, where cytotoxic T cells, T cell-derived lymphokines, and activated macrophages participate (Mason-1984). It would seem that antibodies are usually responsible for hyperacute rejection, but may only play a secondary role in acute rejection.

The ability of alloantibody to cause hyperacute rejection seems to be independent of whether or not the antibodies have been induced by transfusions or rejected transplants. Most of the responsible antibodies probably have anti-HLA class 1 specificity, and are thus reactive with donor T and B cells. These antibodies apparently need to be present at a certain concentration, since positive results in crossmatch assays more sensitive than the standard lymphocytotoxicity test, or only detectable in noncurrent sera, are usually not associated

with hyperacute rejection. Similarly, and with only a few exceptions, a positive B cell but negative T cell crossmatch with current sera usually does not lead to hyperacute rejection. Possibly, the few exceptions have involved high titre antibodies against donor HLA class 2 antigens, which are known to be present on endothelial cells.

Thorsby (1985) suggested that the true significance of alloantibody might be as a marker of prior sensitisation of both humoral and cellular effector mechanisms, indicating an increased risk of irreversible rejection after transplantation. This may be the case particularly when the alloantibody has appeared after rejection of a previous graft, as this will have been accompanied by strong activation of cellular effector mechanisms.There is evidence that donor-reactive alloantibody may not always be accompanied by strongly activated cellular effector mechanisms. Leivestad (1984) has reported unchanged or suppressed donor-specific cellular responses following transfusions. When Taube (1984) removed alloantibody with plasma exchange, hyperacute rejection was not seen and the acute rejection episodes could be reversed. The patients treated by Taube were regarded as high risk, and so received extra immunosuppression. In these plasmapheresis studies the HLA mismatches of earlier rejected grafts were avoided and this may be relevant. Thus donor reactive alloantibody may or may not be accompanied by significantly activated cellular effector mechanisms, perhaps depending upon the mode and type of alloimmunisation. This is probably the most important reason why it has been so difficult to find consistent correlations between donor-specific alloantibodies and acute irreversible rejections. The latter might be expected to depend on the degree and type of accompanying cellular sensitisation (which is not assayed) and the immunosuppressive protocol used. Once again the CTS data has proved helpful and Opelz (1987a) reported that the outcome of both first and second cadaver grafts correlated strongly with the current %PRA, though the second grafts did worse. The effect of HLA matching was stronger with second grafts suggesting indirectly that alloantibody could be a marker for cellular sensitisation.

These points probably apply to the noncurrent positive crossmatch controversy. Following non-current-positive, current-negative crossmatch grafts, irreversible rejections are more often seen in retransplants than in first transplants. This is supported by the CTS data (Opelz-1987), the Council of Europe Study (Bradley-1987), and the Toronto group (Falk-1987).The most likely interpretation is that it is not alloantibody that is responsible, but that donor-specific cellular sensitisation is usually stronger and lasts longer after graft rejection than after blood transfusion. Other factors causing variation in the outcome of non-current-positive, current-negative crossmatches could be alloantibody specificity, the crossmatch sensitivity, and the time from transplantation of the positive historical serum.

1.5 Immunomodulation of the recipient

Other strategies described for transplanting allosensitised patients have included attempts to condition the patient prior to transplantation, either by removing alloantibody, or by using total lymphoid irradiation (TLI) to suppress the immune system. Increasing the immunosuppression used in the post-operative period aims to combat the increased risk of early graft loss by the allosensitised patient. These strategies can be expected to increase the hazards of transplantation relating to infection (Salaman-1987) and malignancy (Penn-1987, Sheil-1987).

1.5.1 The removal of alloantibody

There have been many attempts at preventing hyperacute rejection by removing alloantibody from allosensitised recipients before transplantation. Taube (1984)used plasma exchange and immunosuppression with Pred and cyclophosphamide to remove alloantibody, and prevent its resynthesis in five patients awaiting renal transplantation. After treatment alloantibody titres and reactivities against a panel of donor lymphocytes were considerably reduced, and as a result these patients received transplants. Four were successful, one died as a result of septicaemia with a poorly functioning graft, and another had a life threatening septicaemia. The first five patients treated required urgent transplantation for clinical and social reasons. The regimen was based on that used by Lockwood (1976) to remove and prevent the resynthesis of antiglomerular basement membrane antibody in patients with Goodpasture's syndrome. Approximately six weeks after the start of treatment the patients received an HLA-typed blood transfusion, containing HLA antigens against which they previously had made antibody. If the patients failed to respond to the challenge transfusion by an increase in their antibody titres, they were deemed suitable for transplantation. When transplanted the primary HLA antigens against which the patients had made antibody were avoided. All the patients transplanted experienced acute vascular and cellular rejection and received antilymphocyte globulin. As an alternative the use of CyA instead of cyclophosphamide and Pred was studied in four patients by Taube (1985). Two of the four treated with CyA showed a reduction in panel reactivity and HLA antibody titre. When transplanted one of these kidneys never functioned, the patient was treated by dose reduction of CyA, but the kidney was lost from acute vascular rejection. The other patient developed fulminant pseudomembranous colitis and the immunosuppression was stopped. In the two other CyA treated patients no effect on alloantibody titres was seen despite further treatment with cyclophosphamide. These two patients had multiple HLA antibodies directed against multiple HLA antigens.

This regimen used potentialiy gonadotoxic and marrowtoxic doses of cyclophosphamide, and was associated with one death and one life threatening illness, both from septicaemia. It was not possible to remove all circulating anti-HLA antibody and the reduction in panel

reactivity was achieved in part by the reduction in cross reactions with other HLA antigens. The technique was not useful in patients with multiple antibodies directed against multiple HLA antigens. The experience with CyA was not encouraging.

Fauchald (1987) used a pretransplant protocol of plasma exchange together with cyclophosphamide and Pred for sensitised patients. After grafting the patients were treated with triple therapy (CyA, Pred, cyclophosphamide, the latter being substituted by Aza at four weeks). Thirty-five patients were treated with one death from septicaemia. The %PRA was reduced in 13, unchanged in 19 and increased in three patients. Cadaveric graft survival was 70% at three and twelve months, with four deaths from infectious complications three weeks to five months after transplantation. It is tempting to ascribe the good results in terms of graft function and the worrying number of deaths to the use of triple therapy, rather than to any beneficial effect of plasma exchange.

Palmer (1987) described the use of Protein A for extracorporeal immunoadsorption of alloantibody. Protein A, a constituent of the cell wall of staphylococcus aureus (Cowans strain 1), reacts with the Fc region of immunoglobulin from many mammalian species. In human sera the Ig subclasses reported to bind to Protein A are G_1, G_2, G_4, A_2, and some igM. Extracorporeal perfusion of dog serum (Terman-1980) and human serum (Terman-1981) over purified Protein A immobilised in a collodion charcoal matrix has been described in work on tumour necrolytic responses. Bansal (1978) observed non specific stimulation of the immune system after this form of plasmapheresis. Palmer (1987) reported on six patients in whom the HLA antibody titres and IgG levels dropped significantly after the treatment. Prior to Protein A immunomodulation their %PRA had been consistently above 90% for at least one year. Four patients had rebound of their anti-HLA antibodies despite treatment with Pred and cyclophosphamide. Three of the patients were reimmunoadsorbed and given a course of high dose polyclonal IgG, which preliminary data suggested might delay the alloantibody rebound. Four of the six have been transplanted successfully.

Plasmapheresis together with cytotoxic agents to reduce alloantibody has helped a few allosensitised patients to be transplanted. The technique has not proved helpful to patients with multiple anti-HLA specificities and has only been used in a few centres, combined with more aggressive immunosuppression, which makes it difficult to assess the future value of plasmapheresis. One pointer is that the Guy's group has moved on from plasmapheresis to IgG immunoadsorption by Protein A.

1.5.2 Total lymphoid irradiation

Fractionated high dose total lymphoid irradiation (TLI) is a highly effective technique for producing profound lymphoid depletion and prolonged immunosuppression (Slavin-1985). TLI affects both the humoral as well as the cell mediated immune responses, especially those controlled by T lymphocytes. The ability of a preoperative protocol of TLI to delay greatly the rejection of skin and vascularised solid organ grafts has been demonstrated in rodents, dogs, and primates. Furthermore in allosensitised recipients, prolonged allograft survival rather than accelerated rejection can be seen following TLI. The immunosuppressive effect of TLI is dose dependent and is limited by the toxicity that occurs with higher doses.

Najarian (1982) examined TLI in the preparation of 22 high risk patients for renal transplantation. Twenty had rejected a previous transplant within a few months of transplantation. The median %PRA was 50%. Only seven patients experienced no complications from TLI: one died from pneumonia. Four died after transplantation, two from lymphomas, one from pneumococcal sepsis, and one from a myocardial infarction. The overall graft survival of 74% at two years was excellent, though the patient survival was 78%. The effect of TLI dissipates with time, and the longer the period from completion of TLI to transplantation, the more likely was rejection. The authors suggested that the ideal candidate should have a low %PRA to ensure finding a suitable donor, though this runs contrary to the concept of reserving TLI for the high risk recipient.

Sutherland (1983a) reported a retrospective comparison of preoperative TLI with Aza and Pred (n=20) with CyA and Pred (n=21) for patients regrafted following previous graft failure from rejection within a year of grafting. Unfortunately the %PRA was not described, which further detracted from the study. The graft survival in both groups was 75% at one year, considerably better than historical controls. As CyA was far easier to use than TLI, they have subsequently used CyA for these higher risk patients. Sutherland posed the interesting question as to whether or not an alternative immunosuppression should be considered for the CyA failures? TLI would be a possibility. Molajoni (1987) reported good results with preoperative TLI to patients with a %PRA of greater than 60% and transplanted with CyA. The side effects of TLI were related to the total dose and several deaths were seen with higher doses.

Sampson (1985) reported 21 patients with %PRA of <20% given primary renal allografts. TLI was given pretransplant, with Pred and six doses of ATG to follow. Prednisolone was the sole immunosuppression after two weeks. During a pilot trial with maintenance Aza after TLI, all patients had neutropenia and experienced a rejection episode in the first month, an indication of the potency of TLI and of an additive toxic effect with Aza. In contrast, Najarian (1982) did not report any problems with neutropenia with the combined use of TLI and Aza. The mean number of HLA- A,B & DR mismatches were 1.0 and 0.4 respectively. Twelve

patients had no rejection episodes and only two grafts were lost with a maximum follow-up of 23 months. There was one death from disseminated herpes simplex infection.

With the improvement in results in unsensitised patients with CyA the impetus to use TLI for its immunosuppressive effect alone is not compelling. However, TLI may have a role in protocols which aim to reduce or eliminate maintenance immunosuppression as has been possible in rodents. In addition the allosensitised patient may benefit from protocols employing TLI.

1.5.3 Immunosuppressive protocols for allosensitised recipients

Schleibner (1987) described the use of quadruple drug immunosuppression for a group of 26 immunological high risk patients. CyA, Aza (three weeks), Pred, and ATG (seven days) were used. Twenty-five grafts were functioning with five losses from rejection at one to five months. No deaths and no excess of infections were observed. Gaber (1987) suggested that potent immunosuppression with four drugs (CyA, Pred, Aza, and polyclonal ALG) could overcome the increased risk associated with retransplantation, high %PRA, and historical positive crossmatches. Indeed, Gaber found no difference in the outcome of first cadaver transplants and those with risk factors, though the one year graft survival for first cadaver grafts was only 69%, which is low for this group of recipients.

Allograft rejection, which is associated with vascular damage, is often relatively unresponsive to steroids. This type of rejection is associated with the presence of alloantibody, and the use of plasma exchange in steroid resistant renal allograft rejection has been investigated. In uncontrolled studies, a beneficial effect has been claimed, but in a prospective controlled study Soulillou (1983) observed no beneficial effect from plasma exchange in early graft rejection associated with antidonor antibodies. This lack of benefit was supported by Power (1981) who treated seven patients without success and Kirubakaran (1981) who randomised 24 patients in a prospective study.

1.6 Future experimental strategies for the allosensitised patient

The mouse monoclonal antibody OKT3, which is directed against the T cell antigen T3, is the first monoclonal antibody to be widely used in clinical transplantation. OKT3 is a safe and potent, though expensive, immunosuppressive agent, which may be of benefit in the allosensitised patient. There is at present considerable enthusiasm for trying out new monoclonal antibodies and it is possible that this may benefit allosensitised patients in the future. Interleukin-2 receptor antibodies are being studied in experimental transplantation by Tellides (1987). Taube (1987) has perfused cadaver kidneys with an anti-leucocyte common monoclonal in order to reduce the immunogenicity of the organ. There were only ten kidneys treated but a reduction in the incidence of subsequent rejection episodes was claimed.

Serial monitoring of anti-HLA antibodies in sera from transfused patients frequently shows fluctuations in antibody levels with an apparent loss of certain anti-HLA antibodies. In some allosensitised patients, Reed (1987) has correlated the fall in anti-HLA antibodies with the appearance of anti-idiotypic antibodies to HLA, and furthermore has correlated graft survival with the presence of anti-idiotypic antibodies at the time of transplantation. This report needs confirmation, but it may suggest a potential for blocking anti-donor HLA antibodies.

The study of the role of lipid mediators like prostacyclin, platelet activating factor, and thromboxane in allograft rejection may suggest ways of transplanting allosensitised patients. This approach is reviewed in chapter 7.

1.7 Summary

The allosensitised recipient waits longer for a suitable organ because of the requirement of a negative T-cell crossmatch with current sera. When transplanted, the allosensitised patient is at higher risk of graft loss, particularly within the first three months. The humoral component of allosensitisation can be measured by %PRA, but this is an expression of alloantibody in the circulation and may not reflect the immunological memory. There is at present no satisfactory measure of cellular sensitisation that has been shown to be of use in unrelated renal transplantation (Carpenter-1978). The importance of alloantibody in hyperacute rejection is well proven, but in rejection occurring after the first 24 hours the role of alloantibody is probably of secondary importance compared to that of cellullar mechanisms. This paradox is probably central to the limitations of relating alloantibody, as measured by %PRA and the standard crossmatch test, to the outcome of renal transplantation.

Clinical approaches to this problem can be considered as follows:
1) The avoidance of allosensitisation.
2) Immunomodulation of the recipient.
3) The identification and selection of organs for allosensitised patients.
4) Management and immunosuppressive protocol.

1) The avoidance of allosensitisation.

Allosensitisation is a consequence of exposure to alloantigen from previous transplantation, blood transfusion, and pregnancy. There is both a humoral and cellular component to allosensitisation. Whatever the outcome of the debate on the beneficial relationship between HLA matching and the outcome of transplantation in first cadaver transplants given CyA (Gilks-1987), there can be no doubt that better matching will lead to less allosensitisation as measured by %PRA, and this is probably the simplest way of reducing aliosensitisation.

The benefit of blood transfusion to the outcome of transplantation with conventional immunosuppression (Aza and Pred) is accepted to the extent that most units have elective programs of blood transfusion prior to transplantation. This has led to considerable efforts to reduce the sensitising effect of blood transfusion, by examining the number and timing of blood transfusion in relation to transplantation, by covering the transfusion with an immunosuppressive agent such as Aza, and by modifying the immunogenicity of the blood by using blood components, stored blood, and ultra-violet treated blood. However, Opelz (1987b) for the CTS study has suggested that with CyA the blood transfusion effect may not be significant, and this matter is now the subject of a prospective trial by the CTS group. This implies that patients awaiting transplantation may not need to be electively transfused, and with the increasing popularity of CAPD and the future availability of recombinant erythropoietin (Casati-1987) there is the prospect of chronic renal failure patients receiving much less blood with a beneficial reduction in allosensitisation.

2) Immunomodulation of the recipient

The natural tendency of alloantibody to decline in the absence of further allosensitisation can justify waiting for a crossmatch negative donor, though these patients remain at greater risk than unsensitised recipients. There is some evidence to suggest that the removal of failed grafts, or continuing immunosuppression until retransplantation, reduces allosensitisation. In the special situation of living related donors, repeated donor specific transfusions have occasionally resulted in a positive crossmatch becoming negative.

There exists a small number of patients whose alloantibody titres do not decline spontaneously with time, and in those with antibody with a single specificity significant reductions in %PRA have been reported with plasma exchange and cyclophosphamide. The latest refinement of this is to use Protein A for selective immunadsorption of IgG. The studies so far have been small, had varying success, were uncontrolled, and the recipients usually received a more aggressive immunosuppressive protocol. There exists the hazards of infection and malignancy from the use of cyclophosphamide and the unselective removal of immunoglobulin.

There have been a few, but encouraging, reports of TLI in conditioning allosensitised patients for transplantation.

3) The identification and selection of organs for allosensitised patients.

Organ sharing by improving HLA-matching permits allosensitised patients a better chance of having a negative crossmatch, and as the CTS data compellingly shows there is a strong effect of HLA-matching on graft survival in the allosensitised patient.

The exchange of trays with the sera of highly sensitised patients is a further means of improving the chances of finding a crossmatch negative donor.

Positive crossmatches with current sera on B cells and with historical sera on T cells are acceptable for transplantation, albeit with a higher risk of graft loss than with the unsensitised recipient. Characterisation of alloantibody, its specificity, its autoreactivity, and Ig class may be helpful in enhancing the specificity of the crossmatch test. Despite this, there are limits to the predictive value of the crossmatch test, for example patients who have lost their first kidney early have an increased risk of graft loss from rejection with subsequent transplants.

Techniques to enhance the sensitivity of the crossmatch test have not proved helpful.

4) Management and immunosuppressive protocol.

The allosensitised patient being at increased risk of rejection is watched more closely so that rejection can be diagnosed early. Rejection is most commonly suspected when graft function deteriorates, and thus the diagnosis of rejection is simplified in grafts which are functioning. For this reason immediate function of a renal graft is beneficial. Part of this beneficial effect may be related to the association between alloantibody, the use of CyA, and primary non function (PNF) of the graft. In other words, the association between PNF and alloantibody may have an immunological basis, and thus be an early indication of hyperacute, or accelerated rejection. In contrast, immediate function of a graft to an allosensitised patient may reflect the lack of targets on the graft for the alloantibody

There have been encouraging reports of increased immunosuppression using three or four agents for the immunologically high risk patient. These studies are by their nature, single centre and uncontrolled, thus factors other than increased immunosuppression may account for the improved results. However, the concept of increased immunosuppression in the early stages is an attractive concept for preventing rejection in the allosensitised patient, and is likely to become increasingly popular. Further refinement of the selection procedure by the crossmatch test and alloantibody characterisation could be used to target some patients for more aggressive immunosuppression.

The use of agents to ameliorate the effector arm of the humoral component of graft rejection has perhaps been neglected because of the relative lack of success in studying this problem in xenograft rejection, where there is naturally occurring antibody able to cause hyperacute rejection. The increasing understanding of the role of eicosanoids and platelet activating factor in the rejection process, together with the availability of specific antagonists, suggests the need to study this approach in an experimental model.

Allosensitisation is a heterogeneous problem and it is likely that the application of some of the strategies outlined above will improve results, both by identifying patients at higher risk of rejection and selectively modifying immunosuppressive protocols.

CHAPTER 2

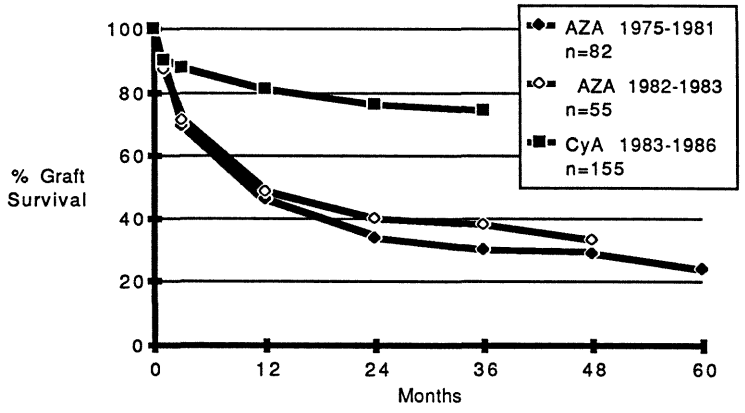
THE LEICESTER EXPERIENCE WITH ALLOSENSITISATION AND RENAL TRANSPLANTATION.

2.1 The impact of Cyclosporin A.
2.2 The use of CyA in Leicester.
2.3 Early rejection episodes - within three months.
2.4 Rejection episodes after three months.
2.5 The benefit of immediate function and no early rejection episodes.
2.6 The allosensitised patient - a case history.
2.7 Discussion.

2.1 The impact of Cyclosporin A

This chapter describes the effects of CyA on the results of cadaveric renal transplantation in Leicester, with special reference to the problem of the allosensitised patient. The first transplant by the Leicester unit was in 1975. Aza and Pred were used for immunosuppression until June 1983, when Cyclosporin A (CyA) and Pred became the standard immunosuppression for all renal cadaver grafts in Leicester. The marked improvement in the results seen with the introduction of CyA is shown in Figure 2.1.

FIGURE 2.1 - ACTUARIAL CADAVER GRAFT SURVIVAL IN LEICESTER 1975-1986



Between June 1983 and the end of 1986, 157 renal cadaver transplants were performed using CyA and Pred. By April 1987, 39 of these grafts had failed, the causes are shown in Table 2.1.

TABLE 2.1- Cause and time after transplantation of graft failure of 157 CyA grafts.

	Time after transplantation	
Cause of failure	Within 3 months	After 3 months
Immune[1]	14	4
Conversion[2]	-	8
Infection	2	1
Cardiac arrest	2	0
Renal vein thrombosis	2	0
Ischaemia[3]	2	0
Cancer[4]	0	4
Total	22	17

1. Histological evidence of either acute or chronic rejection.
2. The switching from CyA and Pred to Aza and Pred at three months. The eight graft failures were from rejection.
3. One case- ischaemia on biopsy with no renal artery lesion on arteriogram, it is not clear whether this was CyA nephrotoxicity or rejection. One presumed renal artery thrombosis.
4. Lymphoma; Kaposi's sarcoma; carcinoma bronchus; adenocarcinoma transplanted from donor.

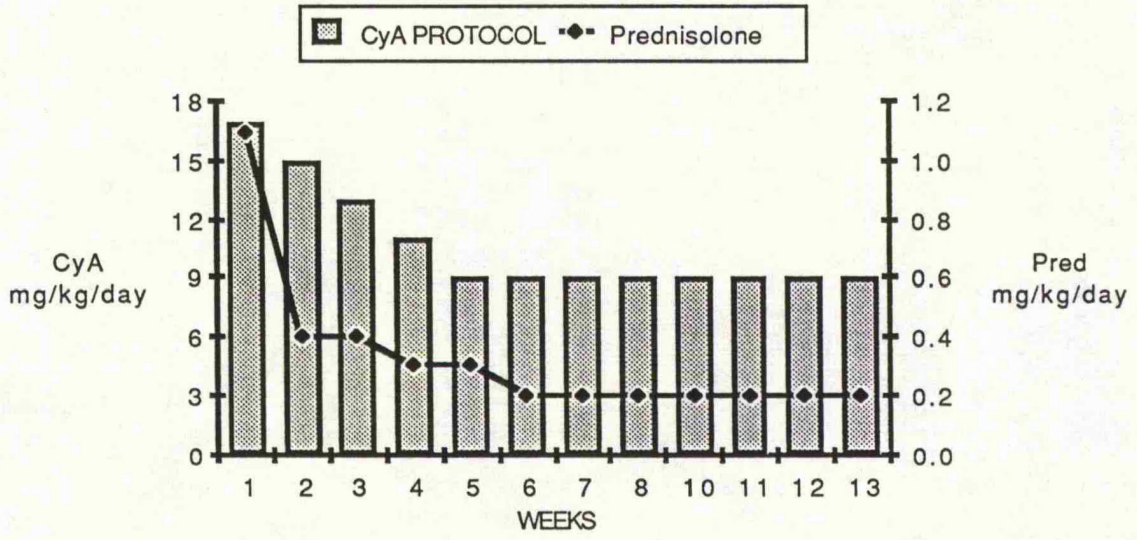
It is apparent that most graft failures occurred within three months of transplantation, and that the main cause of graft failure at all times was from rejection. It therefore appears that the priorities in management remain the avoidance of rejection and the successful treatment of rejection. I have examined these aspects of management in a retrospective study of the first 157 CyA treated renal cadaver transplants. Rather than examine immunological graft failures alone, I examined all episodes of graft dysfunction due to either acute or chronic rejection, occurring before and after three months from transplantation. Most rejection episodes occurred within three months of transplantation, and these were described as early rejection episodes. Rejection episodes after three months were unusual, most were associated with conversion from CyA to Aza at three months, and were called late rejection episodes.

I shall firstly review how CyA has been used in Leicester, before discussing the subject of rejection.

2.2 The use of Cyclosporin A in Leicester

Since June 1983 CyA has been used in a standard way (Figure 2.2) during the first few weeks. Pre-operatively a dose of 8.5mg/kg was given by mouth, and post-operatively CyA was continued at 17mg/kg per day by mouth in two divided doses. The dose is reduced by 2mg/kg per week, down to 9mg/kg/day. Dose reductions were also made for suspected nephrotoxicity due to CyA and for excessively high levels (>1000ng/ml whole blood).

FIGURE 2.2 - PROTOCOL FOR IMMUNOSUPPRESSION IN LEICESTER



At first serum levels of CyA were measured from time to time by a radioimmunoassay (RIA) method. From autumn 1985 whole blood measurements by a high performance liquid chromatography (HPLC) method became the standard. This allowed large numbers of samples to be analysed and in April 1987, I studied the HPLC CyA results in a consecutive series of 58 patients, all of whom had been followed for at least three months. Trough levels were measured daily on all inpatients, and at all clinic visits. However during this time the levels were not reported to the clinicians, except when they were either excessively high (< 1000 ng/ml in whole blood), or when they were asked for in the management of primary non-function or episodes of graft dysfunction. A graft with immediate function (IF) needed no dialysis during the first post operative week, whereas primary non function (PNF) represented the need for any dialysis during the first post operative week.

To define a range of effective immunosuppression patients whose kidney functioned immediately post transplant, and who had no rejection episodes in the first three months were examined. In these patients CyA dosage was managed according to our protocol, without access to levels, except where these were either high, or in episodes of graft dysfunction. Thirty-seven patients fell into this immediate function and no early rejection group. The

range of CyA levels ±1SD at days 3, 7, 14, 30, 90, and 365 was used to define the effective range of immunosuppression. The median CyA levels with one standard deviation from the mean are plotted in Figure 2.3. These patients have excellent renal function as measured by the serum creatinine (Figure 2.4), with a mean serum creatinine of 125 ± 24umol/l at one year.

FIGURE 2.3 - Immediate function with no rejection in first three months
Median CyA level with ± 1SD (n=37)

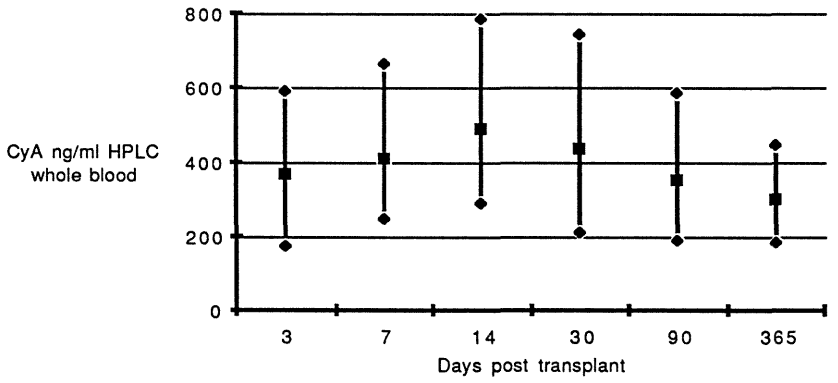
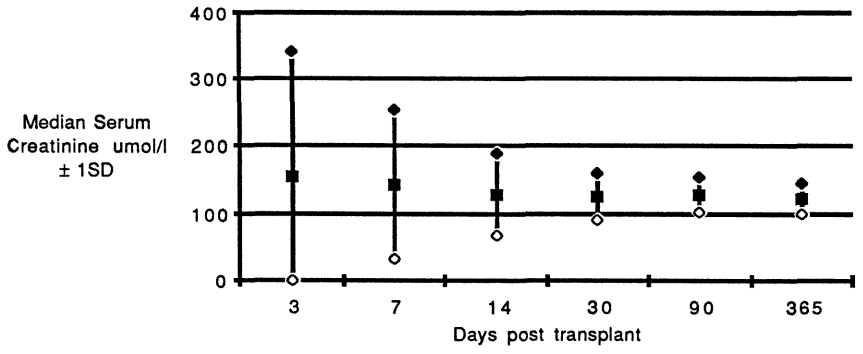


FIGURE 2.4 - Median Serum Creatinine ± 1SD of 37 patients with immediate function and no early rejection episodes



The usefulness of this range of CyA levels as a guide to the CyA dosing of patients was assessed by examining the CyA levels in patients with rejection. Fourteen of the fifty-eight patients experienced an early (< three months), biopsy proven rejection episode, and eight had levels below the effective range on the day of biopsy (Figure 2.5). Five of the thirty-seven patients with IF and no early rejection episodes had levels below the effective range at some time without a rejection episode (Figure 2.5). There were 17 patients with more than one level above the effective range and nine exhibited CyA nephrotoxicity, which was defined as an improvement in serum creatinine following CyA dose reduction (Figure 2.6). There were in addition seven recipients with PNF, whose kidney subsequently functioned, and who did not have an early rejection episode.

FIGURE 2.5 - Scattergram of CyA levels below the effective range of immunosuppression in both rejectors on the day of biopsy and non-rejectors.

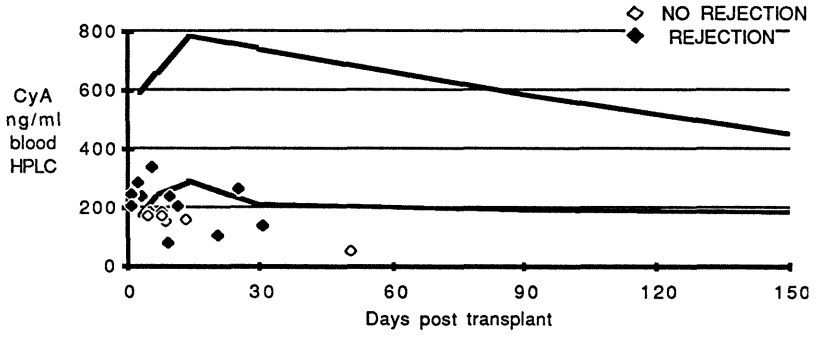
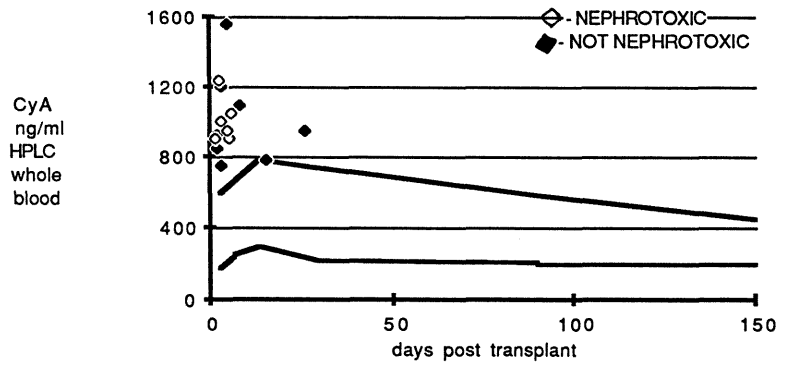


FIGURE 2.6 - Scattergram of CyA levels consistently above the effective range and response to dose reduction



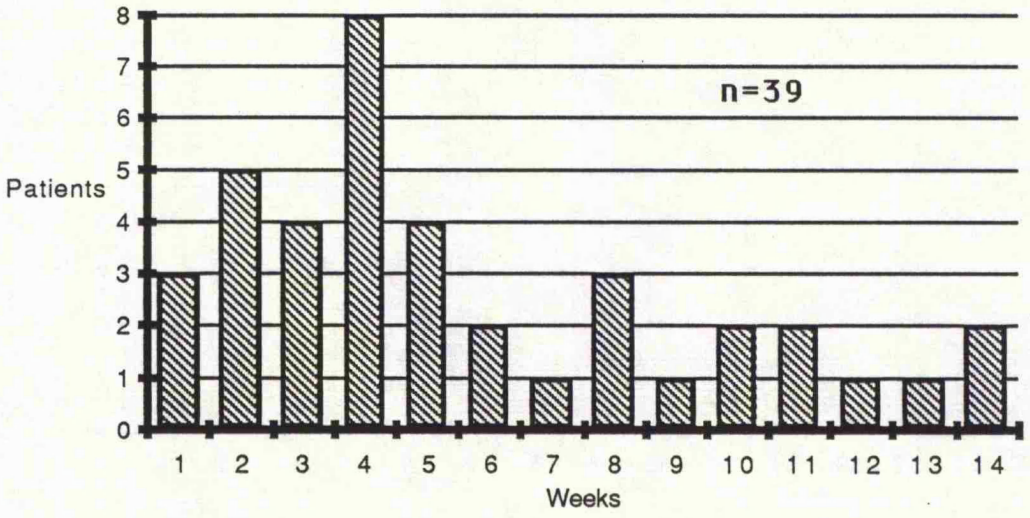
These data indicate that effective immunosuppression can be obtained within a wide range of CyA levels. Kahan (1984) has advocated the use of CyA levels to guide patients through a window of immunosuppression . The window sets a range of CyA levels, and minimises graft dysfunction due to either CyA nephrotoxicity, or to rejection. Our own data provides support for this concept of a window or envelope of effective immunosuppression with CyA. As will be shown, the incidence of rejection episodes decreases with time from transplantation and lower levels of CyA provide adequate immunosuppression. Within the first three months, it is the rejection episodes occurring within the first three weeks that are the most difficult to treat. Reference to Figure 2.5 suggests that the CyA levels with these very early rejection episodes (< three weeks) may be within the so called effective range, and that trying to prevent these rejection episodes with higher doses of CyA might result in more graft dysfunction due to nephrotoxicity. The rejection episodes occurring after three weeks were usually associated with low CyA levels at the time of diagnosis, but these episodes were nearly always treated successfully.

The small numbers prevent firm conclusions about CyA dosage, CyA levels, and the prevention of rejection. The data does provide some support for our policy of high initial dosage of CyA to achieve good levels so as to minimise early rejection of the graft, with subsequent downward adjustment of the levels after the first three months. Whether this policy to minimise rejection in the first three months will improve long term graft survival and function remains to be seen. In the shorter term we are concerned with the increased risk of early graft loss by the allosensitised patient, and as will be shown this occurs in association with rejection in the first weeks after transplantation and primary non function of the graft. Giving these patients more CyA is not, on its own, likely to be a solution to the problem of the allosensitised patient.

2.3 Early rejection episodes

Of 157 patients in receipt of a cadaver graft, 39 had an early rejection episode. Early rejection episodes were those within three months of transplantation. Three of these patients suffered a second early rejection episode within three months of transplantation. There were 20 episodes within four weeks and 19 episodes between five and 14 weeks.(Figure 2.7).

FIGURE 2.7 - Week after transplant of onset of rejection



The recipients with early rejection (n=39) were compared with the recipients without an early rejection episode - the non-rejectors (n=118), (Table 2.2). The factors associated with early rejection episodes were, a history of previous renal transplantation, allosensitisation as defined by %PRA, patient age, and the CyA level. Early rejection was not associated with the number of blood transfusions, number of HLA-DR mismatches, cumulative number of HLA-A, B, DR mismatches, and primary non function (PNF) of the transplant.

TABLE 2.2 . Risk factors for early (within 3 months) rejection episodes.

Risk Factor	Non- Rejector (n=118)		Early Rejector (n=39)
SIGNIFICANT			
Previous Transplant	22		16
		$p < 0.01$ (x^2 test)	
Peak %PRA[1]		$p < 0.0005$	
Pre Transplant %PRA[2]		$p < 0.02$	
		(Mann Whitney U test)	
Recipient Age	44.3 ± 13.8 years		35.2 ± 12.6
Mean ± 1SD		$p < 0.001$ (Mann Whitney U)	
Cyclosporin A Level		Refer to section 2.2	
NOT SIGNIFICANT			
Blood Transfusions	15.8 ± 18.5		18.6 ± 21
Mean ± 1SD		p NS (Mann Whitney U)	
HLA - DR mismatches[3]	0.66		0.89
(Mean)		p NS (Mann Whitney U)	
Cumulative HLA	2.76		2.78
mismatches[4] (Mean)		p NS (Mann Whitney U)	
Primary non function	24		15
		p NS (x^2 test)	

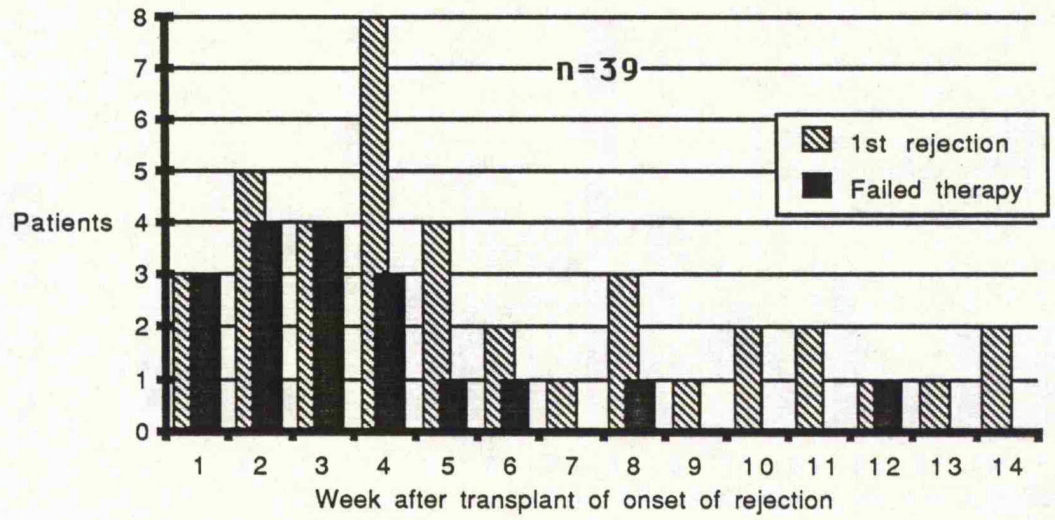
1. Highest %PRA in sera whilst on waiting list.
2. %PRA at time of transplantation.
3. Range is 0,1, or 2.
4. Range 0 - 6.

First rejection episodes were treated by a three day intravenous course of methylprednisolone (MePred), 0.5 grams daily. In addition the dose of CyA was increased if the levels were inappropriately low. Some infected patients were not given MePred. This applied to three patients (two-cytomegalovirus and one-staphyloccal osteomyelitis) who were successfully treated with an increase in CyA alone. A further five patients received no antirejection therapy prior to allograft nephrectomy- one case of hyperacute rejection and four rejecting kidneys which ruptured . All, but these five patients and one other with a wound infection, were biopsied prior to treatment. Second rejection episodes or failed treatment of first episodes were treated in various ways:

- MePred (n=3),
- anti-lymphocyte globulin -ALG (n=1),
- anti-thymocyte globulin - (ATG - ATGAM™ - The Upjohn Company) (n=1),
- OKT3 (Orthoclone OKT3™, Ortho-Cilag Pharmaceutical Ltd) (n=2).

The 39 early rejection episodes are shown by Figure 2.8, together with the proportion of these first rejection episodes that were not successfully treated. Treatment failure was graft failure within three months of rejection (n=15) or a second rejection episode occurring within the next three months (n=3).

FIGURE 2.8 - Week after transplantation of onset of rejection and treatment outcome.



Twenty of these first rejections occurred within four weeks of transplantation, and 19 between five and fourteen weeks after transplantation. In Table 2.3 the outcome of treatment of early rejection episodes, before and after four weeks is compared, and the allosensitisation history of these recipients is compared with the non-rejectors. The recipient status regarding previous transplantation, %PRA and PNF is compared with recipients without an early rejection episode. Rejection episodes within four weeks of transplantation were more difficult to treat successfully than those between five and 14 weeks (30% vs 79%). The successful treatment of rejection after four weeks was not associated with a worse graft outcome compared to non-rejectors (94% vs 88%), whereas the apparently successful treatment of rejection within four weeks was negated by second rejection episodes and late graft failures (63% vs 94% vs 88%). Recipients with rejection within four weeks were more likely to be allosensitised as judged by %PRA and to have had a previous transplant, than patients who rejected later, though these differences were not statistically significant.

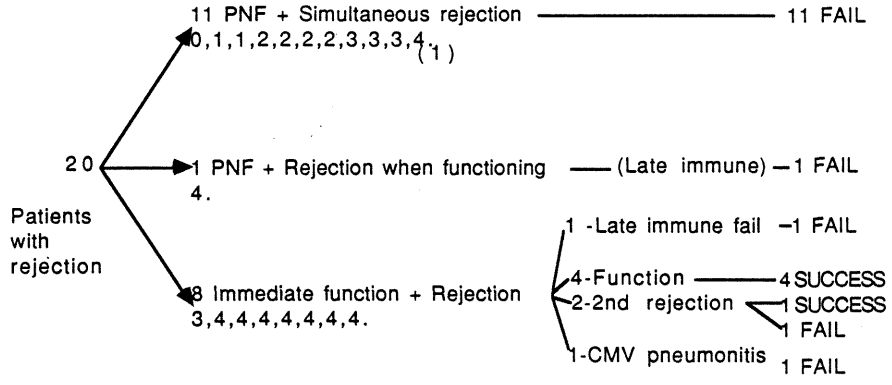
TABLE 2.3 - Graft outcome after early rejection episodes and effects of recipient allosensitisation.

	REJECTION < 3/12		NO REJECTION >3/12
	≤4 weeks (n=20)	≥5 weeks (n=19)	(n=118)
Successful Treatment	6 30%	15 79%	
	p<0.01 (x^2 test)		
Previous Graft	11 55%	7 37%	22 19%
	p NS (x^2 test)		
Peak PRA>50%	11 55%	5 26%	16 14%
	p NS (Mann Whitney U test)		
Acute PRA>50%	8 40%	2 11%	6 5%
	p NS (Mann Whitney U test)		
Primary non function	12 60%	4 21%	29 25%
	p <0.05 (x^2 test)		
Function at three months	8 40%	17 89%	110 93%
Current function as % of function at three months.	5 63%	16 94%	97 88%

Though PNF was not associated with rejection within three months (Table 2.2), the incidence of PNF was significantly greater in rejectors within four weeks than those after four weeks.

This apparent relationship between primary non function of the graft, allosensitisation of the recipient, rejection within four weeks of transplantation, CyA and a poor graft outcome is examined in Figure 2.9.

FIGURE 2.9 - Twenty recipients with rejection within four weeks of transplantation, relationship with primary non function (PNF) and graft outcome.



CMV - cytomegalovirus.
1. Week post transplant of rejection episode.

The simultaneous occurrence of rejection with PNF was associated with 100% failure, none of these grafts ever functioned. Of nine rejection episodes in functioning kidneys, eight were successfully reversed with high dose steroid, the ninth patient having immunosuppression stopped because of CMV pneumonitis. Four have continued to function well, two failed around three years from chronic rejection, and two had second rejections shortly afterwards, one reversed with OKT3 and one steroid. The latter case rejected for a third time and was reversed with OKT3, only to reject terminally four months later.

The effects of allosensitisation and rejection timing on the successful treatment of these 39 rejection episodes within three months are examined in Table 2.4. Treatment failure was significantly associated with early rejection, allosensitisation as measured by peak %PRA, and simultaneous rejection with PNF. This association between alloantibody and early irreversible rejection suggests accelerated rejection in allosensitised patients. The association between alloantibody and failed rejection treatment must not be overstated as 22 out of the 32 patients with a peak %PRA > 50% were successfully transplanted, with 16 having no rejection episodes within three months. No association was seen with either the crossmatch result, or previous transplantation. The association with the acute %PRA was almost significant - p=0.06 (Mann Whitney U Test).

TABLE 2.4 - Effects of allosensitisation, rejection timing, and primary non function with rejection on the outcome of anti-rejection therapy (Rx).

	Rejection within three months			
	Failed Rx. n=18	Success Rx. n=21		
Week of rejection - median	3	5	p<0.005	(Mann Whitney U Test)
Peak %PRA - median	55%	0%	p<0.05	(Mann Whitney U Test)
Acute %PRA - median	21%	0%	p=0.06	(Mann Whitney U Test)
Previous kidney	9	7	p=NS	(x^2 Test)
PNF with rejection	11	0	p<0.001	(Fisher exact Test)
CDC crossmatch result				
Negative	11	15		
Positive B-cell	5	3		
Positive T-cell	2	2		

The risk factors associated with IF and PNF are examined in Table 2.5. The factors associated with immediate function were donor age, cold ischaemia period, source of kidney, and allosensitisation of the recipient. Conversely the warm ischaemia time, anastomosis time, recipient age and a positive B cell crossmatch did not influence IF. Local kidneys were those retrieved by the Leicester unit, "Notts" refers to kidneys from Nottingham and UKTS to kidneys imported from other centres. There were only seven historical T-cell positive crossmatches.

TABLE 2.5 - Risk factors affecting immediate function of renal allografts in Leicester.

Risk Factor	Immediate Function (n=112)	Primary Non Function (n=45)
SIGNIFICANT		
Donor age Mean ± 1SD years	29.9 ± 15.7	39.8 ± 17.1
	$p < 0.002$ (Mann Whitney U test).	
Cold ischaemia Mean ± 1SD hours.	17.6 ± 7.1	21.5 ± 7.7
	$p < 0.005$ (Mann Whitney U test).	
Source of Kidney		
Local v UKTS	$p < 0.02$	
Local v (UKTS + NOTTS)	$p < 0.05$	
Local v Notts	p NS. (x^2 test).	
Previous transplant	22	16
	$p < 0.02$. (x^2 test.).	
Sensitised recipient.		
Peak %PRA	$p < 0.05$.	
Pre Transplant %PRA	p NS (Mann Whitney U test).	
Units of blood Mean ± 1SD	14.0 ±17.8	22.6 ± 21
	$p < 0.001$ (Mann Whitney U test).	
NOT SIGNIFICANT		
Warm ischaemia Mean ± 1SD mins	4.2 ± 4.7	4.4 ± 4.4
	p NS (Mann Whitney U test).	
Anastomosis time Mean ± 1SD mins	31.1 ± 6.8	33.1 ± 5.8
	p NS (Mann Whitney U test).	
Recipient age Mean ± 1SD years	41.3 ± 14.3	43.9 ± 13.3
	p NS (Mann Whitney U test).	
Positive B-cell cross-match.	20	12
	p NS. (x^2 test)	

To summarise, rejection was the main cause of graft loss (26/39) in a series of 157 cadaver grafts. Twenty-five per cent (39/157) of patients had a rejection episode within three months of transplantation. Early rejection was affected by recipient allosensitisation and age, but not by the degree of HLA matching. A distinction can be made between rejection episodes occurring within four weeks of transplantation, and those occurring between five and 14 weeks. The episodes within four weeks were more difficult to treat, particularly when associated with primary non function, and they were associated with second rejection episodes. Of nine rejection episodes in functioning grafts, eight were reversed with steroid, in contrast to no successes in eleven non functioning grafts - four treated with steroid, and two with steroid then ALG . Five were not treated before allograft nephrectomy, one because of hyperacute rejection and four grafts ruptured secondary to rejection. Rejection episodes between five and 14 weeks were associated with low levels of CyA, but were readily treatable, without AS YET any greater risk of graft loss with one to three years follow up.

The basis of this relationship between PNF and simultaneous rejection has important implications for management, as it represented an early graft loss of 7% (11/157). The simplest hypothesis is that the PNF is a consequence of severe rejection occurring in the first four weeks, and that these very early rejection episodes respond badly to treatment because they are early and occurring in allosensitised recipients. In support of this is the data in Table 2.2, which shows that rejection in the first three months was not associated with PNF, though the presence of alloantibody was associated with PNF as shown in Table 2.5. The simultaneous occurrence of rejection with PNF could also be associated with a poor outcome, because PNF may delay the diagnosis of rejection and complicate the assessment of the efficacy of antirejection therapy. Furthermore, as CyA is nephrotoxic, its use is complicated in the rejecting kidney with PNF. An alternative hypothesis could be that PNF predisposes to rejection, possibly by changing the pattern of antigen expression in the graft as a result of tissue damage secondary to ischaemia.

By considering the allosensitisation history of the recipients, it was possible to select most patients who had a rejection episode within the first three weeks of transplantation - Table 2.6. Using the criterion of either a previous failed transplant or a peak %PRA of greater than 50%, 10 of the 12 rejection episodes occurring within three weeks were predicted, though 35 recipients who did not reject were also selected.

TABLE 2.6 - Use of allosensitisation history to select recipients at higher risk of a rejection episode within 3 weeks of transplantation.

	No rejection within 3 weeks n=145	Rejection within 3 weeks n=12(1)
Previous Kidney or Peak PRA >50%	35 (24%)	10 (83%)

1. Number of patients with a rejection episode within three weeks.

Conversely, unsensitised (acute PRA < 10%) first transplant recipients with PNF, immunosuppressed with CyA and steroid were at no risk from rejection before function started - Table 2.7 . This provides some support for the concept of selecting recipients at low risk of early rejection for grafts at high risk of PNF (Table 2.5), thereby avoiding the problem of diagnosing rejection in a non functioning graft.

TABLE 2.7 - Risk of simultaneous rejection with PNF - prediction by history of allosensitisation.

	PNF- No Rejection during period of PNF	PNF+ Simultaneous Rejection
1st Tx +Acute %PRA<10%	24	0
1st Tx + Acute %PRA 10-45%	1	1
2nd Tx	8	7
Acute PRA >45%	2	5
2nd Tx OR Acute PRA >45%	9	10 (53%)

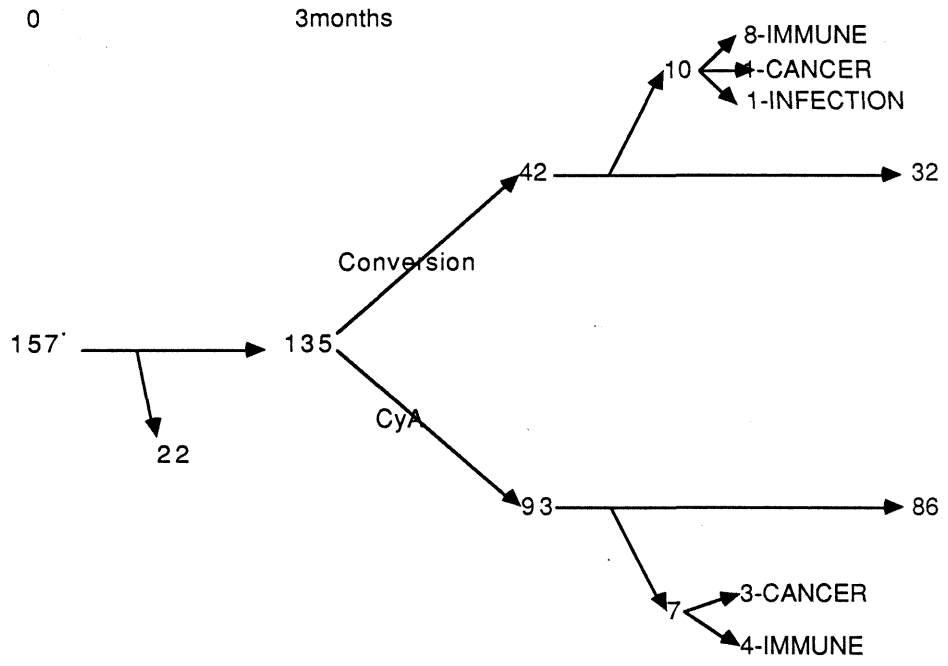
Fifty-three per cent of recipients with PNF having either a 2nd Transplant, or with an acute %PRA >45%, had PNF with simultaneous rejection. The acute %PRA of >45% appeared to be more specific and previous transplantation more sensitive at predicting the risk of PNF with simultaneous rejection. Of the 11 patients with PNF and simultaneous rejection (Figure 2.9), ten were either previous recipients or had an acute %PRA of >45%.

In view of this a more aggressive management might be justified for these recipients (PNF with either a 2nd transplant or acute PRA of >45%). This could include early biopsy (day 2), and rapid optimisation of CyA levels. Steroids have proved to be very unhelpful in the treatment of rejection in these patients, and treatment with a monoclonal antibody might be more effective . OKT 3 seems to be the most effective and safest of these monoclonal antibodies. A case could be made for OKT3 to be given routinely to these high risk patients (PNF with 2nd Tx or current PRA >45%) in an attempt to prevent or ensure early treatment of rejection. It would seem to be helpful to give these allosensitised patients a graft which is likely to function immediately, at the very least this will alert the clinician to the possibility of rejection if PNF ensues, and immediate function might even modify the natural history of a rejection episode.

2.4 Rejection after three months

In common with others we attempted conversion of patients on CyA to conventional immunosuppression at three months because of fears over the long term sequaelae of CyA. Forty-two patients were converted at three months, Aza was substituted for CyA. All, but 14, have had rejection episodes, which have been treated with mixed success, as described by Veitch (1987). This resulted in the abandonment of conversion at the end of 1985. Subsequently, ninety-three patients at three months were left on CyA and low dose steroid. The outcome of these two groups is compared in Figure 2.10. There were 22 graft failures within three months of transplantation.

FIGURE 2.10 - Outcome of renal transplantation to 42 patients converted at three months compared with 93 patients maintained on CyA.



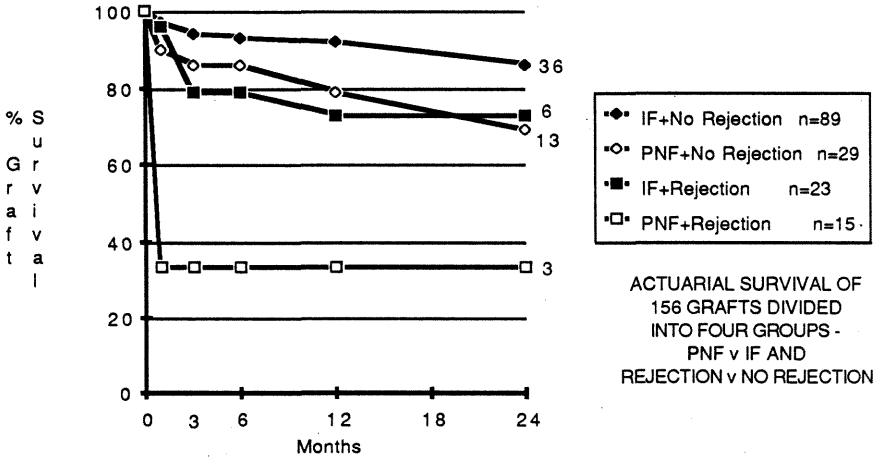
There have been ten graft failures in the 42 conversion patients and seven failures in the 93 CyA patients. Of 42 patients converted at three months, 28 suffered a further rejection episode with eight graft failures to date from rejection. Of 93 maintained on CyA, there were eight whose function deteriorated because of rejection. Four of these patients had had a rejection event in the first three months (three failures) and for the other four it was the first rejection event (1 failure, 3 successes).

In summary, for patients with function at three months the prognosis was excellent, though with our present follow up patients converted to Aza at three months had a ninefold greater risk of subsequent rejection and a fourfold greater risk of graft failure from rejection than patients remaining on CyA.

2.5 The benefit of immediate function and no early rejection.

The beneficial effect of avoiding early rejection episodes and immediate graft function on results is well shown by Figure 2.11 . The recipients of 157 cadaver grafts have been divided into four groups on the basis of the presence and absence of rejection episodes within three months of transplantation and immediate function of the graft. The actuarial graft survival at 2 years for the immediate function and no rejection within three months group (n=89) was 86%. The immediate function group with early rejection had a 20% loss in the first three months, but did well beyond this time. The group with PNF and early rejection did particularly badly, indeed the only survivors were those that rejected after function commenced.

FIGURE 2.11 - PNF AND REJECTION IN THE FIRST THREE MONTHS



ACTUARIAL SURVIVAL OF 156 GRAFTS DIVIDED INTO FOUR GROUPS - PNF v IF AND REJECTION v NO REJECTION

In Table 2.8 the allosensitisation history of the four groups is compared, the associations with rejection within three months are shown in Table 2.2. The maximum HLA mismatch is six antigens,and the mean cumulative number of mismatches did not differ between groups. The results of the CDC crossmatch test did not differ between the four groups.

TABLE 2.8 - Allosensitisation of 155 cadaver grafts with results of CDC crossmatch.

	IF- Rejection	IF + Rejection	PNF - Rejection	PNF + Rejection
Number	89	23	29	15
Graft survival 1 year	92%	73%	79%	33%
Previous transplant	16 (18%)	6 (26%)	6 (21%)	10 (66%)
Blood transfusions (±SD)	14.9 (±19.4)	10.5 (± 8.4)	18.6 (± 15.4)	30.3 (± 28)
Range	(0 -127)	(3 - 30)	(0 - 59)	(5 - 94)
Allosensitisation - % PRA	Peak Pre - Tx	Peak Pre - Tx	Peak Pre - Tx	Peak Pre - Tx
Mean	12 7	30 17	22 8	49 2
Range	0-100 0-100	0-100 0-100	0-100 0-75	0-100 0-91
Median	0 0	0 0	0 0	0 0
HLA mismatches - median (mean)				
A	1 (0.97)	1 (0.91)	1 (1.03)	1 (0.87)
B	1 (1.16)	1 (0.95)	1 (0.97)	1 (1.07)
Dr	1 (0.68)	1 (1.0)	1 (0.62)	1 (0.73)
Mean cumulative mismatch	2.81	2.86	2.62	2.67
CDC Cross match				
All negative	71 = 79%	18 = 78%	21 = 72%	8 = 54%
+ve T	2 = 2%	2 = 9%	1 = 3%	2 = 13%
+ve B	17 = 19%	3 = 13%	7 = 24%	5 = 33%

2.6 The allosensitised patient - A case history.

WV was born in 1941, and is male. He developed end stage renal failure (ESRF) secondary to polycystic kidney disease, commencing haemodialysis in 1979. To date he has received three renal transplants, and is now awaiting a fourth.
Blood group: A Rhesus positive.
Tissue type: A2, A9^{24}, B22, B27, Cw2, Cw3, DR6, DR9.

Transplant (Aza and Pred.) 5.7.81. - this failed after 3 months because of rejection, and was removed two weeks later. Prior to transplantation there was no alloantibody detected against the panel. After graft failure he was persistently sensitised at a level ranging from 45% to 100% of the panel. He had received two units of blood before this transplant.
Donor tissue type: A2, A11, B15, Bw35, Cw3, Cw4.

Transplant (CyA and Pred). 21.11.84. Prior to this transplant he was receiving plasmapheresis whilst on haemodialysis in an attempt to reduce the level of alloantibody. This was not successful. The crossmatch test was negative with donor T-lymphocytes and positive on donor B-lymphocytes, more strongly so at 4 deg. C. The kidney came from a 34 year old donor and was unremarkable, the cold ischaemia period was 21 hours. At operation following release of the clamps the kidney pinked up and some urine was passed. Then over the first 5 minutes the colour of the kidney changed to a dusky blue. As part of another study renal blood flow was being measured with an electromagnetic flow probe placed around the renal artery, and at this time the flow was 100mis/min. The renal artery and its branches were pulsating. Some form of preservation injury was suspected, and a low dose dopamine infusion (3 mcg/kg/min) was started. Twenty minutes later the kidney was flaccid with pulsatile arteries in the hilum, but no recordable blood flow. An injection of twenty mg of papaverine (a potent vasodilator) into the renal artery produced a transient pinking of the kidney. The cortex was incised and there was no significant bleeding from the cortex. The kidney was removed. Our presumption was that the kidney had been hyperacutely rejected. Histologically there was very little to support this diagnosis, but nothing to suggest an alternative diagnosis for the cessation of renal blood flow. After graft failure the %PRA ranged between 90% and 100%.
Donor tissue type: A2, B27, B37, Cw1, Cw6, DR1, DR7.

Transplant (CyA and Pred). 18.5.86. By now he had received 19 units of blood in total. The crossmatch test was negative with donor T- and B- lymphocytes at all temperatures. In view of our interest in prostacyclin we had decided to use this with his next transplant. This was given direct into the transplant renal artery via a catheter tied into a side branch. The prostacyclin was started at 10ng/kg/min before the clamps were released. The initial renal blood flow was 200mls/min. The prostacyclin was continued for seven days. Unfortunately there was never more than a few drops of urine, a course of methyl Pred was given from the sixth to eighth day. On the ninth day renal biopsy showed acute cellular rejection with involvement of arterioles. A course of anti-lymphocyte globulin was started, but a biopsy four days later showed an even worse picture and transplant nephrectomy was performed. Since graft failure the panel reactivity has ranged between 53% and 81%.
Donor tissue type: A2, B7, $B40^{60}$, Cw4, DR2, DRw6.

This case history illustrates the risk of allosensitisation from renal transplantation. This risk is increased by HLA mismatching between donor and recipient. The alloantibody has persisted for over six years, though the level, as judged by the %PRA, has fluctuated. The second transplant was probably hyperacutely rejected despite a negative crossmatch on donor T-lymphocytes. The crossmatch was negative with the third transplant, and there was steroid resistant rejection during the second week. There was never any urine from the graft. No beneficial effect with prostacyclin was seen.

This man is now awaiting his fourth transplant, is this reasonable and how should he be managed?

2.7 Discussion.

The allosensitised recipient is disadvantaged with regard to renal transplantation in the following ways:

- a longer wait for a suitable crossmatch negative donor.
- a greater risk of rejection within three months of transplantation.
- a higher risk of graft loss from rejection.

It is suggested that there is a pattern of early, treatment resistant rejection associated with allosensitisation of the recipient and primary non function of the graft. Therefore attempts at improving the outcome from renal transplantation for these patients must address this problem of allosensitisation.

The justification for examining all rejection episodes and not just the immune failures, was that rejection remains the main cause of graft loss despite CyA. The patients described above received CyA and Pred in a standard manner during the first three months. The crossmatch tests were all performed in Leicester by three workers and the patients were managed by the same small number of clinicians. For whatever reasons, the incidence of rejection in

Leicester in the first three months was lower at 25% than normally reported. This probably helped us to observe the associations between allosensitisation, primary non function, and rejection.

Consider the eleven patients whose graft was lost from treatment resistant rejection starting in the first three weeks. This loss amounted to an early failure rate of 7% (11/157). All eleven were allosensitised as evidenced by a peak %PRA >10% (n=10), or a previous renal allograft (n=1), or both (n=6). There were five negative crossmatches, 4 positive B-cell, and two historical positive T-cell crossmatches. The proportion of negative crossmatches was less than that for the rest of the transplant recipients (45% verses 78%). One possible explanation for the relatively high early failure rate of 7%, was that we in Leicester were proceeding with transplants on the basis of crossmatches, which in other laboratories would contra-indicate transplantation. Nevertheless, the overall incidence of rejection in the first three months did not suggest that we were interpreting the crossmatch results less conservatively than other centres. Furthermore the figure of 7% was comparable to the 6-14% from the Council of Europe Study of High Sensitisation (Bradley-1987) described in chapter one.

The aim of this study was to investigate possible strategies for improving the management of patients with alloantibody. The following approaches could be considered:

1) Conditioning of the recipient so as to render them unresponsive to the relevant donor antigens:
   (a) Removing harmful antibody by plasma exchange.
   (b) Removing the antibody producing cells by the use of irradiation or cytotoxic drug therapy.
   (c) Induction of immunological tolerance using donor antigen.
   (d) Revising the immunosuppressive protocol.

2) Conditioning the donor kidney so as to conceal the relevant antigen sites, eg by removal of dendritic cells.

3) Developing tests for improving the accuracy of the crossmatch test, for example by screening the recipient for antibody against donor endothelial or kidney cells.

Though these strategies can be tested in some form in clinical transplantation, an animal model is needed for detailed and controlled investigation of this clinical problem of alloantibody and renal transplantation.

CHAPTER 3

THE AVOIDANCE OF ALLOSENSITISATION - AN INDICATION FOR ALLOGRAFT NEPHRECTOMY?

3.1 Introduction - The origin of alloantibody

Alloantibody is generated as a result of allosensitisation arising from renal transplantation, pregnancy and blood transfusion. The literature suggests that renal transplantation is the most important cause of allosensitisation, and this is related to the degree of HLA mismatching. The level of allosensitisation as measured by %PRA varies with time, but allosensitisation produced by renal transplantation may be more persistent than that produced by other causes. Furthermore, allosensitised patients wait longer for a transplant, and have lower success rates, particularly if they have rejected a first kidney within three months. Norman (1985) reviewing the Oregon experience of failed grafts found that, either continuing the immunosuppression until the patient was regrafted, or allograft nephrectomy reduced the degree of allosensitisation associated with transplantation. With the long waiting lists for cadaver organs in the UK, a policy of maintaining immunosuppression until the patient was regrafted would be difficult to operate, in view of the risks associated with immunosuppression. On the other hand, removing failed grafts before stopping immunosuppression might be acceptable, providing that allograft nephrectomy was safe. This might benefit patients by reducing allosensitisation, and avoiding the symptoms from graft rejection after stopping immunosuppression.

We reviewed our management of failed renal allografts to see whether the timing of allograft nephrectomy, and the continuation of immunosuppression up to the time of nephrectomy, had any effect on the degree of allosensitisation. The indications for, and safety of transplant nephrectomy were also reviewed. The unit policy was to withdraw immunosuppression as dialysis was recommenced, nephrectomy was reserved for those with symptoms from the failed graft. This yielded three groups, patients with a failed transplant in-situ and off immunosuppression, patients who underwent allograft nephrectomy when the graft became symptomatic some time after stopping immunosuppression, and patients whose graft was removed at the time of graft failure because of symptoms and who were therefore fortuitously immunosuppressed at the time of allograft nephrectomy.

3.2 Methods

All graft failures within the Leicester Unit in the period 1979-85 were examined. This study examined the recipient's allosensitisation, as defined by panel reactivity (%PRA), before grafting and after graft failure. Recipient allosensitisation was measured by the cytotoxic reactivity of serum against a panel of lymphocyte donors and expressed as a percentage. Sera were collected at frequent intervals from all patients, but those from functioning transplants were not routinely tested. Though blood transfusions were documented the relevant samples were not always collected or tested for panel reactivity. With these reservations the %PRA of patients before transplantation and after transplant failure was used as an index of patient allosensitisation.

Patients whose grafts failed were divided into three groups:

Group 1 - Failed transplant in-situ. (Tx. in-situ).
Group 2 - Allograft nephrectomy, not on immunosuppression. (Tx. neph-Imp).
Group 3 - Allograft nephrectomy under immunosuppressive cover. (Tx neph+Imp).

Group 1 patients have remained with a failed transplant in-situ, off immunosuppression, either until regrafted or the end of the study period, November 1986. Patients in group 2 developed symptoms referable to the failed graft some time after stopping immunosuppression and required allograft nephrectomy. Some patients had symptoms from their grafts at the time of allograft failure and thus, coincidentally, they continued immunosuppression up until, but not beyond the time of allograft nephrectomy-group 3.

3.3 Results

The Leicester Unit performed 200 renal transplants during the period 1979-85, including 7 from LRD's (Living Related Donor), in 167 patients. Conventional immunosuppression was used for the first 86, and a combination of low dose steroid with Cyclosporin A for the rest. There have been 83 graft failures (22 on CyA and 61 in Aza treated patients) - Table 3.1.

TABLE 3.1 - Graft failures - Aza verses Cyclosporin A.

	Aza	CyA
Transplants	89	111
Graft failures	61	22
Death with function	4	3
% Functioning	31%	80%

Of the 83 graft failures, seven were due to death of the recipient, and seven died shortly after graft failure, leaving 69 patients that could be studied serologically by %PRA before and after graft failure. The cause of graft failure and immunosuppression are shown in Table 3.2 and no difference in the causes of graft failure was noted with the introduction of CyA.

TABLE 3.2 - Cause of graft failure and immunosuppression.

Cause of graft failure	Aza	CyA
Immune	52 (85%)	14 (77%)
Immune with rupture	0	3
Renal vein thrombosis	2	2
Recurrent disease	3	0
Death with function.	4	3
	61	22

In Table 3.3 the relationship between the time of graft failure and the cause of graft failure is compared between the three groups of graft failures.

TABLE 3.3 - Timing and cause of graft failure compared between the three patient groups.

Days post Transplant (Tx)	Group 3 Tx neph+Imp n=23	Group 2 Tx neph-Imp n=31	Group 1 Tx in-situ n=21
<30	14	2	0
31-60	6	6	1
61-90	0	4	2
>90	3	19	18
Cause of failure			
Immune	16	31	18
Immune+rupture	3	0	0
Renal vein thrombosis	4	0	0
Recurrent disease	0	0	3

The three patient groups in this retrospective study were not comparable. The group 3 patients (allograft nephrectomy under immunosuppressive cover) had more early graft losses (<30 days of transplantation). The rejection process was more acute in group 3 patients, and usually uncontrolled (hence nephrectomy whilst still on immunosuppression), indeed there were three graft ruptures associated with rejection. The four losses from renal vein thrombosis (RVT) were also in group 3 (RVT at days 1,2,7, and 7). Of the 35 patients

whose graft failed within 90 days of transplantation 32 (91%) needed allograft nephrectomy, compared with 22/40 (55%) for those failing after 90 days.

The 31 patients in group 2 developed symptoms referable to their graft from two to 180 days after stopping immunosuppression. The grafts of patients in groups 1 and 2 also functioned for a longer time than those in group 3.

The origin of the alloantibody in the 69 graft failures that could be studied before and after graft failure was examined by comparing the history of previous grafting, sex, pregnancy, and blood transfusion between the unsensitised and allosensitised patients - Table 3.4. In dividing patients into unsensitised and allosensitised all the pretransplant sera were considered, and the median value of %PRA for each patient was defined. The unsensitised patients had a median pre-transplant %PRA of 0%, and allosensitised patients a median %PRA greater than 0%.

TABLE 3.4 - Risk factors for allosensitisation in recipients of 69 grafts which subsequently failed.

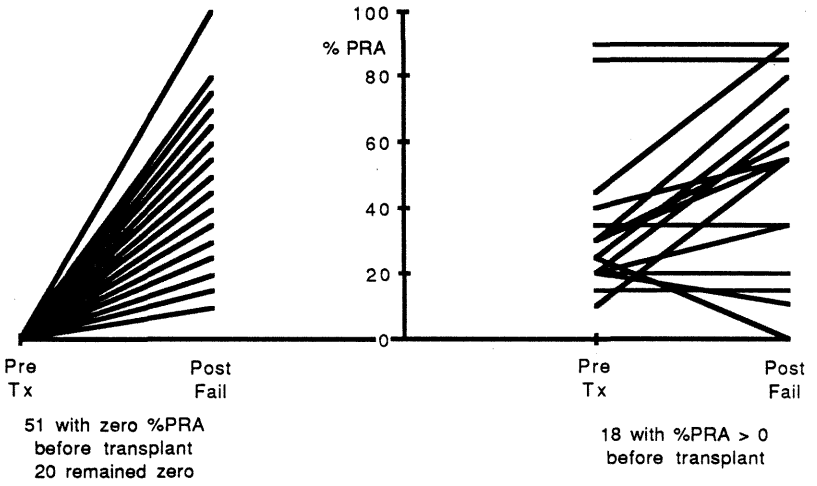
	Unsensitised (n= 52)	Allosensitised (n=17)
Previous graft	5	9
	x^2=12.28 p<0.001	
Female	14	8
	x^2=1.54 p NS.	
Pregnancy	8	6
	p NS (Fisher exact test)	
Blood transfusions (mean ± 1SD)	9.2 ± 8.5	20.3 ± 15.2
	p < 0.002 (Mann Whitney U).	

These factors were also examined with a multivariate analysis by stepwise logistic regression. A history of previous grafting, pregnancy, and blood transfusion were all independently associated with allosensitisation. The strength of the association was greatest with previous grafting and weakest with transfusion.

Following transplantation and graft failure an increase in allosensitisation was observed in 41 of the 69 recipients - Figure 3.1. In Figure 3.1 the median %PRA before transplantation and after graft failure is shown for individual patients. As the pretransplant and after failure %PRA was identical between some recipients, not all the recipients could be represented in

Figure 3.1. The %PRA was expressed as the median of all the results before transplantation and after graft failure, thus positive levels of %PRA are indicative of sustained allosensitisation and not just a peak of %PRA. Of the 51 recipients with a median %PRA of 0% before grafting, only 20 remained at 0% after graft failure.

FIGURE 3.1 - Graft failure and allosensitisation



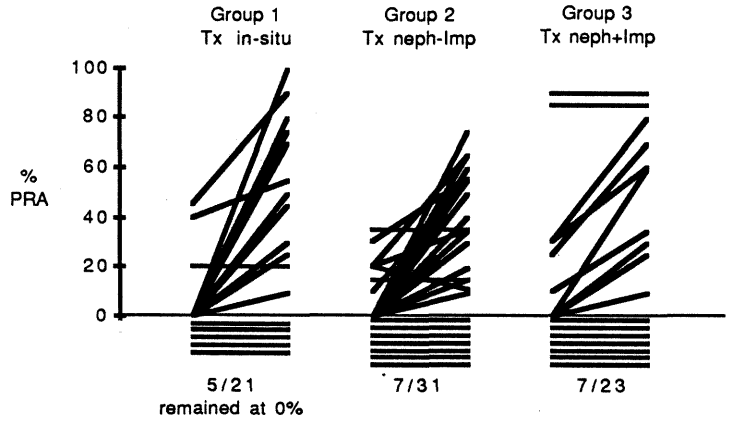
There was an association between the number of HLA -A and B mismatches when comparing those who remained with 0 %PRA and those whose PRA was no longer 0% after graft failure - Table 3.5.

TABLE 3.5. Effect of HLA mismatching on allosensitisation following allograft failure.

	Allosensitisation status by %PRA after allograft failure	
	Remain unsensitised (n=20)	Became allosensitised (n=31)
Median number HLA- A & B mismatches	1	2
Mean number HLA- A & B mismatches (± 1 SD)	1.55 (± 1.14)	1.90 (± 0.89)
	p=0.056 (Mann Whitney U Test- two tail)	

Figure 3.2 shows the individual patient %PRA's before transplantation and after graft failure for each of the three groups of patients. The association between transplantation and an increase in %PRA is clearly shown. Most patients were unsensitised prior to transplantation, 16 in group 1, 23 in group 2, and 12 in group 3. It is the the highly sensitised patient (>50%) that is the greatest problem and there is a trend for the patients in groups 1 and 2 to become more highly sensitised than those in group 3. Whether this is related to leaving the failed graft in-situ and withdrawing immunosuppression (groups 1 & 2) as compared to removing the failed graft under immunosuppressive cover (group 3) cannot be answered from this retrospective study.

FIGURE 3.2 - Patient allosensitisation before and after graft failure



How safe was elective allograft nephrectomy? The 30 day mortality following nephrectomy was three out of 54. The nephrectomies were divided into two groups on the basis of their proximity to the original transplant. As shown in Table 3.3, graft failures within 30 days always required allograft nephrectomy, and this was almost invariably performed as soon as a decision to abandon the graft was made. We called this urgent nephrectomy. In this group of sixteen there were three deaths. Graft failures after 30 days allowed the patient more time to recover from the transplant operation and were usually less urgently needed. This may account for the 38 patients undergoing, so called, elective allograft nephrectomy without mortality.

3.4 Discussion

At present, allograft nephrectomy is reserved for patients with symptoms from the failed graft. These symptoms include pyrexia, malaise, pain and haematuria. Approximately 30% of graft failures required 'urgent' nephrectomy (Group 3) because of the symptoms arising from the graft, and the three perioperative deaths occurred in this group. Following graft failure and the withdrawal of immunosuppression, a further 41% (Group 2) became symptomatic and underwent elective nephrectomy, which in our hands was a safe procedure.

The data on %PRA and allosensitisation shows clearly that transplantation and graft failure are associated with an increase in allosensitisation. Though no significant difference in the degree of HLA mismatching was observed between the three groups, HLA mismatching can be expected to have a major impact on the degree of allosensitisation. This preliminary study offers some evidence suggesting that the increase in allosensitisation associated with transplantation can be minimised by removing failed grafts before stopping immunosuppression. A randomised controlled study is needed to answer this question.

CHAPTER 4

RENAL ALLOGRAFTING IN THE DOG.

4.1 The dog as an experimental animal.

The dog is an ideal experimental model as its size and temperament allow major operations and intensive post operative care. Dogs are useful in investigating biological concepts, after their development in inbred murine strains, in an outbred preclinical animal model. The degree to which the dog can be considered a realistic model for subsequent human studies is often questioned, as it is generally believed that all dogs (in particular dog breeds) are inbred to some degree. This implies the absence of an important experimental group in dogs, namely unrelated outbred donor-recipient pairs. As discussed at the Second International Workshop on Canine Immunogenetics (Vriesendorp-1976) and shown by Raff (1983), unrelated dogs can be found within breeds, when appropriate genetic studies are done. Therefore dogs can be considered as a suitable preclinical model, when the required histocompatibility information is known. The study of histocompatibility in this species has been facilitated by the large family size and relatively short gestation and breeding cycle times (63 and 360 days, respectively). Against these advantages must be set the ethical and financial disadvantages of working with dogs.

The rat was not considered suitable for this study. Morris (1980) observed that a major reason for the rat being a good model in which to produce enhancement of vascularised organ allografts, was the rat's relative resistance to antibody mediated damage in the presence of donor specific lymphocytotoxic antibody. Possible reasons for this could be a defective complement pathway, inadequate fixation of complement by antibody in the rat, and inappropriate MHC antigen density on the target organ. Certainly, hyperacute rejection may be seen after the administration of guinea pig complement together with enhancing antisera, and in the DA to Lewis rat combination without heterologous complement, if large doses of enhancing antisera are given at the time of transplantation.

The DA to Lewis rat renal allograft model represents a strong histocompatibility barrier. Renal allografts to rats allosensitised by skin grafts, with lymphocytotoxic antibody demonstrated by a ^{51}Cr release assay, are only rarely hyperacutely rejected. Homan (1980a) showed that CyA prolonged renal allograft survival in allosensitised rats, though most rats died within 14 days of grafting and graft survival was still significantly less than that of unsensitised controls given CyA. The median graft survival in the unimmunised controls was seven days and six days in the immunised controls. Histologically, arterial fibrinoid necrosis was present in all of the immunised rats, but in only one-half of the unimmunised controls and was less severe. Glomerular necrosis was present in both control groups. Interestingly, CyA was able to eliminate fibrinoid necrosis and reduce glomerular necrosis in both immunised and unimmunised rats. In summary, there were important differences between the allosensitised rodent model and the allosensitised patient in the response to immunosuppressive therapy. These were the small differences in graft survival between the immunised and unimmunised without CyA, the beneficial effect of CyA on graft survival in immunised rats and the dramatic effect of CyA on modifying the histological appearances of vascular rejection.

4.2 The major histocompatibility system of the dog.

4.2.1 The DLA system.

The dog major histocompatibility system has been designated DLA, in analogy to the human HLA system. The D stands for dog, L for leucocyte, and A for the fact that this chromosomal area carries major histocompatibility antigens, and that it was the first genetic region to be discovered that controls leucocyte antigens. Loci within the complex are numbered with consecutive letters starting with A. Alleles receive the prefix DLA followed by a dash and the letter of their locus, followed by the allele number. The chromosome on which the DLA complex is located is not known.

Progress in the field of canine histocompatibility genetics has been facilitated by the organisation of international workshops. The First and Second International Workshops

concluded that there were at least two, and possibly three, genetic loci encoding for serologically defined antigens. There was an additional closely linked locus (DLA-D) encoding for antigens responsible for the reactivity of cells in mixed lymphocyte culture (MLC). As yet there are no known antisera that identify polymorphic antigenic determinants in the DLA-D region. Progress is slow as only a few laboratories actively work in this area - Rotterdam and Maastricht in the Netherlands, Essen and Munich in West Germany, and three in the USA.

The following subdivisions within the DLA complex have been used for loci, their alleles and gene products: SD when they are *s*erologically *d*efined by defined allo-antisera; LD when they are *l*ymphocyte *d*efined by responses in mixed lymphocyte cultures; Ir when they are defined by the presence or absence of a specific humoral *i*mmune *r*esponse; and R when they are involved in the *r*esistance against allogeneic bone marrow grafts (Vriesendorp-1976).

4.2.2 Serologically defined antigens.

Vriesendorp (1977) reported that two different modifications of a microlymphocytotoxicity technique had been in use to define dog leucocyte antigens serologically, namely a one-stage test and a two-stage test. Unfortunately the specificity of some antisera changed, depending on which test was used. Furthermore, in both one- and two-stage testing in dogs, lower reproducibility rates (85-90%) have been reported than those commonly found in man (95-99%). This makes it necessary to type each dog on at least two different occasions.

The Third international Workshop (Buil-1987) addressed the problem of the standardisation of the microlymphocytotoxicity assay for the serological recognition of DLA. The standardised microlymphocytotoxicity test was that previously described for human leucocyte antigen (HLA) typing, with the modification of using only 1µl of rabbit complement rather than the 5µl used with human cells. Briefly, the first stage of the test consisted of combining $1\text{-}2x10^4$ ficoll-hypaque-isolated dog pbl suspended in 1µl of tissue culture medium, with 1µl of the anti-DLA sera under 5µl of nontoxic mineral oil in standard tissue-typing trays. The cells and antisera were incubated at 22deg C for 30 minutes. The second stage consisted of the addition of 1µl of thawed fresh rabbit serum, previously determined as not being cytotoxic for isolated dog lymphocytes. The incubation for the second stage was 60 minutes at 22 deg C. , followed by 2-3µl of 5% eosin, and 5 minutes later 5-8µl 37% buffered formalin, pH 7.4.

Four different immunisation methods have been used to produce dog allo-antisera, organ allografting (including skin), injections of leucocytes, pregnancy sera, and injections of spleen acetone powder. Computer programs were used to cluster dog sera with common reactivity patterns on the basis of a statistical analysis of the reactivities in a population of unrelated animals.

The antigen and gene frequencies of the various DLA alleles in the mixed breeds of the Third Workshop, when compared with the frequencies observed in the Second Workshop, were in general lower - Table 4.1. This presumably reflected the efforts to be more restrictive in the selection of sera, including only sera with a "narrow" reactivity. In the Second Workshop several of the alleles were defined with sera that were crossreactive.

The major difference in the frequencies observed in the Third Workshop, when compared with the Second Workshop, was the increased frequency of "blank" at the DLA-A locus (Table 4.1). This was due to four alleles, DLA-A1, -A7, -A8, and -A10, not being recognised by Third Workshop sera. These antigens in the past were better recognised by sera that were active in the one stage test.

There is strong linkage disequilibrium between some of the alleles of the three loci. This may represent in part, a bottle-neck effect, in that dog history has probably known periods and/or areas in which only a limited number of animals were available for further propagation. The DLA haplotypes of such animals will be over-represented in subsequent generations. Another observation that indicates a relative difference between dogs and humans is the fewer number of alleles per locus of the dog MHC compared to human.

The DLA SD antigens are present on lymphocytes and platelets, but have not been found on erythrocytes. Indirect evidence for their presence in skin, small bowel, pancreas, and heart tissues comes from the observation that recipients of these allografts produced good SD reagents during and after rejection (Vriesendorp-1977).

TABLE 4.1 - Comparison of antigen and gene frequences of DLA-A, -B, and, -C alleles of mixed breeds and beagles in the Third Workshop and those observed in the random bred dogs of the Second Workshop.

Workshop(a)	III		II		III	
Breed	Mixed breeds		Random breeds		Beagles	
No. animals	319		253		152	
Locus	Antigen frequency	Gene frequency	Antigen frequency	Gene frequency	Antigen frequency	Gene frequency
DLA-A1	ND (b)	ND	0.055	0.028	ND	ND
A2	0.201	0.106	0.202	0.106	0.184	0.096
A3	0.176	0.092	0.462	0.267	0.263	0.141
A7	ND	ND	0.352	0.195	ND	ND
A8	ND	ND	0.095	0.049	ND	ND
A9	0.141	0.073	0.225	0.12	0.447	0.256
Aw14	0.169	0.089	ND	ND	ND	ND
Aw15	0.091	0.047	ND	ND	ND	ND
Blank (c)		0.593		0.235		0.507
DLA-B4	0.122	0.063	0.13	0.068	0.105	0.054
B5	0.216	0.115	0.25	0.135	0.086	0.044
B6	0.097	0.05	0.269	0.161	0.342	0.188
B13	0.395	0.222	0.257	0.138	0.105	0.054
Blank		0.555		0.498		0.66
DLA-Cw1	0.135	0.07	0.182	0.096	0.184	0.096
Cw2	0.204	0.108	0.091	0.047	0.565	0.341
Cw3	0.364	0.202	ND	ND	0.217	0.115
Blank		0.62		0.857		0.448

(a) II: Second International Workshop on Canine Immunogenetics
III: Third International Workshop on Canine Immunogenetics
(b) ND: not detected
(c) Blank: product of the subtraction of the sum of the detected gene products from 1.0

4.2.3 Lymphocyte-defined antigens.

The Third Workshop on Canine Immunogenetics (Deeg-1986) involved 80 potentially DLA-D homozygous typing cells obtained from dogs of five different breeds and submitted from five laboratories in Europe and the United States. To exchange the cells between the large distances between individual laboratories, it was necessary to cryopreserve cells and ship them packed in dry ice. Mutual reactivities of all cells were studied in mixed lymphocyte cultures. Ten clusters of homozygous typing cells were defined and accepted on an international level, and they were assigned the specificities Dw1 to Dw10. At least six additional specificities were recognised that were well characterised within laboratories, but required additional testing before workshop specificities could be assigned. The typing cells had been obtained from 54 beagles, 6 foxhounds, 12 dogs of various breeds and 8 mongrels. Raff et al (1983) looked at a population of 160 dogs with a panel of homozygous typing cells, and found a typing response in 59% of laboratory dogs and 39% of random dogs, suggesting that breeds of dog differ in the spectrum and distribution of DLA-D alleles.

Although antigens of class 2 molecular size can be found on canine cells, no seroiogically detectable polymorphism of the DLA-D region has been described so far. No studies on the molecular composition of DLA-D region antigens have yet been reported, and though the circumstantial evidence suggests that the canine Dw specificities correspond to DR specificities described for the human HLA system, there is no formal proof that they are classic class 2 antigens.

4.2.4 Immune response genes.

Immune response (Ir) genes in dogs have been studied by immunisations with random linear copolymers of amino acids-Vriesendorp (1977). The ability of a dog to produce antibodies against a certain copolymer appeared to be polymorphic; i.e. responders and nonresponders have been found. Segregation patterns in family studies are in accordance with linkage between Ir genes and the DLA complex.

4.2.5 Monoclonal antibodies recognising dog MHC antigens.

Monoclonal antibodies directed against murine, rat, and human Ia-like antigens have facilitated investigation of the function of the immune response region and its role in allograft rejection in these species. Canine immunogenetics, by contrast, are not yet well defined. There are few antibodies that have been raised specifically against canine antigens.

Alejandro (1984) has two murine monoclonal antibodies, B1F6 (IgG_{2a}) and B2E8 (IgG_{2b}), which were raised against canine splenocytes. Both antibodies recognised typical Ia-like molecules on both canine and human cells. Direct blocking and additive binding assays

demonstrated that they recognised distinct epitopes. These two monoclonals also recognised porcine Ia, but only B1F6 recognised rat Ia - Shienvold (1986).

Murine monoclonal 7.2 is an IgG_{2b} specific for Ia-like antigens on human cells. Deeg (1982) showed that 7.2 cross-reacted with canine lymphoid cells and recognised an antigen with a molecular structure similar to that of human Ia-like antigens. The distribution of these antigens in dogs was different from that reported for other species in that it was found not only on B lymphocytes and monocytes but also on most normal T-lymphocytes.

Wulff (1982a) described a murine monoclonal antibody (DT-2) raised against canine puppy thymocytes and of immunoglobulin subclass IgG_{2a}. DT-2 activated complement and was reactive with most canine thymocytes, peripheral blood T cells, thoracic duct lymphocytes, bronchoalveolar lymphocytes, and T chronic lymphatic leukaemia cells. Since no standard pan-T cell marker comparable to the sheep red blood cell receptor in humans was available in dogs, it is not possible to say what proportion of canine T cells is recognised by DT-2. DT-2 is nonreactive with B-cells, monocytes, or other blood elements. Wulff(1982b) also described two other murine monoclonals, DLy-1 an IgG2b raised against puppy thymocytes which reacted with virtually all canine blood lymphocytes, thoracic duct lymphocytes, bronchoalveolar lymphocytes, monocytes, and granulocytes. It did not react with erythrocytes, megakaryocytes, or platelets. The other was DLy-6, an IgM antibody raised against bronchoalveolar lymphocytes, which reacted with virtually all nonactivated canine lymphocytes.

Iwaki (1983a) found that monoclonal antibody CIA (IgG_3), produced against human class 2 antigens, and reacting with 20-30% of human peripheral blood lymphocytes, also reacted with more than 90% of dog lymphocytes. Less than half of these CIA positive cells expressed surface immunoglobulin, suggesting that class 2 like antigens were not only present on B lymphocytes.

Krawiec (1984a) described a murine monoclonal antibody (1A1- an IgM immunoglobulin) reactive with canine T lymphocytes, but not with B lymphocytes. Antibodies 1A1 and DT-2 reacted with a similar percentage of thymocytes and peripheral mononuclear cells as. Antibody F3-20-7 (McKenzie-1982) identified a larger population of thymocytes and lymph node cells, recognising the canine Thy1 antigen-equivalent present on all canine T cells. Thy 1 is a glycoprotein expressed by all mouse peripheral T cells. The antigens recognised by these antibodies remains to be identified. Krawiec (1984b) has also produced C6C (IgG_{2b}), which reacted with 13.7% of canine thymocytes.

Szer (1985) described two murine monoclonals E11 (IgG_3) and A5 (IgG_{2b}), both able to activate rabbit complement. Antibody A5 was broadly reactive, recognising all $Thy1^+$ and most $Thy1^-$ peripheral blood mononuclears. Antibody E11 recognised an antigen expressed

on a subset of normal peripheral T lymphocytes (Thy1$^+$) and puppy thymocytes. Szer examined MLC generated suppressor cells, and found that E11 was able to eliminate suppressor activity but not DT-2. This suggested that these two monoclonals, E11 and DT-2, were capable of dissecting functional subgroups of canine T cells.

The majority of dog peripheral blood T lymphocytes express class 2 like antigen recognised by monoclonal antibodies BIF6 (Alejandro 1984) and 7.2 (Deeg 1982). The presence of class 2 like antigen on canine peripheral blood T lymphocytes was not caused by stimulation since the antigens were also found on T cells of specific pathogen free dogs and neonatal thymocytes (Doveren-1985). Doveren showed that two subsets of class 2 like antigen could be detected on canine lymphoid cells. One subset showed an aberrant distribution being expressed on both nonactivated and activated T lymphocytes and on B lymphocytes. The second subset could only be detected on B lymphocytes and activated T lymphocytes, a distribution pattern similar to that found in most other species. Monoclonal 7.2 (Deeg 1982) detected the first subset and monoclonal F3-20-7 (anti-Thy-1) (McKenzie 1982) detected the second subset.

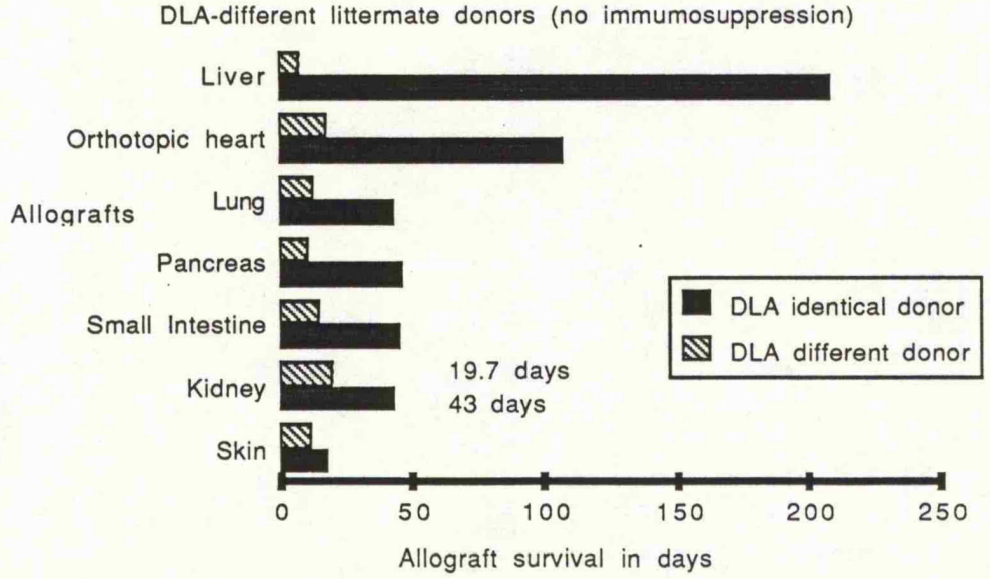
4.3. The transplantation biology of the dog DLA system

In dogs, donor selection for organ grafting and its effect on allograft survival has been studied in two experimental groups: (1) related and (2) unrelated donor recipient pairs.

4.3.1 DLA matching and allograft survival.

A summary of the results obtained by the Rotterdam group is shown in figure 4.1 (Vriesendorp-1977). For all organs a significant influence of DLA matching was observed.

FIGURE 4.1 - Mean survival times of organs of DLA-identical and DLA-different littermate donors (no immumosuppression)



Similar data have been reported from the group working in Cooperstown in the United States for skin, kidney, heart, and liver allografts. The same trend was observed in these data, in that the survival of skin grafts was less affected (mean survival time 24.7 days) by donor selection and that kidney, heart, and liver allografts had a more extended survival of 27.3, 33.9, and 76.7 days, respectively. However, the absolute mean survival times for each organ were not comparable between the two research groups. Possible causes for these variations might have been differences in genetic background of the experimental animals, in operation procedures, in animal care, or in the tissue-typing reagents used.

Variables that might induce differences in survival of different allografts include intrinsic differences between vascular and avascular grafts, e.g., skin versus kidney; differences in immunogenicity of the various organs; a greater vulnerability of some organs to rejection; and the use of blood transfusions at the time of operation.

Westbroek (1972) prospectively tissue typed littermate beagles, and formed three experimental groups:

Group 1. Two DLA haplotype differences between donor and recipient -12 dogs.
Group 2. One DLA haplotype difference between donor and recipient - 10 dogs.
Group 3. No DLA haplotype difference between donor and recipient - 13 dogs.

Recipients had bilateral native nephrectomy, the transplanted kidney was examined histologically when the recipient died or when the serum creatinine level rose above 1000 umol/l. No immunosuppression was given. The mean recipient survival time (± 1SD) in days was:

Group 1: 15.6 (± 7.8) days.
Group 2: 23.8 (± 13.8) days.
Group 3: 43.0 (± 12.5) days.
1 v 3 ($p < 0.001$); 2 v 3 ($p < 0.005$); 1 v 2 (p NS).

One long term survivor (254 days) in group 3 was excluded from the analysis. A significant effect of DLA matching was seen on survival. In the same study, the histology of graft rejection was compared with respect to the donor-recipient relationship. The DLA matching seemed to have an effect on the two most prominent signs of allograft rejection, namely graft arteritis and cellular infiltrate. The cellular infiltrate appeared later and was less aggressive in DLA-identical grafts than in non-identical grafts. The arteritis in DLA-identical grafts occurred for the first time around the third week after grafting, became gradually more intense, and was subsequently the final cause of allograft failure.

Ten of the dogs were tested by Westbroek (1972) for the presence of anti-donor lymphocytotoxicity at the time of death. Alloantibody was only detected after allograft

rejection between DLA-incompatible pairs.

Bijnen (1979a) examined the influence of subregions of the dog MHC on renal allograft survival in recipients without immunosuppression. Results in six beagle littermate donor-recipient pairs, in which either the donor or recipient had a recombination in the MHC, were compatible with the concept of a predominant role for the subregion containing the HLA-D locus in determining allograft survival. Bijnen (1979a) also grafted four different groups of unrelated DLA-typed mongrels without immunosuppression - Table 4.2.

TABLE 4.2 - Effect of SD- and LD- matching on renal allograft survival in unrelated mongrels.

	Mean survival ± 1SD
SD- and LD- matched (n=5).	18.2 ±4.6 days
SD-mismatched and LD-matched dogs (n=5).	13.8 ±3.0
SD-matched and LD-mismatched dogs (n=11).	10.1 ±1.9
SD- and LD-mismatched dogs (n=8).	10.4 ±2.7

The LD- matched individuals survived significantly longer than did the LD-mismatched individuals. SD matching alone did not improve graft survival, but a slight improvement was seen in LD-matched combinations (group 1 versus 2). These results need to be interpreted with care, as matching for defined markers in the MHC may involve unintentional matching for undefined antigens, when linkage disequilibrium between alleles of the defined and undefined genes of the MHC exists. The predictive value of LD-matching was supported by Kahn (1975), who transplanted into each of five beagles two renal allografts, one from a mongrel dog showing high MLC stimulation, and one from a second mongrel showing a low MLC stimulation. Immunosuppression was with Aza and Pred. The high stimulators functioned up to three weeks, and the low stimulators for three to six weeks. Unfortunately there was no information on the SD-antigens.

Bijnen (1979b) went on to assess the relevance of minor histocompatibility systems to renal allograft survival in non-immunosuppressed beagles. Renal allografts between DLA-identical littermates (mean survival time-MST- 43±17) survived significantly longer than those between DLA-identical unrelated beagles (MST 24±14). Bijnen could not attribute this difference to inferior DLA matching in the non-littermate beagles, and a more likely explanation was the presence of non-DLA antigens, of importance to allograft survival. It was suggested that the difference in outcome could be caused by minor histocompatibility antigens (non-MHC). The cumulative effect of these minor histocompatibility antigens was comparable to a one haplotype difference, since the graft survival in the littermate one DLA haplotype different group (MST 24±14 days) was comparable to the graft survival in the SD- and LD-matched non-littermate group (MST 23±5).

Bijnen (1980) repeated this study using Aza and Pred for 100 days, followed by tapered withdrawal over 50 days. Graft survival at 150 days was 88% for DLA-identical littermates and 100% for DLA-identical non-littermates. Graft survival for one-haplotype mismatched littermates was 28% at 150 days and for mismatched unrelated mongrels 5% (one out of 24). Thus it appeared that in moderately immunosuppressed dogs, non-DLA incompatibilities rarely caused rejection, whereas DLA incompatibilities almost always did so. These results contrasted with those obtained without immunosuppression in which non-DLA incompatibilities seemed to be as strong as DLA incompatibilities (one haplotype) in precipitating rejection.

These studies show a highly significant effect of DLA matching in littermate beagles. The lymphocyte defined antigens (DLA-D) appear to have a greater effect on allograft survival than the serologically defined antigens. In unrelated dogs, matching for LD determinants but not SD, determinants slightly prolonged graft survival (10 vs 18 days). The results indicated at best a marginal effect of LD and/or SD matching in unrelated dogs not given immunosuppression. Comparison of the results between LD- and SD- identical beagle littermates and LD- and SD- identical unrelated dogs pointed to the existence of additional loci within the DLA complex, different from the known LD and SD loci and with an important histocompatibility effect. When Aza and Pred were used a highly significant effect of DLA matching was seen in unrelated beagles. which were DLA matched. However there is a high incidence of linkage disequilibrium within dogs, and it is possible that matching for the DLA antigens may also have matched for other, as yet, undefined histocompatibility antigens.

4.3.2 Blood transfusions and graft survival.

With the recognition of the blood transfusion effect in clinical renal transplantation with conventional (Aza & Pred) immunosuppression (Opelz-1980), the Rotterdam group extensively investigated this effect in their beagle model. Of interest to this study was the incidence of allosensitisation and the outcome of transplantation to these allosensitised recipients. Donor specific transfusions (DST) come from the prospective renal allograft donor, whereas third party transfusions (TPT) come from an unrelated dog with a different genotype from the prospective renal allograft donor.

Halasz et al (1964) reported a beneficial effect from small quantities of dog blood on the survival of subsequent donor specific renal allografts. The dogs were mongrels and phenotypic similarities between donor and recipient were avoided. Pretreatment consisted of the subcutaneous injection of 2ml of fresh donor specific (DS) venous blood, 10 and 5 days before allografting. A third group of dogs continued to receive injections of donor blood on every 5th day. No other immunosuppression was given. The dogs were killed after two days of oliguria (<50 mls/day). The results are summarised in Table 4.3.

TABLE 4.3 - Survival of renal allografts following subcutaneous injection of donor blood.

Time blood injected	No. dogs	Survival-mean±SD
Controls - nil injected	10	8.1 ± 1.3
Before grafting	14	29.2 ± 10.9
Before and after grafting	5	16.3 ± 5.7

($p<0.01$ for both experimental groups verses controls)

Halasz consistently induced enhancement with donor specific blood in the absence of immunosuppression. The reduced graft survival with continued antigen therapy cannot be explained. Obertop (1975) gave a single donor specific blood transfusion of 200ml two weeks before renal allografting between the three different DLA match littermate combinations. There was no benefit from this single transfusion, though prolonged survival (>300 days) of two out of ten kidneys between DLA identical littermates was noted. Active enhancement is a possible explanation for these two long term survivors. Other workers have only observed a beneficial effect with blood when immunosuppression was given with the renal allograft. Niessen (1982) observed a beneficial effect with third party blood transfusions when Aza and Pred were given with the renal allograft. Wilson (1969) found that donor specific antigen derived from spleen cells proved effective in prolonging renal allografts between mongrels only when Aza was given with the renal allograft. Similarly, Hall-Allen (1970) found a beneficial effect on renal allograft survival with antigen from donor lymphocytes only when Aza and Pred were given with the renal allograft. The balance of evidence is against the occurrence of active enhancement in dog renal allografting, as observed by Halasz (1964) and Obertop (1975). It must be said though that the timing, route of administration, and amount of antigen have differed between most of the reported experiments, and this could have affected enhancement. Sutherland (1979) repeated Halasz's protocol with two subcutaneous injection of donor specific blood to mismatched DLA typed outbred beagles, and 12 of 19 renal allografts were lost within two days with accelerated rejection, but four of the remainder functioned longer than controls. Thus, the effect of DS blood pretreatment on renal allografts is unpredictable, either enhancement or accelerated graft failure may occur, but the latter is more likely.

Jeekel (1973) immunised one of a pair of DLA-identical littermate beagles (dog B1) with lymphoid cells from beagle littermates differing in one and two haplotypes (dog A) - Table 4.4. After six immunisations serum was taken from the recipient and injected into the other DLA identical member of the pair (dog B2). The kidneys of the lymphoid cell donor were allografted to the pair of recipients.

TABLE 4.4 - Attempts to induce enhancement of dog renal allografts. (Jeekel-1973).

Dog A ——— Dog B1 ——— DogB2

Immunised with lymphoid cells x6.

Serum B1 to B2

B1 & B2 are DLA identical littermates.

Dog A and Dog B differ by either one or two DLA haplotypes.

Results

	Control Not immunosuppressed Mean Survival Time Days	Experimental B2-passively immunised MST (range)
1 DLA haplotype different	18.5 (10-42)	45.5
2 DLA haplotype	12 (9-31)	18 (15-21)

Jeekel demonstrated passive enhancement of renal allografts in tissue typed beagles differing by one DLA haplotype (18.5 vs 45.5). The actively immunised beagles (dog B1) rejected in both DLA combinations in an accelerated manner, though hyperacute rejection was not seen. Morris (1980) transplanted renal allografts without immunosuppression, using either third party kidneys, or kidneys from donors against which hyperimmune antisera had been prepared by injecting mesenteric lymph node lymphocytes into unrelated mongrels. In two of 15 dogs treated with antisera there was a prolongation of graft survival by three to four days over controls, though in seven there was never any function, presumably due to antibody mediated damage. Soulillou (1980) also attempted passive enhancement of mongrel renal allografts to beagles by injection of specific antidonor Ia-like antibodies. Anti Ia-like sera were raised by immunisation with allogeneic splenocytes and subsequently adsorbed on platelets from the immunising dog. Recipients received Aza and Pred with the renal allograft. No significant prolongation of graft survival was noted with the specific antisera. In contrast to Jeekel, neither Morris nor Soulillou were able to produce passive enhancement of renal allografts, which may have been related to the use of one DLA haplotype different littermates by Jeekel.

The effect of DST on renal allograft survival has been investigated in beagle littermates and mongrels, with and without immunosuppression. The results are conflicting. Obertop (1975) gave a single transfusion of 200 ml of donor specific blood 14 days before renal allografting between prospectively DLA tissue typed beagle littermates. A shortened graft survival was noted when, after blood transfusion, a positive cross-match was present. No

beneficial or deleterious influence on graft survival by donor specific blood transfusion was seen when the crossmatch remained negative. Five out of 29 dogs were labeled responders, because they produced lymphocytotoxic and haemagglutinating antibodies against their donors. Four responder dogs consisted of two pairs: the fact that responsiveness occurred in two pairs of littermates and that comparable rejection patterns could be noted in many other pairs would be compatible with responder status being genetically determined. Accelerated rejection occurred in the responder group, with early severe vasculitis, thrombosis and tissue destruction, whereas in the nonresponders the vasculitis was in all cases much less marked and the cellular infiltrate more prominent. No hyperacute rejection was seen. The mean survival times (±1SD) were compared with nonpretreated recipients of renal allografts reported by Westbroek et al. (1972) from the same laboratory - Table 4.5.

TABLE 4.5 - Mean survival time in days of littermate beagles with and without donor blood.

	Untreated	Transfused
Group 1 (2-haplo diff.):	15.6 (± 7.8)	18.9 (± 10.8)
Group 2 (1-haplo diff.) :	23.8 (± 13.8)	20.6 (± 20.0)
Group 3 (DLA-identical) :	43.0 (± 12.5)	(8,12,17,20,31,62,>300, >300)

The slightly longer mean survival time (MST) of transfused recipients (18.9) compared to controls (15.6) in group 1 was not significant. In group 3, two out of eight transfused dogs survived longer than 350 days without any sign of rejection. Dogs with lymphocytotoxic antibodies were present in each group (1, 2, and 2 respectively), the latter pair being DLA identical. Accelerated rejection occurred in this responder group (survival times 3, 7, 8, 8, and 12 days), although hyperacute rejection was not seen. The non-responders did not show any accelerated rejection. In the majority of dogs, antibody production was not detected following a single transfusion, nor was there any deleterious effect of blood transfusion on graft survival. In contrast, Bijnen (1985) showed that three DST improved renal allograft survival in one haplotype different beagle littermates, when Aza and Pred were given with the renal allograft. Despite three of the nine transfused recipients having a positive CDC test, all nine functioned for at least 100 days. Aza and Pred were given for 65 days, thereafter the immunosuppression was tapered off over 50 days.

Iwaki (1983b) gave DST to mongrels with and without Aza followed by a renal allograft with Aza and Pred. Of 28 mongrels receiving from 1 to 3 DST, 21% became crossmatch positive, and renal allografts to these dogs failed within10 days. Once again the grafts were not rejected hyperacutely. The success of grafting to the remaining 79% of dogs with negative crossmatches was higher than could be explained by the removal of the positive crossmatches. That is to say, the 25% graft survival for more than 30 days in control dogs was improved to 55% by DST. The best results were obtained by combining Aza with DST, none of the dogs became crossmatch positive and 78% of grafts survived more than 30 days.

Iwaki (1983b) also performed crossmatches on several sera from transfused dogs, and noted that some became crossmatch negative by the day of transplantation. Some of these functioned for more than 30 days, others less than 10 days.

Roper (1985) gave DST to mongrel dogs which were not DLA-typed. Following two 2ml transfusions given one week apart, 13 out of 20 dogs became CDC positive against donor T lymphocytes - Table 4.6. When the DST was covered by Aza the sensitisation rate was reduced to 10 out of 32. Dogs having positive T cell crossmatches were also positive against donor B cells. Renal allograft survival in nontransfused dogs receiving Aza and Pred was a mean of 19 days. DST significantly prolonged graft survival, 79% functioned for more than 30 days. The addition of Aza to the DST reduced graft survival, though it remained better than those who received no DST.

TABLE 4.6 - Efficacy of donor specific transfusion (DST) and the use of Aza in decreasing sensitisation to donor T-cells in dogs. All given Aza and Pred with renal allografts (Roper-1985).

n=	DST	Aza with DST	CDC test Positive/Total	Grafts surviving ≥30 days
11	none	none	0/11	1 (9%)
8	none	yes	0/8	2 (25%)
20	yes	none	13/20	11(79%) (a)
30	yes	yes	10/32	14 (47%)

(a) six dogs with positive CDC test not transplanted in early part of study.

A positive CDC test did not affect graft survival after DST alone, six of seven dogs given transplants with negative crossmatches survived more than 30 days, as did five of seven of those with positive crossmatches. In contrast, a positive crossmatch after DST with Aza had a deleterious effect upon graft survival, only two out of nine survived 30 days. It was the inclusion of the transplants with positive T cell crossmatches following DST with Aza that accounted for the difference in survival compared to DST without Aza. These results suggested that Aza altered some action of DST, which had resulted in prolonged graft survival despite the development of cytotoxic antibody following DST.

These findings contradict those of Obertop (1975) who observed accelerated rejection in littermate beagles sensitised by a single DST, without any immunosuppression, and Sutherland (1979) and Iwaki (1983b), who observed accelerated graft failure by DLA mismatched dogs with a positive CDC test after DST. Roper (1985) performed mixed lymphocyte cultures between donor-recipient pairs a few days before renal allografting, the results were not used for matching pairs. It was stated that well matched and poorly matched

pairs were evenly distributed in all groups, suggesting that chance matching was not the explanation of the curious results observed in the positive crossmatch recipients.

In summary, DS antigen only improves renal allograft survival in mongrels and one haplotype littermates when Aza is given. Allosensitisation following DST usually leads to accelerated graft failure. Covering DST with Aza reduces allosensitisation, but also reduces the beneficial effect of DST on survival of subsequent renal allografts.

Abouna (1977) gave three TPT to mongrels prior to renal allografting without immunosuppression. Graft survival was slightly prolonged in the transfused dogs - 7±2.2 days vs 4.4±2.2. Fabre (1978) gave mongrels from three to 10 third party blood transfusions; 12 of 18 dogs developed lymphocytotoxicity against either the blood donors or a random panel of six to eight unrelated dogs. In most cases once the antibody appeared it was sustained, though it was not stated for how long. All 18 of these dogs were given a renal allograft from the random donor panel. In five instances transplants were performed across a positive crossmatch because a crossmatch negative donor could not be found. One of these dogs died from haemorrhage, and in the other four there was no function. There were no overt signs of hyperacute rejection at operation and the 30 minute biopsies were normal, but the serum creatinine was greater than 700μmol/l by day 2, and all were dead by day three. In three instances the kidney was totally infarcted, being small and pale with thrombus in the renal artery in two instances and slightly enlarged and cyanotic with thrombi in both renal vessels in the other case. In the fourth dog the kidney was macroscopically unremarkable with patent renal vessels, but histologically it showed extensive fibrin plugging of the glomeruli. Thus only one of the four kidneys transplanted across a positive crossmatch showed histological changes that could definitely be described as antibody mediated. The other three showed infarction with major vessel thrombosis. Whether this pathology was antibody mediated or due to coincidental technical problems was uncertain. It may have been that the pathology of hyperacute rejection in this model was severe vasospasm, with consequent stasis and major vessel thrombosis. Where a negative crossmatch was available, the trend of results was that the transfused dogs had better graft survival than nontransfused dogs similarly treated with Aza and Pred.

Bull (1978) gave TPT of 100ml to tissue typed beagles from a pool of seven mongrel dogs. After transfusion antibodies were found in all dogs, the specificities corresponding with those present on the cells of the transfusion donors. Renal allografts came from littermate donors, without immunosuppression. A positive crossmatch was found once in these experiments, and this graft survived for less than five days. An immune reactivity score for the recipients was derived from the results of lymphocytotoxicity screening against the blood donors, the family members of the recipient (including the kidney donor) and a panel of 20 unrelated dogs. Survival of DLA identical grafts was decreased in some and prolonged in other recipients. There was a correlation between prolonged survival and higher immune

reactivity scores with DLA identical grafts. There was no correlation between survival of mismatched DLA littermate grafts and immune reactivity scores. Overall no beneficial effect of TPT was seen in any of the DLA matching categories, this was in accordance with other work where no immunosuppression was given.

Obertop (1978) transfused pairs of littermate beagles, eight DLA-identical and two 1-haplotype different pairs with three, weekly 100 ml TPT from 7 non-related tissue-typed mongrel donors. Two weeks after the last transfusion both kidneys from one of a group of ten separate mongrel dogs were grafted into each pair of beagles (one transfused, one nontransfused). Aza (2mg/kg body weight) and Pred (1mg/kg body weight) were given daily intravenously. Sera from the recipient beagles were tested against lymphocytes from the kidney donor, blood donors, and a panel of 18 dogs of various breeds. An immune reactivity score against this panel was computed and expressed as the percentage of the maximal obtainable score. After three blood transfusions, 9 of 10 renal allografts survived longer than the comparable allografts transplanted from the same donor into nontransfused dogs. Mongrel dogs receiving kidneys from nontransfused beagles showed no difference in graft survival as compared with nontransfused beagles receiving mongrel kidneys. Random dog kidney grafts from DLA-mismatched donors without immunosuppression survived for significantly shorter periods than any of the above groups. Allogeneic immunisation and pregnancy could be excluded in all beagles used in this study, and no lymphocytotoxic antibodies were detected prior to blood transfusion. All beagles produced antibodies after blood transfusions, but no antibodies were found against prospective kidney donor lymphocytes (i.e. all crossmatch tests were negative).

Bijnen (1982) gave three TPT of 100ml from different DLA mismatched beagle blood donors. All recipients received Aza and Pred after renal allografting. Four groups of beagles received pretransplant blood transfusion:

(1) Littermates-two haplotype difference. n=5.
(2) Littermates-one haplotype difference. n=10.
(3) DLA identical littermates. n=10.
(4) DLA identical non-littermates. n=9.

For each experimental group, a group of nontransfused dogs was used as controls. A beneficial effect of third party transfusion was only found in donor-recipient pairs mismatched for two haplotypes. The littermate and non-littermate DLA identical nontransfused pairs had 100% graft survival with conventional immunosuppression, and survival was slightly reduced with third party transfusions. Immunosuppression was gradually withdrawn after 100 days, thereafter the DLA-identical kidneys in transfused recipients were more frequently rejected than the kidneys in the nontransfused controls.

Of the 19 DLA identical pairs only one developed alloantibody as shown by a positive CDC test

with the prospective donor, though this graft functioned for 170 days. Of the one haplotype different littermates 6 out of ten became CDC positive following the TPT, and 2 out of 5 two haplotype different littermates became CDC positive. The time to graft failure from rejection or continuing graft function are shown in Table 4.7.

TABLE 4.7 - CDC crossmatch result and graft survival following third party beagle blood transfusion. One- and two-haplotype different littermates given Aza and Pred. (Bijnen-1980,1982).

	Positive CDC	Negative CDC
Transfused (n=15)	9,13,41,54,123, 162,>305, >501.	9,33,167,168,>305, >525,>532.
Non-transfused (n=15)		9,12,12,12,12,13,16,16,16,17, 50,121,168,>182,>367.

The benefit of blood transfusion was greater for two haplotype different littermates ($p<0.05$) than for one haplotype different littermates ($p=0.34$). Of particular interest was the lack of an adverse effect of a positive CDC test on graft outcome. Thus in this dog model of Bijnen, the effect of TPT was dependent on the degree of DLA matching. TPT were beneficial in related and unrelated mismatched combinations, but, after discontinuation of immunosuppression, harmful in related and unrelated DLA matched combinations.

These studies showed a beneficial effect of TPT on DLA-different canine renal allografts, mongrels and littermates, when recipients were treated postoperatively with immunosuppression (Aza and Pred). The occurrence of chance histocompatibility in the transfused group of Obertop (1978) was excluded by using DLA tissue-typed sibling pairs as transfused and nontransfused recipients of kidneys from the same donors. In transfused dogs a high immune reactivity score before transplantation was associated with reduced graft survival. However, no accelerated rejection was observed (shortest survival 21 days) by Obertop, and even dogs that rejected their kidney did so later than their nontransfused littermates. Positive CDC tests following TPT generally resulted in accelerated rejection, though Bijnen (1982), who exchanged renal allografts between beagle littermates after TPT, did not observe accelerated rejection.

Van der Linden (1982) showed a similar beneficial effect with a peroperative transfusion of 100ml of third party blood on renal allograft survival in unmatched mongrels. All dogs received post operative immunosuppression with Aza and Pred. The mean survival time in controls was 12.6 days verses 21.0 days for transfused dogs. When the transfusion was given 14 days before grafting no beneficial effect was seen unless immunosuppression was also started 14 days before grafting. Commencing immunosuppression alone 14 days before

grafting made no difference to graft survival. Thus in unmatched pairs a prolonged survival after a single third party transfusion given preoperatively or peroperatively with a renal allograft has only been observed after immunosuppression of the recipient with Aza and Pred.

Niessen (1982) gave three TPT to beagles followed by a mongrel renal allograft. Immunosuppression was either omitted, or consisted of Aza and Pred, or CyA , or Aza, Pred and CyA together. Positive crossmatches were apparently ignored, though their incidence was not stated. There was no accelerated rejection from the stated graft survival times. Pretransplant third party blood transfusions did not enhance the immunosuppressive effect of CyA given with renal allografting, whereas an additive beneficial effect of transfusion and immunosuppression was observed with Aza and Pred. Niessen (1981) observed no beneficial effect of third party transfusions using different doses of CyA (2, 5 and 10mg/kg/day).

Errett (1985) gave mongrel dogs three TPT of 50ml from the same donor. With renal allografting Aza and Pred were given. Three out of five recipients became crossmatch positive with their transfusion donor and one crossmatch positive with their transplant donor. This dog had accelerated rejection and succumbed at day five. All other renal transplant crossmatches were negative: no difference in graft survival was noted between those with and those without alloantibody. There was a trend towards better survival in the transfused dogs with negative crossmatches.

Bijnen (1984) compared the effect of third party thrombocyte and blood transfusions on renal allograft survival, the blood and kidney donors were unrelated mongrels and the recipients beagles. Aza and Pred were used. Three transfusions were given at weekly intervals. It was stated that the same number of lymphocytoxic antibodies were found in beagles whether they had received whole blood or platelets. Crossmatch tests were performed in relation to renal allografting, but the results were ignored. Remarkably the positive crossmatches were not associated with accelerated rejection, survival times of 28-61 days were observed, though these times were shorter than the negative crossmatch recipients.

Marquet (1987) found that splenectomy performed after the administration of third party blood transfusions could abrogate the transfusion effect and that adoptive transfer of spleen cells from transfused dogs to DLA-identical littermates also failed to improve graft survival in dogs receiving Aza and Pred. This would seem to indicate that the transfusion effect was not due to the induction of suppressor cells arising from the spleen.

Esquenazi (1986) gave five TPT to five pairs of DLA identical littermate donors who differed from their prospective littermate donors by one DLA haplotype and were MLC reactive. One member of each DLA-identical recipient pair was given CyA 15mg/kg daily intramuscularly during the transfusion period. Four weeks later renal allografting was performed, without

immunosuppression. Three CyA treated recipients with high daily mean CyA levels (954 ng/ml ±SE 12.3 by RIA) did not develop alloantibody against their respective donors, and had a graft survival that was 7 to 14 days longer than recipients transfused without CyA. Two recipients with lower CyA levels (605 ng/ml ±SE 34.1) developed a positive crossmatch preoperatively, one survived five days longer than its control and the other, two days less than its control. Unfortunately the actual survival times in days were not quoted in the paper. Four of the five recipients transfused without CyA developed a positive crossmatch.

Gores (1987) attempted to render mongrels tolerogenic to a renal allograft by prior administration of class I MHC complex antigen as platelets. The level of leucocyte contamination was reduced to one per $3x10^5$ platelets by incubation with B1F6 (murine monoclonal antibody to dog class 2 products) and rabbit complement. No immunosuppression was used and graft survival was reduced in the platelet treated dogs. Thus, unlike in rats, the administration of donor class I MHC antigen failed to prolong renal allograft survival in the dog.

In general, TPT improve renal allograft survival when Aza is given with the renal allograft. The immunosuppressive effect of CyA is not enhanced by TPT. Renal allografts with positive crossmatches following TPT are usually associated with accelerated graft failure.

4.4 Renal allografting and immunosuppression.

4.4.1 Unmodified renal allografting in the dog.

The gross and histological changes in the dog kidney after allografting are well established, having been recorded by Simonsen (1953) and Dempster (1953). A primary renal allograft survives on average 5-7 days, the kidney swells and urine production ceases at some stage in the rejection process. Histologically there is a mononuclear cell infiltration commencing around the small vessels and glomeruli, which gradually spreads through the cortex. Typically there is not much evidence of cellular infiltration until the second or third day. Later there develops thickening of the endothelium of the small blood vessels and oedema, later still small thrombi, tubular necrosis, focal areas of interstitial oedema and haemorrhage.

It is extremely rare for renal allografts to survive in untreated normal dogs for longer than 20 days, though exceptions have been reported. Jeejeebhoy (1965) saw one dog survive 30 days and Koo (1966) one for 123 days. These longer survivals may have been due to chance DLA compatibility. Zukoski (1960) reported survival of 1,177 days in a bilaterally nephretomised dog with a renal allograft given Pred 30 mg daily for the first 428 days.

4.4.2 Azathioprine and renal allografting in the dog.

Calne (1960) discovered that the survival of dog renal allografts could be prolonged with 6-mercaptopurine. The efficacy of 6-mercaptopurine was confirmed by Zukoski (1960). Diethelm (1968) compared Aza alone with Aza and Pred for renal allografts and observed an improved graft survival with the combined therapy. The addition of postoperative irradiation to the graft was not helpful. Porter (1964) reviewed the histopathology of Aza treated dog renal allografts. In contrast with the unmodified renal allograft where massive celiullar infiltration and tubular necrosis dominated, vascular lesions predominated in the rejection of Aza treated grafts. Of the grafts which functioned beyond 40 days, 83% developed intimal thickening in the interlobular arteries of the graft.

The results of renal allografting in dogs have rarely been studied long term and a recent study has examined this aspect and the application of renal allografting to clinical veterinary practice. Finco (1985) examined allograft survival in transfused and nontransfused sibling beagles. Four donor specific transfusions of 100ml whole blood were given. The DLA relationships of the sibling pairs were not determined, but where possible MLC results were used to pair dogs with the highest response so as to avoid DLA identical matches. Immunosuppression began the day before grafting and consisted of Aza and Pred. Graft rejection was diagnosed by deterioration in function, and treated with dexamethasone (1.5 to

3mg/kg iv daily) for two or three days. The grafts were not biopsied because of the fear of trauma. Dogs that survived a year had the glomerular filtration rate determined, then they were killed. Four of the six transfused dogs were healthy at a year, one dying at day 44 from a renal vein thrombosis and one at day 163 from septicaemia with mild allograft rejection. Of the six controls, three were well at a year, and three lost to rejection at days 32, 45 and 53. Allograft rejection was successfully treated in three dogs. The problems encountered were leucopenia (7), renal vein thrombosis (1), interdigital abscesses (2), chronic cough (2), urinary infection, intestinal intussusception (1) and septicaemia (1). One kidney was transplanted despite incompatible red cell antigens without adverse affect on graft survival at a year. The death of one dog from infection and the occurrence of skin and urinary tract infections reflected the use of immunosuppression. Crowell (1987) observed that the major cause of death of dogs after transplantation was allograft rejection followed by complications resulting from immunosuppressive therapy. With primary renal allografts, acute rejection occurred with, either a predominantly cellular or, a predominantly humoral morphologic pattern, the former being more common. Acute cellular rejection was characterised by widening of the interstitium with oedema and cellular infiltrates. The hallmark of the humoral form was fibrinoid necrosis of the walls of large vessels. Thrombi (primarily fibrin) were in glomerular and interstitial vessels, and concomitant infarction and tubular necrosis were frequently present.

4.4.3 Cyclosporin A and renal allografting in the dog.

The pharmacokinetics of CyA in the dog were reviewed by Ryffel (1982). Absorption of CyA given orally in olive oil occurred rapidly, serum peak levels of 1000ng/ml were measured by radioimmunoassay (RIA). Repeated doses of CyA (5,15, and 45 mg/kg for 52 weeks) did not lead to accumulation, as evidenced by serum concentrations similar to those obtained after a single dose. Using CyA in either oil (olive oil or corn seed oil) or in liposomes gave similar absorption, 50% of the dose being absorbed. Gridelli (1986) examined CyA metabolism after intravenous and oral administration and found evidence of significant metabolism by the gastointestinal tract with oral administration. Studies with tritiated CyA have showed extensive accumulation in skin, fat, liver and kidneys between 4 and 24 hours, the concentration exceeding serum levels by a factor of 3-14. Buice (1985) determined that less than 1% of oral CyA was recovered as parent CyA in the urine, suggesting that renal clearance of CyA was negligible.

In toxicity studies beagles were given oral doses of 20, 60, and 200 mg/kg/day for 13 weeks with little evidence of toxicity. With the higher doses there was decreased weight gain, an increase in liver SGPT (serum glutamic pyruvic transaminase), and lymphopenia. In dogs given 45mg/kg/day for a year there was no hepato- or nephrotoxicity. This is an important difference compared with man.

Renal allografting between unsensitised mongrels using CyA has been studied by several groups. White (1982) showed prolonged survival of mongrel renal allografts with CyA at 25mg/kg/day. Du Toit (1982) observed a sharp dose -response curve, higher doses being more effective. All but one dog on 10mg/kg/day rejected their graft within 36 days, while on a dose of 20mg/kg/day four out of nine dogs still had not rejected by day 100 when the drug was stopped. Dogs in which the CyA therapy was stopped at days 14, 21, or 100 days inevitably rejected their kidneys one to three weeks later, unlike the ability of CyA to induce allograft unresponsiveness when used alone in rodents. There was no evidence of nephrotoxicity in these animals. Du Toit (1982) found that biopsies at day 7 in dogs given CyA in a dose of 10 or 20mg/kg/day showed none of the features of rejection seen at lower doses, except for the persistence of a moderate interstitial infiltrate. Homan (1981) observed that Pred in a low dose when combined with CyA at a suboptimal dose (10mg/kg/day) produced no increased morbidity and no improvement in graft survival. Short-term administration of CyA for 21 days with conversion to conventional Aza and Pred resulted in graft failure at about the time expected for dogs given Aza and Pred alone. Administration of CyA for a longer period of three months with conversion to Aza and Pred produced prolonged survival in the two dogs treated.

CyA has been used with variable success to try and reduce the immunogenicity of a dog kidney prior to transplantation. Toledo-Pereyra (1982) obtained prolonged survival of mongrel allografts that were flushed pretransplant with a solution containing CyA. To observe the effect it was necessary to give Aza after transplantation, and the results were improved when the temperature of the flushing solution was increased from 4 to 25 deg. C. Jeng (1986) repeated these experiments in mongrels and failed to prolong graft survival.

Kelly (1986) compared the abilities of Pred and ATG (anti-thymocyte globulin) at reversing acute rejection in CyA treated (10mg/kg/day) mongrel renal allografts. Acute rejection was diagnosed by a rise in serum creatinine of at least 30μmol/l on each of two consecutive days accompanied by clinical or pathological signs of rejection. Clinical signs were graft swelling, fever and oliguria. Compared to ATG, Pred was more successful in the reversal of primary rejection episodes and produced a quicker return to normal renal function. Therapy with ATG however, resulted in fewer subsequent rejection episodes than Pred therapy. Homan (1980b) showed that CyA given orally in a single dose of 20mg/kg/day from the fourth post-operative day after renal allografting to mongrels delayed rejection in four of six dogs.

Cavallini (1986) has examined the effect of dosage timing on acute renal allograft rejection in mongrels. Dogs were kept in cages in 12 hours light and dark with light-on at 0600 hours. A single daily dose of CyA 12.5mg/kg was given orally either at 0830hours or at 2030 hours. Circadian timing of CyA was shown to delay graft rejection, the mean±SE graft survival times were 38±7.1 days for the treatment at 2030 hours and 15±5.2 days for the

treatment at 0830 hours. The dogs had free access to food and drink which might have affected absorption, but there was no significant difference in the mean trough levels for the two regimens and the area under the curve of CyA profiles. Previously the same group had showed an improved graft survival when the CyA was infused intravenously and peaked during the dark span. These interesting results await confirmation.

Aeder (1983) showed that a combination of CyA (5-10mg/kg/day) and Aza (1.5-2.5mg/kg/day) was synergistic as an immunosuppressive regimen in renal allografts to splenectomised outbred beagles. Splenectomy was done to reduce the neutropenia observed with Aza. Trough whole blood CyA levels by high pressure liquid chromatography were only obtained in ten survivors beyond 200 days. The range of the values was between 110 and 1371 ng/ml with seven of ten between 110 and 345 ng/ml.

Derivatives of CyA have been examined in the search for a less nephrotoxic alternative. Calne (1985) showed that Cyclosporin G (CyG) was at least as immunosuppressive as CyA in dogs with renal allografts. Absorption of CyG following oral dosage appeared to be superior to CyA as judged by the blood levels.

4.4.4 Other modes of immunosuppression.

The pretreatment of donors so as to either reduce graft antigenicity or reduce the immune reactivity of passenger leucocytes has been investigated. Van der Linden (1980) showed that donor pre-treatment with procarbazine hydrochloride and methylprednisolone significantly prolonged renal allograft survival in dogs immunosuppressed with Aza (12.6 verses 26.8 days) Brom (1983) treated mongrel kidney donors five hours before harvesting with cyclophosphamide and Pred. The survival of pretreated kidneys was significantly prolonged in beagle recipients not given immunosuppression (mean survival in days-8.3±2.1 verses 17.0±3.1).

Munda (1982) investigated the effects of Corynebacterium parvum vaccine given in conjunction with Aza and Pred in renal allografts between non-related beagles. The C. parvum vaccine is able to prolong skin allograft survival in mice when given prior to transplantation. There was no effect of C. parvum in beagles given renal allografts without immunosuppression. In immunosuppressed beagles C. parvum vaccine significantly shortened renal allograft survival. Killed suspensions of C. parvum have been shown to stimulate reticuloendothelial system activity and antibody responses while depressing T cell mediated responses. The observation that C. parvum vaccine prolongs skin allograft survival in normal mice but shortens allograft survival in immunosuppressed beagles suggests that macrophage mediated mechanisms become more important and T ceil mediated mechanisms less important during Aza and steroid induced immunosuppression.

Yamamoto (1983) showed that the survival times of mongrel renal allografts were prolonged by perfusion of the donor kidney with a monoclonal antimouse-Ia antibody-HAK-75. The mean survival time of controls was 7.5 days and for the HAK-75 group 15.3 days. HAK-75 is an IgG_{2b} reactive with most of the Ia molecules in mice.

In experimental models total lymphoid irradiation (TLI) has been used either as a preparatory treatment for allogeneic bone marrow transplantation in an attempt to induce tolerance to the bone marrow donor, or combined with pharmacologic immunosuppression. Sunderland (1983b) pointed out that TLI is somewhat of a misnomer, because not all lymphoid tissue is irradiated, and the radiation fields always include at least some extra lymphatic tissue. The areas of the body that are irradiated and the precise regions that are shielded during TLI have also varied from animal model to animal model and investigator to investigator, and the effect of TLI may vary depending on the extent of shielding. Gottlieb (1980) has reported engraftment of allogeneic bone marrow without graft-verses-host disease and with chimerism in mongrel dogs using TLI. Howard (1981) prolonged renal allograft survival in unrelated DLA different beagles with TLI, though TLI alone was not sufficient to prevent rejection indefinitely. Howard gave eight dogs bone marrow from the kidney donor, and in two dogs observed long term (>500 days) survival without immunosuppression. In the one dog tested chimerism was not detected. Raff (1981) found that engraftment of bone marrow was difficult to achieve in dogs treated with TLI alone (two out of 14), and there was evidence of graft-verses-host disease in one of the two chimeras. Thus TLI with or without bone marrow has had little success in inducing tolerance to renal allografts.

In a dog renal allograft model Lewis (1981) gave Aza and TLI. Graft survival times with Aza ranged from 5 to 48 days, with TLI from 8 to 21 days, and with the combination from 40 to 226 days, but 70% of the animals in the latter group died with functioning grafts from Aza induced leucopenia. Though TLI was immunosuppressive, the therapeutic ratio was narrow in this dog model with Aza. Miura (1987) used irradiation to induce long term unresponsiveness to renal allografts between DLA identical beagles. It was not clear whether or not these were littermates. Normal recipients were given DLA-identical renal allografts from donors previously exposed (130-190 days before) to supralethal total body irradiation (SLTBI) followed by reconstitution of the donors with bone marrow from the prospective recipient. Renal allograft survival was prolonged to 55-60 days compared to 13-38 days in recipients of unmodified donor kidneys. It was hypothesised that the treatment by SLTBi with bone marrow reconstitution with long term chimerism was depleting the renal allograft of passenger dendritic cells (DC). Additional conditioning of the recipients with total lymphoid irradiation (TLI) prior to transplantation resulted in long term (>300 days) unresponsiveness of the recipients to such allogeneic DC-depleted renal allografts. No other immunosuppression was given to the recipients after renal allografting.

Supralethal TLI and reconstitution with autologous bone marrow can produce a limited period of altered immunological reactivity during which exposure to alloantigen results in specific unresponsiveness. Rapaport (1978) has treated beagles with supralethal TLI and reconstitution with their own stored autologous bone marrow. Renal allografting from DLA-identical littermates or beagles with DLA identical haplotypes from the same pedigree origin resulted in long term survival in 27 of 44 dogs for 467 to 1,657 days without any further immunosuppression. The fact that only 61% of the recipients developed unresponsiveness led Bachvaroff (1981) to use CyA to improve the response. Interestingly the addition of CyA fully abrogated the capacity of this model to induce allogeneic unresponsiveness in DLA identical dogs.

The mechanism by which donor specific antigen can prolong graft survival is unclear, though this approach has been used in the dog renal allograft model. Rowinski (1981) treated mongrels with horse anti-dog lymphocyte globulin three days prior and for seven days after transplantation with a significant effect on renal allograft survival (9.3 verses 16.2 days). The intravenous administration of platelets eight days before renal allografting made no difference (11 days), but platelets given 11 days after grafting significantly prolonged survival (55.4 days). Hartner (1985) found that treatment of outbred histoincompatible (confirmed by MLC) dogs with rabbit antidog lymphocyte serum and a low density fraction of donor bone marrow was effective in prolonging renal allograft survival. No other immunosuppression was given. Post-transplant MLR's in two long surviving dogs indicated a state of donor specific immunosuppression, the mechanism of which is unknown. Calne (1966) observed no benefit of donor antigen on dog renal allografts using either cellular antigen (whole blood, spleen cells) or semi-soluble antigen prepared from donor spleen.

4.5. The allosensitised recipient.

Dempster (1953) and Simonsen (1953) both observed accelerated rejection of a second allograft from the same donor. Dempster (1953) observed a difference in the histological features between the first kidney (marked cellular infiltrate) and the second kidney (oedema, haemorrhage and virtually no infiltrate).

Altman (1963) sensitised mongrel dogs with donor specific skin grafts and followed these with a renal allograft. Subsequently these dogs were sacrificed and the plasma infused into a dog receiving the second kidney from the original donor. Altman observed four degrees of intensity of the allograft reaction:

(1) Primary allograft reaction with mononuclear infiltrate from day two to three with later vascular and tubular damage, the process taking on average 5-7 days.

(2) An accelerated reaction of the cellular type which occurred after prior sensitisation of the recipient with tissue or whole blood from the kidney donor. The histological picture in less than 24 hours showed a degree of cellular infiltration comparable with that seen in a 3-4 day primary allograft.

(3) A haemorrhagic response with interstitial haemorrhage and oedema, tubular necrosis and infiltration with polymorphs rather than with mononuclear cells. Cessation of function occurring within 12-48 hours, during which the kidney became swollen and grossly congested with blood.

(4) An immediate reaction occurring within 5-20 minutes of transplantation, in which the kidney became increasingly cyanosed, flabby, and urine flow ceased.

Category four would now be called hyperacute rejection, which is primarily antibody mediated. Categories two and three would be called accelerated rejection where both cellular and antibody effector mechanisms participate in the rejection process (Porter 1983). Altman observed a variable outcome with donor specific renal allografts to mongrels sensitised with skin grafts: four had accelerated rejection (<24 hours) of the renal allograft, one hyperacute rejection (20 mins) and two a primary type of response. Hyperacute rejection was seen in three of six dogs perfused with plasma from the sensitised recipient, prior to receiving the second kidney from the sensitising donor.

Altman and Simonsen (1964) demonstrated cytotoxic antibodies against donor leucocytes in the serum of dogs which had received allogeneic transplants of skin and kidney. Almgard (1968) demonstrated cytotoxic antibody against donor marrow cells and kidney cells after renal allografting.

Simpson (1970) sensitised pairs of adult mongrels with repeated skin grafts, 28 of 34 dogs developed lymphocytotoxic antibody against donor lymphocytes. The percentage kill of lymphocytes ranged from 50% to 75%. Attempts were made to deplete the antibodies by perfusing isolated donor organs, namely liver, spleen, and kidneys. Though anti-donor leucoagglutinins fell, there was only a modest fall in lymphocytotoxicity as judged by the CDC test. Indeed the percentage kill of donor lymphocytes did not fall below 50%. Following renal allografting the time to anuria was the rejection time. In five control dogs urine was produced for 24 hours, and hyperacute rejection was not observed. Prior depletion of antidonor antibody by perfusing another donor organ prior to the renal allograft prolonged the production of urine from 50 to 140 hours in four dogs. Once again hyperacute rejection was not consistently observed in an allosensitised dog model, but of interest was the prolongation of function following prior antibody depletion by isolated organ perfusion, despite the continuing presence of lymphocytotoxic antibody.

Lowenhaupt (1969) sensitised dogs with skin grafts prior to renal allografting. Platelet sequestration was observed in the graft but not hyperacute rejection. Pineo (1970) skin

grafted greyhounds, followed later by donor specific renal allografts. Rejection was defined by anuria at 24 hours, and 5 of 21 grafts continued to produce urine beyond 24 hours. The time of cessation of urine production during the first 24 hours was not stated, thus the proportion of grafts that were hyperacutely rejected cannot be determined.

Macdonald (1970) examined heparin and aspirin in the treatment of hyperacute rejection of renal allografts in an allosensitised dog model. Dogs were sensitised by exchanging skin grafts between pairs. Onset of anuria was observed in 3 to 5 hours in four controls, 4 to 36 hours in four kidneys from donors pretreated with aspirin, and 2 to 44 hours in four recipients given Aza 5mg/kg/day from 24 hours before grafting. Five dogs were heparinised, four continued to produce urine at 96 hours when allograft nehprectomy was performed. A further five recipients were given aspirin 600mg daily, three were functioning at 72 hours. The recipients all had one native kidney, but surprisingly 9 of the 12 dogs in the two control groups and the Aza group died within five days of renal allografting. There was no adequate explanation for this. Morphologically the kidneys in the first three groups showed extensive cortical necrosis at 3-4 days. The cortical changes in aspirin treated recipients were attenuated. In the heparin treated dogs cortical necrosis was not present at day 4, but there was a mononuclear infiltrate typical of first set rejection. Thus some protection was offered by heparin against the onset of the features of vascular rejection in this allosensitised renal allograft model. The benefit of aspirin was minimal, and there was no benefit from Aza.

Claes (1972) sensitised mongrel dogs with a single intraperitoneal injection of homogenised spleen together with a skin graft. When the skin graft was rejected after 6 to 10 days, a second skin graft from the same donor was placed. The second graft was rejected within three days and the recipient was considered sensitised. In 18 sensitised dogs given a renal allograft rejection occurred within 24 hours in 14, two rejected after 48 hours, and two had no clinical signs of rejection at 48 hours. Rejection was considered to have occurred when renal blood flow was less than 100ml/min/100g tissue and urine output had ceased. The clinical diagnosis was confirmed histologically. One of the 18 dogs rejected hyperacutely within 45 minutes of revascularisation.

Matthew (1971) sensitised 18 mongrels with four donor specific buffy coat transfusions. Only two developed lymphocytotoxic antibodies and neither of these underwent hyperacute rejection.

Abouna (1977) immunised mongrels with four 150ml blood transfusions pooled from 20 mongrels. Lymphocytotoxic antibody was observed in one out of 24 (4%). In contrast, of the mongrels immunised with four skin grafts and subcutaneous injections of leucocytes pooled from the skin donors, 17 out of 23 (74%) became CDC positive. Aza and Pred were given with renal allografts. The time of onset of graft rejection (serum creatinine >200umol/l) was the experimental endpoint, which was 7 ±2.2 days in the transfused dogs, 3.3 ±0.7 days

in the skin grafted dogs and 4.4 ±2 days in recipients given no antigen pretreatment. Thus third party whole blood did not necessarily produce cytotoxic antibody, whereas immunisation by the subcutaneous route combined with skin grafting usually did. Hyperacute rejection was not seen, but the grafts in the skin grafted group were rejected in an accelerated manner.

Drannik (1979) induced antirenal antibodies in dogs by injecting basal membranes from dog renal glomeruli. No lymphocytotoxic antibodies were demonstrated. Binding of antibody was observed to renal allografts in the capillary loops of glomeruli and the walls of larger vessels. Though these observations may provide evidence for hyperacute rejection being antibody mediated, hyperacute rejection was not observed in these experiments.

Robertshaw (1969) described a curious model of hyperacute rejection of dog renal autografts. A harvested kidney was perfused ex vivo in a sterile chamber using an acellular perfusate. The temperature was not stated, but as urine production was observed the perfusion chamber was presumably at 37 deg C. Following stabilisation, serum from a dog immunised by previous grafting of the contralateral kidney from the same donor, was added to the perfusate. Perfusion was continued for 30 more minutes, during which time urine production continued, before the kidney was autografted back to its original donor. In seven experiments the kidney underwent hyperacute rejection within 15 minutes. Microscopically there was no significant mononuclear infiltrate, though there were neutrophil leucocytes infiltrating the glomerular tufts and the perivascular areas. Apparently a kidney coated with antiserum was no longer recognised by the original donor and was rejected hyperacutely. In another experiment, two kidneys were simultaneously perfused with immune serum directed specifically against one of the pair. When the kidneys were reimplanted into their respective donors, both showed hyperacute rejection. The hyperacute rejection of the kidney perfused with the immune serum not directed against the donor could be explained by the donors sharing antigens. It would have been interesting to know the outcome of a second donor specific renal allograft given to the dog previously sensitised with the other kidney of the pair used to generate the immune serum, as the consistent finding of hyperacute rejection is not in accord with other dog work.

Rapaport (1987a, b) investigated the use of TLI for conditioning allosensitised dogs. Neither TLI nor CyA has the capacity to abrogate secondary immune responses alone. Zan-Bar (1978) demonstrated in mice that TLI could abrogate the primary humoral response to sheep red blood cells, but not the secondary response. Klaus (1983) observed in mice that the function of primed Th cells (helper T cell) was resistant to CyA. Lindsey (1980) showed in rabbits that CyA had little effect on the secondary humoral response to human serum albumin, and Gratwohl (1981) showed that CyA failed to prevent the rejection of skin grafts by pre-sensitised rabbits. Kemeny (1987) showed that CyA prevented the accelerated rejection of murine skin allografts by adoptive transfer of specifically sensitised spleen

cells. The spleen cells were sensitised in vitro by MLC. CyA could not prevent the rejection of skin allografts when the adoptively transferred cells were sensitised in the presence of interleukin 2. Deeg (1980) showed in dogs that CyA (30mg/kg/day) for 21 days was able to prolong the survival of skin grafts undergoing second set rejection and that rejection crises were reversed by additional CyA courses. This surprising finding could have been related to the use of only one immunising skin graft and giving CyA for 21 days with this first skin graft. The effect of CyA alone on renal allografts to allosensitised dogs has not been previously reported.

Rapaport used two different protocols of triple therapy for renal allografts to allosensitised dogs. Pairs of beagles differing by one DLA haplotype were selected and the prospective recipient sensitised with multiple skin grafts and subcutaneous injections of buffy coat cells. Six of these fourteen pairs were littermates. Anti-DLA specific antibody titres reached a maximum within one week of the second buffy-coat injection. Titres fell during the 90-160 days to renal allografting, reaching a low two days after renal allografting and peaking again around the time of graft failure. The crossmatches were all positive prior to renal allografting. Four allosensitised dogs received total lymphoid irradiation (TLI). The technique of Slavin (1979) was used for TLI. A total dose of 1760 ±35 cgy was given over 9 to 11 weeks, providing either 70, 75 or 100 cgy per day for three days each week. Following this, a course of rabbit anti-dog thymocyte globulin (RATG) was given over fifteen days. Some hundred days later the dogs received a renal allograft from a donor bearing the same DLA antigens as the sensitising donor without further immunosuppression. Five dogs received TLI as before, and then CyA 5mg/kg and methylprednisolone (MePred) 2.5mg/kg intravenously beginning 3 to 10 days before renal allografting. The recipients own kidneys were removed at the time of transplantation. Five unmodified allosensitised dogs rejected the graft in 2, 4, 5, 6, & 6.5 days with a vascular or humoral type of rejection process, including haemorrhage, fibrinoid necrosis and disruption of small vessels, intravascular leucocyte-fibrin deposits and renal cortical necrosis. In contrast, first set allografts under the same conditions of DLA-incompatibility had a mean survival time of 12.4 days. In the TLI-RATG group of allosensitised dogs renal allografts survived 7, 8, 14 & 17 days. The graft rejecting at 17 days showed predominantly cellular rejection while the grafts lost earlier showed features of humoral and cellular rejection. Survival in the TLI-CyA/MePred group was 7, 8, 20, 62 & 227 days. The three longer surviving grafts showed predominantly cellular rejection and the two early failures predominantly a vascular picture. Of the two regimens, TLI-CyA/Mepred and TLI-RATG, the former was more effective in delaying allograft rejection in this allosensitised model. The specific anti-DLA response (secondary humoral) was diminished by both protocols following renal allografting, again the TLI-CyA/MePred was more effective than TLI/ATG. The reduction of the alloantibody response may have been the mechanism by which allograft survival was prolonged, though prolonged survival was seen despite the presence of significant titres of circulating alloantibody (1/3 to 1/243). The use of TLI was time consuming though not reported to be

associated with morbidity in this series. Though the graft survival was prolonged by TLI, all bar one of the grafts was lost by day 62 despite a dose of CyA of 5mg/kg/day intravenously. CyA levels were not quoted. These regimens may only have postponed the increased risk of graft loss in allosensitised recipients. The results indicated that the combined use of TLI and CyA/MePred constituted a uniquely effective synergistic combination for attenuation of the state of allosensitisation produced with DLA-incompatibie skin grafts and buffy coat injections. This protocol also inhibited secondary humoral responses in these recipients. The apparent capacity of renal allografts to retain function in the face of significant levels of antibodies directed against the graft's DLA specificities is particularly intriguing.

4.6 The Leicester approach.

4.6.1 Allograft verses xenograft model.

It is evident from this review that the state of knowledge of canine immunogenetics is many years behind that in man, the rat, and the mouse. This relates to the relatively small number of laboratories working in the field, with the consequence that the majority of workers opt for dog studies in non DLA-typed animals.

The dog is an outbred model suitable because of its size and temperament for studies on allosensitisation and renal allografting. Despite the polymorphism of the dog DLA being less than that of the human HLA system, and the degree of linkage disequilibrium being greater the effects of DLA matching (littermate, unrelated beagles and mongrels), blood transfusion (third party and donor specific), and immunosuppression with Aza show remarkable parallels with clinical renal transplantation. Furthermore, the suspicion of a reduced effect on renal allograft survival of HLA matching (Hardy-1987) and third party blood transfusions (Opelz-1987, Groth-1987) with CyA in clinical practice is paralled by observations in the dog with third party blood transfusions and CyA (Niessen-1982). The relationship between CyA and DLA matching in unrelated dogs has not been examined.

Considerable progress has been made with the clinical problem of alloantibody by determining the specificity and class of the antibody and using these results to reinterpret "intelligently" the results of the cytotoxic crossmatch test. This has enabled many patients with alloantibody to be successfully transplanted. The dog literature is almost devoid of any mention of this approach to organ allografting. There has not been much interest in studying the natural history of alloantibody in the dog beyond the needs of DLA typing. There have been reports of attempted T and B cell crossmatching, though the crossmatches were positive on both. Clearly the distribution of class 2 like antigen in the dog seems to differ from humans. For the present this approach in the dog does not look promising.
Perhaps the dog is more suitable for studies that involve removing alloantibody prior to transplantation and those trying to ameliorate the consequences of antigen/antibody

interaction after transpiantation. Along these lines there have been attempts to abrogate the dog hyperacute xenograft rejection, though none has achieved any lasting success (more than one day). Though the xenograft rejection may be mediated by similar mechanisms to hyperacute allograft rejection there are important differences, for example the antibody participating in xenograft rejection is naturally occurring (Hammer-1973) and DLA matching is irrelevant. One of the more difficult parts of alloantibody studies is generating the alloantibody and using a xenograft model removes this step. A tantalising aspect of hyperacute rejection is its unpredictable occurrence, emphasising perhaps the importance of the target antigen. Thus the xenograft model can be used to evaluate protocols for ameliorating the effects of antigen/antibody interaction, but the effect is so strong and dramatic that it has thus far proved resistant to treatment - Table 4.8.

Table 4.8 - Summary of attempts to modulate the pig kidney to dog xenograft model

	Control Survival Mean±SD (mins)	Experimental Mean±SD (mins)	Reference
Conditioning dog recipient by removing "anti-pig" immunoglobulin.			
1. Organ perfusion prior to renal transplantation.			
Liver	9.8±1.1	147±204	Giles-1970
Liver	(5-8 mins)	(60-240)	Moberg-1971
Spleen	9.8±1.1	18.3±15.7	Giles-1970
Kidney	9.8±1.1	25.7±20.5	Giles-1970
2. Plasmapheresis.	(5-8 mins)	(45-180)	Moberg-1971
	10	(90-180)	Merkel-1971
3. Haemodilution.	15	815	Messmer-1971
Induction of tolerance by the administration of low dose soluble antigen.			
Liver	15	387.5	Messmer-1971
The depletion of complement.			
Oxidation by hypochlorite	(5-10 mins)	61±25	Bier-1971
Sodium citrate	(3-10 mins)	85	Kux-1971
Ethylenediaminetetra-acetate	(10-12)	73	Belitsky-1973
The inhibition of platelets.			
Rabbit anti-platelet serum	5.4±1.8	6.1±3	Slapak-1971
The depletion of leukocytes.			
Azathioprine	(10-12)	(<20 mins)	Perper-1966
The inhibition of coagulation.			
Heparin	5.4±1.8	9.4±6	Slapak-1971
Arvin	5.4±1.8	6.1±3	Slapak-1971
Arvin	(5-8 mins)	(7-12 mins)	Moberg-1971

Given these observations, it seemed desirable to use an allograft rather than a xenograft model to study antibody and renal transplantation. The first aim of this study was to study the generation of alloantibody in dogs, and subsequently to give renal allografts to allosensitised recipients with the aim of provoking hyperacute rejection of the allograft. Subsequent experiments would examine strategies for transplanting these allosensitised recipients.

Previous work indicated that the donor-recipient pairs would have to be DLA mismatched in order to generate alloantibody. There are cases described above of positive CDC crossmatches between DLA identical littermate pairs, though the antibody did not affect graft function adversely.

As the review above illustrates the transplantation of dogs with a renal allograft against a positive crossmatch is more commonly associated with accelerated rejection than hyperacute rejection. Indeed the latter event is relatively uncommon event in the dog - Table 4.9. Kux (1971) and Mundy (1980) provide the only two reports of consistent hyperacute rejection, both groups allosensitised the dogs with skin grafts and a kidney, before a second donor specific renal allograft. The numbers were small, three and six dogs.

TABLE 4.9 - Hyperacute rejection (HR) and the allosensitised dog. The proportion of total renal allografts undergoing hyperacute rejection with the time from transplantation is indicated.

	Antigen for allosensitisation	Survival of first renal allograft	Survival of second renal allograft
Egdahl-1955	Kidney	3-14 days	2/12 -no urine
Altman-1963	Skin	1/7 - 20 mins 4/7 - <24 hours	
McDonald-1970	Skin	3-5 hours (n=5)	
Simpson-1970	Skin	24 hours (n=5)	
Kux-1971	Skin	<24 hours	14, 20, and 35 mins (n=3)
Claes-1972	Skin+buffy coat	13/18 <24 hours 1/18 - 45mins.	
Abouna-1977	Skin	0/9 (74% +ve CDC test)	
Mundy-1980	Skin	14/26 <4 hours	6/6 <4 hours
Rapaport-1987	Skin+buffy coat	2-6 days (n=5)	

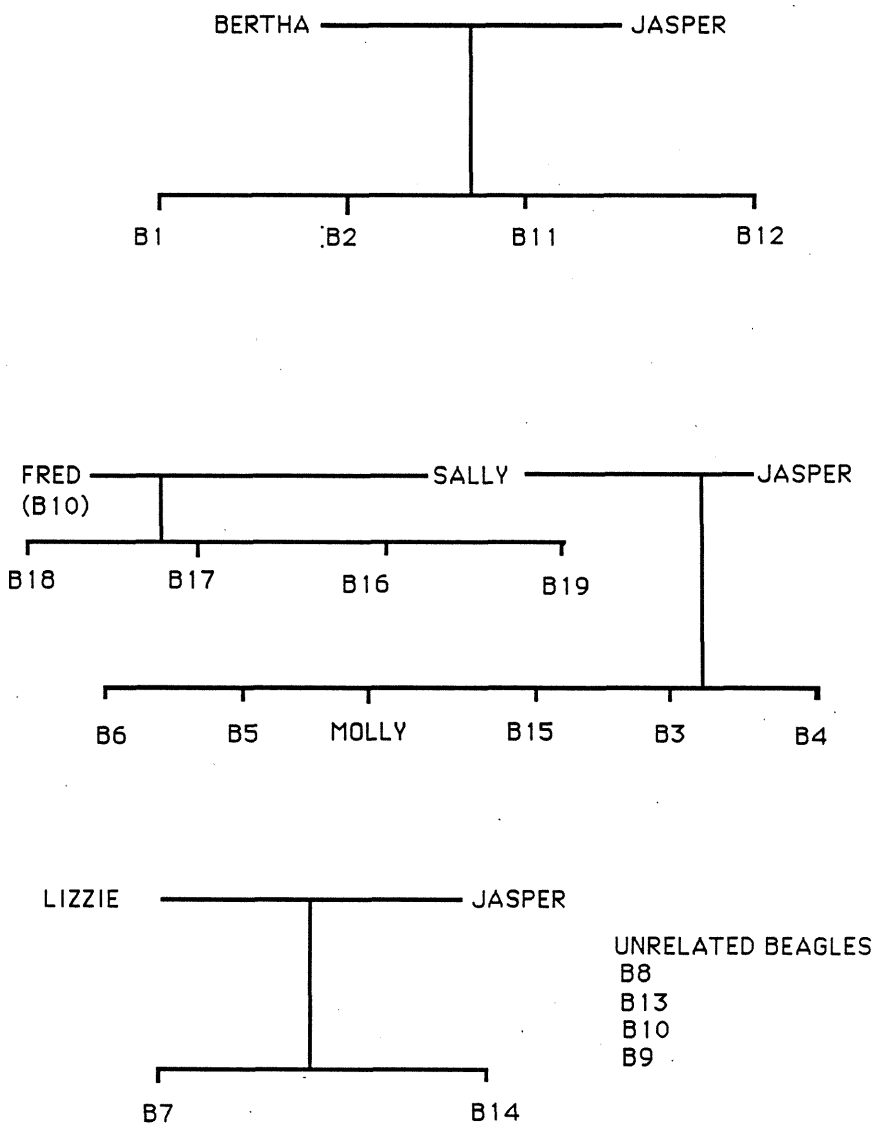
The explanation for this could lie in the specificity of the alloantibody in the recipient and the target against which it is directed on the donor kidney, for example antibody to donor endothelium might be important in the pathogenesis of hyperacute rejection. In the event we did not observe hyperacute rejection and our dog model was one of accelerated rejection.

Was it necessary to tissue type the dogs, given the need to transport frozen cells to one of the laboratories able to DLA type? For alloantibody generation DLA mismatching was important, but the polymorphism of the dog-DLA and avoidance of family pairs should have ensured DLA mismatching. Clearly the dog renal allograft model is a severe test, with little effect of DLA matching in the absence of immunosuppression. However when immunosuppression is added there is a significant matching effect on graft survival. Thus by selecting unrelated pairs, observing alloantibody production and the results of renal allografting with and without immunosuppression it was planned to infer retrospectively that the dogs were DLA mismatched, thus obviating the need for DLA typing.

4.6.2 The Leicester dogs.

A colony of beagles was bred within the University of Leicester so as to avoid the considerable expense of buying in dogs. A male beagle (Jasper) and three females (Bertha, Sally and Lizzie) were purchased from a single source, but were unrelated. Several litters were sired by the male stud dog, see figure 4.2, before he became sterile. Subsequently four other male beagles were purchased at a later date, one (Fred B10) being used as a stud dog. Figure 4.2 shows the pedigree of the beagles.

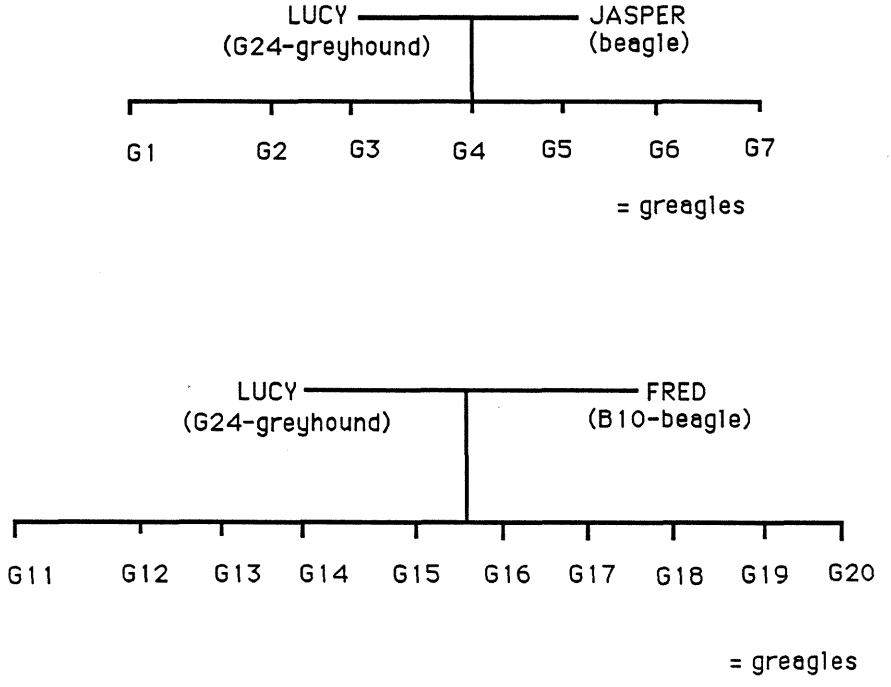
FIGURE 4.2 - The beagle pedigree.



As many of the experimental beagles were related it was necessary to find another source of dogs to be donors. The dogs were be bred in house, partly on grounds of cost and partly to comply with the new Home Office regulations. A greyhound within the Department of Biomedical Services was crossed with a beagle to produce a crossbreed, which we called a

greagle. These were smaller and less spindly than greyhounds, which made them easier to keep in runs and they also ate less. Figure 4.3 shows the pedigree of the two greagle litters with a common greyhound dame, and beagle sires. Of note is the fact that the beagle sires also sired the beagle colony thus reducing the DLA polymorphism between donors and recipients. Any possible effect this might have had on the results will be discussed after presentation of the results.

FIGURE 4.3 - The greagle pedigree.



Joachim Deeg kindly examined some of the allosera generated in this study. The results are shown in Table 4.10. Serial dilutions of seven allosera were tested against the cells from 7 dogs from four different families. His interest in our sera stemmed from his involvement in the Canine Immunogenetics Workshops and the search for further allosera for defining DLA antigens. A numerical system of scoring lymphocytotoxicity was used (Bull-1978), 4 being 25-50% kill, 6 being 50-75% kill and 8 75-100% kill. I do not know the DLA types of the panel of dogs and therefore how representative they were of the polymorphism of the dog DLA. The Leicester allosensitised beagles do not appear to be highly immunised against the panel of Washington dogs, though alloantibody was detected in all of these sera against our own panel of donors - Appendix 4. This difference may be relevant to why we did not see hyperacute rejection of renal allografts by our allosensitised beagles.

In chapter five there is a detailed description of our dog model of accelerated renal allograft rejection. In chapter six the use of cyclophosphamide and cytosine arabinoside to immunomodulate the allosensitised recipient is described. In chapter seven the effect of prostacyclin on accelerated renal allograft rejection is examined.

TABLE 4.10 - Typing results with Leicester beagle sera on 7 dogs from four different families in Seattle, Washington.

SERUM	DILUTION		C277				C279					
	1/1	1/2	1/4	1/8	1/16	1/32	1/1	1/2	1/4	1/8	1/16	1/32
PC	8	8	8				8	8	8			
NC	1	1	1				1	1	1			
2	1	1	1	1	1	1	1	1	1	1	1	1
235	4	4	1	1	1	1	1	1	1	1	1	1
264	1	1	1	1	1	1	1	1	1	1	1	1
230	1	1	1	1	1	1	1	1	1	1	1	1
295	6	6	6	4	2	1	1	1	1	1	1	1
240	1	1	1	1	1	1	1	1	1	1	1	1
280	2	4	2	1	1	1	2	4	2	1	1	1
	C322						C325					
PC	8	8	8				8	8	8			
NC	1	1	1				1	1	1			
2	1	1	1	1	1	1	1	1	1	1	1	1
235	1	1	1	1	1	1	1	1	1	1	1	1
264	1	1	1	1	1	1	1	1	1	1	1	1
230	2	4	4	1	1	1	1	1	1	1	1	1
295	2	4	2	1	1	1	1	1	1	1	1	1
240	1	1	1	1	1	1	1	1	1	1	1	1
280	6	4	2	1	1	1	2	2	1	1	1	1
	C326						C327					
PC	8	8	8				8	8	8			
NC	1	1	1				1	1	1			
2	1	1	1	1	1	1	1	1	1	1	1	1
235	1	1	1	1	1	1	1	1	1	1	1	1
264	1	1	1	1	1	1	1	1	1	1	1	1
230	1	1	1	1	1	1	1	1	1	1	1	1
295	1	1	1	1	1	1	1	1	1	1	1	1
240	1	1	1	1	1	1	1	1	1	1	1	1
280	1	1	1	1	1	1	6	8	6	1	1	1
	B851											
PC	8	8	8									
NC	1	1	1									
2	6	1	1	1	1	1	2	B2-unsensitised				
235	4	1	1	1	1	1	235	B10-two kidneys				
264	1	1	1	1	1	1	264	B1-skin grafts				
230	6	1	1	1	1	1	230	B3-skin grafts				
295	8	6	1	1	1	1	295	B13-two kidneys				
240	1	1	1	1	1	1	240	B11-blood				
280	6	4	1	1	1	1	280	B14-blood				

PC=Positive control
NC=negative control
C277,279,322,325,326,327,B851.-Designation of typing cells-University of Washington

CHAPTER 5

THE ALLOSENSITISED DOG MODEL

THE ALLOSENSITISED DOG MODEL

The special features of this model were the use of a subcutaneous groin pouch to facilitate biopsies of the renal allograft and the preservation of the native kidneys so that the allosensitised recipient was available for further study. Two questions for consideration are firstly, how best to follow the course of renal allograft rejection and secondly, what bearing does this model have on the management of the allosensitised patient?

5.1 The technique of canine renal transplantation

5.1.1 The recipient

5.1.1.1 Anaesthesia and preparation

The recipient was fasted overnight, and given an intramuscular injection of acetylpromazine maleate (ACP™; C-Vet Ltd.) 0.125mg/kg., 30 minutes before induction of anaesthesia with thiopentone. Venous access was via the cephalic vein with a 20G Abbocath. The dog was intubated, placed on the ventilator, and maintained with nitrous oxide, oxygen, and halothane. Saline (0.9% sodium chloride), 25 ml/kg, was infused during the operation in order to encourage a diuresis. In later experiments alphaxalone and alphadolone acetate (Saffan™-Glaxovet Limited) were used for induction. The abdomen was shaved and the skin prepared with povidone iodine (Betadine ™), followed by chlorhexidine in alcohol.

5.1.1.2 Surgery

The recipient was positioned supine, with the pelvis rotated, and gentle traction to the hind leg so as to open up the selected groin. The operations were always performed by an aseptic technique.

A lower midline incision was made, extending up from the symphysis to just below the umbilicus. The subcutaneous plane was dissected down to the selected groin. Considerable care was taken with haemostasis. The femoral artery and vein were mobilised, and branches ligated and divided such that vascular clamps could be used to isolate a length of vessel. The kidney was implanted by anastomosing the renal vein to the side of the femoral vein, and the renal artery end to end with the femoral artery, the latter having been ligated distally with Nurolon™ (braided polyamide - Ethicon Ltd). A continuous 7/0 Prolene suture was used for the vascular anastomoses. The clamps were released. The femoral artery in the dog can be divided in the groin without causing ischaemia of the leg.

The ureter was either brought out as a ureterostomy or anastomosed to the bladder. For the ureterostomy the ureter was brought through a stab incision in the groin, then spatulated and

the ureteric mesentery ligated at the end of the ureter with 6/0 plain catgut. The ureter was sutured to the skin with four interrupted 6/0 plain catgut sutures. For the neo-ureterocystostomy the bladder was exposed by deepening the original midline incision through the linea alba into the peritoneal cavity. A tunnel was easily fashioned through the femoral canal with Lahey forceps, and the ureter brought through the femoral canal to the bladder. Using a scalpel the bladder muscle was incised for one centimetre, down to the mucosa. The mucosa was picked up with fine clips and incised. The ureteric mesentery was ligated, the ureter spatulated, and then anastomosed direct to the bladder mucosa with multiple interrupted 6/0 plain catgut sutures. The layer of bladder muscle was then loosely approximated over the anastomosis with interrupted 3/0 plain catgut.

The abdominal muscles was closed with a nylon suture. The kidney was positioned with regard to the best lie of the vessels and the subcutaneous space surrounding the kidney was partly closed using interrupted catgut sutures. Room was left for some movement and expansion of the kidney within the subcutaneous pouch. The midline wound was closed with a continuous 2/0 chromic suture to the subcutaneous layer and interrupted Supramid™ (pseudo monofilament polyamide - B. Braun Melsungen AG) sutures to the skin.

Beagles receiving a third allograft had to have a groin re-explored. Providing the femoral vein was patent (one out of ten thrombosed), then a length of artery was readily made available by mobilising the external iliac artery deep to the inguinal ligament.

5.1.1.3 Post operative care

The dog was returned to a heated pen and maintenance fluids were given intravenously for the first twelve hours or until the dog pulled out the line. By the next morning dogs were usually starting to walk, and were given their normal diet. The daily food consumption, body weight, and character of the motions were recorded, and comments made on the general well being of the dog by the staff. The dogs were routinely examined twice daily, temperature, pulse, and respiration being recorded. The kidney was also palpated, its size assessed, and urine production from the ureterostomy observed. Vaseline was applied around the ureterostomy so as to protect the skin from the urine. Skin sutures were removed after 7 days.

Pain relief: Subcutaneous buprenorphine (Temgesic™ - Reckitt & Colman), at 0.003mg/kg was given as the dog was extubated and prior to leaving the operating room. This was repeated on the evening of the operation, and afterwards as required, normally for two to three days.

Antibiotics: All recovery dogs were given antibiotic cover for five days: one ml. of duplocillin (150mg procaine penicillin and 112.5mg benzathine penicillin per ml) and 40mg of trlbrissen 80 (trimethoprim 40mg and sulphadiazine 200mg) prior to operation.

Post-operatively the duplocillin was repeated on the 3rd and 5th day. Trivetrin 0.5mls (Each ml contains trimethoprim 40mg and sulfadoxime BP 200mg) was given 12 hourly intramuscularly, changing to a half tablet of tribrissen 80 12 hourly as soon as feeding started.

Blood tests: The packed cell volume and white blood count of the dogs were routinely checked monthly, but following an operation these indices were checked weekly. The serum biochemistry was estimated monthly.

Complications: Forty-one dog kidney transplants were performed on eighteen dogs in this study. The operations were well tolerated by the dogs, which may have been due to most of the operation being performed in a subcutaneous plane.

The most common complication was swelling of the hind leg beyond the transplanted kidney. This occurred to some degree following 32 of the transplants. The extent of the swelling was closely related to the degree of swelling of the graft. Following transplant nephrectomy the swelling of the leg always resolved rapidly. Other complications seen included two wound infections, both of these occurred in wounds where the transplant had undergone infarction because of rejection.

There was one renal artery thrombosis in an unsensitised recipient on CyA. There were five instances of significant haemorrhage. Both dogs receiving heparin (group 9) died as a result of haemorrhage, one from a rupture of the kidney and one from a tear in the renal vein at the anastomosis where too large a bite of vein had been included in the vascular anastomosis. Three dogs receiving prostacyclin had significant haemorrhage, two from a rupture of the cortex and the third was managed conservatively by transfusion. In the prostacyclin treated dogs extra efforts were made to close the subcutaneous space around the allograft with interrupted catgut sutures, and these three ruptures may have resulted from these efforts by restricting movement of the allograft when the dog started to mobilise. Certainly there were no more kidney ruptures when the subcutaneous space was closed less completely. All dogs receiving prostacyclin had an excess of cutaneous bruising both at the operation site and beyond the margins of dissection.

5.1.1.4 Transplant nephrectomy

Twenty-eight transplant nephrectomies were performed under general anaesthesia. These were performed through an incision over the kidney in the groin, usually via an extracapsular dissection. The renal artery and vein were individually ligated. The ureter was ligated and divided, it was not necessary to follow the ureter down to the bladder. The kidney was removed, and the subcutaneous space closed with interrupted catgut sutures and the skin with Supramid. The same antibiotic protocol as for transplant operations was used. One dog

developed a wound infection which drained spontaneously and one required two aspirations of a seroma. One dog died under anaesthesia during removal of a rejecting kidney; histological evidence of a myocarditis was found, but no other cause of death identified.

Nineteen dogs were pyrexial prior to nephrectomy, presumably due to rejection of the transplant, as their fever subsided rapidly after operation. In three dogs there was no need to remove the transplant, because of absence of symptoms, and it was left in situ. A further eight dogs were not recovered at the time of nephrectomy.

5.1.2 The donor

5.1.2.1 Anaesthesia and preparation

See 5.1.1.1.

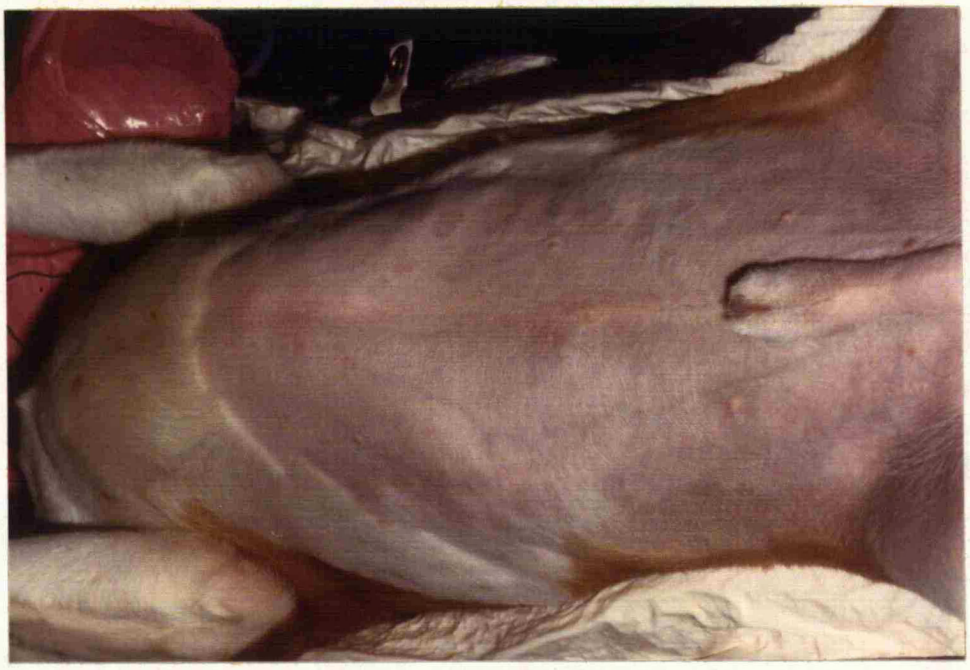
5.1.2.2 Surgery

The abdomen was opened in the midline, the posterior peritoneum incised, and the kidney was then easily mobilised . In the dog, the kidneys lie retroperitoneally, and are not enveloped by fat. In all dogs only a single renal artery was present, though the first division was sometimes very close to the aorta. Dissection in the hilum of the kidney was minimised so as to avoid damaging the blood supply to the ureter. By this stage the kidney had been sufficiently mobilised so that it could be lifted from its bed, together with the mobilised renal vessels still attached to their origins. The ureter was then mobilised, preserving its blood supply, down to the pelvic brim and divided. The renal artery and vein were clamped and divided, freeing the kidney, which was transferred to a sterile bowel and immediately perfused via the renal artery with saline at 0 deg C. The kidney was perfused until it felt cold, and the venous effluent was clear (about 150 mls). The kidney was left immersed in the cold saline and the bowel surrounded by ice. As the period of cold ischaemia was always less than three hours, the kidneys were flushed with a normal saline, rather than a special preservation fluid.

Finally some mesenteric lymph nodes were removed for harvesting of lymphocytes.

The model is illustrated in illustrations 1-9.

Illustration1- The anaethetised recipient supine, abdomen shaved and skin prepared with chlorhexidine in alcohol.



Ilustration 2 - Renal allograft perfused via renal artery with ice cold saline.

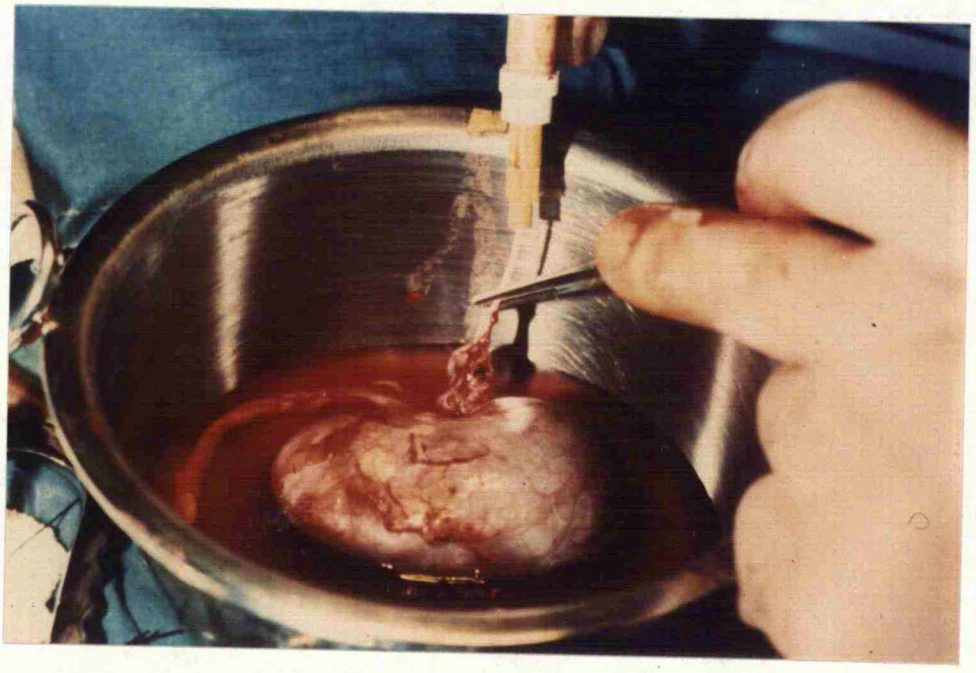


Illustration 3 - Superficial groin pouch right side. The prepared femoral vessels, the divided artery ligated distally with Neurilon and a clamp applied proximally. The vein lies medially and a length has been isolated between two clamps.

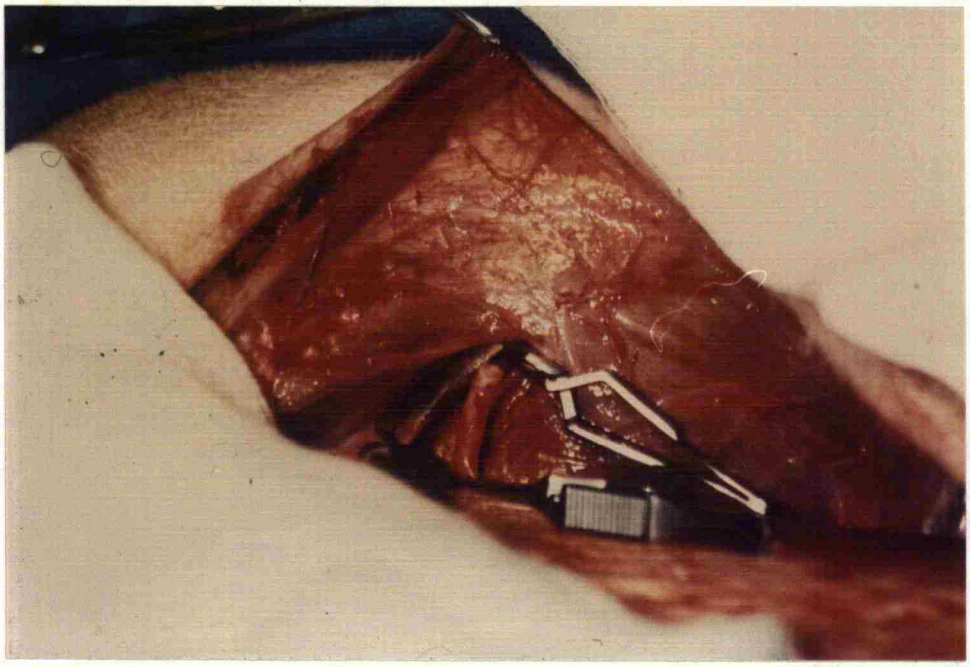
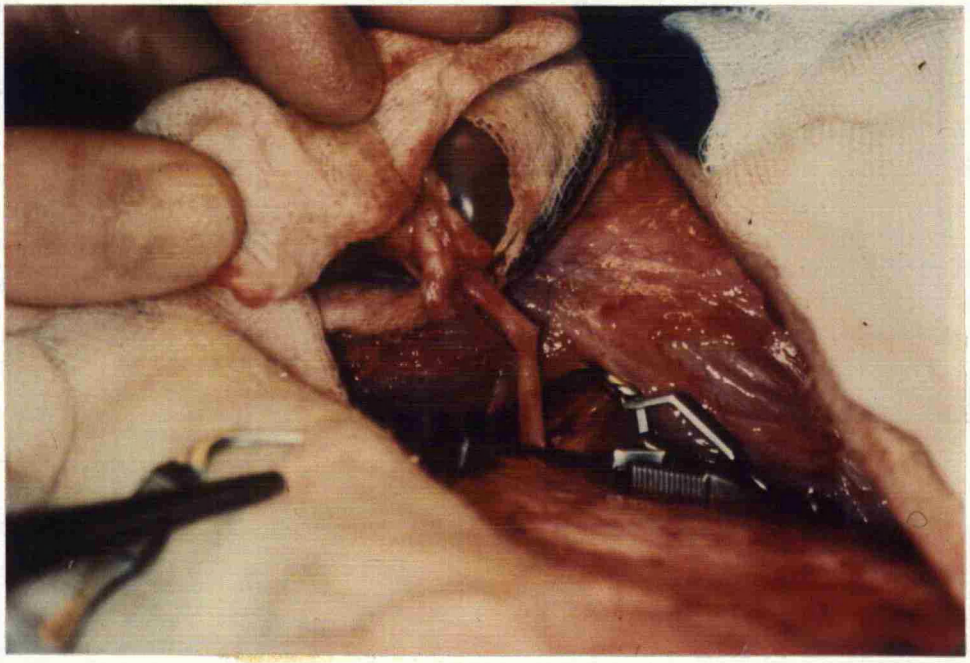
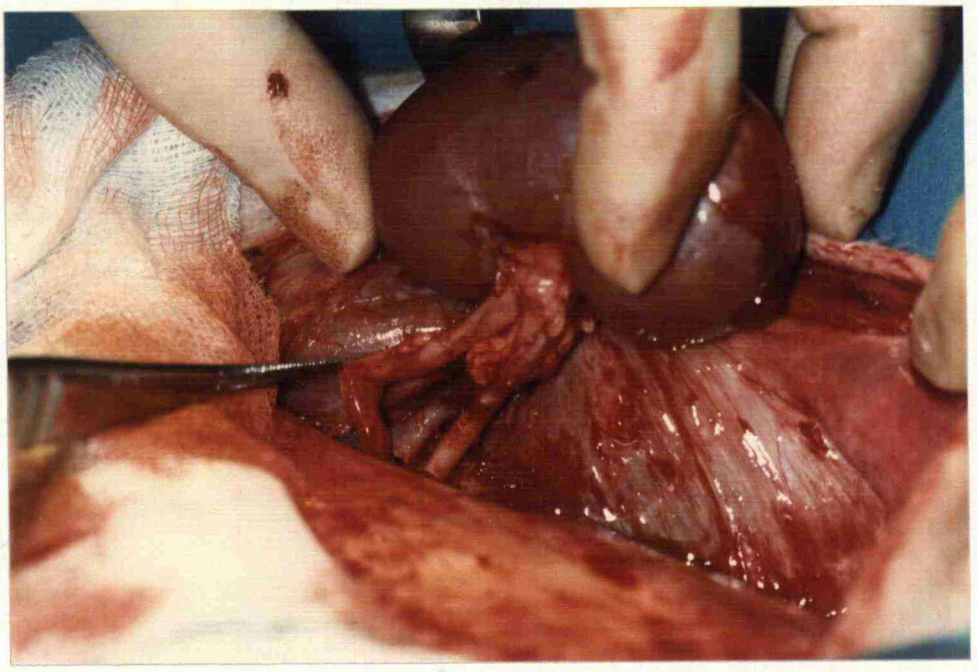


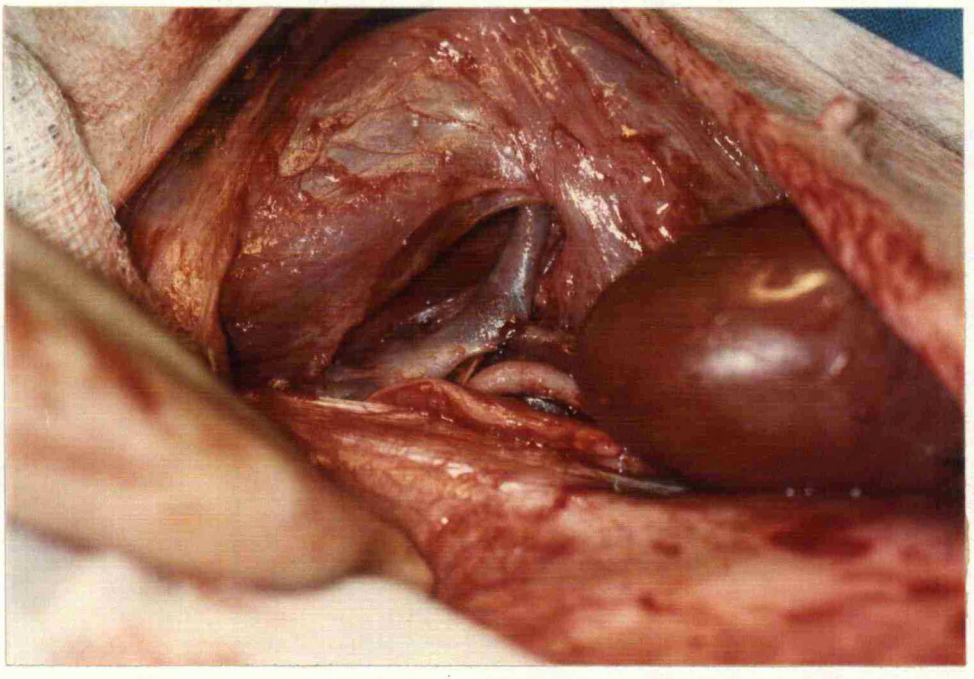
Illustration 4 - Completed vascular anastomoses with clamps in place. Renal allograft wrapped in cold guaze swab. Renal artery end to end with femoral artery with continuous 7/0 Prolene. Renal vein end to side with femoral vein with continuous 7/0 Prolene.



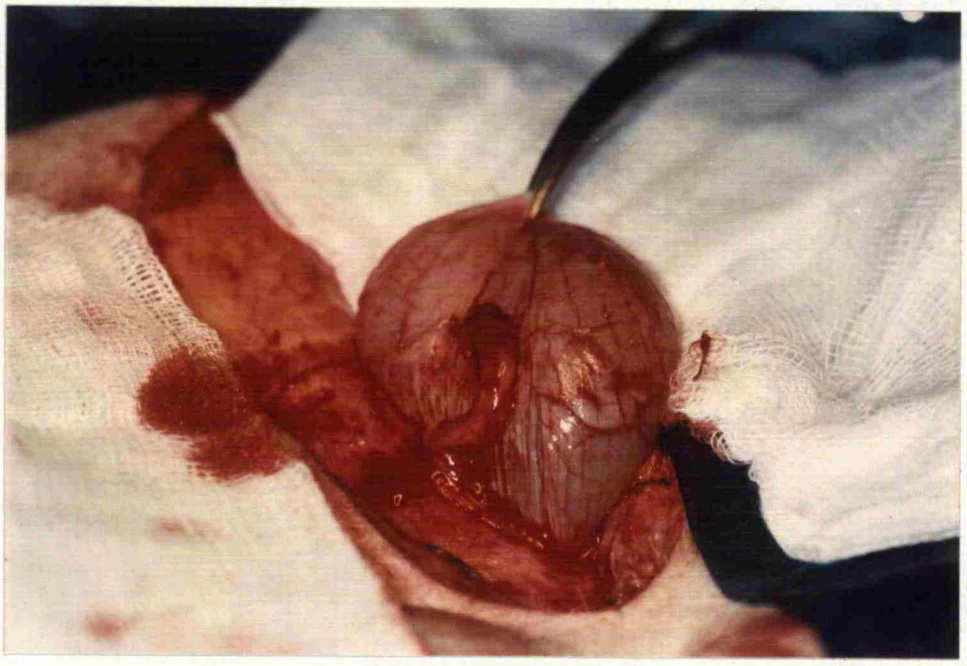
Ilustration 5- Reperfused renal allograft. The ureter is being held medially with a haemostat to display the anastomoses.



Ilustration 6- Renal allograft in groin pouch. The ureter is seen running medially, before disappearing under the free border of the inguinal ligament into the femoral canal.



Ilustration 7- The delivery of the bladder through the midline incision is possible because of the intraperitoneal lie. The spatulated ureter has been sewn to bladder mucosa with interrupted 6/0 catgut.



Ilustration 8- Bladder musculature approximated over the anastomosis with interrupted catgut.

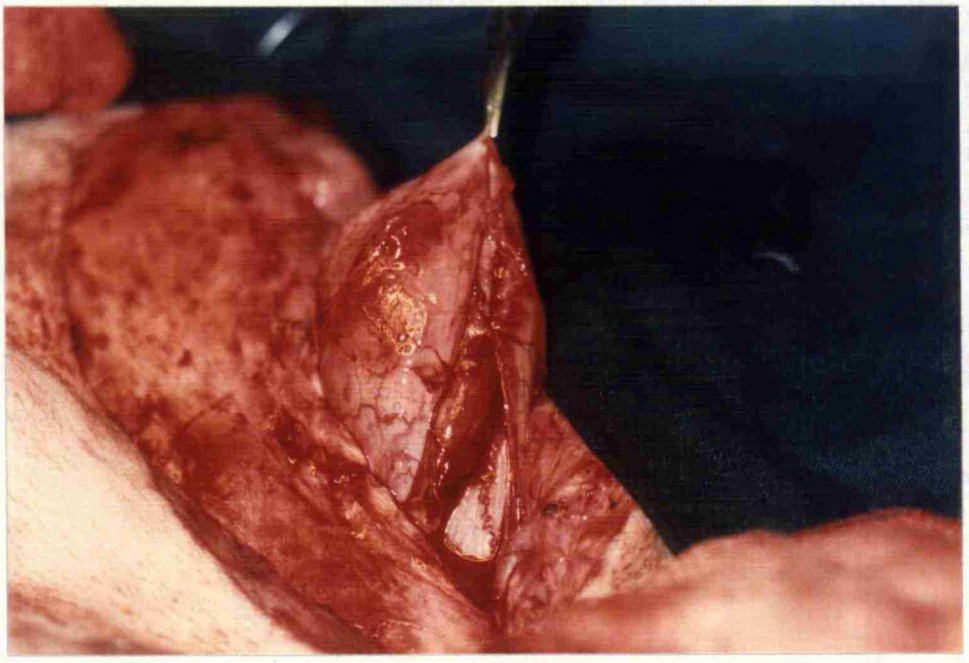
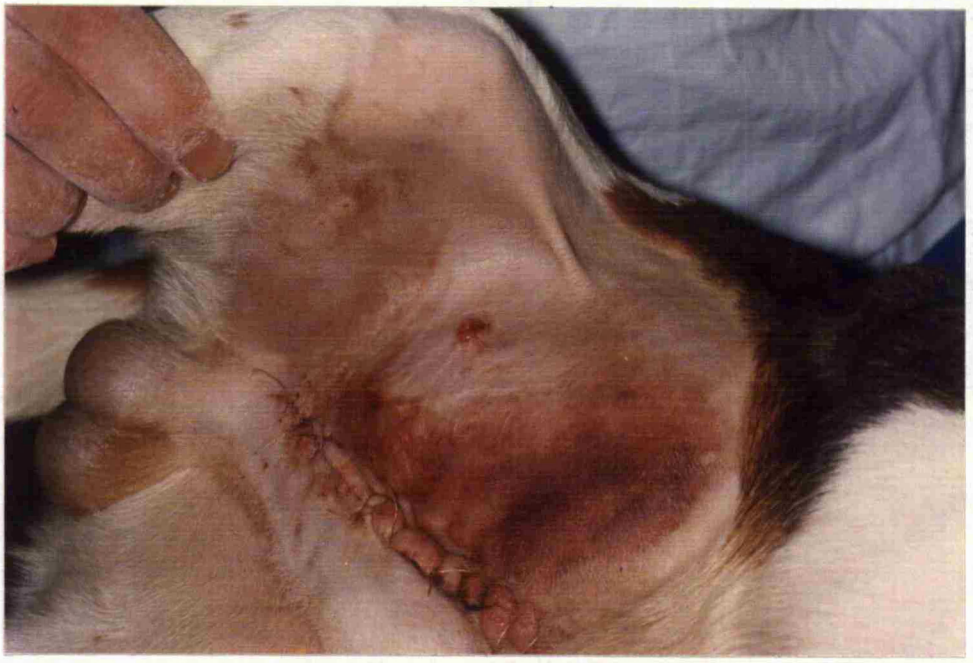


Illustration 9- The allograft ureter emerging just proximal to the groin crease, the kidney in the subcutaneous pouch between the ureterostomy and the midline, and a drop of urine below the midline incision. There is some subcutaneous bruising.



5.2 Monitoring of graft function and the experimental end points

5.2.1 Monitoring of graft function

In experimental models the transplanted kidney is conventionally monitored by removing the recipients own kidneys at the time of transplantation. The function of the transplant is simply followed by measuring serum creatinine. The experiment end point is defined, eg serum creatinine >1000umol/l. The disadvantages are that the recipient may undergo distressing side effects, such as persistent vomiting, before death. These side effects can be reduced by setting a lower level of serum creatinine, at which the experiment is ended. The main disadvantage of removing the native kidneys is that the dog is not available for follow-up studies of allosensitisation, and the experimental aims for this study dictated that the recipients stay alive after graft failure. Consequently the native kidneys were left in-situ, and the function of the transplant had to be studied in other ways.

Intravenous urography (IVU) was used to demonstrate perfusion and function of the graft. This yielded no quantitative information of function, and with impaired allograft function, the contrast was preferentially excreted by the native kidneys. Similarly, arteriography would have shown perfusion, but minimal information of function. Radioisotopes can show perfusion and function by imaging with a gamma camera following injection of a suitable radionuclide, eg technetium-^{99} conjugated with dimercaptosuccinic acid (-^{99}Tc-DMSA).

Volume and clearance studies can accurately define function, and Stacy (1966) used Chromium-51 ethylenediaminetetraacetate (Cr^{51}-EDTA) to measure glomerular filtration rate concurrently with iodine-125 orthoiodohippuric acid for estimation of effective renal plasma flow (ERPF). Unfortunately, it was difficult to collect transplant urine separately from native kidney urine. Collection of urine from a ureterostomy was attempted, but the dogs frustrated attempts at urine collection into an adhesive bag over the groin. External urine collection might have been more successful with training.

Without access to a gamma camera the measurement of graft function in the presence of the native kidneys was impractical. Graft function was followed by intravenous urography where there was a neocystoureterostomy, and by simply observing the urine from the ureterostomies. It was anticipated that many kidneys would be rejected either hyperacutely, or undergo accelerated rejection within days. In the event of prolonged survival the native kidneys could be removed to demonstrate function of the graft.

As the biopsy results were only available later, practical criteria were needed to determine the end point of each experiment. In the presence of these features transplant nephrectomy was performed:

a) The dogs general condition, malaise, anorexia, vomiting, behaviour, and pyrexia.
b) Cessation of urine production.
c) Swelling of the graft.

Each kidney was examined histologically. By performing a series of biopsies on each renal allograft it was hoped to ascertain the cause of graft failure, and compare the time course of the rejection process between recipients. Placing the renal allograft in a subcutaneous groin pouch facilitated repeated biopsies.

5.2.2 Biopsy of the graft.

5.2.2.1 Introduction

It was hoped that renal allografts to allosensitised dogs would be rejected hyperacutely, and histology would offer confirmation. In the event, hyperacute rejection was not seen with the recipient/donor combination used, but renal allografts to allosensitised recipients were rejected in an accelerated manner compared with allografts to unmodified recipients. The function of the allograft was difficult to follow because of the presence of the native kidneys. This led to the evaluation of histology on serial biopsies in differentiating between the course of renal allografts in the allosensitised and unsensitised recipients.

Rejection is often loosely qualified by terms such as hyperacute, accelerated, vascular, and humoral. Porter (1983) stated that hyperacute rejection occurs within a few hours and accelerated rejection in 12 to 48 hours of grafting. Both are usually associated with the

presence of pre-existing antidonor antibodies, and are resistant to treatment. A renal allograft undergoing acute rejection may show a spectrum of changes with varying degrees and combinations of cellular and antibody mediated damage. The pattern will depend on factors such as the genetic disparity between donor and recipient, prior allosensitisation, immunosuppressive policy, and time from grafting. Acute cellular rejection is characterised by oedema and cellular infiltrates. Fibrinoid necrosis of arterial walls, thrombosis in glomerular capillaries and interstitial haemorrhage are features associated with antibody mediated damage, or so called humoral rejection.

Magil (1980) showed that renal allograft survival in patients on conventional immunosuppression and with steroid resistant rejection was significantly related to the presence of vascular pathology on biopsy. These changes included endothelial swelling, intimal mononuclear cell proliferation or infiltration, sub-endothelial vacuolation, medial necrosis of arterial walls, and plugging of glomerular capillaries with platelets and fibrin. The terms vascular and humoral are both used to qualify allograft rejection with these features. Jeannet (1970) described a significant correlation between vascular changes and the presence of humoral antibodies in clinical renal allografting.

In the dog Matthew (1971) placed renal allografts into both non immunised and dogs immunised by four injections of donor buffy coat. Only two of 18 immunised dogs developed detectable donor specific lymphocytotoxic antibodies, and by the time of transplantation one of these had become crossmatch negative. Vascular lesions were consistently observed in the immunised dogs. Thrombosis of glomerular capillaries and occasional afferent arterioles was seen in 15 out of 18 immunised dogs, and 14 had fibrinoid necrosis in vessels. These changes were seen in about one half of the control dogs, but were less marked. The incidence of vascular lesions was highest in biopsies taken at the early sign of rejection, and in some dogs these features had disappeared by the time of necropsy some days later. No significant differences in the survival times of the dogs was seen, nevertheless it was suggested that the vascular features were associated with allosensitisation, though alloantibody was only detected in two of eighteen recipients.

The division of allograft rejection into cellular and humoral is a gross simplification. Hayry (1986) emphasised the interconnection between the classical humoral and cellular arms of the immune effector system. Nevertheless this simple division is of some practical use clinically, as the features of vascular or humoral rejection occur earlier in allosensitised patients and this morphological pattern is associated with a poor response to any form of anti-rejection therapy. Therefore, there is a need for an experimental model to investigate the relationship between allosensitisation, the features of vascular rejection, treatment resistance and early graft failure as a means to improve the management of allosensitised patients. The model could be used to investigate whether modifying the appearance of vascular features could be associated with improved graft survival. Dempster (1953), Simonsen

(1953), and Egdahl (1955) have all observed that primary allograft rejection in the dog is a vigorous affair accompanied by vascular features and it was therefore necessary in this study to contrast this primary rejection response with the allosensitised recipient and the immunosuppressed recipient.

The finding of immunoglobulin and complement in vessels of rejecting allografts supports the concept that vascular rejection is primarily antibody mediated, and this fits with the poor response to treatment of rejection with these vascular features. Busch (1971) noted in renal allografts that failed within two months of transplantation, the almost invariable presence of extensive thrombosis, fibrinoid necrosis of vessels, intimal thickening of arteries and deposits of IgG, complement, and fibrinogen in cortical vessels by fluorescence. This morphologic pattern of vascular rejection was observed, both immediately after transplantation and at various times during the first two months. Hyperacute rejection is antibody mediated, and occurs within hours of transplantation. The dominant histoiogical feature is fibrin in the capillary lumens with blocking of the intrarenal capillaries and arterioles by microthrombi (Porter-1983).

In clinical transplantation percutaneous allograft needle biopsies are used for the diagnosis of graft dysfunction, but in addition may provide guidance on future allograft function. Herbertson (1977) reported on needle biopsies taken from 154 renal allografts during the first 90 days after transplantation. The relationship between the presence and severity of particular lesions in these biopsies and subsequent graft function at 1, 3 and 5 years was examined. The particular changes examined included mononuclear cell infiltration, various vascular lesions and tubular necrosis. The presence or absence of six common structural changes in every needle biopsy was recorded and their severity estimated on the basis of a simple grading scheme:

1) Mononuclear cell infiltration in interstitial tissue.
   Grade 0 = none.
   Grade 1 = slight to moderate focal and/or diffuse infiltration.
   Grade 2 = substantial focal and diffuse infiltration.

2) Mononuclear cell adherence to arterial endothelium and infiltration of arterial intima.
   Grade 0 = none.
   Grade 1 = slight focal or diffuse involvement of one or a few vessels.
   Grade 2 = substantial lesions in one or more vessels.

3) Medial necrosis of arteries and/or arterioles.
   Grade 0 = none.
   Grade 1 = small focal lesion(s) affecting a single vessel or a few vessels only.
   Grade 2 = larger focal lesions or diffuse lesions affecting several or many vessels.

4) Glomerular thrombosis and/or necrosis.
   Grade 0 = none.
   Grade 1 = small lesions affecting up to 25% of glomeruli.
   Grade 2 = larger lesions affecting more than 10% of glomeruli or smaller lesions involving over 25% of glomeruli.

5) Interstitial haemorrhage.
   Grade 0 = none.
   Grade 1 = haemorrhage involving interstitial tissue of less than 25% of total area of section prepared.
   Grade 2 = haemorrhage involving interstitial tissue of more than 25% of total area of section prepared.

6) Tubular necrosis.
   Grade 0 = none.
   Grade 1 = patches of tubular cell necrosis involving less than 25% of total area of the sections prepared.
   Grade 2 = substantial tubular cell necrosis involving more than 25% of total area of the sections prepared.

Herbertson found that the presence of medial necrosis of arteries, glomerular thrombosis and/or necrosis, and interstitial haemorrhage, either separately or together in needle biopsies during the first 90 days proved to be a very sensitive and accurate indicator of early graft failure. Less than 10% of the 65 grafts with one or more of these lesions, regardless of grade, were capable of supporting life at one year and none of the 20 in which all three lesions were present were functioning at one year. On the basis of the clinical and

pathological features acute allograft rejection was thought to be the cause of these three types of lesion in the vast majority of grafts. These changes seem to be fundamentally similar expressions of severe immunological damage at arterial, arteriolar, glomerular capillary and peritubular capillary levels. It is likely that antibody mechanisms often play a major role in their development. However, causes other than rejection, such as severe hypertension, poor preservation, renal vein thrombosis, and infection may sometimes be responsible for these lesions. This is especially true of interstitial haemorrhage.

A significant association was seen between poor subsequent graft function and mononuclear cell infiltration of the intima of arteries. No clear relationship existed between graft function and the degree of mononuclear cell infiltration of the interstitial tissue. Tubular necrosis has many causes, poor preservation and rejection being the most important. Grade 1 tubular necrosis was common, and had little adverse effect on future graft function. With severe tubular necrosis future graft function was affected, though the vast majority of these also had arterial or glomerular lesions, and appeared to belong to the group of allografts undergoing severe acute rejection.

Based on this information a similar grading system was used in this study. Certain features were taken as representing severe rejection and a poor future graft function. These were called class 1 features, and were scored on a 0, 1 and 2 scale giving a cumulative score out of six. Class 1 features were:

1) Medial necrosis of arteries and/or arterioles.
2) Glomerular thrombosis and/or necrosis.
3) Interstitial haemorrhage.

The three other morphological features of Herbertson were labeled as class 2, 3 and 4 on the basis of a presumed decreasing importance both in the specific diagnosis of rejection and as indicators of duration of graft function. No connection with class 1 and 2 MHC antigens is implied by this terminology.

Class 2: Mononuclear cell adherence to arterial endothelium and infiltration of arterial intima.
Class 3: Mononuclear cell infiltration in interstitial tissue.
Class 4: Tubular necrosis.

Figure 5.1 shows the format of the biopsy report form used in this study. Illustrations 10-15 show examples of biopsies, with two completed biopsy report forms. Pig to dog renal xenografts undergo hyperacute rejection, and several of these were performed.

A potential problem with applying Herbertson's criteria to the present study is that CyA was used in the dog study, and Aza in the Cambridge patients. In clinical practice, adverse effects

of CyA have included renal and hepatic dysfunction, hypertension, hirsuitism and gingival hyperplasia. Whiting (1985) observed that in most animal studies in which therapeutic doses of CyA were used to prolong allograft survival very few adverse effects of the drug have been reported. In clinical practice CyA has been associated with vascular changes, such as arterial and glomerular thrombosis, endothelial damage with fibrin deposition (Keown-1985) and arterial thickening (Wallace-1985). There is evidence in clinical transplantation that CyA may produce some of these morphological features in the absence of rejection. However these features have not been described in canine renal transplantation with CyA.

FIGURE 5.1 - The biopsy report form.

DOG RENAL TRANSPLANT BIOPSY.

LAB REF NO DATE OF BIOPSY DATE OF TX

HISTOLOGICAL FEATURES

GLOMERULI

Present 0 1 Number ____

Necrosis 0 1 2 Sclerosis - focal 0 1 2

- diffuse 0 1 2

Capsule dilated 0 1 Capillary perfusion 0 1

Cellularity 0 1 2 Number Polys per glom ___

TUBULES

Normal 0 1 Atrophic 0 1 2 Necrosis 0 1 2

Regeneration 0 1 2 Dilated 0 1 2 Casts 0 1 2

INTERSTITIUM

Normal 0 1 Oedema 0 1 2 Fibrosis 0 1 2

Haemorrhage 0 1 2 Cellular Infiltrate (Degree) 0 1 2

Cellular Infiltrate (Type) ____

VESSELS

Normal 0 1 Oedema 0 1 2 Intimal lymphocyte adherence 0 1 2

Intimal fibrosis 0 1 2 Cellular infiltrate 0 1 2 Necrosis 0 1 2

GENERAL

Infarction 0 1 Obstruction 0 1 Ischaemia 0 1

CONCLUSION

KEY

0=No 1=Yes

0=None

1=Slight

2=Substantial

Illustration 10 - Normal dog kidney, a glomerulus and arteriole. (H & E x400).

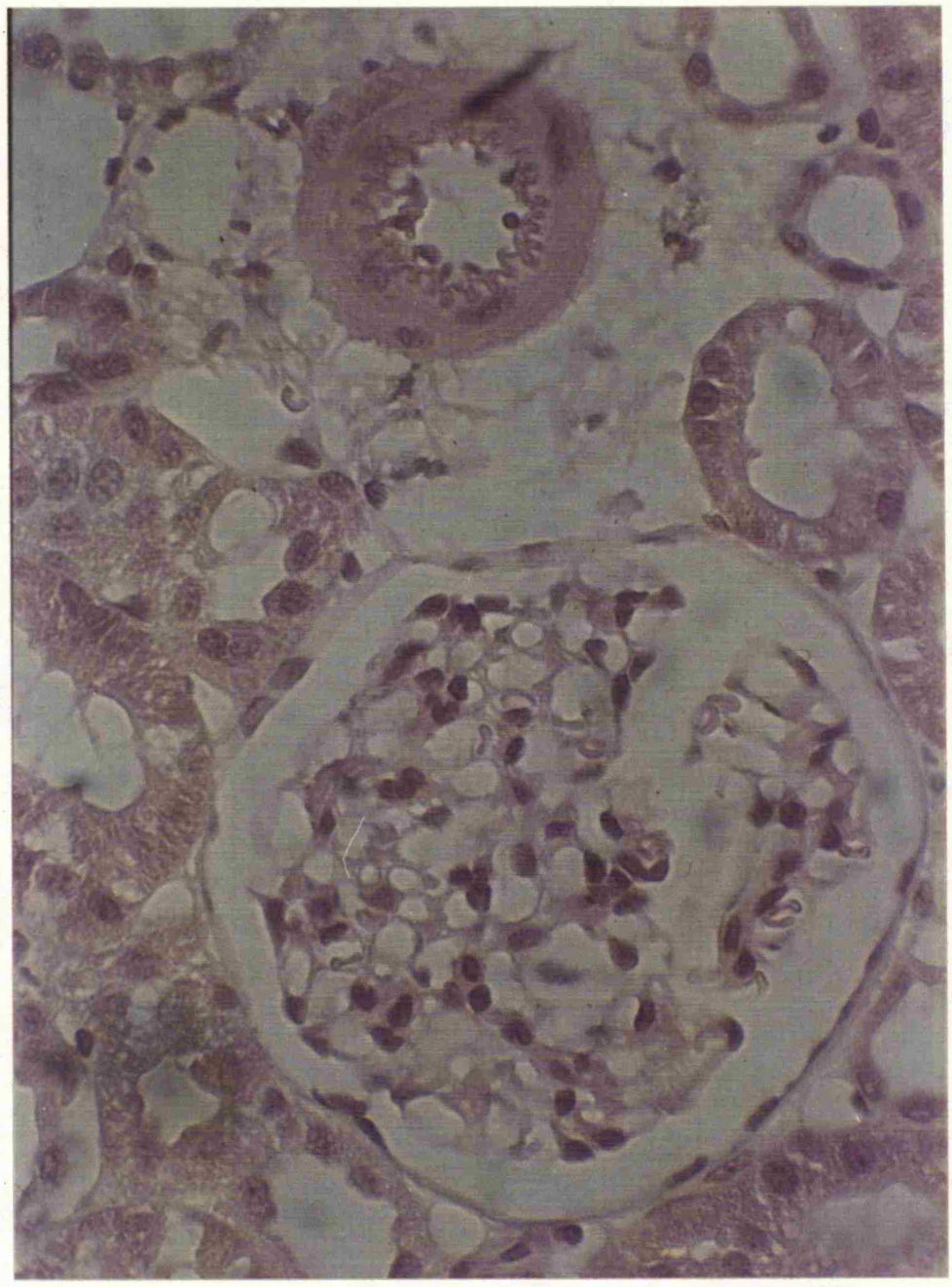


Illustration 11 - Pig kidney xenograft to dog at 50 minutes. Thrombosis in arteriole. (H & E x400).

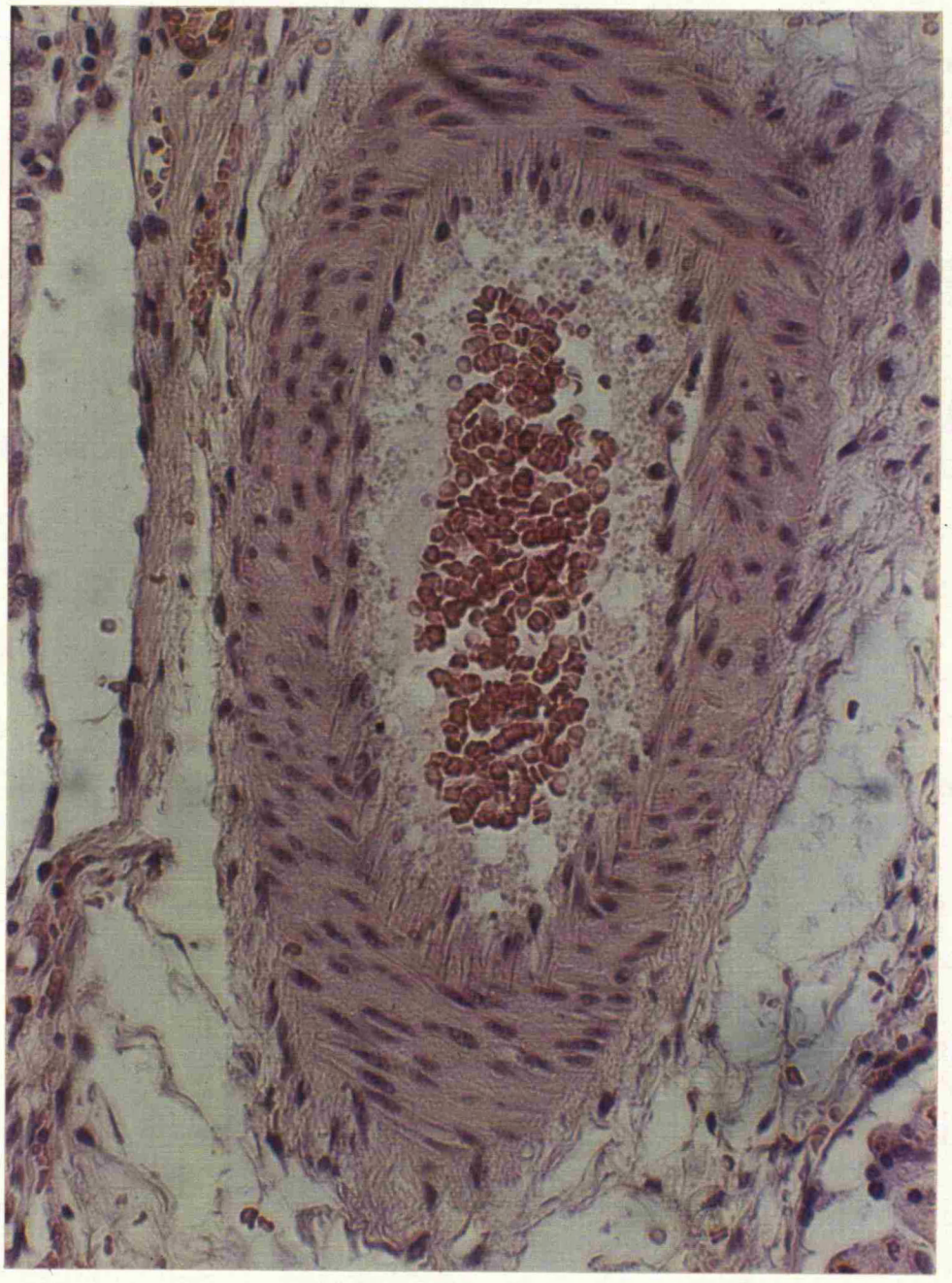


Illustration 12 - Pig kidney xenograft to dog at 10 minutes. Glomerular thrombosis characteristic of hyperacute rejection. (H & E x400).

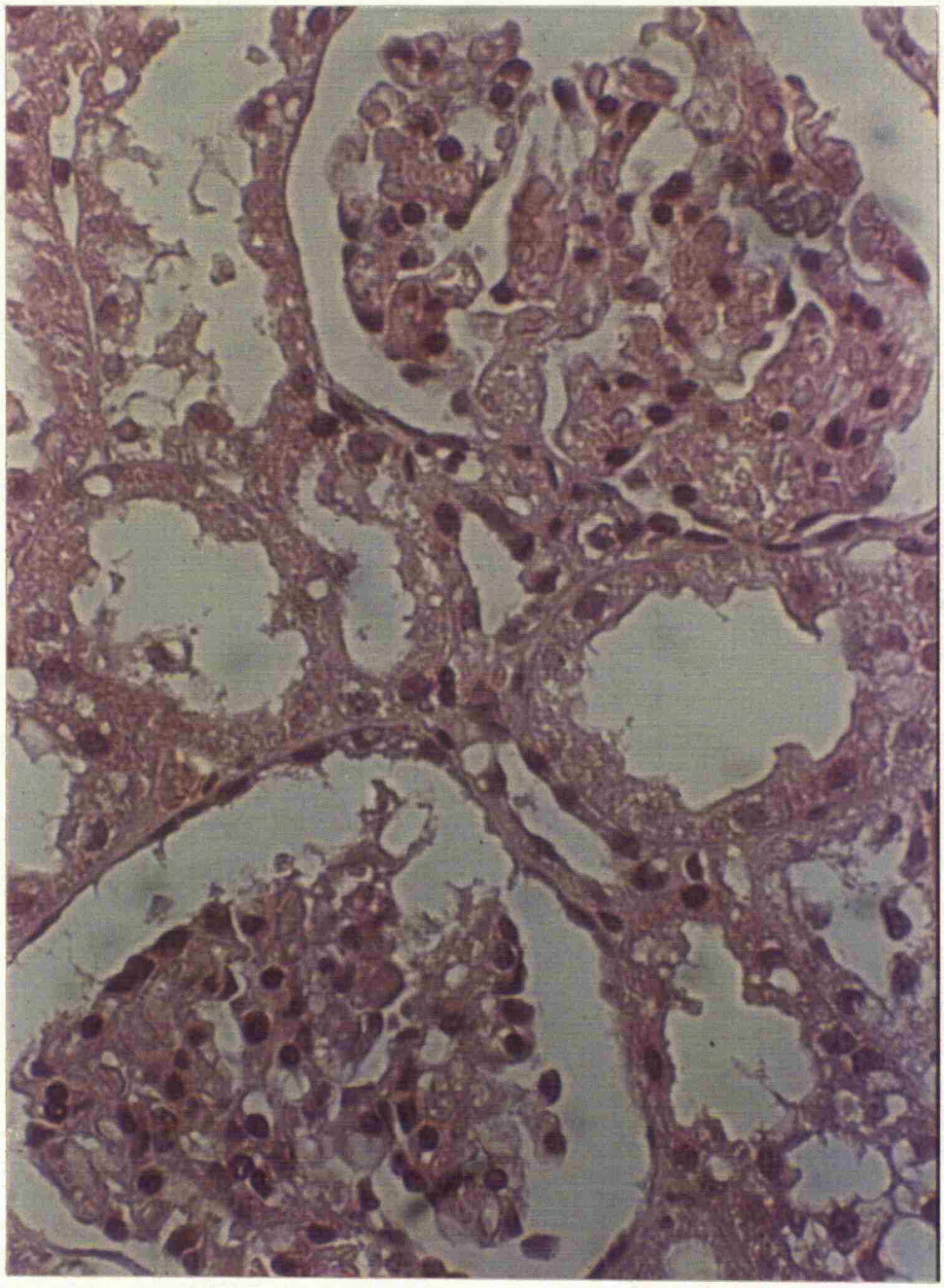
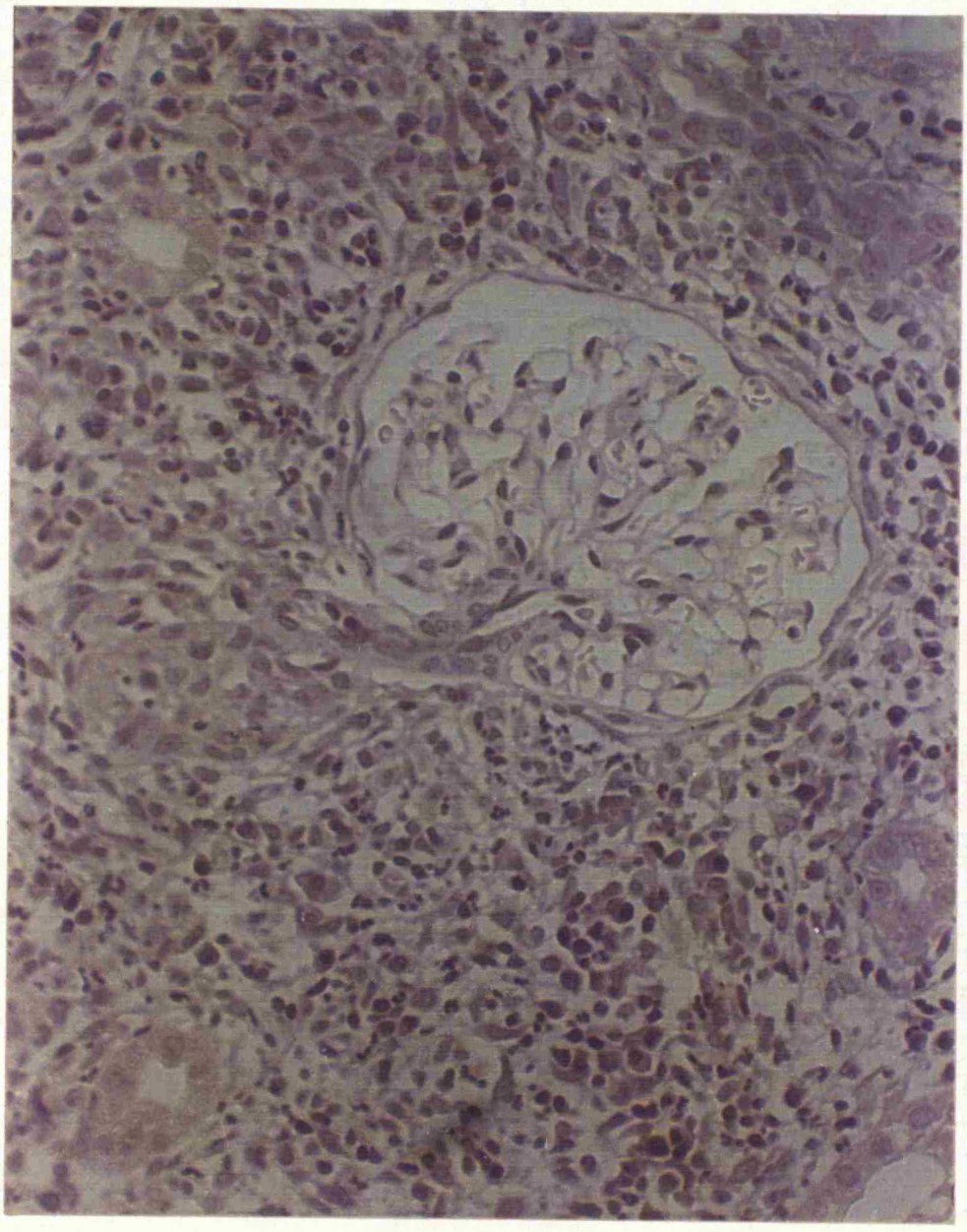


Illustration 13 - Primary dog renal allograft at six days. CyA at 20mg/kg/day. Laboratory reference number-15. Severe cellular rejection with lymphocyte infiltrate around glomerulus. (H & E x400).



DOG RENAL TRANSPLANT BIOPSY.

LAB REF NO 15 DATE OF BIOPSY 6.11.84 DATE OF TX 31.10.84

HISTOLOGICAL FEATURES

GLOMERULI

Present 0 1 Number 24 Necrosis 0 1 2

Sclerosis - focal 0 1 2 Capsule dilated 0 1 Capillary perfusion 0 1

- diffuse 0 1 2 Cellularity 0 1 2 (normal) Number Polys per glom nil

TUBULES

Normal 0 1 Atrophic 0 1 2 Necrosis 0 1 2

Regeneration 0 1 2 Dilated 0 1 2 Casts 0 1 2

INTERSTITIUM

Normal 0 1 Oedema 0 1 2 Fibrosis 0 1 2

Haemorrhage 0 1 2 Cellular Infiltrate (Degree) 0 1 2

Cellular Infiltrate (Type) lymphocytes, few plasma cells.

VESSELS

Normal 0 1 Oedema 0 1 2 Intimal lymphocyte adherence 0 1 2

Intimal fibrosis 0 1 2 Cellular infiltrate 0 1 2 Necrosis 0 1 2

GENERAL

Infarction 0 1 Obstruction 0 1 Ischaemia 0 1

CONCLUSION Severe cellular rejection

KEY

0=No 1=Yes

0=None

1=Slight

2=Substantial

B8 PODGE - Group 2.

Day of biopsy. (Biopsy number)	Score: 6	8	10	12	Conclusion:
6- (15)	0	1	3	4	Severe cellular rejection

Illustration 14 - Primary dog renal allograft at six days. CyA at 20mg/kg/day. Laboratory reference number-15. Severe cellular rejection with lymphocyte infiltrate and intimal lymphocyte adherence to arteriole. (H & E x400).

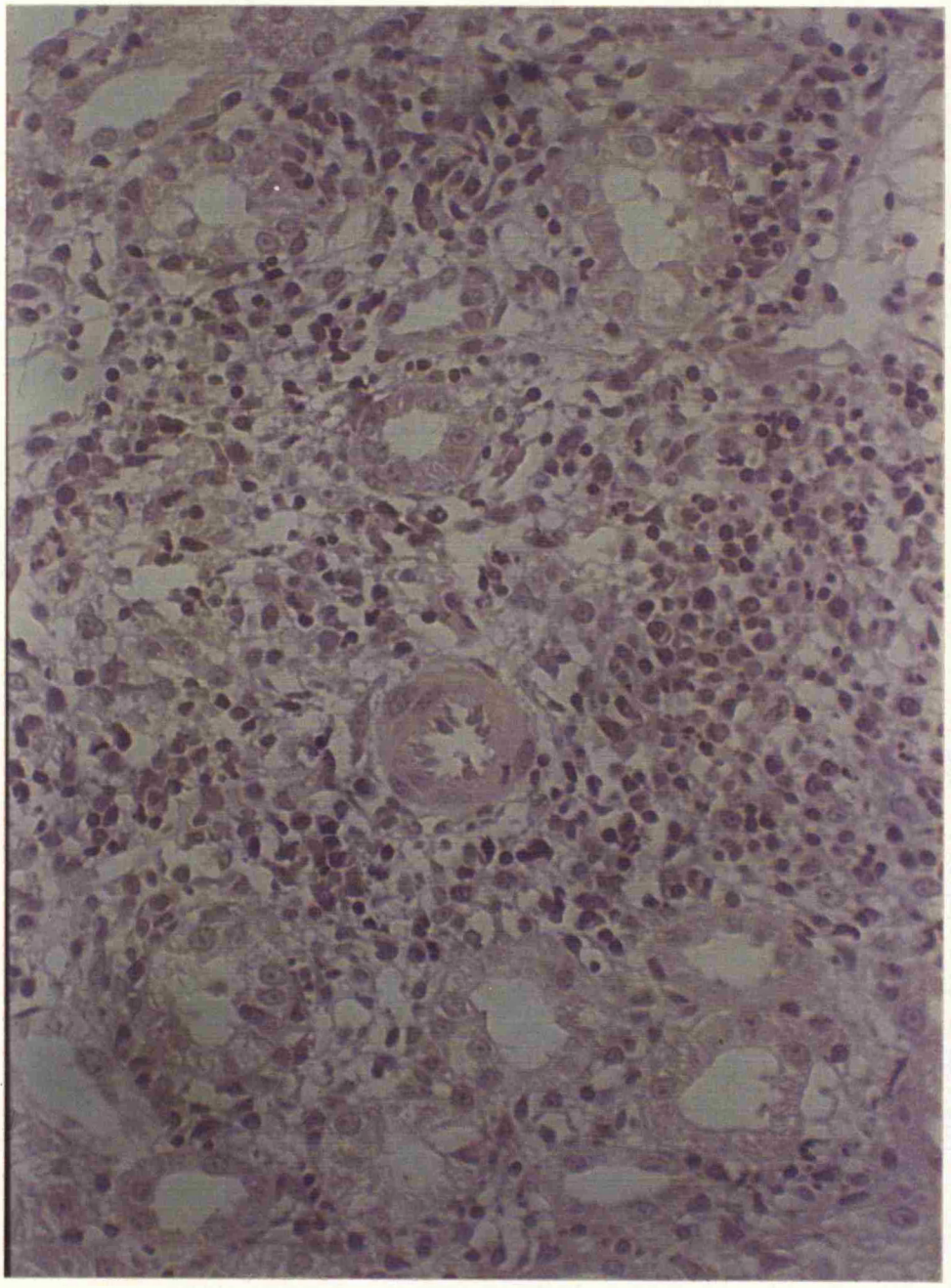
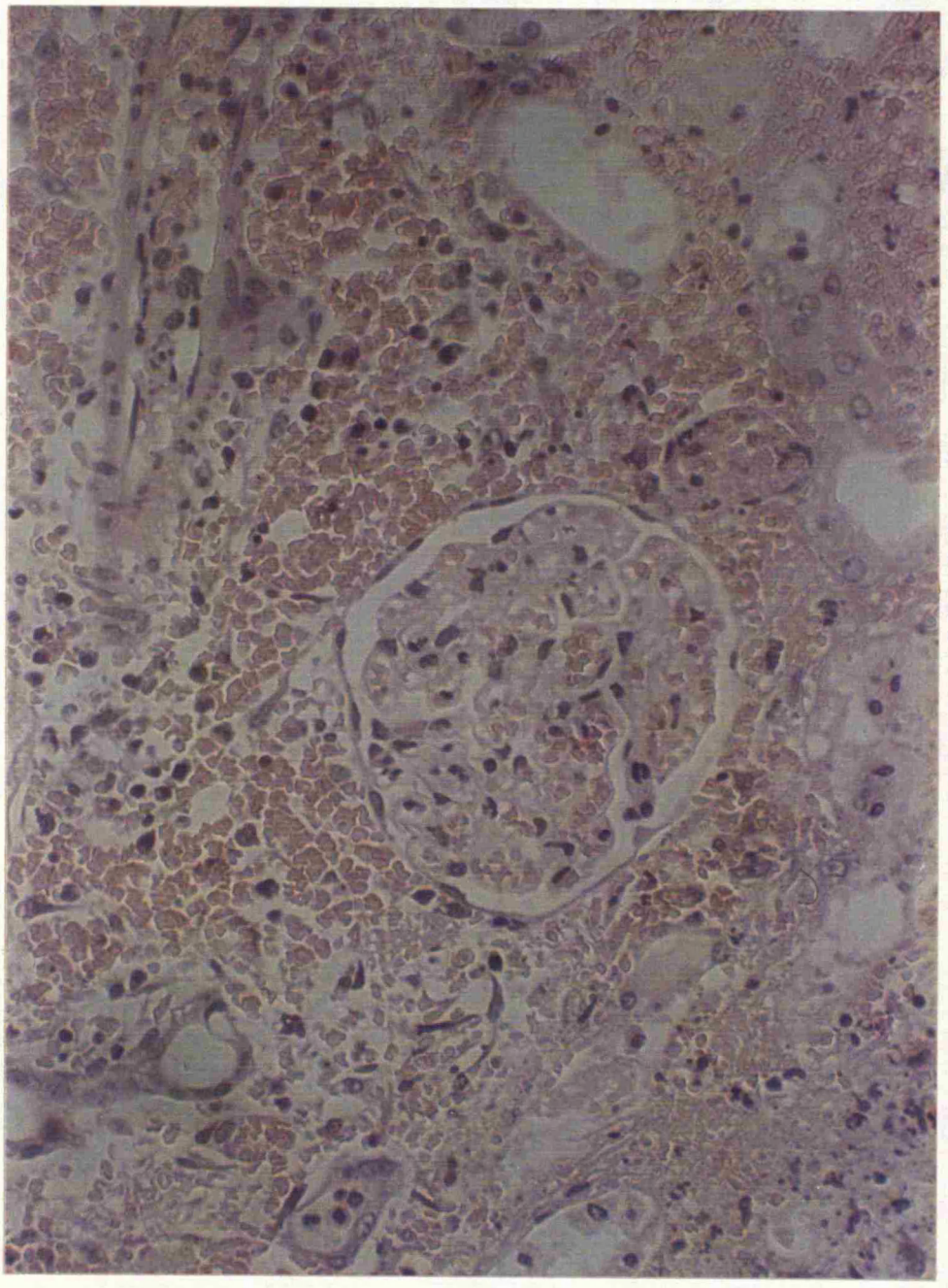


Illustration 15 - Second donor specific dog renal allograft at five days. No immunosuppression. Laboratory reference number-60. Interstitial haemorrhage with necrotic glomerulus. (H & E x400).



DOG RENAL TRANSPLANT BIOPSY.

LAB REF NO 60 DATE OF BIOPSY 4.3.85 DATE OF TX 27.2.85

HISTOLOGICAL FEATURES

GLOMERULI

Present 0 1 Number ++ Necrosis 0 1 2

Sclerosis - focal 0 1 2 Capsule dilated 0 1 Capillary perfusion 0 1 many thrombosed

- diffuse 0 1 2 Cellularity 0 1 2 Number Polys per glom <1

TUBULES

Normal 0 1 Atrophic 0 1 2 Necrosis 0 1 2

Regeneration 0 1 2 Dilated 0 1 2 Casts 0 1 2

INTERSTITIUM

Normal 0 1 Oedema 0 1 2 Fibrosis 0 1 2

Haemorrhage 0 1 2 Cellular Infiltrate (Degree) 0 1 2

Cellular Infiltrate (Type) mainly neutrophils, some plasma cells

VESSELS

Normal 0 1 Oedema 0 1 2 Intimal lymphocyte adherence 0 1 2

Intimal fibrosis 0 1 2 Cellular infiltrate 0 1 2 (polys) Necrosis 0 1 2 + thrombosis

GENERAL

Infarction 0 1 Obstruction 0 1 Ischaemia 0 1

CONCLUSION Suggests acute haemorrhagic rejection plus infarction.

KEY

0=No 1=Yes

0=None

1=Slight

2=Substantial

B9 EBONY - Group 3.

Day of biopsy. (Biopsy number)	6	Score: 8	10	12	Conclusion:
5 - (60)	5	5	7	9	Acute haemorrhagic rejection + infarction.

5.2.2.2 Method.

Dogs were anaethetised with a combination of etorphine hydrochloride (IMMOBILON ™ - C-Vet Ltd) and midazolam (HYPNOVEL™ - Roche Products Ltd). An incision was made over the kidney and two cores of kidney tissue were taken under direct vision using a Tru-Cut needle. One core was placed in formalin and the other in liquid nitrogen. There was often bleeding from the cortex of the kidney, but this was always stopped by direct pressure applied for 5 minutes. A single suture was placed in the skin.

It was soon apparent that the friendly nature of the dogs would permit renal biopsy without general anaesthesia, and the technique of biopsy under local anaesthesia was used successfully on over 150 occasions. Dogs were pre-medicated with twice the normal dose of ACP, intramuscularly, 30 minutes before the procedure. The dog was brought to the operating room and made comfortable on the table. The biopsy site was shaved and cleaned . One ml of 1% lignocaine was infiltrated subcutaneously with a 25G needle. This frequently caused transient discomfort as the lignocaine was being injected. One minute later a small stab incision (2-3mm) was made through the skin with a number 11 scalpel blade, to allow the biopsy needle to pass through the skin without pressure. The kidney was steadied with one hand, the needle advanced into the kidney with the other hand, and the two biopsies taken. Providing the $3^1/2$ inch needle was used, the biopsy needle could be readily operated with one hand. Direct pressure was then applied until haemostasis was satisfactory. The wound was sprayed with a plastic spray dressing. Antibiotic prophylaxis was not used. Illustrations 16 - 22 show the technique of renal allograft biopsy.

5.2.2.3 Results

There were 180 needle biopsies performed and reported in this study. Forty-five were taken at the time of transplantation before wound closure. In these circumstances haemostasis was obtained by direct pressure. When the biopsy was taken before reperfusion the kidney capsule was repaired with a 7 /0 Prolene stitch. Forty-one biopsies were taken at the time of transplant nephrectomy. The remaining 94 biopsies were taken by percutaneous biopsy and at least two passes were made on each occasion so as to obtain two cores. On two occasions a brisk haemorrhage occurred down the ureter. On both occasions the bleeding responded to pressure. As the kidney lies in a superficial pouch it was readily palpable, and thus bleeding into the ureter was uncommon, because the needle could be aimed so as to traverse the kidney cortex.

Illustration 16 - Beagle recipient prior to renal allograft biopsy. Sedated with acetylpromazine maleate.

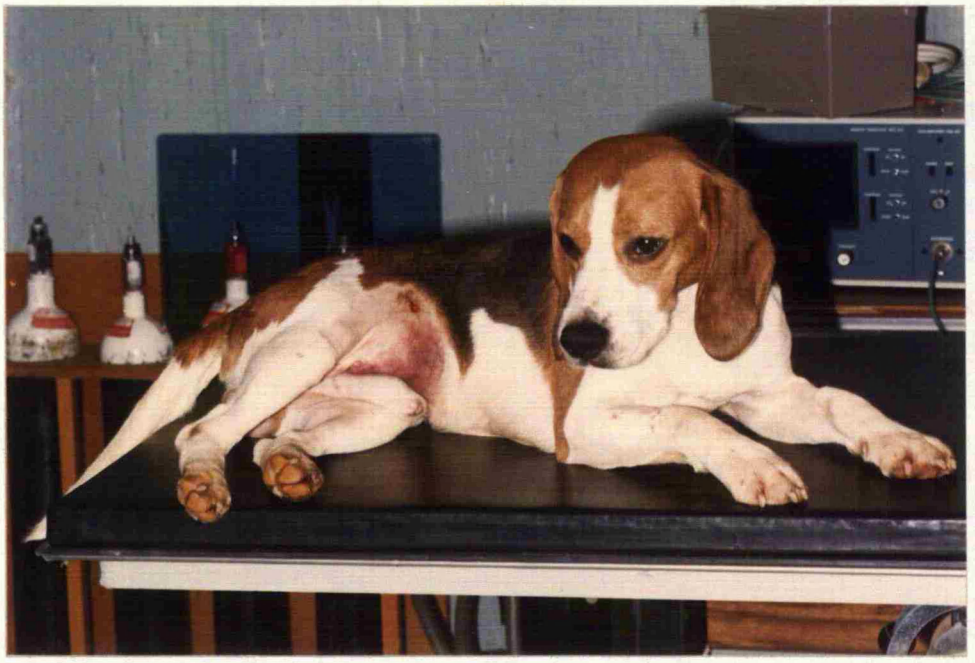


Illustration 17 - Infiltration of the skin with 1% lignocaine through a 25 G needle.

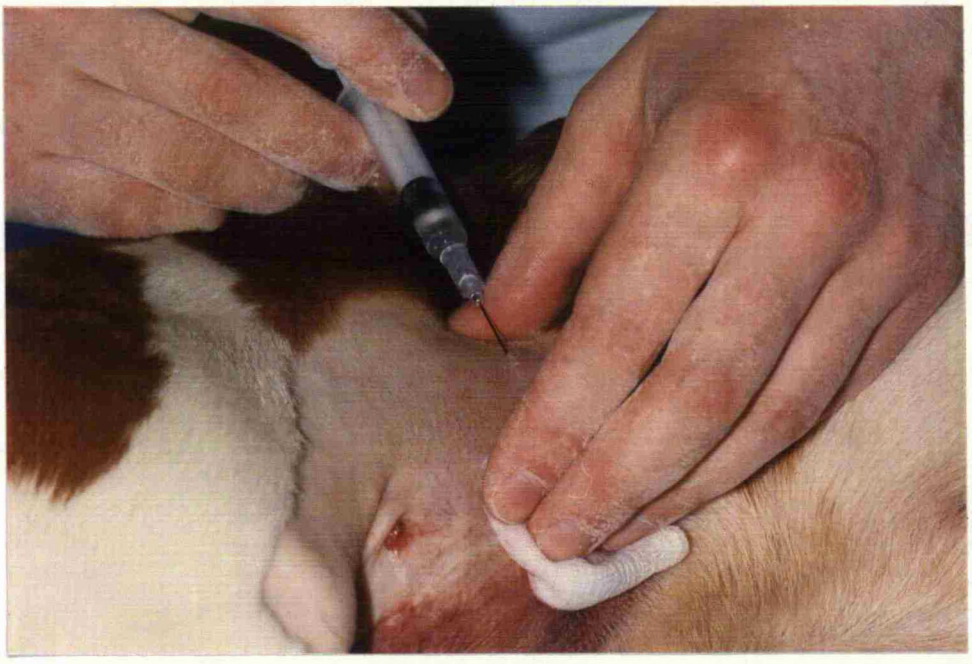


Illustration 18 - Stab incision with number 11 scalpel blade.

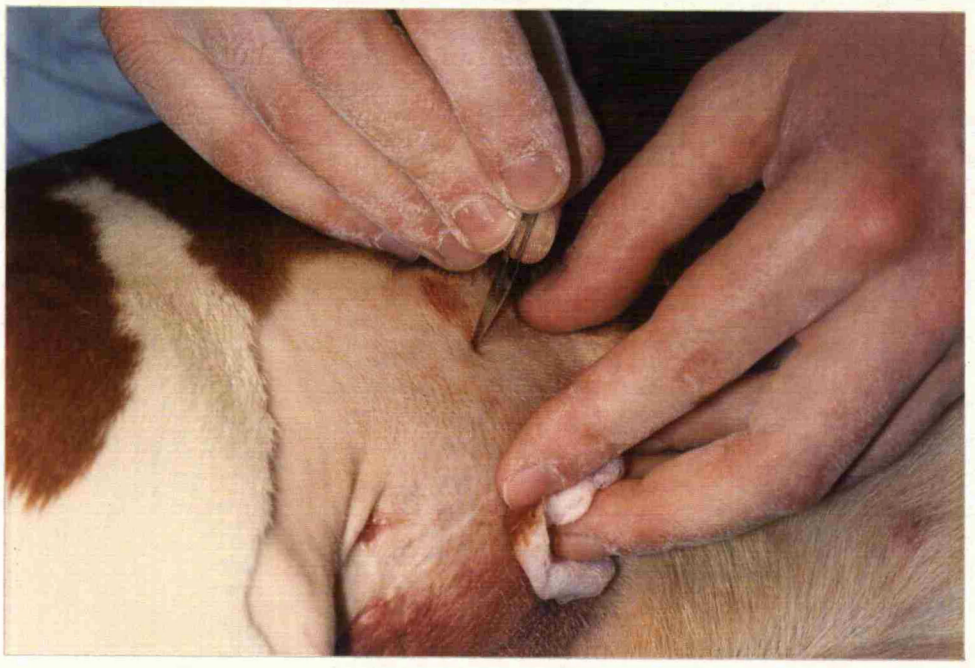


Illustration 19 - Tru-Cut needle, three and a half inches long needle, and renal allograft in subcutaneous groin pouch.

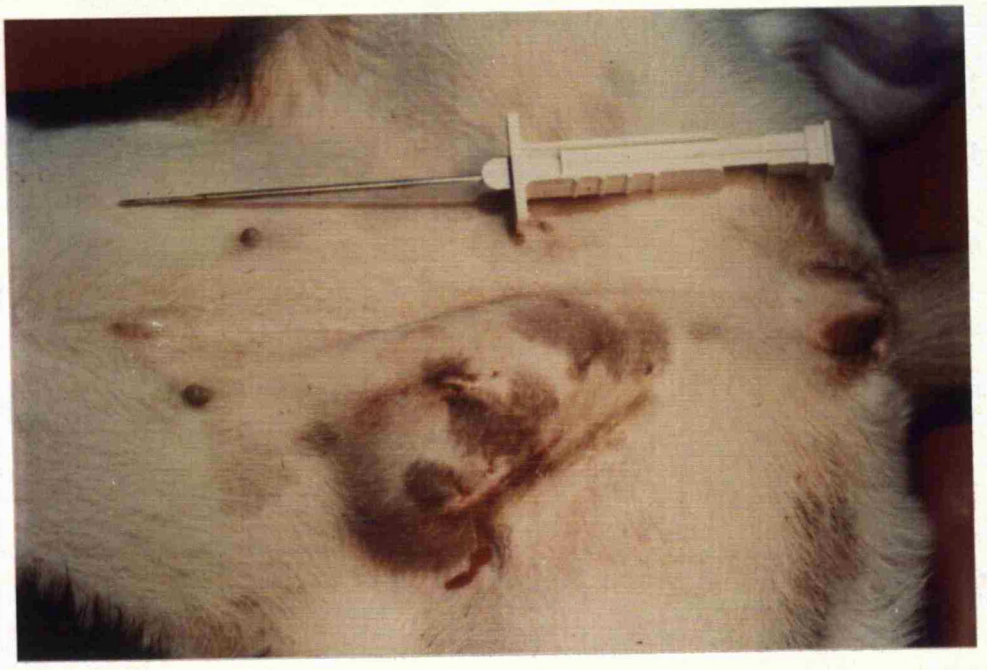


Illustration 20 - Biopsy needle aligned for pass through kidney cortex.

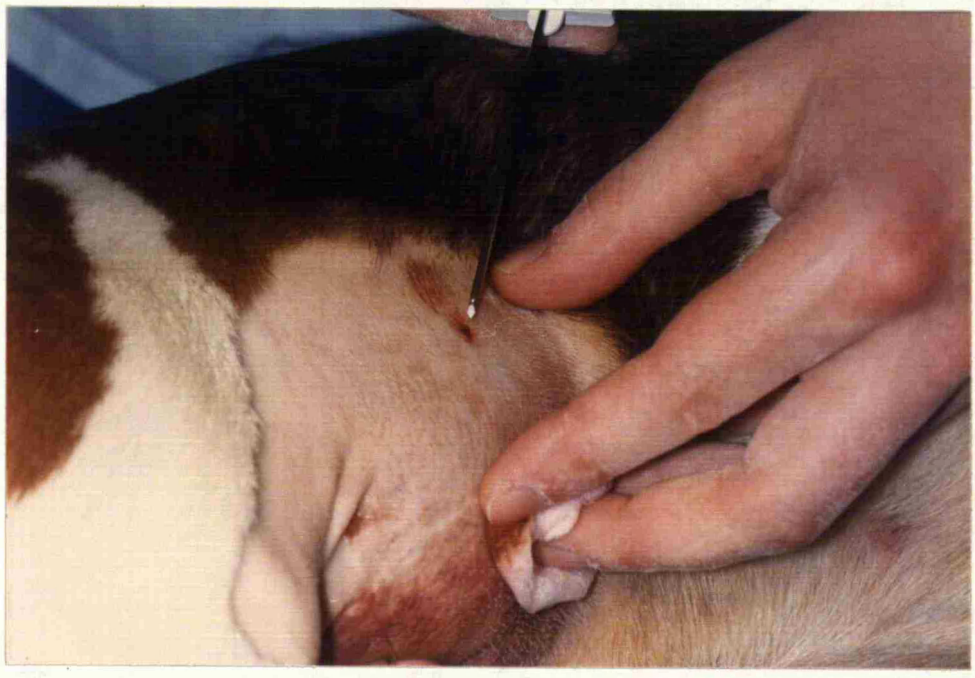


Illustration 21 - Core of allograft kidney.

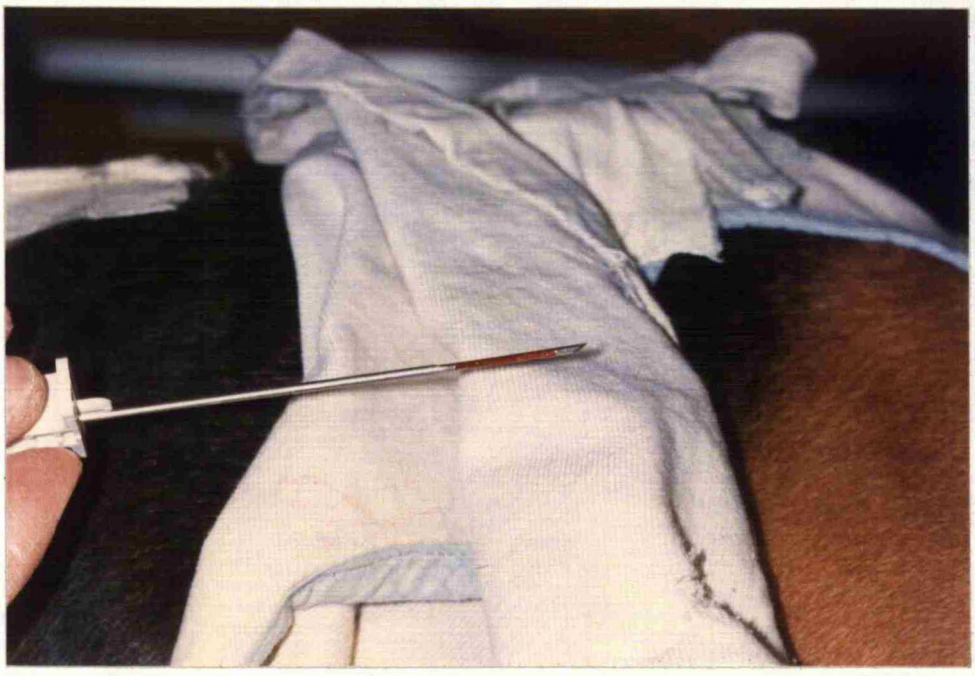
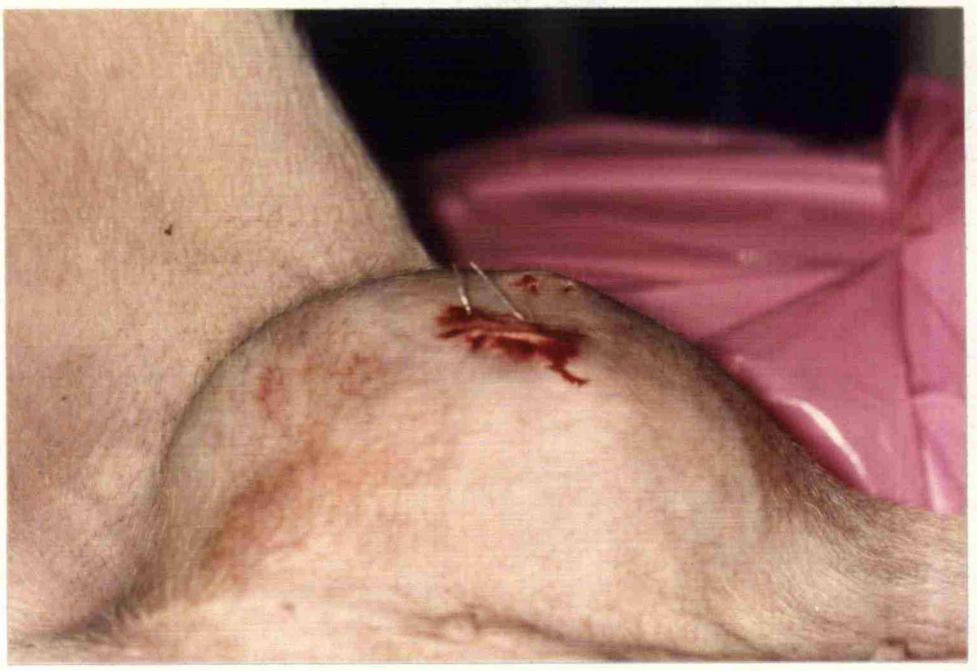


Illustration 22 - An enlarged rejecting renal allograft. Biopsy site has been sutured.



5.2.3 Cyclosporin A

CyA was indicated as the basic immunosuppression in this study, because in general allosensitised patients do better with CyA than Aza. Steroids were not given, as their affect is marginal in preventing rejection in clinical practice.

CyA was given once daily in the morning, by syringe into the back of the mouth. The dogs did not appear to be affected, apart possibly from occasional diarrhoea. This emphasised the importance of monitoring CyA levels as the absorption of CyA may have been variable. Initially the CyA levels in serum were measured by a radioimmunoassay (RIA) method. Trough samples were taken, just before the next dose was given.

Nine months after starting this work the laboratory switched to a high performance liquid chromatography (HPLC) method for CyA monitoring, using whole blood as opposed to serum. Several profiles were done on the beagles, with regular blood samples taken during the day (Figures 5.2-5).

Figure 5.2- CyA profile of beagle B18 after two doses of oral CyA.
Time 0 = 0845h on 5.11.86.

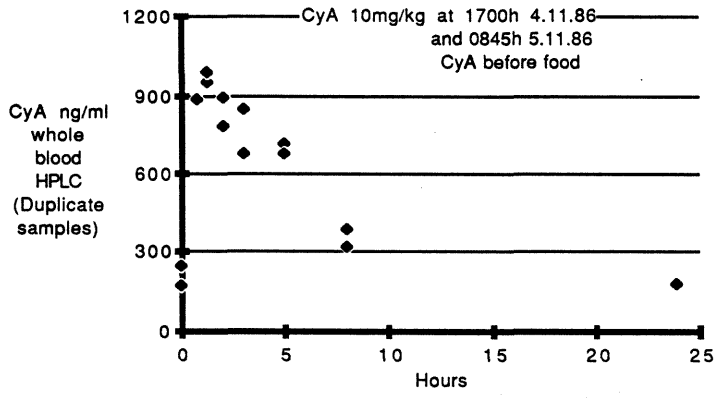


Figure 5.3- Two 24 hour whole blood CyA profiles in beagle B17 on days 1 and 26. CyA dose was 25mg/kg/day orally.

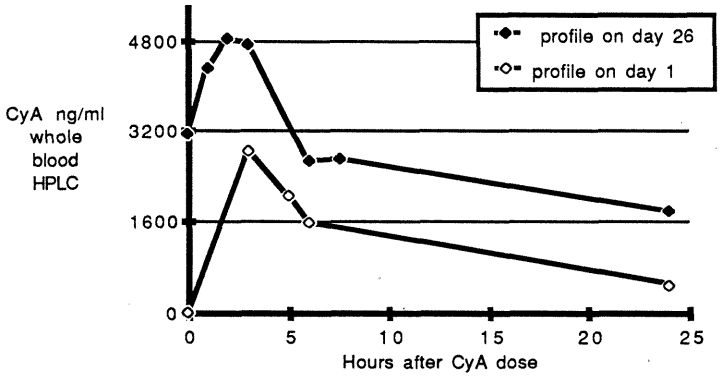


Figure 5.4- Two 24 hour whole blood CyA profiles in beagle B2 on days 1 and 26. CyA dose was 25mg/kg/day orally.

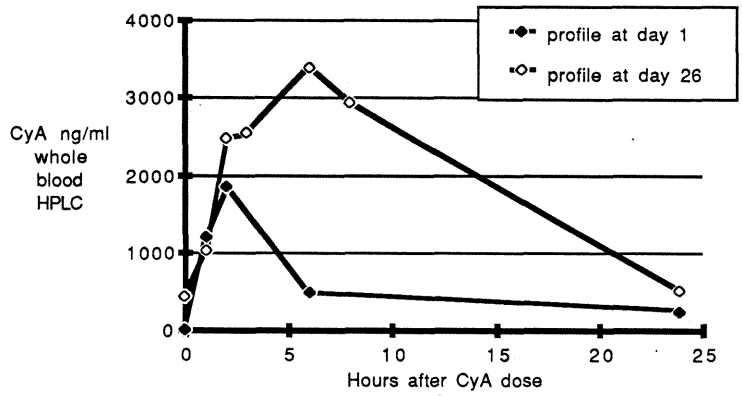
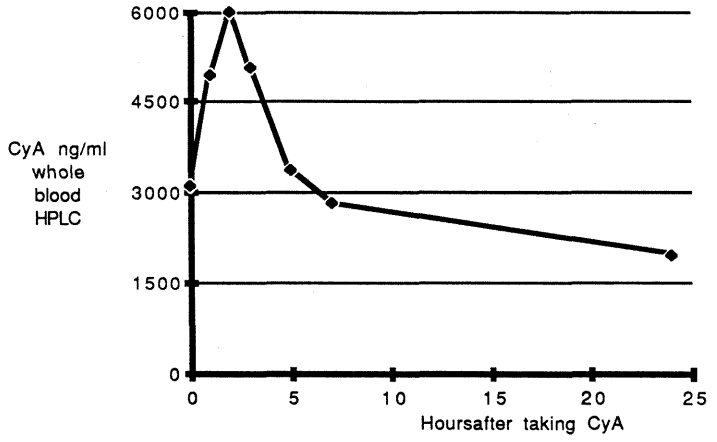


Figure 5.5- 24 hour profiles for B19 on day 12 of CyA at 25mg/kg/day orally.



These profiles indicate that oral CyA at 25mg/kg/day produced excellent CyA levels as determined by HPLC. The literature shows that there is a dose response relationship between CyA and primary renal allograft survival, though the therapeutic range of CyA levels has not been examined in the dog as extensively as it has been in man.

5.3 The generation of alloantibody in the dog.

Three methods were used to generate alloantibody in the beagles against the donor greagles:

1) Skin "grafting".
2) Blood transfusion.
3) Kidney graft.

Recipients were screened for alloantibody against donor peripheral blood lymphocytes (pbl) with a complement mediated lymphocytotoxicity assay (appendix-1).

5.3.1 Skin Grafting

The use of repeated skin grafting to generate alloantibody has been well described. The more rapid rejection of successive grafts provides visual confirmation that the recipient has become sensitised to the donor. For this to be achieved the skin graft must take, ie it must become vascularised by the ingrowth of capillaries from the donor.

Methods:

Skin grafting the young beagle dogs was a battle of wills, won by the beagles. They were grafted at a playful age (six months), and made short work of getting rid of dressings. Three different methods of skin grafting were employed, before resorting to the successful technique of injecting diced skin subcutaneously.

1) Primary full thickness graft (PSG).
2) Delayed full thickness graft (DSG).
3) Buried delayed full thickness graft.
4) Subcutaneous injection of diced skin (DSsc).

Primary full thickness graft.

The donor greagle was anaesthetised with a combination of etorphine hydrochloride (IMMOBILON ™ - C-Vet Ltd) and midazolam (HYPNOVEL™ - Roche Products Ltd) and two full thickness ellipses of skin (5cm x 2cm) were taken. The incision was closed in two layers with catgut and Supramid. The skin was thoroughly defatted with a scalpel. The donor was revived with diprenorphine hydrochloride (REVIVON™ - C-Vet Ltd), and given 1ml of Duplocillin intramuscularly.

The back of the recipient's neck was prepared for the graft, because this was the least accessible site to the recipient beagle, and a dressing could be applied so as to resemble a collar, with the least disturbance to the dog. The recipient was anaesthetised with etorphine hydrochloride and midazolam, and 1ml of Duplocillin given. An ellipse of skin was removed from the back of the neck, together with underlying fat and fascia so as to give a muscle bed. The graft was anchored with interrupted 4/0 Prolene sutures, and two stabs made through the graft with a number 11 blade so as to prevent accumulation of fluid under the graft. A dressing was placed over the graft so as to immobilise the graft. The graft was inspected on days 3, 6, 8 & 10.

Delayed full thickness graft.

The graft bed was created in the recipient seven days before skin grafting. The graft was therefore placed on a granulating wound. As well as the neck, a foreleg was also used as described by McKeever (1978). A site on the craniolateral surface of the forelimb, just below the elbow, was prepared. To protect the site, the leg was encased in a lightweight waterproof plaster cast. Unfortunately these proved an irresistible challenge to the beagles, who had the casts off within 36 hours.

Buried delayed full thickness graft.

A granulating site was prepared on the back of the neck. Prior to grafting the margins of recipient skin were undermined so that the recipient skin could be closed over the skin graft. This provided a secure biological dressing, but prevented inspection of the graft.

Subcutaneous injection of diced skin.

The donor was anaesthetised, and an ellipse of skin taken from the flank. The wound was closed in two layers. The skin was then chopped up into little pieces so that it could be injected from a 2ml syringe through a 14G needle. This injection was readily given subcutaneously to the recipient without any preparation. The recipient received 1 ml of Duplocillin intramuscularly.

The outcome of the skin grafts was assessed in two ways:

1) by visual inspection.
2) screening sera from the recipient for alloantibody against the donor pbl.

The grafts were inspected daily after the third day, and viability assessed by the colour of the graft. Grafted skin was either white, reddish blue, black, or pink in colour. A white colour was indicative of an avascular graft, which had started to undergo necrosis and slough. A reddish blue colour indicated that revascularisation (reddish) and dry ischaemic necrosis (blue) were occurring. These grafts had partial survival. Black indicated that dry ischaemic necrosis had occurred, this skin became leathery and sloughed. A pink colour indicated revascularisation. Providing the graft was at least partially revascularised one could expect allosensitisation of the recipient to occur. The survival of successive grafts from the same recipient should be reduced as a consequence of this allosensitisation.

Results:

Donor (Greagle). Recipient (Beagle).

G1 to B1 & B2 .

G2 to B3 & B4 .

Primary full thickness skin graft to unprepared site (PSG): n=6

In the following tables the recipients are placed horizontally with the time from grafting vertically. The state of the skin graft is defined using the following key with percentage areas - P=pink; RB=reddish black; B=black; W=white.

	Recipients:			
18.9.84 = Day 0.	B1	B2	B3	B4
Day 3	50% RB 50% P	50% RB 50% P	50% RB 50% P	50% RB 50% B
Day 6	100% RB	100% B	100% RB	100% B
Day 8	100% B	100% B	100% B	100% B

24.10.84 = Day 0	B1	B2
Day 3	100% RB	100% RB
Day 7	100% B	100 % B

Parts of some grafts were possibly viable at day 3. As the recipients were unsensitised at the outset, one might have expected to see signs of viability with a primary skin allograft at days 6 and 8. The appearance of the grafts at these times suggested that they had not been revascularised. This, however, might not preclude some sensitisation of the recipient. The results of sensitisation by skin grafting are shown in figure 5.6, and no alloantibody was detected after PSG.

Delayed full thickness skin graft (DSG): n=8

To neck.

6.11.84=Day 0	B1	B2	B3	B4
Day 3	50% P 50% RB	100% B	100% RB	100% RB
Day 6	100% B		100% B	100% B

To neck.

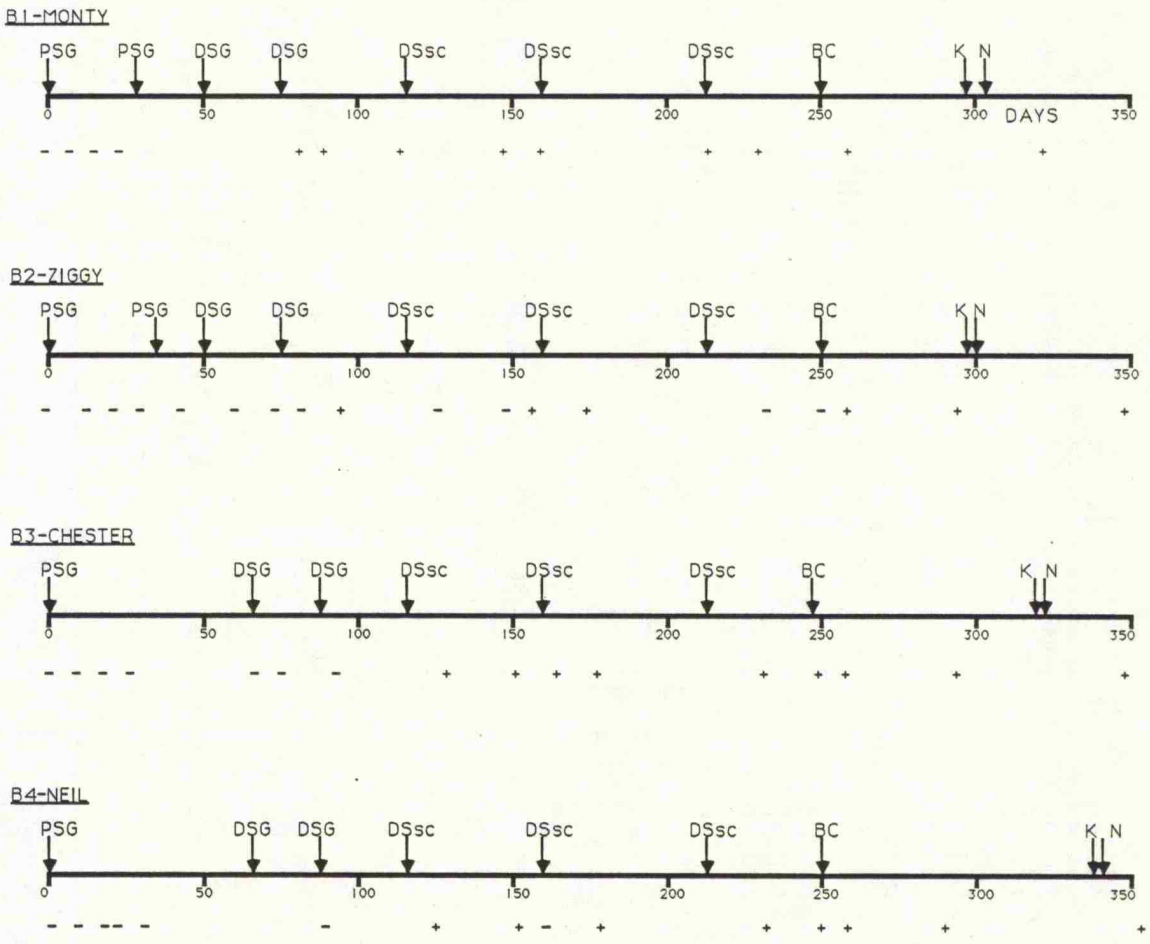
4.12.84= Day 0	B1	B2
Day 3	100% RB	100% B
Day 6	100% B	

To leg

22.11.84= Day 0	B3	B4
Day1	Dressing and graft removed by dog.	

Referring to figure 5.6, alloantibody was detected after DSG in B1 and B2, but not in B3 and B4.

FIGURE 5.6 SENSITISATION BY SKIN GRAFTING - FOUR BEAGLES.



PSG= PRIMARY FULL THICKNESS SKIN GRAFT

DSG= DELAYED FULL THICKNESS SKIN GRAFT

DSsc= DICED SKIN INJECTED SUBCUTANEOUSLY

BC = DONOR SPECIFIC BUFFY COAT GIVEN INTRAVENOUSLY

K = DONOR SPECIFIC KIDNEY ALLOGRAFT

N = TRANSPLANT NEPHRECTOMY

\- = NO DONOR SPECIFIC ALLOANTIBODY AGAINST PBL's BY CDC ASSAY

± = LESS THAN 50% KILL OF DONOR PBL's

\+ = DONOR SPECIFIC ALLOANTIBODY AGAINST PBL's BY CDC ASSAY

Burying full thickness graft under a flap of recipient skin. (n=4):

To neck	B1	B2	B3	B4
4.12.84	*	*		
14.12.84			*	*

(* Graft not visible).

These results were discouraging. In non of the grafts was there convincing evidence of revascularisation. McKeever (1978) using autogenous skin grafts reported a success rate of 90% for delayed partial thickness grafts and 59% for full thickness grafts. Their dogs were housed in individual cages, which may have helped the skin grafts to stay in place. With full thickness grafts of the size used here one would have expected half the grafts to have taken using the technique of delayed grafting. There was some take of DSG's, but this was unpredictable, only being observed in 2 out of 4 beagles. This was probably related to the mobility and determination of these young recipient beagles to remove any dressings. It is likely that partial thickness grafts would have been more likely to become revascularised, but this would still have left the problem of dressings.

With subcutaneous grafting it was not possible to assess the graft. A simple alternative seemed to be to simply inject diced skin (DSsc). This was simple, only the donor required an anaesthetic. The demonstration of sensitisation was now dependent on the detection of alloantibody, and not the demonstration of second set allograft rejection. The injection of diced skin might have presented antigen differently to the recipient compared with a vascularised skin graft. It was decided not to use an adjuvant at first, and it indeed proved possible to get sensitisation without the use of an adjuvant. Figure 5.6 shows that all four beagles were consistently sensitised following DSsc. From this time on skin grafting was abandoned and diced skin was given by subcutaneous injection.

These four beagles subsequently received a renal allograft from their skin donors, and comprised experimental group 4, as described in the summary.

5.3.2 Blood Transfusion

The dog blood groups have been designated by capital letters of the alphabet, beginning with A, in the order in which the systems were encountered. Swisher (1961) observed in contrast to man, that reciprocal antigens and isoantibodies do not occur regularly in the dog. A single transfusion can nearly always be safely performed between dogs without reactions. Compatibility for the canine A factor is probably of greatest importance as the canine anti-A is a powerful haemolytic antibody, and the A factor is the most antigenic dog erythrocyte

antigen. Dogs lacking the A factor will regularly be immunised by transfusion as the A factor is present in 63% of dogs with the risk of transfusion reactions with subsequent blood transfusions. These reactions are rarely fatal in dogs particularly in conscious dogs. These difficulties are most easily avoided by using A-negative donors only as donors.

No association between red cell compatibility and the pattern of renal allograft rejection in dogs has been demonstrated.

We did not red cell type our donor/recipient combinations, though red cell crossmatches were performed. We detected no haemolysins and saw no evidence of transfusion reactions.

Method:
Blood was taken from the donor and 30-50 mls injected intravenously into the recipient. The blood transfusion was repeated until lymphocytotoxic antibodies against donor pbl could be detected in the serum of the recipient.

These four beagles subsequently received a renal allograft from their blood donors, and comprised experimental group 5, as described in the summary.

5.3.3 Kidney grafting

See section 5.1.1 above for the methods. The three beagles in receipt of a renal allograft comprised experimental group 1, as described in the summary. Subsequently they received a second renal allograft from the same donor, experimental group 3. Beagles receiving a primary renal allograft with CyA comprised experimental group 2.

5.3.4 Results of allosensitisation- the detection of alloantibody.

Skin (Figure 5.6):
No alloantibody was detected after one or two primary skin grafts (PSG). Beagles B1 & B2 were given 2 PSG's and 2 delayed skin grafts (DSG), alloantibody was detected after the fourth graft. Beagles B3 & B4 were given 1 PSG, 2 DSG's, and 1 subcutaneous injection of skin (DSsc), alloantibody was detected after the fourth graft. Each beagle was given one injection of donor buffy coat in an attempt to further boost alloantibody levels. All four beagles had donor specific alloantibody prior to renal allografting. Following allograft nephrectomy, alloantibody was still present from 25 to 50 days later, after which time the beagles received more alloantigen.

Blood (Figure 5.7):
With B11 & B12 alloantibody was detected after the second blood transfusion, both then received two more transfusions. Prior to renal allografting B12 was negative and the sera from B11 was not screened. Both B11 and B12 were positive after allograft nephrectomy. B14 was positive after two transfusions, B15 remained negative despite three transfusions. After allograft nephrectomy B14 and B15 had persistent alloantibody 150 days later, after which time they again were given more alloantigen.

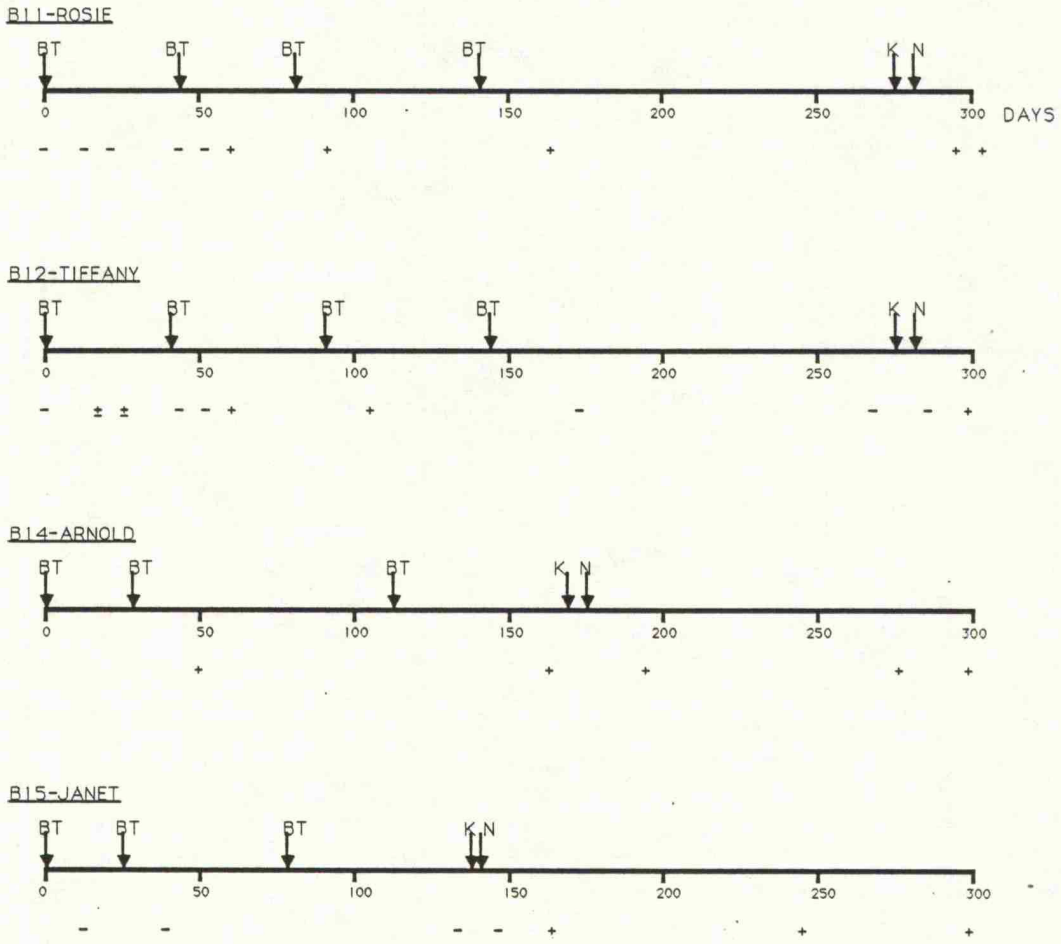
Kidney (Figure 5.8):
Following a renal allograft without immunosuppression all three beagles became allosensitised. Alloantibody was still detected some 50 days after nephrectomy when a further kidney was grafted from the same donor. Following this second kidney from the same donor as the first kidney, two survivors had detectable alloantibody some 200 days later, despite allograft nephrectomy shortly after transplantation.

Kidney with CyA (Figure 5.9):
Following a primary renal allograft CyA was given orally for 21 days, and all kidneys remained in situ beyond this time. Only B6 failed to develop alloantibody, this could have been related to the presumed renal artery thrombosis, which occurred within the first five days in this beagle. In the three other dogs alloantibody persisted at 250 -300 days after allograft nephrectomy.

It is difficult to draw many conclusions from these serological studies on the natural history of canine alloantibody. The data presented represents a tremendous amount of screening work. It can be seen that a renal allograft was highly effective at generating alloantibody, which was shown to persist for at least 250 days after allograft nephrectomy. Skin "grafting", where the skin was given by subcutaneous injection also proved effective at alloantibody generation. The effect of blood transfusion was variable, and the alloantibody response may not have been persistent. It was not surprising that CyA for 21 days did not prevent allosensitisation where the allograft remained in situ beyond this time.

In the experiments on allosensitisation described above, each recipient was exposed to alloantigen from a particular donor, and then screened against the same donor. The allosensitised recipients then went on to receive a kidney from the same donor (see section 5.5). Subsequently recipients were exposed to antigen from several potential donors, and recipients were screened against panels of prospective donors so as to select appropriate recipient-donor pairs for the later experimental groups (groups 6, 7, 8, and 9).

FIGURE 5.7 SENSITISATION BY BLOOD TRANSFUSION - FOUR BEAGLES.



BT = BLOOD TRANSFUSION

K = DONOR SPECIFIC KIDNEY ALLOGRAFT

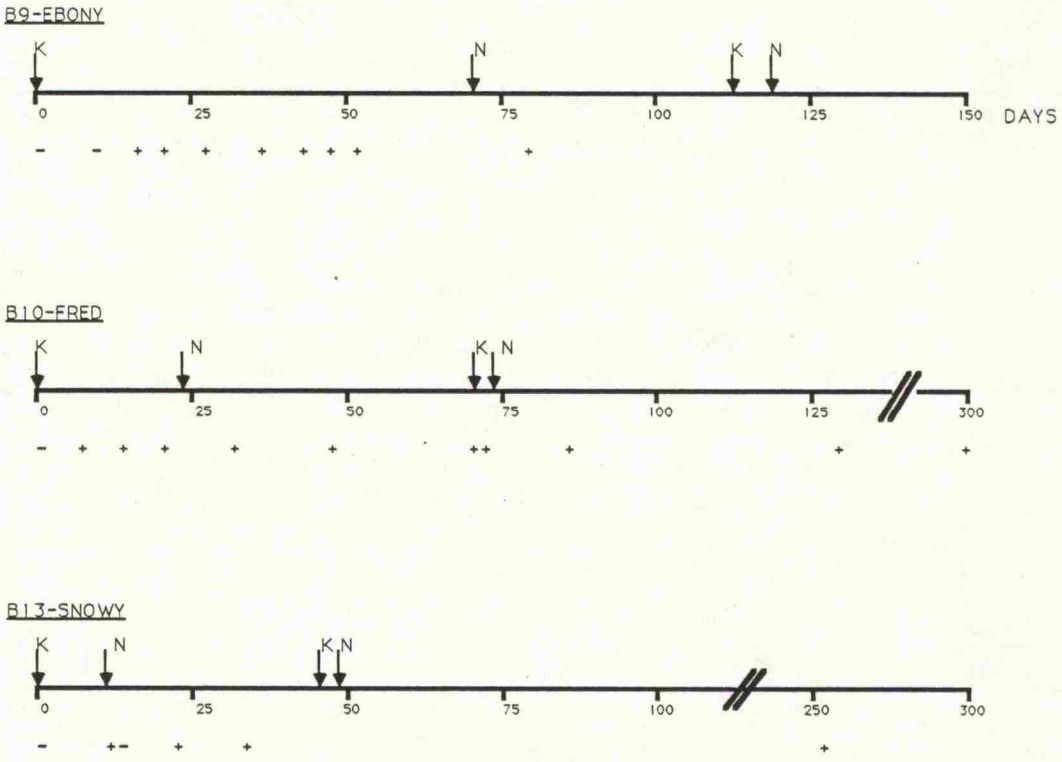
N = TRANSPLANT NEPHRECTOMY

- = NO DONOR SPECIFIC ALLOANTIBODY AGAINST PBL's BY CDC ASSAY

± = LESS THAN 50% KILL OF DONOR PBL's

+ = DONOR SPECIFIC ALLOANTIBODY AGAINST PBL's BY CDC ASSAY

FIGURE 5.8 SENSITISATION BY KIDNEY ALLOGRAFT - THREE BEAGLES.



K = DONOR SPECIFIC KIDNEY ALLOGRAFT

N = TRANSPLANT NEPHRECTOMY

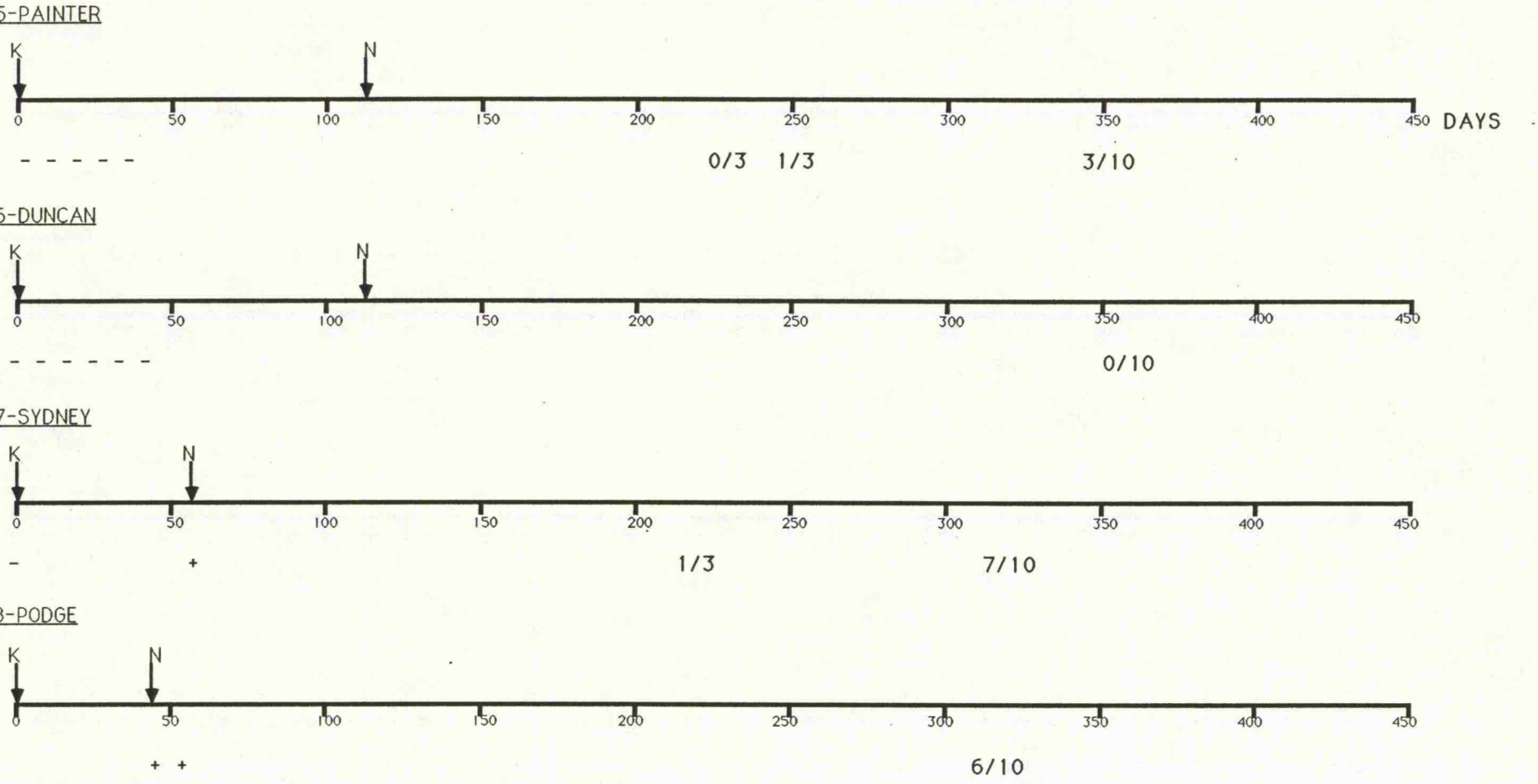
\- = NO DONOR SPECIFIC ALLOANTIBODY AGAINST PBL's BY CDC ASSAY

\+ = DONOR SPECIFIC ALLOANTIBODY AGAINST PBL's BY CDC ASSAY

FIGURE 5.9 SENSITISATION BY KIDNEY ALLOGRAFT - FOUR BEAGLES.

ORAL CYA GIVEN FOR 21 DAYS.

K = Kidney allograft
N = Transplant nephrectomy



5.4 Transplantation of unsensitised recipients

5.4.1 Introduction

The first dog renal allografts were to unsensitised recipients with a negative complement dependent lymphocytotoxicity crossmatches (CDC test), and compared the outcome in dogs receiving CyA as sole immunosuppression (Group 2-unsensitised, negative CDC, and CyA) and dogs receiving no immunosuppression (Group 1-unsensitised, negative CDC, and no immunosuppression). Recipients had not been exposed to alloantigen, and were not sensitised against their prospective donor as judged by a negative CDC test against donor pbl.

5.4.2 Methods

In this study all the recipients were beagles, each beagle had a number with the prefix 'B' and a name. All but five of the donors were greagles, the remainder were greyhounds. They were identified by the prefix 'G', a number and name. This means of identifying the experimental animals was used throughout this study.

Group 1 comprised three beagles, who each received one renal allograft from a different greagle, without immunosuppression. The results of alloantibody production in these beagles was described above - figure 5.8. Group 2 comprised seven beagles, who each received one renal allograft from a different greagle, and CyA as immunosuppression. The first four dogs in group 2 were given CyA orally at a dose of 20 mg/kg in a single daily dose starting on the day of surgery. The CyA was stopped 21 days after transplantation. The biopsy results and CyA trough levels (RIA-serum) suggested that this dose of CyA was inadequate when given in this way. In the remaining three beagles in group 2, and in all subsequent groups, CyA was given at a dose of 25 mg/kg/day, starting one day before transplantation. A 21 day course of CyA was given because the period of greatest risk to the allosensitised recipient is the first few weeks after transplantation. Thus if useful function persisted at 21 days in an allosensitised recipient, then the protocol used might have a clinical application.

Beagles were transplanted and managed as described in section 5.1.1 . Where only one kidney was removed from a greagle donor, the dog was recovered.

Each biopsy was identified by a number, and this was the only information that the reporting pathologist was given. The sections were stained with haematoxylin and eosin. The biopsy results are shown in a tabular format, with the day from transplantation, the biopsy number, the biopsy scores, and conclusion. As previously described each biopsy was scored for the three class 1 features out of 6, for class 1 and 2 features out of 8, for class 1, 2, and 3 features out of 10, and for class 1, 2, 3, and 4 out of 12. It was not always possible to score all the features, usually because of infarction of the graft, and only rarely because of

an inadequate biopsy. The histology scores are presented graphically, with the score on the 'y' axis and day after transplantation on the 'x' axis. The four scores (6, 8, 10, and 12) were individually plotted, and it was the score out of 6 for class 1 features alone which gave the best separation of the experimental groups. Only the graphs for the class 1 features are shown.

The timing and frequency of the biopsies necessitated a compromise between the ideal of daily biopsies and avoiding swamping the Department of Histopathology. In the unsensitised recipient biopsies were performed weekly, partly because these first biopsies were done on anaethetised dogs. It was planned to use the information from these biopsies to choose a suitable biopsy protocol for the allosensitised recipients.

5.4.3 Results

GROUP ONE

PRIMARY KIDNEY ALLOGRAFT. NO CyA. NEGATIVE CDC CROSSMATCH. N=3.

B9 EBONY

Day of biopsy. (Biopsy number)	Score: 6	8	10	12	Conclusion:
0-pre reperf (18)	0	0	0	0	Normal kidney
6- (21)	5	7	9	11	Severe acute cellular rejection
14- (24)	-	-	-	-	Totally infarcted kidney
20- (28)	6	-	7	9	Total acute infarction
33- (35)	5	5	-	7	Total infarction- no cellular detail visible
70- (44)	6	-	-	-	Totally infarcted

pre reperf=biopsy before revascularisation.

B10 FRED

0- (23)	0	0	0	0	Normal kidney
6- (27)	3	5	7	9	Severe acute on chronic rejection.
13- (31)	-	-	-	-	Total infarction of kidney, some cellular infiltrate.
19- (34)	-	-	-	-	No renal tissue.
23- (38)	4	-	-	6	Totally necrotic

B13 SNOWY

0- (41)	0	0	0	0	Normal kidney.
2- (42)	0	0	1	0	Non specific, not typical rejection or renal disease.
9- (45)	6	8	10	11	Intense interstitial cellular infiltrate + haemorrhage. Severe rejection.

GROUP TWO

PRIMARY RENAL ALLOGRAFT. PLUS CyA. NEGATIVE CDC CROSSMATCH. N=7.

NB CyA dose was 20mg/kg/day for B1, B2, B3, & B4.

CyA dose was 25mg/kg/day for B5, B6, & B7.

B5 PAINTER

Day of biopsy.	Score:				Conclusion:
(Biopsy number)	6	8	10	12	
0-pre-reperf (1)	0	0	0	0	No significant abnormality
5 - (2)	0	0	0	0	Minimal abnormality
13-(4)	0	1	3	3	Moderate low grade rejection
23- (6)	0	1	1	1	Minimal abnormality
28- (9)	0	1	2	2	Mild cellular/possibly vascular rejection.
37- (14)	5	5	7	9	Severe acute on chronic rejection
48- (20)	-	-	-	-	Total acute ischaemic infarction. No cellular detail remaining.
79- (36)	5	5	-	7	Total haemorrhagic infarction
112- (43)	3	-	4	6	Infarcted + necrotic. Some glomeruli relatively well perfused.

B6 DUNCAN

0-pre-reperf (1)	0	0	0	0	No significant abnormality
5- (3)	4	4	5	7	Total ischaemic infarction
13-(5)					Inadequate-capsule only.
23- (7)	5	-	-	7	Total recent ischaemic infarction
28- (10)	4	4	-	6	Total recent infarction-only ghosts remain.
37- (13)	-	-	-	-	Total acute ischaemic infarction
41- (16)	-	-	-	-	Total acute ischaemic infarction

The biopsy appearances at day five suggested a renal artery thrombosis, supported by the absence of a nephrogram on the IVU.

B7 SYDNEY					
0- post reperf (8)	0	1	0	0	No evidence rejection
9- (12)	0	1	3	4	Moderately severe chronic cellular rejection.
14- (17)	0	1	3	4	Severe cellular rejection
20- (19)	1	2	4	6	Severe cellular rejection
27- (25)	0	1	3	4	Severe cellular rejection
41- (32)	1	3	5	7	Severe chronic cellular rejection
51- (37)	1	2	4	5	Severe chronic cellular rejection
56- (40)	3	3	5	7	Severe chronic cellular rejection
B8 PODGE					
0-pre reperf (11)	0	0	0	0	No evidence rejection
6- (15)	0	1	3	4	Severe cellular rejection
13- (22)	1	2	4	6	Severe cellular rejection
22- (26)	2	4	6	8	Severe acute on chronic rejection
27- (29)	2	4	6	8	Severe end stage chronic rejection
34- (30)	-	-	-	-	Total infarction
40- (33)	-	-	-	-	Total infarction
42- (39)	4	-	6	8	Virtually totally necrotic
B17 TIZER					
0- pre (96)	0	0	0	1	Collapsed capillaries,
0- post (95)					Inadequate specimen
2-(97)	0	0	0	1	Early ATN.
7- (99)	0	0	1	1	? low grade cellular rejection
23- (105)	0	0	0	1	Minimal cellular rejection
35- (108)	0	0	0	1	Early ATN picture
77-	Nephrectomy- clinically rejecting. No histology.				
B18 MUTTLEY					
0 post reperf- (110)	1	0	0	0	Normal, damaged.
5- (112)	1	1	2	2	Early cellular rejection
23- (116)	0	1	3	4	Sub-acute cellular rejection
					Kidney left in, no other biopsies

ATN- acute tubular necrosis

B19 KIM

0 post reperf- (102)	0	1	1	1	Virtually normal, ? reduced glomerular perfusion.
2- (104)	0	0	1	1	Suggests low grade rejection
22- (114)	0	1	2	2	Very early cellular rejection
36- (115)	0	1	3	4	Severe cellular rejection
38- (117)	4	6	8	9	Severe cellular rejection

Table 5.1 summarises the results of group 1 (PRIMARY RENAL ALLOGRAFT. NO CyA. NEGATIVE CDC CROSSMATCH) and group 2 (PRIMARY RENAL ALLOGRAFT. PLUS CyA. NEGATIVE CDC CROSSMATCH). The day of onset of malaise and fever is noted, and the day of cessation of urine production. The urine production was easily followed with a ureterostomy, but it was not possible to follow urine production readily with the intravenous urogram. The transplant and the bladder were close together and the bladder rapidly opacified from the native kidneys. By seeing a nephrogram of the transplant it was possible to infer that there was some function of the transplant, though this was not a quantitative measure. As some dogs experienced no malaise, the day of the last biopsy or day of nephrectomy was taken as the end point when comparing the onset of malaise between groups. The day post transplant that a biopsy first showed class 1 features, those associated with vascular rejection was noted. A score of 2 or more out of six was required. The day of nephrectomy was recorded. The results from B6-Duncan were excluded from the analysis because of the presumed renal artery thrombosis and the vascular features on the biopsies would have distorted the results.

TABLE 5.1 - Effect of CyA on dog renal allograft survival. Comparison between group 1 (PRIMARY RENAL ALLOGRAFT. NO CyA. NEGATIVE CDC CROSSMATCH) and group 2 (PRIMARY RENAL ALLOGRAFT. PLUS CyA. NEGATIVE CDC CROSSMATCH).

	Malaise	Fever (days)	Urine (Day ceased)	Vascular rejection (Day Dx)	Nephrectomy (Day)
Group 1					
B9-Ebony	6	6 - 8	-	6	70
B10-Fred	5	5 - 7	-	6	23
B13-Snowy	4	3	-	9	9
Group 2					
(CyA20mg/kg/day					
B5-Painter	None	(8-9) + (28-48)	-	37	112
B6-Duncan	None	2	-	5	41
B7-Sydney	51	51	-	56	56
B8-Podge	28	(8-13) + (26-31)	-	22	44
(CyA25mg/kg/day					
B17-Tizer	None	59	-	77	77
B18-Muttley	None	(6-7)+22+25	30	None	Never
B19-Kim	None	(15-16) + (36-38)	36	38	38

Notes:

1) Day post transplant of onset of malaise, not always present.

2) Day post transplant of onset of fever.

3) (-), day of urine cessation uncertain as ureter joined to bladder.

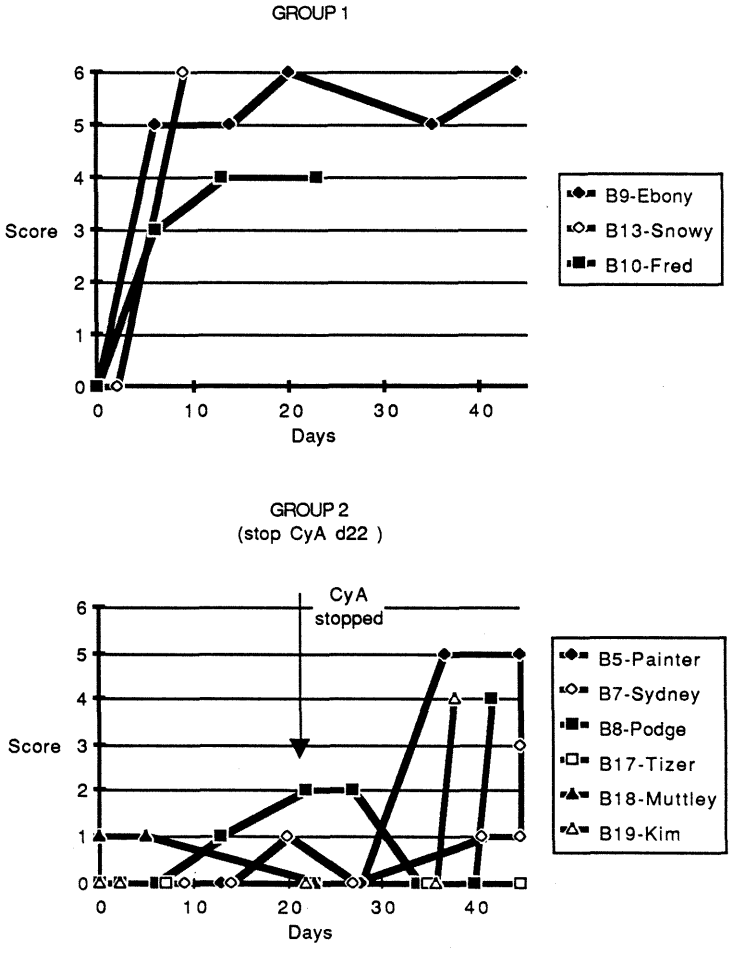
4) Day of onset of vascular rejection is the day that a biopsy is scored greater than 1 out of 6.

5) All figures are day of onset after transplantation. Some recipients were pyrexial over more than one time period.

	Group 1	Group 2	
Malaise	4,5,6,	23,28,37,38,39,51,	p<0.05
Fever	3,5,6,	6,8,8,15,51,59,	p<0.05
Vascular Rej.	6,6,9,	22,37,38,56,77,	p<0.05

(Mann - Whitney U Test - two tail.)

FIGURE 5.10: Results of histology, score out of six for Class 1 features, comparing group 1 (PRIMARY RENAL ALLOGRAFT. NO CyA. NEGATIVE CDC CROSSMATCH) and group 2 (PRIMARY RENAL ALLOGRAFT. PLUS CyA. NEGATIVE CDC CROSSMATCH).



Reviewing the CyA levels in group 2 beagles is made difficult by the change from measurement from radioimmunoassay in serum (Figure 5.11) to HPLC in whole blood (Figure 5.12). Considering the serum measurements, it is B5 who had the highest levels and least rejection. The levels in B7 and B8 are low, and these dogs had acute cellular rejection on biopsy, though with minimal class 1 features. It is unreliable to make comparisons

between the two methods of measurement for CyA, nevertheless beagles B17, B18, and B19 probably had higher levels of CyA, and this is in keeping with the histological findings and the higher CyA dose.

FIGURE 5.11 - GROUP 2. Serum CyA trough levels by RIA.

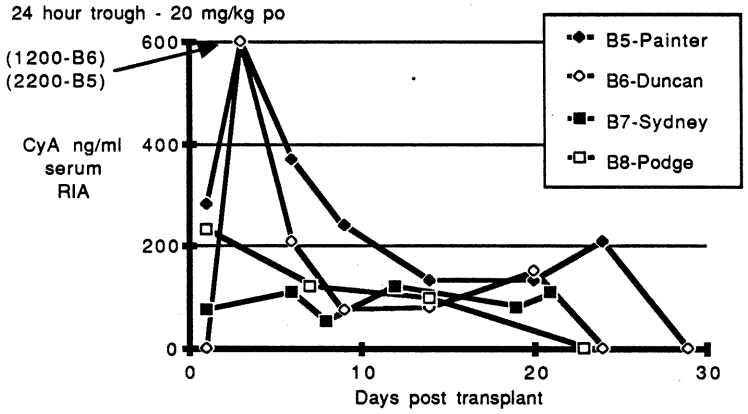
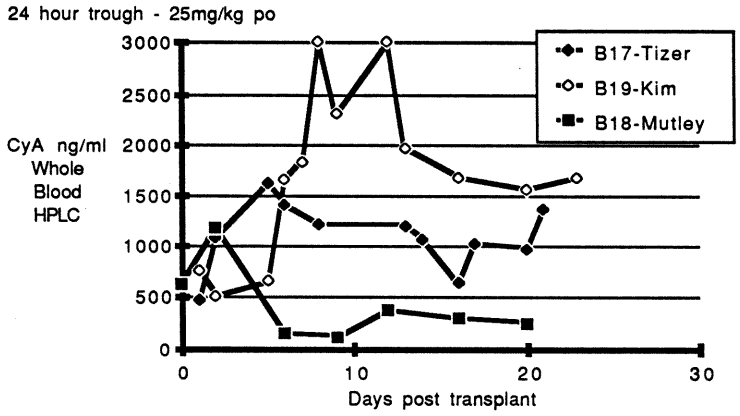


FIGURE 5.12 - GROUP 2. Whole blood CyA trough levels by HPLC.



5.4.4 Discussion

The results from group 1 indicate that this is a severe model of allograft rejection in the unsensitised recipient not given immunosuppression. The recipients developed malaise from days 4 to 6, and fever from day 3 to 6. All three dogs showed features of severe vascular rejection by days 6 to 9 (figure 5.10). Beagle B13 required nephrectomy at day 9 because of symptoms, B10 had an infarcted kidney removed at day 23, though not unwell at the time, and B9 had an elective nephrectomy at day 70.

The limited value of intravenous urography is illustrated by beagle B9, in which a nephrogram was seen on day 14. This contrasts with the histology, which indicated infarction of the kidney

Group 2 differed from group 1 by the addition of CyA for at 21 days. Following withdrawal of CyA, the recipients were on no immunosuppression. The renal allograft to B6 underwent ischaemic infarction, probably as a result of a renal artery thrombosis. The kidney slowly atrophied and was adsorbed. This dog was therefore excluded from the statistical comparisons. It can be seen that CyA delayed the onset of malaise and fever. The appearance of the class 1 vascular features (score≥2/6) on the biopsy was prevented in all recipients. However some grafts were reported as acute cellular rejection on the basis of the mononuclear cell infiltrate. In clinical terms such an infiltrate would be regarded as treatable in contrast with rejection associated with class 1 features. Nevertheless three further beagles were transplanted with a higher dose of CyA, and this was successful in reducing the mononuclear infiltrate. Following the withdrawal of CyA class 1 features appeared in beagles B5, B7, B8, and B19. Both B17 and B18 rejected some days after stopping CyA as judged by swelling of the graft, cessation of urine production, and symptoms, but unfortunately no histology was available to confirm this. The time to the appearance of class 1 features or clinical rejection after stopping CyA was variable - (B5-11 days), (B17-33 days), (B18-6 days), and (B19-14 days).

The contrast in the timing of the appearance of vascular features (class 1) in these two groups of unsensitised recipients was so great, that the timing and frequency of biopsies was not crucial. However, it was anticipated that with allosensitised recipients, events would move more quickly and the timing of biopsies would be crucial.

5.5 Transplantation of allosensitised recipients with either a positive or negative CDC crossmatch.

5.5.1 Introduction

This section examines renal allografting to dogs with alloantibody, generated as described in section 5.3 above. Renal allografts to allosensitised recipients with a positive crossmatch against the selected donor were performed, with and without CyA. Then allosensitised recipients were given a crossmatch negative renal allograft.

5.5.2 Methods

Renal allografts were grafted to beagles, which had been allosensitised by either a renal allograft (Group 3 - ALLOSENSITISED (KIDNEY)+SECOND DONOR SPECIFIC KIDNEY, +ve CDC X-MATCH, NO CYA), or skin (Group 4 - ALLOSENSITISED (SKIN) + DONOR SPECIFIC KIDNEY, +ve CDC X-MATCH, NO CyA), or blood (Group 5 - ALLOSENSITISED (BLOOD) + DONOR SPECIFIC KIDNEY, +ve CDC X-MATCH, NO CyA), as described in section 5.3 above. Each recipient received a kidney from the same donor that had been the source of immunising antigen. At the time of renal allografting most of the recipients had antibody lymphocytotoxic for donor pbl as detected by the crossmatch test. Alloantibody was not detected in the current sera of two of the transfused beagles (Figure 5.7). Group 6 (Group 6, ALLOSENSITISED + KIDNEY, +ve CDC X-MATCH, PLUS CyA) beagles received a renal allograft from positive crossmatch donors with the addition of CyA 25mg/kg/day, starting on the day before surgery. Group 7 (Group 7 -ALLOSENSITISED + KIDNEY, -ve CDC X-MATCH, PLUS CyA) beagles had alloantibody against the panel of greagles at the time of allografting, but received a kidney from crossmatch negative greyhound donor.

Methods were as described in sections 5.1-4.

5.5.3 Results

GROUP THREE

ALLOSENSITISED (KIDNEY)+SECOND DONOR SPECIFIC KIDNEY (+ve CDC X-MATCH).

NO CyA.

N=3.

B10 FRED

Day of biopsy. (Biopsy number)	6	Score: 8	10	12	Conclusion:
0 post reperf (46)	0	1	1	1	Slightly dilated tubules
0+60min (47)	0	1	1	1	Probably normal.
1-(48)	1	1	2	3	Tubular cell vacuolation, ?ischaemic
4- (49)	3	4	6	7	Intense interstitial haemorrhage
0+60min- bipsy 60 minutes after reperfusion					
B13 SNOWY					
0 pre reperf- (50)	0	0	0	1	Early ATN
0+50min- (51)	0	0	0	1	Mild ischaemic changes. ?significance of vascular endothelial changes
0+5min (52)	0	0	1	2	Mainly ATN ? early acute rejection large vessel.
0+50min (53)	1	1	1	2	Not obviously rejecting, trauma may account for interstitial haemorrhage.
5- (54)	1	1	3	5	Subcapsular zone of intense interstitial infiltrate. Rest infarcted with virtually no cellular infiltrate, and glomeruli looking more or less normal.
B9 EBONY					
0 pre reperf- (55)	0	0	0	1	? pre Tx biopsy
0+60min- (56)	0	0	0	0	Normal
0+30min (57)	0	0	0	1	Normal, ?early ATN, ?poorly perfused.
0+55min (58)	0	0	0	1	No evidence rejection, ?mild ATN
1 - (59)	1	0	3	4	? nearby infarction, mainly polys
5 - (60)	5	5	7	9	Acute haemorrhagic rejection + infarction.

GROUP FOUR

ALLOSENSITISED (SKIN) + DONOR SPECIFIC KIDNEY

(+ve CDC X-MATCH).

NO CyA.

N=4

B1 MONTY

Day of biopsy.	Score:				Conclusion:
(Biopsy number)	6	8	10	12	
70 - (pre-reperf)	0	0	0	1	Ragged biopsy. Tubules suggest ATN
71 - (60min)	0	0	0	1	Ragged, early ATN.
74 - (1)	0	1	2	3	Early cellular rejection + ATN
76 - (5)	1	1	3	5	Severe cellular rejection, only 1 arteriole
78 - (7)	6	7	9	11	Cortex totally infarcted, medulla partly infarcted
B2 ZIGGY					
72 - (pre-reperf)	0	0	0	1	Totally empty vessels, early ATN
73 - (45min)	0	0	0	0	ATN - no evidence rejection
75 - (1)	0	0	1	2	Early cellular rejection, more polys than normal
77 - (5)	6	7	8	10	Virtually totally infarcted
B3 CHESTER					
79 - (pre)	0	0	0	0	Nil
81 - (3)	2	2	4	5	acute cellular rejection
B4 NEIL					
80 - (0)	0	0	0	1	Possible early ATN
82 - (3)	4	4	4	6	Totally infarcted

GROUP FIVE
ALLOSENSITISED (BLOOD) + DONOR SPECIFIC KIDNEY
(+ve CDC X-MATCH).
NO CyA.
N=4

B11 ROSIE

Day of biopsy. (Biopsy number)	6	Score: 8	10	12	Conclusion:
0 pre reperf- (83)	0	0	0	0	Focal glomerular sclerosis
0+30 min- (84)	0	0	0	0	Nil specific
5- (88)	0	1	3	3	Acute cellular rejection
B12 TIFFANY					
0 pre reperf- (85)	0	0	0	0	Some tubular cell swelling
0+60min- (86)	0	0	0	0	Tubular cell vacuolation ?ATN
2- (87)	6	7	8	10	Totally infarcted
B14 ARNOLD					
0 pre-reperf (89)	0	0	0	0	Thickened capillaries.
0+30min- (90)	0	0	0	0	Thickened capillaries.
5- (94)	4	4	6	8	Largely infarcted
B15 JANET					
0 pre reperf- (91)	0	0	0	0	Normal
0+60min- (92)	0	0	0	0	Glomerular congestion only
5- (93)	1	2	4	-	Acute cellular rejection

To compare the outcome of renal allografting to unsensitised (negative CDC crossmatch) and allosensitised (positive CDC crossmatch) recipients in the absence of immunosuppression the results of group 1 were compared with groups 3 (kidney), 4 (skin) and 5 (blood). Table 5.2 summarises the results in the same way as described above in section 5.4. The allosensitised dogs were biopsied twice in the first week. Biopsies were earlier, because the clinical tempo of the rejection episodes was more rapid. Nevertheless, it could be said that the apparent earlier appearance of vascular features in the allosensitised dogs was because they were biopsied earlier. The earlier occurrence of fever and malaise (not significant) is against this, but the dogs in group 1 were not biopsied sufficiently frequently in the first week to refute the suspicion.

TABLE 5.2- Comparison of group 1(PRIMARY RENAL ALLOGRAFT. NO CyA. NEGATIVE CDC CROSSMATCH) with groups 3, 4, & 5.(Group 3 - ALLOSENSITISED (KIDNEY)+SECOND DONOR SPECIFIC KIDNEY, +ve CDC X-MATCH, NO CYA), (Group 4 - ALLOSENSITISED (SKIN) + DONOR SPECIFIC KIDNEY, +ve CDC X-MATCH, NO CyA), (Group 5 - ALLOSENSITISED (BLOOD) + DONOR SPECIFIC KIDNEY, +ve CDC X-MATCH, NO CyA),

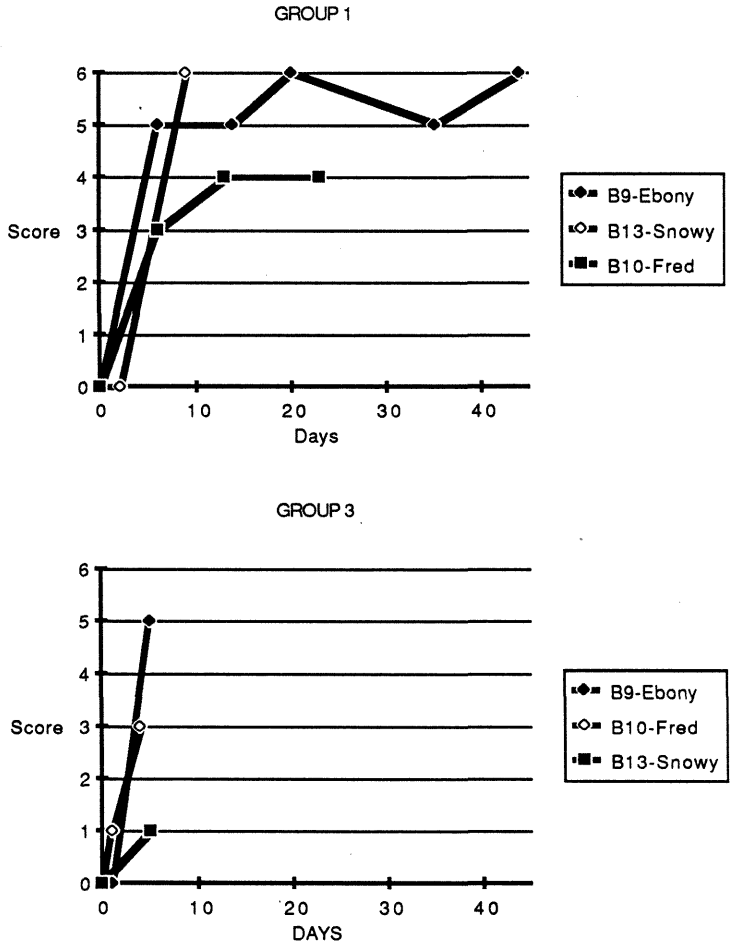
	Malaise	Fever (days)	Urine (Day ceased)	Vascular rejection (Day Dx)	Nephrectomy (Day)
Group 1					
B9-Ebony	6	6 - 8	-	6	77
B10-Fred	5	5 - 7	-	6	23
B13-Snowy	4	3	-	9	9
Group 3(Kidney)					
B9-Ebony	5	5	-	1	5
B10-Fred	3	3	-	4	4
B13-Snowy	2	4	-	5	5
Group 4 (Skin)					
B1-Monty	6	(4 - 6)	6	5	7
B2-Ziggy	4	(2 - 5)	4	5	5
B3-Chester	3	3	3	3	3
B4-Neil	3	3	2	3	3
Group 5 (Blood)					
B11-Rosie	5	5	-	5	5
B12-Tiffany	2	2	-	2	2
B14-Arnold	5	(1 - 5)	2	5	5
B15-Janet	5	(1 - 5)	4	5	5

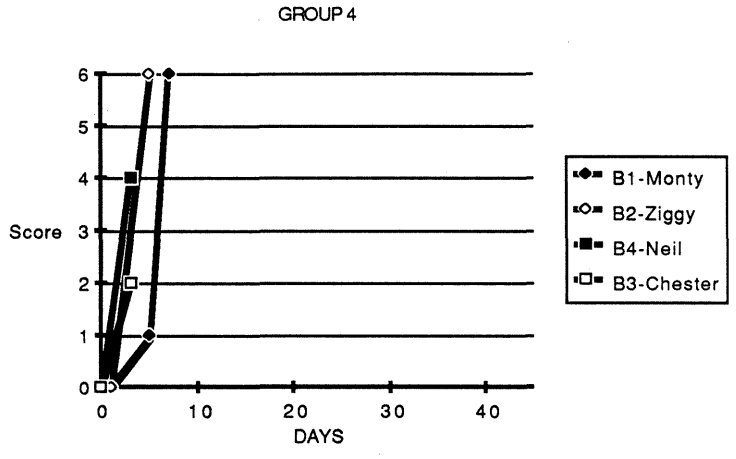
Group 1 vs Groups 3, 4, & 5.

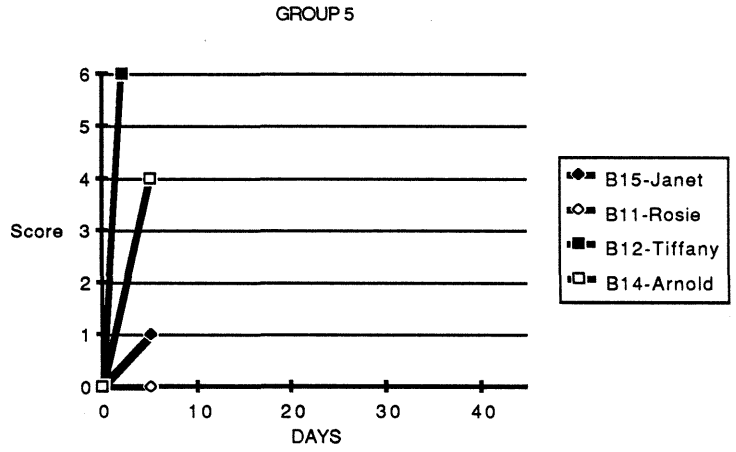
	Group 1	Groups 3, 4, & 5.	
Malaise	6,5,4,	5,3,2,6,4,3,3,5,2,5,5,	NS
Fever	3,5,6,	5,3,4,4,2,3,3,2,5,1,1,	NS
Vascular Rej.	6,6,9,	1,4,5,5,5,3,3,5,2,5,5, (Mann Whitney U test)	$p<0.05$

The day of onset of malaise and fever did not differ significantly, it was not possible to determine the day of cessation of urine production in beagles with a ureteroneocystostomy. The day of diagnosis of vascular rejection (class 1 features score $\geq$ 2 out of six) was earlier with allosensitised recipients.

FIGURE 5.13 - Results of histology, score out of six for Class 1 features, comparing group 1 (PRIMARY RENAL ALLOGRAFT. NO CyA. NEGATIVE CDC CROSSMATCH) with groups 3, 4, & 5.(Group 3 - ALLOSENSITISED (KIDNEY)+SECOND DONOR SPECIFIC KIDNEY, +ve CDC X-MATCH, NO CYA), (Group 4 - ALLOSENSITISED (SKIN) + DONOR SPECIFIC KIDNEY, +ve CDC X-MATCH, NO CyA), (Group 5 - ALLOSENSITISED (BLOOD) + DONOR SPECIFIC KIDNEY, +ve CDC X-MATCH, NO CyA).







Regarding differences between the routes of sensitisation (kidney, skin, or blood), there was no clear cut difference in the outcome of renal allografting to the allosensitised dogs.. Of the eleven beagles in groups 3, 4 & 5 nine were allosensitised immediately before transplantation (Figures 5.6-8). Two beagles (B12 and B15) in the donor specific transfused group were not allosensitised at the time of transplantation as judged by the CDC crossmatch test. Referring to figures above there was no obvious association between the presence and absence of alloantibody in the transfused group (group 5) and the appearance of vascular rejection. It is possible that more frequent biopsies between two and five days would have shown a greater difference in histopathology between allosensitised and unsensitised dogs. The lack of any beneficial effect with donor specific blood transfusion in these unrelated, nonimmunosuppressed dogs was as expected.

GROUP SIX

ALLOSENSITISED + KIDNEY (+ve CDC X-MATCH).

PLUS CyA.

N=5

B2 ZIGGY

Day of biopsy. (Biopsy number)	Score: 6	8	10	12	Conclusion:
0 pre reperf- (96)	0	0	0	0	Collapsed capillaries
2-(98)	5	6	7	9	Acute rejection++, with infarction
7- (100)	-	-	-	-	Totally necrotic
23- (106)	2	2	3	5	Severe ischaemic changes
35- (107)	4	4	5	7	Near total infarction.

B4 NEIL

0+30min (101)	0	0	0	0	Normal
2- (103)					Medulla only ?Systemic leucocytosis and/or rejection.
4-(111)	3	4	5	5	acute cellular rejection

B3 CHESTER

0 post reperf- (109)	0	0	0	0	Normal
6- (113)	3	4	6	7	Intense interstitial haemorrhage, focal necrosis.

B14 ARNOLD

0- (121)	0	0	0	0	?primary glomerular disease
2- (124)	1	1	2	3	Acute rejection
8- (130)	6	6	8	10	Advanced acute rejection, with infarction.

B8 PODGE

0+60min- (128)	0	0	1	0	Possible early ATN/acute rejection
6- (132)	6	6	7	9	Infarction due to cellular rejection.
9- (134)	-	-	-	-	Total infarction, probably following rejection.

Group 6 recipients were allosensitised, had a positive crossmatch with alloantibody against donor pbl, and received CyA. The CyA trough levels for group 6 beagles are shown in figure 5.14 . During the first seven days the levels were comparable to group 2 beagles -figure 5.12. Table 5.3 shows that CyA delayed the onset of fever in allosensitised recipients given a cross match positive kidney, but had no significant effect on malaise, urine production, or vascular rejection.

FIGURE 5.14 - GROUP 6. Whole blood CyA trough levels by HPLC.

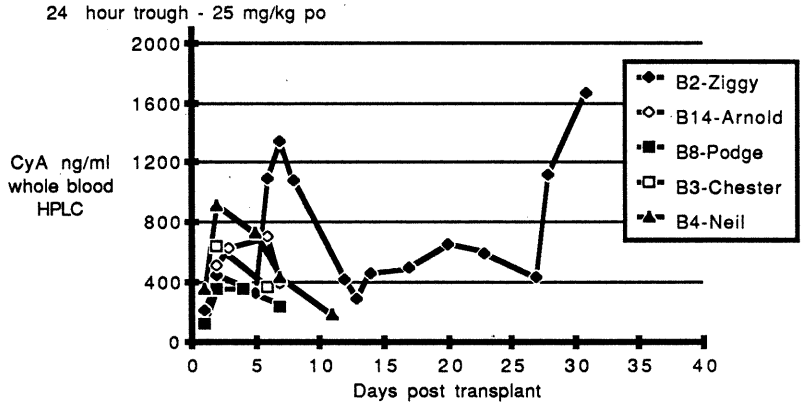


TABLE 5.3 - Comparison of group 6 (Group SIX, ALLOSENSITISED + KIDNEY, +ve CDC X-MATCH, PLUS CyA) with groups 3, 4, and 5 (Group 3 - ALLOSENSITISED (KIDNEY)+SECOND DONOR SPECIFIC KIDNEY, +ve CDC X-MATCH, NO CYA), (Group 4 - ALLOSENSITISED (SKIN) + DONOR SPECIFIC KIDNEY, +ve CDC X-MATCH, NO CyA), (Group 5 - ALLOSENSITISED (BLOOD) + DONOR SPECIFIC KIDNEY, +ve CDC X-MATCH, NO CyA).

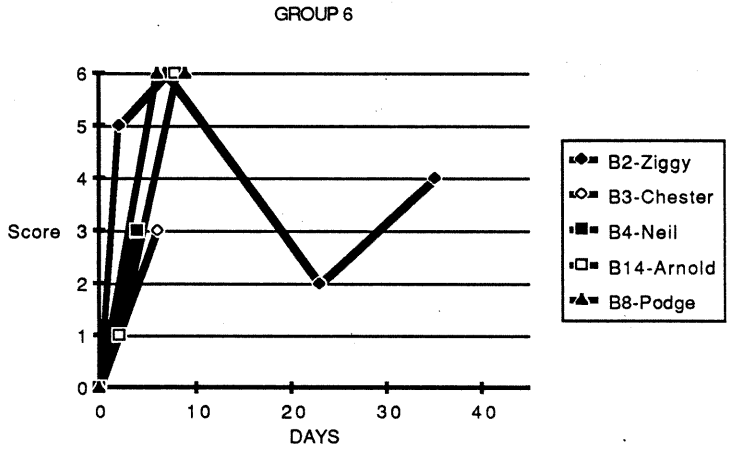
	Malaise	Fever (days)	Urine (Day ceased)	Vascular rejection (Day Dx)	Nephrectomy (Day)
Group 6 (CyA25mg/kg/day)					
B2-Ziggy	None	(32-34)	-	2	23
B3-Chester	6	6	5	6	6
B4-Neil	4	4	4	4	4
B14-Arnold	6	5	6	6	8
B8-Podge	9	4	6	6	9
Group 3 (Kidney) (No Immunosupp.)					
B9-Ebony	5	5	-	1	5
B10-Fred	3	3	-	4	4
B13-Snowy	2	4	-	5	5
Group 4 (Skin) (No immunosupp.)					
B1-Monty	6	(4-6)	6	5	7
B2-Ziggy	4	(2-5)	4	5	5
B3-Chester	3	3	3	3	3
B4-Neil	3	3	2	3	3
Group 5 (Blood) (No immunosupp.)					
B11-Rosie	5	5	-	5	5
B12-Tiffany	2	2	-	2	2
B14-Arnold	5	(1-5)	2	5	5
B15-Janet	5	(1-5)	4	5	5

Group 6 vs Groups 3, 4, & 5.

	Group 6	Groups 3,4, & 5.	
Malaise	4,6,6,9,	2,2,3,3,3,4,5,5,5,5,6,	NS
Fever	4,4,5,6,32,	1,1,2,2,3,3,3,4,4,5,5,	$p<0.05$
Urine	4,5,6,6,	2,2,3,4,4,6,	NS
Vas. Rej.	2,4,6,6,6,	1,2,3,3,4,5,5,5,5,5,5,	NS
		(Mann Whitney U test)	

The group 6 recipients were biopsied twice in the first week at comparable times to groups 3, 4, & 5, and thus the lack of effect of CyA in delaying the vascular features of the rejection process is genuine. The plot of class 1 features shows the rapid appearance of class 1 features in group 6. This was comparable to the time of appearance in groups 3,4, & 5.

FIGURE 5.15 - Plot of class 1 features for group 6 (Group SIX, ALLOSENSITISED + KIDNEY, +ve CDC X-MATCH, PLUS CyA).



Groups 3, 4, & 5 - see figure 5.13.

GROUP SEVEN

ALLOSENSITISED + KIDNEY (-ve CDC X-MATCH).

PLUS CyA.

N=3

NB CyA given at 25mg/kg/day continuously till nephrectomy.

B7 Sydney

Day of biopsy.	Score:				Conclusion:
(Biopsy number)	6	8	10	12	
0 - (148)	0	0	0	0	Normal
4 - (154)	0	0	1	0	?low grade cellular rejection.
14 - (165)	6	-	-	8	Total haemorrhagic infarction consistent with severe cellular rejection

B12 TIFFANY

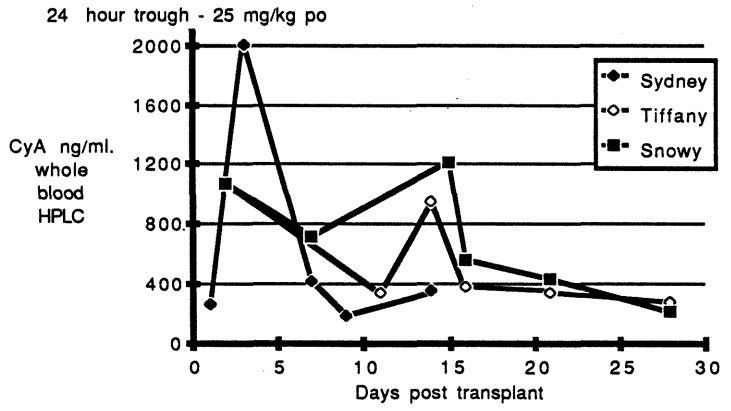
0 - (157)	0	0	1	0	Virtually normal
4 - (160)	0	0	1	2	Inadequate biopsy, but suggests cellular rejection
14 - (172)	0	1	3	4	Moderately severe cellular rejection
22 - (176)	1	-	3	4	Severe cellular rejection, inadequate specimen
29 - (180)	0	0	2	3	Severe chronic cellular rejection (surprisingly normal vessels)

B13 SNOWY

0 - (158)	0	0	0	1	?early ATN
4 - (161	0	0	1	1	Low grade cellular rejection.
14 - (174)	0	1	3	4	Severe cellular rejection, single giant cell
22 - (177)	0	0	2	3	Severe cellular rejection, with occasional giant cell.
29 - (179)	0	0	2	2	Extreme tubular atrophy with thick tubular BM and very odd cellular infiltrate. Chronic rejection.

Groups 2, 6 & 7 all received CyA. Beagles in group 2 were unsensitised, and those in groups 6 and 7 allosensitised. Groups 2 and 7 received negative CDC crossmatch grafts and group 6 received positive CDC crossmatch grafts. Figure 5.16 shows that CyA levels in group 7 beagles were comparable with beagles in groups 2 and 6.

FIGURE 5.16. - GROUP 7. Whole blood CyA trough levels by HPLC.



Comparing groups 2 and 6, CyA did not prevent a significantly more rapid onset of vascular rejection in the allosensitised recipients. The production of urine and onset of vascular rejection were prolonged in sensitised recipients given a crossmatch negative graft (group 7) compared with a crossmatch positive graft (group 6). No differences were seen in symptoms, urine output or vascular rejection between group 2 (unsensitised and negative CDC crossmatch) and group 7 (sensitised and negative CDC crossmatch). This makes an interesting parallel with the practice in clinical transplantation of giving allosensitised patients a crossmatch negative kidney.

TABLE 5.4- Comparison of group 7 (Group 7 -ALLOSENSITISED + KIDNEY, -ve CDC X-MATCH, PLUS CyA) with group 6 (Group 6 - ALLOSENSITISED + KIDNEY, +ve CDC X-MATCH, PLUS CyA) with group 2 ((PRIMARY RENAL ALLOGRAFT. PLUS CyA. NEGATIVE CDC CROSSMATCH).

	Malaise	Fever (days)	Urine (Day ceased)	Vascular rejection (Day Dx)	Nephrectomy (Day)
Group 2					
(CyA20mg/kg/day					
B5-Painter	None	(8-9) + (28-48)	-	37	112
B6-Duncan	None	2	-	5	41
B7-Sydney	51	51	-	56	56
B8-Podge	28	(8-13) + (26-31)	-	22	44
(CyA25mg/kg/day)					
B17-Tizer	None	59	-	77	77
B18-Muttley	None	(6-7)+22+25	30	None	Never
B19-Kim	None	(15-16) + (36-38)	36	38	38
Group 6					
(CyA25mg/kg/day)					
B2-Ziggy	None	(32-34)	-	2	Never
B3-Chester	6	6	5	6	6
B4-Neil	4	4	4	4	4
B14-Arnold	6	5	6	6	8
B8-Podge	9	4	6	6	9
Group 7					
(CyA25mg/kg/day)					
B7-Sydney	14	14	13	14	14
B12-Tiffany	None	6	28	22	29
B13-Snowy	None	6	28	29	29

Group 2 vs Group 6 vs Group 7.

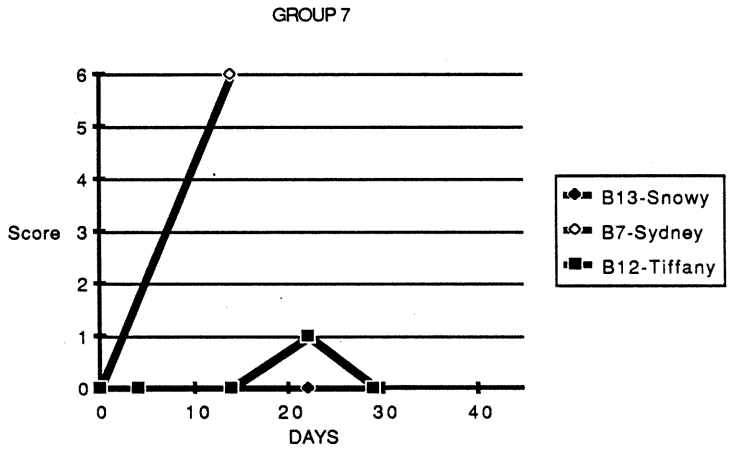
	Group 2	Group 6	Group 7
Malaise	28,51,	4,6,6,9,	14
Fever	6,8,8,15,51,59	4,4,5,6,32	6,6,14,
Urine	30,36,	4,5,6,6,	13,28,28,
Vascular Rej.	22,37,38,56,77,	2,4,6,6,6,	14,22,29,

	Gp.2 vs Gp.6	Gp.6 vs Gp.7	Gp.2 vs Gp.7
Malaise	NS	NS	NS
Fever	NS	NS	NS
Urine	NS	p<0.05	NS
Vascular Rej.	p<0.05	p<0.05	NS

(Mann Whitney U test)

Figure 5.17 confirms the similarities between groups 2 and 7, though one dog did show vascular features despite CyA.

FIGURE 5.17 - Plot of class 1 features for group 7 (Group 7 -ALLOSENSITISED + KIDNEY, -ve X-MATCH, PLUS CyA).



5.6 Summary

A dog model of accelerated renal allograft rejection by allosensitised recipients has been described. The features of this model include the use of a subcutaneous groin pouch with implantation to the femoral vessels and preservation of the native kidneys.The urine can be drained either via a ureterostomy in the groin, or to the bladder by directing the ureter through the femoral canal. The procedure was well tolerated with a low complication rate. The superficial position of the kidney permitted palpation and frequent safe needle biopsy of the kidney, which was readily done with local anaesthesia and sedation.

CyA in a once daily dose of 25mg/kg/day produced good trough levels with effective immunosuppression in unsensitised recipients. A lower dose of 20mg/kg/day was less effective. The course of the graft was followed by histopathology on needle biopsies, using a scoring system for the biopsies. The scoring method was used successfully to differentiate the course of renal allografts to unsensitised recipients with and without immunosuppression. In this comparison the timing of the biopsies was not critical as the differences between the two unsensitised groups was so great with CyA.

Three different protocols for inducing alloantibody production were studied, using either a vascularised kidney graft, "skin grafts", or blood transfusions. The renal allograft resulted in alloantibody in three beagles not given CyA and in three out of four beagles given a kidney

with CyA for 21 days - the kidney remaining in-situ after stopping the CyA (the fourth beagle had an early renal artery thrombosis). Skin grafting by subcutaneous injection resulted in alloantibody production. Blood transfusions were less efficient at inducing ailoantibody, though the numbers were small.

Following allograft nephrectomy persistence of alloantibody was observed 225 days (B10) and 200 days (B13) later. Further follow-up was limited by the need for further antigen exposure so as to ailosensitise the recipients to other potential donors.

It was with a considerable sense of anticipation that in January 1985 the first allosensitised beagle with a positive CDC crossmatch was transplanted (group 3). For whatever reason, hyperacute rejection was not observed, possible reasons for this were discussed in chapter 4. Some of the beagle recipients (B1,2, 3, 4, 5, 6 & 7) and greagle donors shared a common sire, whilst other beagles (B8, 9, 10 &13) were unrelated to the greagles and yet hyperacute rejection was not observed. It is likely, that the formal histopathology described here, was not the most sensitive way of picking up the early changes that might have occurred following antigen/antibody interaction. However, as urine production was observed at 24 hours in all allosensitised recipients given a crossmatch positive kidney with a ureterostomy, it can be said with confidence that none of these grafts were hyperacutely rejected. In the event, accelerated rejection with class 1 features, those associated with vascular rejection, was observed in these allosensitised recipients. A small, but significant difference was observed between allosensitised (groups 3,4, & 5) and unsensitised recipients (group 1). The results in group 1 indicate that the particular donor recipient combination used produced a severe model of primary allograft rejection. In differentiating the outcome between primary (negative crossmatch) and allosensitised recipients with a positive crossmatch (not immunosuppressed) the timing of biopsies in the day two to six region was crucial and insufficient biopsies were done in the first six days.

The most convincing evidence that a useful model of accelerated renal allograft rejection in allosensitised recipients has been described comes from the use of CyA in allosensitised recipients given a crossmatch positive kidney (group 6). No beneficial effect of CyA in either delaying or preventing rejection was seen. This was in marked contrast to the results obtained with CyA in unsensitised recipients (group 2). Furthermore allosensitised beagles given a crossmatch negative kidney behaved more like unsensitised than allosensitised recipients. Crucially, this obvious difference between group 2 and group 6 indicated the potential applicability of this model for assessing further protocols for transplanting allosensitised recipients. both in conjunction with CyA and in the absence of CyA - table 5.5.

TABLE 5.5 -Comparison between group 2, group 6 , and group 3,4, and 5 .

	Malaise	Fever (days)	Urine (Day ceased	Vascular rejection (Day Dx)	Nephrectomy (Day)
Group 2					
(CyA20mg/kg/day					
B5-Painter	None	(8-9) + (28-48)	-	37	112
B6-Duncan	None	2	-	5	41
B7-Sydney	51	51	-	56	56
B8-Podge	28	(8-13) + (26-31)	-	22	44
(CyA25mg/kg/day)					
B17-Tizer	None	59	-	77	77
B18-Muttley	None	(6-7)+22+25	30	None	Never
B19-Kim	None	(15-16) + (36-38)	36	38	38
Group 6					
(CyA25mg/kg/day)					
B2-Ziggy	None	(32-34)	-	2	Never
B3-Chester	6	6	5	6	6
B4-Neil	4	4	4	4	4
B14-Arnold	6	5	6	6	8
B8-Podge	9	4	6	6	9
Group 3 (Kidney)					
B9-Ebony	5	5	-	1	5
B10-Fred	3	3	-	4	4
B13-Snowy	2	4	-	5	5
Group 4 (Skin)					
B1-Monty	6	(4-6)	6	5	7
B2-Ziggy	4	(2-5)	4	5	5
B3-Chester	3	3	3	3	3
B4-Neil	3	3	2	3	3
Group 5 (Blood)					
B11-Rosie	5	5	-	5	5
B12-Tiffany	2	2	-	2	2
B14-Arnold	5	(1-5)	2	5	5
B15-Janet	5	(1-5)	4	5	5

	Group 2	Group 6	Groups 3, 4, & 5,
Malaise	28,51	4,6,6,9	2,2,3,3,3,4,5,5,5,5,6
Fever	6,8,8,15,51,59	4,4,5,6,32	1,1,2,2,3,3,3,4,4,5,5,
Urine	30, 36	4,5,6,6	2,2,3,4,4,6
Vascular Rejection	22,37,38,56,77	2,4,6,6,6	1,2,3,3,4,5,5,5,5,5,5
	Gp 2 vs gp 6	Gp 2 vs gps 3,4, &5	Gp 6 vs gps 3, 4, & 5
Malaise	NS	NS	NS
Fever	NS	$p<0.005$	$p<0.05$
Urine	NS	$p=0.072$	NS
Vas. Rej	$p<0.05$	$p<0.005$	NS

In answer to the questions posed at the beginning of this chapter, these experiments have shown a relationship between vascular features of renal allograft rejection, allosensitisation, and CyA in the allosensitised dog model. In this dog model CyA failed to prevent rejection with vascular features in the allosensitised dogs, and this was reproducible. To complete the analogy with the clinical situation it would be useful to demonstrate the ineffectiveness of high dose steroids in treating vascular rejection in allosensitised dogs given CyA.

In respect of the positive CDC crossmatch, this model was a more extreme than the clinical situation, where a patient must have a negative crossmatch on T cells. Hyperacute and accelerated rejection undoubtedly occur clinically despite a negative CDC crossmatch, though this is unpredictable. The experimental model clearly needed predictable accelerated rejection, and this was achieved with a positive CDC crossmatch. The absence of hyperacute rejection of dog renal allografts with a positive CDC test could have been expected from the experience of others, though this difference from the clinical situation has not been explained. The beagles reliably hyperacutely rejected pig kidneys, suggesting that the reason does not lie with the dog complement system.

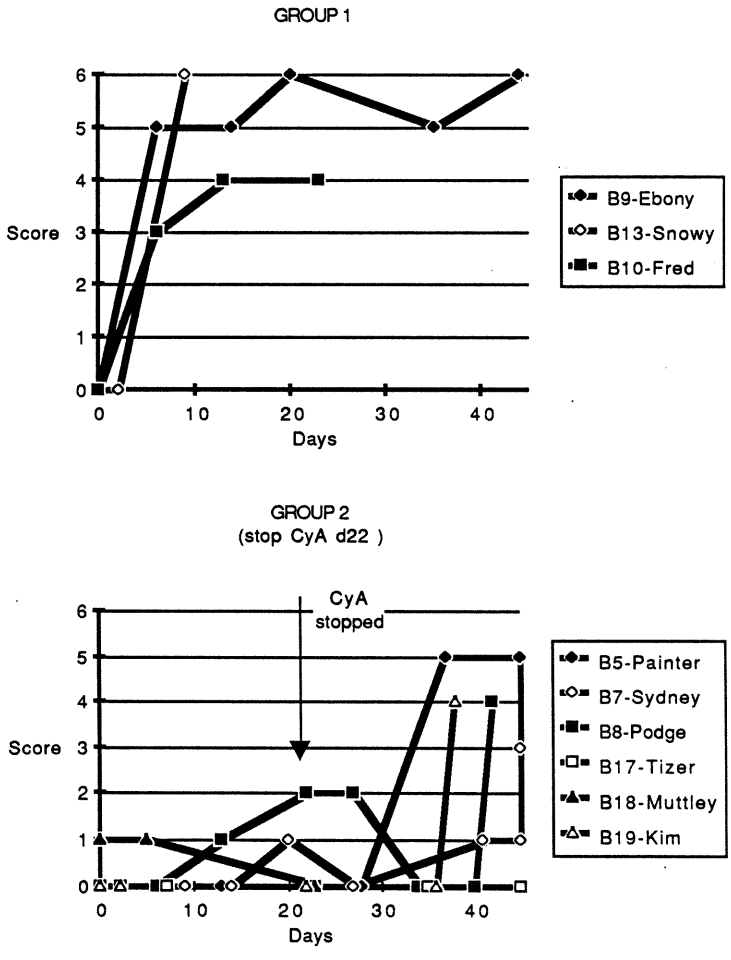
Histopathology on serial biopsies was successfully used to differentiate the experimental groups. A parallel to the clinical situation was demonstrated by the beneficial effect of CyA in the allosensitised recipient given a CDC crossmatch negative kidney. Would it have been useful to study the relationship between vascular rejection and early graft failure by removing the native kidneys? Our dog resources would not have permitted this, and instead we used vascular rejection as an indication of graft function and graft survival.

The absence of CyA nephrotoxicity in the dog model could be considered a disadvantage in view of the clinical relationship between primary non function (PNF) and treatment resistant rejection in allosensitised recipients. My own view is that the PNF in the clinical situation reflects accelerated rejection by allosensitised recipients and that the importance of the nephrotoxic effect of CyA in PNF has been overstated. The apparent absence of an effect of CyA nephrotoxicity in this dog model can therefore be considered an advantage.

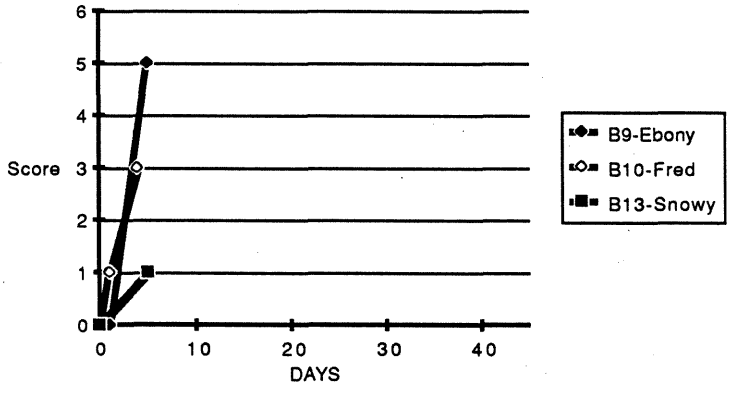
The improved management of the allosensitised patient is being investigated by characterising alloantibody specificity and class, crossmatching on T and B cells, and increasing the sensitivity of the crossmatch test. These approaches rely on a large pool of donors and recipients to find suitable grafts for particular allosensitised recipients. This approach does not lend itself to the limited numbers of an experimental model. Separate crossmatches on T and B cells in dogs have not been helpful, and this may reflect a different distribution of MHC antigens as compared to man.

In summary, an experimental model of accelerated renal allograft rejection with vascular features on biopsy has been described. CyA at 25mg/kg/day prevented the appearance of vascular features in unsensitised dogs, but there was no beneficial effect of CyA on either function or vascular rejection in allosensitised dogs given a CDC crossmatch positive renal allograft.

FIGURE 5.18 - Summary of histology of the eight experimental groups.



GROUP 3



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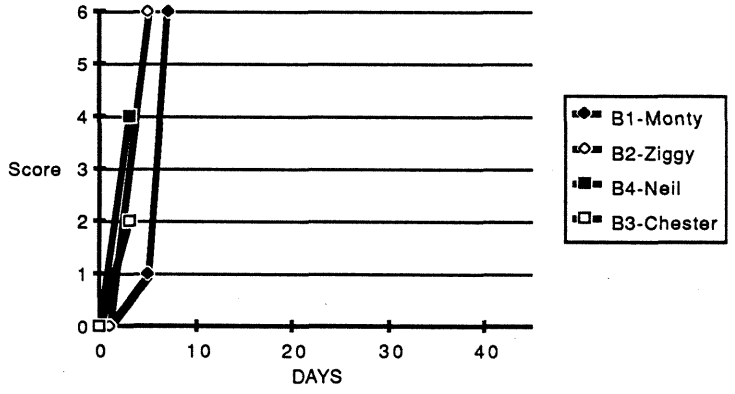

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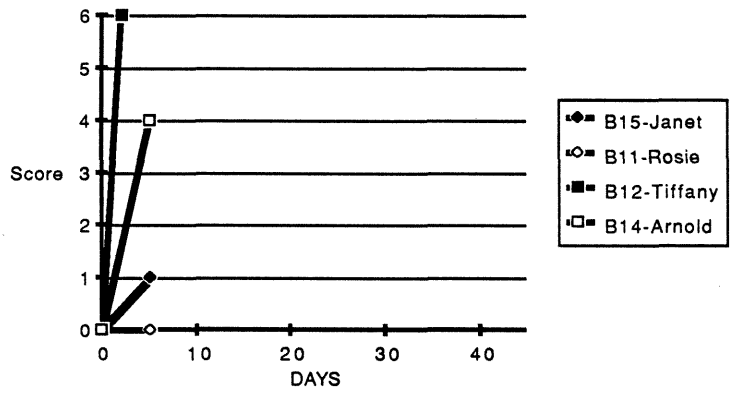

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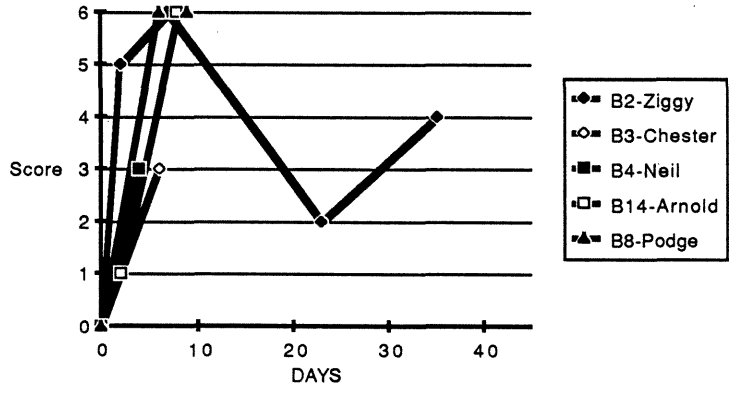

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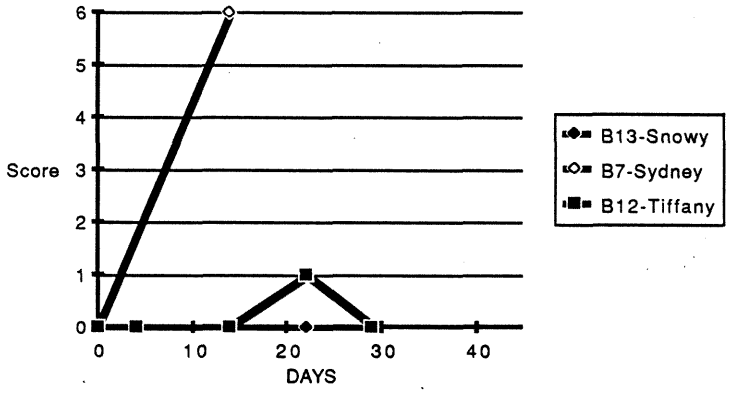
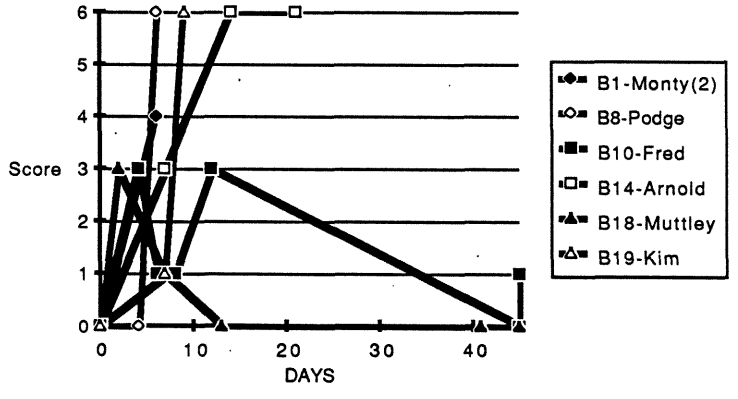


GROUP 8



CHAPTER 6.

THE EFFECT OF CYCLOPHOSPHAMIDE AND CYTOSINE ARABINOSIDE ON ALLOANTIBODY PRODUCTION IN DOGS.

6.1 Introduction

The aim of these experiments was to modify the alloantibody response in allosensitised beagles so as to permit kidney transplantation without accelerated rejection. Allosensitised recipients with a positive crossmatch donor were pretreated with two cytotoxic agents. The response to treatment was followed by repeated crossmatches against the original donor. As this approach might be expected to be more successful where the alloantibody was against one specificity rather than multiple specificities, the alloantibody responses were examined in parallel with the effect on antibody production to tetanus toxoid.

The protocol of cytotoxic drug administration was similar to that described in mongrel dogs by Terman (1978), who specifically removed circulating bovine serum albumin (BSA) antibodies with a BSA collodion charcoal extracorporeal immunoadsorbent. A rebound and overshoot of specific antibody levels followed within seven days after perfusion. Attempts were made to modify this post-immunoadsorption antibody rebound with cytotoxic agents given in the post-perfusion period - Table 6.1.

TABLE 6.1 - Cytotoxic agents for suppressing antibody rebound after extracorporeal immunoadsorption in dogs; protocols and results Terman (1978).

Single agents:	Drug protocol	Effect on anti-BSA.
Cyclophosphamide	5mg/kg iv Day 1-3	delayed rebound.
Cytosine arabinoside	10mg/kg iv Day 0-7	no change in rebound.
Methotrexate	0.5mg/kg iv Day 1-3	delayed rebound.
Two agents in combination:		
Cyclophosphamide	5mg/kg iv Day 1-3	arrest of rebound, with specific
Cytosine arabinoside	10mg/kg iv Day 0-7	decline to day 43.
Three agents in combination:		
Cyclophosphamide	5mg/kg iv Day 1-3	arrest of rebound, with specific decline to day 11, when dog died.
Cytosine arabinoside	10mg/kg iv Day 0-3	
Cytosine arabinoside	5mg/kg iv Day 4-6	
Methotrexate	0.12mg/kg iv Day 4-6.	

Terman used only six dogs. Cyclophosphamide, cytosine arabinoside and methotrexate given individually failed to prevent rebound to pre-perfusion values. In contrast, cytosine arabinoside given together with cyclophosphamide in the post perfusion period, produced a marked attenuation of BSA antibody rebound compared to the rebound in the same dogs following immunoadsorption alone. Two dogs treated with two agents were followed to 23 and 43 days, but it was not stated what happened to the antibody level beyond this. The dog given three agents died at day eleven with diarrhoea. The effect on antibody titres of the extracorporeal immunoadsorbent as well as the chemotherapeutic treatment were specific, since only the level of BSA antibody was reduced and not that to human serum albumin (HSA), which was used as a control. A slight decline in leucocyte and platelet counts was seen, this was greater in dogs receiving a combination of cytotoxic agents, as was fever and diarrhoea. Terman's study suggested that a similar protocol of cytotoxic agents could be investigated for the removal of alloantibody in the allosensitised beagle.

The mechanism of post-perfusion antibody rebound is unknown. As Terman arrested the rebound with cytotoxic agents, it is likely that the observed post-perfusion rebound was associated with lymphocyte proliferation, making these cells susceptible to the combination of cycle-dependent agents used.

Hengst (1984) reviewed the immunomodulatory role of cyclophosphamide. At therapeutically employed doses the most important action of cyclophosphamide is probably disruption of DNA synthesis. Cell cycle specificity refers to the killing properties of an agent relative to the mitotic activities of the target cells. Phase-specific drugs exert maximum immune inhibitory effects when administered during the S (DNA synthesis) phase of the

cycle. The cycle-specific agents have broader cell killing properties, being toxic for both intermitotic (G_0) and proliferating cells. Winkelstein (1984) described cytosine arabinoside as a phase-specific drug and cyclophosphamide as a cycle-specific drug. Plasmapheresis has been used clinically, in combination with cytotoxic agents, to remove alloantibody. In addition to diluting the alloantibody, plasmapheresis stimulates the immune system specifically and non-specifically, thereby rendering B-lymphocytes more susceptible to cytotoxic agents (Bansal-1978). In these canine experiments there was no readily available means of specifically removing alloantibody as a stimulus to alloantibody production. Ex-vivo perfusion of isolated donor organs was considered, but was deemed unsuitable, both as it was a remote possibility in the clinical situation, and because of difficulties in obtaining suitable canine donor organs. As an alternative to using plasmapheresis to stimulate alloantibody production, the simpler alternative of the administration of donor alloantigen was investigated. The diluting effect of plasmapheresis on alloantibody was thus lost.

6.2 Methods

Sixteen beagle dogs were sensitised with blood, skin and kidney from third party and specific donor greagles and tetanus toxoid (TT). Alloantibody responses were assayed by a complement mediated cytotoxicity test (CDC) against donor peripheral blood lymphocytes (see appendix 1), and anti-TT antibody responses were assayed by an enzyme linked immunosorbent assay (ELISA - see appendix 2).

In setting up the ELISA method, the responses to two antigens, tetanus toxoid and BSA, were examined. Beagle B16 was immunised with an intramuscular injection of 0.5ml of tetanus toxoid into a hind leg (Merieux Tetovax™- Institute Merieux). Twenty-six days later the tetanus toxoid injection was repeated. Beagle B14 was given O.5ml of BSA in Freund's Incomplete Adjuvant into both hind legs (each ml contained 50mg of BSA emusilfied with 0.5ml of Freund's incomplete Adjuvant). A second dose of 25mg BSA was given intravenously at twenty-six days. Within seconds the dog vomited, defecated, and became agitated. The pulse was weak. Anaphylaxis was suspected, intravenous steroid (Betsolam™), chlorpheniramine maleate, adrenaline, dopamine (Dopram™), and Haemacell™ successfully resuscitated the dog. The third dose of 25mg BSA was given intramuscularly, preceded by an intravenous injection of chlorpheniramine maleate (1mg/kg), without ill-effect.

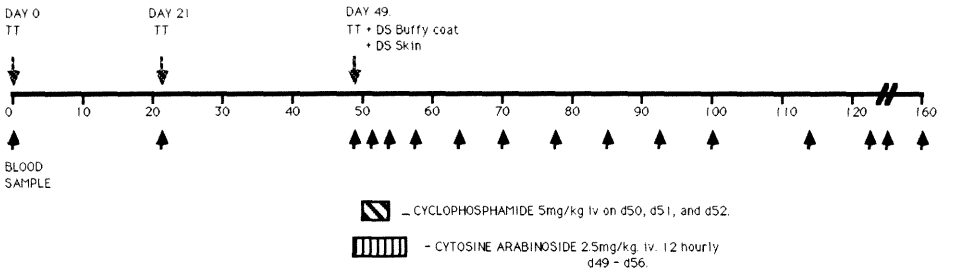
The ELISA gave better separation of the primary and secondary responses to TT compared to BSA (Figures 6.2 & 6.4). In view of the anaphylactic response to BSA, and the greater experience within the laboratory of assaying anti-TT with the ELISA, TT was used in subsequent experiments.

The experimental protocol is shown in Figure 6.1. All sixteen beagles had alloantibody as

FIGURE - 6.1: EXPERIMENTAL PROTOCOL FOR STUDYING THE EFFECT OF CYCLOPHOSPHAMIDE AND CYTOSINE ARABINOSIDE ON THE ANTI-TT RESPONSE IN ALLOSENSITISED DOGS

CONTROL GROUP -------- (no treatment)---------- n= 8

EXPERIMENTAL GROUP ---(cyclophosphamide and cytosine arabinoside)---- n= 8



TT---------- = 0.5 ml tetanus toxoid im.

DS Buffy coat--- = Donor specific buffy coat

DS Skin--------- = Donor specific skin

judged by a positive CDC test with a panel of greagles. They were immunised with TT at day 0 and day 21. Following a further immunisation with donor specific (ds) buffy coat, ds skin and TT, 8 dogs were given cyclophosphamide (5mg/kg iv, day 1,2 and 3) and cytosine arabinoside (5mg/kg iv day 0; 2.5mg/kg iv 12 hourly, day 1-7). The dose equivalent in terms of surface area was 130 mg/m^2. The dose of cyclophosphamide was identical to that used by Terman (1978), but the dose of cytosine arabinoside was halved to lessen the neutropenia observed by Terman. In the event significant neutropenia was still observed in this study. The doses of these two agents employed in this study were comparable to those described by Owen (1977) for initial treatment of canine lymphosarcoma.

Eight control dogs were similarly immunised, but did not receive cytotoxic agents.

Frequent serum samples were collected in the ensuing 130 days to follow the antibody responses to TT and donor pbl. To spread the sampling load the experimental group started on day 54 and the control group on day 49, but for clarity both are shown as starting on day 49 in figure 6.1. In subsequent figures and tables the actual days are used, with the exception of figure 6.11, where the experimental and control groups are directly compared. Twice weekly, the haematocrit, platelet count, and differential white cell count were measured.

6.3 Results

Beagle B16 (Misty) was immunised with TT and sera screened for anti-TT as indicated in Table 6.2. The sera examined were pre-immunisation, 26 days after primary immunisation, 7 days after secondary immunisation, and four days after a tertiary immunisation. The controls were buffer alone and a known human anti-TT IgG. Serial dilutions of the test serum were examined. The tests were repeated using diphtheria vaccine as a third party antigen. Figures 6.2 & 6.3 show a very satisfactory separation of the test sera with TT and no response to diphtheria vaccine. An intermediate reaction was observed with the human anti-TT IgG, suggesting some cross reaction with the rabbit anti-dog peroxidase conjugate used in the ELISA.

FIGURE 6.2 - Misty B16: antibody response to TT by ELISA (see TABLE 6.2)

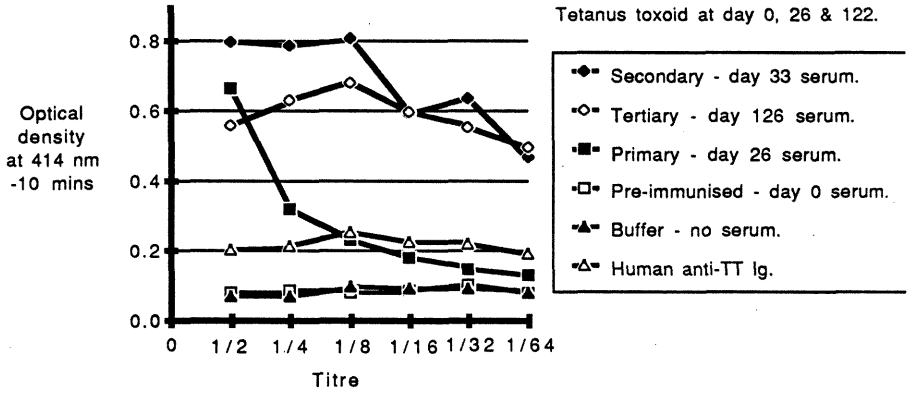
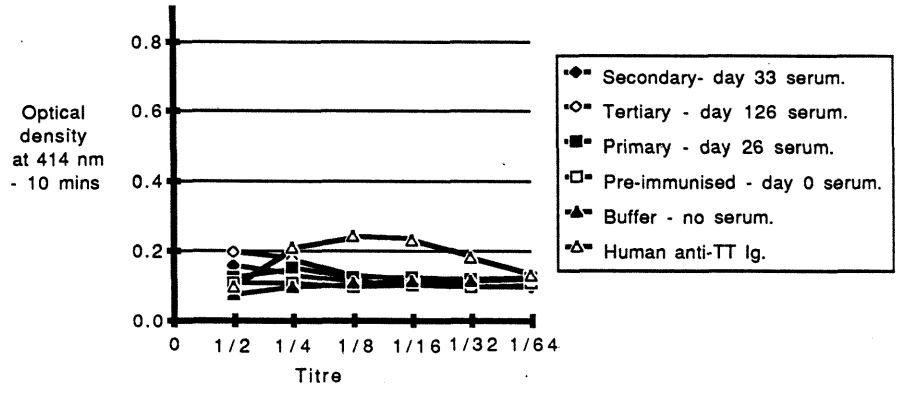


FIGURE 6.3 - Misty B16: antibody response to Diphtheria vaccine by ELISA (see TABLE 6.2)



Beagle B14 (Arnold) was immunised with BSA and the sera screened as indicated in Table 6.3. Figure 6.4 shows that the separation of the sera was less satisfactory than seen with anti-TT, in particular the pre-immunisation serum. Using an alternative substrate (Ortho-phenylenediamine - OPD) rather more satisfactory results were seen with the anti-BSA sera (not shown). Figure 6.5 shows the lack of response to human serum albumin (HSA).

FIGURE 6.4 - Arnold B14- Antibody response to BSA by ELISA (see TABLE 6.3)

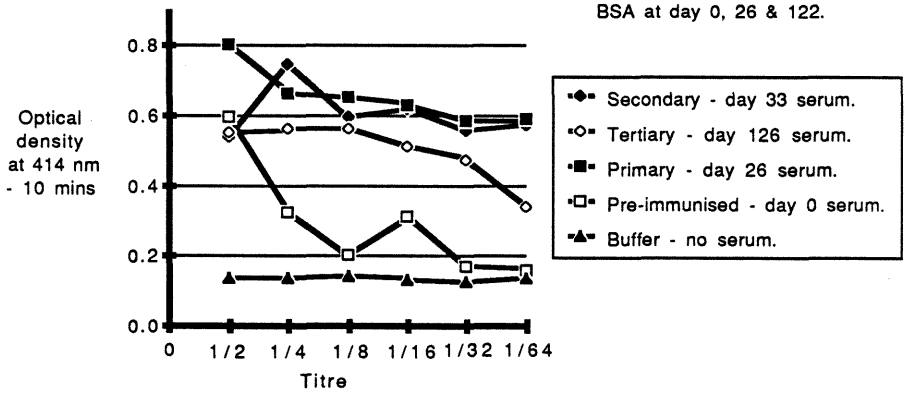


FIGURE 6.5 - Arnold B14: antibody response to HSA by ELISA (see TABLE 6.3)

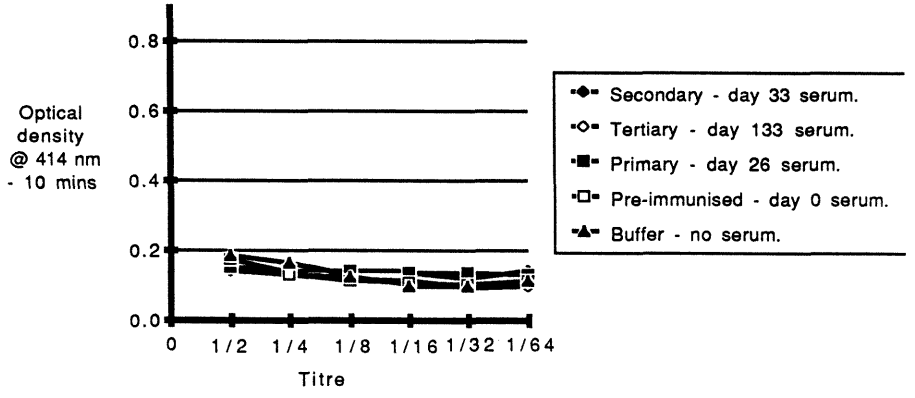


TABLE 6.2 - MISTY B16 - Antibody response to tetanus toxoid by ELISA.
Diphtheria vaccine used as control.

Tetanus toxoid (TT) 0.5 ml im
21.3.86 = Day 0
16.4.86 = Day 26
21.7.86 = Day 122

Test antigen = TT	Serial dilution of beagle test serum Optical density at 414 nm at 10 mins.					
	1/2	1/4	1/8	1/16	1/32	1/64
Pre-immunised (Day 0)	0.076	0.079	0.076	0.08	0.099	0.073
Primary response (Day 26-serum 369)	0.66	0.318	0.229	0.178	0.145	0.127
Secondary response (Day 33-serum 371	0.797	0.786	0.806	0.59	0.635	0.466
Tertiary response (Day 126 serum 392)	0.556	0.628	0.681	0.595	0.551	0.495
Buffer - no serum	0.067	0.067	0.093	0.087	0.086	0.077
Human anti TT IgG	0.25	0.222	0.218	0.189	0.094	0.204

Test antigen = Diphtheria vaccine	1/2	1/4	1/8	1/16	1/32	1/64
Pre-immunised (Day 0)	0.106	0.105	0.093	0.099	0.09	0.101
Primary response (Day 26-serum 369)	0.123	0.148	0.119	0.122	0.112	0.121
Secondary response (Day 33-serum 371	0.152	0.128	0.111	0.098	0.094	0.094
Tertiary response (Day 126 serum 392)	0.195	0.173	0.128	0.113	0.114	0.113
Buffer - no serum	0.069	0.095	0.105	0.111	0.108	0.118
Human anti TT IgG	0.094	0.204	0.236	0.226	0.177	0.128

TABLE 6.3 - ARNOLD B14 - Antibody response to bovine serum albumin (BSA) by ELISA.
Human serum albumin (HSA) as control.

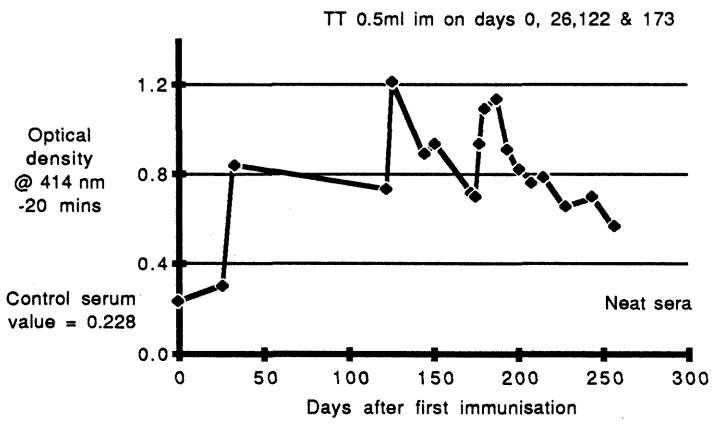
BSA
21.3.86 = Day 0 - 50mg BSA im
16.4.86 = Day 26 - 25 mg BSA iv
21.7.86 = Day 122 - 25 mg BSA im

Test antigen = BSA	Serial dilution of beagle test serum Optical density at 414 nm at 10 mins.					
	1/2	1/4	1/8	1/16	1/32	1/64
Pre-immunised (Day 0)	0.594	0.323	0.2	0.311	0.163	0.152
Primary response (Day 26-serum 370)	0.799	0.663	0.649	0.631	0.585	0.588
Secondary response (Day 33-serum 372	0.539	0.747	0.593	0.62	0.554	0.573
Tertiary response (Day 126 serum 391)	0.553	0.56	0.562	0.51	0.472	0.34
Buffer - no serum	0.134	0.131	0.138	0.128	0.118	0.134

Test antigen = HSA	1/2	1/4	1/8	1/16	1/32	1/64
Pre-immunised (Day 0)	0.169	0.125	0.108	0.102	0.096	0.108
Primary response (Day 26-serum 370)	0.149	0.135	0.137	0.133	0.132	0.126
Secondary response (Day 33-serum 372	0.143	0.137	0.135	0.134	0.116	0.139
Tertiary response (Day 126 serum 391)	0.136	0.124	0.115	0.109	0.088	0.094
Buffer - no serum	0.183	0.158	0.122	0.095	0.093	0.107

Beagle B16 (Misty) received four doses of TT and Figure 6.6 shows a response to each immunisation. The antibody response to tetanus toxoid was sustained between days 33 and day 122 without further antigen challenge. The optical density of the day 0 serum was comparable to that seen in the quality control test summarised in Table 6.4, where with pooled dog serum the mean ± 1SD was 0.244 ±0.003.

FIGURE 6.6 - MISTY-B16: anti TT antibody profile by ELISA



The anti-TT activity in the control and test sera was tested in a single run of the ELISA test. Optical density readings were made at 10 and 20 minutes. Table 6.4 shows a quality control test obtained with various combinations of the reagents in the absence of a test serum. The values obtained were remarkably consistent, though there was some cross-reactivity observed with the pooled dog serum (pdg). The values at 20 minutes were greater, and it is these values that are presented below as they give a greater numerical separation of the control and experimental groups. However, the significant differences between the controls and experimental beagles occurred at the same times regardless of whether 10 or 20 minute values were examined.

Table 6.4 - Control values of optical density in the ELISA using different combinations of reagents

	Optical density at 414 nm at 20 minutes, six separate wells.						
	1	2	3	4	5	6	Mean ± 1SD
Ag (TT) + Buffer + Subst.	0.186	0.185	0.192	0.185	0.183	0.186	0.186±0.003
Ag (TT) + FCS + Subst.	0.164	0.182	0.179	0.184	0.183	0.181	0.180±0.008
Ag (TT) + FCS + Subst. + Conj.	0.185	0.184	0.168	0.183	0.186	0.185	0.182±0.007
Ag (TT) + FCS + Subst.	0.176	0.183	0.179	0.182	0.177	0.177	0.179±0.003
Ag (TT) + FCS + Subst. + Conj.	0.193	0.18	0.181	0.187	0.183	0.19	0.186±0.005
Ag (TT) + pdg + Subst. + Conj.	0.246	0.24	0.244	0.246	0.243	0.247	0.244±0.003

Ag (TT) = Antigen, tetanus toxoid
FCS = Foetal calf serum
Substrate = ABTS
Conjugate = Anti-dog peroxidase conjugate
pdg = Pooled dog serum

Figure 6.7 shows the individual responses to tetanus toxoid of 8 beagles given three doses of tetanus toxoid. The individual values are shown in Table 6.5. Day 0 was the day of first administration of TT as indicated in Figure 6.1. An increase in anti-TT occurred in response to each dose of TT, the time of administration is represented by the vertical arrows on the 'x' axis in Figure 6.7. The means (±1SD) of these values are plotted in Figure 6.8. The anti-TT persisted from the time of the third injection at 49 days to 130 days.

FIGURE 6.7 - Control. Anti-TT antibody response in beagle dogs as measured by ELISA.

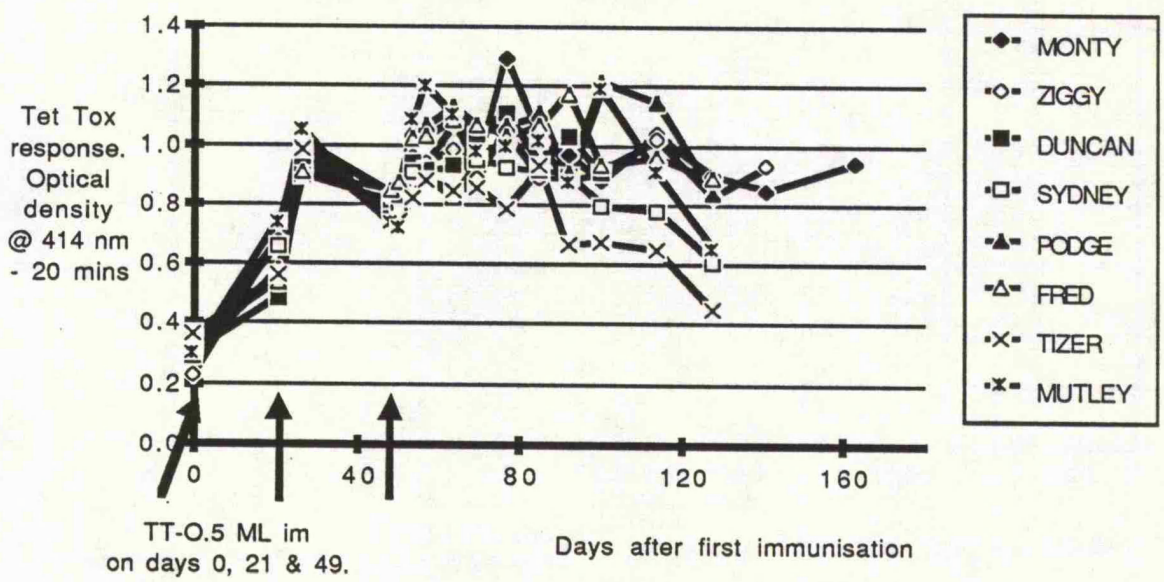


FIGURE 6.8 - Control beagles: anti-TT antibody response by ELISA, Mean ± 1SD.

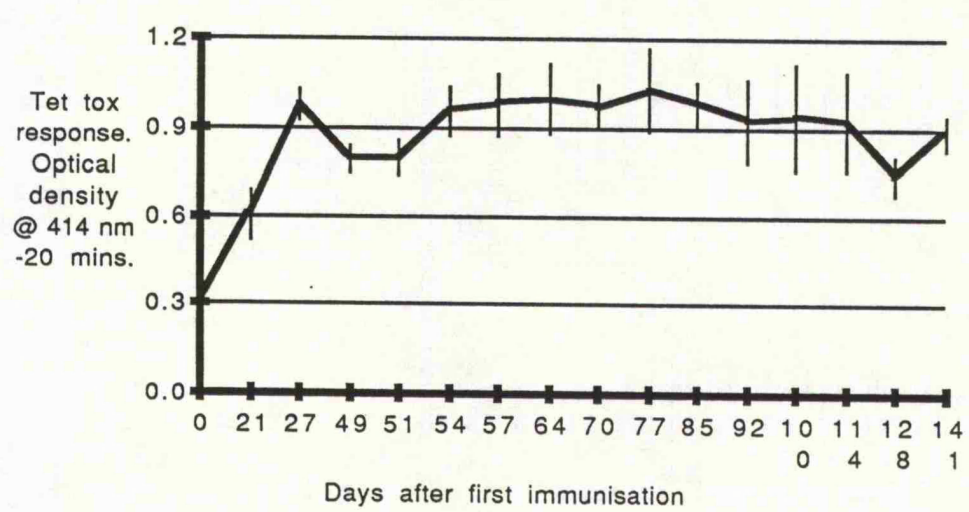


Figure 6.9 shows the individual anti-TT responses in the 8 beagles receiving cytotoxic agents, with the individual values in Table 6.16. Again a response was seen to each TT dose. The means (±1SD) of these values are plotted in Figure 6.10.

FIGURE 6.9 Experimental - anti-TT antibody response in beagle dogs as measured by ELISA

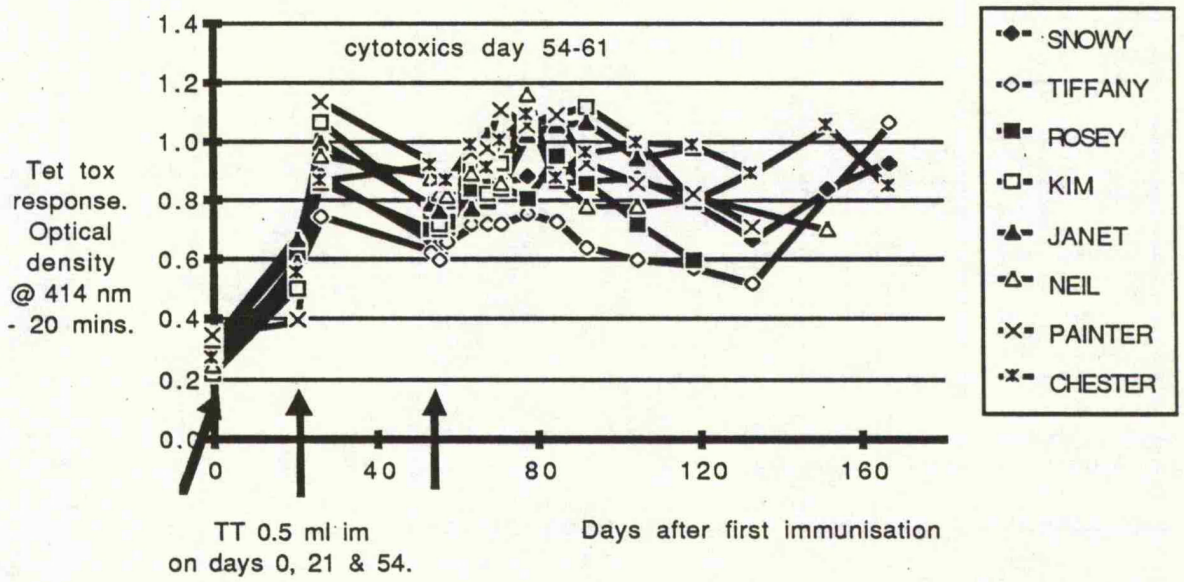


FIGURE 6.10 - Experimental beagles: anti-TT antibody response measured by ELISA - Mean ± 1SD

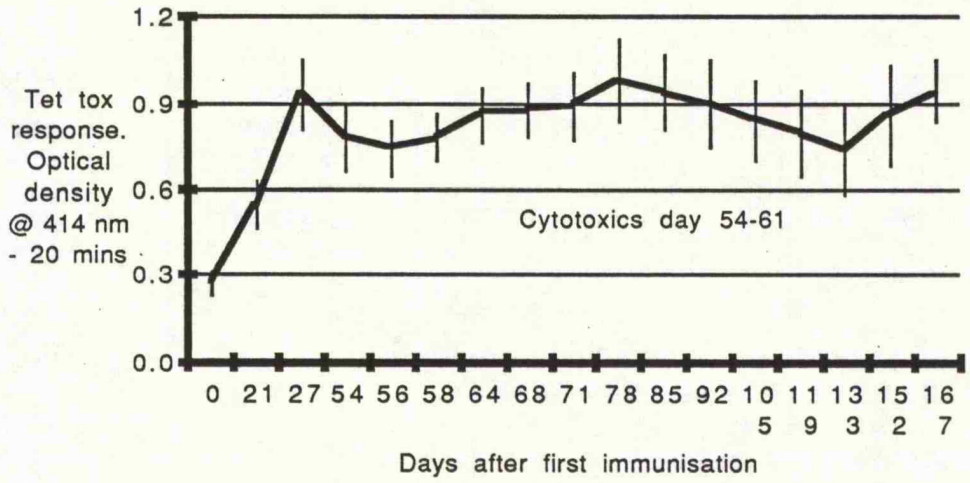


TABLE 6.5 - CONTROL GROUP. Optical density of neat sera at 20 minutes in dogs given TT on days 0, 21 & 49.

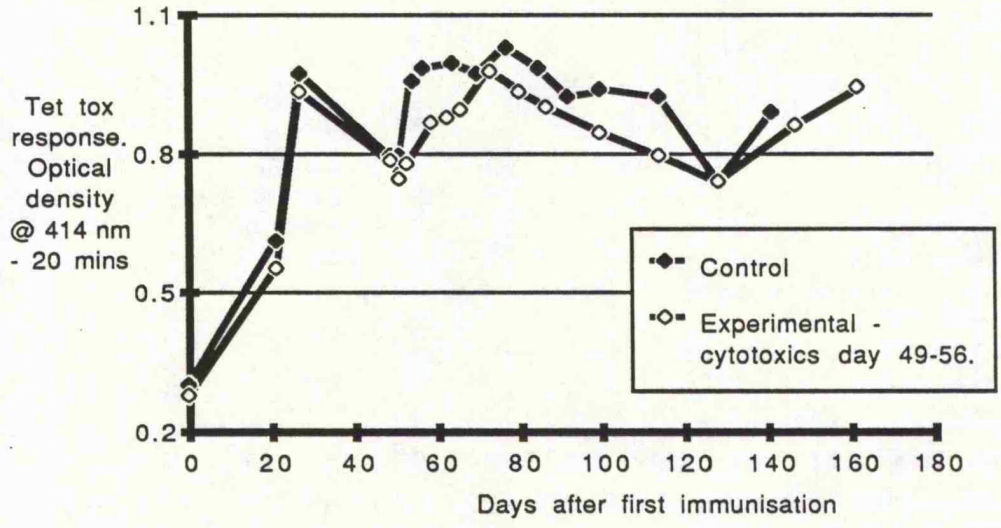
DAYS	MONTY	ZIGGY	DUNCAN	SYDNEY	PODGE	FRED	TIZER	MUTLEY	MEAN±1SD
0	0.32	0.221	0.297	0.282	0.287	0.344	0.364	0.288	0.3±0.044
21	0.669	0.674	0.479	0.659	0.576	0.535	0.557	0.738	0.611±0.087
27	0.999	0.994	0.946	0.901	1.016	0.905	0.99	1.051	0.975±0.053
49	0.842	0.759	0.78	0.814	0.841	0.843	0.733	0.736	0.794±0.047
51	0.862	0.749	0.784	0.798	0.826	0.872	0.744	0.713	0.794±0.057
54	0.896	0.961	0.943	0.905	1.043	1.02	0.821	1.081	0.959±0.086
57	0.943	9.941	0.921	0.883	1.064	1.029	0.883	1.198	0.983±0.108
64	1.071	0.99	0.931	0.825	1.126	1.083	0.843	1.099	0.996±0.118
70	0.937	0.887	1.03	0.948	1.065	1.06	0.859	0.98	0.971±0.077
77	1.286	1.051	1.109	0.923	1.068	1.026	0.788	0.998	1.031±0.144
85	1.012	0.888	0.938	0.913	1.104	1.061	0.935	1.008	0.982±0.076
92	0.97	0.885	1.027	0.9	0.91	1.167	0.662	0.877	0.925±0.144
100	0.878	0.917	0.905	0.798	1.214	0.932	0.677	1.187	0.939±0.182
114	1.039	1.025		0.774	1.142	0.962	0.65	0.907	0.921±0.168
128	0.899	0.83		0.607	0.841	0.892	0.45	0.651	0.739±0.065
141	0.848	0.933							0.890±0.060
163	0.946								

TABLE 6.6 - EXPERIMENTAL GROUP. Optical density of neat sera at 20 minutes in dogs given TTon days 0, 21 & 54, cyclophosphamide on days 55-57 and cytosine arabinoside on days 54-61.

DAYS	SNOWY	TIFFANY	ROSEY	KIM	JANET	NEIL	PAINTER	CHESTER	MEAN±1SD
0	0.269	0.272	0.297	0.21	0.32	0.236	0.347	0.265	0.277±0.044
21	0.504	0.56	0.623	0.498	0.667	0.583	0.398	0.549	0.548±0.083
27	0.885	0.741	0.86	1.065	0.999	0.949	1.132	0.867	0.937±0.126
54	0.667	0.622	0.702	0.762	0.79	0.875	0.929	0.919	0.783±0.116
56	0.648	0.599	0.697	0.718	0.761	0.819	0.865	0.864	0.746±0.099
58	0.687	0.654	0.725	0.793	0.842	0.813	0.861	0.865	0.78±0.081
64	0.886	0.713	0.837	0.921	0.768	0.887	0.959	0.983	0.869±0.093
68	0.872	0.72	0.792	0.819	1.017	0.91	0.974	0.905	0.876±0.097
71	0.868	0.716	0.838	0.923	0.835	0.854	1.112	1	0.893±0.12
78	0.878	0.754	0.808	1.057	1.017	1.156	1.054	1.094	0.977±0.145
85	0.852	0.728	0.952	1.089	1.058	0.861	1.089	0.869	0.937±0.132
92	0.863	0.64	0.855	1.115	1.067	0.775	0.928	0.964	0.901±0.154
105	0.875	0.592	0.718	1	0.94	0.778	0.852	0.996	0.844±0.142
119	0.788	0.572	0.593	0.791	0.981	0.812	0.82	0.985	0.793±0.152
133	0.668	0.52		0.893			0.711	0.894	0.737±0.159
152	0.836					0.698		1.058	0.864±0.182
167	0.926	1.063						0.844	0.944±0.111

Figure 6.11 compares the production of anti-TT, as determined in the ELISA by optical density measurements at 20 minutes, between the experimental and control beagles. Values expressed are the means. Anti-TT titres were significantly depressed in the experimental group at days 4 ($p<0.01$) and 14 ($p<0.05$) after commencement of the cyclophosphamide and cytosine arabinoside (Table 6.16 - Mann Whitney U Test). Beyond this time the anti-TT levels were comparable in the two groups, with a modest decline between days 70 and 120. The dogs received a further renal allograft, the controls between days 85-148 and the experimentals day 119-148. No more TT was given. An increase in anti-TT was observed in both groups, suggesting an amnestic response.

FIGURE 6.11 - Mean anti-TT antibody response as measured by ELISA in control and experimental beagles.



The mean white cell count ±1SD (wcc) is shown for the control (Figure 6.12; Table 6.7) and experimental beagles (Figure 6.13; Table 6.8). The cytotoxic agents significantly depressed the wcc at days 2 ($p<0.01$), 4 ($p<0.02$), 10 ($p<0.001$), and 14 ($p<0.002$) after starting these agents (Table 6.16 - Mann Whitney U Test). There was a rebound in the wcc, which peaked at day 85 (26 days after the finish of the cytotoxic agents), but the wcc was not significantly greater than in controls. Three beagles experienced malaise and fever 12-14 days after starting the cytotoxic agents. Two of these had a wcc of $0.9x10^9/l$ at this time. The three were treated with an injection of Duplocillin 1ml and Trivetrin 0.5ml. They all recovered within three days of the onset of symptoms. Other than neutropenia no untoward effects were seen in the other beagles given cytotoxics.

FIGURE 6.12 - Control beagles - mean wcc ±1SD (TABLE6.7)

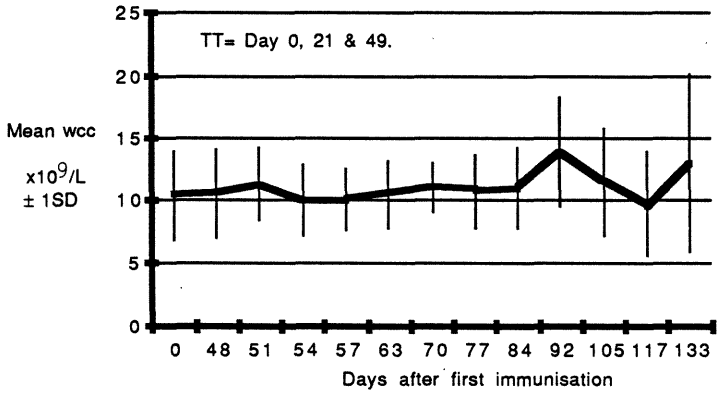


FIGURE 6.13 - Experimental beagles - mean wcc ±1SD (TABLE 6.8)

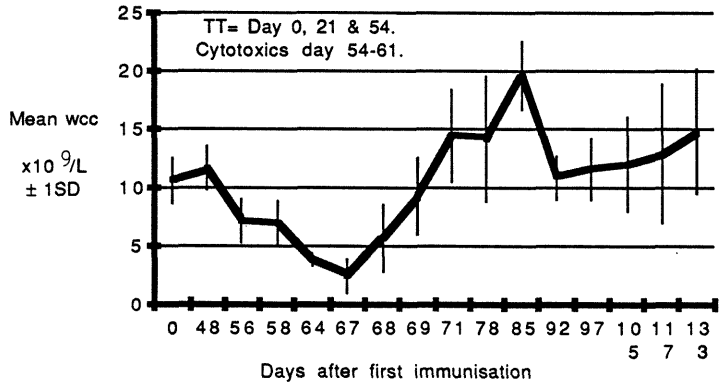


TABLE 6.7 -CONTROL GROUP -White cell count x 10^9/l in dogs given TT on days 0, 21 & 49.

DAYS	MONTY	ZIGGY	DUNCAN	SYDNEY	PODGE	FRED	TIZER	MUTLEY	MEAN±1SD
48	8.5	7.4	13.9	7.1	10.4	8	17.8	10.6	10.6±3.5
49									
51	10.4	11	11.3	10.5	11.3	6.5	16.1	10.6	11.3±2.9
54	8.6	9.6	13.9	7.8	9.8	5.5	14.6	10	10±2.8
57	10.6	7.1	11.1	9.3	8.3	7.5	14.6	10.2	10.1±2.4
63	14.4	11	9.3	6.5	12.9	6.5	12.5	12.6	10.6±2.7
70	10.6	10.8	13.4	7.4	12.3	8.7	13.4	10.5	11.1±2
77	12.9	11.7	10	10.3	13.8	4.5	15	10.8	10.8±2.9
84	10.4	13.9	15.5	8.8	11.2	6.3	13.5	8.5	11.1±3.2
89						7.7		11	
92	15.6	10	15.6	17.5	10.9	6	17.4	20.6	13.9±4.4
105	14.8	13.8	8	9.8	14.8	5.8	10.1	11.3	11.5±4.3
117	10.7	5.5		8.3	8.6	9.2	16.1	10.2	9.6±4.3
133	10	5		12.6	21.8	12.6	11.5	9.8	13.1±7.1

TABLE 6.8 - EXPERIMENTAL GROUP -White cell count x 10^9/l in dogs given TT on days 0, 21 & 54, cyclophosphamide on days 55-57 and cytosine arabinoside on days 54-61.

DAYS	SNOWY	TIFFANY	ROSEY	KIM	JANET	NEIL	PAINTER	CHESTER	MEAN±1SD
48	10	8.8	11.2	10.6	14.3	12.3	13.5	12.8	11.7±1.9
54									
56	5.3	6.6	5.9	10.7	9.7	6.8	6.3	6.5	7.2±1.9
58	4.5	6.2	5.4	7.7	9.8	6.4	8.2	7.8	7±1.9
64	3.8	3.5	3.1	4.3	3	3.8	3.7	4.3	3.7±0.5
65					1.2				1.2
66			0.8		0.9				0.9
67			1.2		4.1	2			2.4±1.5
68	4.3	5.5	4.5	6.3	12.2	2.9	6.4	3.7	5.7±2.9
69	6	12.2	5.4	12.9	5.6	12.9	12.8	8.2	9.3±3.3
71	13.9	11.7	8.6	21.9	13.8	21.9	12.7	17.8	14.5±4
78	12.5		13.9	24.8	10.2	24.8		10.7	14.2±5.4
85				21.8	17.6	21.8			19.7±3
92	10.3		9.5	12.7	10.6	12.7	13.3	11.9	10.9±1.9
97	7.7	12.1		11.8	15.2	11.8		9.6	11.6±2.6
105	11.5	10.9	11.2	9.5	21.6	9.5	11.5	8	12±4.1
117	9.9	10.5		11.5	25	11.5	11.9	8.5	12.9±6
133	10.7	9.3		14.6		14.6		23.2	14.8±5.4

The mean platelet count ±1SD is shown for the control (Figure 6.14; Table 6.9) and experimental beagles (Figure 6.15 Table 6.10). The cytotoxic agents significantly depressed the platelet count at days 2 ($p<0.002$), 10 ($p<0.001$), and 14 ($p<0.001$) after starting these agents (Table 6.16 - Mann Whitney U Test).

FIGURE 6.14 - Control beagles: mean platelet count ± 1SD (Table 6.9)

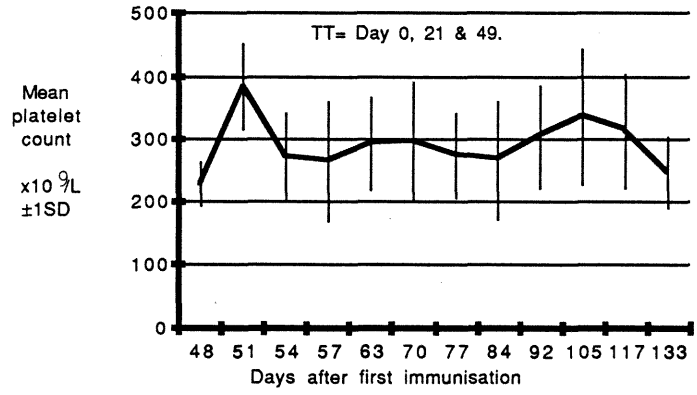


FIGURE 6.15 - Experimental beagles: mean platelet count ± 1SD (Table 6.10)

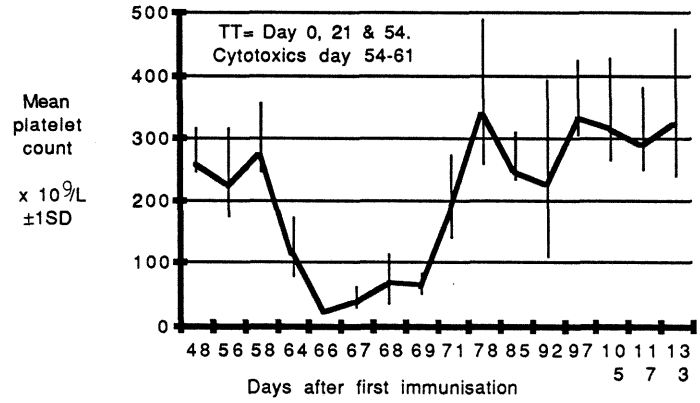


TABLE 6.9 - CONTROL GROUP -Platelet count x 10^9/l in dogs given TT on days 0, 21 & 49.

DAYS	MONTY	ZIGGY	DUNCAN	SYDNEY	PODGE	FRED	TIZER	MUTLEY	MEAN±1SD
48	160		249		257	244	215	262	229±35
49									
51	456	414	378	344	298	328	358	446	384±69
54	281	436	226	289	247	231	297	197	271±70
57	227	306	424	182	200	214	186	217	264±96
63	338	336	328	255	141	223	268	344	292±73
70	322	350	455	257	125	196	264	335	297±94
77	280	296	274	272	142	261	195	359	274±67
84	290	402	267	253	140	431	246	246	267±94
92	390	321	320	263	124	319	336	366	305±82
105	125	417	357	277	273	396	276	503	337±108
117	410	123		256	311	262	338	337	313±90
133	242	264		158	168	263	224	264	246±56

TABLE 6.10 - EXPERIMENTAL GROUP -Platlet count x 10^9/l in dogs given TT on days 0, 21 & 54, cyclophosphamide on days 55-57 and cytosine arabinoside on days 54-61.

DAYS	SNOWY	TIFFANY	ROSEY	KIM	JANET	NEIL	PAINTER	CHESTER	MEAN±1SD
48	289					250		225	255±32
54									
56	279	168	177	277	147	215	327	195	223±64
58	226	302	348	278	326	260	207	234	273±50
64	177	117	83	161	53	83	139	92	113±43
65					84				84
66			19		21				20±1.4
67			23		30	59			37±19
68	109	45	28	105	46	114	61	31	67±36
69			46		76	64			62±15
71			254		146	160			187±59
78	386	208	402	387	394	474	248	196	337±104
85	234	200		2895			234	272	245±34
92	457		223	116	123		165	273	226±128
97		279		387	321				329±54
105	385	257	212	415	265	258	364	329	311±73
117	259	268			255	232	400	300	286±60
133	242	480		342		330		212	321±105

The mean absolute lymphocyte count ±1SD is shown for the control (Figure 6.16; Table 6.11) and experimental beagles (Figure 6.17; Table 6.12). The cytotoxic agents significantly depressed the absolute lymphocyte count at days 2 ($p<0.002$) and 4 ($p<0.05$), after starting these agents (Table 6.16 - Mann Whitney U Test).

FIGURE 6.16 - Control beagles: mean lymphocyte count ±1SD. (Table 6.11)

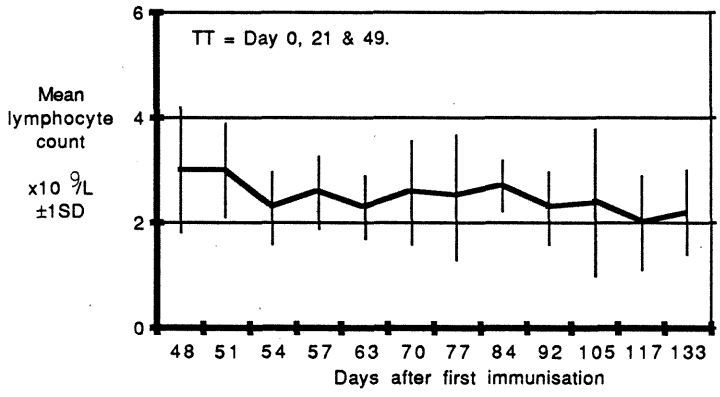


FIGURE 6.17 - Experimental beagles: mean lymphocyte count ±1SD

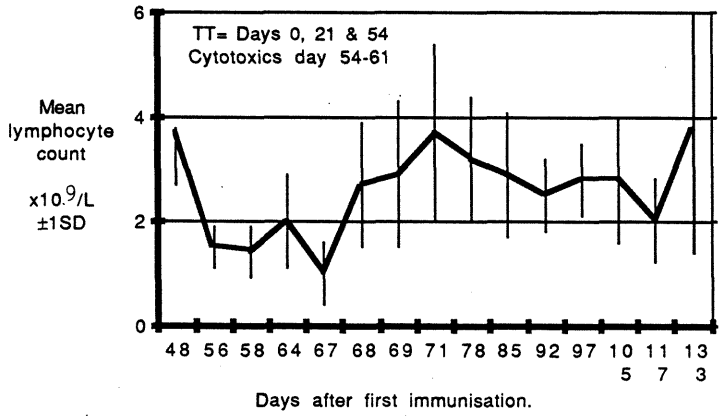


TABLE 6.11 - CONTROL GROUP -Lymphocyte count x 10^9l in dogs given TT on days 0, 21 & 49.

DAYS	MONTY	ZIGGY	DUNCAN	SYDNEY	PODGE	FRED	TIZER	MUTLEY	MEAN±1SD
48	2.1	1.6		2.3	4.2	2.2	5.1	3.6	3±1.2
49									
51	2.4	2.1	3.4	2.9	3.8	1.6	4.3	3	3±0.9
54	1.4	2.6	2.9	2.3	2.3	1	4	2	2.3±0.7
57	2	2.4	2.2	2.6	2.2	2.1	4.2	3.2	2.6±0.7
63	1.8	3	1.5	1.9	2.5	1.4	2.7	3	2.3±0.6
70	1.4	2.6	1.7	1.7	3	2	4.2	4	2.6±1
77	2.4	1.5	1.8	1.7	3.6	0.5	4.4	2.9	2.5±1.2
84	2.2	3.2	3	2	2.4		3.5	2.8	2.7±0.5
92	2.6	2.3	2.4	2.3	1	1.3	1.9	2.5	2.3±0.7
105	2.4	3.7	1.1	2.2	3.5	1.2	1.6	1.4	2.4±1.4
117	1.7	0.6		1.5	1.5	2.1	3.8	2.5	2±0.9
133	1.5	0.8		3	3.5	2.1	2.1	2	2.2±0.8

TABLE 6.12 - EXPERIMENTAL GROUP -Lymphocyte count x 10^9l in dogs given TT on days 0, 21 & 54, cyclophosphamide on days 55-57 and cytosine arabinoside on days 54-61.

DAYS	SNOWY	TIFFANY	ROSEY	KIM	JANET	NEIL	PAINTER	CHESTER	MEAN±1SD
48	3.5			3.5	5.4	2.6		3.6	3.7±1
54									
56	1.6	0.9	1.3	2.3	1.9	1.2	1.4	1.4	1.5±0.4
58	1.5	1	1	2.5	1.5	0.8	1.8	1.2	1.4±0.5
64	1.4	2.5	1.3	3.7	2.3	1.1	2.4	1.4	2±0.9
66			0.5		0.5				0.5
67			0.4		1.3	1.4			1±0.6
68	2.9	2.7	2.3	4.7	4.3	1.1	1.5	2.3	2.7±1.2
69			2		2.2	4.5			2.9±1.4
71			2.2		3.2	5.6			3.7±1.7
78	1.9	4.5	2.9	4.8	1.8	3.6	4	2.3	3.2±1.2
85	4.6	1.7		3.8			2.2	2.2	2.9±1.2
92	2.8		1.9		2.5	1.6	3.4	2.8	2.5±0.7
97		2.4		3.6	2.3				2.8±0.7
105	2.8	2.6	1.9	3.4	5	1.4	3.6	1.4	2.8±1.2
117	2.3	2.9			3	1.5	1	1.5	2±0.8
133	3.1	2.4		2.9		2.9	2.4	8	3.8±2.4

The allosensitisation history of the beagles at the commencement of these experiments is summarised in Table 6.13 . These beagles had been repeated challenged with alloantigen. All beagles were allosensitised to at least one member of the panel of seven greagles, indeed thirteen of the sixteen were sensitised against six or more of the greagle panel.

TABLE 6.13 - Summary of alloantigen exposure prior to the administration of third injection of TT and cytotoxics.
The day 27 serum, six days after second TT injection was used for the panel screen for lymphocytotoxic alloantibody.

CONTROLS	IMMUNISATION - (number) SKIN	BLOOD	KIDNEY	PANEL SCORE (7 GREAGLES)
B1-MONTY	10	5	1	7/7
B2-ZIGGY	9	4	2	6/7
B6-DUNCAN	6	5	1	6/7
B7-SYDNEY	6	4	1	7/7
B8-PODGE	6	4	1	7/7
B10-FRED	6	3	2	6/7
B17-TIZER	2	-	1	1/7
B18-MUTLEY	2	-	1	4/7
EXPERIMENTAL	IMMUNISATION - (number) SKIN	BLOOD	KIDNEY	PANEL SCORE (7 GREAGLES)
B13-SNOWY	6	4	2	7/7
B12-TIFFANY	6	8	1	6/7
B11-ROSEY	6	8	1	7/7
B19-KIM	2	0	1	4/7
B15-JANET	6	8	1	7/7
B4-NEIL	8	4	2	6/7
B5-PAINTER	6	5	1	7/7
B3-CHESTER	8	5	2	6/7

Alloantibody was measured by performing CDC crossmatch tests on sera from each beagle, testing each beagle against a selected greagle to which lymphocytotoxic alloantibody had previously been shown. The results are presented in Table 6.14. The results of the CDC test were divided into three categories:

(1) 10-30% kill- this was described as a negative (-) result as it was comparable to the kill observed with pooled dog sera.
(2) 40-50% kill - a weak positive (±) result.
(3) 60-90% kill - a strong positive (+) result.

The sera examined were those before the administration of cytotoxics, and 10,17, 31, and 64 days after starting the cytotoxics. Comparable sera were examined from the control group. The results are presented in Table 6.14 . Unfortunately, the day 0 sera in the experimental sera showed less cytotoxicity against prospective donor greagles than the control sera. Nevertheless by day 31 a reduction in cytotoxicity was seen in seven of the experimental group, compared with no reduction in the control group by day 36. At this time, five out of eight of the experimental group were CDC crossmatch negative compared with none in the control group. However, by day 6$\frac{5}{5}$ the CDC tests in the experimental group were all positive, suggesting a rebound in the alloantibody levels. None of the beagles were exposed to alloantigen in the 6$\frac{5}{5}$ days from the commencement of the cytotoxic agents.

TABLE 6.14 - Results of CDC crossmatch test against single prospective donor selected from the greagle panel on the basis of an 80-90% kill in the panel screen shown in Table 6.13. The level of kill is graded as shown below this table. The day of testing is shown both from the day of first exposure to TT and in parentheses from the day of the third dose of TT and commencement of cytotoxics.

CONTROL BEAGLES	GREAGLE TARGET	DAY 49 (Day 0)	DAY 57 (Day 8)	DAY 64 (Day 16)	DAY 85 (Day 36)	DAY 114 (Day 65)
B1-MONTY	G16	+	+	+	+	+
B2-ZIGGY	G16	+	+	+	+	+
B6-DUNCAN	G16	+	+	+	+	
B7-SYDNEY	G16	+	+	+	+	+
B8-PODGE	G20	+	+	+	+	+
B10-FRED	G20	+	+	+	+	+
B17-TIZER	G16	±	+	+	±	-
B18-MUTLEY	G11	+	+	+	+	±
EXPERIMENTAL BEAGLES	**GREAGLE TARGET**	**DAY 54 (Day 0)**	**DAY 64 (Day 10)**	**DAY 71 (Day 17)**	**DAY 85 (Day 31)**	**DAY 119 (Day 65)**
B13-SNOWY	G11	±	±	±	-	+
B12-TIFFANY	G11	+	+	±	±	+
B11-ROSEY	G15	±	±	±	-	+
B19-KIM	G15	+	+	+	+	+
B15-JANET	G14	±	±	±	-	+
B4-NEIL	G12	±	-	-	-	+
B5-PAINTER	G12	±	-	-	-	+
B3-CHESTER	G14	+	+	+	±	+

+ = 80-90% CELL KILL
± = 40-50% CELL KILL
- = 10-30% CELL KILL

The outcome of subsequent renal allografts to these beagles is summarised in Table 6.15. In the experimental group, six were given a positive CDC crossmatch kidney, and despite receiving what has previously been documented as adequate immunosuppression with CyA in this model with crossmatch negative kidneys, four had early vascular rejection, and two ruptured. Rupture in the latter two dogs was probably related to attempts to close the dead space around the graft rather than accelerated rejection (see chapter 5). Two of the experimental group were given CDC crossmatch negative greyhound kidneys (though still CDC crossmatch positive with greagles) and vascular rejection was delayed. Of the controls, seven were grafted with a positive CDC crossmatch, and all had vascular rejection despite CyA. The eighth was given a CDC negative crossmatch greyhound kidney (positive CDC test with greagles) and had delayed vascular rejection. It is difficult to compare the outcome of renal allografting to the experimental and control groups because of the variable pre-transplant crossmatch result, but considering only the positive CDC crossmatches there was no significant difference in day of diagnosis of vascular rejection:

	Day of diagnosis of vascular rejection - see Table 6.15.
Control	2,4,5,6,6,11.
Experimental (cytotoxics)	1,5,6,9.

p NS (Mann Whitney U Test).

TABLE 6.15 - Outcome of subsequent renal allografts to the allosensitised beagles used in these TT experiments. The time of grafting is shown in days both from the first TT injection and, in parentheses, from the third TT injection and commencement of cytotoxics. The result of the pre-transplant CDC test and immunouppression used are also shown.

RECIPIENTS CONTROLS	Donor+Day Transplanted (Day0= 1st TT)	CDC-test Pre-Tx	Immunosupp (Experimental group)	Day after grafting of diagnosis of vascular rejection
B1-MONTY	G16- day 99 (50)	+	CyA+PGI2 (8)	Day 5
	G11- day 146 (97)	+	CyA+PGI2 (8)	Day 6
B2-ZIGGY	G23- day 138 (89)	+	CyA+PGI2 (8)	Day 11
B6-DUNCAN				
B7-SYDNEY	G21- day 131 (82)	-	CyA (7)	Day 14
B8-PODGE	G20- day 121 (72)		CyA (6)	Day 6
	G21- day 131 (82)	+	CyA+PGI2 (8)	Day 6
B10-FRED	G20- day 131 (82)	+	CyA+PGI2 (8)	Day 4
B17-TIZER				
B18-MUTLEY	G18- day 85 (36)	±	CyA+PGI2 (8)	Day 2
EXPERIMENTAL- cytotoxics given				
B13-SNOWY	G22- day 138 (84)	-	CyA (7)	Day 29
B12-TIFFANY	G23- day 138 (84)	-	CyA (7)	Day 22
B11-ROSEY	G15- day 119 (65)	+	CyA+Heparin (9)	Day 5
B19-KIM	G15- day 119 (65)	+	CyA+PGI2 (8)	Day 9
B15-JANET	G14- day 127 (73)	+	CyA+Heparin (9)	Rupture day 2
B4-NEIL	G17- day 139 (85)	+	CyA+PGI2 (8)	Day 6
B5-PAINTER	G21- day 133 (79)	±	CyA+PGI2 (8)	Day 1 (Rupture day 1)
B3-CHESTER	G14- day 127 (73)	+	CyA+PGI2 (8)	Rupture day 1

Grading of CDC crossmatch result:
+ = 80-90% CELL KILL
± = 40-50% CELL KILL
- = 10-30% CELL KILL

Renal allografts were given to the following groups:
Group 6 - Allosensitised, positive CDC test and CyA.
Group 7 - Allosensitised, negative CDC test and CyA.
Group 8 - Allosensitised, positive CDC test and CyA and prostacyclin (PGI2).
Group 9 - Allosensitised, positive CDC test and CyA + Heparin.

6.4 Discussion

The combination of cyclophosphamide and cytosine arabinoside has been shown to be capable of modulating the production of alloantibody and anti-TT in this canine model in the short term. The cytotoxic agents were given using a protocol described by Terman (1978), except that the dose of cytosine arabinoside was halved. The marrow toxicity seen here indicates that it would not have been safe to prolong the use of these two agents at the dose employed. Anti-TT was significantly reduced at four and 14 days by these two cytotoxics, but there was no difference from controls by day 24, which was 17 days after finishing the cytotoxics.

The two cytotoxic agents reduced the alloantibody level as determined by the CDC crossmatch test. The greatest reduction in lymphocytotoxicity was seen 31 days after starting the two agents (24 days after their finish) - Table 6.14. There was some reduction at days 10 and 17. However, the sera screened at 65 days showed no reduction in lymphocytotoxicity suggesting a recovery of alloantibody levels to pre-treatment levels. The beagles were grafted after this alloantibody rebound, with disastrous results. In retrospect, the outcome might have been different had they been grafted around 31 days. The recovery of anti-TT (24 days) appeared earlier than that of alloantibody (after 31 days) as estimated by lymphocytotoxicity, though it would be necessary to quantitate more accurately the alloantibody response before being confident of this. This may reflect differences in the nature and route of administration of the antigens.

Though reductions in alloantibody were observed, they were negated by a delayed rebound after discontinuing the cytotoxics. There was no beneficial effect with the cytotoxics on subsequent renal allograft survival, though the dogs were grafted after the alloantibody rebound. Continuing the cytotoxics beyond seven days, as permitted by marrow toxicity, and better timing of renal allografting might have yielded better results. The greagle pbl that each beagle was tested against as shown in Table 6.14 were not usually the source of the renal allograft as shown in Table 6.15, though the greagles in this part of the study were littermates (G11-G20, see chapter 4.6.2).

The two antibody responses measured were not comparable. The anti-TT response measured by the ELISA test was a quantitative result for a single specificity, whereas the detection of alloantibody by the CDC crossmatch test was an all or none response, that may have been the sum of many specificities.

These results suggest that alloantibody responses in dogs may be susceptible to cytotoxics, but that the efficiency of such treatment depends on many factors such as type of immunogen, potency, antibody titre and length of treatment.

Table 6.16 - Summary of comparisons between experimental and control beagles.

Control vs Experimental	Days after start of cytotoxics Control	Experimental	Anti-TT	wcc	lymphs	platelet
Day 0 vs Day 0			NS			
21 vs 21			NS			
27 vs 27			NS			
49 vs 54	0	0	NS	NS	NS	NS
51 vs 56	2	2	NS	p<0.01	p<0.002	p<0.002
54 vs 58	5	4	p<0.01	p<0.02	p<0.05	NS
57 vs 64	8	10	NS	p<0.001	NS	p<0.001
64 vs 68	15	14	p<0.05	p<0.002	NS	p<0.001
70 vs 78	21	24	NS	NS	NS	NS
77 vs 78	28	24	NS	NS	NS	NS
85 vs 92	36	38	NS	NS	NS	NS
92 vs 97	43	43	NS	NS	NS	NS
100 vs 105	51	51	NS	NS	NS	NS
114 vs 119	65	65	NS	NS	NS	NS
128 vs 133	79	79	NS	NS	NS	NS

Mann Whitney U Test - two tail

CHAPTER 7

PROSTACYCLIN AND THE ALLOSENSITISED DOG MODEL

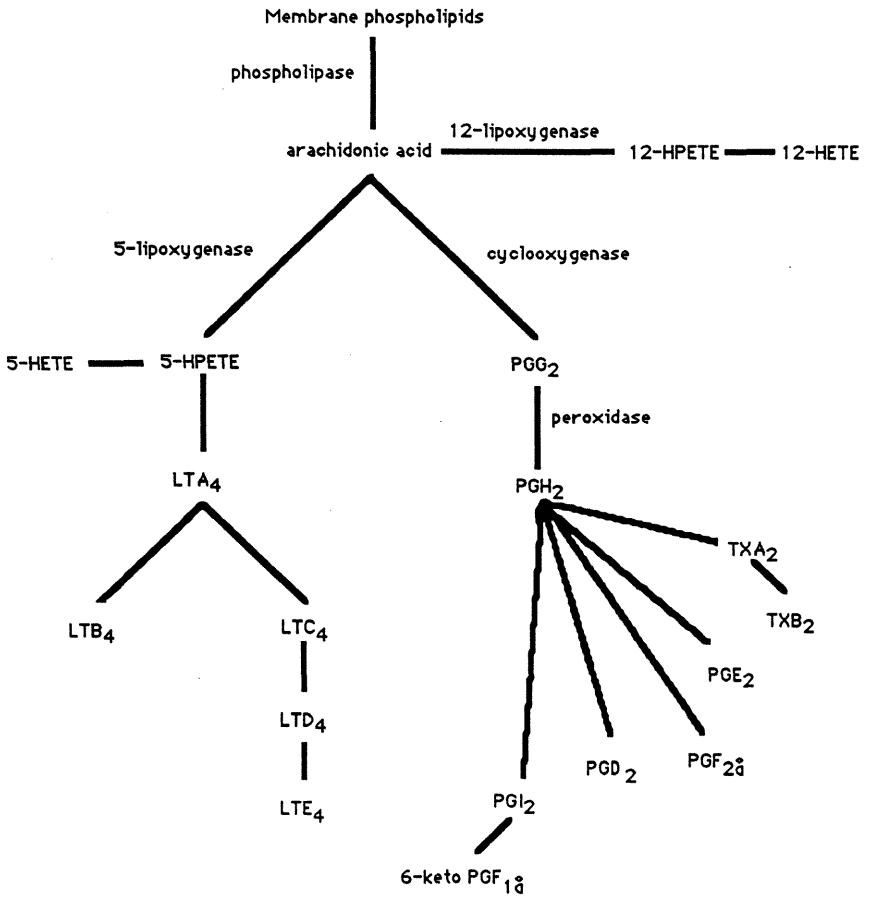
7.1 Introduction

Evidence is accumulating that lipid mediators modulate both the affector and effector arms of the immune cellular response in organ transplantation. The biologically active lipids include the eicosanoids, platelet activating factor (PAF), diacyl glycerol, phospholipids, and the polyphosphoinositides. Eicosanoids are defined as compounds derived from the same eicosaenoic (*eicosa* = 20-carbon; *enoic* = containing double bonds) acid precursors. Thromboxane (TXA_2), prostacyclin (PGI_2), hydroperoxyeicosatetraenoic acids (HPETE's), hydroxyeicosatetraenoic acids (HETE's) and the leukotrienes are eicosanoids, which have in common a pathway originating with the oxygenation of arachidonic acid, but their structures and actions differ markedly. PAF is not an eicosanoid, for although 2-arachidonyl PAF contains an eicosanoid precursor, PAF is not an oxidation product of such a fatty acid. The oxidation of arachidonic acid is catalysed by cyclooxygenase, various lipoxygenases, and by P-450 enzymes. Consequently some 50 to 100 products can be identified, and the number of metabolites of these products is far higher (Ramwell-1986).

Endogenously generated eicosanoids are synthesised at the cell membrane. The appropriate stimulus may vary, depending on the cell type. The cascade of prostaglandin synthesis begins with the release of arachidonic acid from phospholipids via activation of phospholipase, primarily phospholipase A_2. This rate limiting step is initiated by hormonal, ischaemic, neural, inflammatory, or other stimuli varying from cell to cell. Once released the arachidonic acid is available for enzymatic oxidation. One type of enzyme is a series of lipoxygenases that oxidise arachidonic acid at different carbon atoms, eg, 5 & 12. Different

lipoxygenases have been described in different tissues. The 12-lipoxygenase in platelets produces the unstable 12-hydroperoxyeicosatetraenoic acid (12-HPETE), which is reduced by peroxidase to the stable 12-hydroxyeicosatetraenoic acid (12-HETE). By a similar mechanism, 15-lipoxygenase in leukocytes forms 15-HPETE and 15-HETE.

FIGURE 7.1 - Eicosanoid biochemical pathways



HETE = hydroxyeicosatetraenoic acid

LT (A_4, B_4, C_4, D_4, E_4) = leukotriene

PG (D_2, E_2, $F_{1\alpha}$, $F_{2\alpha}$, G_2, H_2, I_2) = prostaglandin

Tx (A_2, B_2) = thromboxane

HPETE = hydroperoxyeicosatetraenoic acid

Oxidation of arachidonic acid by cyclooxygenase at C 11 produces a cyclopentane ring and a cyclic endoperoxide, prostaglandin G2 (PGG_2), which is rapidly converted by peroxidase activity to PGH_2, the unstable common intermediate of prostaglandins and thromboxanes. Conversion of PGH_2 to thromboxanes or various prostaglandins is effected by enzyme systems, the distribution of which varies between cell types. Examples are PGE_2 and $PGF_{2\hat{a}}$, which are present in many tissues, and have diverse possible physiological actions including smooth muscle contraction and as pyrogenic and inflammatory mediators. Prostacyclin (PGI_2) is prominent in vascular endothelium and PGD_2 is a product of mast cells and brain tissue.

Leukotriene formation, which predominates in polynuclear leucocytes and some mononuclear cells, results from 5-lipoxygenase activity forming 5-HPETE and conversion to an epoxide moiety, the unstable common intermediate, leukotriene (LT) A_4. LTA_4 can react with water to form the potent chemotactic leukotriene, LTB_4. Alternatively, LTA_4 reacting with glutathione leads to the peptide derivatives LTC_4, LTD_4 and LTE_4.

A problem in defining the pathophysiological roles of eicosanoids is that, different eicosanoids are frequently released simultaneously from activated cells, and these products often display diametrically opposite biological properties. Furthermore, not all the products have the same half life. A further problem is that enzyme inhibitors may divert precursors into other metabolic pathways, where the products possess potent biological properties of their own. This appears to be the case with cyclooxygenase and thromboxane synthetase inhibitors. Therefore, receptor antagonists are of crucial importance in helping to identify the precise role of eicosanoids. A further problem is the availability of assay procedures to measure these lipids. Zipser (1985) defined the methodological problems as including, poor antiserum specificity in early radioimmunoassays, the myriad of unidentified prostaglandin metabolites that may cross-react in these assays, poor sensitivity and specificity of bioassays, the potential for artifactual generation of eicosanoids during sample collection, and the short half-life of many eicosanoids.

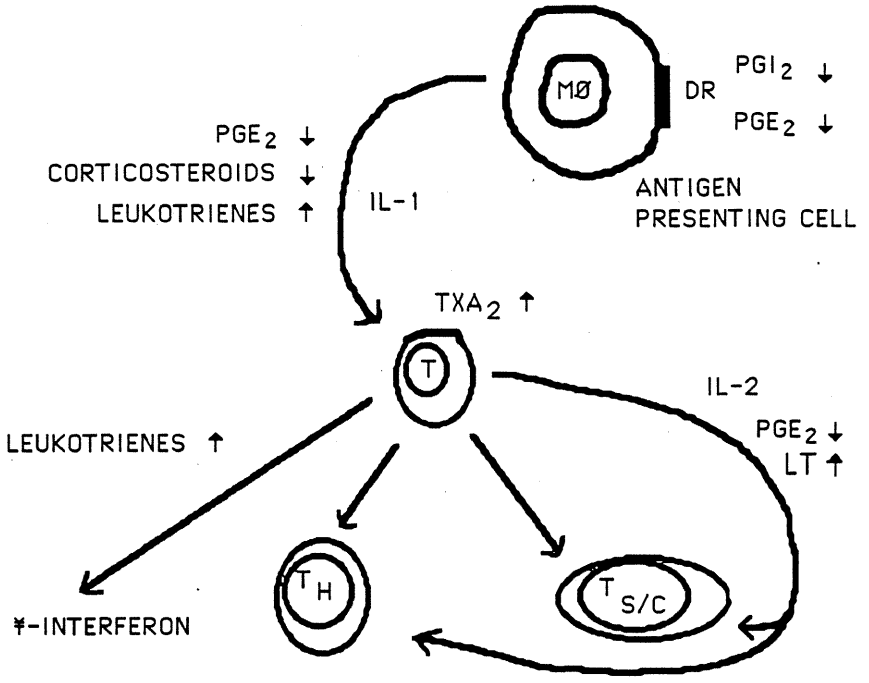
Mundy (1980) described the beneficial effect of a high dosage prostacyclin (PGI_2) infusion on short term (six hours) survival of renal allografts in a dog model, in which the recipients were specifically allosensitised against the donor. We have attempted to extend this work to our own allosensitised canine renal allograft model, looking at the effect of a prostacyclin infusion given over six days in preventing rejection of a renal allograft by an allosensitised recipient with a positive CDC crossmatch.

7.2 Lipid mediators in organ transplantation

7.2.1 The eicosanoids as modulators of leucocyte function.

Prostaglandin E_2 (PGE_2) inhibits interleukin 1 (IL-1) and IL-2 production (Rappaport-1982), whereas leukotrienes, indirectly, have been shown to promote IL-1 production (Farrar-1985). PGE_2 , via its stimulatory effect on adenylate cyclase, is a negative signal to IL-2 regulated proliferation and differentiation of both human and murine lymphocytes (Farrar-1986). Farrar showed in-vitro that both the proliferation of human activated T lymphocytes and the differentiation of lymphokine-activated killer (LAK) cell activity stimulated by IL-2 were negatively controlled by PGE_2. Leukotriene B_4 (LTB_4) has been reported to promote lymphocyte proliferation (Goodwin-1986, Rola-Pleszczynski-1986) and migration (Jordan-1986). A thromboxane A_2 (TXA_2) analogue (U 46619) has been shown by Ceuppens (1985) to promote mitogen-induced lymphocyte proliferation and to reverse the inhibition of lymphocyte proliferation obtained with a TXA_2 synthetase inhibitor (dazoxiben). PGE_2 is a potent inhibitor in-vitro of the expression of Ia-antigen (murine class 2 antigen) on murine macrophages (Snyder-1982). PGI_2 decreases Ia antigen expression on mouse kidney treated with bacterial lipopolysaccharide (Jephthah-Ochola - 1987). Leukotrienes B_4, C_4 and D_4 are capable of increasing the release of gamma interferon (INF-¥) from murine T-helper cells in-vitro (Johnson-1984). INF-¥ increases class 1 and 2 antigen expression in-vitro on a wide variety of cell types (Basham-1983). Observations like these suggest the possible involvement of the eicosanoids in immune responses - Figure 7.2.

FIGURE 7.2 - Modulation of macrophage-lymphocyte interaction by eicosanoids after Foegh (1986a).



LT = leukotrienes
MØ = macrophage
T = T lymphocyte
T_H = T helper cell
$T_{S/C}$ = T suppressor/cytotoxic cell

Goldyne (1986) has recently reviewed the conflicting evidence for the regulation by eicosanoids of the immunological reactivity of human lymphocytes and Table 7.1 summarises the conclusions for two eicosanoids.

TABLE-7.1: Summary of the observed effects of LTB_4 & PGE_2 on human lymphocyte reactivity.

PGE_2	Reactivity	LTB_4
↑ ↓	Blastogenic proliferation	↑ ↓ —
↑	Suppressor/cytotoxic T cell generation	↑ ↓
↑ ↓	Natural killer cell activity	↑
↑ ↓	Antibody dependent cellular cytotoxicity	
↓	Interleukin 2 generation	
↑ ↓	Lymphokine secretion	
↓	Mixed lymphocyte reaction	

Key. ↑, enhancement; ↓, suppression; —, no effect.

The fact that some of the specific reactivities show variation in the response to PGE_2 or LTB_4 reflect different experimental conditions or in the case of lymphokines, the type of lymphokine being measured.

Human peripheral blood monocytes and macrophages can generate PGE_2, $PGF_{2\alpha}$, TXA_2 5-hydroxyeicosatetraenoic acid (5-HETE), LTB_4, and leukotriene C_4 (LTC_4). In addition, PGI_2 has been recovered from human peritoneal macrophages and monocytes. There are conflicting reports concerning the ability of the normal human lymphocyte to produce arachidonic acid metabolites. Goldyne (1986) argues that lymphocytes do not normally produce eicosanoids, and that the production of eicosanoids correlates with the presence of either contaminating platelets, or monocytes, or neutrophils. In contrast Goodwin (1986) argues that LTB_4 is produced in phytohaemagglutinin (PHA) stimulated cultures of human peripheral blood T cells. In addition, the presence of exogenous arachidonic acid profoundly suppressed LTB_4 production by PHA-stimulated T cells. Thus the addition of arachidonic acid to the incubation media could explain why some workers found no eicosanoid production by T cells. Rola-Pleszczynski (1986) found that LTB_4 enhanced the binding of effector lymphocytes to susceptible target cells. LTB_4 was also shown to augment the lytic efficiency of natural killer cells when it was present during effector-target conjugate formation.

Jordan (1986) tested the ability of arachidonic acid metabolites to modulate differentially the responses of T-lymphocyte clones with specifically defined effector functions. T-lymphocyte clones were derived from MLC between two mice strains. Clones were analysed for proliferative and cytolytic capacity and Lyt (cell surface markers on murine T cells)

phenotype. It was observed that the effector phase of the cytotoxic T cell (CTL) response was not affected by PGE_2 at physiological concentrations (10 to 100 ng/ml). The in-vitro migration of T-helper (T_h) lymphocyte clones was inhibited by PGE_2, whereas migration of T-cytotoxic clones was not affected by even the highest concentration of PGE_2 (1000 ng/ml). Using murine recombinant IL-2 to induce proliferation of T lymphocyte clones, the proliferation of T_h but not T cytotoxic clones was inhibited by concentrations of PGE_2 (10 to 100 ng/ml), which were also found to inhibit helper cell migration. Consistent with these observations it was seen that T cytotoxic clones could respond chemokinetically to LTB_4, even in the presence of high concentrations of PGE_2. In contrast, the inhibitory effects of PGE_2 on helper cell migration predominated in the presence of LTB_4. Although the mechanism governing differential responses to PGE_2 is unknown, there exists the possibility of a role for the immunoreactive metabolites of arachidonic acid in the regulation of T cell responses. Furthermore, as the in-vitro inhibitory effects of PGE_2 occur at concentrations detected at sites of inflammation in-vivo, there is reason to speculate on an immunoregulatory role for PGE_2. Since PGE_2 is thought to act in part by stimulating cellular cAMP, Jordan (1987a) examined the effects of the cAMP-active agents theophylline and dibutyrl cyclic adenosine monophosphate (dbcAMP) on lymphocyte migration. In contrast to the subset specific effects of PGE_2, theophylline and dbcAMP inhibited both CTL and Th migration. These observations suggest that cAMP directly modulates lymphocyte function regardless of cell effector function but that PGE_2 alone somehow fails to transmit this signal to CTL. As yet the mechanism of this selectivity of PGE_2 remains unknown. Jordan (1987b) has examined the effect of nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor, on the in-vitro functions of clones of allosensitised T cells. NDGA inhibited proliferation of Th and CTL clones. NDGA also inhibited CTL and natural killer cytotoxicity. This inhibition was not altered by indomethacin. NDGA was less effective if added after the initiation of lymphocyte proliferation and had no effect once effector target binding occurred. Thus the lipooxygenase inhibitor NDGA suppresses T cell function without selectivity with respect to clone effector function. As it works in the presence of indomethacin it is unlikely that it works by diverting arachidonic acid metabolism towards the cyclooxygenase pathway.

There is evidence for the eicosanoids modulating both the formation and action of monokines and lymphokines, as well as antigen expression. Eicosanoids are able to modulate differentially the responses of T cell clones with specifically defined effector functions. In the interactions between lymphocytes and macrophages, the role of the endogenously generated eicosanoids as opposed to exogenously introduced eicosanoid remains to be determined.

7.2.2 Eicosanoid synthesis and allograft function

Foegh (1987) has tested an extensive number of agents related to arachidonic acid metabolism in a rat cardiac allograft model. The effect of these agents was compared with that of CyA + Pred and Aza + Pred. The dose of CyA was 0.5 mg/kg/day intramuscularly, and azathioprine 5 mg/kg/day. Neither dose by itself prolonged allograft survival. None of the agents tested prolonged graft survival on their own. Table 7.2 summarises the results:

TABLE 7.2: Drugs that do and do not prolong rat cardiac allograft in combination with either low dose CyA or Azathioprine. Foegh-1987.

Prolong survival.

Acyl hydrolase inhibitor
- Prednisolone

Thromboxane synthetase inhibitor
- OKY 1581 (Ono, Japan).

Thromboxane receptor antagonist.
- L640,035 (Merck-Frost, Canada).
- AH 23848 (Glaxo UK).

5-Lipoxygenase inhibitor.
- EP 10045 (Beaufour Institute, France).
- SC 33579 (G.D. Searle).
- Dipyridamole (Boehringer-Ingelheim).
- L 751,392 (Merck-Frost, Canada).

Prostacyclin analogue.
- Iloprost (Schering AG, Germany).

Do not prolong survival

Cyclooxygenase inhibitor
- Indomethacin

Leukotriene D4 antagonist.
- SC 39070 (G.D. Searle).

The improvement in results with these agents was not startling. The untreated controls survived 8.5 days, and the Pred and CyA group 18 days. The substitution of Pred by one of these agents produced survivals of up to 21 days (TXA_2 receptor antagonist). These data, in the view, of Foegh provide a basis for considering the use of TXA_2 synthetase inhibitors,

TXA_2 antagonists, prostacyclin analogues, and 5-lipoxygenase inhibitors as agents that would permit decreased doses of CyA or Aza and as agents that might replace corticosteroid immunosuppressive therapy. As LTB_4 promotes lymphocyte proliferation and NK activity it will be interesting to test an LTB_4 antagonist in this model.

PGE_2, PGD_2, and PGI_2 all have properties that attenuate cell-mediated rejection. PGI_2 is more potent than PGE_2, but PGE_2 may be more relevant since the human macrophage produces far more PGE_2 than PGI_2. A further property of PGE_2 is inhibition of lymphocyte migration. The non-immune related effects, vasodilatation and platelet antiaggregatory activity of these prostaglandins may be important in maintaining allograft function during rejection.

Prolongation of rat renal allograft survival by PGE_1 was reported by Strom (1983). Rowles (1986) found that a PGI_2 analogue, iloprost, exerted a synergistic effect with CyA in a rat cardiac transplant model. Imura (1987) showed that the PGE_1 analogue 15(s)-15-methyl PGE_1 produced a significant prolongation survival of rat cardiac allografts. However the PGE_1 analogue was still markedly less effective than CyA. Aziz (1986) also observed that CyA and 15-methyl-PGE_1 each resulted in prolongation of rat cardiac allograft survival. However, with combination therapy the graft survival was much improved.

Jephthah-Ochola (1987) examined the ability of a relatively stable analogue of prostacyclin (6a-carba-PGI_2) to modulate inducible MHC expression in the mouse kidney. Class 1 and class 2 MHC product expression were induced in mice with bacterial lipopolysaccharide (LPS). The measurement of MHC product expression were determined by three methods: 1) radioimmunoassay, 2) immunoperoxidase staining, and 3) renal messenger RNA specific for class 1 and class II antigens, which was determined by northern blot analysis. The 6a-carba-PGI_2 suppressed LPS-induced class 1 and 2 antigen expression at the mRNA level. Whether this was by a direct action on pre-existing mRNA stability or on transcription from MHC genes remains unknown. Thus under these experimental conditions, when a state of enhanced MHC expression exists, PGI_2 was shown to have the potential to regulate the immune response by modulating the amount of MHC product expression.

TXA_2 and the leukotrienes may act as 'prorejection' compounds, promoting both mediator release and initiating lymphocyte proliferation by increasing cytosolic calcium formation and preventing an increase in cAMP levels. In addition, these products have vascular effects that are similar to rejection related events such as oedema and a reduction in graft blood flow. Lowry (1987) showed that rat cardiac allografts released more PLA_2, PAF, TXB_2, 6-oxo-$PGF_{1\text{-alpha}}$, and LTC_4 than normal hearts. Whilst PAF is a very potent inflammatory mediator, it was not possible to say whether or not the levels of PAF measured in rejecting heart allografts would suffice to exert direct tissue injury. In the rat heterotopic cardiac transplant model, both the inhibition of TXA_2 synthesis and a TXA_2 antagonist prolonged graft

survival.

Coffman (1986) observed with a rat model of acute renal allograft rejection that urinary TXB_2 excretion directly correlated with renal thromboxane production measured in-vitro in the isolated perfused kidney. There were also significant increases in the renal production of both LTC_4 and PAF compared with isografts. Leukotrienes increase vascular permeability.

Mangino (1986) has shown a significant increase in TXB_2 levels in the cortex of rejecting dog renal allografts not receiving immunosuppression. No concomitant change in PGI_2 production was observed. A close association between the degree of rejection as assessed histologically and the rejection induced increase in TXB_2 production was seen. This supports the possibility that local thromboxane release may participate in the tissue damage seen during acute rejection, possibly by inducing vasoconstriction and intrarenal thrombosis. Production of both 12-HETE and 15-HETE was greater in allograft renal cortex undergoing rejection than in normal cortex. Allografted cortex generated roughly 50 times greater amounts of LTB_4 than did normal cortex. There was no significant change in eicosanoid production in rejecting renal medullary tissue.

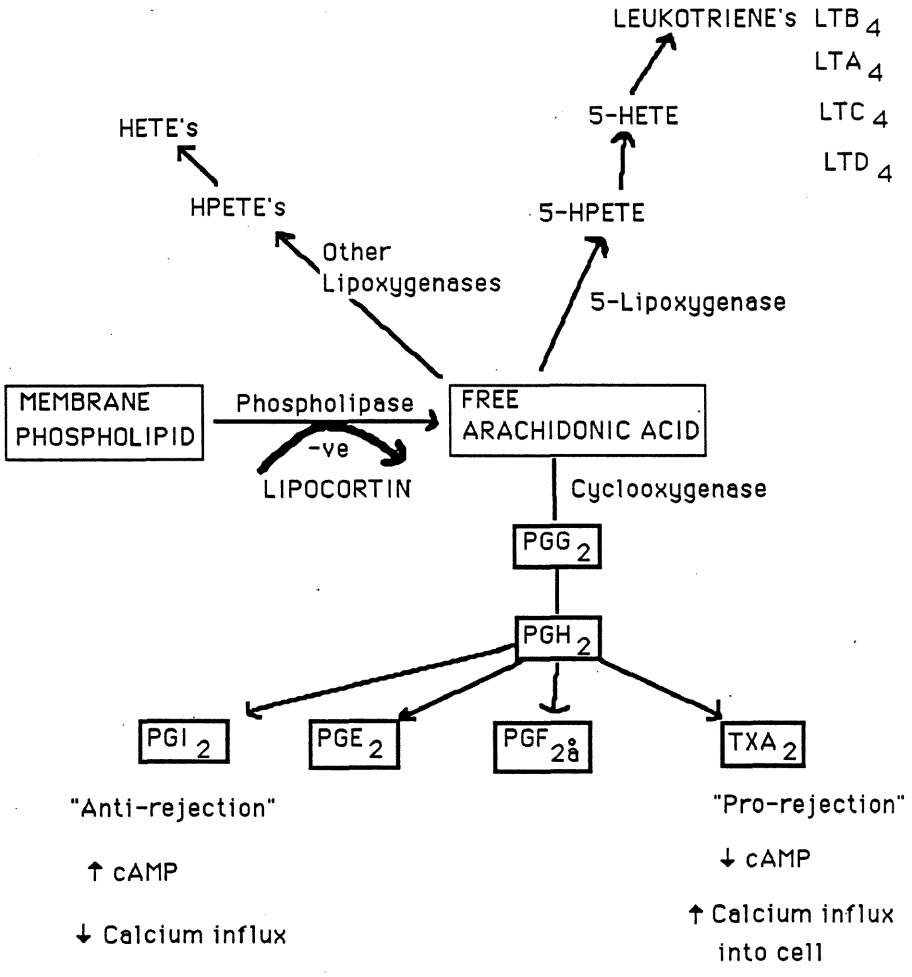
Kawaguchi (1987) found that urinary excretion of immunoreactive TXB_2 ($iTXB_2$) was an indicator of rat cardiac allograft rejection in both immunosuppressed and nonimmunosuppressed rats. In the strain of rats used (Fisher, CyA increased $iTXB_2$ excretion, though the increase was less than that seen in allograft rejection. In contrast, azathioprine and prednisolone appeared to attenuate urine $iTXB_2$ production during rejection. Steinhauer (1986) observed an increase in $iTXB_2$ in the urine with clinical allograft rejection. No increase in iTXB2 was seen over controls with CyA treatment. An increase in $iTXB_2$ was seen following cytomegalovirus infection or renal vein thrombosis. A single dose of aspirin (3mg/kg) reduced the increase in urinary $iTXB_2$ excretion in allografted patients during acute rejection. As platelets do not possess the capacity for de novo protein synthesis, acetylation by aspirin of the cyclooxygenase results in long lasting inhibition of platelet eicosanoid production, whereas endothelial cells and macrophages resynthetise cyclooxygenase after acetylation within 24 to 48 hours. This provides some evidence that platelets could be the source of increased TXB_2 in acute interstitial rejection.

The cells responsible for producing arachidonic acid metabolites in rejecting renal tissue remain unknown, as does the significance of enhanced eicosanoid production in the cortex of rejecting renal allografts. Renal parenchymal cells are able to produce eicosanoids in rejecting cortex, though the eicosanoids could also originate from cells infiltrating the graft, eg platelets or monocytes. LTB_4 promotes neutrophil chemokinesis, and may serve to initiate or maintain the infiltration of immune competent cells into the graft. Mast cell and neutrophil degranulation is stimulated by 12-HETE, and 12-HETE may be involved in the attachment of macrophages to glomeruli. Thromboxane production could mediate tissue

damage in allograft rejection. Lim (1987) has taken FNAB's from humans and correlated the cellular TXB_2 production with the number of monocytes/macrophages. The production of TXB_2 formed by the aspirate on incubation also correlated with urinary TXB_2. This provides further support for the role of thromboxane in allograft rejection.

Foegh (1986a) has attempted to link together these observations - Figure 7.3. Those arachidonate products that activate adenylate cyclase and increase intracellular cAMP, such as prostacyclin and PGE_2, attenuate the immune response. This effect may be due to the effect of cAMP in maintaining low cytosolic ionic calcium. Other products of cyclooxygenase (TXA_2) and the products of 5-lipoxygenase (leukotrienes B_4, C_4, D_4, E_4) all appear to facilitate immune responses. This may take place by facilitating calcium entry and preventing adenylate cyclase activation. TXA_2 and leukotrienes increase T lymphocyte clonal expansion, natural killer cell activity, and DR expression. The prostaglandins have opposite effects. Corticosteroids block both cycloxygenases and lipoxygenases by preventing arachidonate deesterification from phospholipid. The important therapeutic effect of corticosteroid is more likely to be related to lipoxygenase product synthesis, since the cyclooxygenase route is blocked by indomethacin, which does not improve graft survival. If however the thromboxane synthetase component of the cyclooxygenase route is blocked, then increased graft survival is seen. The concomitant redirection of the cyclic endoperoxides to the prostaglandins is a further consequence of inhibiting TXA_2 synthetase.

FIGURE 7.3 -Lipid mediators in organ transplantation after Foegh (1986a).



7.2.3 Immunosuppressive agents and arachidonate metabolism.

The mechanisms of action of the main immunosuppressive agents and the possible relationships with the eicosanoids are now considered. Strom (1987) has recently reviewed the immunopharmacology of graft rejection without making any mention of arachidonic acid metabolites. Foegh (1986) discussing the same subject referred almost exclusively to arachidonic acid metabolites. The reality may lie someway between these two opinions.

The corticosteroids prevent transcription of IL-1 encoding messenger RNA. This blocks IL-1 dependent release of IL-2 from antigen activated T cells. Steroids also inhibit phospholipase activity through the formation of inhibitory proteins, called lipocortins, that block the formation of leukotrienes, prostaglandins, and thromboxane. The anti-inflammatory action of corticosteroid has been proposed to be associated with induction of synthesis of phospholipase-inhibitory proteins (lipocortins). Increased levels of lipocortin inhibit the release of arachidonic acid. Lipocortin has been gene cloned, and has a molecular weight of around 40,000. It is more specific for phospholipase A_2 than for phospholipase C, including phosphatidylinositol specific phospholipase C. The suggested mechanism by which lipocortin inhibits phospholipase A_2 is that 1 mole lipocortin binds to the calcium ion binding (active) sites of 1 mole of phospholipase A_2. Hirata (1986) reviewed the effects of lipocortins, the second messenger proteins of corticosteroids, and their possible effects on events occurring during allograft rejection:

1. Lipocortin blocks neutrophil chemotaxis, PAF formation, and arachidonic acid release by inhibiting phospholipase A_2.
2. Lipocortin can induce suppressor T cells, thus leading to inhibition of immunoglobulin synthesis by B cells.
3. Lipocortin exerts a dose dependent inhibitory effect on the acute phase (cytolytic reaction) of CTL as well as on the antibody dependent cellular cytotoxicity

activity of NK cells. Lipocortin also inhibits the generation of alloreactive CTL. This inhibition appears to be attributable to reduction in the production and/or release of IL-1 and IL-2. Lipocortin appears to suppress the proliferation of T helper/inducer cells (T4+: mouse monoclonal antibody recognising the Th subset of human T lymphocytes) and promote the maturation of T suppressor cells (T8+:mouse monoclonal antibody recognising the Ts/c subset of human T lymphocytes).

Lipocortin does not mimic all the actions of corticosteroid. It has only weak effects on responses such as lysozyme secretion, free radical oxygen production in neutrophils, and non T cell-mediated immunoglobulin synthesis by B cells. This may be because exogenously added lipocortin can not penetrate through cell membranes, or that phospholipases not sensitive to lipocortin are involved.

Azathioprine blocks mitosis and proliferation of cells. In the liver azathioprine is cleaved to 6-mercaptopurine and imidazole. Imidazole is a weak thromboxane synthetase inhibitor, and this could relate to the superiority of intravenous azathioprine over 6-mercaptopurine in improving dog renal allograft survival.

CyA blocks IL-2 release from activated helper T-lymphocytes, probably by blocking transcription of the IL-2 gene. Under the influence of CyA, cytotoxic T cells, helper T cells, B cells, and macrophages are not fully activated due to a lack of necessary helper cell stimulants. The release of other lymphokines is also inhibited, including gamma interferon,

B cell stimulating factor, and cytotoxic differentiation factor. Whisler (1984) showed that human monocytes exposed to CyA released PGE_2.

In summary since corticosteroids and CyA exert their effects through inhibition of IL-1 and IL-2, it might be possible to replace corticosteroids or decrease the dose of CyA or azathioprine in the immunosuppressive regimen with drugs that prevent the formation of the "prorejection" eicosanoids (TxA_2, LTB_4, LTC_4, LTD_4, and LTE_4) or promote the synthesis of the "antirejection" compounds (PGE_2, PGD_2, 6-keto PGE_1, and PGI_2).

7.2.4 Prostacyclin. (PGI_2)

PGI_2 is the arachidonic acid metabolite generated by vascular endothelium. It is a potent vasodilator and the most potent inhibitor of platelet aggregation yet discovered - Moncada (1980). Unlike many other prostaglandins, it is not metabolised during passage through the pulmonary circulation. The cardiovascular effects disappear within 30 minutes of the end of infusion. It inhibits platelet aggregation by elevating platelet cyclic adenosine monophosphate (cAMP). The action is dose related following intravenous administration at above 2 nanograms/kg/min. Significant inhibition of aggregation induced by adenosine diphosphate is observed after intravenous administration of 4 or more nanograms/kg/min. Effects on platelets usually disappear within 30 minutes of discontinuing infusion of PGI_2. Higher doses of PGI_2 (20ng/kg/min) disperse circulating platelet aggregates and increase by up to twofold the cutaneous bleeding time. PGI_2 reduces platelet procoagulant activity and the release of heparin neutralising factor. At normal physiological pH and temperature, PGI_2 is hydrolysed with a half life of 2 - 3 minutes to 6-keto prostaglandin $F_{1\grave{a}}$. PGI_2 is a general inhibitor of platelet aggregation, blocking aggregation evoked by a variety of stimuli, including thrombin, ADP, and adrenaline. This is in contrast to cyclooxygenase inhibitors, such as aspirin, which inhibit the aggregation evoked by only a subset of specific stimuli, eg, aspirin will block the aggregation evoked by collagen, but its effect is readily overridden by thrombin. Thrombin induced aggregation can take place by a mechanism that is independent of thromboxane A_2. PGI_2 is produced in far smaller quantities by other cells, such as macrophages. In the renal circulation of the dog PGI_2 infused intravenously reduces renal vascular resistance and increases blood flow and urinary excretion of sodium, potassium, and chloride ions - Moncada (1979). PGI_2 induces renin release when infused intrarenally into dogs.

Mundy (1980a) showed that PGI_2, in the short term, effectively abrogated hyperacute rejection in allosensitised dogs. Pairs of mongrel dogs were allosensitised by the cross-transfusion of 50ml of whole blood three times weekly for three weeks. Allosensitisation was confirmed by demonstrating lymphocytotoxic antibody. Kidneys were exchanged between pairs, and renal blood flow continuously measured by means of an electromagnetic flow probe in the renal artery. The time to rejection was when renal blood flow ceased. Only the donor-

recipient combinations with a control kidney rejection time of four hours or less were studied further by the exchange of the second kidney. Fourteen donor-recipient combinations of the original twenty-six satisfied these criteria. Thirty minutes before transplanting a second kidney, eight of these hyperacutely rejecting recipients were started on an intra-aortic infusion of prostacyclin (0.25mcg/kg body weight/min). This infusion was continued for the duration of the experiment. The other six pairs were controls, receiving an infusion of glycine buffer without prostacyclin. At four hours the renal blood flow was normal in the prostacyclin treated group, but had ceased in the controls. In three of the prostacyclin treated dogs the prostacyclin infusion was stopped at four hours. In these three, renal blood flow remained stable for 20-60 minutes and then began to fall, eventually to cease as in the controls. The renal arterial and peripheral venous platelet counts for each dog did not change during the entire procedure. However, the renal venous platelet count was reduced in the control dogs. All kidneys in both groups produced urine when the renal blood flow was greater than 1ml/gram of kidney/min, and the volume produced was not different between the two groups. When renal blood flow fell below 1ml/gm/min in the control group urine output ceased. Histologically, kidneys in the control group showed varying degrees of platelet and red cell aggregation in most glomerular and peritubular capillaries with corresponding degrees of vascular occlusion. Cortical arteries and arterioles appeared contracted and many had occlusive thrombi, platelet aggregates, red cell stasis and endothelial damage. Prostacyclin treated kidneys appeared normal at four hours apart from focal areas of red cell stasis and neutrophil margination. These changes were also found in autografts after four hours of perfusion in addition to multifocal thrombi and platelet aggregates within collapsed capillary loops, not seen in prostacyclin treated allografts.

Cross-species renal transplantation usually results in rapid hyperacute rejection. The xenograft reaction has many similarities with the hyperacute rejection of an allograft by an allosensitised recipient. Mundy (1980b) reported that as long as a prostacyclin infusion was maintained in a dog recipient, kidneys transplanted from a cat donor survived and produced urine. In these experiments prostacyclin was given for up to eight hours. Untreated dogs rejected xenografted kidneys within 25 minutes. When the prostacyclin was discontinued in three dogs at 3, 5, and 6 hours after revascularisation there was a time lag of 30, 95 and 120 minutes respectively before the rejection process started. Light microscopy of kidneys from prostacyclin treated recipients appeared to be virtually normal, although peritubular capillary congestion and tubular dilation were seen in some sections. The dose of prostacyclin used was 1000ng/kg/min.

Mundy thus showed that prostacyclin in the short term (four hours), could effectively abrogate hyperacute rejection in allosensitised dogs. The mechanism by which antigen-antibody reactions cause graft failure in hyperacute rejection is not fully understood. Complement consumption occurs, but decomplementation with cobra venom factor does not modify the rejection process. Heparin infusions and anti-platelet drugs such as aspirin and

sulphinpyrazone have been successfully used, but only to delay the rejection, not to inhibit it. In these experiments, prostacyclin was presumed to be achieving its effect by inhibiting platelet aggregation and the formation of occlusive thrombi within the renal vasculature. Whether or not platelet aggregation was primarily involved following the antigen-antibody reaction in hyperacute rejection of allografts in allosensitised recipients, or was alternatively a byproduct or an end result of a sequence of injurious events, it would appear that this platelet phenomenon was of major importance in ultimate graft failure.

In these experiments of Mundy the kidneys were transplanted to extracorporeal shunts in the femoral vessels, and the recipients were kept anaesthetized. This limited the duration of the experiment to a few hours. Although prostacyclin proved to be the most efficient agent at abrogating hyperacute rejection so far described, this effect only lasted for as long as the prostacyclin was administered. This was not surprising in view of the short half life of prostacyclin. Furthermore, the dose of prostacyclin used was approximately 25 times the therapeutic dose in man - 10/kg/min. Thus further experiments were needed to define the duration of suppression of hyperacute rejection by prostacyclin, and whether smaller doses would be effective.

7.2.5 Platelet activating factor (PAF).

Platelet activating factor has been implicated as a mediator of allograft rejection. PAF has direct effects, but it also stimulates arachidonic acid release, which depending on cell type leads to leukotriene, prostaglandin, or thromboxane synthesis. PAF is a phospholipase A_2 sensitive phospholipid, and is identified as 1-alkyl-2(R)-acetyl-glycero-3-phosphoryl-choline 1.

The pharmacology of PAF has recently been summarised by Braquet (1986). A variety of stimuli trigger the release of PAF by rabbit basophils, circulating polymorphonuclear leucocytes and monocytes. PAF induces aggregation and degranulation of platelets, may cause either vasoconstriction or vasodilatation as a consequence of a direct activity on smooth muscle cells, enhances vascular permeability, and participates in the recruitment of inflammatory cells. PAF induces aggregation, chemotaxis, and granular secretion of neutrophils and monocytes. No information is available on the possible release of PAF from activated T-cells. Natural killer cells are able to release PAF after specific stimulation. In addition, macrophages or endothelial cells stimulated by lymphokines may be involved in the release of PAF in cell-mediated hypersensitivity reaction.

When hyperacute renal allograft rejection was induced in rabbits by preimmunisation with skin or skin and renal allografts by Ito (1984), PAF was present in the renal venous effluent. PAF was also detected in some of the rabbits with transient signs of rejection, but was absent in rabbits without macroscopic and histological signs of rejection. Camussi

(1987) detected PAF release from rabbit hearts perfused in-vitro with alloantibody and fresh rabbit serum as a source of complement. The release of PAF was detected in the coronary effluent within one minute, increased to a maximum between 10 and 20 minutes and declined slowly thereafter. The release of PAF did not occur with complement inactivated rabbit serum. This suggested that the release of PAF could occur in the absence of inflammatory cells, possibly from endothelial cells or macrophages. The observed reduction in coronary blood flow was partially reversed by the PAF receptor antagonist, SRI 63-072 (Sandoz, East Hanover, NJ). The PAF receptor antagonist was given 20-25 minutes after beginning the perfusion with alloantibody and complement. It restored coronary blood flow to 70% of initial levels in 1-2 minutes. Though cessation of the heart action was delayed, it was still observed within 50 minutes of beginning perfusion with the alloantibody and complement. it would have been interesting to know the effect of giving the PAF receptor antagonist before starting perfusion with alloantibody and complement. The release of PAF from the allograft during antibody mediated rejection led Camussi (1986) to suggest that PAF release in-vivo might contribute to the recruitment of inflammatory cells and to organ failure.

Treatment of rat cardiac allograft recipients by Foegh (1986b) with PAF antagonist BN 52021 (10mg/kg, im daily), alone or in combination with either Aza or CyA significantly delayed graft rejection.. The combination of Aza and PAF antagonist was more effective in prolonging graft survival than immunosuppression with Aza and Pred. The combination of BN 52021 and CyA improved allograft survival to the same degree as Pred and CyA. The protective effect of BN 52021 did not appear to be mediated through a reduction in platelets in the graft, as demonstrated by the accumulation of ^{111}- Indium labeled platelets - Khirabadi (1987). This was not unexpected in view of the known absence of PAF receptors on rat plateiets.

The data implies that PAF might be involved in both cell and antibody mediated allograft rejection. PAF may be involved with a variety of other pathophysiological conditions, including arterial thrombosis, acute inflammation, endotoxic shock, and acute allergic diseases. Several human cell types have been reported to synthesise PAF, including circulating monocytes, alveolar macrophages, PMN's, platelets, and endothelial cells. Zimmerman (1985) reported that the synthesis of PAF by cultured human endothelial cells was stimulated by specific agonists, namely human thrombin and the calcium ionophore A23187. In addition, a variety of potential agonists (Table 7.3) that had vasoactive or proinflammatory properties, did not cause PAF production.

TABLE 7.3: Potential mediators of vascular effects that did not induce the synthesis of PAF by cultured endothelial cells.

Endotoxin (lipopolysaccharide from E. coli).	Serotonin
Angiotensin II	Adrenaline
Concanavalin A	Collagen
Acetycholine	Vasoactive
Fibrinogen	Intestinal peptide.
Fibrin	Insulin
Urokinase	Gastrin
Trypsin	

The fact that these agents did not stimulate PAF accumulation under these experimental conditions indicates that the production of PAF by endothelial monolayers requires the action of specific agonists. Histamine, bradykinin, and ATP are agonists for PAF synthesis by human endothelial cells and they appear to stimulate PAF production by interacting with specific receptors. The production of PAF by stimulated endothelial cells was enhanced by inhibition of cyclooxygenase and inhibited by treatment of the monolayers with prostacyclin. This finding suggests an interaction between arachidonic acid metabolism and PAF production in endothelial cells.

7.2.6 Summary.

There is evidence to support a role for these lipid mediators in allograft rejection. Possible strategies for improving graft survival might be:

1) Increase the synthesis or expression of PGE_2, PGD_2, and PGI_2.
2) Inhibit the synthesis or expression of TXA_2.
3) Inhibit the synthesis or expression of the leukotrienes.
4) Use of a PAF antagonist to reduce production of TXA_2 and leukotrienes by reducing arachidonate release.

7.3 Prostacyclin and the allosensitised dog model

The aims were to examine PGI_2 in the allosensitised dog model, hoping to extend the work of Mundy by using lower doses and prolonged infusions.The mechanism of the beneficial effect of PGI_2 is not known, though an effect on platelets or the immune response are possibilities. The hypothesis was that if PGI_2 could block or delay the consequences of alloantibody and alloactivated cells for the renal allograft, thereby delaying accelerated rejection, this might enable the graft to become tolerant of the circulating alloantibody, perhaps by target antigens on cells becoming internalised. Alternatively it might give time for other approaches, such as the removal of alloantibody by plasmapheresis. The rat work suggested that it would be neccessery to combine the PGI_2 with another immunosuppressive agent, such as CyA.

Allosensitised beagles received a crossmatch positive renal allograft with oral CyA, an infusion of prostacyclin into the renal allograft artery for six days, and a small dose of aspirin - Group 8 (ALLOSENSITISED + KIDNEY, +ve X-MATCH, PLUS CyA & PROSTACYCLIN).

7.3.1 Problems with the use of prostacyclin

There are practical problems to the therapeutic use of prostacyclin, which will be discussed under these headings:

1) Stability.
2) Tolerance.
3) Side effects.

1) Stability.

At physiological pH and temperature the half life of prostacyclin in the circulation is only 2-3 minutes. The short biological half life of PGI_2, and the return of platelet activity within 30 minutes of stopping PGI_2 means a continuous intravascular infusion. PGI_2 is presented in vials containing 500 mcg freeze-dried PGI_2 as the sodium salt. The contents of the vial have the appearance of a white, or off white fluffy solid. Each vial is accompanied by 50 ml of sterile diluent containing Sodium Chloride BP 0.147% w/v and Glycine BP 0.188% w/v in clear solution. The alkalinity of the diluent has been adjusted to pH 10.5 $\pm$0.3 by the addition of sodium hydroxide. Solutions of PGI_2 in this glycine buffer when diluted to a maximum of 1:6 with Sodium Chloride Intravenous Infusion BP 0.9% w/v will retain 90% of initial potency for at least 12 hours at room temperature. In effect its half life is enhanced to about 100 hours at 25 deg C. - Moncada (1979). Whether PGI_2 is infused into the venous or arterial circulation should not matter as the action on platelets is independent of intravascular concentration. Nevertheless, it was decided to give the PGI_2 direct into the renal artery, so as to achieve the highest concentrations within the transplant kidney. This required a peristaltic pump capable of injecting continuously against arterial pressure. As the infusion was planned to last for several days the pump ideally had to be fixed to the dog to permit mobilisation. The alternative of delivery from a remote pump by means of an umbilical cord to the dog was not considered practical, as it was thought that they would not tolerate this.

Campbell (1984) described constant intrarenal infusion of PGE_1 into a dog renal allograft using a totally implantable pump. PGE_1 was stored in the reservoir of the implantable pump and delivered continuously in high doses direct into the renal transplant artery. PGE_1 was infused at 2-4 mls/24 hours for six days. Under the experimental conditions PGE_1 was stable over the six day period, and thus access to the reservoir in the pump was not required once the pump had been implanted at the time of transplantation. However, with PGI_2 access

was required to the reservoir at ieast every 24 hours. A further problem was the expense of the implantable pump (£3,000), and it was not possible to borrow one. For our experiments we turned to the ACT-A-PUMP 1000 manufactured by Pharmacia Nu Tech. The company were abie to iend us two pumps for our work. The pump weighs 425 grams, and is 9.5 cm wide x 10.5 cm high x 3.8 cm thick. The peristaltic pumping action is achieved by a rotary mechanism which continuously rolls along a silicone tubing displacing the fluid from the pump tubing. Connected to the tubing is a 75 ml reservoir located in the lid. This pump provides a flow rate accuracy of ± 5% of any flow rate selected between 2 ml and 110 mi per 24 hours. It can operate at output pressures of up to 2000 mm Hg. The pump is a self contained unit with no exposed control knobs or electrical connections and the fluid reservoir is protected inside the pump lid. Using this pump it was planned to deliver the PGI_2 continuously into the renal transplant artery from a pump strapped to the dog and lying within a jacket. This would permit access to the pump and contained reservoir as often as required, and yet at the same time permit the dog to make a near normal postoperative recovery.

To circumvent the problem of stability, prostacyclin analogues have been synthesised:

i) OP-41483 (Ono Pharmaceutical Co., Osaka, Japan).
15-cyclopentyl-*w*-pentanor-5(E)-6,9 metano PGI_2 is chemically stable for more than 48 hours at pH 7.4 and 37 deg.C. It is 3-10 times less powerful than PGI_2 in terms of the inhibitory effect on platelet aggregation, and four times less powerful than PGI2 in terms of the vasodilatory effect - Tobimatsu (1987).

ii) Ciprostene (Upjohn).
Ciprostene calcium (9-beta-methyl-carbacyclin calcium) is a stable derivative of PGI_2. Ciprostene is about 15 times less potent than PGI_2- Linet (1986).

iii) Iloprost (ZK 36 374) - (Schering, Berlin, West Germany).
A carbacyclin derivative of PGI_2, which has been shown to be a more potent than PGI_2 as an anti-platelet agent but induces less vasodilatation - Yardumian (1985).

As none of these analogues were available to us, we used PGI_2 in our experiments.

2)Tolerance

Sinzinger (1984) reported a decreased sensitivity of platelets to PGI_2 in patients treated for 7 days continuously at an infusion rate of 5 ng/kg/min. This observed "intra-infusion rebound" was measured by platelet protein release, platelet aggregation, peripheral platelet count, plasma thromboxane B_2, and platelet sensitivity to the antiaggregatory prostaglandins (PGI_2, PGE_1, and PGD_2), despite continuation of PGI_2 infusion. This rebound started between the second and fourth day of PGI_2 administration, and reaching its maximum

between days 6 and 10 and leveling off thereafter. The mechanism of this rebound is unclear. It may result from a re-setting of the balance between pro-aggregatory TXA_2 and anti-aggregatory PGI_2 in favour of the former: this would result in reduced platelet cyclic AMP production and hyperaggregability occurring due to excess TXA_2 production. Yardumian (1985) observed with prolonged ZK 36374 infusions in patients a decreased platelet sensitivity, rebound hyperaggregability, spontaneous aggregation, and raised serum thromboxane B_2 levels. The rebound hyperaggregability during and after infusions of ZK 36374 could be prevented by platelet cyclooxygenase inhibition with aspirin or indomethacin therapy.

3)Adverse effects

In clinical practice facial flushing is commonly seen with PGI_2 . Headache and gastrointestinal symptoms including nausea, vomiting and abdominal colic have occurred in some conscious individuals. PGI_2 is a potent vasodilator, and may cause hypotension. Haemorrhagic complications have not been encountered during surgery. This is surprising in view of the platelet actions of PGI_2, and the prolongation of cutaneous bleeding time. In our dogs an increase in subcutaneous bleeding was seen despite meticulous haemostasis during surgery. No other adverse effects were seen in the dogs with the dosage of PGI_2 used - 20ng/kg/day.

7.3.2 Methods

As previously described adult beagle dogs were allosensitised with donor blood and tissue (skin, kidney) until they developed strong complement dependent lymphocytotoxicity (CDC) against the prospective donor pbl. Kidneys were transplanted to these allosensitised recipients, with CyA 25mg/kg/day orally starting 24 hours before transplantation together with PGI_2, which was infused into the renal artery at 20ng/kg/min for six days - Group 8. The PGI_2 was started ten minutes before the clamps were released. Dogs receiving PGI_2 also received aspirin 100mg daily for the duration of the PGI_2 infusion. Whole blood CyA 24 hour trough levels were monitored daily by HPLC. Rejection was monitored by twice weekly Tru-Cut needle biopsies, which were scored for vascular damage and cell infiltrate after Herbertson (1977).

The difficulties lay in the fixation of the pump to the dog and the delivery of prostacyclin into the renal artery. The pump delivery tube was a thick walled silastic tube with a narrow bore. Initially the end of the silastic tube was joined to the hub of the needle of a 27G intravenous catheter placement unit, with a second thin walled silastic tube placed over the needle tip. The latter was machined so as to remove the sharp edges at the tip. This second tube was implanted into a side branch of the femoral artery, these being readily found. Unfortunately leaks occurred around the junction of the needle and secondary silastic tube. Plumbing the silastic pump delivery tube directly into a large side branch of the artery was

more successful. This was usually the internal iliac artery, which was readily exposed by retracting the inguinal ligament.

The pump was positioned on the dog's back behind the neck, where it proved to be comfortable and not accessible to the dog. The silastic tube was easily tunneled subcutaneously from the groin to the back, and the pump held in place within a dog jacket. Two jackets were required for each dog to allow changing and washing every 12 hours. To discontinue the PGI_2 the silastic tube was tied at the exit point on the back and the cut end allowed to retract under the skin. With the needle hub arrangement the dog was anaesthetized and the needle hub removed.

Illustrations 23-34 demonstrate these practical details.

The action of PGI_2 was compared with that of heparin in group 9. The reasons for this were twofold, firstly a beneficial effect of PGI_2 could be attributed to the anticoagulant effect of prostacyclin and secondly the work of Macdonald (1970), who observed a small beneficial effect with heparin in an allosensitised dog renal allograft model. The dogs received a crossmatch positive kidney with CyA and a heparin infusion into the renal artery with the Act-A-Pump. Heparin therapy was monitored using the Activated Coagulation Time (ACT) as determined with the Hemochron ™ system. This system does away with the major sources of error in measuring the clotting time of whole blood:

1. Inconstant activation of the intrinsic system (factors XI and XII) can lead to a wide variation in clotting time. The Hemochron system has a diatomaceous powder (Celite ™) to give maximal area for surface contact.
2. There can be a great variation in time between the formation of the first visible clot in a tube and the solid coagulation of all the blood in the tube. It is therefore essential to observe the formation of the first clot and use this as the end point. The Hemochron system automatically detects the first fibrin clot.

We established the normal range for the ACT in our dogs - Table 7.4. Four separate venepunctures were done on two consecutive days in 10 dogs (7 beagles and 3 greagles). The ACT was determined using the Hemochron system.

TABLE 7.4 - Normal range for ACT by the Hemochron system in our dog population.

	ACT in seconds				Mean ±SD
Beagle					
Monty	*44*	85	94	(21)	61 ±34.4
Sydney	95	(19)	90	91	73 ±35.6
Podge	84	89	96	93	90 ±5.2
Chester	103	98	101	94	99 ±3.9
Ziggy	*51*	93	98	82	81 ±21.1
Janet	105	88	101	92	96 ±7.8
Kookie	*55*	*48*	102	92	74 ±26.7
Greagle					
Rambo	106	103	106	82	99 ±11.6
Dusty	109	(23)	106	101	84 ±40.9
Scooby	95	94	95	80	91 ±7.3
			Mean for all dogs:		85 ±24.1

(21) = No clot seen when tube examined

51 = Difficulty bleeding from leg vein, only 1ml of blood by 60 seconds

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The results were skewed by 7 of the 40 estimations. In three of these there were no clots to be seen when the tube was examined at the apparent end point. In three females only 1 ml of blood had been obtained at sixty seconds. Subsequently these three were bled from a jugular puncture. Omitting these 7 samples from the analysis the mean ACT was 95 seconds (SD±7.6). The time from commencement of the venepuncture to filling the Hemochron tube was noted. The mean was 21 seconds (Range 8-60).

Our normal ACT was slightly longer than that reported by Wilkerson (1984) for dogs - 75 ±5.5 seconds. For consistent results it would seem to be important to keep the interval from beginning the venepuncture to injecting the blood into the Hemochron tube to no more than 60 seconds. In 3 of 40 determinations a low value of around 20 seconds was obtained. On these occasions no clots were seen suggesting a false result. In these circumstances the test should be repeated.

Wilkerson stated that adequate heparinisation of dogs is achieved if the ACT is prolonged to 1.5 to 2.0 times normal. In our experiments 2,500 units of heparin given over 24 hours prolonged the ACT to this extent.

Only two dogs were included in group nine mainly because of the need to achieve numbers in the prostacyclin group resulted in us running out of dogs. A second reason was that both dogs in group 9 died as a result of haemorrhage from kidney rupture, one at 36 hours and one at five days. The results of these two experiments are described in Appendix 5, and are not otherwise mentioned.

Illustration 23 - The ACT-A-Pump 1000 and housing. The 75 ml reservoir with peristaltic pumping mechanism is shown.

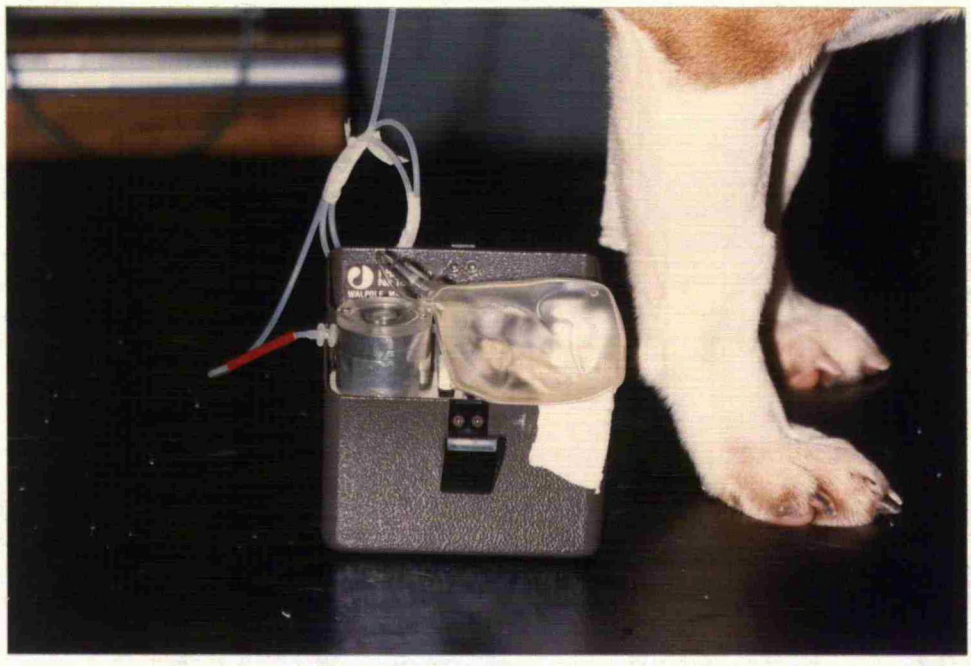


Illustration 24 - Sealed pump unit with silastic delivery tube emerging.

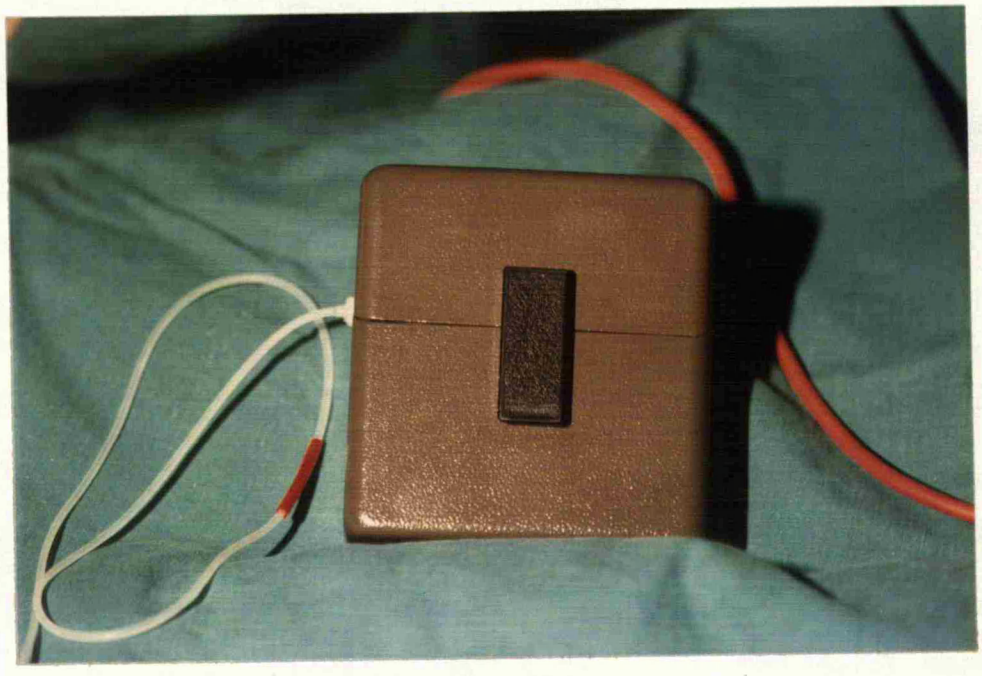


Illustration 25 - Silastic delivery tube plumbed into the divided internal iliac artery and held in place with a 7/0 Prolene tie.

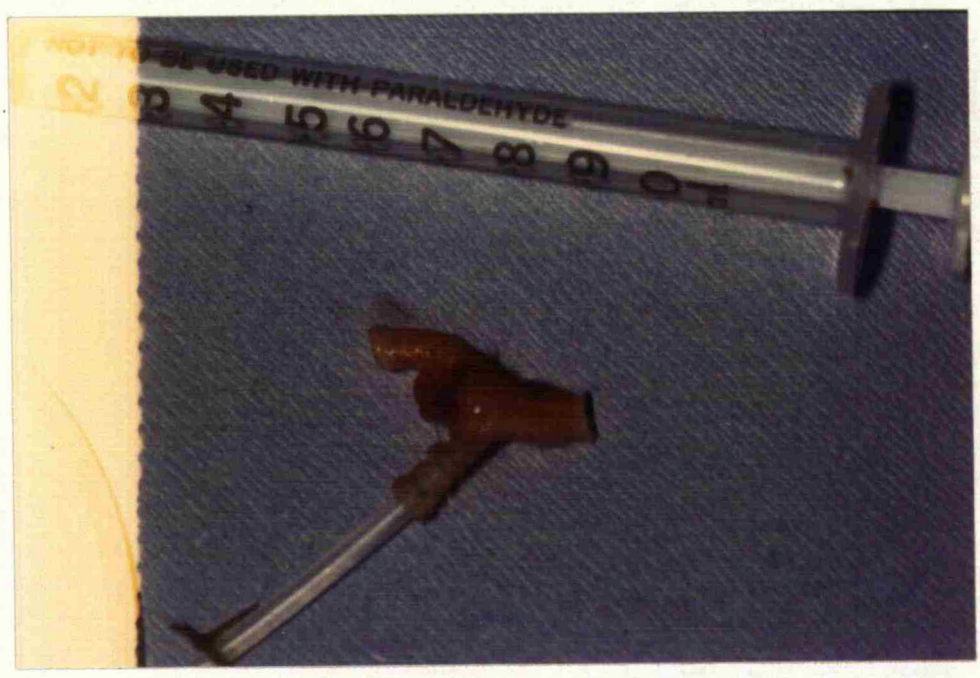


Illustration 26 - Silastic delivery tube plumbed into internal iliac artery, with forceps on external iliac artery. The silastic tube has been loosely coiled to avoid traction on the intra-arterial end.

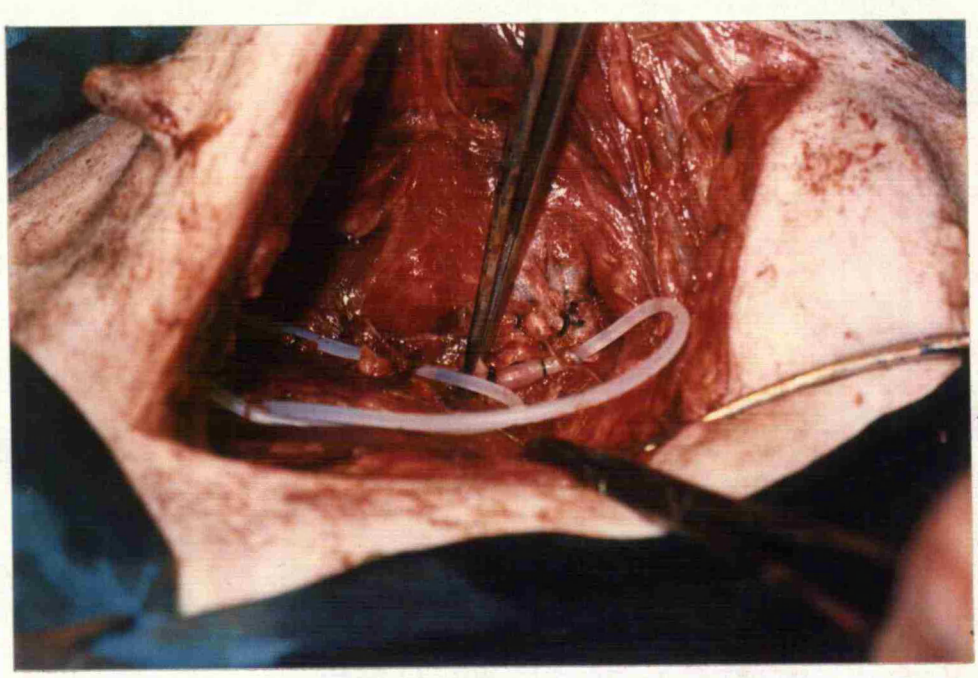


Illustration 27 - Alternative method for delivery of PGI_2 into the renal artery. The silastic delivery tube is joined to the hub of a 27G intravenous catheter placement needle, with a fine silastic tube from the needle tip to a side branch of the artery. The method was abandoned because of leaks where the silastic tube joined the needle. The silastic tube from the pump is seen emerging from the subcutaneous tunnel.

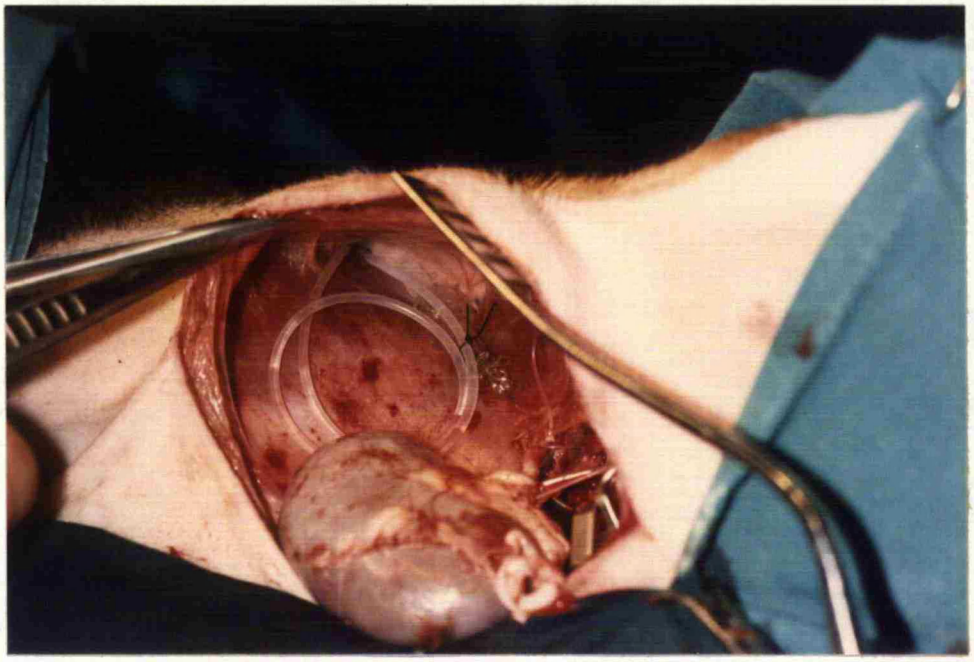


Illustration 28 - Silastic tube emerging on the beagle's back, firmly fixed plastic spray dressing and elastoplast.

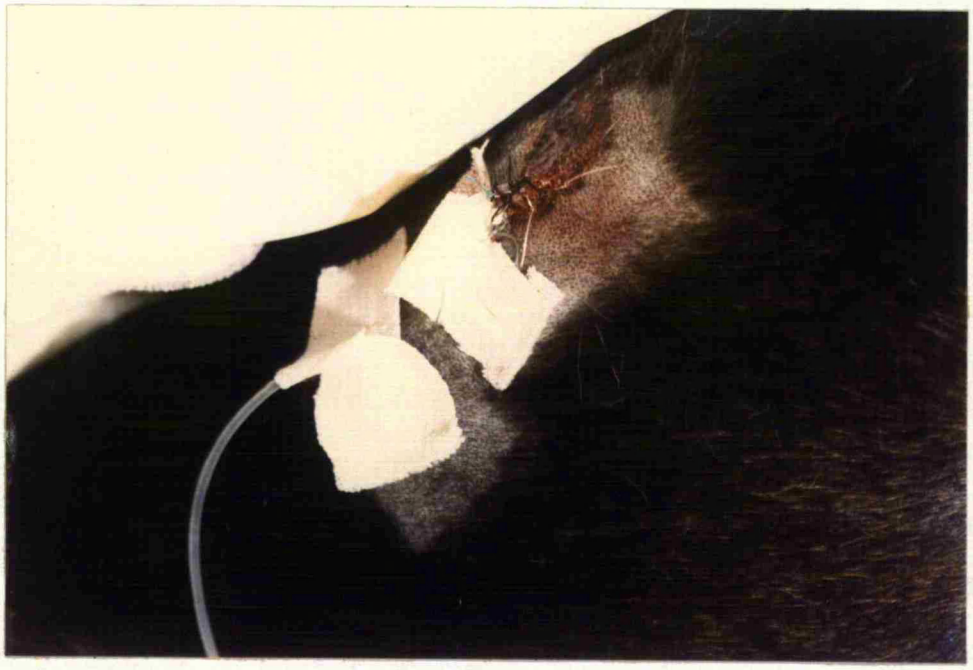


Illustration 29 - Recipient with jacket, the pump lies just behind the neck.

Illustration 30 - Daytime jacket made from orthapaedic stocking.

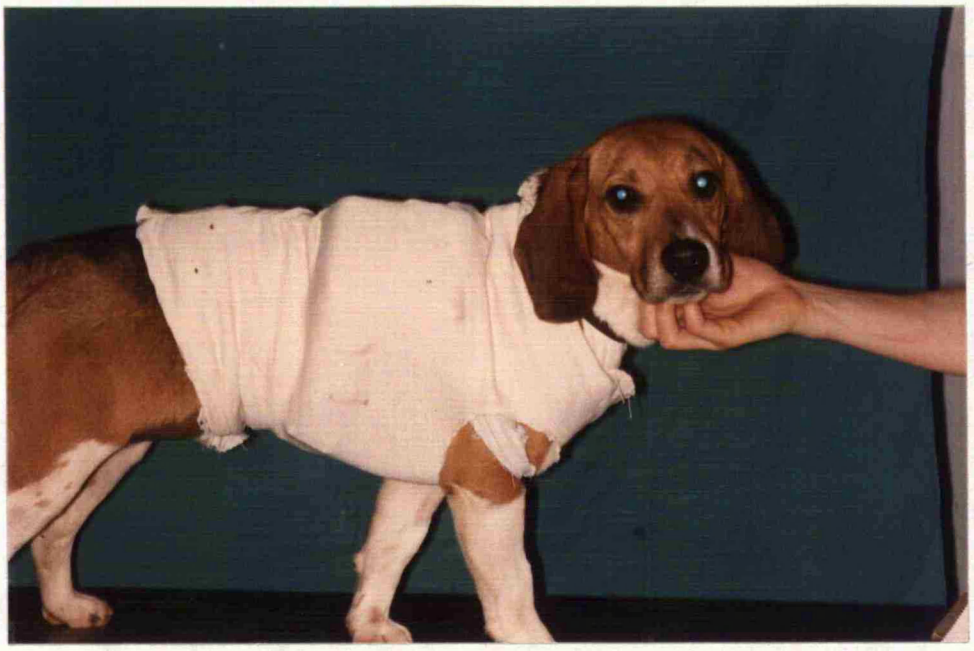


Illustration 31 - Exit site of silastic tube on back, with pump behind the neck and inside a pouch. Proximal to the hindleg subcutaneous bruising in association with the renal allograft is seen.

Illustration 32 - Renal allograft at 24 hours, with urine from ureterostomy. Prostacyclin was associated with more subcutaneous bruising, in contrast to illustration 9.

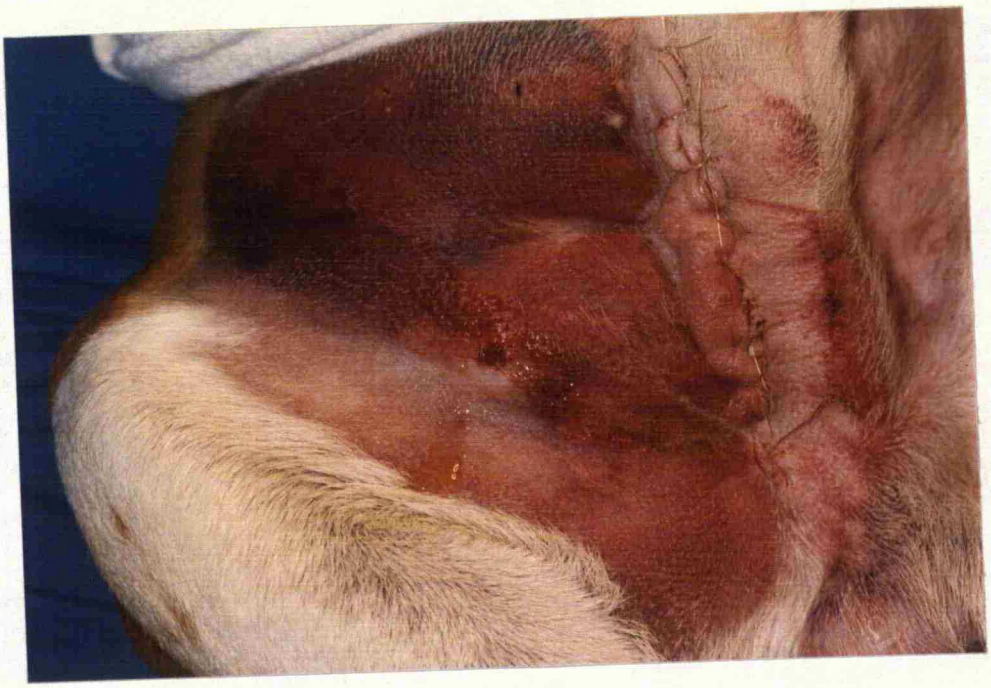


Illustration 33 - Renal allograft at three days with PGI_2. The bruising extends beyond the plane of dissection into the scrotum and abdominal wall.

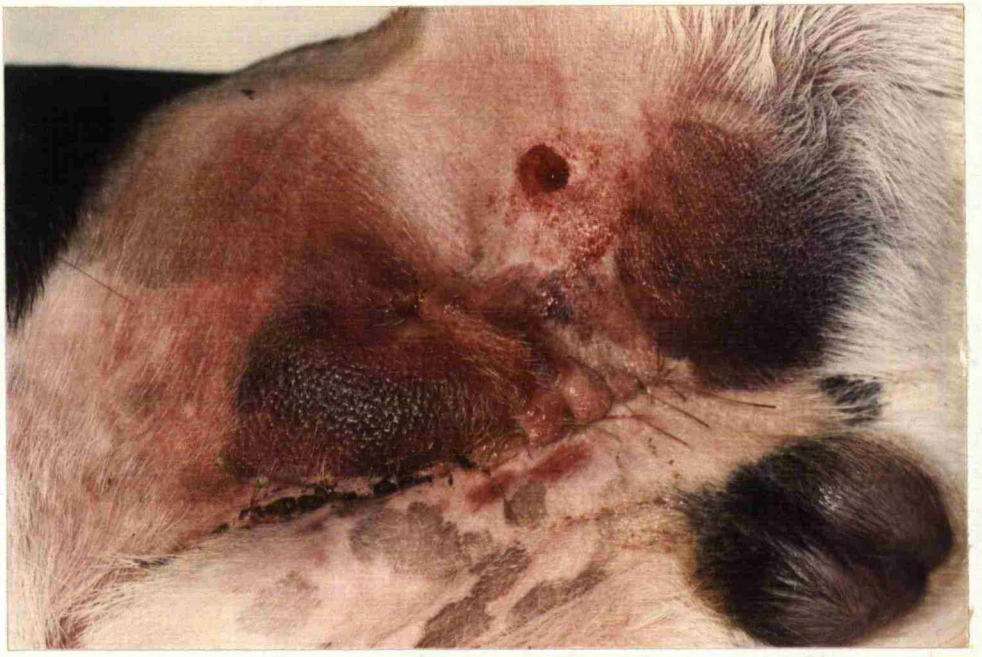
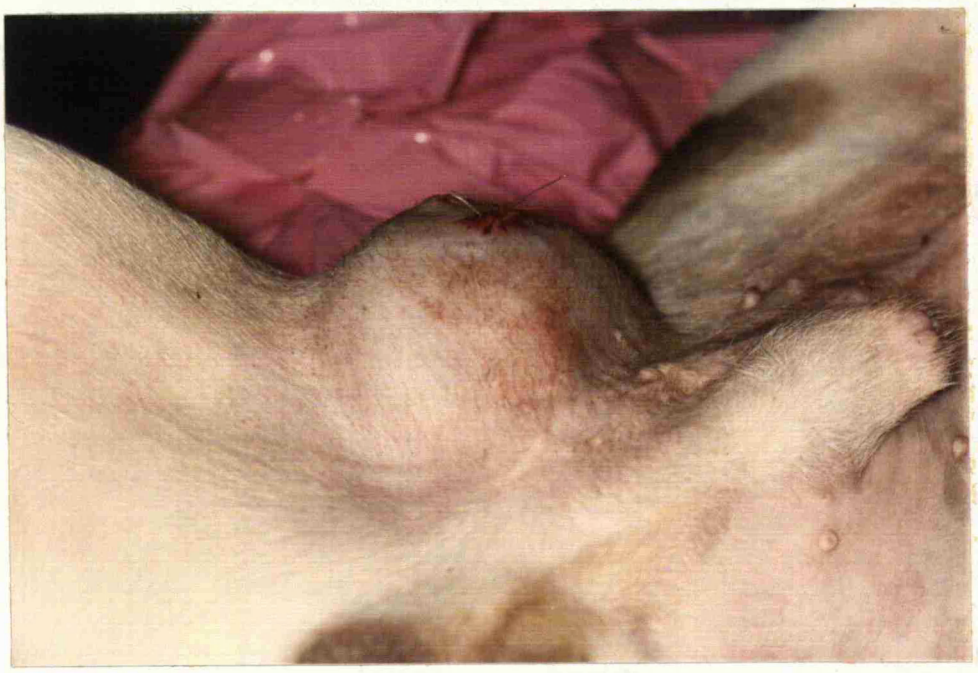


Illustration 34 - Typical appearance of renal allograft at three days without PGI_2.



7.3.3 Results

GROUP EIGHT
ALLOSENSITISED + KIDNEY (+ve X-MATCH).
PLUS CyA (25mg/kg/day) & PROSTACYCLIN.
N=11

1) B1 MONTY
PGI_2 for three days only owing to leak.

Day of biopsy. (Biopsy number)	Score: 6	8	10	12	Conclusion:
Pre-op					Not done
5 - (135)	6	6	8	10	Almost totally infarcted, mainly polymorphs.
2) B1 MONTY					
0 - (167)	0	0	0	1	Swollen tubular cells, early ATN
6- (173)	4	5	7	9	Almost completely infarcted, mainly neutrophils

3) B2 ZIGGY
PGI_2 for one day at reduced dose (2ng/kg/min) due to pump malfunction. This was probably due to a few drops of water spilt on the pump mechanism, as the pump functioned faultlessly when allowed to dry.

0 - (156)	0	0	0	0	No diagnostic changes.
7 - (163)	0	1	3	4	Severe cellular rejection
11 - (168)	3	3	5	7	Severe cellular rejection, total tissue necrosis
14 - (171)	4	-	-	6	Completely infarcted

4) B3 CHESTER
PGI_2 for12 hours, when the kidney ruptured. This was probably non-immunological.

0 - (142)	0	0	0	1	Possible ATN ?pre Tx biopsy
12 hours - (144)	0	0	1	2	Neutrophils. Not rejection.

5) B4 NEIL

PGI_2 for 5 days with haematuria from day one. At nephrectomy on day 8 there was a renal vein thrombosis. The histology suggested that this was a secondary event to rejection.

0 - (159)	1	0	0	2	Marked ATN, poor glom perfusion,
6 - (162)	6	-	-	8	Total haemorrhagic infarction, probably post rejection.
8 - (166)	6	-	-	8	Total haemorrhagic infarction consistent with rejection.

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6) B5 PAINTER

PGI_2 for 12 hours, when the kidney ruptured.

0 - (149)	0	0	0	1	Normal
1 - (152)	1	2	2	3	Acute rejection (?humoral) + early ATN.

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7) B8 PODGE

PGI_2 for six days with anuria from day five.

0 - (147)	0	0	0	0	Normal
4 - (153)	0	1	3	4	Cellular rejection+some ATN
6 - (155)	6	6	7	9	Total haemorrhagic infarction including quite large vessels. Acute rejection

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8) B10 FRED

PGI_2 for six days. The day four biopsy showed haemorrhagic infarction, but on medulla only. The day six biopsy showed modest cellular rejection only.

15min - (118)			0	0	Inadequate biopsy-medulla.
4- (119)	3	3	4	5	Haemorrhagic infarction, ?acute rejection. Haemorrhage++, and polymorphs. Medulla only.
6 - (120)	1	2	3	3	Acute rejection.
8 - (123)	1	2	3	3	Mod severe cellular rejection?ischaemic glomerular sclerosis
12 - (127)	3	4	6	7	Acute rejection superimposed on chronic sclerosis.
18 - (131)					Inadequate biopsy
47 - (139)	0	0	1	2	Chronic cellular rejection ?large vessel disease
55 - (151)	0	0	2	2	Chronic rejection (severe)
71 - (181)	1	2	4	-	Extensive interstitial infiltrate and fibrosis.

9) B14 ARNOLD

PGI_2 for four days when a leak occurred into pump.

0 - (167)	0	0	0	1	Early ATN
7 - (170)	3	4	6	7	Acute rejection, ?mixed cellular/humoral
14 -(175)	6	6	7	9	Haemorrhagic infarction
21- (178)	6	-	-	-	Total haemorrhagic infarction

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10) B18 MUTTLEY

PGI_2 for seven days and day 7 biopsy with mild cellular rejection.

0 - (122)	0	0	0	0	Non specific ?glomerular protein leak.
2 - (125)	3	-	3	5	Haemorrhagic infarction - presumed rejection.
7- (129)	1	1	2	2	Mild cellular rejection, fibroblastic response + haemorrhage at previous biopsy sites in medulla.
13 - (133)	0	0	2	2	Chronic rejection (cellular) +previous biopsy site scar.
41 - (140)	0	0	2	3	Severe cellular rejection
49 - (150)	0	1	3	4	Severe cellular rejection
61 - (164)	0	1	3	4	Severe late cellular rejection

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11) B19 KIM

PGI_2 for six days.

0 - (136)	0	0	0	0	Virtually normal
7 - (141)	1	-	2	3	Acute rejection, medulla only
9 - (145)	6	7	8	10	Almost totally infarcted, focal lymphoid

PGI_2 was given to eleven allosensitised recipients. In seven dogs (experiments 2,5,7,8,9,10 &11) PGI_2 was given for a mean of 6 days {4,5,5,6,6,6,7}. In the other experiments there were two renal allograft ruptures at 12 and 24 (experiments 4 &6) hours, and two technical failures, pump malfunction and silastic tube leak (experiments1&3).

For this analysis groups 3, 4, & 5 (GROUP THREE - ALLOSENSITISED (KIDNEY) + SECOND DONOR SPECIFIC KIDNEY, +ve X-MATCH, NO CyA), (GROUP FOUR - ALLOSENSITISED (SKIN) + DONOR SPECIFIC KIDNEY, +ve X-MATCH, NO CyA), (GROUP FIVE - ALLOSENSITISED (BLOOD) + DONOR SPECIFIC KIDNEY, +ve X-MATCH, NO CyA) were analysed together, and then compared with group 6 (GROUP SIX - ALLOSENSITISED + KIDNEY, +ve X-MATCH, PLUS CyA) and group 8 (GROUP EIGHT - ALLOSENSITISED + KIDNEY, +ve X-MATCH, PLUS CyA & PROSTACYCLIN)

TABLE 7.5: Group 8 vs Group 6 vs Groups 3, 4, & 5.

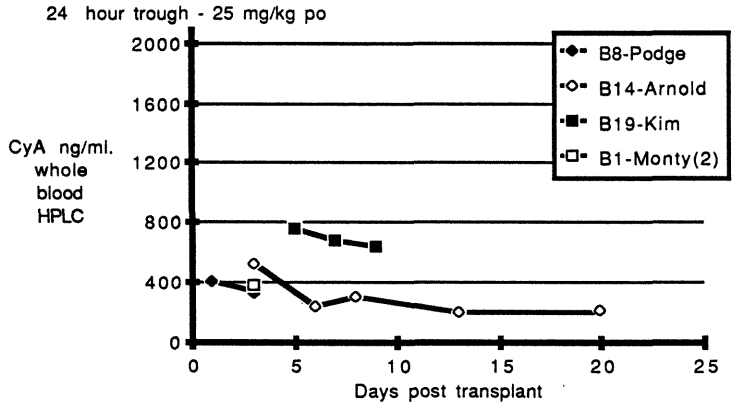
	Malaise	Fever (days)	Urine (Day ceased)	Vascular rejection (Day Dx)	Nephrectomy (Day)
Group 8					
(CyA25mg/kg/day+					
PGI2 + Aspirin)					
B1-Monty	3	(3-6)	-	6	6
B4-Neil	None	None	6	6	8
B8-Podge	6	5	6	6	6
B10-Fred	None	None	12	12	71
B14-Arnold	None	(5-7)	15	7	14
B18-Muttley	None	(11-12)	40	2	61
B19-Kim	None	9	8	9	9
Group 6					
(CyA25mg/kg/day)					
B2-Ziggy	None	(32-34)	-	2	23
B3-Chester	6	6	5	6	6
B4-Neil	4	4	4	4	4
B14-Arnold	6	5	6	6	8
B8-Podge	9	4	6	6	9
Group 3 (Kidney)					
B9-Ebony	5	5	-	1	5
B10-Fred	3	3	-	4	4
B13-Snowy	2	4	-	5	5
Group 4 (Skin)					
B1-Monty	6	(4-6)	6	5	7
B2-Ziggy	4	(2-5)	4	5	5
B3-Chester	3	3	3	3	3
B4-Neil	3	3	2	3	3
Group 5 (Blood)					
B11-Rosie	5	5	-	5	5
B12-Tiffany	2	2	-	2	2
B14-Arnold	5	(1-5)	2	5	5
B15-Janet	5	(1-5)	4	5	5

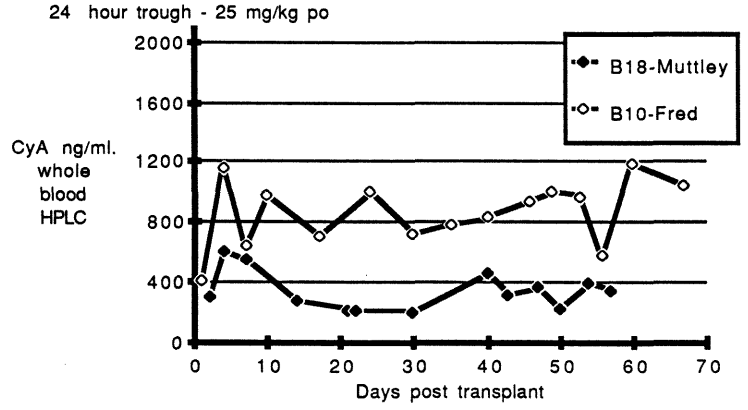
	Group 8 (CyA+PGI2 +Aspirin)	Group 6 (CyA)	Groups 3,4, & 5. (No immunosupp.)
Malaise	3,6,	4,6,6,9,	2,2,3,3,3,4,5,5,5,5,6,
Fever	3,5,5,9,11,	4,4,5,6,32,	1,1,2,2,3,3,3,4,4,5,5,
Urine	6,6,8,12,15,40,	4,5,6,6,	2,2,3,4,4,6,
Vas. Rej.	2,6,6,6,7,9,12,	2,4,6,6,6,	1,2,3,3,4,5,5,5,5,5,5,
	Gp.8 vs Gp.6	Gp.6 vs Gps. 3, 4, & 5.	Gp.8 vs Gps 3,4, &5.
Malaise	NS	NS	NS
Fever	NS	$p<0.05$	$p<0.05$
Urine	$p<0.05$	NS	$p<0.05$
Vas. Rej.	$p<0.05$	NS	$p<0.05$

No difference was observed in the day of onset of malaise. Fever was significantly delayed in those dogs given CyA (groups 6 and 8). CyA alone did not prolong urine production, but the combination of CyA+PGI_2 did prolong urine production compared with the other groups (group 8 vs 3, 4, 5, and 6). CyA alone did not delay the onset of vascular rejection as compared with untreated dogs. However CyA+PGI_2 appeared to caused a slight but significant delay in the onset of vascular rejection compared with CyA and with no treatment.

The CyA levels in group 8 were comparable to those observed with groups 2 and 6.

FIGURE 7.4 - Group 8. Whole blood CyA trough levels by HPLC.





7.3.4 Discussion

The dog model of accelerated rejection was described in chapter 5. The onset of vascular rejection was slightly earlier in the allosensitised as compared to the unsensitised recipient. CyA was shown to be effective in preventing vascular rejection in unsensitised recipients when given for 21 days, and on stopping CyA all allografts were rejected. However, CyA failed to prevent vascular rejection in allosensitised recipients with a positive CDC crossmatch. Vascular rejection was significantly delayed in allosensitised recipients allografted with a CDC negative crossmatch kidney.

The combination of CyA, PGI_2 and aspirin appeared to produce a small but significant delay in the onset of vascular rejection in allosensitised recipients. However despite continuing the CyA after stopping PGI_2 at day six the delay in terms of days was small. Any beneficial effect of PGI_2 could be explained by the dogs in group 8 not being biopsied as often or as early as the dogs in other groups (groups 3, 4, 5 & 6). This reflected a reluctance to biopsy the dogs because of the risk of bleeding. It can be safely said though that the PGI_2 did not delay rejection to an extent that would be clinically useful. Many of the technical problems associated with the PGI_2 were related to the attempts to perfuse the renal artery direct and less trouble could have been expected with an intravenous infusion.

Campbell (1984) continuously delivered PGE_1 into the renal transplant artery of canine allografts for six days, using a dose of 1250ng/min. The sensitisation status was not mentioned, but presumably the recipients were unsensitised. No immunosuppression was given, Renal blood flow and function as shown by I^{-131} Iodohippuran scanning diminished similarly in both PGE_1 treated and untreated dogs. No graft was perfused by day 6. There were striking histological differences. In the controls the findings were typical of lymphocyte mediated acute rejection, whereas PGE_1 infusion resulted in the appearance of large numbers of polymorphonuclear leucocytes and relatively few lymphocytes. The native kidneys were normal.

Tobimatsu (1987) treated dogs in receipt of a renal allograft with the stable PGI_2 analogue OP-41483, which was given for four hours at 30ng/kg/min during the day of operation, and at 40mcg/kg (27ng/kg/min) for the next four days. No other immunosuppression was used. The PGI_2 analogue prolonged graft survival to a mean of 12.8 $\pm$1.6 days compared to 6.1 $\pm$1.4 in the controls. Renal blood flow (hydrogen washout technique), serum creatinine, and urine production were all significantly prolonged by the PGI_2 analogue. The four day biopsies in the controls showed a marked diffuse mononuclear infiltrate, and fibrinoid necrosis of renal cortical small vessels. Destruction of tubules and collapse of glomeruli was also seen. In the treated dogs there was some perivascular mononuclear infiltrate and medial hypertrophy of renal cortical arteries, but no fibrinoid necrosis. The tubules and glomeruli were unremarkable. The reduction in vascular lesions at day four suggests that the PGI_2

analogue might be protecting the vascular endothelium. However,PGI_2 has also been reported to suppress cell mediated immunity, and this could be an alternative mode of action in this model.

In these two different models of canine renal allografting to unsensitised recipient PGE_1 was shown not to delay rejection, though the cell infiltrate was modified. A PGI_2 analogue on the other hand delayed allograft rejection. In both models no other immunosuppression was used. In numerous rat studies both a PGE_1 analogue (15-methyl-PGE_1) (Strom-1983; Aziz-1986; Zhao-1984) and a PGI_2 analogue (Iloprost) (Rowles-1985) have shown an additive effect with CyA in prolonging graft survival.

Makowka (1987) gave preliminary data in an oral presentation on the use of SR-63-441 (Sandoz), the most specific PAF antagonist to date. This agent was examined in the xenograft model of a pig kidney to dog, and compared with PGI_2 and PGE_2. Used singly none of these agents were effective. This contrasts with the experience of Mundy with PGI_2 in the xenograft model, though Mundy used vast doses of PGI_2. The dose of PGI_2 used by Makowka was not stated. SR-63-441 was administered intravenously in a single dose 4 minutes before reperfusion of the graft. When SR was combined with either PGI_2 or PGE_2 function of the xenograft was seen to 300 minutes. A rat model of hyperacute rejection was also examined. Rats were allosensitised with three skin grafts, and then given a cardiac graft. The hearts were hyperacutely rejected, but with SR given before revascularisation survival to three to four days was produced. The histology in the three day grafts showed an aggressive cellular rejection, instead of the expected picture of vascular rejection. This led to experiments combining SR with FR 900506 (FR). FR is a new immunosuppressive agent, which was first isolated from Streptomyces tsukubaensis in 1984. The chemical structure belongs to macrolides, and the molecular weight is 822. The agent is a potent inhibitor of IL-2 production, and is approximately 100 times as potent as CyA - Ochai (1987). Returning to the allosensitised rat model, the combination of FR and SR-63-441 prolonged graft survival beyond 30 days. However histology still showed some evidence of cell mediated damage.

Teraoka (1987) has examined the treatment of chronic vascular rejection in man with Ticlopidine, a prostacyclin analogue (OP-41483), and a thromboxane synthetase inhibitor (OKY-046). The data is very preliminary with a small number of patients (25) and follow up of six to twelve months. However the beneficial effect with each of these three agents on CyA and prednisolone patients with chronic vascular rejection was encouraging. All three agents can be given by mouth. Isai (1987) reported on the use of ticlopidine in a canine model of renal transplantation. Ticlopidine inhibits platelet aggregation and has potent antithrombotic activity. Maximal inhibition is observed when it is given by mouth. It has no or minimal influence on all other coagulation and fibrinolysis tests, and has no immunosuppressive actions. The survival of dogs given no immunosuppression was increased

from a mean of 8 days to 19 days with ticlopidine. Histology of the treated group showed cellular rejection, though the changes in the arteries were less marked than in the untreated controls.

Leithner (1981) treated 8 patients with chronic rejection of their renal allograft with a prostacyclin infusion at 5ng/kg/day for 5 days. A slight improvement in serum creatinine was observed and a reduction in platelet sequestration, as tested with ^{111}indium-oxine labeled platelets. Whether this improvement was due to an improvement in renal blood flow or preventing platelet involvement in the chronic rejection process was not determined. Furthermore there was no follow up on the outcome beyond the five day infusion. These patients were treated with azathioprine and prednisolone.

The study described above is the first to investigate an allosensitised dog model of renal allografting with CyA and PGI_2. The benefit from the PGI_2 was very small in contrast with the technical problems of infusing PGI_2. The rejection process in the allosensitised beagles was so rapid and overwhelming that it was asking a lot of a mediator such as PGI_2 to have any significant beneficial effect. The failure of PGI_2 must be seen in the context of the failure of all other strategies in the allosensitised dog other than TLI. In unsensitised models the lipid mediators have shown some beneficial effect. This model is a particularly severe one, given that in the clinical situation the allosensitised patient usually has a negative T-cell crossmatch on the current sera and in this situation PGI_2 might still be of use. In the future, experiments with specific PAF antagonists may be more productive.

CHAPTER 8

Conclusions:

8.1 The management of the allosensitised patient.
8.2 The dog model of accelerated renal allograft rejection.
8.3 Prospects for future research.

8.1 The management of the allosensitised patient.

Three areas of controversy in renal transplantation today are the shortage of organ donors, the best use of immunosuppression so as to maximise the success and safety of transplantation, and the management of the allosensitised patient.

The CTS organised by Opelz from Heidelberg is now the largest data base on renal transplantation, collecting information on allosensitisation history, crossmatch results, immunosuppression, and long term follow up. The CTS indicates that the allosensitised patient has a reduced graft survival, which is correlated with the level of %PRA. This deleterious effect is not removed by CyA, is most marked in the first three months after transplantation, and is reduced by better HLA matching. Allosensensitised patients accumulate on the waiting lists because they wait longer for a transplant. Of the patients on the UK waiting list, 32% have a peak %PRA greater than 50% and 20% a current %PRA greater than 50%. The CTS data demonstrates the benefit of organ sharing, which improves matching for allosensitised patients and minimises the risk of becoming allosensitised through graft failure.

The challenge posed by allosensitisation is to achieve results comparable to those in the unsensitised patient. The standard crossmatch test finds acceptable patients for a particular donor, but overall has the effect of excluding allosensitised patients so that they accumulate on the renal transplantat waiting list. There are reports of hyperacute rejection and more frequently accelerated rejection in allosensitised patients with negative crossmatches, but conversely not all positive crossmatch kidneys fail in allosensitised patients. Increasing the sensitivity of the crossmatch test can pick up some of the grafts that will be lost by allosensitised patients despite an acceptable standard crossmatch. Unfortunately this information is not specific enough to make the decision not to transplant these so called false negative crossmatches, as the majority of these grafts are successful. Thus to date, techniques that enhance the sensitivity of the crossmatch test have not had the specificity to be useful in avoiding transplanting highly allosensitised patients. They may be of use in predicting grafts which are more likely to undergo rejection.

The specificity of the crossmatch test as a predictor of graft outcome has been enhanced by the definition of acceptable positive crossmatches, as it is clear that not all lymphocytotoxic antibodies are harmful to the graft. Thus positive B cell crossmatches with current and historical sera, and positive T cell crossmatches with historical sera, permit successful transplantation in allosensitised patients, though the results are not quite as good as to unsensitised patients. The class and specificity of the alloantibody are helpful. Generally IgM alloantibody is not harmful, whereas IgG is more often harmful than not. Where the positive crossmatch is due to autoantibodies the result can be ignored. Antibodies directed against MHC class I antigens are generally harmful, though the effect will depend on the titre and specificity of the antibody.

Thus the sera of allosensitised patients on the waiting list should be studied so as to define the class and specificity of the alloantibody. Where there is antibody to an HLA-specificity then this antigen can be avoided when selecting particular donors and in addition this may identify those patients suitable for immunomodulation by some technique of alloantibody removal. When donor material becomes available for the crossmatch then time is short, as the kidneys will have been removed from the donor and will be on ice, which leaves only 24-48 hours for the recipient to be selected and the transplant performed. There is therefore a time limit to the selection process with the present methods of organ preservation.

Given the importance of trying to avoid allosensitisation, strategies which minimise blood transfusion, improve HLA matching and maximise the success of first grafts can all be expected to reduce allosensitisation. Donors are a national resource and used nationally will improve matching quality and lessen allosensitisation.

The allosensitised patients comprise a heterogeneous group and are not all equally difficult to manage. It is the highly allosensitised patient that poses the clinical problem. Most authorities chose a cut off of 80%PRA, though without stating whether this is on current or on historical sera. The CTS data indicates that a cut off of 50%PRA with current sera is more appropriate. The source of the alloantigen may be important, as the alloantibody arising from graft failure can be expected to be more persistent than that from other causes and this is of relevance because one strategy is to wait for the alloantibody titres to decline. The prospects of finding a suitable kidney for the allosensitised patient can be improved by finding an HLA matched graft and also testing against as many suitable donors as possible. Alternatives to this passive strategy are immunomodulation of the patient by removal of alloantibody. In cases of sensitisation against multiple specificities, then TLI may be helpful. This is controversial because of the side effects of TLI, furthermore the best time for the transplant is at the time of completing the TLI course, for unless the TLI continues there is a rebound production of alloantibody. For the present, the most practical option clinically is to monitor

the transplanted allosensitised patient more closely, treat rejection early and possibly use extra immunosuppression in the perioperative period.

The Leicester experience showed a marked improvement in results with the introduction of CyA, though rejection remained the main cause of graft loss. This led to a study of the factors influencing the incidence of allograft rejection and the successful treatment of rejection. The starting dose of CyA at 17mg/kg/day was higher than the normally quoted range of 5-15mg/kg/day. This difference may not be as great as is it seems, since most centres claim to adjust the dose according to the results of the monitoring of CyA levels. Measurements of trough CyA levels in whole blood by HPLC were used to define an effective range of immunosuppression in the Leicester patients. Levels outside this range were associated with graft dysfunction due to CyA nephrotoxicity and rejection. This pattern of immunosuppression was associated with rejection episodes in 25% of patients in the first three months. The episodes after three weeks tended to be associated with low CyA levels but they were nearly always successfully treated. Rejection episodes in the first three weeks were usually in allosensitised patients and occurred despite levels of CyA within the therapeutic range. The factors associated with rejection episodes within three months were, a history of previous renal transplantation, allosensitisation as defined by panel reactivity, younger recipients, and the level of Cyclosporin A in the recipient. Early rejection was not associated with the number of blood transfusions, number of HLA-DR mismatches, cumulative number of HLA-A, B, DR mismatches, and primary non function of the graft. Rejection in the first three weeks was more difficult to treat, particularly in a kidney that was not functioning. This was probably related to the severity of the rejection, rather than the need to adjust CyA levels downwards because of suspected CyA nephrotoxicity.

The incidence of rejection in the first three months was well below that quoted in most other series and this may have made it easier for us to observe the deleterious effect of allosensitisation in the first three weeks. The benefit of immediate function and avoiding rejection was well demonstrated. Beyond three months the course of CyA treated grafts with an uncomplicated first three months has been excellent, particularly compared with those converted to conventional therapy with Aza.

The avoidance of allosensitisation is best achieved by good HLA matching and avoiding graft failure, and this was confirmed by the retrospective study of the management of allograft failure in Leicester. The data on %PRA and allosensitisation clearly showed that transplantation and graft failure were associated with an increase in allosensitisation. The study provided some support for the hypothesis that transplant nephrectomy under immunosuppressive cover might reduce alloantibody as determined by %PRA. Another aspect of the study was the safety of elective transplant nephrectomy. A randomised controlled study

is needed to answer this question of the role of transplant nephrectomy in the avoidance of allosensitisation.

Consider WV the allosensitised patient who has rejected three allografts within three months and who remains with a %PRA >80%. The best hope for this patient at present is a fully matched HLA graft, a negative crossmatch, and immediate function.

In conclusion the allosensitised recipient is disadvantaged with regard to renal transplantation in the following ways:

- a longer wait for a suitable crossmatch negative donor.
- a greater risk of rejection within three months of transplantation.
- a higher risk of graft loss from rejection.

It is suggested that there is a pattern of early (first three weeks), treatment resistant rejection associated with allosensitisation of the recipient. Therefore attempts at improving the outcome from renal transplantation for these patients must address the problem of allosensitisation.

8.2 The dog model of accelerated renal allograft rejection.

There is a need for experimental models to study the problem of the allosensitised patient. A primate model would be closest to man. The blood transfusion effect and the role of TLI in renal transplantation have both been studied in primates, but they are difficult to handle and prohibitively expensive. The costs of working with dogs are formidable, but they are easier to handle and there are centres studying dog transplantation biology. Inbred rodent and murine models have the advantage of well defined histocompatibility mismatches, but results in inbred small animal models may not be applicable to the clinical situation. For example, treatment resistant accelerated rejection is not easily reproduced in rodent and murine models.

The literature review of dog renal allografting confirmed the large experience of renal allografting to unsensitised dogs with and without immunosuppression. Though the study of the dog DLA system is behind that in man and small animals, this area has received a large impetus from international workshops. The dog DLA system has serologically defined (class 1) and lymphocyte defined (class 2) antigens with considerable polymorphism at each of the loci. Tissue typing has demonstrated the polymorphism within breeds, indicating the possibility of performing non-DLA matched grafts between unrelated members of the same dog breed. The relevance of DLA matching has been confirmed in both related and unrelated dogs. The presence of a blood transfusion effect has also been confirmed in related and unrelated dogs. These observations parallel the clinical situation and support the suitability of dog models for studying new strategies in clinical transplantation.

The situation as regards the allosensitised dog is different. The literature contains many descriptions of allosensitisation protocols, but there has been little published work on the characterisation of the alloantibody produced and its duration. Transplantation of these allosensitised recipients across a positive crossmatch is usually accompanied by accelerated rejection, but the incidence of hyperacute rejection is unpredictable and relatively uncommon. In the clinical situation, transplantation across a positive T cell match is usually accompanied by hyperacute rejection. These observations were made in retrospective studies in the late 1960, and Patel (1969) suggested a risk of 75% with a positive crossmatch. For obvious reasons, these studies have not been repeated prospectively. There have been no reports in dogs of a sensitisation protocol that consistently produces hyperacute rejection, the best was that of Mundy who produced hyperacute rejection in 60% following allosensitisation by blood transfusion. Generally the reported incidence was considerably lower than this, though more common when mongrels were used. The effect of CyA alone on renal allograft rejection by allosensitised dogs does not appear to have been published. This may be explained by the weight of clinical and experimental evidence against a beneficial effect of CyA against secondary immune responses.

To what extent has this study answered questions on the allosensitisation produced by different alloantigens, the duration of the alloantibody produced, the results of renal allografts to allosensitised recipients, and the effect of crossmatching and CyA on the outcome? Three different allosensitisation protocols were compared, using either skin, blood, or a kidney as the source of alloantigen. The skin and kidney produced allosensitisation consistently as evidenced by the presence of alloantibody causing lymphocytotoxicity to donor pbl. The effect of blood transfusion was less predictable. The addition of CyA for 21 days did not prevent alloantibody production, though the kidney did remain in-situ after discontinuing CyA. Despite avoiding further alloantigen, the allosensitised dogs still had detectable alloantibody one year later. Renal allografts to the allosensitised dogs were rejected in an accelerated manner, and no beneficial effect on graft survival was noted with CyA.

In this model histology was used to monitor the progress of the graft. Ultimately, life supporting function is the test for a transplanted organ. In practice this means removing the native kidneys and using the serum creatinine to set the experimental end point. This has the disadvantage that the dog dies and is not available for the subsequent study of allosensitisation by following the persistence of alloantibody and further renal transplants. As these were aims of the study, it was necessary to find other experimental end points, which were compatible with the dogs keeping their native kidneys. To this end, histology with a scoring system was investigated. This necessitated having the transplant accessible for frequent biopsies and this was successfully achieved with the subcutaneous groin pouch and

the Tru-Cut needle biopsy. The biopsies confirmed that the rejection of a primary renal allograft in the dog was a vigorous response with features of vascular rejection. In the aliosensitised recipient these vascular features appeared earlier, though the timing of the biopsies was critical in order to detect this. The addition of CyA in adequate doses prevented a vascular pattern of rejection in the unsensitised recipient but had no delaying effect in the allosensitised recipient. There was some benefit seen with CyA in the case of allosensitised recipients given a crossmatch negative kidney.

There were no cases of hyperacute rejection seen in this study despite the strenuous efforts to produce allosensitisation. The beagles were capable of hyperacutely rejecting pig kidneys in xenografts experiments suggesting that the absence of hyperacute rejection in the allograft experiments was related to the degree of allosensitisation. At the outset we aimed to exchange kidneys between two breeds so as to increase the degree of DLA mismatching. This seemed more practical than attempting to tissue type our colony of beagles. It subsequently transpired that the beagle sire crossed with the greyhound to produce the donors had also served as a stud dog for most of the beagle dames. This increased the likelihood of DLA matching occurring subsequently with several possible consequences. This may have contributed to hyperacute rejection not being seen, though it is likely that DLA mismatching was still present in view of the rapid graft rejection seen in the non immunosuppressed recipients. The tempo of rejection was comparable to the results in the literature with DLA mismatched grafts between non-littermates. However, if a strategy tested in this model had produced a significant improvement in graft survival then it would have been necessary to confirm DLA mismatching, whether by typing, MLC testing, or controls in the same donor recipient combination.

The strategies to immunomodulate the allosensitised recipients were unsuccessful. Prostacyclin given over a period of six days was of minimal benefit and it is difficult to see how this could be taken further. The use of cytotoxic agents given over seven days produced significant neutropenia, and reduced the production of antibody to tetanus toxoid as measured by the ELISA test and the production of alloantibody as measured by the lymphocyte crossmatch test. This effect was maximal within the two to three weeks of stopping the cytotoxic agents, following which there was a rebound of alloantibody and anti-tetanus toxoid antibody above that in the control group. Subsequently no beneficial effect on graft outcome was observed when the treated dogs were given renal allografts. In retrospect the outcome might have been different if they had been transplanted within 30 days of completion of the cytotoxics, before the rebound in alloantibody production was detected.

In this experimental model renal allografts against a positive CDC crossmatch test consistently produced accelerated vascular rejection despite CyA. This is a more severe test than the clinical situation where the allosensitised patient has at least a negative T-cell

crossmatch with the current sera. Nevertheless the clinical picture of treatment resistant rejection within three weeks was paralleled by the outcome in the allosensitlsed dog with a positive crossmatch test. The severlty of this dog model of accelerated rejection is ideal for the investigation of some strategies that might be of clinical benefit to the allosensitised patient.

It has been shown that this dog model of accelerated renal allograft rejection with histology to monitor the progress of the graft is a useful adjunct to the study of the problem of the allosensitised patient. Of the strategies tested, the use of a crossmatch negative kidney prolonged graft survival, but immunomodulation as described with either cytotoxic agents or prostacyclin was unhelpful.

8.3 Prospects for future research.

At the same time as this study was undertaken Rapaport's group was examining the beneficial effect of TLI in a model of renal transplantation to allosensitised dogs. A combination of pretransplant TLI with post-transpiant CyA and ATG has produced promlsing results, and it is important that this should be confirmed. In clinical practice there are considerable reservations about the use of TLI, as it is such a potent immunosuppressive tool, with the risks of infection and malignancy.

The present clinical approach to allosensitisation involves characterising the specificities of alloantibody and using the crossmatch test to select a suitable donor for the allosensitised recipient. This approach requires a large pool of donors and does not lend itself to the smaller numbers in experimental models. Furthermore, the immunological probes available for studying alloantibody clinically are not yet available for the dog. I do not foresee that the dog model will be helpful for exploring this approach of improving donor selection. Similarly, while the immunoadsorptlon of alloantibody by Protein A is being evaluated clinically, there seems less point in examining this strategy in the dog.

Recent work has demonstrated the release of PAF from hyperacutely rejecting rabbit kidneys and the beneficial effect of the most potent PAF antagonist to date (SR-63-441) was impressive in an allosensitised rat model. It would be of considerable interest to test this agent in the allosensitised dog model.

A better understanding of the relationship between alloantibody, cellular sensitisation and rejection should improve the selection of grafts for allosensitised patients. A major drawback of immunomodulation before transplantation is the necessity to continue the process until a donor kidney becomes available so as to prevent a rebound of alloantibody production. If it were possible to store cadaver kidneys for several days then this might

permit more specific immunomodulation directed at being able to transplant a particular kidney.

The problem of allosensitisation is a heterogeneous one and it is likely that differing combinations of strategies will be needed for different patients and that centres which take a special interest in the problem are likely to achieve better results.

APPENDIX 1

COMPLEMENT MEDIATED LYMPHOCYTOTOXICITY ASSAY (CDC TEST)

Materials:

1) Hanks balanced salt solution. (HBSS, Gibco Ltd.).

2) Polystyrene tubes with caps. (144AS, Sterilin Ltd.).

3) Ficoll-paque. (Pharmacia Ltd.).

4) Terasaki plates. (Sterilin Ltd.).

5) Paraffin oil. (BDH Chemical Co. Ltd.).

6) Rabbit compiement (Buxted Rabbit Co. Ltd.). The complement was preabsorbed with dog blood and stored at -70 deg. C.

7) Propidium iodide. (P5262, Sigma Chemical Co. Ltd.). The stock solution was at 1mg/ml with distiled water, and stored in the dark at 4 deg. C.

8) Invert fluorescence microscope. (E. Leitz Ltd.).

Methods:

1) Dilute heparinised dog blood 1 in 3 with HBSS. Five mls of diluted blood is under layered with two mls of lympo-paque in conical tubes. The blood is centrifuged at 400g for 25-30 minutes.

2) The cells at the interface (mononuclear lymphocytes) are removed with a pasteur pipette. These cells are diluted several fold with HBSS and centrifuged at 400g for 10 minutes. The pellet of cells is gently resuspended in HBSS and washed three times at 200g for 10 minutes. The cells are counted on a haemocytometer and their concentration adjusted to 4×10^6 cells/ml.

3) Terasaki plates are lightly oiled and 2ul of control or test sera are dispensed in each well in sextupletes. The controls are non-immunised pooled canine sera and rabbit anti-dog lymphocyte serum.

4) One microlitre of cells are added to the wells and plates incubated at 21 deg. C. for 30 minutes. After 30 minutes 5ul of 1:2 diluted complement containing propidium iodide (15ul per ml of complement) is introduced to each well. The cell viability is assessed under green excitation (530-560nm range) one hour after complement is added. Dead cells are stained orange-red by propidium iodide.

APPENDIX 2

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Introduction.

The principal of the enzyme linked immunosorbent assay is based on a three stage reaction. Firstly, the specific antibody (Ab) and its antigen (Ag) are coupled. The next step in the assay is to link a second antibody (AbE) to the antibody antigen matrix. This second antibody is usually coupled to an enzyme such as horse radish peroxidase or alkaline phosphatase. Finaliy, the presence of antigen antibody complex is visualised by the development of a colour reaction when a suitable substrate/dye conjugate is added to the system.

The assay can be summarised as follows:

(1) Ag + Ab = Ag/Ab.

(2) Ag/Ag + Ab-E = Ag/Ab-Ab-E.

(3) H_2O_2 + Dye H_2 = $2H_2O$ + Dye.

Pale Green — Dark Green.

Materials

1) Polyvinylchloride microtitre plates, round bottom, (Flow Laboratory Ltd).

2) Antigens:

Tetanus toxoid- (Institute Merieux)

Diptheria toxoid- (Wellcome Foundation Ltd.).

3) Buffers.

a) Bicarbonate/carbonate buffer (Stock 0.5M). 10.59 gm of sodium bicarbonate, and 8.40 gm sodium bicarbonate dissolved in 200 mls of distiled water, pH adjusted to 7.2 with 2M. hydrochloric acid. Stock solution diluted 1:10, to make a 50mM bicarbonate/carbonate buffer.

b) Phosphate buffer saline (Mercia Brocades Ltd). Concentrate diluted 1 in 20 to make up a single strength.

c) 0.05% Tween 20 (Sigma Chemicals Ltd) in phosphate buffer saline (PBS).

d) 10% horse serum (Flow Laboratory Ltd) in PBS.

4) Conjugated anti dog peroxidase (Miles Laboratory). Diluted 1 in 500 in PBS/horse serum.

5) Substrate ABTS (2,2'-azino-dye[3-ethylbenzthioline sulphonate] - Sigma Chemical Co. Ltd.). 548.7mg of ABTS dissolved in 100ml distiled water, pH adjusted to 7.0 with 2M sodium hydroxide. Hydrogen peroxide added to make a final concentration of 0.003% (ie 0.5ml H_2O_2[0.3%] to 50ml ABTS).

6) Multiscan plate reader (Flow Laboratory Ltd).

Screening for canine anti-tetanus toxoid antibody.

1) Microtitre plates are coated with 50ul per well of 1:10 diluted tetanus toxoid in 50 mM bicarbonate/carbonate buffer, or with Diptheria toxoid as third party controls.

2) The plates are incubated overnight at 4 deg. C.

3) The microtitre plates are washed three times in PBS containing 0.05% Tween 20.

4) The non specific binding sites on plastic surfaces are blocked by adding to each well 250ul of 10% horse serum (HS) in PBS and incubating for 30 minutes at room temperature. The plates are then emptied and 50ul of 10% HS-PBS is added to each well.

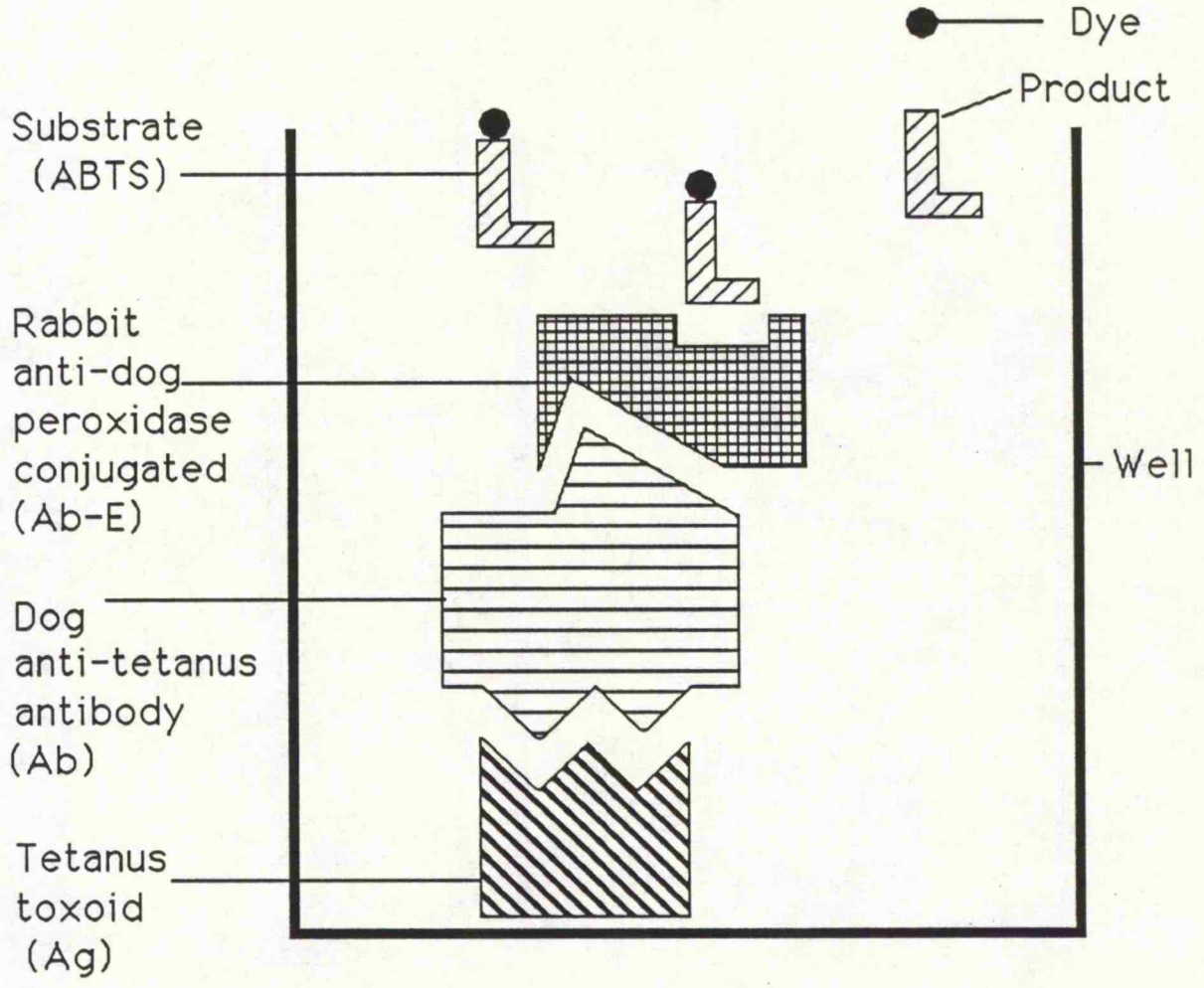
5) The test and control sera (non-immunised) are diluted serially from 1:2 to 1:64, in the wells, and incubated at 37 deg. C. for 2 hours. The surplus proteins are removed by washing the plates 5 times in PBS/Tween.

6) The second antibody is introduced to each well by adding 100ul of anti-dog peroxidase, diluted 1/500 with PBS/HS, and incubated for 1 hour at 37 deg. C.

7) The plates are washed 5 times with PBS/tween to remove any excess peroxidase conjugated antibody and then 100ul of substrate ABTS is added to each well.

8) The colour reaction is measured at 414 nm on a multiscan plate reader 10 and 20 minutes after addlng the substrate.

Enzyme linked immunosorbent assay (ELISA)



(1) $Ag + Ab \longrightarrow Ag/Ab$

(2) $Ag/Ab + Ab\text{-}E \longrightarrow Ag/Ab\text{-}Ab\text{-}E$

(3) $H_2O_2 + Dye\,H_2 \xrightarrow{Ag/Ab\text{-}Ab\text{-}E} 2H_2O + Dye$

APPENDIX 3

DRUG LIST.

ACETYLPROMAZINE MALEATE - C-Vet Ltd.
ACP injection 2mg/mi contains Acepromazine Maieate equivalent to 2mg/ml acepromazine base per mi. This is used for anaesthetic premedication. It can be given by intramuscular or slow intravenous injection. The dose is 0.125-0.25mg/kg body weight.

BUPRENORPHINE - Reckitt & Colman.
Temgesic injection contains 0.3mg/ml buprenorphine as the hydrochloride, in a 5% dextrose solution, adjusted to a pH range 3.5 - 5.5. A strong analgesic.

CYCLOSPORIN A - Sandoz Pharmaceuticals.
Clear, yellow, oily solution containing 100mg/ml. Each ml of concentrate for intravenous injection contains 50mg of cyclosporin in an oily solution containing 650mg polyethoxylated castor oil and 33% ethanol by volume.
A cycloic polypeptide consisting of 11 amino acids. It is a potent immunosuppressive agent. Cyclosporin inhibits the development of cell mediated reactions. It appears to block the resting lymphocytes In the G_0 or early G_1 phase of the cell cycle, and also inhibits lymphokine production and release, including interleukin 2.

CYCLOPHOSPHAMIDE - Boehringer Ingelheim Ltd.

CYTOSINE ARABINOSIDE - Upjohn Ltd.

DIPHTHERIA VACCINE - Welcome.
Adsorbed Diphtheria vaccine is a suspension of highly purified toxoid prepared from the exotoxin of Corynebacterium diphtherla adsorbed onto hydrated aluminium phosphate. Thiomersal BP is added as a preservative to a concentration of 0.01%. Each 0.5 ml. dose contains not less than 30IU.

DUPLOCILLIN - Gist-Brocades Animal Health.
An injectable aqueous suspension containing 150mg procaine peniciiiin and 112.5mg benzathine peniciliin per ml. The procaine peniciliin achieves a high initial penicillin blood level and the more slowly adsorbed benzathine penicillin maintains a therapeutic blood level for three to four days.

EPOPROSTENOL SODIUM - (Prostacyclin) - Wellcome.
Each vial contains 500mcg freeze dried epoprostenol (formerly known as prostacyclin) as the sodium salt. Each 50ml vial of diluent contains Sodium Chlorlde BP 0.147% w/v and Glycine 0.188% w/v in clear solution. The alkalinity of the diluent has been adjusted to pH 10.5 $\pm$ 0.3 by the addition of sodium hydroxide.

HAEMACCEL™ - Hoechst UK Ltd.
A colloidal infusion solution based on geiatin.

HYPNOVEL™ - (Midazolam - Roche Products Ltd)
Each vial contains 10mg of midazolam base as the hydrochloride in 2 ml aqueous solution.

IMMOBILON / REVIVON - C-Vet Ltd.
Small animal immobilon, each ml contains etorphine hydrochloride 0.074mg (0.068 mg/ml etorphine base), methotrimeprazine 18mg, chlorocresol 0.1% w/v sodium citrate, citric acid, sodium metabisuiphite, ascorbic acid, disodium EDTA.
Small animal revivon, each ml contains diprenorphine 0.272mg as hydrochloride, methylene blue 0.001% w/v, chlorocresol 0.1% w/v.
Immobilon is used to produce reversible neuroleptanalgesia prior to minor operations in dogs.

PIRITON - Allen & Hanburys Ltd.
Chlorpheniramine maleate is a potent antihistamine.

SAFFAN - Glaxovet Limited.
An injectable steroid anaesthetic, which is a clear solution of neutral pH. The active constituents are two pregnanedione derivatives, alphaxalone and alphadolone acetate, solubilised in saline by 20% w/v polyoxyethylated castor oil. Each ml of Saffan contains 12 mg of total steroids composed of 9 mg alphaxalone and 3mg alphadolone acetate.

TETANUS TOXOID- Institute Merieux.
Merieux Tetovax is a sterile aqueous suspension of purified tetanus toxoid prepared by treating the toxin of Clostridium tetani with formaldehyde. The toxoid is adsorbed onto aluminium hydroxide and thiomersal is added as preservative.
Each 0.5ml dose contains not less than 40 IU of tetanus toxoid.

TRIBRISSEN 80 - Coopers Animal Health Ltd.
Each tablet contains Trimethoprim 80mg and Sulphadiazine 400mg.

TRIVETRIN INJECTION- Coopers Animal Health Ltd.
Each ml contains Trimethoprim 40mg and Sulfadoxime BP 200mg.

Appendix 4

Appendix 4 documents the alloantigen history of the nineteen beagle recipients on the right side. On the left the results of alloantibody testing of the recipient sera are set out. Each serum sample is characterised by a number with the day from commencing an experiment below. Recorded vertically are the sources of donor test pbl, the date of testing, and the results. A '+' indicates lymphocytotoxicity in the recipient test serum against donor pbl. The results of all the donor-recipient crossmatches are recorded.

BEAGLE RECIPIENTS.

B1 MONTY	B2 ZIGGY	B3 CHESTER
B4 NEIL	B5 PAINTER	B6 DUNCAN
B7 SYDNEY	B8 PODGE	B9 EBONY
B10 FRED	B11 ROSIE	B12 TIFFANY
B13 SNOWY	B14 ARNOLD	B15 JANET
B16 MISTY	B17 TIZER	B18 MUTTLEY
B19 KIM		

GREAGLE and GREYHOUND DONORS.

G1 DOUGLAS	G2 SIMON	G3 TERRY
G4 PATSY	G5 MYRTLE	G6 SYBIL
G7 CAESAR	G8 REBEL (greyhound)	G9 TESSA
G11 SCOOBY	G12 DUSTY	G13 SEB
G14 RAMBO	G15 PANDORA	G16 ELSA
G17 KELLY	G18 TIPSY	G19 SHANDY
G20 PRUDENCE	G21 CHAUCER (greyhound)	
G22 MILLY (greyhound)	G23 TILLY (greyhound)	
G24 LUCY (greyhound)		

Recipient		Donor
B1-Monty		G1-Douglas
Date	Day	
18.9.84	0	Skin graft
24.10.84	32	Skin graft
6.11.84	49	Skin graft
4.12.84	77	Skin graft
11.1.85	115	Diced Skin sc
26.2.85	161	Diced Skin sc
18.4.85.	212	Diced Skin sc
24.5.85	248	Buffy coat iv
10.7.85	295	Kidney
17.7.85	302	Nephrectomy
		G24-Lucy +
		Molly beagle
14.11.85	422	Blood 7ml sc
25.11.85	433	Blood 7ml sc
6.12.85	444	Blood 7ml sc
11.12.85	449	Blood 7ml sc
21.3.86	549	Diced Skin sc-G11
	Blood	7ml sc-G24+B10
23.7.86	673	Tet Tox 0.5ml im
28.7.86	678	Diced Skin sc-G15
13.8.86	694	Diced Skin sc-G16
		+Tet Tox 0.5ml im
10.9.86	722	G16-Diced Skin sc
+ Buffy coat 5ml iv + Tet tox im		
30.10.86	772	G16-Kidney
4.11.86	777	Nephrectomy
16.12.86	819	G11-Kidney
22.12.86	825	Nephrectomy

Panal of 4 Greyhounds-
Tilly, Milly, Chaucer, Cassie.

	Sera															
	Number	1	5	13	21	29	37	46	58	70	84	101	115	130	143	
	Day	0	6	9	13	18	20	23	27	42	49	63	70	77	84	
Date	Target PBL's															
10.10.84	G1-Douglas	-	-	-	-	-	-									
19.10.84	G2-Simon					-										
30.10.84	G1+G2								-							
30.10.84	G1+G2	51Cr Assay-Sera diluted1/10							-							
18.12.84	G1-Douglas														+	
26.6.85	G1-Douglas	-	-	-	-	-	-	-	-						+	
	Number	156	188	207	220	229	236	246	251	257	264	267	268	284	304	
	Day	90	125	149	160	175	231	249	253	276	294	315	319	356	456	
Date	Target PBL's															
24.1.85	G1-Douglas	-	-	51Cr Assay-Sera diluted1/20												
7.2.85	G1-Douglas	-	+													
27.2.85	G1-Douglas			+												
26.3.85	Panal of 9					9/9	Panal-3 Greagles, Beagles, Greyhounds									
30.5.85	Panal of 9								6/9							
4.6.85	Panal of 3							3/3	3/3							
26.6.85	G1-Douglas	+	+		+	+	+	+	+	+	Visual CDC + 51Cr Assay					
12.9.85	Panal of 6			Panal-3 Beagles, Greyhounds										4/6		
10.10.85	Panal of 10 Greagles													4/10		
19.12.85	Panal of 10 Greagles													10/10		
13.3.86	Panal of 7 Greagles													5/7		
	Number			322	364	375	393	411	429	439	449	475	493	519	537	
	Day			477	531	673	694	700	722	724	727	730	737	743	750	
Date	Target PBL's															
9.1.86	Panal of 7 Greagles			8/9												
13.2.86	Panal of 7 Greagles			6/8												
4.3.86	Panal of 7 Greagles				4/7											
5.9.86	Panal of 7 Greagles							7/7								
23.10.86	G16-Elsa								+			+		+		
	Number	555		573	591	609	627	644	650							
	Day	758		765	778	792	806	819	840							
Date	Target PBL's															
23.10.86	G16-Elsa	+														
6.11.86	Panal of 4 Greyhounds			3/4												
1.12.86	Panal of 5 Greagles					5/5										

Recipient		Donor			Sera														
B2-Ziggy		G1-Douglas			Number	2	6	14	22	30	38	47	57	71	89	102	116	131	144
Date	Day				Day	0	6	9	13	16	20	23	27	42	49	63	70	77	84
18.9.84	0	Skin graft		Date	Target PBL's														
24.10.84	36	Skin graft		10.10.84	G1-Douglas	-	-	-	-	-	-								
6.11.84	49	Skin graft		19.10.84	G2-Simon					-									
4.12.84	77	Skin graft		30.10.84	G1+G2								-						
11.1.85	115	Diced Skin sc		30.10.84	G1+G2	51Cr Assay-Sera diluted1/10							-						
26.2.85	161	Diced Skin sc		18.12.84	G1-Douglas														-
18.4.85.	212	Diced Skin sc		4.7.85	G1-Douglas	-	-	-	-	-	-	-	-	-	-	-	-	-	
24.5.85	248	Buffy coat iv																	
10.7.85	295	Kidney			Number	157	190	208	221	234	237	247	252	258	263	265	266	269	285
15.7.85	300	Nephrectomy			Day	90	125	149	160	175	231	249	253	276	288	294	315	319	356
				Date	Target PBL's														
		G24-Lucy +		24.1.85	G1-Douglas	-	-		51Cr Assay-Sera diluted1/20										
		Molly beagle		7.2.85	G1-Douglas	-	+												
14.11.85	422	Blood 7ml sc		27.2.85	G1-Douglas				-										
6.12.85	444	Blood 7ml sc		26.3.85	Panal of 9					1/9		Panal-3 Greagles, Beagles, Greyhounds							
11.12.85	449	Blood 7ml sc		30.5.85	Panal of 9								5/9						
22.1.86	491	G13-Kidney		4.6.85	Panal of 3							0/3	2/3						
23.7.86	673	Tet Tox 0.5ml im		4.7.85	G1-Douglas	+	-	-	+	+	-	-	+		+				
28.7.86	678	G15-Diced Skin sc		12.9.85	Panal of 6			Panal-3 Beagles, Greyhounds											4/6
13.8.86	694	G16-Diced Skin sc		10.10.85	Panal of 10 Greagles														7/10
		+Tet Tox 0.5ml im		13.3.85	Panal of 7 Greagles														5/7
10.9.86	722	G16-Diced Skin sc																	
+ Buffy coat 5ml iv + Tet tox im																			
8.12.86	811	G23-Kidney																	
22.12.86	825	Nephrectomy																	
					Number			305	323	338	376	394	412	430	440	450	476	494	520
					Day			456	477	491	673	694	700	722	724	727	730	737	743
				Date	Target PBL's														
				12.12.85	Panal of 10 Greagles			8/10											
				9.1.86	Panal of 9 Greagles				8/9										
				13.3.86	Panal of 7 Greagles					7/7									
				25.4.86	G12-Dusty					+		(Neat; 1/10; 1/20)							
										(T+B)									
				5.9.86	Panal of 7 Greagles								6/7						
				23.10.86	G16-Elsa									+			+		+
					Number	538	556	574	592	610	628	646							
					Day	750	758	765	778	793	806	825							
				Date	Target PBL's														
				23.10.86	G16-Elsa		+												
				6.11.86	Panal of 4 Greyhounds			4/4											
				1.12.86	Panal of 5 Greagles					4/5									

Recipient		Donor		Sera														
B3-Chester		G2-Simon		Number	3	7	15	23	31	39	45	53	113	125	147	158	192	209
Date	Day			Day	0	6	9	13	16	20	23	27	65	70	87	90	125	149
18.9.84	0	Skin Graft	Date	Target PBL's														
22.11.84	65	Skin Graft	19.10.84	G2-Simon							-							
14.12.84	87	Skin Graft	30.10.84	G2-Simon							-							
11.1.85	115	Diced skin sc	30.10.84	G2-Simon	51Cr Assay-Sera diluted 1/10						-							
26.2.85	161	Diced skin sc	24.1.85	G2-Simon	51Cr Assay-Sera diluted 1/20											-	+	
18.4.85	212	Diced skin sc	7.2.85	G2-Simon												-	+	
24.5.85	248	Buffy coat iv	4.7.85	G2-Simon	-	-	-	-	-	-	-	-	-	-	-	-	+	+
21.8.85	337	Kidney																
24.8.85	340	Nephrectomy		Number	222	230	238	248	254	261	270	272	278	286	306	324	338	365
				Day	160	175	231	249	253	288	337	340	349	356	456	477	491	532
		G24-Lucy +	Date	Target PBL's														
		Molly beagle	27.2.85	G2-Simon	+													
14.11.85	422	Blood 7ml sc	26.3.85	Panal of 9		7/9		Panal-3 Beagles, Greyhounds, Greagles										
25.11.85	433	Blood 7ml sc	30.5.85	Panal of 9					4/9		Panal-3 Beagles, Greyhounds, Greagles							
6.12.85	444	Blood 7ml sc	4.6.85	Panal of 3				1/3	2/3		Panal- 1 Beagle, Greyhound, Greagle							
11.12.85	449	Blood 7ml sc	4.7.85	G2-Simon	+	+	+	+	+	+								
			12.9.85	Panal of 6		Panal- 3 Beagles, Greyhounds								4/6				
26.2.86	526	G19-Kidney	10.10.85	Panal of 10 Greagles										2/10				
4.3.86	532	Nephrectomy	19.12.85	Panal of 10 Greagles											9/10			
			9.1.86	Panal of 9 Greagles												8/9		
23.7.86	673	Tet Tox 0.5ml im	13.3.86	Panal of 7 Greagles										5/7				
28.7.86	678	Diced Skin sc-G15																
13.8.86	694	Diced Skin sc-G16		Number	377	395	413	451	467	485	495	511	529	547	557	575	593	611
		+Tet Tox 0.5ml im		Day	673	694	700	727	729	731	737	741	744	751	758	765	778	792
15.9.86	722	G14-Diced Skin sc	Date	Target PBL's														
+ Buffy coat 5ml iv + Tet tox im			5.9.86	Panal of 7 Greagles			6/7											
26.11.86	799	G14-Kidney	23.10.86	G14-Rambo				+			+		+		+			
27.11.86	800	Nephrectomy	6.11.86	Panal of 4 Greyhounds												4/4		
			1.12.86	Panal of 5 Greagles														3/5
				Number	629	647	651											
				Day	819	825	840											

Recipient		Donor		Sera														
B4-Neil		G2-Simon		Number	4	8	16	24	32	40	48	54	112	126	148	159	193	210
Date	Day			Day	0	6	9	13	16	20	23	27	65	70	87	90	125	149
18.9.84	0	Skin Graft	Date	Target PBL's														
22.11.84	65	Skin Graft	19.10.84	G2-Simon							-							
14.12.84	87	Skin Graft	30.10.84	G2-Simon							-							
11.1.85	115	Diced skin sc	30.10.84	G2-Simon	51Cr Assay-Sera diluted 1/10						-							
26.2.85	161	Diced skin sc	24.1.85	G2-Simon	51Cr Assay-Sera diluted 1/20											-	+	
18.4.85	212	Diced skin sc	7.2.85	G2-Simon												-	+	
24.5.85	248	Buffy coat iv	26.6.85	G2-Simon	-	-	-	-	-	-	-	-	-	-	-	-	+	+
21.8.85	337	Kidney																
24.8.85	340	Nephrectomy		Number	223	231	239	249	253	262	271	273	279	287	307	325	351	378
				Day	160	175	231	249	253	288	337	340	349	356	456	477	512	673
		G24-Lucy +	Date	Target PBL's														
		Molly beagle	27.2.85	G2-Simon	-													
14.11.85	422	Blood 7ml sc	26.3.85	Panal of 9		9/9		Panal-3 Beagles, Greyhounds, Greagles										
6.12.85	444	Blood 7ml sc	30.5.85	Panal of 9					4/9		Panal-3 Beagles, Greyhounds, Greagles							
11.12.85	449	Blood 7ml sc	4.6.85	Panal of 3				2/3	3/3		Panal- 1 Beagle, Greyhound, Greagle							
			26.6.85	G2-Simon	-	+	+	+	+	+								
12.2.87	512	G17-Kidney	12.9.85	Panal of 6		Panal- 3 Beagles, Greyhounds								5/6				
16.2.86	516	Nephrectomy	10.10.85	Panal of 10 Greagles										7/10				
			12.12.85	Panal of 10 Greagles											7/10			
23.7.86	673	Tet Tox 0.5ml im	9.1.86	Panal of 9 Greagles												8/9		
28.7.86	678	Diced Skin sc-G15	13.2.86	Panal of 8 Greagles													3/8	
13.8.86	694	Diced Skin sc-G16	13.3.86	Panal of 7 Greagles										5/7			7/7	
		+Tet Tox 0.5ml im														-		
15.9.86	722	G12-Diced Skin sc																
+ Buffy coat 5ml iv + Tet tox im				Number	396	414	452	468	486	496	512	530	548	558	576	594	612	630
9.12.86	812	G17-Kidney		Day	694	700	727	729	731	737	741	744	751	758	765	778	792	819
17.12.86	820	Nephrectomy	Date	Target PBL's														
			5.9.86	Panal of 7 Greagles		6/7												
			23.10.86	G12-Dusty			+			+		+		-				
			6.11.86	Panal of 4 Greyhounds											4/4			
			1.12.86	Panal of 5 Greagles													3/5	

	8		Donor			Sera													
85-Painter			G3-Terry			Number	9	17	25	33	41	49	55	59	65	72	79	85	94	103
Date		Day				Day	0	1	5	8	12	15	19	23	28	34	37	41	48	55
26.9.84		2	Kidney		Date	Target PBL's														
16.1.85		114	Nephrectomy		7.11.84	G3-Terry	-	-	-	-	-	-	-	-	-	-				
					7.11.84	G3-Terry	-	-	-	-	-	-	-	-	-	-		51Cr Assay		
			G24-Lucy+																	
			Molly beagle																	
14.11.85		416	Blood 7ml sc																	
25.11.85		427	Blood 7ml sc																	
6.12.85		438	Blood 7ml sc																	
11.12.85		443	Blood 7ml sc			Number	117	132	149	160	173	184	195	214	244	255	288	308	326	340
15.1.86		480	G12+Yapper beagle			Day	62	69	79	82	104	113	118	145	230	245	348	448	469	504
			-Diced skin sc		Date	Target PBL's														
22.1.86		487	G13-Diced skin sc		4.6.85	Panal of 3	Panel-1Beagle, Greyhound, Greagle								0/3	1/3				
28.1.86		493	G18-Diced skin sc		12.9.85	Panal of 6	Panel-3Beagles, Greyhounds										2/6			
21.3.86		543	G11-Diced skin sc		10.10.85	Panal of 10 Greagles											3/10			
			+Blood scB10+G24		19.12.85	Panal of 10 Greagles												9/10		
23.7.86		667	Tet tox 0.5ml im		13.2.86	Panal of 8 Greagles														0/8
28.7.86		672	G15-Diced skin sc		4.3.86	Panal of 7 Greagles											1/7			
13.8.86		688	G16-Diced skin sc		13.3.86	Panal of7 Greagles											0/7			7/7
			+Tet tox 0.5ml im																	
15.9.86		716	G12-Diced skin sc			Number	363	379	397	415	453	469	487	497	513	531	549	559	577	595
+Buffy coat 5ml iv+Tet tox im						Day	523	665	688	694	721	723	725	731	735	738	745	752	759	772
3.12.86		800	G21-Kidney		Date	Target PBL's														
4.12.86		801	Nephrectomy		5.9.86	Panal of 7 Greagles				7/7										
					23.10.86	G12-Dusty					+			+		+		+		
					6.11.86	Panal of 4 Greyhounds													0/4	
						Number		613	631											
						Day		787	800											
					Date	Target PBL's														
					1.12.86	Panal of 5 Greagles		4/5												

Recipient			Donor			Sera													
86-Duncan			G3-Terry			Number	10	18	26	34	42	50	56	60	66	73	81	86	95	104
Date		Day				Day	0	3	7	10	14	17	21	25	30	36	39	43	50	57
26.9.84		2	Kidney		Date	Target PBL's														
16.1.85		114	Nephrectomy		7.11.84	G3-Terry	-	-	-	-	-	-	-	-	-	-				
					7.11.84	G3-Terry	-	-	-	-	-	-	-	-	-	-	-	51Cr Assay		
			G24-Lucy+																	
			Molly beagle																	
14.11.85		416	Blood 7ml sc																	
25.11.85		427	Blood 7ml sc																	
6.12.85		438	Blood 7ml sc																	
11.12.85		443	Blood 7ml sc			Number	118	133	150	161	176	185	196	215	289	309	327	341	362	380
15.1.86		480	G12+Yapper beagle			Day	64	71	81	84	106	115	120	147	350	450	471	506	525	665
			-Diced skin sc		Date	Target PBL's														
22.1.86		487	G13-Diced skin sc		12.9.85	Panal of 6	Panel-3Beagles, Greyhounds								0/6					
28.1.86		493	G18-Diced skin sc		10.10.85	Panal of 10 Greagles									0/10					
21.3.86		543	G11-Diced skin sc		19.12.85	Panal of 10 Greagles										8/10				
			+Blood scB10+G24		9.1.86	Panal of 9 Greagles											5/9			
23.7.86		667	Tet tox 0.5ml im		13.2.86	Panal of 8 Greagles												7/8		
28.7.86		672	G15-Diced skin sc		4.3.86	Panal of 7 Greagles													5/7	
13.8.86		688	G16-Diced skin sc		13.3.86	Panal of 7 Greagles									0/7			7/7		
			+Tet tox 0.5ml im																	
10.9.86		716	G16-Diced skin sc																	
+Buffy coat 5ml iv+Tet tox im						Number			398	416	431	441	454	477	498	521	539	560	578	596
						Day			688	694	716	718	721	724	731	737	744	752	759	772
					Date	Target PBL's														
					5.9.86	Panal of 7 Greagles				6/7										
					23.10.86	G16-Elsa					+			+		+		+		
					6.11.86	Panal of 4 Greyhounds													0/4	

Recipient		Donor		Sera														
B7-Sydney		G4-Patsy		Number	67	74	80	87	96	105	119	134	151	162	166	167	170	177
Date	Day			Day	0	6	9	13	20	28	34	41	51	54	56	58	70	76
24.10.84	0	Kidney	Date	Target PBL's														
19.12.84	56	Nephrectomy	20.12.84	G7-Caesar											+			
		G24-Lucy+																
		Molly beagle																
14.11.85	386	Blood 7ml sc		Number	197	216	245	250	290	310	328	342	361	381	399	417	432	442
6.12.85	408	Blood 7ml sc		Day	90	117	202	217	320	420	441	476	495	637	658	664	686	688
11.12.85	413	Blood 7ml sc	Date	Target PBL's														
15.1.86	448	G12+Yapper beagle	30.5.85	Panal of 9				0/9		Panal -3 Beagles, Greyhounds, Greagles								
		-Diced skin sc	4.6.85	Panal of 3				1/3										
22.1.86	455	G13-Diced skin sc	12.9.85	Panal of 6					4/6		Panal-3Beagles, Greyhounds							
28.1.86	463	G18-Diced skin sc	10.10.85	Panal of 10 Greagles					7/10									
21.3.86	513	G11-Diced skin sc	19.12.85	Panal of 10 Greagles						9/10								
		+Blood scB10+G24	9.1.86	Panal of 9 Greagles							8/9							
23.7.86	637	Tet tox 0.5ml im	13.2.86	Panal of 8 Greagles								1/8						
28.7.86	642	G15-Diced skin sc	4.3.86	Panal of 7 Greagles									7/7					
13.8.86	658	G16-Diced skin sc	13.3.86	Panal of7 Greagles					5/7			7/7						
		+Tet tox 0.5ml im	5.9.86	Panal of 7 Greagles												7/7		
10.9.86	686	G16-Diced skin sc	23.10.86	G16-Elsa													+	
		+Buffy coat 5ml iv+Tet tox im																
1.12.86	768	G21-Kidney																
15.12.86	782	Nephrectomy		Number	455	478	499	522	540	561	579	597	615	632				
				Day	691	694	701	707	714	722	729	742	756	770				
			Date	Target PBL's														
			23.10.86	G16-Elsa		+		+		+								
			6.11.86	Panal of 4 Greyhounds							4/4							
			1.12.86	Panal of 5 Greagles									3/5					

Recipient		Donor		Sera														
B11-Rosie		G7-Caesar		Number	123	138	145	164	180	182	191	212	224	232	240	274	277	293
Date	Day			Day	0	7	14	20	45	50	55	79	90	105	161	273	279	286
28.11.84	1	Blood 30ml	Date	Target PBL's														
11.1.85	45	Blood 20ml	18.12.84	G1-Douglas			-											
15.2.85	80	Blood 25ml	20.12.84	G7-Caesar	-	±	-	-										
18.4.85	142	Blood 50ml	24.1.85	G7-Caesar				-	-	+			51Cr Assay- sera 1/20					
28.6.85	274	Kidney	7.2.85	G7-Caesar				-	±	±								
2.9.85	279	Nephrectomy	27.2.85	G7-Caesar									+					
			26.3.85	G7-Caesar									+					
		G24-Lucy+	26.3.85	Panal of 9									4/8					
		Molly beagle	30.5.85	G7-Caesar											-			
14.11.85	352	Blood 7ml sc	30.5.85	Panal of 9		Panal-3Beagles, Greyhounds, Greagles									3/9			
6.12.85	374	Blood 7ml sc	4.6.85	G7-Caesar											+			
11.12.85	379	Blood 7ml sc	4.6.85	Panal of 3											1/3			
15.1.86	414	G12+Yapper beagle	12.9.85	Panal of 6												0/6	0/6	5/6
		-Diced skin sc	20.3.86	Panal of 7 greagles		0/7	0/7	0/7	0/7	2/7	4/7	5/7	4/7	6/7	6/7			5/7
22.1.86	421	G13-Diced skin sc																
28.1.86	428	G18-Diced skin sc																
21.3.86	479	G11-Diced skin sc		Number			296	300	313	331	345	358	367	384	402	420	458	470
		+Blood scB10+G24		Day			294	328	386	407	442	461	476	603	624	630	657	659
23.7.86	603	Tet tox 0.5ml im	Date	Target PBL's														
28.7.86	608	G15-Diced skin sc	10.10.85	Panal of 10 greagles			7/10											
13.8.86	624	G16-Diced skin sc	19.12.85	Panal of 10 greagles					10/10									
		+Tet tox 0.5ml im	9.1.86	Panal of 9 greagles														
15.9.86	657	G15-Diced skin sc	13.2.86	Panal of 8 greagles							1/8							
		+Buffy coat 5ml iv+Tet tox im	4.3.86	Panal of 7 greagles								4/7						
19.11.86	722	G15-Kidney	13.3.86	Panal of 7 greagles			5/7				7/7							
25.11.86	728	Nephrectomy	20.3.86	Panal of 7 greagles			6/7	7/7	5/7	6/7	7/7	7/7	6/7					
			5.9.86	Panal of 7 Greagles												7/7		
			23.10.86	G15-Pandora													+	
				Number			488	502	514	532	550	564	582	600	618			
				Day			671	667	671	674	681	688	695	708	722			
			Date	Target PBL's														
			23.10.86	G15-Pandora				+		+		+						
			6.11.86	Panal of 4 Greyhounds									2/4					
			1.12.86	Panal of 5 Greagles											5/5			

Recipient		Donor		Sera															
B8-Podge		G4-Patsy		Number	75	88	97	111	120	135	140	152	154	168	171	174	198	217
Date	Day			Day	0	6	13	22	27	34	40	44	47	51	63	69	83	110
31.10.84	0	Kidney	Date	Target PBL's														
14.12.84	44	Nephrectomy	18.12.84	G1-Douglas								+	+					
		G24-Lucy+ Molly beagle																
14.11.85	379	Blood 7ml sc		Number			291	311	329	343	360	382	400	418	433	443	456	479
6.12.85	401	Blood 7ml sc		Day			313	413	434	469	488	630	652	658	680	682	685	688
11.12.85	406	Blood 7ml sc	Date	Target PBL's														
15.1.86	441	G12+Yapper beagle	12.9.85	Panal of 6			3/6											
		-Diced skin sc	10.10.85	Panal of 10 Greagles			6/10											
22.1.86	448	G13-Diced skin sc	19.12.85	Panal of 10 Greagles				7/10										
28.1.86	456	G18-Diced skin sc	9.1.86	Panal of 9 Greagles					5/9									
21.3.86	506	G11-Diced skin sc	13.2.86	Panal of 8 Greagles						2/8								
		+Blood scB10+G24	13.3.86	Panal of 7 Greagles			4/7			7/7								
23.7.86	630	Tet tox 0.5ml im	4.3.86	Panal of7 Greagles							0/7							
28.7.86	635	G15-Diced skin sc	5.9.86	Panal of 7 Greagles										7/7				
13.8.86	651	G16-Diced skin sc	23.10.86	G20-Prudence											+			+
		+Tet tox 0.5ml im																
10.9.86	679	G20-Diced skin sc																
		+Buffy coat 5ml iv+Tet tox im		Number	500	523	541	562	580	598	616	633						
21.10.86	720	G20-Kidney		Day	695	701	708	716	723	736	750	764						
30.10.86	729	Nephrectomy	Date	Target PBL's														
1.12.86	761	G21-Kidney	23.10.86	G20-Prudence		+		+										
7.12.86	767	Nephrectomy	6.11.86	Panal of 4 Greyhounds					4/4									
			1.12.86	Panal of 5 Greagles							4/5							

Recipient		Donor		Sera														
B9-Ebony		G5-Myrtle		Number	90	98	106	121	136	141	163	175	186	199	218	226	226	227
Date	Day			Day	0	7	15	21	28	34	41	43	49	77	104	112	114	118
7.11.84	1	Kidney	Date	Target PBL's														
16.1.85	71	Nephrectomy	18.12.84	G1-Douglas						+								
27.2.85	113	Kidney	12.2.85	G5-Myrtle	-	-	+	+	+	+	+	+	+	+				
4.3.85	118	Nephrectomy																

Recipient		Donor		Sera														
B10-Fred		G6-Sybil		Number	107	122	137	142	153	155	169	172	178	200	202	203	204	205
Date	Day			Day	0	6	13	19	23	26	30	42	48	62	70	71	74	79
21.11.84	0	Kidney	Date	Target PBL's														
14.12.84	23	Nephrectomy	18.12.84	G1-Douglas				+	+	+	Neat serum & 1/10							
30.1.85	70	Kidney	1.2.85	G6-Sybil	-		+		+		+				+	+	LN cells	
3.2.85	74	Nephrectomy	1.2.85	G6-Sybil	-		+		+		+				+	+	Spleen	
			22.3.85	Panal of 4	0/4		Sera diluted 1/10 to 1/800										4/4	
		G24-Lucy+	29.3.85	G2-Simon	-		+		+		+		+		+	51 Cr Assay		
		Molly beagle	4.7.85	G1 + G2		+		Sera 1/10										
14.11.85	358	Blood 7ml sc		Number	211	235	259	292	312	330	344	359	383	401	419	434	444	457
6.12.85	380	Blood 7ml sc		Day	85	127	212	292	392	413	448	467	609	630	636	658	660	663
11.12.85	385	Blood 7ml sc	Date	Target PBL's														
15.1.86	420	G12+Yapper beagle	29.3.85	G2-Simon	+	+		51Cr assay										
		-Diced skin sc	12.9.85	Panal of 6				3/6										
22.1.86	427	G13-Diced skin sc	10.10.85	Panal of 10 greagles				5/10										
28.1.86	433	G18-Diced skin sc	19.12.85	Panal of 10 greagles					9/10									
21.3.86	485	G11-Diced skin sc	9.1.86	Panal of 9 greagles						4/9								
		+Blood scB10+G24	13.2.86	Panal of 8 greagles							1/8							
23.7.86	609	Tet tox 0.5ml im	4.3.86	Panal of 7 greagles							3/7							
28.7.86	614	G15-Diced skin sc	13.3.86	Panal of 7 greagles				7/7			7/7							
13.8.86	630	G16-Diced skin sc	5.9.86	Panal of 7 Greagles											6/7			
		+Tet tox 0.5ml im	23.10.86	G20-Prudence												+		
10.9.86	658	G20-Diced skin sc																
+Buffy coat 5ml iv+Tet tox im				Number			480	501	524	542	563	581	599	617	634			
9.10.86	687	G20-Kidney		Day			666	673	679	686	694	701	714	729	742			
19.12.86	758	Nephrectomy	Date	Target PBL's														
			23.10.86	G20-Prudence			+		+		+							
			6.11.86	Panal of 4 Greyhounds								4/4						
			1.12.86	Panal of 5 Greagles										5/5				

16.1.85	G6-Sybil	107	122	137	142	169	172			
	Neat	-	+	+	+	+	+	C' 1/4 Buxted		
	1/10	-	+	+	+	+	+	107 = Pre Transplant ? C' toxicity		
	1/20	±	+	+	+	+	+			
	1/40	±	+	+	+	+	+			
	1/80	±	+	+	+	+	+			
	1/160	±	+	+	+	+	+			
	1/320	+	+	+	+	+	+			

Recipient		Donor
B12-Tiffany		G7-Caesar
Date	Day	
28.11.84	0	Blood 30ml
11.1.85	43	Blood 20ml
15.2.85	80	Blood 25ml
18.4.85	142	Blood 50ml
28.8.85	274	Kidney
30.8.85	279	Nephrectomy
		G24-Lucy+
		Molly beagle
14.11.85	352	Blood 7ml sc
6.12.85	374	Blood 7ml sc
11.12.85	379	Blood 7ml sc
15.1.86	414	G12+Yapper beagle
		-Diced skin sc
22.1.86	421	G13-Diced skin sc
28.1.86	428	G18-Diced skin sc
21.3.86	479	G11-Diced skin sc
		+Blood scB10+G24
23.7.86	603	Tet tox 0.5ml im
28.7.86	608	G15-Diced skin sc
13.8.86	624	G16-Diced skin sc
		+Tet tox 0.5ml im
15.9.86	657	G11-Diced skin sc
+Buffy coat 5ml iv+Tet tox im		
8.12.86	741	G23-Kidney
6.1.87	770	Nephrectomy

	Sera																
	Number	124	139	146	165	181	183	189	213	225	233	241	275	276	294		
	Day	0	7	14	20	45	50	55	79	90	105	161	273	276	286		
Date	Target PBL's																
18.12.85	G1-Douglas			-													
20.12.84	G7-Caesar	-	±	±	±												
24.1.85	G7-Caesar					-	-	+			51Cr	Assay-	sera	1/20			
7.2.85	G7-Caesar					-	±	±									
27.2.85	G7-Caesar									+							
26.3.85	G7-Caesar									+							
26.3.85	Panal of 9									6/9							
30.5.85	G7-Caesar											-					
30.5.85	Panal of 9											0/9					
4.6.85	G7-Caesar											-					
4.6.85	Panal of 3											0/3					
12.9.85	Panal of 6													0/6	0/6		
	Number			297	301	314	332	346	357	368	385	403	421	459	471		
	Day			294	328	386	407	442	461	476	603	624	630	657	659		
Date	Target PBL's																
10.10.85	Panal of 10 greagles			10/10													
19.12.85	Panal of 10 greagles					7/10											
9.1.86	Panal of 9 greagles						3/9										
13.2.86	Panal of 8 greagles							1/8									
4.3.86	Panal of 7 greagles								1/7								
13.3.86	Panal of 7 greagles			7/7				7/7									
5.9.86	Panal of 7 Greagles												6/7				
23.10.86	G11-Scooby													+			
	Number			489	503	515	533	551	565	583	601	619	636				
	Day			661	667	671	674	681	688	695	718	723	736				
Date	Target PBL's																
23.10.86	G11-Scooby				+		+		+								
6.11.86	Panal of 4 Greyhounds									4/4							
1.12.86	Panal of 5 Greagles											3/5					

Recipient		Donor
B13-Snowy		G8-Rebel
Date	Day	
9.1.85	0	Kidney
18.1.85	9	Nephrectomy
20.2.85	42	Kidney
25.2.85	47	Nephrectomy
		G24-Lucy+
		Molly beagle
14.11.85	309	Blood 7ml sc
6.12.85	331	Blood 7ml sc
11.12.85	336	Blood 7ml sc
15.1.86	371	G12+Yapper beagle
		-Diced skin sc
22.1.86	378	G13-Diced skin sc
28.1.86	384	G18-Diced skin sc
21.3.86	436	G11-Diced skin sc
		+Blood scB10+G24
23.7.86	560	Tet tox 0.5ml im
28.7.86	565	G15-Diced skin sc
13.8.86	630	G16-Diced skin sc
		+Tet tox 0.5ml im
15.9.86	614	G11-Diced skin sc
+Buffy coat 5ml iv+Tet tox im		
8.12.86	698	G22-Milly
6.1.87	727	Nephrectomy

	Sera																
	Number	179	187	194	201	206	219	228	295	315	333	356	386	404	422		
	Day	0	9	12	21	30	40	54	243	343	364	418	560	581	587		
Date	Target PBL's																
12.2.85	G8-Rebel	-	-	-	-												
14.2.85	G8-Rebel	-	+	-	+	+											
12.9/85	Panal of 6								4/6								
10.10.85	Panal of 10 greagles								6/10								
19.12.85	Panal of 10 greagles									8/10							
9.1.86	Panal of 9 greagles										6/9						
13.2.86	Panal of 8 greagles										3/8						
4.3.86	Panal of 7 greagles											2/7					
13.3.86	Panal of 7 greagles								4/7		7/7						
5.9.86	Panal of 7 Greagles														7/7		
	Number			460	472	490	504	516	534	552	566	584	602	620	637		
	Day			614	616	618	624	628	631	638	645	652	665	679	693		
Date	Target PBL's																
23.10.86	G11-Scooby			+			+		+		+						
6.11.86	Panal of 4 Greyhounds											1/4					
1.12.86	Panal of 5 Greagles												3/5				
	Number	648	653														
	Day	712	727														
Date	Target PBL's																

Recipient		Donor		Sera														
B14-Arnold		G9-Tessa		Number	234	235	242	280	282	298	302	316	334	348	355	370	372	373
Date	Day			Day	14	30	48	166	173	190	215	273	294	329	348	392	399	488
20.3.85	0	Blood 30ml	Date	Target PBL's														
18.4.85	29	Blood 50ml	30.5.85	Panal of 9			7/9											
10.7.85	112	Blood 50ml	4.6.85	Panal of 3			0/3											
4.9.85	168	Kidney	12.9.85	Panal of 6				4/6	2/6									
9.9.85	173	Nephrectomy	10.10.85	Panal of 10 Greagles						1/10								
			19.12.85	Panal of 10 Greagles								7/10						
		G24-Lucy+	9.1.86	Panal of 9 Greagles									1/9					
		Molly beagle	13.2.86	Panal of 8 Greagles										1/8				
14.11.85	239	Blood 7ml sc	4.3.86	Panal of 7 Greagles											2/7			
25.11.85	250	Blood 7ml sc	13.3.86	Panal of 7 Greagles						5/7			7/7					
6.12.85	261	Blood 7ml sc																
11.12.85	266	Blood 7ml sc																
15.1.86	301	G12+Yapper beagle																
		-Diced skin sc		Number			391	405	423	435	445	461	481	505	525	543	567	585
22.1.86	308	G13-Diced skin sc		Day			492	511	517	539	541	544	547	554	560	567	573	582
28.1.86	314	G18-Diced skin sc	Date	Target PBL's														
28.7.86	495	G15-Diced skin sc	5.9.86	Panal of 7 Greagles					6/7									
13.8.86	511	G16-Diced skin sc	23.10.86	G18-Tipsy						+			+		+		+	
			6.11.86	Panal of 4 Greyhounds														4/4
10.9.86	539	G18-Diced skin sc																
		+Buffy coat 5ml iv+Tet tox im		Number			603	621	638	645	654							
15.10.86	574	G18-Kidney		Day			595	609	610	636	657							
23.10.86	582	Nephrectomy	Date	Target PBL's														
16.12.86	636	G11-Kidney	1.12.86	Panal of 5 Greagles				4/5										
6.1.87	657	Nephrectomy																

Recipient		Donor		Sera														
B15-Janet		G9-Tessa		Number	243	256	260	261	283	299	303	317	335	349	354	366	387	
Date	Day			Day	19	41	74	137	144	161	186	244	265	300	319	334	461	
18.4.85	0	Blood 50ml	Date	Target PBL's														
15.5.85	27	Blood 60ml	30.5.85	Panal of 9	0/9													
10.7.85	83	Blood 50ml	4.6.85	Panal of 3	0/3	0/3												
4.9.85	139	Kidney	12.9.85	Panal of 6				0/6	0/6									
9.9.85	144	Nephrectomy	10.10.85	Panal of 10 Greagles						9/10								
			19.12.85	Panal of 10 Greagles								7/10						
		G24-Lucy+	13.2.86	Panal of 8 Greagles										2/8				
		Molly beagle	4.3.86	Panal of 7 Greagles											0/7			
14.11.85	210	Blood 7ml sc	13.3.86	Panal of 7 Greagles						7/7				7/7				
6.12.85	232	Blood 7ml sc																
11.12.85	237	Blood 7ml sc																
15.1.86	272	G12+Yapper beagle		Number	406	424	462	473	491	506	517	535	553	568	586	604	622	
		-Diced skin sc		Day	482	488	515	517	519	525	529	532	539	546	553	566	581	
22.1.86	279	G13-Diced skin sc	Date	Target PBL's														
28.1.86	285	G18-Diced skin sc	5.9.86	Panal of 7 Greagles		7/7												
21.3.86	337	G11-Diced skin sc	23.10.86	G14-Rambo			+			+		+		+				
		+Blood scB10+G24	6.11.86	Panal of 4 Greyhounds											0/4			
23.7.86	461	Tet tox 0.5ml im	1.12.86	Panal of 5 Greagles														4/5
28.7.86	466	G15-Diced skin sc																
13.8.86	482	G16-Diced skin sc																
		+Tet tox 0.5ml im																
15.9.86	515	G14-Diced skin sc																
		+Buffy coat 5ml iv+Tet tox im																
27.11.86	588	G14-Kidney																
29.11.86	590	Nephrectomy																

Recipient		Donor		Sera												
B16-Misty		G24-Lucy+		Number	318	336	350	353	369	371	374	392	407	425	436	446
		Molly beagle		Day	0	21	56	75	119	126	215	219	238	244	266	268
14.11.85	-	Blood 7ml sc	Date	Target PBL's												
25.11.85	-	Blood 7ml sc	10.10.85	Panal of 10 Greagles	0/10											
6.12.85	-	Blood 7ml sc	12.12.85	Panal of 10 Greagles	0/10											
11.12.85	-	Blood 7ml sc	13.3.86	Panal of 7 Greagles			0/7									
15.1.86	28	G12+Yapper beagle	5.9.86	Panal of 7 Greagles										0/7		
		-Diced skin sc	23.10.86	G16-Elsa											+	
22.1.86	35	G13-Diced skin sc														
28.1.86	41	G18-Diced skin sc														
21.3.86	93	Tet tox im		Number	463	482	507	526	544	569	587	605	623	640		
16.4.86	119	Tet tox im		Day	271	274	281	287	294	302	309	322	337	350		
21.7.86	215	Tet tox im	Date	Target PBL's												
28.7.86	222	G15-Diced skin sc	23.10.86	G16-Elsa		+		+		+						
13.8.86	238	G16-Diced skin sc	6.11.86	Panal of 4 Greyhounds							4/4					
			1.12.86	Panal of 5 Greagles									1/5			
10.9.86	266	G18-Diced skin sc														
		+Buffy coat 5ml iv+Tet tox im														

Recipient		Donor		Sera												
B17-Tizer		G13 - Seb		Number	319	339	388	408	426	437	447	464	483	508	527	545
Date	Day			Day	0	35	217	238	244	266	268	271	274	281	287	294
22.1.86	35	G13-Kidney	Date	Target PBL's												
9.4.86	112	Nephrectomy	10.10.85	Panal of 10 Greagles	0/10											
			12.12.85	Panal of 10 Greagles	0/10											
23.7.86	217	Tet tox im	5.9.86	Panal of 7 Greagles					0/7							
28.7.86	222	G15-Diced skin sc	23.10.86	G16-Elsa						+			+		+	
13.8.86	238	G16-Diced skin sc														
		+Tet tox im														
10.9.86	266	G18-Diced skin sc														
		+Buffy coat 5ml iv+Tet tox im														
				Sera												
				Number	570	588	606	624	641							
				Day	302	309	322	168	350							
			Date	Target PBL's												
			23.10.86	G16-Elsa	+											
			6.11.86	Panal of 4 Greyhounds		0/4										
			1.12.86	Panal of 5 Greagles				0/5								

Panal of 7 Greagles
G15-Pandora
G14-Rambo
G16-Elsa
G12-Dusty
G18-Tipsy
G11-Scooby
G20-Prudence

Recipient		Donor		Sera														
B18 Muttley		G19 Shandy		Number		320	353	389	409	427	438	448	465	484	509	528	546	
Date	Day			Day		0	70	217	238	244	266	268	271	274	281	287	294	
26.2.86	70	Kidney	Date	Target PBL's														
			10.10.85	Panal of 10 Greagles		0/10												
23.7.86	217	Tet tox im	12.12.85	Panal of 10 Greagles		0/10												
28.7.86	222	G15-Diced skin sc	5.9.86	Panal of 7 Greagles						4/7								
13.8.86	238	G16-Diced skin sc +Tet tox im	23.10.86	G11-Scooby							+			+		+		
10.9.86	266	G18-Diced skin sc		Number		571	589	607	625	642								
+Buffy coat 5ml iv+Tet tox im				Day		301	309	322	337	350								
15.10.86	301	G18-Kidney	Date	Target PBL's														
15.12.86	362	Nephrectomy	23.10.86	G11-Scooby		+												
			6.11.86	Panal of 4 Greyhounds			4/4											
			1.12.86	Panal of 5 Greagles					1/5									

Recipient		Donor		Sera														
B19 Kim		G17 Kelly		Number		321	352	390	410	428	466	474	492	510	518	536	554	
Date	Day			Day		0	56	217	238	244	271	273	275	281	285	288	295	
12.2.86	56	Kidney	Date	Target PBL's														
25.3.86	97	Nephrectomy	10.10.85	Panal of 10 Greagles		1/10												
			12.12.85	Panal of 10 Greagles		0/10												
23.7.86	217	Tet tox im	13.3.86	Panal of 7 Greagles			0/7											
28.7.86	222	G15-Diced skin sc	5.9.86	Panal of 7 Greagles						4/5								
13.8.86	238	G16-Diced skin sc +Tet tox im	23.10.86	G15-Pandora							+			+		+		
15.9.86	271	G15-Diced skin sc																
+Buffy coat 5ml iv+Tet tox im				Number		572	590	608	626	643								
19.11.86	336	G15-Kidney		Day		302	309	322	336	350								
28.11.86	345	Nephrectomy	Date	Target PBL's														
			23.10.86	G15-Pandora		+												
			6.11.86	Panal of 4 Greyhounds			1/4											
			1.12.86	Panal of 5 Greagles					4/5									

1.12.86 Panal of 5 Greagles
G12-Dusty
G22-Milly
G11-Scooby
G21-Chaucer
G23-Tilly

Appendix 5.

GROUP NINE

SENSITISED + KIDNEY (+ve X-MATCH).

PLUS CyA (25mg/kg/day) & HEPARIN.

N=11

B15 JANET

Died at 36 hours from haemorrhage from a ruptured kidney.

Day of biopsy.	Score:				Conclusion:
(Biopsy number)	6	8	10	12	
Pre-op - (143)	0	0	0	1	Pre-Tx biopsy.
2 - (146)	0	0	1	2	Mild ATN with early cellular rejection

B11 ROSIE

Died during the fifth night from haemorrhage from a ruptured kidney.

Pre-op - (137)	0	0	0	0	Pre-Tx biopsy.
5 - (138)	5	5	7	9	Acute haemorrhagic rejection with glomerular and vascular thrombosis.

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