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THESIS

SPECIFIC AND NON-SPECIFIC SUPPRESSION OF RENAL ALLOGRAFT REJECTION IN THE RAT

by

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ABSTRACT

In this thesis certain clinically relevant aspects of specific and non-specific suppression of renal allograft rejection were examined in a rat renal allograft model.

The potentiation of the partial immunosuppressive effect of passive enhancement was found to be possible with suboptimal doses of antilymphocyte serum or cyclophosphamide but not azathioprine and prednisolone. Moreover, the use of high doses of azathioprine with enhancing serum resulted in significantly shorter survival times than those seen with enhancing serum alone. It was concluded that potentiation of enhancement required a significant but suboptimal level of non-specific immunosuppression with the appropriate agent to be applied during the induction of enhancement.

The administration of antidonor antiserum to rat kidney allograft recipients does not usually result in damage to the graft but it is likely to occur in human allograft recipients. The enhancing capacity of the non-complement-fixing, $F(ab')_2$ fraction of enhancing antibody was investigated and found to be 100 x less effective than whole antibody in the induction of enhancement. However, $F(ab')_2$ was found to be capable of blocking antibody-mediated damage to allografts caused by whole antibody and guinea-pig complement, without blocking the latter's specific immuno-suppressive effect. Red cell absorption of enhancing serum to produce a putative, "anti-Ia-like" serum, resulted in the loss of the serum's ability to produce immediate antibody-mediated

damage to a renal allograft without removing its ability to enhance. Both strategies could, with appropriate modifications, be applied to clinical practice.

Antilymphocyte serum and cyclophosphamide, which were thought to have relatively selective actions on cellular and humoral immunity respectively, were found to have no such selectivity in the suppression of renal allograft rejection. When used in combination their effects were found to be additive but not synergistic.

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ABBREVIATIONS

ACT	Tris-ammonium chloride
ACTH	Adrenocorticotrophic hormone
ADCC	Antibody dependent cellular cytotoxicity
ALS	Antilymphocyte serum
ARA	Antireceptor antibody
ARCO	Antigen reactive cell opsonisation
ATS	Antithymocyte serum
B cell	Bone marrow derived cell
BCG	Bacille-Calmette-Guerin
Bn	Brown-Norway
B.P.	British Pharmacopoeia
"B" rat	Lethally irradiated, thymectomised rat reconstituted with bone marrow from thoracic duct drained isogenei donor
BSA	Bovine Serum Albumin
CMI	Cell mediated immunity
CML	Cell mediated lympholysis
cpm	counts per minute
^{51}Cr	Chromium-51
DAB	Dulbecco's A and B solution
DNA	Deoxyribonucleic acid
DNCB	Dinitrochlorobenzene
DNFB	Dinitrofluorobenzene
EDTA	Ethyldiamine triacetic acid
Fc	Fraction crystalisable portion of immunoglobulin molecule
FCA	Freund's complete adjuvant
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GBM	Glomerular basement membrane
GVH	Graft versus host
H and E	Haematoxylin and Eosin

Ia	Immune associated
i.m.	Intramuscular
i.p.	Intraperitoneal
Ir	Immune response
KLH	Keyhole limpet haemocyanin
LD	Lymphocyte defined
LDA	Lymphocyte dependent antibody
LD_{50}	Lowest dose killing 50% of experimental animals
ma	Milliampere
mg	Milligram
MHC	Major histocompatibility complex
ml	millilitre
MLC	Mixed lymphocyte culture
MLR	Mixed lymphocyte reaction
mm	Millimetre
m.w.	Molecular weight
NaOH	Sodium Hydroxide
ng	nanogram 10^{-9} gram
NS	Not significant
N/Saline	Normal saline = Sodium Chloride 9g/l or 150mmol/L.
PBL	Peripheral blood lymphocyte
PFc	C-terminal portion Fc region immunoglobulin molecule
PHA	Phytohaemagglutinin
PMN	Polymorphonuclear (leucocyte)
PPD	Purified protein derivative
PTa	Peripheral T cell antigen
PWM	Pokeweed mitogen
RNA	Ribonucleic acid
SD	Serologically determined or Standard deviation
SDS	Sodium dodecylsulphate
SG	Specific gravity
SPF	Specific pathogen free
SRBC	Sheep red blood cell

T cell	Thymus derived cell
T la	Thymus leukaemia antigen
w/v	weight/volume
µCi	Microcurie
µg	Microgram 10^{-6} gram

GENERAL INTRODUCTION

Despite more than a decade of experience, intensive patient assessment and monitoring, improved tissue typing, and reliable surgical techniques, the results of renal transplantation in man have reached a plateau which is substantially below the goal of uniform success. The major problem remains rejection and its control.

Current immunosuppressive regimens used to suppress rejection are neither sufficiently effective nor sufficiently safe. Indeed, the optimum results with cadaver kidney donors of 60% one year, and 40% five year graft survival, are obtained at the cost of a significant incidence of infective complications and systemic side effects (The 12th report of the Human Renal Transplant Registry, 1975). Not the least of these is the 100 fold increase in the risk of developing malignant disease (reviewed Penn, 1977).

The experiments described in this thesis are related to both of these problems.

1. Safety

The immunosuppressive agents currently used i.e. azathioprine, prednisolone, and in some centres anti-lymphocyte globulin, although primarily directed at the immune system, have many systemic side effects. It is, however, their non-selective action on immunity which renders them so dangerous. While suppressing the immune

response to the allograft they seriously weaken the host's defences against infection. Research has therefore been concentrated on a clinically applicable form of antigen-specific immunosuppression. Although this has been achieved within the artificial environment of the laboratory, application to clinical practice has been delayed by the practical problems encountered in the transition. Two of the problems which have been anticipated in the application of one form of specific immunosuppression, passive enhancement, have been examined in this thesis. Firstly, since passive enhancement is not uniformly successful in suppressing graft rejection, non-specific immunosuppressive agents will also be used. Ideally such agents should not interfere with the specific immunosuppressive measures, and should be effective at safe dose levels. The standard immunosuppressive agents were combined with passive enhancement in a model in which this provides only partial suppression of rejection. This was done to identify which agents would potentiate, and which would antagonise the specific immunosuppressive effect of enhancing serum.

Secondly, the administration of donor-specific antiserum is likely to cause immediate antibody-mediated damage to human allografts. I have investigated various ways of avoiding this complication, without forfeiting the specific immunosuppressive action of the enhancing antiserum.

2. Efficacy

In a separate group of experiments the effect of the combined use of two non-specific agents, one directed primarily at the cell-mediated immune response, (ALS) and the other at the humoral response (cyclophosphamide) was explored. This combination was suggested by the observation that human and rat renal allograft rejection appeared to be mediated by both humoral and cellular effector mechanisms and that humoral rejection was not easily controlled by azathioprine and prednisolone.

CHAPTER 1

TRANSPLANTATION IMMUNOLOGY

Introduction
Transplantation Antigens
Minor Histocompatibility Antigens
The MHC of the Rat
The MHC differences between DA(Ag-B^4) and Lewis (Ag-B^1) strains
The antigenic component of the graft
The site of sensitisation
Cellular events
The role of antibody in allograft rejection
Rejection patterns in vivo
Acute renal allograft rejection in the rat

INTRODUCTION

The immune system which is a functional not a morphological unit, attempts to maintain the balance between the individual host and the multitude of foreign agents with which it comes into intimate and perpetual contact. It evolved presumably to maintain the functional integrity of the individual threatened by pathogenic micro-organisms and, though this is more controversial, malignant cells.

It has both specific and non-specific components. The latter includes the non-specific inflammatory response. The former component, the lymphoid system, has the following cardinal characteristics:-

1. It can discriminate self from non-self.
2. It produces specific antibodies and cells which are capable of destroying or neutralising the agent to which they are directed.
3. It has a memory system by which secondary encounters with the same agent result in an amplified response.

In the course of a specific immune response the non-specific components act in concert to protect the host.

This basic outline of the immune system has been stated to reinforce the point that the reaction to an allograft is a predictable biological event in which the same processes which follow more natural intrusions upon the individual, are evident. This became apparent at the

time of the first allograft experiments (reviewed Wu and Mann, 1934) but allograft rejection was most carefully scrutinised and described by Medawar in (1944). He concluded, "the mechanism by which foreign skin is eliminated belongs to the general category of actively acquired immune reactions. The inflammatory process which accompanies it has in all likelihood the character of local anaphylaxis. The inflammatory process includes vascular and lymphatic proliferation; a massive invasion of the grafts by lymphocytes and monocytes of native origin through the walls of the vessels within them; oedema of such severity that the graft bed may be distended by tracks of free fibrinous matter; and a general mobilisation of mesenchyme cells. Inflammation reaches its peak and passes into necrosis, with the stagnation and obliteration of the vascular system of the graft and the death of every cellular element with it."

Transplantation antigens

The immune system responds to foreign agents after recognising unfamiliar molecular determinants. Theoretically, therefore, any cell surface protein, whatever its function should act as a transplantation antigen if it exists in polymorphic forms. This is most obvious in xenotransplantation systems but applies equally to allotransplantation. However, the products of one genetic complex appear to have a dominant influence on the survival of allografts and it

has therefore been called the major histocompatibility complex (MHC). Although the study of this genetic complex has developed with transplantation research its true function is not yet understood. In addition to determining transplant survival and related processes such as the graft-versus-host reaction (GVH), in vitro cell-mediated lympho-cytotoxicity (CML), mixed lymphocyte culture reactions (MLR), (reviewed Bach and van Rood, 1976), it is involved in a number of other processes and phenomena. These include cell-cell interaction, for example in the killing of the virus infected cells (Doherty & Zinkernagel, 1975; McMichael et al., 1977); education of the thymus as to what constitutes self and non-self (Zinkernagel et al., 1978); immune responsiveness (Benacerraf and McDevitt, 1972) which may account for the association of various diseases in man with the MHC (reviewed McMichael and McDevitt, 1977), and the levels of certain complement components (Meo et al., 1975).

Homologous MHC's have been identified in many species, including the rat, the mouse and man (reviewed Klein, 1977a). It is the mouse MHC(H-2) that has been most carefully dissected and it has therefore become the reference point to which other MHC's are compared.

The products of the mouse MHC(H-2) can be considered in three classes (Klein, 1977a). The class 1 regions code for what are commonly called serologically-determined or SD antigens, a term which has proved to be a misnomer. In the mouse these are coded for by three loci, K, D, and G.

In man there are apparently three such loci, A, B, and C. The antigens are proteins consisting of a pair of non-covalently linked chains, the larger of which (m.w. 45,000) carries the antigenic determinant and is coded for by a gene within the MHC. It is linked to B_2 microglobulin (m.w. 12,000) which is coded for by a gene on a different chromosome (reviewed Barnstable et al., 1979). The SD antigens provoke strong humoral responses, hence their name, and are thought to be an important stimulus and target for cytotoxic T-cells (Bach et al., 1976). The SD antigens are highly polymorphic and are widely distributed on most tissues, including erythrocytes in mice and rats but not man.

The class 11 region, otherwise called the I region, contains the immune response (Ir) genes; the genes coding for the lymphocyte defined (LD) antigens which are of dominant importance in stimulating the MLR and the stimulation of helper T-cells; and the genes coding for the (Immune-associated) Ia antigens. Ia antigens have a restricted tissue distribution in the mouse being found on B-lymphocytes, macrophages, epidermal cells, spermatocytes, suppressor T-cells, some thymocytes but they are absent from red cells, muscle, and helper T-cells (reviewed Hämmerling, 1976). Their presence on kidney is controversial. Hämmerling et al. (1975) were not able to find them on mouse kidney tissue but their test system was inadequate. Davies and Butcher (1978) and Hart and Fabre (1978) have

found that Ia-like antigens are present in large amounts on rat kidney tissue. Their presence on kidney is consistent with the ability of kidney cells to stimulate in the MLR (Mashimo et al., 1976). The Ia antigens are also glycoproteins but they are not homologus with the class 1 region antigens. They consist of two polypeptide chains the larger of which is thought to carry the antigenic determinant and they are not linked to B_2 microglobulin (reviewed Cullen et al., 1976).

Class 111 region genes code for various complement components, and a number of other less important antigens in and around the MHC, e.g. the mouse Tla antigen found on thymocytes and certain mouse leukaemia cells. The H-2 G locus codes for a red cell alloantigen. The Ss and Slp loci code for a serum protein like human C4.

The participation of individual classes of MHC antigens in the allograft reaction has not yet been clearly defined. Bach et al. (1976) have proposed a simple model for the triggering of the immune system in the allograft reaction. They suggest that the various T-cell subpopulations (which can be separated functionally by Ly alloantigens (Cantor and Boyse,1975a and b) are triggered by different MHC antigens. Ly^{1+} helper T-cells are triggered by LD antigens whereas killer cells, $Ly^{2+\ 3+}$, are triggered by SD antigens and their development into killer cells, which are directed at SD antigens, is enhanced by the presence of helper T-cells.

However, it is clear that rejection can take place when either only an I region difference or, only an SD difference between host and donor is present. Klein (1977b) has shown that differences in the mouse at H-2 K, H-2 D or the A subregion of the I region result in skin graft, heart graft, and tumour graft rejection. GVH reactions will also take place across these differences. Similar results for mouse skin grafts were obtained by McKenzie and Henning (1977a & b) who ranked the H-2 loci in the order of their immunogenicity as follows: H-2K = H-2I> H-2D> H-2IC.

Gallico and Butcher (1978) have recently shown that in congenic rat strains both an A region (the homologue of mouse K and D regions) and a B region (homologue of the mouse I region) differenceare required for complete rejection of a renal allograft.

Examination of the importance of various antigens of the human MHC to transplantation is obviously more difficult. Matching for A and B locus antigens in donors and recipients improves the prognosis in cadaver transplants by 10 to 15% at 1 year and in family combinations the difference is more striking (reviewed Morris, 1978b). More recently it has been possible to match for the DRw antigens (putatively the human equivalent of antigens coded by the mouse I region) and the preliminary results suggest that the region may be as, if not more important than the A, B and C loci (Ting and Morris, 1978; Persijn et al., 1978).

Minor Histocompatibility Systems

Minor histocompatibility antigens are capable of stimulating rejection albeit more slowly than the major histocompatibility complex antigens. In rats minor differences can provoke skin graft rejection although kidneys are often not rejected (reviewed Günther and Štark, 1977). However, sensitisation to minor antigens can give rise to accelerated kidney graft rejection (Fabre and Morris, 1975).

The MHC of the Rat (reviewed Günther and Štark, 1977)

This is not as well defined as the mouse MHC but appears to be functionally homologous. It has two nomenclatures, H-1 (Štark and Křen, 1969) and Ag-B (Elkins and Palm, 1966).

Two regions in the rat MHC, an A region and a B region have now been defined by recombination (Butcher and Howard, 1977a). The A region corresponds to the mouse K and D regions and the B region to the mouse I region. Gill and Kunz (1976) have suggested a tentative model for the rat MHC with two loci controlling A region antigens and unlike the mouse, the B region outside these two, as in man.

Anti-Ag-B sera which are absorbed with red cells or platelets to remove anti-A region antibodies cannot strictly be called anti-Ia as they may contain antibodies

to minor specificities not expressed on red cells. Just such an antigen is the PTa antigen (Butcher and Howard, 1977b). Red cell absorbed sera, although haemagglutinin negative do react against a subpopulation of lymphocytes (Davies and Alkins, 1974), inhibit EA rosette formation (Soulillou et al., 1976) and in the Lewis anti-DA strain combination detect a product of the MHC as shown by back-cross experiments (Hart and Fabre, 1978).

The MHC differences between DA ($Ag\text{-}B^4$) or ($H\text{-}1^a$) and Lewis ($Ag\text{-}B^1$) or ($H\text{-}1^1$) strains

The strains used in the experiments described in this thesis were DA, Lewis, and (DA X Lewis)F_1 hybrids. In addition to their differences at the major histo-compatibility complex these strains also differ at a number of other loci (Palm et al., 1977) including the PTa (Peripheral T-cell antigen) locus (Butcher and Howard, 1977b). There are a number of important differences in the reaction between these two strains. The DA response to a Lewis kidney transplant is weaker than the Lewis response to a DA transplant. Graft rejection is more difficult to suppress both with specific immunosuppressive measures (Fabre and Morris, 1974a) and with non-specific immunosuppressive agents (Mottram and Fabre, unpublished). The Lewis strain produces stronger antibody responses both after transplantation and after hyperimmunisation.

Moreover, whereas DA anti-Lewis antisera are mostly directed against Ia-like antigens (Gallico and Mason, 1978) only about a quarter of Lewis anti-DA sera are so directed (Hart and Fabre, 1978). Parodoxically the DA strain responds more strongly to the Lewis in mixed lymphocyte culture and in GVH assays than vice versa (Wilson, 1969; Silber et al., 1976).

The Antigenic component of the graft

Precisely which component of any grafted tissue triggers the allograft responses is unknown. The distribution and concentration of histocompatibility antigens varies from tissue to tissue, e.g. (Hart and Fabre, 1978).

Passenger leucocytes increase the immunogenicity of organ grafts (reviewed Billingham, 1971; Guttmann et al., 1969a; Guttmann and Lindquist, 1969) but they are not an essential part of the stimulus (Stuart et al., 1971b). An important role for passenger leucocytes in triggering endocrine graft rejection has been proposed by Sollinger et al. (1977) and Lafferty and Woolnough (1977). Both showed that cultured thyroid allografts enjoyed prolonged survival when compared to fresh allografts. The effect could be reversed by the addition of passenger cells. Pretreatment of human renal allograft donors has not yet been shown to improve results (Leader Br.Med.J. 1977).

Both kidney cells (Mashimo et al., 1976) and endothelial cells (Hirschberg et al., 1975) are able to stimulate in the MLR confirming that the graft without passenger leucocytes itself should be sufficiently immunogenic to evoke a rejection response.

The Site of sensitisation

Whether activation of the immune system to an allograft is central or peripheral may depend on the type of graft. Skin grafts appear to sensitise mainly via the draining lymph node whereas lymphocyte activation against a kidney allograft is within the graft itself. This view is based on the observation of lymph flow from the two types of graft. Whereas the afferent lymphatic from the skin graft of a sheep has a normal lymph flow, the efferent lymphatic leaving the draining node shows not only an increase in flow but the lymph contains blast cells (Hall, 1967). The afferent lymphatic draining a kidney allograft on the other hand shows a dramatic increase in lymph flow after transplantation and also contains blast cells (Pedersen and Morris, 1974a). Surprisingly, drainage of this lymph to the exterior makes no difference to the tempo of graft rejection (Pedersen and Morris, 1970). These experiments are consistent with those of: Barker and Billingham (1968) and Tilney and Gowans (1971) who demonstrated that skin graft survival was prolonged, but not indefinitely, if the graft was maintained on an alymphatic pedicle;

Strober and Gowans (1965) who showed that lymphocytes perfused through an allogenic kidney ex vivo became sensitised during their passage; and Hume and Egdahl (1955) who showed that kidney allografts were destroyed even if lymphatic connections were prevented from forming.

Cellular events (reviewed Gowans, 1977)

The dominant role of T-cells in the rejection of allografts is now well established. Rejection of skin grafts is suppressed after neonatal thymectomy (Arnason et al., 1962), in athymic nude mice (Wortis, 1971) and in "B" rats (Rolstad and Ford, 1974). More recently Hall et al. (1978) have shown that it is the long-lived rapidly recirculating small T-lymphocyte which is responsible for graft rejection in the rat. The lymphocyte pool has considerable reserve capacity for effecting rejection. It has been estimated by Ford (1973) that 7 to 12% of T-lymphocytes in the rat are capable of responding to a particular Ag-B haplotype. Dorsch and Roser (1974) found that only 3×10^7 cells (about 2% of the recirculating pool (Gowans and Knight, 1964))were able to restore skin graft rejection to a normal tempo in irradiated rat recipients. Similar work with rat cardiac allografts has shown that 5×10^8 TDL restored rejection to a first set time in irradiated recipients (Hall et al., 1978).

Although most of the cells which infiltrate the graft are the products of recent division (Gowans et al., 1962) only a minority are specifically sensitised cells (Prendergast, 1964). Surprisingly there is no dramatic homing of sensitised cells into allografts (Prendergast, 1964; Tilney and Ford, 1974; Tilney et al., 1978b). Thus, much of the cellular infiltrate seen within allografts must be non-specifically attracted.

Whether it is the T-cells which are the effectors which actually destroy the graft is not certain. Examination of the cellular infiltrate in human kidney grafts (Strom et al., 1975b) and rat cardiac allografts has revealed a remarkably heterogeneous population of cells. Approximately 40% of the cells infiltrating rejected cardiac allografts in rats were Ig^{+ve}, 14% were macrophages and the remainder were presumed to be T-cells (Tilney et al., 1975). The possible role of K cells (or at least Fc receptor +ve cells) which are 30% of the cells which are present in rejecting human renal allografts has not been established (Strom et al., 1975b).

Although there is a considerable macrophage infiltration (Nabarra and Descamps, 1976; Billingham et al., 1977), and traffic through allografts (Pedersen and Morris, 1974a; MacPherson et al., 1977) they cannot be a critical component, for Hall et al. (1978) have shown that adoptive transfer of B-cell depleted TDL alone is able to return first set heart allograft rejection time to normal, in irradiated

recipients. In contrast to delayed hypersensitivity reactions, which also require bone marrow cells, lymph node cells alone were able to transfer allograft immunity to tolerant rats (Lubaroff, 1973).

The role of antibody in allograft rejection (reviewed Carpenter et al., 1976).

The role of circulating anti-donor antibody in all but hyperacute rejection remains controversial. It has recently been demonstrated by Hall et al. (1978) that pure T-cells alone can restore the first set rejection time of cardiac allografts to normal in irradiated hosts, and that the addition of B-cells, although resulting in a plasma cell infiltrate into the graft and a humoral response did not alter the tempo of rejection. They also found that reconstitution of these animals with B-cells and a dose of T-cells, which alone was not able to restore first set graft rejection time to normal, did not result in rejection although the reconstituted hosts produced a strong humoral response to the graft. This is cogent evidence for the view that T-cells alone are sufficient to effect rejection, and that B-cells alone cannot do so. A more difficult problem is the elucidation of the role of antibody when T-cell immunity is relatively depressed as, for example, in human renal allograft recipients.

The belief in a role for humoral immunity in acute rejection is based on the following evidence:-

1. Passive transfer of antiserum results in damage to grafts and the histological appearances may be similar to those seen in acute rejection. e.g. (Straus et al., 1971; Pedersen and Morris, 1974b).

2. Deposits of IgG, IgM and complement can be identified within rejecting allografts significantly more often than in non-rejecting kidneys (McKenzie and Whittingham, 1968). In particular it is seen in the media of arterioles showing fibrinoid necrosis (Busch et al., 1971; Abbas et al., 1974b) and in the obliterative lesions of chronic rejection (reviewed Brent and Porter, 1975). After elution from rejecting kidneys the antibody can be shown to react against donor antigens (Pedersen and Morris, 1974b).

3. There is a temporal relationship between the development of vascular lesions and the appearance of antibody in the serum and lymph of sheep allograft recipients (Pedersen and Morris, 1974a). Circulating antibody can often not be detected in graft recipients while the graft is in situ but the appearance of antibody after graft removal suggests that the antibody may have been taken up by the graft (Milgrom et al., 1966; Morris et al., 1968).

4. Mononuclear cell infiltration may be scanty in acute renal allograft rejection (Porter, 1976), and the degree of mononuclear cell infiltration in biopsies taken from acutely rejecting human renal allograft does not correlate with prognosis (Herbertson et al., 1977). In fact the prominent lesion in allograft biopsies from immunosuppressed human renal allograft recipients undergoing acute rejection were found to be in the glomeruli, afferent arterioles and interlobular arteries (Kincaid-Smith, 1967).

5. Many of the vascular lesions and the glomerular lesions are associated with complement deposition, (Lindquist et al., 1971; Abbas et al., 1974b).

In summary, T-cells alone can mediate acute rejection but antibody may well contribute to tissue damage.

Rejection patterns in vivo.

Patterns of rejection in vivo have been classified according to their relationship to the time of transplantation.

1. Hyperacute rejection occurs immediately after transplantation in recipients with circulating anti-donor antibody directed against certain donor antigens (Kissmeyer-Nielsen et al., 1966; Williams et al., 1968) e.g. in man antibodies to ABO antigens

(Starzl et al., 1964) and HLA A,B, and C antigens (Patel and Terasaki, 1969) but not B-cell allo-antigens (Ettenger et al., 1976; Morris et al., 1977). The reaction of antibody with antigen on the graft endothelium results in complement fixation and secondary platelet aggregation, polymorph infiltration and vascular thrombosis.

2. Accelerated rejection. This is a second set rejection of a different type to hyperacute rejection. It is the severe form of rejection which occurs earlier than normal in individuals probably with undetectable sensitisation (Lucas et al., 1970a), and has a poor prognosis (Berne et al., 1976). Sensitisation appears to give rise to an enormous clonal expansion. Hall et al. (1977) showed that whereas 5×10^8 TDL were required to restore first set heart graft rejection to normal in irradiated rat recipients, only 5×10^6 cells were required if TDL were harvested from sensitised animals. This 100 fold amplification explains why immuno-suppression is likely to be less effective in sensitised recipients.

3. Acute rejection. Classic acute rejection in virgin recipients occurs after a short period of stable graft function. This was noted in the classic experiments of Medawar (1944) who showed that allo-grafts behaved as isografts for only 2 to 3 days

after transplantation before undergoing rejection. In unmodified experimental animals acute rejection is characterised by mononuclear cell infiltration but this is not usually the case in immunosuppressed human renal allograft recipients (Kincaid-Smith, 1967; Porter, 1976).

4. Chronic rejection occurs in two different circumstances. Firstly, it occurs when the difference between donor and host is only at minor histocompatibility loci and secondly it occurs in partly immunosuppressed individuals. It is gradual in onset and may be incomplete. Because biopsies of chronically rejecting renal allografts show a trivial cellular infiltrate and prominent vascular lesions with immunoglobulin deposition this form of rejection has been thought to be a manifestation of antibody-mediated damage (Jeannet et al., 1970).

Acute renal allograft rejection in the rat.

The morphology of acute renal allograft rejection in the rat has been most carefully dissected by Guttmann et al. (1967a)and Abbas et al. (1974a and b). Both groups demonstrated an intense cellular infiltrate into the graft reaching a maximum by day 5 and associated with a rise in the in vitro lymphocyte-mediated cytotoxicity (Strom et al., 1975a). There was, in addition, a severe vasculitis with IgG and complement deposition in the media of medium-sized arterioles and glomeruli. Glomerular cell necrosis

appeared at day 5 and progressed rapidly to almost complete necrosis by day 7. Lindquist et al. (1971) examined the glomerular lesion in rat renal allografts and showed IgG and complement deposition with polymorphonuclear leucocyte invasion. The endothelial cells were destroyed and the capillaries then occluded by platelets and fibrin. Although they demonstrated IgG deposition in the vascular and glomerular lesions, Abbas et al. (1974a and b) could not correlate this with the appearance of IgG antibodies in the serum. The experiments of Burgos et al. (1974) showed that the early antibody response detected in the serum was IgM.

My own unpublished results showed that deterioration in rat renal allograft function coincided not with the development of vascular and glomerular lesions which were most prominent at day 7, at the time of death, but the time of maximal cellular infiltration which was at day 5.

The morphological appearances of renal allograft rejection in the rat appears therefore to include "humoral" and "cellular" immune components. This consensus was the basis of certain experiments described in this thesis in which attempts to suppress both cellular and humoral immunity were made.

CHAPTER 2

NON-SPECIFIC SUPPRESSION OF THE ALLOGRAFT RESPONSE

Introduction

Problems with experimental immunosuppression

Miscellaneous immunosuppressive measures

- Irradiation
- Thymectomy
- Splenectomy
- Thoracic duct drainage
- Uraemia

Immunosuppressive drugs

- Azathioprine
- Corticosteroids
- Cyclophosphamide
- Antilymphocyte serum

INTRODUCTION

Non-specific immunosuppression is achieved by assaulting the lymphoreticular system either by ablating its components or inhibiting its function. Most methods are not specific for the lymphoid system and none are antigen-specific.

It is hazardous on both accounts. The therapeutic range for immunosuppression almost always overlaps the toxic range so that an immunosuppressive effect is seldom achieved without cost. The degree of overlap often determines the clinical usefulness of a particular agent. Systemic side effects notwithstanding, the indiscriminate suppression of immune responses compromises the host's resistance to infection.

This non-specific action has a further consequence, namely disruption of the homeostatic mechanisms controlling the immune response which may therefore be exaggerated rather depressed (Chanmougan and Schwartz, 1966; Turk et al., 1972; Lagrange et al., 1974).

The characteristics of an ideal non-specific immunosuppressive agent have been proposed by Turk (1967).

(1) There should be a large margin of safety between toxic and therapeutic doses.

(2) There should be a selective action on lymphoid cells.

(3) The agent should be directed against those cells involved in the immunological processes.

(4) It should be possible to administer the agent for a limited period.

(5) It should be active against established immune responses.

It is clear that there are no agents in current use which meet this ideal definition.

PROBLEMS WITH EXPERIMENTAL IMMUNOSUPPRESSION (reviewed by Berenbaum, 1974).

The conduct and interpretation of experiments on non-specific immunosuppression are complicated by a number of factors.

(1) The doses of agents used in many animal experiments and *in vitro* models often bear no relation to therapeutic levels.

(2) Extrapolation of data obtained in animals to humans should take into account innate differences in susceptibility to immunosuppressive agents, for example, corticosteroids (Claman, 1972) and differences in pharmacokinetics. Comparison of drug doses should be performed on the basis of amount of drug per unit surface area and not per unit weight because drug metabolism is dependent on the metabolic rate which is related to surface area and not to body weight (Freireich et al., 1966).

(3) Drug effects depend on the timing of administration in relation to antigenic challenge, the size of the dose, and the frequency and duration of treatment. Many reports describe effects of single large doses of immunosuppressive agents, whereas in human clinical practice regular small doses are administered. The experiments in this thesis have in general involved clinically applicable dose schedules.

(4) Data from human experiments are further complicated by the lack of standardisation, the underlying conditions for which the subjects were treated, and the concurrent administration of other immunosuppressive drugs.

(5) In addition to effects on the lymphoid system many immunosuppressive agents have anti-inflammatory properties.

(6) Assessment of synergism.

Many immunosuppressive regimens involve the use of more than one agent. The effect of two agents is often compared and found to be greater than the effect of either agent used alone and this increased effect is then cited as a demonstration of synergism. Berenbaum (1977) has recently demonstrated that such a conclusion is inadmissible unless an adequate dose response curve for each agent is constructed. The proof of synergism depends on demonstrating that the effect of the two agents combined, is greater than would be expected from their sum.

MISCELLANEOUS IMMUNOSUPPRESSIVE MEASURES.

Irradiation was one of the earliest non-specific immunosuppressive measures to be discovered. Dempster et al. (1950) showed that rabbit skin allograft rejection was delayed after irradiation of the recipients. It was used in early human renal allograft recipients (Hamburger et al., 1962) but was found to be unacceptably toxic and was therefore abandoned in favour of azathioprine when it became available (Hamburger et al., 1965). There has been a vogue for local graft irradiation and extracorporeal blood irradiation, e.g. (Murray et al., 1968) as part of the treatment of acute rejection but the value of both of these measures has been questioned. In a controlled trial Godfrey and Salaman (1977) showed no effect following the administration of 150 rads to the graft on alternate days, in an attempt to reverse first acute rejection episodes nor was the subsequent course of the transplant modified.

More recently a dramatic prolongation of H-2 incompatible skin allograft survival has been reported by Slavin et al. (1977) after total lymphoid irradiation(TLI) with protection of the marrow by lead shielding of the tail and skull. This affect appeared to be a consequence of a profound T-lymphocytopenia. Recent experiments have confirmed the potent effect of TLI in rats and dogs. When TLI was combined with donor bone marrow infusion, GVH did not occur and a state of specific transplantation tolerance developed (Slavin et al., 1979).

Thymectomy Although neonatal thymectomy produces profound immunosuppression (Miller, 1961), adult thymectomy alone does not. The latter has been shown to delay immunological recovery in irradiated mice (Miller, 1962) and ALS treated rats (Jeejeebhoy, 1965). Tilney and Bell (1976) showed that the survival of Ag-B incompatible cardiac allografts was prolonged from 7.4 to 14.9 days in neonatally thymectomised rats but adult thymectomy made no difference to survival. Thymectomy was performed in some human renal transplant centres but because no benefit was demonstrated the practice was abandoned (Starzl et al., 1970).

Splenectomy There has been no proven benefit from splenectomy in human renal transplantation although it may increase tolerance to azathioprine (reviewed Leader British Medical Journal, 1974). Recently there have been reports of an increased incidence of septicaemia in splenectomised renal transplant patients and more particularly children (Schröter et al., 1977; McEnery & Flanagan, 1977).

However, Fabre and Batchelor (1975a) showed that splenectomy both pre-transplant and at the time of transplant prolonged renal allograft survival in the (AS x Aug)F_1 to AS strain combination, an effect which was associated with a marked reduction in the lymphocytotoxic antibody response. Although splenectomy made no difference to survival when the homozygous August donor was used, lymphocytotoxic antibody responses were also markedly suppressed. Reconstitution of the splenectomised animals with sensitised spleen cells

but not normal spleen cells restored the ability to reject the graft suggesting that the spleen provides an essential part of the environment required for mounting an immune response to the graft. This is supported by the findings of Silber et al. (1976) who showed that renal allograft rejection was less severe in the (DA x Lewis)$F_1 \longrightarrow$DA model in splenectomised recipients given 1.5 x 10^6 isogeneic spleen cells 24 hours earlier. Unfortunately no group underwent splenectomy alone without reconstitution. No benefit from splenectomy has been demonstrated in the DA $\longrightarrow$ Lewis combination although a slight effect was apparent in the weak (DA X Lewis)$F_1 \longrightarrow$DA combination. The combination of splenectomy and a suboptimal dose of antilymphocyte serum prolonged survival very significantly in this latter combination (Evans et al., 1978).

Thoracic duct drainage This was shown to prolong skin graft survival across both weak and strong histocompatibility barriers in rats, although in the latter case the delay in rejection was only a few days (McGregor and Gowans, 1964). It has been used in human renal transplant recipients but with rather modest benefit for the trouble involved (Tilney et al., 1970; Franksson et al., 1976).

Uraemia Associated with the metabolic derangement of uraemia is a significant alteration in immunological reactivity resulting in a higher incidence of infection

in uraemic patients (Montgomerie et al., 1968). The precise reasons for the high incidence of infection have been difficult to identify because the patients studied were malnourished, poorly dialysed, and subject to a number of procedures likely to introduce infective organisms. The significance of the immunosuppressive effect of uraemia was first suggested because one of the earliest human transplant recipients survived for 5½ months with a functioning cadaver graft despite having received no immunosuppression (Hume et al., 1955). The phenomenon has been examined experimentally. Skin allograft survival is prolonged in uraemic rats (Morrison et al., 1963), rabbits (Smiddy et al., 1961) and man (Dammin et al., 1957). Kidney allograft survival was reported to be prolonged in dogs made uraemic by perfusion of their kidneys with puromycin (Mannick et al., 1960). However, in this study the control animals did not undergo a sham procedure.

Delayed hypersensitivity is suppressed in uraemic individuals (Wilson et al., 1965; Byron et al., 1976) and PHA responses were found to be reduced in the majority of dialysis patients in the studies of Morris (1978a), and McIntosh et al. (1976). Similar findings were reported by Newbury and Sanford (1971) and Elves et al. (1966) who concluded that the depression of reactivity was mediated by a dialysable factor. Birkeland (1976) on the other hand, did not find any difference between the PHA responsiveness of lymphocytes harvested before or after dialysis. In contrast to uraemic rats, uraemic humans are lymphocytopenic.

Quadracci et al. (1976) found that both B and T-cell numbers were reduced in uraemic patients. McIntosh et al. (1976) showed that although PMN phagocytosis was normal in uraemic patients the bactericidal capacity of PMN leucocytes was depressed. In the same study they were unable to show any relationship between the level of depression of immune function before transplant on subsequent graft survival. d'Apice and Morris (1974) have shown that K-cell function is depressed in uraemic individuals.

Uraemia may well be a significant factor in the outcome of renal allografting in the rat. The timing of recipient nephrectomy was found to make a difference to the survival times of DA recipients of (DA X Lewis)F_1 renal allografts (Winearls unpublished). Whereas the majority of untreated recipients which were immediately nephrectomised survived acute rejection to become long survivors, the majority of those undergoing a delayed, 7 day right nephrectomy, died. Improved survival of rat renal allografts related to longer ischaemia times of the donor kidney may also be related to uraemia (Schanzer et al., 1974; White et al., 1977). Souhami et al. (1973) found that rats made uraemic by a 5/6 nephrectomy took longer to reject cardiac allografts and that the prolongation of survival was even more marked if they were treated with 8mg/kg/day of azathioprine, a dose which given to non-uraemic animals had no effect on graft survival.

Uraemia has also been shown to alter hypersensitivity reactions in guinea pigs although similar effects were produced by making the animals ill in other ways such as ileal ligation (Kroe and Vasquez, 1967).

IMMUNOSUPPRESSIVE DRUGS

Azathioprine

Azathioprine is part of the standard immunosuppressive treatment used in human renal transplant recipients, usually at a dose of 2.5mg/kg/day. It was developed as a masked form of 6-mercaptopurine (6-MP) by Elion, Hitchings and their co-workers (Elion et al., 1961). 6-mercaptopurine is itself capable of prolonging rabbit skin allograft survival (Schwartz and Dameshek, 1960) and dog renal allograft survival (Calne, 1960). Later Calne et al. (1962) compared 6-mercaptopurine and azathioprine in a dog renal allograft model and concluded that azathioprine was as effective as 6-mercaptopurine but less toxic. The experiments were not adequately controlled and the results do not justify this conclusion. Although azathioprine appears to be more effective than 6-mercaptopurine as an inhibitor of in vitro immune reactions the distinction is not so clear in vivo (reviewed Elion, 1977). Spreafico et al. (1973) have shown that some of the differences between 6-mercaptopurine and azathioprine depend on the route of administration. Whereas azathioprine was more effective than 6-mercaptopurine when administered orally, the converse was true of intraperitoneal or intravenous administration.

Both azathioprine and 6-mercaptopurine are converted to thioinosinic acid which, because it is stereochemically similar to inosinic acid, inhibits enzymes required in purine biosynthesis. Another product of 6-mercaptopurine,

6-methylmercaptopurine ribotide inhibits an even earlier stage of purine biosynthesis. 6-mercaptopurine is also converted to 6-thioguanine ribotide which is incorporated into DNA and RNA as a fraudulent base. Although the critical biochemical locus of action is not known, both these effects inhibit cell proliferation (reviewed Berenbaum, 1975). It has been suggested that an additional immunosuppressive effect of azathioprine is its ability to inhibit T-cell recognition. (reviewed Fournier et al., 1973).

Azathioprine can inhibit lymphocyte proliferation in vitro e.g. Brown et al. (1976) but this is difficult to equate with the in vivo situation in which similar drug levels are only transiently maintained.

Because most transplant recipients receive azathioprine and prednisolone the effects of azathioprine alone have not been clearly identified in man. However, Maibach and Epstein (1965) showed that azathioprine in doses of 100mg/day hardly altered the primary antibody response to plague vaccine in healthy volunteers but the treated subjects failed to produce a secondary type response to an antigen boost. Dinitrochlorobenzone (DNCB) contact hypersensitivity could, however, be induced in these subjects. In contrast to this study is that of Swanson and Schwartz (1967) who showed that 3mg/kg of azathioprine given to patients with various "immunological" disorders delayed the primary immune response which was almost exclusively IgM. Secondary responses to diptheria toxoid were also suppressed as was the delayed hypersensitivity response to keyhole limpet haemocyanin (KLH).

Azathioprine appears to have a striking effect in depressing the number of effector K-cells required for the ADCC reaction in both man (Campbell et al., 1974) and mice (Purves and Berenbaum, 1975).

The immune responses of long-surviving transplant recipients receiving azathioprine and prednisolone have been reviewed by Morris (1978a). In summary, the evidence for depressed cell-mediated immunity in these patients is: their failure to reject the allograft; an increased incidence of viral, fungal and intracellular bacterial infections; depressed T-lymphocyte counts; and depressed skin reactions to ubiquitous antigens. Surprisingly, however, PHA responsiveness, mixed lymphocyte reactivity and the response to primary sensitisation with DNCB were normal in the majority. Thus, cell-mediated immune function is relatively but not absolutely depressed in these patients.

Humoral responses are also altered. The primary response of IgM antibody production may be normal, but secondary responses to the same antigen are depressed tending once again to be IgM rather than IgG. Thus there is a failure to develop memory. Secondary responses to antigens encountered before transplantation, for example cytomegalovirus, are normal in most patients.

Polymorphonuclear leucocyte function is qualitatively normal although some patients may develop leucopenia on standard dose regimens (Gotch et al., 1977). Clearance studies suggest that the function of the reticulo-endothelial system is not significantly altered in transplant patients

(Palmer et al., 1971). The special efficacy of azathioprine in suppressing renal allograft rejection is not yet fully explained.

In rats azathioprine has been shown to suppress rejection of kidneys allografts (Tinbergen, 1968) heart allografts (van Bekkum et al., 1969) and islet cell allografts (Marquet and Heystek, 1975b), with varying degrees of success. There are also reports showing only a trivial effect in the suppression of organ graft rejection in the rat e.g. kidney allografts (Shehadeh et al., 1970) islets (Finch, 1977) hearts (Souhami et al., 1973). Laden (1972) showed that even in doses of 48mg/kg azathioprine made no difference to skin graft rejection in the Ag-B compatible Slonaker → Wistar combination. The general impression is that it is much less effective in this species than in man and it is certainly less effective than cyclophosphamide or antilymphocyte serum. Thus, results obtained in the rat may not be particularly relevant to man.

Corticosteroids

Suppression of allograft rejection by corticosteroids was first described by Billingham et al. (1951). Zukoski et al. (1965) showed that prednisolone alone was capable of prolonging dog renal allograft survival. They are now an essential constituent of the immunosuppressive regimens used in the management of human renal transplant recipients, both as part of maintenance therapy and, in high doses, in attempts to reverse acute rejection (Hamburger et al., 1972; Bell et al., 1971).

The consequences of their glucocorticoid action, such as their effects on skeletal collagen and glucose homeostasis, and their mineralocorticoid effects on sodium balance and blood pressure, are an important cause of morbidity in human renal transplant recipients (reviewed Gray et al., 1978). Despite the widespread use of corticosteroids, most regimens are still designed empirically and the precise mechanisms of action are incompletely understood. Although the mineralocorticoid side effects can be reduced by using corticosteroids with predominantly glucocorticoid effects such as prednisolone (Berenbaum, 1975) the glucocorticoid effects can only be reduced by keeping doses as low as possible. de Andrada et al. (1964) showed that side effects were few if maintenance therapy could be kept below 10mgs/kg/day. Conversely there are a number of reports which associate the risk of infection in renal transplantations with total corticosteroid doses (Bach et al., 1973; Anderson et al., 1973). Minimum dose levels have not been established for human transplant recipients but alternate day therapy (Hamburger et al., 1972) can reduce the side effects.

Even if specific immunosuppression does not suppress rejection completely when applied clinically, it is the hope that dose requirements of steroids will be lowered. Any steroid sparing effect would reduce morbidity significantly.

Any comparisons of corticosteroid effects should always take into account differences in potency between preparations (Thorn & Lauler, 1972; Fauve & Pierce-Chase, 1967;

Bach, 1975), and animal species (Shewell and Long, 1956; Claman, 1972). Indeed it is relevant to this thesis that rats are considered steroid-sensitive and humans steroid-resistant. The effects of steroids are also dependent on rates of degradation, binding to plasma proteins, the route of administration, and the timing of administration in relation to antigenic challenge (Elliott & Sinclair, 1968; de Sousa & Fachet, 1972).

The immunosuppressive effects of corticosteroids can be approached by examining their effects on the distribution, viability and function of lymphoid cells.

Spry (1972) noted a fall in thoracic duct lymphocyte output in rats after distress or prednisolone infusion and a delay in the recovery of labelled cells in the thoracic duct after injection of ACTH. The changes could not be attributed wholly to changes in recirculation because total recovery of labelled cells was less than for untreated animals. Cohen (1972) demonstrated sequestration of T-cells in the marrow of hydrocortisone treated mice. Redistribution of lymphocytes also appears to occur in man whether after single doses of corticosteroids or during chronic treatment (reviewed Fauci, 1975).

The lymphocytolytic action of corticosteroids is most obvious in steroid-sensitive species (Shewell & Long, 1956; Claman et al., 1971). The immunosuppressive effect must depend on more than lymphoid depletion in the so called steroid-resistant species. The effect on subpopulations in

the mouse (a steroid-sensitive animal) has been summarised by Cohen (1971). Thymic helper T-cells ,committed B-cells and GVH reactive cells were found to be steroid-resistant whereas B-cell precursors and cortical thymocytes were found to be steroid-sensitive. The GVH reaction could be suppressed by treating the F_1 but not the parental animal with corticosteroids.

In man, corticosteroids can suppress lymphocyte proliferation in response to PHA in vitro (Heilman et al., 1973) and in vivo (Clarke et al., 1977). Both the sensitisation and lytic components of the CML reaction have been shown to be inhibited by steroids in vitro (Rosenberg and Lysz, 1978). However, earlier work had shown that although the presence of steroids reduced the number of effectors generated, the killing capacity of those that were generated was not reduced (Balow et al., 1977a) and in some cases it was increased (Cohen et al., 1970). Rosenau and Moon (1962) showed that effector cells were still able to recognise target cells although they were not able to effect lysis.

Humoral responses are not depressed by steroids in man (Butler, 1975) although serum IgG levels do fall. The effects on humoral responses in mice were found to be dependent on the timing of drug administration in relation to antigenic challenge. (Elliott & Sinclair, 1968). Pretreatment with corticosteroids could depress the 19S response but treatment after antigenic challenge affected the 7S response.

At least some of the effects of steroids on immune responses appear to be due to anti-inflammatory properties (Ebert & Barclay, 1952; de Sousa & Fachet, 1972; Thompson & van Furth, 1970).

The effect of steroids on allograft rejection in the rat seems to be unexpectedly weak in view of the fact that it is "steroid sensitive" (Claman, 1972). Neither Tinbergen (1968), Shehadeh et al. (1970) or Bell et al. (1971) were able to show any dramatic effect on graft survival.

In summary, the action of steroids in inhibiting the allograft response appears to be through depression of lymphocyte proliferation, altering lymphocyte recirculation, inhibiting target cell lysis and attenuating the inflammatory reaction.

Cyclophosphamide

Cyclophosphamide is an alkylating agent. It causes cross linking of complementary DNA strands thus preventing them from separating and acting as a template for the production of new DNA, thereby inhibiting cell replication (Roberts et al., 1971).

The drug is inactive until it is oxidised in the liver (Brock et al., 1971) but its products are excreted by the kidney, so toxicity can be increased in the presence of poor renal function (Mellett, 1963; Cohen et al., 1971).

Its side effects, apart from those which result from immunosuppression, include leucopenia, alopecia, cystitis, gonadal atrophy, nausea and vomiting (reviewed Lancet Editorial, 1976). In general, azathioprine has been preferred to cyclophosphamide in transplantation because of the lower incidence of side effects exhibited by the former (Currey, 1974).

Effects on the immune system

Cyclophosphamide has diverse effects on the immune system. It can suppress and exaggerate immune response depending on the timing and doses of its administration. The immunosuppressive effects result in part from inhibition of cell replication in response to antigen but resting cells are also thought to be damaged (Aisenberg, 1967).

Balow et al. (1977b)have shown that lymphocyte function in vitro may be depressed by activated cyclophosphamide. ADCC and PHA induced cytotoxicity were both reversibly suppressed by incubation of effector cells with activated cyclophosphamide. Hunninghake and Fauci (1977) have shown that alveolar macrophages have diminished effector capacity in ADCC reactions if harvested from mice treated five times with 20mg/kg of cyclophosphamide, but not after a single dose of 100mg/kg.

There is good evidence that cyclophosphamide has a predilection for eliminating B-cells. The B-dependent areas of the spleen and lymph nodes are depleted by a single

dose of cyclophosphamide in both mice and guinea pigs (Turk & Poulter, 1972a)and the proportion of ● positive cells in spleen and lymph nodes is increased in mice (Poulter & Turk, 1972). These findings have been confirmed in mice by Stockman et al. (1973). These workers also demonstrated a greater depression of lymphocyte responses to pokeweed mitogen (PWM) than phytohaemagglutinin (PHA). Antibody responses to KLH, but not the lymphocyte transformation response to KLH in vitro, was diminished by the administration of cyclophosphamide after immunisation. However, it is clear that cyclophosphamide can depress T-cell function too e.g. it prolongs skin graft survival in guinea pigs (Berenbaum, 1965), and mice (Berenbaum & Brown, 1963). Turk has suggested that the drug acts preferentially on cells which are turning over rapidly (Turk & Poulter, 1972b) and that in the resting state B-cells are more sensitive to the drug because they are turning over more rapidly. If the drug is given after immunisation this selectivity may be lost (Turk & Stone, 1963; Lagrange et al., 1974). Schwarze(1977) has shown that cyclophosphamide can inhibit helper T-cell function in mice injected with PHA and SRBCs. A reduction in antibody responses following cyclophosphamide treatment should not necessarily therefore be considered evidence for suppression of B-cells.

Depending on the test system, cyclophosphamide has been shown to depress both cellular and humoral immunity in a number of species. In man DNCB sensitisation can be prevented (Townes et al., 1972) and the response to bacterial

antigens can be abolished (Santos et al., 1964). In mice skin rejection (Berenbaum & Brown, 1963) and the antibody response to TAB (Berenbaum & Brown, 1964) can be suppressed. Currey (1971) showed that in the rat cyclophosphamide was able to suppress the development of adjuvant arthritis, and the haemolysin response to SRBC.

Cyclophosphamide also has an anti-inflammatory effect (Stevens & Willoughby, 1969) but this is more obvious in acute than chronic inflammatory responses (Arinoviche & Loewi, 1970).

Effects on immune regulation

Turk and his co-workers have shown that pretreatment of guinea pigs with a single dose of cyclophosphamide can result in increased and prolonged delayed hypersensitivity reactions to a chemical sensitiser dinitrofluorobenzene (DNFB). They considered that this effect was due to elimination of a population of "suppressor B-cells". They have also shown that cyclophosphamide could abrogate the state of reversible tolerance induced in guinea pigs with dinitrobenzenesulphonate (reviewed Turk et al., 1976).

The timing of cyclophosphamide administration appears to be critical to its effect on immune regulation. Pre-treatment appears to inhibit suppressor cell activity (Sy et al., 1977), but suppressor cells generated after antigenic stimulation may be cyclophosphamide resistant (Bonavida, 1977).

The heterogeneity of suppressor cell sensitivity to cyclophosphamide is further shown by the experiments of Ramshaw et al. (1977) who have shown that cyclophosphamide given after horse red blood cells (HRBC) immunization results in the development of "repressor" T-cells which suppress humoral immunity but not delayed hypersensitivity to the antigen. Indeed, delayed hypersensitivity was increased. Recently, Ferguson and Simmons (1978) have demonstrated that suppressor cell precursors are more sensitive to cyclophosphamide than cytotoxic cell precursors.

Lagrange et al. (1974) attributed the increase in delayed type hypersensitivity (DTH) to SRBC which follows cyclophosphamide pretreatment to suppression of a controlling humoral response. They too found that the enhancing effect was highly dependent on the timing of cyclophosphamide treatment. The complexity of the effect of cyclophosphamide on immune regulation is such that its action in a clinical situation could be either beneficial or deleterious.

The effect of cyclophosphamide on allograft rejection

Although cyclophosphamide can suppress allograft rejection in mice (Fox, 1964; Berenbaum & Brown, 1963) guinea pigs (Berenbaum, 1965) rabbits (Jones et al., 1962) and man (Fox, 1964) its clinical use has been confined to patients who are sensitive to azathioprine and to the first few days after liver transplantation. There is no reason to believe that it is superior to azathioprine but

because the side effects appear to be more troublesome, there has been no systematic comparison of the two agents. Starzl et al. (1971) used cyclophosphamide in their renal transplant programme and concluded that it was equivalent in its effect to azathioprine in its therapeutic role and effectiveness. The infection rate between the two groups was not different but alopecia was more common in patients treated with cyclophosphamide.

Reluctance to use the drug in man results in part from the earlier disappointing results in experimental dog renal transplantation (Reams, 1963; Zukoski et al., 1963). Moreover, Calne et al. (1962) showed that the combination of azathioprine and cyclophosphamide resulted in a lower mean survival rate because of marrow toxicity. Motivated by the belief that cyclophosphamide preferentially suppresses humoral immunity, Putnam et al. (1975) re-examined the use of cyclophosphamide in dog renal transplantation. They showed no effect on graft survival although the drug did reduce the incidence of arterial fibrinoid necrosis, considered a marker for humoral damage. It was no more effective than azathioprine in this respect and the use of both drugs together provided no advantage.

The use of cyclophosphamide in the rat

Cyclophosphamide is a highly effective immunosuppressant in the rat (Currey, 1971). In contrast to azathioprine the effect was obtained at doses below the LD_{50}. Skin allograft survival was shown to be prolonged by

cyclophosphamide by Silvers et al. (1967). Shehadeh et al. (1970) showed that 10mg/kg/day of cyclophosphamide resulted in normal renal function 7 days after transplantation in the (Lewis X Brown Norway) to Lewis model and Kawabe et al. (1972) showed in the same combination that a single dose of 35mg/kg on day 2 or 3 post-transplant modified acute rejection markedly. Continuous drug administration at 5mg/kg/day, although less effective in suppressing acute rejection, resulted in a greater number of long survivors. Husberg (1972) showed that cyclophosphamide was able to reduce both cell-mediated cytotoxicity and antibody mediated cytotoxicity in rat renal allograft recipients. Finch and Morris (1977) have shown that the rejection of islet cell allografts may be prevented by cyclophosphamide provided a larger than usual number of islets is transplanted.

In addition to its immunosuppressive effects, cyclophosphamide is also anti-inflammatory. Currey (1971) showed that although antilymphocyte serum was able to suppress the PPD reaction if given on both the day of sensitisation or the day of testing, cyclophosphamide was only able to abolish the response if given at the time of testing.

Brock et al. (1971) have shown that the handling of cyclophosphamide is similar in man and rats although man eliminates the drug more slowly.

Cyclophosphamide and the induction and maintenance of specific immunosuppression

This aspect is covered in detail in Chapter 6 part ii describing experiments in which cyclophosphamide was combined with passive enhancement.

Despite reports of the induction of specific immunosuppression with donor antigen and cyclophosphamide in mice (Nirmul et al., 1973) and rats (Bramis et al., 1977), the general view is that cyclophosphamide does not augment specific immunosuppression (Koene et al., 1975) and may antagonise it (Myburgh & Smit, 1972; Zola, 1975). Marquet and Heystek (1975a) have shown that it can both augment and antagonise enhancement depending on the dose used and the rat strain combination tested.

There are reports too of the administration of cyclophosphamide abrogating states of specific immunosuppression e.g. in autoenhanced mouse kidney graft recipients (Russell et al., 1978) and mouse skin graft recipients (Kilshaw et al., 1975).

Antilymphocyte Serum

This is the antiserum obtained when animals of one species are immunised with lymphoid cells or cell fractions from animals of another species (Lance et al., 1973).

Although "serum antileukocytaire" had been used in the 19th century to investigate inflammation (Metchnikoff, 1899) it was only 60 years later that the immunosuppressive

properties of ALS were carefully examined. Inderbitzin (1956) showed that it could depress tuberculin sensitivity in guinea pigs and then Waksman et al. (1961) reported that it could delay skin allograft rejection. This latter property was further investigated by the transplantation pioneer, Woodruff, who showed that ALS could delay skin allograft rejection in the rat and that the effect was increased by draining the thoracic duct of the host (Woodruff and Anderson, 1963). The immunosuppressive action was confirmed in mice (Gray et al., 1964) and in man by Monaco et al. (1967). The demonstration that ALS was capable of abrogating second set rejection (Levey & Medawar, 1966b), deleting memory (Levy & Medawar, 1966a) and delaying xenograft rejection (Monaco et al., 1966) must have reinforced the view that ALS was the most powerful and promising immunosuppressive agent available. The clinical use of ALS soon followed but the results were hard to assess and certainly not dramatic (Starzl et al., 1967).

In addition to practical aspects of ALS production such as the best antigen source, host species, immunisation schedules and purification (reviewed Lance et al., 1973), its fundamental properties have also been investigated.

The dominant immunosuppressive component of ALS is an IgG antibody (James & Medawar, 1967), the $F(ab')_2$ fraction of which is inactive (Lance et al., 1973; James, 1970). In vitro it binds to lymphocytes (Woodruff et al., 1967) and is cytotoxic in the presence of complement

(Gray et al., 1966). However, the effect of ALS depends on something other than its capacity to bind to, or lyse lymphocytes (Williams & Fabre unpublished). They produced an ALS directed against a common leucocyte antigen which had excellent cytotoxicity and binding but was less effective than a standard ALS.

Both cellular and humoral immunity are depressed by ALS but it is more effective against the former (Lance, 1968; Lance & Batchelor, 1968). Although second set skin rejection can be delayed (Levey and Medawar, 1966b) secondary humoral responses are not sensitive to ALS (Monaco et al., 1966; James, 1970).

The prevailing view of the mechanism of action of ALS is that it selectively depletes the host of thymus-derived lymphocytes belonging to the long-lived recirculating pool (reviewed Lance, 1970). This view is based on: the morphological evidence showing depletion of T-dependent areas of lymphoid organs in ALS treated animals (Lance, 1970); loss of GVH reactivity in appropriate hybrids of spleen cells from ALS treated animals (Lance, 1968); the labelling studies of Denman et al. (1968) showing a striking reduction in long-lived cells; a reduction in the number of θ positive and PHA reactive cells in ALS treated mice (Raff, 1969; Tursi et al., 1969); and the fact that the immunosuppressive effect of ALS far outlasts the duration of its presence in vivo (Lance, 1968).

The active ALS antibody is rapidly cleared from the circulation (Lance, 1969) and appears to act within the circulation rather than in the lymphoid organs (Levy & Medawar, 1967; Taub & Lance, 1968).

The way in which ALS causes depletion of the recirculating pool is uncertain though it may be by cytotoxicity or by opsonisation of the cells while in the circulation. Lymphocytes exposed to ALS in vitro and injected into syngeneic hosts fail to home to the lymph nodes but are diverted to the liver (Martin & Miller, 1967; Taub & Lance, 1968).

The effect of ALS on primary humoral responses is probably through the suppression of helper T-cells (Mitchison, 1970). Whereas ALS can suppress the response to T-dependent antigens, the humoral response to T-independent antigens is not suppressed (Barth et al., 1973) and may even be increased (Baum et al., 1969). Antibody responses to histocompatibility antigens are T-dependent, but Lance and Batchelor (1968) were able to detect a haemagglutinin response in mice treated with ALS, bearing healthy allogeneic skin grafts, suggesting that suppression of graft rejection could be achieved without suppression of this helper function.

Although ALS is immunosuppressive in man (Monaco et al., 1967) it has not been shown unequivocally to be useful in the management of human renal allograft recipients (reviewed Monaco et al., 1977). The role of ALS in human transplant practice will be definitively assessed when a reagent of defined purity and immunosuppressive potency can be prepared.

ALS is a potent immunosuppressive agent in the rat prolonging both skin (Woodruff & Anderson, 1963) and renal allograft survival (Guttmann et al., 1967b). Currey (1971) showed that in addition to its immunosuppressive effect ALS was also a potent anti-inflammatory agent in the rat.

The effect of ALS can be increased by subjecting the treated animal to thoracic duct cannulation (Woodruff & Anderson, 1963) pre-graft irradiation (Levy & Medawar, 1966a), thymectomy (Jeejeebhoy, 1965), hydrocortisone treatment (Levy & Medawar, 1966a) and some cytotoxic agents (Floersheim, 1969).

The use of ALS and cyclophosphamide is discussed in detail in Chapter 7.

CHAPTER 3

SPECIFIC SUPPRESSION OF REJECTION BY ENHANCEMENT

Specific immunosuppression.

Specific immunosuppresion may be defined as a state of wholly or partly suppressed immunological reactivity to a specific antigen. It was usually thought of as occurring in two forms - tolerance and enhancement.

Tolerance was considered as a state of unresponsiveness resulting from the deletion or irreversible paralysis of the critical lymphocyte clone, whereas enhancement was thought to be a state of unresponsiveness resulting from continuous active restraint of a potentially reactive lymphocyte clone by antibody (Fabre, 1976).

The original concept of transplantation tolerance as a central failure of the immune response due to clonal deletion as exemplified by the acceptance of incompatible skin grafts by mice exposed to donor strain tissue in utero (Billingham et al., 1953) has been modified (Brent et al., 1976). Tolerance is more complex than it was first supposed. It can be induced in adults (Shapiro et al., 1961), can be partial (Cantor & Simpson, 1975) and can be transferred by T-cells (Dorsch & Roser, 1975). Moreover Kilshaw et al. (1975) have described a state of non-reactivity which can be transferred by T-cells which has some characteristics of tolerance and others incompatible with tolerance.

Enhancement is equally complex and it is safest to think of specific immunosuppression as a spectrum of phenomena and I shall not dwell on the distinctions between the two. This thesis is concerned with enhancement, and in particular passive enhancement of rat allografts and it is on this latter subject that I shall concentrate.

Enhancement

Historical outline

The discovery of enhancement is attributed to Flexner and Jobling (1906) who described increased tumour growth after inoculation of rats with heated tumour emulsion. The phenomenon of increased tumour growth after immunisation was only called "enhancement" in 1932 by Casey. The systematic analysis of its properties was made by Kaliss and his co-workers and summarised by Kaliss (1958). Enhancement of tumour growth could be evoked by pretreatment with normal tissues as well as tumour tissue, and could be transferred by the globulin fraction of the serum of pre-treated animals. He recognised too that pretreatment could give rise to either enhanced tumour growth or immunity to the tumour. In 1954, Snell showed that enhancement in mice was related to the H-2 complex.

Enhancement of normal tissue allografts as opposed to tumours was observed in rabbits by Billingham and Sparrow (1955) who showed that intravenous administration of epidermal cells or blood delayed the rejection of a subsequent skin graft in some but not all experiments. Heslop (1966) demonstrated enhancement of rat skin allografts after pretreatment of the hosts with donor spleen cells, an effect that she was able to transfer with serum in some cases.

However, it was the demonstration of prolonged survival of renal allografts in the rat by Stuart et al. (1968) and French and Batchelor (1969) that must have stimulated the enormous volume of research into experimental

organ graft enhancement.

Definition Perhaps the least contentious of definition of enhancement is that proposed by Voisin (1971). "It is the mechanism whereby an antibody (or an immune reaction) promotes the persistence of the corresponding antigen and the integrity of the cytological structures which support it, by preventing it from inducing or undergoing (or both) immune rejection."

Enhancement is usually divided into active and passive enhancement. In active enhancement the recipient is treated with preparations containing donor histocompatibility antigens and in passive enhancement with antisera directed against these antigens. There are several studies in which both active and passive enhancement have been combined,with results suggesting that this combination may be the most potent means of inducing enhancement (Stuart et al., 1968; Myburgh & Smit, 1972; Tilney et al., 1978a). What is common to both active and passive enhancement is that prolonged allograft survival follows from the presence in the recipient at the time of grafting of antibody against donor histocompatibility antigens.

Active enhancement Although active enhancement with donor tissues has certain advantages such as automatic cover of all incompatible histocompatibility antigens, there are two major obstacles to its clinical application. Firstly, there is the problem of predicting whether antigenic pretreatment

will lead to sensitisation or to enhancement, and secondly, there is the problem of the delay required between pretreatment and performing the actual transplant. Recently, Batchelor (1978) has suggested that it should be possible to induce enhancement with histocompatibility antigens without the risk of inducing immunity.

Passive enhancement Passive enhancement of whole organ allografts requires the administration of antisera directed against donor histocompatibility antigens. The circumstances methods and outcome of passive enhancement experiments have varied greatly. The current state of knowledge is summarised as follows:

(1) The class of antibody involved.

Although the enhancing capacities of various classes of antibody have often been compared, interpretation of the experiments is complicated by the lack of precise information on the purity of the classes in the preparations, the actual quantities of specific antibody in the preparations and dose related differences in effect between classes. Interpretation is further complicated by the fact that in some cases small doses of antibody can cause enhancement whereas large doses may curtail survival e.g. of tumours (Rubinstein et al., 1974) or renal allografts (Fabre & Morris, 1974a).

IgM, IgA, IgG, and IgG_2 have all been reported to be able to enhance tumour allografts (Fuller & Winn, 1973;

Voisin et al., 1969; Kinsky et al., 1972; Takasugi & Hildemann, 1969; Rubinstein et al., 1977).

Using a mouse skin graft model Jansen et al. (1975a) showed that both IgG_1 and IgG_2 could enhance, but only IgG_2 was able to induce hyperacute rejection of established skin grafts.

Experiments in rat renal allograft enhancement have shown that enhancing antibodies are of the IgG class. IgM was shown to be ineffective by Strom et al. (1975a) and in the experiments of Hildemann and Mullen (1973) it actually curtailed survival. van Breda Vriesman et al. (1975) were, however, able to show some enhancing effect with an IgM antibody prepared from early post-transplant sera.

Thus, it seems that any class or subclass of antibody has the potential to enhance provided it binds to the specific antigen (Rubinstein et al., 1977). The point is not academic because certain subclasses of IgG bind complement more effectively than others, a difference which could be exploited to avoid producing complement-dependent antibody-mediated graft damage (Smit & Myburgh, 1974; Jansen et al., 1975a).

Species variability Passive enhancement has been most consistently demonstrated in inbred rats and mice. Because neither species suffer hyperacute damage following antibody administration, most basic research into passive enhancement

has been performed in these species. It has been demonstrated in baboon liver allograft recipients (Myburgh & Smit, 1972) rabbit kidney allograft recipients, (McDowall et al., 1973). Fabre (1978) has demonstrated a slight but definite delay in rejection in dog renal allograft recipients provided the early antibody-mediated damage was avoided. Recent attempts to induce enhancement in rhesus monkeys have yielded disappointing results with only a trivial delay in the onset of rejection (Marquet et al., 1978). Early experience in man gave equivocal results (French & Batchelor, 1972).

Organ variability There is a marked variability in the success of passive enhancement depending on the nature of the organ allograft. Using the (AS X Aug)$F_1 \longrightarrow$AS rat combination Nash et al. (1977) showed that passive enhancement resulted in indefinite survival of kidney allografts but only delayed skin graft rejection for 3 days. Intermediate results were obtained for heart and pancreatic islet allografts. In the (DA X Lewis)$F_1 \rightarrow$ DA combination Fabre and Morris (1975) have shown only a trivial prolongation of skin allograft survival with enhancing serum despite complete suppression of renal allograft rejection. In the same combination Finch and Morris (1976) have shown that pancreatic islet enhancement is significantly more difficult to achieve than kidney allograft enhancement.

These differences between organs can be attributed in part to the definition of rejection and the sensitivity of

the organ to a mild rejection response. Enhanced kidneys usually undergo a reversible rejection episode but because survival is possible even with very poor graft function, the death end point may not be reached. The end point in cardiac and islet allograft experiments is not death but a cessation of detectable function. This point is illustrated by the experiments of Nash et al. (1977) who showed that islet allografts functioned for a mean of 3.2 days, kidney allograft recipients survived for a mean of 8.8 days and heart allografts contracted for a mean of 7.9 days. In this context it is interesting that Finch and Morris (1977) showed better results in cyclophosphamide treated islet recipients if large numbers of islets were transplanted showing that rejection would not be detected if there was sufficient reserve tissue. Other indirect evidence that the immune response to islets may be similar in intensity to that to kidneys is that of Garvey et al. (1978) who showed that the same doses of ALS were required to suppress islet rejection as kidney rejection.

The difference between skin allograft and renal allograft enhancement is more difficult to explain though it may depend on the way the graft immunises the host. Wustrack et al. (1975) detected an effect of enhancing serum only when lymphatic reconstitution was delayed and vascular reconstitution was immediate. They suggested that enhancing serum was less successful in prolonging graft survival if sensitisation took place via the lymphatic route.

Skin specific alloantigens (Lance et al., 1971) are unlikely to be the main reason as many enhancing sera are raised by skin graft immunisation (Fabre & Morris, 1975). The observation that skin allografts are rejected by animals which accept a simultaneous kidney allograft (Mullen et al., 1973) suggests that there are more important explanations which allow kidneys to be enhanced but skin to be rejected. Fabre and Morris (1975) suggested that skin may have a greater density of antigen making it more susceptible to low affinity lymphocytes than kidney.

Variations across histocompatibility barriers. Although it has been beneficial in most strain combinations tested, there is a puzzling variation in the ease with which passive enhancement can be achieved depending on the histocompatibility barrier. A clear example of this difference are the models used in this thesis. Lewis kidneys survive indefinitely in DA recipients after passive enhancement but in the reverse direction rejection can be delayed only a few days (Fabre & Morris, 1974a).

Fabre and Morris (1975) suggested that Lewis rats may have clones with high affinity receptors for DA antigens but not vice versa. Recently Gallico and Mason (1978) have shown that the serological response of DA rats to Lewis antigens is almost exclusively directed at Ia-like antigens. Indeed it seems to be a non-responder to SD-like antigens. Hart and Fabre (1978) have confirmed this finding and also

shown that the Lewis response to DA is predominantly to SD-like antigens. If enhancement depends on blocking of helper cell induction by blocking Ia antigens on passenger leukocytes, then an already weak DA anti-Lewis response would presumably become undetectable. The Lewis anti-DA response may be less critically dependent on helper cell induction which would mean that enhancement would be expected to have a less powerful effect.

It has also been observed that hybrid donor organs are more easily enhanced than those of homozygote donors (French & Batchelor, 1969). This can be attributed to the reduced sensitivity of heterozygous target cells to damage by rejection mechanisms and possibly the smaller dose of immunising antigen.

Antigen specifities important in enhancement

Fabre and Morris (1974b) and Fabre and Batchelor (1975b) have demonstrated the remarkable lack of specificity of enhancing sera and have shown that cover of all incompatible specificities may not be required for enhancement. This conflicted with the finding that cover of all incompatibilities was required for optimal tumour graft enhancement (Moller, 1963).

A further controversy has arisen about the importance of Ia antigens as targets for enhancing antibody. Davies and Alkins (1974) demonstrated that red cell absorption of a rat enhancing serum removing reactivity to 'SD' antigens and

leaving reactivity to 'Ia-like' antigens did not diminish the enhancing capacity of the serum. These results were confirmed by Soulillou et al. (1976) in rat renal allografts, in heart allografts by Marquet (1978), and in mouse skin grafts by Staines et al. (1974). It was therefore suggested that the critical antigenic determinants in enhancement were the Ia-like antigens (Davies & Staines, 1976). This has now been found to be an oversimplification. Marquet (1978) found that a serum raised with pure erythrocytes could enhance and that activity was nearly completely removed by red cell absorption. Jeekel et al. (1977) found anti-SD antibodies enhanced rat kidney allografts. Gallico and Butcher (1978) have examined this question using recombinant rat strains and showed that sera directed at A region (SD), B region (Ia) or both could enhance a kidney mismatched for both subregions.

In the mouse Davis (1977) has shown that antibody to H-2 D antigens enhanced survival of H-2 D incompatible split heart allografts. What is not certain is whether such an antibody would have enhanced in the presence of an I-region difference as well. McKenzie and Henning (1977a) were unable to induce enhancement of mouse skin allograft with antibodies to H-2 K antigens whether or not an I-region incompatibility existed. Staines et al. (1977) did find a slight enhancing effect with an anti-H-2 K serum when the only incompatibility was in the K region.

Thus it seems that the I-region may be the more important if not the exclusive target for enhancing antibodies.

The effect in sensitised hosts. Fabre and Morris (1975) have shown that passive enhancement confers no benefit in the presensitised host in both the (DA X Lewis)F_1 → Lewis and DA models. In both cases sensitisation with skin grafts resulted in prolonged graft survival in spite of an early acute rejection reaction which was not influenced by the administration of enhancing serum. It is interesting that sera from the sensitised animals were themselves enhancing.

Abrogation of enhancement. The state of enhancement is difficult to abrogate. Neither adoptive transfer of sensitised lymphocytes nor parabiosis with a sensitised syngeneic partner resulted in rejection of an enhanced kidney (Bowen et al., 1974). Although Enomoto and Lucas (1973) found that splenectomy 4 days after transplantation and administration of enhancing serum prevented enhancement, Fabre and Batchelor (1975a) and Tilney and Bell (1974) found it made no difference to enhancement. Recently, Russell et al. (1978) have shown that the state of autoenhancement which develops in certain mouse kidney allograft recipients could be broken by the administration of donor antigen, BCG and cyclophosphamide. Wood et al. (1977) have recently reported rejection of long enhanced cardiac allografts in rats after

the administration of 100mg/kg of levamisole and Corynebacterium parvum 7mg i.p. which they attributed to stimulation of both lymphocyte and macrophage function.

The Mechanisms of Passive Enhancement.

There is as yet no full and clear explanation for the enhanced survival of allografts after administration of enhancing serum.

The problem is best approached by dividing enhancement into stages. The induction stage occurs immediately after transplantation and is characterised by an active but incompletely effective response to the allograft. This is followed by the maintenance stage in which the animal reaches a stable relationship with the allograft and rejection is not detectable.

The induction phase

The immune response of enhanced and non-enhanced recipients early after transplantation have been compared in a number of ways.

The cell-mediated immune response as measured by in vitro target cell lysis assays have shown that the CMI response is delayed but the amplitude of the response is similar to controls (Biesecker et al., 1973b; Burgos et al., 1974; Strom et al., 1975a;Tilney et al., 1978). Only Mullen et al. (1973) have shown any diminution in the amplitude of the CMI response.

Abbas et al. (1974a) compared the histological appearances and showed that there was the same degree of mononuclear cell infiltrate in enhanced as control rejecting grafts but the peak time of the infiltration was delayed. The cellular infiltrates extracted from enhanced and control grafts were compared and found to be similar by Tilney et al. (1977).

Some suppression of humoral immunity is apparent in most systems (Biesecker et al., 1973b; Burgos et al., 1974). Although Strom et al. (1975a)could show no difference in the lymphocytotoxic antibody response between control and enhanced rats, their immunohistological studies showed that the critical difference between the two groups was the absence of an IgG-mediated vasculitis in the enhanced group (Abbas et al., 1974b). Burgos et al. (1974) compared the IgG and IgM responses and showed that both were suppressed in enhanced animals but it was the IgM depression which was most marked.

To explain these alterations in the host response Batchelor and Welsh (1976) have proposed that enhancing antibody blocks the recognition of donor antigenic specifities by subsets of host T and B-cells.

The maintenance stage

The state of non-reactivity seen in long enhanced animals has been examined in a number of ways. Such animals accept a fresh donor type kidney allograft (Stuart et al., 1970).

Donor strain skin graft rejection is delayed (French and Batchelor, 1972) but this depends on the time after the original renal transplant that the skin graft is placed. If placed at the time of renal allografting no delay is seen but if placed at 100 days, skin graft survival is markedly prolonged (Mullen et al., 1973). Poole et al. (1976) have shown that whole leg allografts are accepted by long enhanced animals but they themselves cannot be enhanced do novo - a clear demonstration the difference between the induction and maintenance of enhancement.

Lymphocytes from enhanced animals are able to respond in GVH reactions to donor antigens (French et al., 1971; Mullen et al., 1973) and in MLC (Lucas et al., 1970b) though the response may be lower than controls (Batchelor et al., 1977a).

Although only low levels of CMI were detected by Strom et al. (1975a) Biesecker et al. (1973a) showed that the result depended on the assay used. Using a ^{51}Cr release assay only low level cytotoxicity could be detected but with a microcytotoxicity assay prolonged high levels of CMI could be found in enhanced animals.

The state of non-reactivity is not due to clonal deletion because adoptive transfer of sensitised cells or parabiosis with a sensitised partner does not result in rejection (Bowen et al., 1974). Challenge with donor lymphocytes results in a primary CMI response in enhanced rats but further challenge results in a fall in CMI and not

a secondary response (Batchelor et al., 1977a).

Although enhanced animals do produce an early humoral response this wanes to negligible levels in the maintenance stage (French and Batchelor, 1972). Challenge with donor lymphocytes results in an IgM antibody response (Burgos et al., 1974) and does not appear to cause any damage to the graft (French and Batchelor, 1972). Sequential challenges with donor cells results in a reduction in antibody response with each immunisation (Batchelor et al., 1977a).

A number of factors have been suggested to contribute to the failure of long enhanced animals to reject their allografts.

Long enhanced kidneys are more slowly rejected than fresh kidneys (Stuart et al., 1970) and not at all in the weak (DA X Lewis)$F_1 \longrightarrow$DA combination (Winearls unpublished). This suggested that the grafts may become less immunogenic by adaptation. They do however have antigenic sites available (Fine et al., 1973). Graft adaptation cannot be the major factor accounting for long survival for it does not explain the failure of long enhanced animals to reject a fresh (non-adapted) graft. A more likely explanation for the delayed rejection of long enhanced kidneys is that they are free of immunogenic passenger leucocytes (Guttmann et al., 1969; Millar & Salaman, 1974).

"Blocking factors" have been proposed as a mechanism by which cytotoxic T-cells are prevented from damaging allografts in enhanced animals. Peripheral inactivation of T-cells is an attractive explanation for the failure of sensitised cells to damage grafts (Bowen et al., 1974) or the secondary CMI response in enhanced animals challenged with donor lymphocytes to cause any damage to the graft (Burgos et al., 1974).

Stuart et al. (1971a) and Strom et al. (1975a)have demonstrated factors in sera from enhanced animals which block CMI in vitro. The results of Biesecker et al. (1973a) make these in vitro assays difficult to interpret because they found that sera from enhanced animals could block killing in a microcytotoxicity assay but not in a short-term ^{51}Cr release assay.

Hutchinson and Zola (1977) have proposed an elegant explanation by which blocking factors could work. They showed that radiolabelled antigen reactive cells (ARC) were opsonised in enhanced animal but not in rejecting rats and that sera from enhanced rats could cause opsonization of ARC in normal animals.

The failure of serum from long enhanced animals to transfer enhancement e.g. (Fabre and Morris, 1972b) has long been thought to exclude a role for blocking factors. There has been one report of successful enhancement with such sera (Tilney and Bell, 1974). This is not an adequate reason to discount blocking factors, for they are proposed

as a mechanism involved in the maintenance stage and not the induction stage.

Suppressor T-cells appear to play a role in several systems of specific immunosuppression (reviewed Steinmuller, 1978). In particular they have been shown to mediate the strain-specific state of unresponsiveness in adult mice induced by donor tissue extract, Bordatella pertussis vaccine and ALS (Kilshaw et al., 1975). However, early attempts to transfer enhancement by adoptive transfer of cells from enhanced animals to syngeneic hosts have not shown any striking effect (Fabre & Morris, 1972b;Stuart et al., 1976; Marquet, 1978). Batchelor et al. (1977b) closely imitated the transfer experiments of Kilshaw et al. (1975) by treating the recipients with ALS before transferring cells from long enhanced kidney allograft recipients. However, they used a skin graft to test the state of non-reactivity. Since skin grafts are notoriously difficult to enhance, even in the presence of a long-surviving kidney graft, this may have been too stringent a test for suppressor cells. Although no mention is made in this paper of the survival of skin grafts on the suppressor cell donors, previous work had shown prolonged skin graft survival (French & Batchelor, 1972) and leg allograft survival (Poole et al., 1976). The experiments of Bowen et al. (1974) although not intended as a search for suppressor cells are interesting in this context. They showed that after parabiosis of a sensitised syngeneic partner with a long enhanced recipient, the sensitised animal had a reduced capacity to reject a renal allograft.

Recently Hall et al. (1979) have shown that spleen T-cells from enhanced DA recipients of HO cardiac allografts suppress the ability of lymph node cells to restore the capacity of irradiated hosts to reject the specific allograft. They also showed that there was no quantitative reduction in the number of lymph node cells or thoracic duct cells, which can effect rejection, in enhanced animals.

The role of auto-anti-idiotype immunity in enhancement is yet to be resolved. Certainly, antibodies directed against alloantigen receptors can modify transplantation immune responses. (reviewed Binz and Wigzell, 1976). Although Lucas and Enomoto (1973) were able to induce modest prolongation of renal graft survival with anti-receptor antibody (ARA), Stuart et al. (1976) failed to do so. Batchelor and Welsh (1976) have also failed to prolong the survival of (Aug X AS)F_1 renal allografts in AS recipients that had been immunised with AS anti August serum. Thus the role of ARA in inducing enhancement remains controversial. Stuart et al. (1976) showed that Lewis rats treated with $LBnF_1$ spleen cells and Lewis anti BN serum responded by producing auto-anti-idiotype antibody. If renal transplantation was performed at the time when the auto-anti-idiotype antibody peaked there was little evidence of rejection functionally and in CMI assays. If transplantation was performed at the time of treatment with donor cells and alloantibody mild rejection occurred. They could not detect auto-anti-idiotype antibody in long-enhanced animals however. Thus auto-anti-idiotype antibody may be involved in the prevention of early rejection of grafts in enhanced animals but does not account for their long term depressed reactivity.

In summary it is not yet possible to reconcile the available data within a single hypothesis to explain the phenomenon of passive enhancement.

In general it seems that the administration of enhancing serum alters the initial response to the graft, allowing it to survive and giving time for the development of the maintenance phase. Batchelor (1978) proposes that antiserum prevents the generation of helper cells e.g. by opsonising passenger leucocytes, and the graft antigens alone, without these cells, cannot induce a full immune response. Instead they induce a state of non-responsiveness in the precursors of the reactive B- and T-cell clones. Certainly platelets alone were found to be unable to induce a primary immune response (Welsh et al., 1977) and under certain circumstances were able to induce a depression in the immune response (Batchelor et al., 1977). The adoptive transfer experiments of Hall et al. (1979) suggest that enhanced animals retain their full complement of alloreactive cells and so one must propose an active mechanism by which they are held in check e.g. suppressor cells. Although this hypothesis may explain the failure of long enhanced animals to produce normal CMI and antibody responses to the graft it does not explain how these animals escape the effect of adoptively transferred sensitised cells. Blocking factors frustrating the effect of cytotoxic T-cells or causing their removal (Hutchinson & Zola, 1977) remain the obvious candidates.

CHAPTER 4

PASSIVE ENHANCEMENT AND THE PROBLEM OF IMMEDIATE ANTIBODY-MEDIATED ALLOGRAFT DAMAGE

Introduction.

Possible strategies to avoid antibody-mediated damage.

The use of $F(ab')_2$ to induce enhancement

- (a) Is $F(ab')_2$ capable of inducing enhancement?
- (b) The use of $F(ab')_2$ to block antibody-mediated damage
- (c) The effect of $F(ab')_2$ on antibody responses

The use of antibodies directed against Ia-like antigens in enhancement.

INTRODUCTION

Passive enhancement of renal allografts in the rat has usually been achieved without producing any immediate damage to the grafted kidney by the donor-specific serum. This has been an advantage in that basic research into the phenomenon of passive enhancement has been possible without this restriction. It is also a weakness of the model because donor organs in higher species, including man, are unlikely to be insensitive to the effects of large doses of passively administered serum.

The resistance of the rat to immediate antibody-mediated damage is not absolute. Fabre and Morris (1974a) reported dose-dependent hyperacute damage to homozygous DA(Ag-B^4) renal allografts in Lewis (Ag-B^1) recipients after administration of hyperimmune Lewis anti-DA serum. No such damage occurred when the heterozygous (DA x Lewis)F_1 donor was used. French (1972) showed that the simultaneous administration of AS anti-August enhancing serum and xenogeneic complement resulted in immediate damage to August grafts in AS recipients. Chavez-Peon et al. (1971) showed that rat kidneys were hyperacutely rejected by mice given mouse anti-rat serum and that rats were able to reject mouse or rabbit kidneys hyperacutely if given rat anti-mouse or rat anti-rabbit serum. Guttman (1976) has demonstrated hyperacute rejection of cardiac allografts following skin graft sensitisation in some, but not all Ag-B host-donor combinations.

These experiments show that rat organ grafts are sensitive to immediate antibody-mediated damage and that rat hosts are capable of mediating it. There is no single deficient component of the hyperacute rejection process in the rat which explains the infrequent occurrence of antibody-mediated damage following serum administration. Alloantigen density may be critical as shown by the difference between heterozygous and homozygous donors in the DA to Lewis model. Rat complement may be inefficient in mediating hyperacute rejection of allografts but this is not the case with xenografts (Chavez-Peon et al., 1971). Enhancing serum undoubtedly contains complement-fixing antibodies but the specificities recognised by the sera may also be important in determining the outcome. Although hyperacute damage occurs in the DA to Lewis combination, it does not occur in the reverse direction, Lewis to DA. Hart and Fabre (1978) using quantitative absorptions have shown that DA anti-Lewis alloantisera differ from Lewis anti-DA alloantisera in that most of the antibody is directed against non-SD, Ia-like antigens. In the Lewis anti-DA sera the converse is true. Gallico and Mason (1978) have recently shown that even with a variety of immunising tissues the serological response is directed almost entirely against Ia-like antigens. This is relevant to the genesis of antibody-mediated damage because Ia-like antigens may not be the target antigen in this reaction.

In summary, there is some peculiarity in the interaction of passively administered rat alloantibody with alloantigens and rat complement which must account for the insensitivity of this species to passively administered enhancing serum.

Hyperacute rejection is the form of rejection which occurs in recipients presensitised to donor histocompatibility antigens (Kissmeyer-Nielsen et al., 1966; Williams et al., 1968; Patel and Terasaki, 1969) or in the presence of ABO incompatibility (Starzl et al., 1964). It is not certain whether this form of rejection will follow passive administration of a limited amount of antidonor serum to human renal allograft recipients, but experimental evidence from work in species other than the rat and some circumstantial evidence in man, suggests that immediate antibody-mediated damage will occur.

Damage to organ allografts by passively administered anti-donor antibody has been reported in rabbits by Klassen and Milgrom (1971), Holter et al. (1972), McDowall et al. (1973) and Sutherland et al. (1973); in dogs following intra-arterial administration of serum by Altman (1963) and Dubenhard et al., (1968) and following i.v. administration by Fabre (1978); in sheep by Pedersen and Morris (1974b); in goats by Cochrum et al. (1969); and in baboons by Smit and Myburgh (1974). Cross et al. (1974) reported the hyperacute rejection of a pair of human kidneys which had accidently been perfused with plasma containing donor-

specific anti-HLA antibodies. Curiously, there are also reports in which no damage was noted after antibody administration; in dogs by Wren et al., (1974) and Abouna et al. (1977) both of whom used polyspecific antisera; in rhesus monkeys (Marquet et al., 1978) and in a human case by Batchelor et al. (1970).

These variations notwithstanding, those involved in attempts to apply passive enhancement clinically have assumed the worst case, namely that the administration of unmodified or unselected anti-donor sera will have deleterious consequences. The clinical results of renal transplantation are now sufficiently good to make it essential that any innovation is shown not only to be potentially beneficial, but also unequivocally safe.

Possible strategies to avoid antibody-mediated damage

Hyperacute antibody-mediated damage follows the interaction of circulating allo- or xeno-antibody with allo- or xeno-antigens on the graft endothelium. Complement fixation leads to platelet aggregation and degranulation, vascular spasm, triggering of the coagulation cascade, fibrin deposition, polymorph aggregation and eventually vascular thrombosis and infarction of the graft (reviewed Porter, 1976).

Since passive enhancement also requires the inter action of antigen and antibody, this must be allowed to

occur without triggering the processes listed.
The possibilities are:

1. Establishing the state of specific immunosuppression before performing the definitive transplant. This approach has been successfully demonstrated by Tilney et al. (1978) who showed that the injection of donor antigen and antibody 10 days before transplantation prolonged the survival of subsequent heart allografts. However, circulating antibody was found to be present at the time of transplantation. In clinical practice it would be necessary to devise protocols in which it was possible to transplant at a time when antibody was no longer present in the circulation. Any approach which requires the use of donor-specific antigen prior to transplantation has limited applicability to cadaver kidney transplant practice.

2. Since complement fixation triggers the events leading to hyperacute rejection, another approach is to avoid complement fixation without affecting the induction of enhancement. The possibilities include-

(a) depleting the host of complement by, for example, the use of cobra venom factor (Cochrane et al., 1970) during the administration of the alloantiserum.

Cobra venom factor has been found to delay hyper-acute rejection of cardiac allografts in the sensitised rat (Whittum and Lindquist, 1977) but not in monkeys (Kobayashi et al., 1972). Boyer et al. (1975) have shown that it is possible to deplete dogs of complement with hypochlorite but that this also reduces the levels of coagulation factors.

(b) Forbes et al. (1977) demonstrated the critical role of platelets in rat cardiac allograft hyperacute rejection and succeeded in delaying it with an antiplatelet serum. Platelet depletion in human renal transplantation would create too great a risk to be acceptable.

(c) the use of non-complement fixing antibody preparations such as $F(ab')_2$, or succinylated or papain digested antibody (Batchelor et al., 1970; Smit and Myburgh (1974).

(d) selecting antibodies within a class which does not bind complement efficiently (Smit and Myburgh, 1974; Jansen et al., 1975a).

(e) using enhancing antibodies directed against antigens which are not targets for the hyperacute rejection reaction (Jansen et al., 1975b; McKenzie and Henning, 1978).

Two of these possibilities have been selected for study:- the use of $F(ab')_2$ and selection of antibodies directed at antigens which are not targets in the hyperacute rejection reaction.

The use of $F(ab')_2$ to induce enhancement

Pepsin cleaves the antibody molecule on the carboxy terminal side of the heavy chain disulphide bond leaving a divalent $F(ab')_2$ fragment and the remains of the Fc portion of the molecule (reviewed Porter, 1973).

Pepsin degradation substantially reduces the ability of the antibody molecule to fix complement (Taranta and Franklin, 1961). Before adopting the strategy two questions must be answered.

1. Is pure $F(ab')_2$ capable of inducing enhancement and, if so, how does its efficacy compare with whole antibody?
2. If it is effective, will it induce antibody mediated damage?

There is the remote possibility that $F(ab')_2$ might still be damaging, for Simpson et al. (1975) have shown that the $F(ab')_2$ fraction of an anti-GBM antibody is still capable of inducing nephrotoxic nephritis in guinea pigs and, moreover, $F(ab')_2$ may activate complement via the alternate pathway (Sandberg et al., 1971).

Although a number of experiments have been performed in an attempt to answer these questions, an unequivocal conclusion cannot be reached from the available data. A clear answer depends on comparing quantitatively a $F(ab')_2$ preparation of known purity and antigen-binding capacity, with undigested antibody. If the $F(ab')_2$ preparation is effective it is essential to demonstrate that the effect cannot have been caused by contaminating IgG. The minimum effective dose of whole antibody was not established in any of the reported studies. The minimum effective dose of enhancing antibody can be extremely low, for example Fabre and Morris (1973) demonstrated that 10µl of enhancing serum was sufficient to enhance a (DA X Lewis)F_1 renal allograft in a DA recipient. If the $F(ab')_2$ preparation is found to be ineffective it must be shown that digestion did not alter the antigen-binding capacity of the antibody.

(a) Is $F(ab')_2$ capable of inducing enhancement?

Organ allografts

Some experimental work has been performed in rat renal allograft enhancement by French and Batchelor (1972) and Stuart (1973). Both groups concluded that $F(ab')_2$ was able to induce enhancement but that it was less effective than whole antibody. However, in neither case were the experiments formally described and the conclusions are expressed in reviews of a general nature. Shaipanich et al. (1971) showed prolonged graft survival in the (Lewis X Brown Norway)F_1 to Lewis combination if the donor

kidney was perfused with anti-donor $F(ab')_2$ and the recipient received $F(ab')_2$ intravenously after the operation. These experiments are difficult to interpret because the purity of $F(ab')_2$ was not established, and whole IgG failed to produce any effect. Subsequent experiments using an anti-spleen cell serum and a $F(ab')_2$ prepared from it also failed to show an effect with undigested antibody (Shaipanich et al., 1973).

Schilling and Svehag (1976) showed that a $F(ab')_2$ preparation was able to enhance mouse split cardiac allografts but failed to demonstrate the purity of their preparation except to say that it had less than 1% of the cytotoxic capacity of undigested serum. The effect was highly dose dependent and the experimental design made quantitative comparison with unmodified antibody impossible.

Wren et al. (1974) showed prolonged kidney graft survival in dogs after perfusion of the donor kidney with $F(ab')_2$ and treatment of the recipients with azathioprine and prednisolone in suboptimal doses. One can draw no conclusions about the effect of $F(ab')_2$ from their study because there was no group treated with azathioprine and prednisolone alone. Abouna et al. (1973) were able to show only a very trivial effect of $F(ab')_2$ in their dog transplant model.

In 1972 Holter et al. reported that the administration of anti-donor IgG to rabbits caused immediate

allograft destruction but that the $F(ab')_2$ prepared from this IgG did not. They were, however, unable to show any effect of the $F(ab')_2$ in prolonging graft survival either. Later, this group showed that infusion of anti-donor $F(ab')_2$ before administration of IgG not only prevented the hyperacute rejection seen in animals treated with IgG alone, but also caused a modest prolongation of graft survival (Holter et al., 1973). A short report by Sutherland et al. (1973) recorded that a daily administration of $F(ab')_2$ resulted in a delay in the onset of uraemia in allografted rabbits to a mean of 15.8 days compared to 6.0 days in controls.

Myburgh and Smit (1972) found that a $F(ab')_2$ preparation from a polyspecific alloantiserum prolonged the survival of liver allografts in baboons but it was less effective than whole serum. Humoral damage to the liver allografts was noted in some animals treated with poly-specific IgG but was less frequent in those treated with $F(ab')_2$ (Smit and Myburgh, 1974).

The first attempts to secure kidney graft enhance-ment in man were reported by Batchelor et al. (1970). The full series of patients who received living related donor kidneys was reviewed later by French and Batchelor in 1972. Sera raised within the families were used and partly degraded with papain in one case and pepsin in the others. The preparations used had low but definite cytotoxic titres

and reversible renal shutdown following the $F(ab')_2$ administration was common. The subsequent course of these patients was satisfactory and they concluded that these attempts at the induction of enhancement had resulted in a reduction in non-specific immunosuppressive drug requirements. It is rather difficult to assess this study firstly because of the small number of patients involved and secondly because the excellent results of living related donor transplants make the detection of anything other than a dramatic improvement in graft survival extremely difficult.

Tumour graft enhancement with $F(ab')_2$

Despite the conflicting evidence in organ allografting, $F(ab')_2$ fragments of whole antibody are thought to be able to enhance tumour allografts in mice (Chard, 1968; Kaliss et al., 1976) and xenografts in guinea pigs (Broder and Whitehouse, 1968). The experiments of Kaliss et al. (1976) are difficult to evaluate because the amounts of antibody in the IgG and $F(ab')_2$ preparations were not standardised. The results obtained by Chard (1968) cannot be criticised, for unequivocal enhancement was obtained with a pure non-cytotoxic $F(ab')_2$ preparation. Even Fab fragments enhanced tumour growth in this model confirming earlier work (Chard et al., 1967). In this model rejection is only delayed suggesting that the antibody fragments may have had only a transient antigen blocking effect.

(b) The use of $F(ab')_2$ to block antibody-mediated damage

In vitro $F(ab')_2$ is able to block complement-dependent cell lysis by whole antibody (Whitehouse and Broder, 1968; Welsh & Creswell, 1971). Infusion of $F(ab')_2$ before whole antibody is theoretically therefore an attractive strategem for blocking antibody-mediated damage in vivo. For example, Smith et al. (1971) demonstrated that prior perfusion of autografts with $F(ab')_2$ before whole alloantibody protected the kidney from damage after re-implantation. In 1972, Kobayashi et al. demonstrated a delay in hyperacute rejection in sensitised monkeys if the kidneys were perfused with anti-donor $F(ab')_2$ but no protection was found in the pig to dog xenograft system. (Habal et al., 1972). Corman et al. (1973) tried to protect a human renal allograft from hyperacute rejection in a sensitised recipient with anti-donor $F(ab')_2$ but without success. However the blocking of hyperacute rejection in sensitised recipients is a different situation to the blocking of antibody-mediated damage following the administration of a limited amount of anti-donor antibody. It was Holter et al. (1973) who showed that the infusion of anti-donor $F(ab')_2$ before passively administered IgG could block antibody-mediated damage in rabbits and in three out of five animals graft survival was actually prolonged.

(c) <u>The effect of $F(ab')_2$ on antibody responses</u>.

Strom et al. (1975a) have suggested that the protective effect of passive enhancement is in part due to prevention of an IgG-mediated vasculitis though they were not able to demonstrate any consistent differences in the lymphocytotoxic antibody responses between controls and enhanced animals. Although the importance of suppression of the antibody respone to an allograft by enhancing serum is uncertain, comparative effects of $F(ab')_2$ and whole antibody in suppression of humoral responses are still worth considering.

Sinclair et al. (1970) showed that the ability of $F(ab')_2$ to suppress the primary humoral response and prevent immunological priming is much weaker than that of intact antibody. However, after extensive purification they were able to show that $F(ab')_2$ itself possessed definite suppressive activity i.e. the activity could not be attributed to contamination with IgG. By increasing the amount of $F(ab')_2$ used and the frequency of administration to compensate for the rapid excretion of $F(ab')_2$ described by Spiegelberg and Weigle (1965), they were able to produce greater but not equivalent suppression with the $F(ab')_2$ and concluded that rapid excretion alone could not account for the difference between whole antibody and $F(ab')_2$.

Experiments of Tao and Uhr (1966) showed that $F(ab')_2$ was less effective than IgG in suppressing the humoral response to bacteriophage 174 in guinea pigs.

By delaying administration of the antibody or $F(ab')_2$ the difference between the two preparations was accentuated. The purity of the $F(ab')_2$ preparation used in these experiments was not established. Cerottini et al. (1969) showed that despite similar binding capacities a single dose of $F(ab')_2$ was ten times less effective than IgG in suppressing anti-KLH antibody production in the rabbit but with repeated doses of $F(ab')_2$ they were able to achieve the same degree of suppression as with IgG. To exclude the problem of rapid excretion of $F(ab')_2$, Wason and Fitch (1973) investigated suppression of antibody responses in a mouse spleen cell tissue culture system and confirmed that $F(ab')_2$ was ten times less effective than IgG in suppressing the response to sheep red blood cells (SRBC). Feldmann & Diener (1972) on the other hand, found no difference in the effect of $F(ab')_2$ and IgG on the suppression of antibody responses to flagellin. It is possible that the antigen involved is critical as SRBC's are a T dependent antigen whereas flagellin is T-independent.

<u>The use of antibodies directed against Ia-like antigens in enhancement</u>.

In 1974 Davies and Alkins showed that the removal of "anti-SD" antibody from a rat enhancing serum by red cell absorption did not alter the enhancing capacity of the serum and they suggested that the target antigen for

enhancement was "Ia-like". The enhancing efficacy of similar anti-Ia-like antibodies has been confirmed in rat kidney allografts by Soulillou et al. (1976); in mouse skin grafts by Staines et al. (1974), Jansen et al. (1975b)and McKenzie and Henning (1977b). Jansen et al. (1975b)showed that the enhancing serum from which the SD antibodies had been removed by absorption not only had undiminished enhancing capacity but, even in high doses, was unable to mediate hyperacute rejection of established skin grafts when administered with rabbit complement. These findings have been confirmed by McKenzie and Henning (1978). These experiments suggested that the targets for enhancement and antibody-mediated damage were not the same and that by separating the relevant antibodies from the serum it would be possible to induce enhancement safely. Further support for such a strategy came from the report of Ettenger et al. (1976) who showed that in human cadaver renal transplantation, a positive crossmatch against donor B cells did not result in hyperacute rejection provided the cross-match against donor T-cells was negative. Similar results were reported by Morris et al. (1977). Although some of these antibodies were auto-anti-B-cell antibodies many were true allo-antibodies and these patients did at least as well as those who had a completely negative cross-match (Ting and Morris, 1977). It was felt that because Ia antigens were not found to be expressed on mouse kidney (David et al., 1973; Hämmerling et al., 1975) it

would be reasonable to use anti-Ia-like antibodies in an attempt to induce passive enhancement in human clinical transplantation. The presence of Ia-like antigens on the target organ for enhancement may not even be relevant to the avoidance of antibody-mediated damage for Jansen et al. (1975b) showed that Ia-like antibodies did not damage skin grafts although skin does express Ia antigens (Hammerling et al., 1975; Klaveskog et al., 1977). Other organs may of course behave differently depending on the cells on which the antigen is expressed and the concentration on the cell surface.

The situation has become more complicated by more recent work. Davies and Butcher (1978) and Hart and Fabre (1978) have shown that Ia-like antigens are expressed on rat kidneys in substantial amounts but neither group has established which cells express the antigen. The ability of kidney homogenates to absorb anti-Ia-like antibody is not due to passenger leucocyte contamination. Whether human kidney expresses Ia-like antigen has yet to be determined. Jeekel et al. (1977) have shown that SD-like antibodies enhanced cardiac allografts and that red cell absorption of enhancing serum resulted in the loss of enhancing capacity. Gallico and Butcher (1978) have now shown that antibodies directed at both A and B regions of the rat MHC are able to enhance kidney allografts. Lastly, Marquet et al. (1978) have shown only trivial enhancement of kidney allografts in rhesus monkeys with an anti-Ia-like serum, despite the additional administration of suboptimal

doses of azathioprine and prednisolone. However, they were unable to demonstrate accelerated rejection with an anti-SD IgG preparation.

Although Ia antigens have yet to be demonstrated on human kidney, the sensitivity of rat renal allografts to hyperacute rejection with high doses of anti-Ia-like antibody has been investigated in this thesis.

CHAPTER 5

MATERIALS AND METHODS

1. Experimental animals
2. Renal Transplantation
3. Preparation of alloantisera
4. Biopsy and histological assessment
5. Lymphocytotoxic antibody titrations
6. Binding Assay
7. Polyacrylamide gel electrophoresis
8. Statistical methods

1. Experimental Animals

The rats used in three experiments were inbred DA, Ag-B^4 or H-1^a, Lewis Ag-B^1 or H-1^1 and (DA X Lewis)F_1 hybrids.

The 5 original breeding pairs of DA rats were obtained from the Dunn School of Pathology, South Parks Road, Oxford and were the 7th generation of inbreeding in that institution. The 3 original Lewis breeding pairs were obtained from St. Mary's Hospital, Paddington, London and were the 10th generation of inbreeding in that institution. The F_1 hybrids were raised by crossing DA females with Lewis males.

The rats were bred and maintained in the Radcliffe Infirmary Animal House.

Later, a commercial firm of breeders, Banton and Kingman, Hull, England were contracted to breed specific pathogen free (SPF) Lewis and DA rats. Their colonies were started with pairs from the Radcliffe Infirmary Animal House.

There was a marked variation in the state of health of the rats. A common problem was the development of pneumonia after stress. The SPF rats were not used as recipients in experiments in which survival times were compared.

Male rats were used throughout for transplant experiments. When there was a shortage of male rats females were used as spleen or target cell donors.

2. Method of renal transplantation

The method used was a modification of that described by Fabre et al. (1971) and required an assistant and a binocular operating microscope. (Zeiss OPMi 11 Operating microscope).

(a) Anaesthesia

The donor and recipient each received 0.7mls chloral hydrate solution (5% w/v) i.p. followed 5 minutes later by 0.4mls atropine sulphate (0.1 mg/ml) subcutaneously. The latter drug prevents the excessive bronchial secretion provoked by the ether which was administered as the main anaesthetic using a nose-cone. The safety of the anaesthetic was increased by keeping the animals warm with a heated operating board.

(b) Procedure

The left renal vessels in both donor and recipient were isolated, cleaned of fat and small branches tied with 7/0 silk. Scoville-Lewis clamps (10mm x 1mm) were applied to the recipient artery and vein and,after cutting the ureter near the renal pelvis,the kidney was removed. The donor was then heparinised by administering 1ml of normal saline containing 200 units/ml heparin

intravenously. Two minutes later the aorta was clamped and after the kidney had blanched it was removed and placed in a petri-dish of normal saline to remove excess donor blood.

The arteries were anastomosed using 8 to 10 interrupted 10/0 nylon sutures (Ethicon 10/0 on a 6mm round bodied needle). The vein was anastomosed second and required 12 to 14 sutures. To avoid stitching the back wall to the front wall the field was regularly flushed with saline which floated the vein walls apart.

Ischaemia times ranged from 20 to 35 minutes.

Initially immediate bilateral nephrectomy was performed and I attempted to make a urine-tight ureteric anastomosis over a small length of polythene cannula (external diameter 0.5mm; internal diameter 0.2mm, Dural Plastics, Australia). This technique had a significant failure rate from urine leakage and hydronephrosis and was later replaced by a 4 stitch, one at each quadrant, anastomosis. If this was done the recipient right nephrectomy was delayed until 7 days after the transplant. This technique was associated with a minimal failure rate. I have termed these the immediate and delayed nephrectomy models respectively. In the group of experiments involving hyperacute rejection, assessment of immediate graft function was necessary and therefore an immediate urine-tight ureteric anastomosis was performed using special 10/0 nylon with a delicate 4mm-3/8 needle. (S + T "Vascular"

by Acland supplied by John Weiss, London, England). This required 6 to 8 sutures and was more difficult than the 4 quadrant anastomosis and was only used in the appropriate experiments.

The kidney was tethered to the posterior abdominal wall and the abdomen closed with 4/0 chromic catgut for the muscle layer and 4/0 silk to the skin.

One hundred thousand units of benzylpenicillin were administered i.m. at the end of the operation.

The delayed right nephrectomy was performed on the seventh day after transplantation through the same abdominal incision as the transplant. In the immediate nephrectomy experiments the right nephrectomy was performed immediately after completion of the transplant anastomoses.

The outcome following transplantation was assessed using the following parameters: animal survival, blood urea levels, histological changes in graft renal biopsies, and lymphocytotoxic antibody titres. Survival beyond 20 weeks was considered indefinite and no attempt was made to discriminate beyond this period.

Blood samples were taken for blood urea and lymphocytotoxic antibody titres at weeks 1, 2, 3, 4, 6, 8, 12, 20. When delayed nephrectomy was performed, the first blood urea estimation was performed at day 10 post-transplant.

(c) Blood urea estimation (Henry, 1964)

Blood ureas were measured using urease and the Berthelot reaction. Urease splits urea into ammonia and carbon dioxide. The ammonia can be determined photometrically by the phenol-hypochlorite reaction of Berthelot. Twenty µl of sample or urea standard were added to 200µl of buffered urease solution (150mg urease and 1.0 gm EDTA/100ml aqueous solution adjusted to pH 6.5 with 1M NaOH). The samples were then incubated at 50°C for 5 minutes. One ml of phenol colour reagent was then added and mixed and immediately followed by the addition of 1ml alkaline hypochlorite reagent and mixed and incubated for 5 mins at 50°C. Eight mls of distilled water were then added to each test tube.

The absorbance was then read on a Unicam SP 500 Series 2 spectrophotometer at 630mµ. Samples were then compared to a linear curve using urea standards of 25mg, 50mg and 100mg/ml. Concentrated samples were diluted before reading.

(d) Isografts

A set of 5 Lewis ⟶ Lewis isografts was performed and the results are shown in Appendix 1.
The position of the kidney after completion is shown in the photograph (Fig. i) at the end of the Chapter.

3. Preparation of enhancing alloantisera

These were prepared using the basic method of Fabre and Morris (1975).

Spleens were removed from freshly killed donor strain animals and most of the fat and connective tissue removed. The spleens were then finely minced with scizzors and then pressed through a fine sieve with a spatula. The spleen cells were then suspended in normal saline and centrifuged at 900g for ten minutes. After tipping off the supernatant the cells were resuspended and allowed to stand for three minutes to allow coarse aggregates to settle. The cells still in suspension were then removed, centrifuged at 900g and then made up to 0.5mls/spleen. If Freund's complete adjuvant (FCA) was to be used, an equal volume was added to the spleen cell suspension and the mixture shaken until a pale thick emulsion was obtained. When FCA was not used an equal volume of normal saline was added. The Freund's complete adjuvant was Lot 25 obtained from Miles Research Laboratories, USA.

Under ether anaesthetic the host strain rats were immunised by injection with 0.1mls of the spleen cell suspension into each footpad. This immunisation was equivalent to 1/5 spleen/rat. The immunization schedules for each serum are given below.

In certain sera, immunizations were performed by injecting the spleen cell suspension i.p. For one serum, OAS-56 the spleen cells were supplemented with cervical lymph nodes.

After bleeding the sera were separated and stored at -40°C. Bleeds were pooled before aliquotting.

The sera used in the experiments were:

1. OAS-53 Lewis anti-DA
2. OAS-55 Lewis anti-DA
3. OAS-56 Lewis anti-DA
4. OAS-57 Lewis anti-DA
5. OAS-58 DA anti-Lewis
6. OAS-59 Lewis anti-DA

1. <u>OAS-53</u> Lewis anti-DA

Week 0: 20 Lewis male rats immunised with 1/5 DA spleen in FCA/rat.

6: Boosted with 1/5 spleen in normal saline/rat.

9: Boosted with - as for week 6.

10: Rats bled out by aortic puncture.

The lymphocytotoxic antibody titre in a one-stage ^{51}Cr release assay was 1/64

2. <u>OAS-55</u> Lewis anti-DA

Identical protocol to OAS-53

The lymphocytotoxic antibody titre in a one-stage ^{51}Cr release assay was 1/256

3. OAS-56 Lewis anti-DA (prepared by Miss J. McKenzie)

Week 0: 30 Lewis rats injected i.p. with 1/5 DA spleen in 1ml normal saline/rat.

2: Boosted with 1/5 spleen and 1/5 cervical nodes from a DA rat in 1ml normal saline i.p.

4: as for week 2.

5: bled by aortic puncture.

The lymphocytotoxic antibody titre in a two-stage ^{51}Cr release assay was 1/128.

4. OAS-57 Lewis anti-DA

Week 0: 30 Lewis rats were immunised with 1/5 DA spleen in FCA/rat.

3: Boosted with 1/5 DA spleen in normal saline/rat.

6: as for week 3.

7: Cardiac puncture of 4mls/rat.

8-9: 3 further cardiac punctures/rat and then bled out by aortic puncture.

The lymphocytotoxic antibody titre in a two-stage ^{51}Cr release assay was 1/256.

5. OAS-58 DA anti-Lewis

Week 0: 18 DA rats injected 1/5 Lewis spleen in FCA/rat.

3: Boosted with 1/5 spleen in normal saline/rat.

7: Boosted with 1/5 spleen in normal saline/rat.

8: Cardiac puncture of 4mls/rat.

9: Cardiac puncture of 4mls/rat.

10: Cardiac puncture of 4mls/rat.

11: Boosted i.p. 1/5 Lewis spleen/rat (14 rats surviving).

12: Boosted 1/5 Lewis spleen/rat.

13: Cardiac puncture of 4mls/rat.

14: Cardiac puncture of 4mls/rat.

14 + 3 days: Bled out by aortic puncture.

The lymphocytotoxic antibody titre in a two-stage ^{51}Cr release assay was 1/10.

6. <u>OAS-59</u> Lewis anti-DA

Week 0: 30 Lewis rats injected with 1/5 spleen in FCA/rat.

4: Boosted with 1/5 spleen in normal saline/rat.

7: Boosted with 1/5 spleen in normal saline/rat.

8: Cardiac puncture of 4mls/rat.

8 + 4 days: Cardiac puncture of 4mls/rat.

9: Bled out by aortic puncture.

The lymphocytotoxic antibody titre in two-stage ^{51}Cr release assay was 1/256.

4. Method of Biopsy and Histological Assessment

Further information about the effects of various treatment schedules was obtained by examination of tissue from the allografts. Initially I attempted to compare groups by examining post-mortem specimens. This had several disadvantages:-

1. The number of animals which died, and the interval between the time of transplantation and death varied from group to group making comparisons unreliable.
2. Knowledge of the treatment administered made observer bias probable.
3. My own experience of the histopathology of the allograft rejection was limited.
4. Post-mortem autolysis impaired the quality of the specimens.

To avoid these disadvantages the following method was adopted:

1. Fresh renal allograft tissue was obtained at a fixed time after allografting. Routine biopsies were taken at the time of 7 day right nephrectomy. In certain experiments additional biopsies were taken at a predetermined interval after transplantation. Post-mortem samples were also processed and assessed.

2. Cortical renal biopsies were taken using a Tru-cut biopsy needle laid on the surface of the kidney after stripping the capsule at the site selected for biopsy. The biopsy site was then sealed with a small piece of absorbable gelatin sponge (Sterispon No. 1, Allen and Hanburys Ltd., England). Haemorrhage was never a problem using this technique.

3. The samples were fixed in 10% w/v formalin solution, processed and embedded in paraffin wax and 4μ sections were stained with haematoxylin and eosin (H & E) by the departmental histology technician.

4. The sections were then examined "blind" by a histopathologist with a special interest in transplant pathology, Dr. P.R. Millard. The biopsies were found to be of good quality and contained 2-12 glomeruli per section.

Changes were quantitated purely subjectively and scored 0 to 4+ for the following features:-

1. <u>Mononuclear cell and polymorphonuclear leucocyte infiltrate</u> scoring was based on an impression of the number of cells infiltrating the entire specimen irrespective of their exact distribution.

2. <u>Oedema</u> was assessed by the separation of tubules and the scoring was based on the extent not the degree.

3. Haemorrhage was assessed according to extent. For both oedema and haemorrhage, a 4+ score was given if the whole section was involved and 0 if there was no involvement. Intermediate degrees were scored 1+ 2+ or 3+.

4. Fibrinoid necrosis was always patchy within any affected artery and so the scoring reflected the number of vessels involved.

5. Subintimal mononuclear cell infiltrate was scored according to the number of vessels involved.

6. Glomerular capillary necrosis was scored by the number of glomeruli involved and not the extent.

7. Tubular necrosis and atrophy were scored by the number of tubules involved and not according to severity.

After the biopsies had been scored, the number of +'s for each specimen were added together giving a histological score. The scores for various groups of animals and various features were then compared.

These features were considered the most reliable evidence of rejection. Herbertson et al. (1977) have shown that interstitial haemorrhage, medial necrosis of arteries, acute glomerular lesions and subintimal mononuclear cell infiltration are reliable prognostic features in human allograft biopsies. Mononuclear cells and oedema have been regarded as evidence of tissue rejection in all types of organ grafts since the classic experiments of Medawar (1944).

A set of 5 Lewis ⟶ Lewis isografts was performed. Biopsies were taken at day 7 and day 14. The results are shown in Appendix 2.

5. Lymphocytotoxic antibody titrations

A complement dependent microlymphocytotoxicity Chromium-51 (^{51}Cr) release assay described by Goodman (1961) and Welsh and Creswell (1971) was used.

Target cells

Peripheral blood lymphocytes

Blood was taken by aortic puncture and defibrinated for ten minutes using glass beads. The blood was then diluted 1:2 with normal saline and underlaid with Triosil-Ficoll (SG = 1.077) and centrifuged at 700g for 20 minutes (Boyum, 1974). The cells were washed by diluting the cells removed from the interface in saline and centrifugation at 225g for 5 minutes. Red cell contamination was reduced by incubating the cells in ACT for 10 minutes at 37°C after which they were centrifuged at 60g for 5 minutes (Boyle, 1968). The cells were then washed in medium and resuspended in 1ml medium. Two hundred μCi ^{51}Cr (obtained from the Radiochemical Centre, Amersham, England) was then added and the cells incubated for 2 hours at 37°C. After labelling, the cells were centrifuged at 225g for 5 minutes over foetal calf serum, resuspended in 10mls of medium and allowed to stand at 4°C

for 20 minutes to allow diffusion of non-bound ^{51}Cr. After two washes the cells were counted and adjusted to 2×10^6/ml.

Red Cells

Chromium-51 labelled red cells were prepared as follows. Heparinised blood was washed twice with normal saline and the buffy coat removed. One hundred µl of washed, packed red cells were added to 900µl of medium and 200 µCi of ^{51}Cr and incubated at 37°C for 2 hours. The red cells were then washed in the same way as peripheral blood lymphocytes and adjusted to a concentration of 5×10^7/ml.

Spleen cells and thymocytes

These were prepared by gently teasing the organs apart in medium with two No.19 gauge hypodermic needles. The cell suspension was strained through cotton wool to remove debris and then left to stand for 2 - 5 minutes to allow lumps to settle. The cells were then washed once with medium and then centrifuged over Triosil-Ficoll (SG = 1.077). In this and all subsequent steps their preparation was identical to that used for peripheral blood lymphocytes.

Media (All obtained from Gibco Bio-cult, Middlesex, Ltd)

In some experiments RPMl 1640 buffered with Hepes and supplemented with 10% foetal calf serum (FCS) was used. Because cell viability varied from test to test I changed to using Hanks Balanced Salt Solution buffered with sodium bicarbonate and supplemented with 10% foetal calf serum. This too gave rise to the occasional technical failure and so Medium 199 with 10% FCS was eventually selected.

Complement

A large batch of guinea-pig serum was obtained from Tissue Culture Services, Slough, England, thawed and aliquotted into 2ml amounts and stored at -80°C. The serum was toxic to rat red cells but not lymphocytes. When red cell targets were used, the complement was absorbed with red cells as follows: heparinised rat blood taken by aortic puncture was centrifuged at 225g for 10 minutes and the plasma and buffy coat removed. The red cells were washed twice by resuspension in saline, centrifugation and removal of the buffy coat. The packed cells were then aliquotted and stored at 4°C. Using cooled glassware, 2mls of the guinea-pig complement was added to 0.66mls packed red cells, mixed and allowed to stand for 15 minutes at 4°C, then centrifuged at 250g at 4°C for ten minutes. The absorption procedure was performed three times.

Sera

Sera for testing, either post-graft samples or hyperimmune enhancing sera were diluted in Cooke microtitre plates using medium, saline or heat-inactivated foetal calf serum as diluent.

Assay

The assay was performed in Terasaki microtest plates (Falcon Plastics), humidified with wet filter paper strips. Duplicate 2µl aliquots of serum or dilution were added to the wells using a Hamilton syringe. 2µl of the target cells were added and the plates incubated for 30 minutes at 37°C after which 5µl guinea-pig complement was added. After a further 60 minutes incubation the plates were centrifuged at 250g at 4°C and 5µl of supernatant removed from each well using a Lang-Levey pipette. The samples were then counted on a Hewlett Packard Autogamma scintillation spectometer. The counting time was calculated as follows:-

6 samples containing 2µl of the labelled cells were counted until they reached 1000 counts. The mean counting time was then adjusted so that 1000 counts were equivalent to 100% cell death, allowing for the fraction of the supernatant harvested (5/9), the amount of ^{51}Cr released by dead cells (approximately 75%) (Goodman, 1961).

Maximum release (M) was determined using a known positive alloantiserum. The negative control (C) was

complement added to normal non-immune rat serum. If C, the complement background was greater than 25% the test was repeated.

Specific cytotoxicity was calculated as follows:-

$$\text{Specific cytotoxicity} = \frac{E - C}{M - C} \times \frac{100}{1}$$

where E = Experimental ^{51}Cr release

C = Complement background release

M = Maximum release

The antibody titre for post-graft sera was the last dilution to give 20% or greater specific cytotoxicity.

In the early experiments a one-stage test was performed. Complement was added immediately after the addition of cells and the incubation period was 30 minutes. Because this was found to be a relatively insensitive method (Sanderson, 1965) the two stage test was adopted.

For the hyperimmune enhancing sera the titre was the last dilution to give $>50\%$ cell death.

6. Indirect antibody-binding assay

The assay used in these experiments was the indirect antibody-binding assay developed by Dr. A.F. Williams (Morris and Williams, 1975). It involves the binding of ^{125}I-labelled anti-Ig antibodies to cells previously incubated with a test antiserum. The assays described in

this thesis were strength assays in which targets were in excess, the test serum was limiting and the second antibody was saturating.

Target Cells

(a) Lymph node lymphocytes

An animal of the appropriate strain was bled out by aortic puncture. Mesenteric and cervical lymph nodes were then dissected and placed in a petri-dish of (Dulbecco's A and B solution) DAB at room temperature. The nodes were then minced and pressed through a fine sieve. The cells were then filtered through fine cotton-wool to remove clumps and debris and then washed by suspending them in 0.5% BSA/DAB and centrifugation at 400g for 10 minutes at 20°C. The cells were then incubated in ACT for 10 minutes at 37°C, washed and again gently filtered through cotton-wool, washed and centrifuged at 4°C. The cells were then resuspended, counted and their viability checked with trypan blue. Viability was always $>70\%$. After a final centrifugation at 400g for 10 minutes the cells were then resuspended in 0.5% BSA/DAB at a concentration of 1×10^8/ml.

(b) Red Cells

Blood was taken by aortic puncture into a heparinised syringe, diluted 4 x with DAB and spun at 400g for ten minutes at 20°C. The plasma and the buffy coat were removed and the cells diluted in twice their own volume in DAB

and spun over Triosil-Ficoll (SG = 1.090) at 1700g for 15 minutes at 20°C. The cells were then resuspended in DAB and centrifuged at 400g for 10 minutes at 4°C. They were then resuspended, counted, centrifuged and resuspended in DAB at a concentration of 10^9ml.

Test Sera

These were always, heat-inactivated and then microfuged for 5 minutes at 4°C. Dilutions of 1/10, 1/20, 1/40, 1/80, 1/160 were made in 0.5% BSA/DAB. 25µl of neat test serum, IgG or $F(ab')_2$ and the respective dilutions were added to LP3 tubes (Luckham Ltd. Sussex, England) on ice.

Second Antibody

The second antibody was an affinity column purified rabbit $F(ab')_2$ anti-rat $F(ab')_2$ (RAR) which had been prepared by Dr John Fabre and Dr D.N.J. Hart. RAR was made up to a concentration of 25µg/ml and then 1/100th volume of ^{125}I labelled RAR at 12µg/ml were added. One hundred µl of the trace-labelled RAR was counted for 1 minute to establish the cpm = 2.5µg RAR bound. This allowed different assays to be compared.

Assay (performed at 4°C throughout)

25µl cells were added to each dilution of test antibody using a Hamilton syringe. The cells and antibody were then incubated for 60 minutes with a thorough shake

at 30 minutes. Unbound antibody was removed by washing as follows. After the addition of 1.6mls of 0.1% BSA/DAB the tubes were centrifuged at 1000g for 10 minutes and the supernatant removed. A further 1.6mls of 0.1% BSA/DAB was added and the cells resuspended by vortexing. After centrifugation and removal of the supernatant 100µl of the RAR was then added to the cell button and incubated for 60 minutes with a 30 minute shake. Unbound antibody was then removed by the same washing procedures as for the first antibody. The cell buttons were then resuspended in 0.6mls DAB and transferred to fresh tubes for counting. Each tube was counted for 1 minute on a Hewlett-Packard Autogamma scintillation spectrometer.

Expression of results

The results are expressed either as cpm against serum dilution or ng RAR bound vs serum dilution. The latter method was used when different assays were plotted on the same graph.

7. Polyacrylamide gel electrophoresis

The method of Fairbanks et al. (1971) was used to examine the $F(ab')_2$ preparations for IgG contamination.

20mls of 5.6% gels were prepared by mixing:-

2.8mls solution A (40g acrylamide; 1.5g bisacrylamide; made up to 100mls with distilled H_2O, filtered and stored in dark bottle at 4^oC).

2.0mls solution B (40mls 1MTris; 10mls 2M Na acetate; 10mls 0.2M Na EDTA; adjusted to pH 7.4 with glacial acetic acid made up to 100mls and stored at 4°C in a dark bottle).

1.0mls solution C (20% w/v sodium dodecylsulphate, (SDS) stored in a dark bottle at 4°C).

12.2mls distilled water

2.0mls solution D (1.5% w/v ammonium persulphate, made up fresh each week. Stored in dark bottle at 4°C).

After de-aeration for 30 minutes 5µl 0.5% v/v Temed (freshly made) was added. The gel was then poured into glass tubes (9.5cms long, internal diameter 0.5cms and external diameter 0.7cms) to a height of 8.5cms. The tubes had been sealed with parafilm and stood in rubber bungs. Distilled water was gently layered on to the surface of the gels to flatten them after which they were allowed to set overnight. The gels were then placed in the electrophoresis chamber filled with buffer (100mls solution B; 10mls solution C; 890mls H_2O).

Samples were prepared for electrophoresis as follows:

(a) <u>without reduction</u>

20µl of sample at 1µg/µl was added to 20µl solution 1 (8M urea; 2% SDS; 0.004M sodium iodiacetamide; made up to 10mls with distilled H_2O).

(b) <u>with reduction</u>

20µl sample at 1µg/µl was added to 20µl solution 2. (8M urea; 2% SDS; 2% Dithiothreitol DTT).

In both cases the mixtures were placed in glass tubes, sealed with tin-foil and suspended in boiling water for 2 minutes. After boiling, 10µls of a solution of, 100µl 0.2% bromphenol blue and 100µl 1.5M sodium iodoacetamide were added to each sample.

The samples were then loaded on to the gel surface using a 50µl microcap.

The samples were then electrophoresed for 2-3 hours until the dye reached the bottom of the gel, using a current of 4ma/tube.

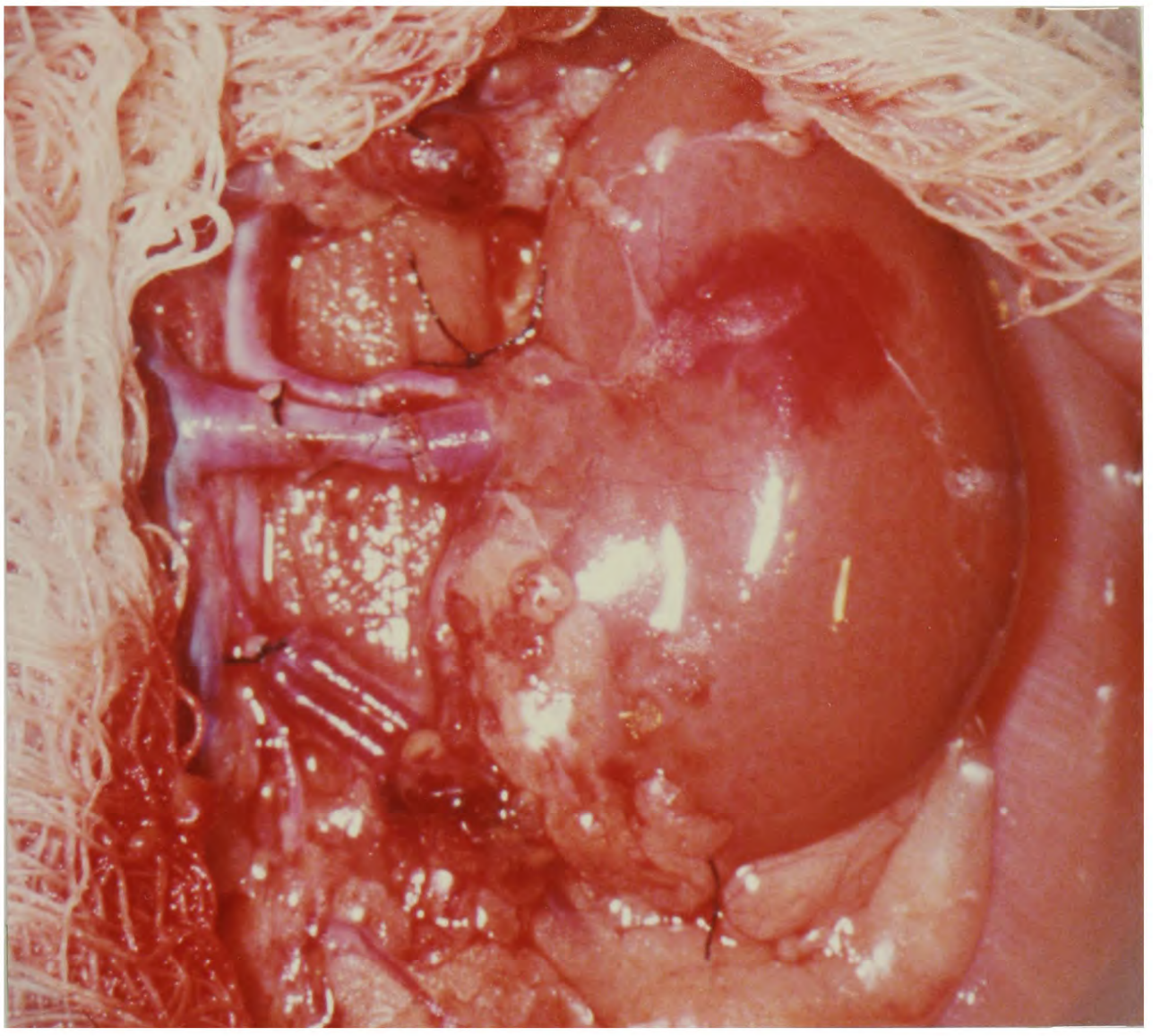
The gels were then removed from the glass tubes and stained for 2 hours in a straining solution (1gm Coomassie brilliant blue; 182mls methanol, 182mls distilled water, 36.8mls glacial acetic acid; filtered before use).

The gels were then destained for 30 minutes in a Shandon Southern transverse gel rod destainer Mk III. using 7% acetic acid and a current of 900ma.

8. Statistical methods

Groups were usually compared using a two-tailed Wilcoxon rank sum test (Wilcoxon, 1945). The sums were compared using tables in Documenta Geigy. In certain experiments where a two-tailed Fishers exact test was more appropriate, it was used instead of the rank sum test (Fisher, 1934). Differences were considered significant if $p \leq 0.05$.

Fig.1



Renal isograft after completion of the anastomoses.(×5)

CHAPTER 6

EXPERIMENTS ON THE USE OF NON-SPECIFIC IMMUNOSUPPRESSIVE AGENTS WITH PASSIVE ENHANCEMENT

General Introduction

(i) Azathioprine and prednisolone

(ii) Cyclophosphamide

(iii) Antilymphocyte serum

GENERAL INTRODUCTION

Because human clinical transplantation involves an "outbred" population, donors and recipients differ at both major and minor histocompatibility loci. The expectation is that passive enhancement alone will therefore be insufficient to control rejection and that non-specific immunosuppressive agents will also be required.

The aim of these experiments was to identify agents, firstly which could be used to potentiate, and secondly which might antagonise, the effect of enhancement.

In certain strain combinations indefinite survival of rat renal allografts can be produced across the major histocompatibility barrier by passive enhancement e.g. (French & Batchelor, 1969; Strom et al., 1975a;Fabre & Morris, 1973). However the (DA X Lewis)F_1 to Lewis is a particularly strong combination in which even with hybrid donors the administration of enhancing serum results only in a delay in rejection (Fabre & Morris, 1972a). This model was chosen because the effect of passive enhancement is always restricted. No matter what dose schedule is employed all animals undergo delayed but severe rejection.

The agents studied were: azathioprine, prednisolone, cyclophosphamide and antilymphocyte serum. The last agent has previously been shown to potentiate passive enhancement in this model by Batchelor et al. (1972).

The experiments will be described in the order in which they were performed. Changes in the experimental approach were made as more experience with the use of drugs in rats was gained.

Chapter 6 section (i)

AZATHIOPRINE and PREDNISOLONE

These two agents are the standard immunosuppressive drugs used in human renal transplant recipients. The usual azathioprine dose is 2.5mg/kg/day given indefinitely although adjustments are made if leucopenia or thrombocytopenia develop. Prednisolone dose schedules vary widely but commonly start at 100mg/day with a reduction to 10mg/day by three months after transplantation. Rejection crises are treated by increasing the dose of corticosteroids e.g. three boluses of 1 gram of methylprednisolone at 12 hourly intervals.

These drugs were studied first because any clinical trial of passive enhancement is unlikely to be attempted without their use. Although French and Batchelor (1972) suggested from a small pilot experiment that suboptimal doses of the drugs acted synergistically with passive enhancement, a systematic study has not been performed. In their experiments the drugs were used with suboptimal doses of enhancing serum in a model in which passive enhancement alone was able to suppress rejection completely.

MATERIALS AND METHODS:

Renal transplantation. (DA X Lewis)F_1 kidneys were transplanted into Lewis recipients. Immediate bilateral nephrectomy was performed in the first experiments but in the later experiments right nephrectomy was delayed until day 7. (see Chapter 5 section 2).

Enhancing serum. Two batches of serum were used:

1. OAS-53 : 500µl given on days 0, 1, 3 and 5.
2. OAS-55 : 500µl given on days 0 and 1.

(for details of their preparation see Chapter 5 section 3)

Drugs.

Azathioprine for injection (Burroughs Wellcome Berkhamstead, England) and methylprednisolone sodium succinate (Upjohn, Crawley, Sussex, England) were prepared freshly each day. The first injections were given i.v. but all subsequent injections were given i.p. The toxicity of azathioprine in Lewis rats was tested by administering 15, 30, 60 and 75mg/kg/day to four animals in each group for two weeks. At the end of the treatment period the animals were sacrificed, weighed, and smears prepared from the femoral marrow. These were stained with Wrights Romanowsky stain and examined without prior knowledge of the treatment by a haematologist, Dr. A.A. Sharp, F.R.C.Path.

Lymphocytotoxic antibody titres

In the first experiments (a) using low doses of azathioprine and prednisolone a one-stage test was used. Because this was found to be relatively insensitive the two-stage assay was used in experiments (b) and (c). (see Chapter 5 section 5).

Histology

Because most specimens were obtained at autopsy groups were compared without formal quantitation.

Experimental groups

(a) Low doses of azathioprine and methylprednisolone with immediate nephrectomy.

The doses chosen were based on previous reports of the use of azathioprine and prednisolone in rats (Tinbergen, 1968; Shehadeh et al., 1970).

1. No treatment
2. Azathioprine 4mg/kg/day x 6 weeks
3. Azathioprine 8mg/kg/day x 6 weeks
4. Methylprednisolone 4mg/kg/day x 3 weeks, then 2mg/kg/day x 3 weeks.
5. Methylprednisolone 8mg/kg/day x 3 weeks, then 4mg/kg/day x 3 weeks.
6. Azathioprine 8mg/kg/day x 6 weeks plus methylprednisolone 8mg/kg/day x 3 weeks, then 4mg/kg/day x 3 weeks.
7. OAS-53 500µl i.v. days 0, 1, 3 and 5.
8. OAS-53 as in (7) plus azathioprine 8mg/kg/day as in (3)
9. OAS-53 as in (7) plus methylprednisolone 4mg/kg/day as in (4)

(b) High doses of azathioprine with immediate nephrectomy

The dose of azathioprine was chosen after performing the toxicity study.

1. No treatment
2. Azathioprine 30mg/kg/day x 14 days
3. OAS-55 500µl i.v. days 0 and 1.
4. OAS-55 as in (3) plus azathioprine 30mg/kg/day x 14 days.

(c) High doses of azathioprine and methylprednisolone with delayed nephrectomy.

1. No treatment
2. Azathioprine 30mg/kg/day x 21 days
3. Methylprednisolone 16mg/kg/day x 21 days
4. Azathioprine 30mg/kg/day plus methylprednisolone 16mg/kg/day both for 21 days
5. OAS-55 500µl i.v. days 0 and 1.
6. OAS-55 as in (5) plus azathioprine 30mg/kg/day x 21 days
7. OAS-55 as in (5) plus methylprednisolone 16mg/kg/day x 21 days
8. OAS-55 as in (5) plus azathioprine 30mg/kg/day x 21 days plus methylprednisolone 16mg/kg/day x 21 days

Results

Experiment (a). Low doses of azathioprine (Tables 1 and 2 and Appendices 3 and 4).

The administration of azathioprine at 4mg/kg/day made no difference to blood urea levels at day 7 nor did it prolong animal survival. At a dose of 8mg/kg/day rejection

was slightly less severe in that blood urea levels were lower at 7 days ($p < 0.02$) and the rats survived longer ($p < 0.05$).

Enhancing serum (OAS-53) modified rejection markedly. The blood urea levels at day 7 were significantly lower than controls ($p < 0.01$) and survival times were markedly prolonged ($p < 0.01$). Most animals survived over 100 days. Elevated blood urea levels and histology showed that all grafts had been damaged by rejection.

The concurrent administration of azathioprine 8mg/kg/day to enhanced animals did not influence survival, nor was there any modification of the low grade rejection seen in enhanced animals. Neither dose of azathioprine made any difference to lymphocytotoxic antibody titres at day 7 when compared to controls (Table 2). Sera from enhanced animals showed virtually no activity and this was not altered by the use of azathioprine.

Low doses of methylprednisolone (Tables 3 and 4 and Appendices 3 and 4).

The administration of methylprednisolone 4mg/kg/day resulted in lower 7 day blood urea levels ($p < 0.05$) and significantly longer survival ($p < 0.05$) than controls. The use of methylprednisolone 8mg/kg/day also resulted in lower blood urea levels at day 7 ($p < 0.01$) and longer survival ($p < 0.05$). However, four of six animals treated with 4mg/kg/day survived beyond 50 days, compared to

only one of six treated with 8mg/kg/day. Enhancing serum was therefore combined with the 4mg/kg/day schedule. There was no evidence of either a synergistic or an inhibitory effect on renal function and survival with the combined treatment.

The administration of methylprednisolone did not suppress the lymphocytotoxic antibody dose at day 7 nor did it modify the antibody levels in enhanced animals.

Low dose methylprednisolone plus azathioprine (Results included in Tables 3 and 4 and Appendices 3 and 4).

Because both drugs are administered to human renal transplant recipients, a single group was given azathioprine 8mg/kg/day and methylprednisolone 8mg/kg/day to establish whether they were synergistic. Although the administration of the combination resulted in lower 7 day blood urea levels ($p < 0.01$) and longer survival ($p < 0.01$) than controls, the effect was no greater than that obtained with either of the drugs administered alone.

Conclusion

These results showed that suboptimally effective doses of azathioprine and prednisolone did not potentiate the effect of enhancing serum in this model.

However, the doses chosen were thought to be inappropriately low. Drug metabolism is related not to weight but to surface area, and so the human doses in mg/kg

should be multiplied by a factor of 6 or 7 to reach the equivalent dose in mg/kg for the rat. (Freireich et al., 1966). In the case of azathioprine the rat dose equivalent to the human dose of 2.5mg/kg is of the order of 15mg/kg/day. This is consistent with the dose which is considered the maximum which can be tolerated by rats in the long term. (Burroughs Wellcome). On this basis the doses of azathioprine used in the first experiments, 8mg/kg/day and 4mg/kg/day were approximately 50% and 25% of equivalent human doses.

A toxicity study of azathioprine was performed to test the validity of this correction factor and to select an effective but sublethal dose.

A similar approach cannot be used for corticosteroid doses because the level of the human dose has been chosen empirically. There are no obvious constraints on maximum doses such as bone-marrow depression as in the case of azathioprine. Moreover, humans are considered to be steroid resistant and rats steroid sensitive (Claman, 1972). Nevertheless, the dose of methylprednisolone was increased to 16mg/kg/day in the hope of being in drug excess.

Results of azathioprine toxicity study (Table 5)

Weight loss over the 2 week period of administration was only apparent in the animals treated with 60mg/kg and 75mg/kg/day. The bone marrow smears showed a range of features from mild depression of the myeloid series at the lower doses to frank aplasia with the higher doses. The

dose of 30mg/kg/day, which resulted in moderate myeloid depression, was chosen for the next transplant experiments. This is approximately double the equivalent human dose.

Experiment (b). The use of high doses of azathioprine with enhancing serum and immediate nephrectomy. (Tables 6 & 7 and Appendices 5 & 6).

In the previous experiments the effect of the enhancing serum (OAS-53) had been variable (median blood urea ± SD at 7 days was 130 ± 109mg/100ml, range 74-355mg/100ml). This variability may have been caused by antibody-mediated damage to the graft in some cases. Each animal received a total of 2mls of serum. To avoid this possibility, I decided to give OAS-55 in smaller doses, 500µl on days 0 and 1. With this regimen the effect was less variable. The median blood urea level at 7 days was 85 ± 50mg/100ml (range 49-185mg/100ml), clearly a significant protective effect, but by 14 days all animals showed a marked deterioration in renal function (median blood urea ± SD = 325 ± 144mg/100ml).

The 30mg/kg dose of azathioprine modified acute rejection considerably. Although all grafts underwent acute rejection, the blood urea levels at 7 days were significantly lower than controls ($p < 0.01$) and survival times were significantly prolonged ($p < 0.01$). The combined use of azathioprine 30mg/kg/day with enhancing serum appeared to prevent the deterioration in renal function which was seen in animals treated with enhancing serum alone. However, when

the drug was withdrawn after 14 days all animals experienced severe acute rejection resulting in the death of 5 of 6 animals, in contrast to the enhanced group in which only one out of 6 animals died. This is not quite significant using a two-tailed Fishers exact test, $p = 0.068$).

Azathioprine at 30mg/kg/day did not depress the lymphocytotoxic antibody responses at 7 days.

Titres in the enhanced group were significantly lower than controls but azathioprine did not depress these further.

Experiment (c). High doses of azathioprine for 21 days. (Table 8 and Appendix 7).

The rapid deterioration in renal function after withdrawal of azathioprine at day 14 led to this next experiment in which azathioprine was administered to enhanced animals for 21 days. In addition, delayed nephrectomy at 7 days was introduced to decrease the number of technical failures from urine leaks. All other conditions of the experiment were the same except that the first blood urea measurement was at 10 days, 3 days after nephrectomy.

The immunosuppressive effect of azathioprine alone, noted in the immediately nephrectomised animals, was not seen when nephrectomy was delayed. Survival times and blood urea levels at 10 days were similar to untreated controls.

Despite this difference the drug still prevented the deterioration in renal function seen at 14 days in the enhanced group. The median blood urea $\pm$ SD at this time for

the combination group was 51 ± 23mg/100ml compared to 232 ± 110mg/100ml for the enhanced group ($p < 0.01$). However, continuing the drug for a further week had no further protective effect. Indeed as in the previous experiment rejection was progressive and fatal in the majority of animals ($p = 0.026$ using a two-tailed Fishers exact test).

High dose methylprednisolone (Table 8 and Appendix 7).

No immunosuppressive effect was apparent when methylprednisolone at 16mg/kg/day was used alone, nor did it alter the course of rejection when used with enhancing serum.

High dose methylprednisolone and high dose azathioprine. (Table 8 and Appendix 7).

The two drugs together were ineffective in suppressing rejection, but when used in combination with enhancing serum OAS-55, excellent renal function was maintained in all animals for two weeks, and in three out of five for three weeks. However, these three suffered severe rejection episodes at week 4. Moreover, this drug combination appeared toxic, the animals looking unwell during treatment and one died at day 21 with multiple abscesses.

Lymphocytotoxic antibody responses in the high dose azathioprine and methylprednisolone experiments with delayed nephrectomy. (Table 9 and Appendix 8).

Neither azathioprine 30mg/kg/day nor methylprednisolone 16mg/kg/day made any significant difference to the lymphocytotoxic antibody titres at 7 days. However, when the two drugs were used together the titre was significantly reduced. Animals treated with enhancing serum alone had low titres at 7 days, which rose at day 10 and then waned by the fourth week. This delayed rise was suppressed when both azathioprine, 30mg/kg/day, and methylprednisolone, 16mg/kg/day, were given in addition but not when either agent was used alone.

Histology (see plates at the end of this Chapter).

The grafts from untreated animals at 7 days and at the time of death showed patchy fibrinoid necrosis of glomeruli and arterioles with obliteration of the glomerular capillary lumina, patchy tubular necrosis, and interstitial oedema, haemorrhage and a cellular infiltration. These cells included lymphocytes, plasma cells and neutrophil polymorphs and were seen in the interstitium and the walls of the vessels. These features appeared in both delayed and immediately nephrectomised groups, although more severe changes were seen in the delayed nephrectomy group.

The grafts of animals treated with enhancing serum showed markedly different features. At 7 days the glomeruli

and tubules were normal but there was a marked cellular infiltrate consisting of lymphocytes and plasma cells. At day 14 there was disorganisation of the tissue architecture with disruption of the tubules and focal necrosis of glomeruli. At twenty weeks the glomeruli had become sclerosed, the interstitium fibrotic, the tubules atrophic and a mild cellular infiltrate persisted.

Azathioprine and prednisolone when used alone or together in both low and high doses did not result in any modification of the histological picture of acute rejection. In particular it was noted that the classic glomerular lesion seen in controls was unmodified.

However, when both drugs were used in high doses with enhancing serum there was attentuation of the rejection reaction seen in enhanced animals. Although the degree of cellular infiltrate was unaltered the tissue architecture was better preserved and in the three animals studied the glomerular lesion seen in enhanced animals at day 14 was absent. When these animals died they showed a severe rejection reaction similar to that seen in untreated enhanced animals.

DISCUSSION

These experiments failed to show any significant synergistic effect of azathioprine or prednisolone with passive enhancement and indeed suggested an antagonistic effect of azathioprine in certain situations. The results were unexpected in view of the clear synergy between ALS and enhancing serum in this model (Batchelor et al., 1972).

In this model untreated recipients experience an early irreversible acute rejection reaction which is invariably fatal. The pattern of rejection is modified by the use of enhancing serum. Renal function remains good for the first week, after which all animals show marked functional and morphological deterioration. In these experiments most animals survived indefinitely but with elevated blood urea levels and small scarred kidneys which showed the features of chronic rejection. Burgos et al. (1974) and Strom et al. (1975a)have both suggested that the delayed rejection seen in enhanced rat renal allograft recipients is mediated by the delayed cell-mediated response. This may also be the case in the (DA X Lewis)$F_1 \longrightarrow$Lewis model. If this is so, the delayed cell mediated rejection is as resistant to the effects of azathioprine and prednisolone as the rejection response in non-enhanced animals.

Azathioprine was found to have little effect on acute rejection although with higher doses in the immediate nephrectomy model, survival was significantly prolonged. Only one in vitro measurement of the immune response was made - the lymphocytotoxic antibody response. Azathioprine had no effect on this either suggesting that it would not have had any effect on any humoral component of rejection. This minimal immunosuppressive action of azathioprine should be seen in the light of the relative resistance of the rat to the immunosuppressive actions of the drug (see Chapter 2). Husberg (1973) showed that azathioprine at a dose of 30mg/kg made no difference to the cell-mediated cytotoxicity

of thoracic duct lymphocytes measured in Brown Norway recipients of Wistar Furth renal grafts nor was the number of thoracic duct lymphocytes reduced. There was,however, a slight reduction in antibody levels and 'blocking factor' production. In the same model ALS was found to cause a profound reduction in the cell-mediated cytotoxicity. Shehadeh et al. (1970) found that Lewis recipients of (Lewis X Brown Norway)F_1 renal allografts had little significant renal function at day 7 after transplantation when treated with azathioprine in doses ranging from 8-40mg/kg/day. In contrast to these findings are those of Tinbergen (1968) who showed that azathioprine at a dose of 4mg/kg/day prolonged the survival of Wag recipients of Brown Norway kidneys. Unfortunately, no functional or morphological data was presented. Currey (1971) showed that in the rat, doses of azathioprine which exceed the LD_{50} were required to obtain suppression of primary antibody responses, tuberculin skin reactivity and adjuvant arthritis. Thus it would appear that to suppress the in vivo manifestations of the immune responses in the rat with azathioprine may require doses exceeding the acceptable level of toxicity. The drug is not a placebo in rats for my toxicity studies showed that at a dose of 30mg/kg/day, Lewis rats showed some retardation of growth, and their bone marrows showed depressed myelopoiesis. On the basis of the correction factor discussed earlier this dose is equivalent to 5-6mg/kg/day in humans. Thus it appears that the toxic effects of azathioprine in man and the rat may run in parallel but that

this is not reflected in comparable immunosuppressive effects. It may be relevant that the effect of azathioprine varies according to the route of administration (Spreafico et al., 1973). Humans take the drug orally whereas the rats were given it i.p.

Despite the trivial immunosuppressive effects that were obtained with azathioprine alone, some effect was seen when the large dose, 30mg/kg, was used with passive enhancement. However, rejection was rapid and irreversible when it commenced so that the number of long term survivors was less in the group given both azathioprine and passive enhancement, when compared to passive enhancement alone. If the results of both experiments with OAS-55 and azathioprine 30mg/kg are pooled the difference is statistically highly significant. Using a two-tailed Fisher exact test $p < 0.01$. Thus at this dose azathioprine may have inhibited the long-term effects of passive enhancement, perhaps by preventing the development of auto-enhancement. It is unlikely that the higher mortality in the combination group was due to toxicity because infections were not found at autopsy and the allografts showed features of severe rejection. The survival curves are shown in Fig. 1.

An unexpected finding was the difference in survival times between the two groups of animals treated with azathioprine alone depending on the timing of nephrectomy. Immediately nephrectomised animals survived significantly longer than those undergoing 7 day nephrectomy. This difference can be attributed to the additional non-specific

immunosuppressive effect of uraemia (see Chapter 2). An additive effect of uraemia and azathioprine in rats has been reported by Souhami et al. (1973). It is interesting that the median lymphocytotoxic titre at day 7 in the immediately nephrectomised (uraemic) control animals was significantly lower than in the delayed nephrectomy (non-uraemic controls). It was 1:4 in the uraemic group and 1:256 in the non-uraemic group (see Tables 7 & 9). Azathioprine did not add to this difference however. The drug effect could also have been increased by poor excretion during the early rejection crisis. The pharmacokinetics of azathioprine in the rat have not been fully studied, but the mouse has been shown to excrete in the urine a significant fraction of azathioprine both as free drug and as 6-mercaptopurine (Elion et al., 1961).

Rats are considered steroid sensitive animals (Claman, 1972) and yet high doses of methylprednisolone had little effect on acute rejection or on the rejection experienced by enhanced animals. Although the small dose of 4mg/kg/day prolonged survival significantly, all the animals experienced a severe acute rejection, and the improved survival may have been due to the non-specific effects of uraemia superimposed on a marginal effect of the drug. Earlier experiments with prednisolone in other rat allograft models yielded disappointing results. Bell et al. (1971) failed to prolong heart allograft survival in the A/gus to Wistar combination with 3mg/kg/day although

one dose of 3mg given on day 5 post-transplant did prolong survival slightly. Shehadeh et al. (1970) found that 2 out of 5 Lewis rats treated with 8mg/kg/day of prednisolone had normal renal function at day 7 after receiving (Lewis X BN)F_1 renal allografts. 12mg/kg was found to be no more effective but more toxic.

If the early rejection crisis is partly antibody mediated then the failure of methylprednisolone to prevent acute rejection is understandable. The antibody responses at 7 days were unaltered. Currey (1971) demonstrated that doses of 100mg/kg/day of hydrocortisone (equivalent to 20mg/kg/day of prednisolone) suppressed the haemolysin response to SRBC to only 70% of control levels. The same dose did however suppress tuberculin sensitivity. Neifield et al. (1975) showed that 10mg/kg/day of methylprednisolone hemisuccinate prolonged the survival of Lewis skin grafts on Sprague-Dawley recipients from 9 to 15 days. Husberg (1973) showed that 12mg/kg/day of methylprednisolone caused a significant reduction of thoracic duct lymphocyte cell-mediated cytotoxicity and cytotoxic antibody production in Brown Norway recipients of Wistar-Furth kidneys. Thus the dose used in my experiments should have been in the effective range for depressing the cell-mediated response. The finding that prednisolone fails to augment the effect of enhancement differs from that of Chutná (1971) using a mouse skin graft model. He demonstrated an additive effect if hydrocortisone was given with passive enhancement.

Koene et al. (1975), however, showed no synergistic effect of prednisolone when used with enhancing serum in their mouse skin graft model, although they did demonstrate synergism with ALS.

It is possible that the form of corticosteroid and route of administration may have been inappropriate in my experiments. Fauve and Pierce-Chase (1967) found that succinylated prednisolone was much less potent than cortisone acetate in depressing immunity in mice. The duration of action of corticosteroid is prolonged if acetate formulations are administered subcutaneously. The action of the highly soluble prednisolone hemisuccinate given i.p. may have been too brief.

When both azathioprine and prednisolone were given in conjunction with enhancing serum normal renal function was maintained for three weeks in 3 out of the 5 animals. This suppression was not permanent for just as in the azathioprine experiments the withdrawal of the drug was followed by severe acute rejection.

Thus these experiments failed to show any significant synergistic effect of azathioprine and prednisolone with passive enhancement, and indeed suggested that if an augmentation of the effects of passive enhancement is to be obtained with these agents in clinical practice then the normal dose schedules would have to be used. The validity of this conclusion can be challenged because of the difficulties inherent in comparisons of the effects of drugs in rats with those in uraemic man.

An explanation for the apparent antagonistic effect of azathioprine on passive enhancement would require a clearer understanding of the homeostatic mechanisms involved in the maintenance phase of passive enhancement. The possible mechanisms have been discussed in Chapter 3.

Experiment (a)

TABLE 1: The effect of low doses of azathioprine on enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts

(Appendix 3)

Treatment	No.of Rats	Median blood urea ± SD (mg/100ml) at week: 1	2	3	4	6	8	12	20	Median survival in days (range) and comparison with controls
1. NIL	6	634±114	-	-	-	-	-	-	-	7 (7 - 10)
2.Azathioprine 4mg/kg/day x 6 weeks	6	450±103	-	-	-	-	-	-	-	9.5 (7 - 10) NS
3.Azathioprine 8mg/kg/day x 6 weeks	6	390±75	-	-	-	-	-	-	-	10.5 (8 -111) p < 0.05
7.OAS-53 500µl days 0,1,3,5	6	130±109	135±74	223±140	270±110	175±50*	145±29*	132±26	127±160	>140 (23 - >140) p<0.01
8.OAS-53 500µl days 0,1,3,5 plus azathioprine 8mg/kg/day x 6 weeks	6	124±206	145±47	100±201	176±136*	162±36*	109±35*	88±22*	65±29*	>26 >140 (9 - >140 p<0.01 NS different vs enhancement alone.

NS = Not significant * Less than 4 values

Experiment (a)

TABLE 2: The effect of low doses of azathioprine on lymphocytotoxic antibody responses* of enhanced and non-enhanced Lewis recipient of (DA X Lewis)F_1 renal allografts

(Appendix 4)

Treatment	No.of Rats	Median lymphocytotoxic antibody titres at week: 1	2	3	4	6	8	12	20	Comparison with controls
1. NIL	5	1/4	-	-	-	-	-	-	-	-
2.Azathioprine 4mg/kg x 6 weeks	4	1/32	-	-	-	-	-	-	-	NS
3.Azathioprine 8mg/kg x 6 weeks	5	1/16	-	-	-	-	-	-	-	NS
7.OAS-53 500µl days 0,1,3,5	6	0	0	0	0	ND	ND	ND	ND	$p<0.01$
8.OAS-53 500µl days 0,1,3,5 plus azathioprine 8mg/kg/day x 6 weeks	6	1/1	0	0	0	0	0	0	0	$p<0.01$

ND = Not done

* One-stage assay

Experiment (a)

TABLE 3: (Appendix 3) The effect of low doses of prednisolone on enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts.

Treatment	No.of Rats	Median blood urea ± SD (mg/100ml) at week: 1	2	3	4	6	8	12	20	Median survival in days (range) and comparison with controls
1. NIL	6	634±114	-	-	-	-	-	-	-	7 (7 - 10)
4. Prednisolone 4mg/kg/day	6	397±170	125±63	143±44	120±34	113±26	83±16	90±16*	69±10*	>62 <140 (9 - >140) p < 0.05
5. Prednisolone 8mg/kg/day	6	229±89	-	-	-	-	-	-	-	9.5 (9 - >140) p < 0.05
6. Prednisolone 8mg/kg/day plus azathioprine 8mg/kg/day	6	234±115	-	-	-	-	-	-	-	12.5 (8 - >140) p < 0.01
7. OAS-53 500µl days 0,1,3,5	6	130±109	135±74	223±140	270±110	175±50*	145±29*	132±26	127±160	>140 (23 - >140) p < 0.01
9. OAS-53 500µl days 0,1,3,5 plus prednisolone 4mg/kg/day x 3 weeks then 2mg/kg/day x 3 weeks	7	106±152	134±97	101±95	78±25*	89±63*	46±116*	91±127	86±165	> 140 (9 - >140) p < 0.01 NS different vs enhancement alone

* Less than 4 values

Experiment (a)

TABLE 4: (Appendix 4) The effect of low doses of methylprednisolone on lymphocytotoxic antibody responses* of enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allograft.

Treatment	No.of Rats	Median lymphocytotoxic antibody titres at week: 1	2	3	4	6	8	12	20	Comparison with controls
1. NIL	5	1/4	-	-	-	-	-	-	-	
4. Prednisolone 4mg/kg/day	6	1/4	1/2	1/2	1/1	0	0	ND	ND	NS
5. Prednisolone 8mg/kg/day	6	1/4	1/1	-	-	-	-	-	-	NS
6. Prednisolone 8mg/kg/day plus azathioprine 8mg/kg/day	6	1/2	-	-	-	-	-	-	-	NS
7. OAS-53 500µl days 0,1.	6	0	0	0	0	0	ND	ND	ND	$p<0.01$
9. OAS-53 500µl days 0,1 plus prednisolone 4mg/kg/day	7	0	0	0	0	0	0	0	0	$p<0.01$

ND = Not done * = One stage assay

TABLE 5: Azathioprine toxicity after 14 days treatment

Group	No.of Rats	Mean Weight Change ± SD	Bone Marrow
Controls 1ml normal saline i.p. daily	4	+5.8% SD=1.9%	All four were normal
Azathioprine 15mg/kg	4	+3.7% SD=2.4%	One animal only had mild myeloid depression.
Azathioprine 30mg/kg	4	+2.0% SD=1.1%	Three animals had moderate myeloid depression.
Azathioprine 60mg/kg	4	-3.3% SD=2.2%	Three animals had severe myeloid depression and megaloblastic erythropoiesis. One animal was aplastic.
Azathioprine 75mg/kg	4	-5.2% SD=0.7%	Two animals showed severe myeloid depression and megaloblastic erythropoeisis. Two were aplastic.

SD = Standard deviation

Experiment (b)

TABLE 6: (Appendix 5) The effect of azathioprine 30mg/kg/day x 14 days on enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts with immediate nephrectomy.

Treatment	No.of Rats	Median blood urea ± SD (mg/100ml) at week: 1	2	3	4	6	8	12	20	Median survival in days (range) and comparison with controls
1. NIL	6	634±114	-	-	-	-	-	-	-	7 (7 - 10)
2. Azathioprine 30mg/kg/day x 14 days	5	365±106	193±46	240±176*	360±115*	230±81*	185±28*	135±43*	185±84*	77 (17 - >140) p<0.01
3. OAS-55 500µl days 0,1.	6	85±50	325±144	285±103	212±81	177±63	192±54	145±54	97±89*	133 (16 - >140) p<0.01
4. OAS-55 500µl days 0,1 plus azathioprine 30mg/kg/day x 14 days	6	52±10	85±37	565±186*	-	-	-	-	-	20 (18 - 91) p<0.01 p = 0.068' vs enhancement alone

* Less than 4 values

' Two-tailed Fishers exact test

Experiment (b)

TABLE 7: The effect of azathioprine 30mg/kg/day x 14 days on the lymphocytotoxic antibody responses of Lewis recipients of (DA X Lewis)F_1 renal allografts with immediate nephrectomy.
(Appendix 6)

Treatment	No.of Rats	Median antibody titre at week: 1	2	3	4	6	8	12	20	Comparison' with controls
1. NIL	5	1/4	-	-	-	-	-	-	-	-
2.Azathioprine 30mg/kg/day x 14 days	5	1/2-4	0-1/1	1/1	ND	1/1	1/1	0	0	NS
3.OAS-55,500µl i.v. days 0,1	6	1/1	1/2	0	0	0	0	0	0	$p<0.01$
4.OAS-55,500µl i.v. days 0,1 plus azathioprine 30mg/kg/day x 14 days	6	1/1	1/1-2	0	0	-	-	-	-	$p<0.01$

' Comparison of 7 day titres

Experiment (c)

TABLE 8: (Appendix 7) The effect of high doses of azathioprine and prednisolone on enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts with delayed nephrectomy.

Treatment	No.of Rats	Median blood urea ± SD (mg/100ml) at week: 10	2	3	4	6	8	12	20	Median survival in days (range) and comparison with controls
1. NIL	6	530±114	-	-	-	-	-	-	-	11(11 - 13)
2. Azathioprine 30mg/kg/day x 21 days	5	592±98	-	-	-	-	-	-	-	10(10 - 12) NS
3. Prednisolone 16mg/kg/day x 21 days	5	500*±63	-	-	-	-	-	-	-	10(10 - 11) NS
4. Azathioprine 30mg/kg/day plus prednisolone 16mg/kg/day x 21 days	6	550±98	-	-	-	-	-	-	-	10.5(10 - >140) NS
5. OAS-55 500µl days 0,1.	6	45±7	232±110	270±100	155±44	165±28	145±18	112±75	135±23	>140 (16 - >140) p<0.01
6. plus azathioprine 30mg/kg/day x 21 days	5	47±130	51±23	190±150	-	-	-	-	-	28 (21 - 49) p<0.01 p = 0.026' vs enhancement alone
7. plus prednisolone 16mg/kg/day x 21 days	6	38±16	107±41	185±152	343±186	225*±198	175*±85	150*±142	120*±106	(18 - >140) p<0.01 NS different from enhancement alone
8. plus azathioprine 30mg/kg+prednisolone 16mg/kg/day x 21 days		50±2*	55±4	64±168	323±210	263*±88	283*±60	253±138	-	32 (21 - >140) p<0.01 NS different from ennancement alone

* Less than 4 values

' Two-tailed Fishers exact test

Experiment (c)

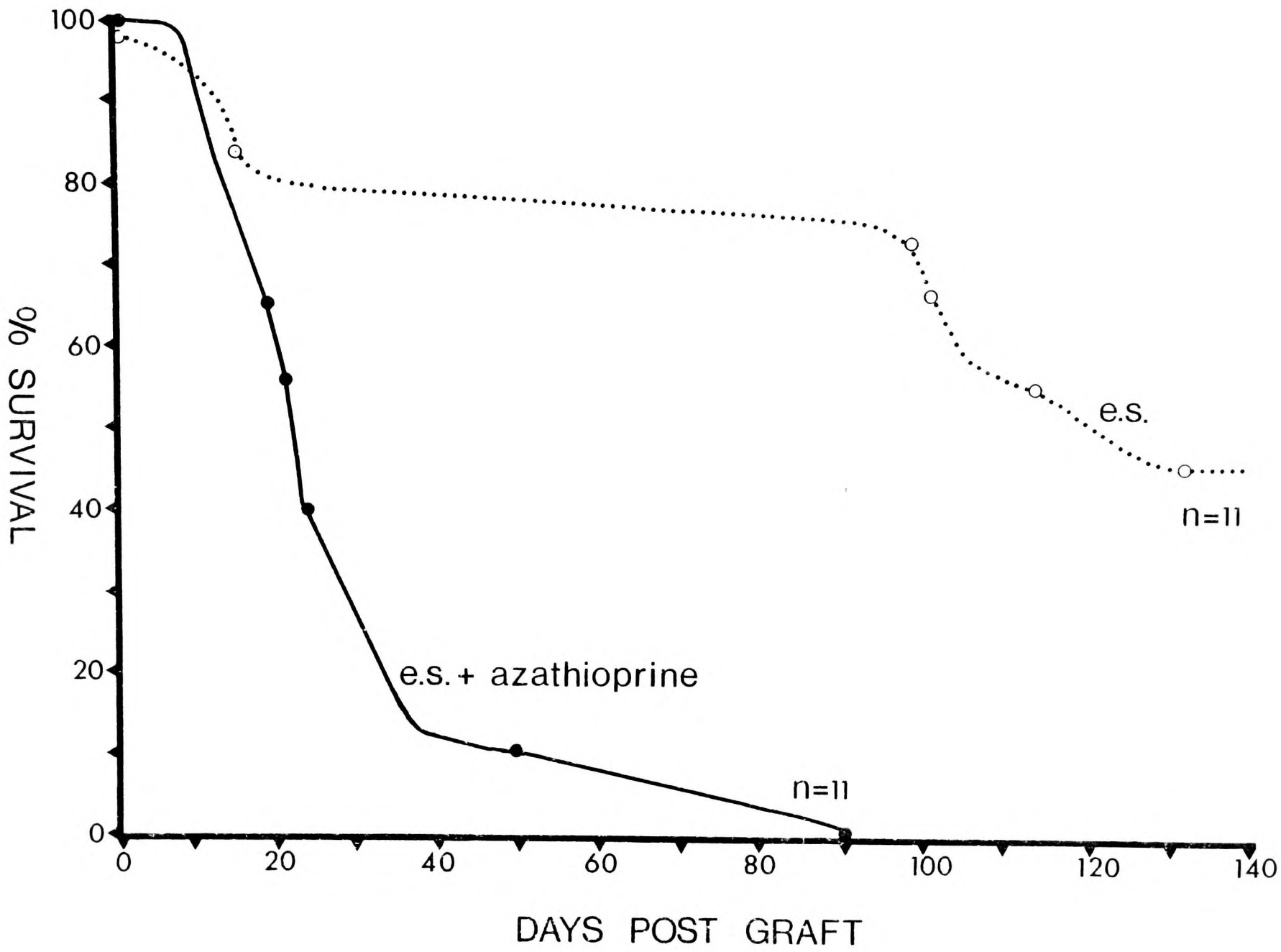
TABLE 9: (Appendix 8) The effect of high doses of azathioprine and prednisolone on the lymphocytotoxic antibody responses of enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts with delayed nephrectomy.

	Treatment	No.of Rats	Median lymphocytotoxic antibody titre at week: 1	10d	2	3	4	6	8	12	20	Comparisons with the controls at 7days
1.	NIL	7	1/256	1/32	-	-	-	-	-	-	-	-
2.	Azathioprine 30mg/kg/day x 21 days	5	1/256	1/128	-	-	-	-	-	-	-	NS
3.	Methylprednisolone 16mg/kg/day x 21 days	5	1/512	1/256	-	-	-	-	-	-	-	NS
4.	Azathioprine 30mg/kg/day plus methylprednisolone 16mg/kg/day x 21 days	6	1/16-32	0	-	-	-	-	-	-	-	$p<0.05$
5.	OAS-55 500µl days 0,1	6	1/1	1/4-8	1/4	1/1	0	0	0	0	0	$p<0.01$
6.	plus azathioprine 30mg/kg/day x 21d	5	TF	1/2	1/4	0/1/1	-	-	-	-	-	ND
7.	plus methylprednisolone 16mg/kg/day x 21 days	6	TF	1/8	1/4	1/2-4	1/1	0	0	0	0	ND
8.	plus azathioprine 30mg/kg/day plus methylprednisolone 16mg/kg/day x 21 days	5	1/1	0	1/1	0	0	-	-	-	-	$p<0.01$

TF = Technical failure

Fig.1

EFFECT OF AZATHIOPRINE ON PASSIVE ENHANCEMENT



Chapter 6 section (ii)

CYCLOPHOSPHAMIDE

In the previous experiments, azathioprine and prednisolone were shown to have only a modest and temporary effect on the delayed rejection reaction seen in the (DA X Lewis)F_1 → Lewis enhancement model. This was unexpected in view of the synergistic effect of ALS and enhancing serum in this combination.

The effect of cyclophosphamide was studied for two reasons. Firstly, cyclophosphamide, unlike azathioprine, has well-defined immunosuppressive properties in the rat (Currey, 1971), thus allowing quantitation of the dose requirements to provide complete suppression of rejection. Secondly, cyclophosphamide has been thought to have a relatively selective immunosuppressive effect on B-cells. If humoral factors are involved in the maintenance state of enhancement then this selective action could antagonise the protective effect of passive enhancement, rather than augment it. Moreover, there are a number of circumstances in which the immune response has been shown to be increased by the administration of the drug (reviewed Turk et al., 1976).

MATERIALS AND METHODS

Rats, transplantation, histological assessment, lymphocytotoxic antibody titres: see Chapter 5.

Transplants were performed in the (DA X Lewis)F_1→Lewis model with delayed nephrectomy. The two-stage ^{51}Cr release assay was used for measuring the lymphocytotoxic antibody titres.

Enhancing alloantisera (for details see Chapter 5 section 3).

OAS-55 (raised with Freund's complete adjuvant) 500µls were given intravenously on days 0 and 1.

OAS-56 (raised without Freund's complete adjuvant) 500µls were given intravenously on days 0, 1 and 3.

Cyclophosphamide:

In the first experiments Cyclophosphamide B.P. (W & B Pharmaceuticals) was used. In the second set of experiments "Cyclophosphamide for injection", Endoxana®, (W & B Pharmaceuticals), obtained directly from the Radcliffe Infirmary pharmacy, was used. This change was made firstly to ensure continuity of supply and secondly because "Cyclophosphamide for injection" contains 450mgs of sodium chloride per 1069mg of Cyclophosphamide B.P. making it more readily soluble than Cyclophosphamide B.P. In all experiments the doses quoted are for Cyclophosphamide B.P. but are not identical because of the different formulations. In both cases the drugs were aliquotted and made up immediately before use and used within 2 hours of reconstitution. Cyclophosphamide B.P. was dissolved in normal saline with gentle warming. Cyclophosphamide for injection was dissolved in water for injection. The first dose was given intravenously at the time of operation but all subsequent doses were given by the intraperitoneal route.

Graft-versus-host (GVH) assay:

This was performed using the method of Ford et al. (1970). Spleen cells were prepared by gentle mincing in Dulbecco's A + B solution (DAB), washed and then incubated in ACT for 10 minutes, washed twice before adjusting the concentration to 10^8/ml. Viability was greater than 85% as checked by trypan blue exclusion. One hundred µl (10^7 cells) were injected subcutaneously into the hind foot pads of six to eight week old (DA X Lewis)F_1 males and the popliteal lymph nodes weighed seven days later. The GVH reactivity of spleen cells of two animals treated with OAS-56 and cyclophosphamide 5.3mg/kg for 28 days was tested approximately one month after discontinuing the drug. The animals, 2415 and 2417, were transplanted after the main experiment but they behaved similarly to animals in the main experiment. The results were compared to those obtained using spleen cells from (i) a normal Lewis male (ii) a normal (DA X Lewis)F_1 male.

Statistical Comparisons

The survival times and blood urea levels of treated groups were compared to controls using a two-tailed Wilcoxon rank sum test. Comparisons between the enhanced groups, with and without cyclophosphamide, were performed using a two-tailed Fisher's exact test.

Experimental Groups:

A. Enhancing serum, OAS-55, plus cyclophosphamide B.P.

1. Controls (no treatment).
2. Cyclophosphamide 5mg/kg/day x 14 days.
3. Cyclophosphamide 10mg/kg/day x 14 days.
4. OAS-55, 500µl i.v. on days 0 and 1.
5. OAS-55 (as in 4) plus cyclophosphamide 5mg/kg/day x 14 days.

B. Enhancing serum, OAS-56, plus cyclophosphamide B.P. (as Cyclophosphamide for injection).

1. Controls (no treatment).
2. Cyclophosphamide 3.5mg/kg/day x 28 days
3. Cyclophosphamide 5.3mg/kg/day x 28 days
4. Cyclophosphamide 7.0mg/kg/day x 14 days
5. Cyclophosphamide 10.6mg/kg/day x 14 days
6. OAS-56 500µl i.v. on days 0,1 and 3
7. OAS-56 (as in 6) plus cyclophosphamide 3.5mg/kg/day x 28 days
8. OAS-56 (as in 6) plus cyclophosphamide 5.3mg/kg/day x 28 days

RESULTS

Experiment A. (Table 1 and 2, Appendices 9 and 10)

Two doses of cyclophosphamide were used. At the higher dose, 10mg/kg/day, rejection was almost completely suppressed but there was evidence of early mild rejection.

The median blood urea ± S.D. at day 10 was 75 ± 15mg/100ml. The biopsies at day 7 showed a 2+ mononuclear cell infiltrate. There was, however, little evidence of rejection by day 14. Blood urea levels had returned to normal (median blood urea ± S.D. = 52 ± 7mg/100ml) and biopsies showed only a few scattered mononuclear cells and a little interstitial fibrosis. Survival times were significantly prolonged when compared to controls ($p < 0.01$) and 4 of 6 animals survived with normal renal function for more than 140 days.

At the lower dose, 5mg/kg/day, early rejection was severe. Blood urea levels at day 10 were not significantly lower than controls. 4 of 5 animals survived this acute rejection and three survived for more than 140 days. Compared to controls survival was significantly prolonged ($p < 0.01$).

Enhancing serum OAS-55 had a consistent effect, delaying the onset of rejection significantly. Blood urea levels at day 10 were low but they rose sharply by day 14. The majority of animals survived this rejection crisis to become long survivors but in all cases blood urea levels remained elevated (This is the same group as that in section (i) of this chapter).

Six animals were treated with enhancing serum and cyclophosphamide 5mg/kg/day for 14 days (total dose 70mg/kg) representing 50% of the dose which suppressed rejection completely. In contrast to the group treated with enhancing

serum alone, 5 out of 6 of these animals had normal blood urea levels at 14 days. However, all 5 of these had severe rejection episodes after the drug was discontinued. Although the median survival in this group (37 days) was shorter than in the group treated with enhancing serum alone, the difference was not statistically significant ($p > 0.05$).

When it became apparent that rejection would follow discontinuing the drug, one animal, 1622, was treated for 28 days (total dose = 140mg/kg). This animal survived beyond 140 days and did not reject the allograft after withdrawal of the drug. Indeed the blood urea remained normal throughout.

This result suggested that although a lower dose level of cyclophosphamide could be used to prevent rejection in enhanced animals, the same total dose of non-specific agent might need to be administered to ensure lasting suppression of rejection. (As there was insufficient OAS-55 to test this hypothesis, experiment B using OAS-56 was performed).

The histological appearances can be summarised as follows. At day 7 the untreated animals showed patchy areas of infarction with necrotic glomeruli and tubules. Only a minimal cellular infiltrate was present. At both doses of cyclophosphamide, 7 day biopsies showed a dense mononuclear cell infiltrate but only the higher dose (10mg/kg) prevented the development of glomerular necrosis.

By day 14 the 10mg/kg group showed little evidence of rejection, the only features being a few scattered mononuclear cells and a little interstitial fibrosis. Seven day biopsies of animals treated with enhancing serum showed a 2+ mononuclear cell infiltrate and normal glomeruli but by day 14, biopsies showed a fierce rejection reaction characterised by a dense mononuclear cell infiltrate, oedema, and focal glomerular necrosis. This fierce reaction was not seen in the animals treated with enhancing sera and cyclophosphamide 5mg/kg/day although it did develop when the drug was withdrawn. In the one recipient treated for 28 days this progression did not occur.

Ten mg/kg/day of cyclophosphamide suppressed the lymphocytotoxin response at day 7 completely. No data are available for the 5mg/kg because of a technical failure with the assay (Table 2). Enhanced animals had low levels of antibody at day 7, rising by 10 and falling by 4 weeks. The addition of cyclophosphamide suppressed this low level response and there was no rebound rise when the animals rejected their renal allografts after withdrawal of the drug.

Experiment B. (Tables 3,4,5 & 6, Appendices 11 and 12).

The dose response results for cyclophosphamide given as "Cyclophosphamide for injection", Endoxana® showed that 10.6mg/kg/day caused a significant and constant suppression of rejection as measured by 10 day blood urea levels ($p < 0.01$). At lower doses the effect was variable

and incomplete. In contrast to the effect on renal function, the low doses of the drug suppressed the antibody response significantly (Table 4). No antibody was detectable in any of the animals treated with cyclophosphamide at doses $\geqslant$5.3mg/kg/day. In this dose study none of the rats treated with the higher dose of cyclophosphamide (10.6mg/kg/day) became long survivors. Autopsies showed that they had died of infection (three cases) and perforation of the gut with haemorrhage (two cases). In all five the transplanted kidneys showed only scattered mononuclear cells and a little interstitial fibrosis. This difference from experiment A was attributed to the variation in the condition of the experimental animals.

OAS-56 which had been raised without Freund's adjuvant was less effective than OAS-55, but the pattern of rejection was the same. Blood urea levels at day 10 were significantly lower than controls ($p < 0.01$) but all animals underwent severe rejection by day 14, (median blood urea $\pm$ S.D. = 682 $\pm$ 124 mg/100ml), resulting in the death of 4 of 6 animals.

The use of cyclophosphamide 3.5mg/kg/day, which alone made no difference to rejection, was insufficient to suppress rejection completely and consistently in enhanced animals. Although blood urea levels at two weeks in the combination group were significantly lower than in the enhanced group ($p < 0.01$), 4 out of 7 had rejection episodes and survival was not significantly prolonged ($p > 0.05$).

This dose of cyclophosphamide represents 33% of the dose which alone suppressed rejection completely.

At a dose of 5.3mg/kg/day, 50% of the effective dose, suppression of rejection in enhanced animals was almost complete. Blood urea levels were normal in all 7 animals at 2 and 4 weeks and rejection did not occur after discontinuing the drug, although there was one unexplained death at 35 days. Survival times were significantly longer than those in animals treated with enhancing serum alone ($p < 0.05$).

The lymphocytotoxic antibody responses are shown in Table 4. There was a striking suppression of antibody production at all doses of cyclophosphamide (Table 4). Animals treated with enhancing serum alone showed a rise in titre at day 10 but this rise was suppressed when cyclophosphamide (at both dose levels) was added. Animals treated with cyclophosphamide at 5.3mg/kg/day had no detectable antibody at day 7 whereas those treated with the same dose of cyclophosphamide and enhancing serum had titres of 1:1 presumably due to the passively administered antibody. Passively administered antibody was easily detectable in the three untransplanted Lewis rats given a course of enhancing serum.

The histological features are shown in Table 5. At day 7 control allograft biopsies showed the features of infarction. Both tubules and glomeruli were necrotic. When whole kidney allografts were examined at 7 days, a

polymorph and mononuclear cell infiltrate, interstitial oedema and haemorrhage, and arterial fibrinoid necrosis could be seen. Histological results in the treated groups should be compared with the control biopsies since the sampling method was the same. The pattern of infarction suggested a predominantly vascular pathogenesis for the rejection changes. Three control allografts were examined at day 5 and showed an intense mononuclear cell infiltrate without glomerular or vascular lesions. Thus, the infarction appears to have followed cellular rejection. The histological pattern in the treated groups showed both qualitative and quantitative differences from controls. With low doses of cyclophosphamide (3.5mg/kg and 5.3mg/kg/day) rejection was less severe but both tubular and glomerular damage were still apparent. At the higher doses 7.0mg/kg and 10.6mg/kg/day glomeruli and tubules were not damaged although an interstitial mononuclear cell infiltrate and oedema were still present. Seven day allograft biopsies from enhanced animals with and without cyclophosphamide treatment showed intact glomeruli and tubules but a marked interstitial mononuclear cell infiltrate. Although there was significant differences in renal function between treated and untreated enhanced animals by day 14, the differences in histological appearances were subtle. A similar degree of cellular infiltration and oedema were seen in both. Two features distinguished the groups: animals treated with enhancing serum alone showed tubular atrophy and focal glomerular necrosis whereas the treated

group did not. These features are illustrated in the photographic plates at the end of the chapter.

The results of the GVH assay are shown in Table 6. The GVH reactivity of spleen cells from the two animals treated with OAS-56 and cyclophosphamide 5.3mg/kg/day for 28 days was tested approximately one month after discontinuing the drug. Blood ureas were normal at the time of assay. The results showed that the two treated animals were still able to react against DA antigens in the GVH assay. The lymph node weights were, however, lower than those which resulted from injection of normal Lewis spleen cells.

DISCUSSION

These results demonstrate that cyclophosphamide can augment the effect of enhancing serum in the suppression of rejection of rat renal allografts. The rejection reaction mounted by enhanced animals was suppressed at a dose level of cyclophosphamide of about 50% of that which suppressed rejection completely when administered alone.

Although the dose level in mg/kg/day could be reduced, the total amount of drug required to suppress rejection completely may have been unaltered. Ten mg/kg/day of cyclophosphamide given for 14 days (a total dose of 140mg/kg of cyclophosphamide) resulted in complete suppression of rejection and prolonged survival with normal blood ureas in 4 of 6 animals. However, when 5mg/kg/day was administered with enhancing serum for 14 days (total dose 70mg/kg) all 6 recipients ultimately rejected their grafts (Experiment A).

When the period of drug administration was extended to 28 days (5.3mg/kg/day for 28 days making a total dose of 148mg/kg) renal function remained normal even after the drug was withdrawn (Experiment B).

Even the dose of 3.5mg/kg/day (33% of the optimal dose) modified the rejection reaction in enhanced animals in that blood urea levels at both day 10 and 14 were lower than in enhanced animals, but the effect was incomplete and inconsistent.

Thus, to augment the specific immunosuppressive effect of enhancing serum to allow complete suppression of rejection, cyclophosphamide had to be given for a definite time period and at a definite dose level.

These experiments do not provide an explanation for this synergistic effect. Serial measurement of various parameters of cellular and humoral immunity to the allograft may have revealed the mechanism. Attempts to establish an assay for CMI in this model were unsuccessful.

Burgos et al. (1974), Strom et al. (1975a) and Abbas et al. (1974a and b) working with similar enhancement models to the one used in these experiments, have shown that enhanced rats have a delayed but otherwise unimpaired cell-mediated response to renal allografts. This finding is compatible with the histological appearances of 7 day and 14 day biopsies of the enhanced kidneys in these experiments. Suppression of the cell-mediated response is the most likely

mechanism by which cyclophosphamide exerted its effect. Currey (1971) showed that in rats a dose of 10mg/kg/day of cyclophosphamide suppressed tuberculin reactivity and the development of adjuvant arthritis, both of which are manifestations of cell-mediated immunity. Although a depression in CMI might have been expected to be reflected in a diminution in the cellular infiltrate into the grafts this was not the case. It was only the consequences of rejection; tubular and glomerular damage, that were absent in the animals treated with enhancing serum and cyclophosphamide.

The role of antibody in the delayed rejection in enhanced animals is not clear. Both 3.5mg/kg/day and 5.3mg/kg/day doses suppressed the humoral response in enhanced animals but only the higher dose consistently suppressed rejection.

The maintenance state of enhancement is thought to reflect a balance between the suppressive effect of enhancing factors and the allograft response. The augmentation of the effect of enhancing serum by cyclophosphamide in these experiments suggests that the drug was able to tip the balance in the favour of enhancement, possibly by reducing the size of the effector cell alone, without a concomitant reduction in the enhancing factors.

An alternative explanation is that cyclophosphamide eliminated the antigen reactive clone altogether making any central enhancing mechanism superfluous. Such an explanation could account for the observation that a limited

course of cyclophosphamide alone, 10mg/kg/day x 14 days, induced a prolonged state of unresponsiveness to the renal allograft. An immune reaction was evident in the graft at day 7 but this had disappeared by 14 days suggesting that the antigen reactive clone had been eliminated. It was to clarify this point that the GVH assay of spleen cells from rats 2415 and 2417 was performed. Both animals were able to react against donor DA antigens but the reactivity appeared rather lower than that obtained with normal Lewis spleen cells. Without the necessary controls (i) reactivity against third party antigens (ii) the effect of cyclophosphamide alone on non-transplanted Lewis recipients, (iii) the use of different cell doses, one cannot draw any useful conclusions about the degree or specificity of this immunosuppressive effect. Nevertheless one can conclude that cyclophosphamide did not eliminate the antigen reactive clone.

These results conflict with two previous reported attempts to augment the effect of passive enhancement with cyclophosphamide. Myburgh and Smit (1972) found that the addition of a single dose of 20mg/kg of cyclophosphamide to a regimen of polyspecific antidonor serum and donor bone marrow which gave optimal prolongation of survival after liver allografting in baboons, reduced the effect to that of polyspecific serum alone. Koene et al. (1975) showed no further prolongation of mouse skin graft survival if cyclophosphamide was given in a single dose on day 2 with an effective enhancing serum regimen. In neither set of

experiments were other dose schedules of cyclophosphamide tested. Although cyclophosphamide is more effective when administered in large infrequent doses, (Kawabe et al., 1972) the effect of a single dose so early after transplantation may be insufficient to inhibit the delayed allograft response seen in enhanced animals. Wustrack et al. (1975) on the other hand, were able to show an additive effect of cyclophosphamide and enhancing serum in the suppression of skin graft rejection provided vascular continuity was immediately established and lymphatic connections were delayed.

In my experiments there was no evidence to suggest that cyclophosphamide was antagonistic to passive enhancement. In the first experiments, using OAS-55, animals treated with enhancing serum alone entered a stable state after a delayed but severe rejection crisis. The median survival was greater than 100 days. The additional use of 5mg/kg/day of cyclophosphamide for 14 days resulted in a shorter median survival (37 days) but this difference was not statistically significant. Survival results do not necessarily indicate differences in rejection patterns because survival may have been shortened because of infections which were more common in animals treated with cyclophosphamide.

In the second experiment only two of the six animals treated with enhancing serum alone survived the delayed rejection to become long survivors. This precluded any assessment of the effect of cyclophosphamide on the stable state. Indeed it was only with the use of cyclophosphamide

that a stable state developed.

There is convincing evidence that cyclophosphamide can antagonise specific immunosuppressive measures. Zola (1975) showed that cyclophosphamide prevented active enhancement of a mouse tumour graft. Marquet and Heystek (1975a) showed that cyclophosphamide at a dose of 50mg/kg abolished the protective effect of blood transfusion on heart allograft survival in the Brown Norway $\longrightarrow$WAG combination but a dose of 100mg/kg resulted in a remarkable degree of specific immunosuppression such that even Brown Norway skin grafts were accepted. In the reverse combination WAG $\longrightarrow$Brown Norway, in which blood transfusion led to accelerated rejection, the addition of cyclophosphamide resulted in enhanced survival. There are also reports of the induction of specific immunosuppression with donor antigen and cyclophosphamide. Nirmul et al. (1973) showed that mouse skin graft survival was prolonged if the recipients were pretreated with donor spleen cells and 200mg/kg of cyclophosphamide. Bramis et al. (1977) showed that rat kidney allograft survival was prolonged by pretreatment of recipients with donor platelets and 75mg/kg of cyclophosphamide.

Although there is controversy about the role that suppressor cells play in the maintenance phase of enhancement of rat renal allografts (Fabre and Morris, 1972; Batchelor et al., 1977b; Hall et al., 1979) they are reported to be more sensitive to cyclophosphamide than cytotoxic T-cells (Ferguson & Simmons, 1978).

They appear to be the mechanism maintaining a state of specific non-reactivity to skin allografts which can be induced in mice, with donor liver extract, Bordatella pertussis and ALS. In this model of specific immunosuppression the state of unresponsiveness can be broken by the administration of cyclophosphamide (Kilshaw et al., 1975). The results of Russell et al. (1978) are also pertinent in this context. They found that the state of auto-enhancement in mice kidney allograft recipients could be abrogated by the simultaneous administration of cyclophosphamide, BCG and donor antigen.

There is good evidence to support a role for blocking factors as the mechanism responsible for the maintenance state of enhancement in some rat renal allograft models (Stuart et al., 1971a;Tilney & Bell, 1974; Strom et al., 1975a;Hutchinson & Zola, 1977) but not in this model. In my experiments the dose of cyclophosphamide which augmented the effect of enhancing serum completely inhibited the lymphocytotoxic antibody response, suggesting that in this situation humoral factors are unlikely to play a critical role.

In summary, suboptimal doses of cyclophosphamide in conjunction with enhancing serum were able to induce prolonged renal allograft survival provided treatment was administered for 4 weeks. The suppression of the humoral response to the allograft in these animals is not compatible with a critical role for humoral factors in maintaining the state of non-reactivity.

Experiment (a)

TABLE 1: The effect of Cyclophosphamide on enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts.
(Appendix 9)

Treatment	No.of Rats	Median blood urea ± SD (mg/100ml) at week: 10d	2	3	4	6	12	20	Median survival and range (days)	Comparison of survival times with controls
1) NIL	7	530±114	-	-	-	-	-	-	11(11-13)	-
Cyclophosphamide										
2) 5mg/kg/day x 14d	5	475±123	92±57	115±156	75*	97*	75*	67*	>140(13->140)	p<0.01
3) 10mg/kg/day x 14d	6	75±15	52±7	55±4	50±7	55±12	60±3	55±21	>140(15->140)	p<0.01
4) OAS-55 500µl days 0,1	6	45±7	232±110	270±100	155±44	165±28	112±75	135±23	>140(16->140)	p<0.01
5) OAS-55 500µl days 0,1 plus Cyclo 5mg/kg/day x 14 days	6	50±50	53±64	155±110	275±158	260*	167*	157*	37(20->140)	p<0.01 NS different from enhancement alone
	Rat No. 1622'	60	55	50	37	50	47	54	>140	

' Cyclophosphamide 5mg/kg/day x 28 days

NS = Not significant

* Less than 4 values

Experiment (a)

TABLE 2: The effect of cyclophosphamide on the lymphocytotoxic antibody response of enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts.

(Appendix 10)

	Treatment	No.of Rats	Median lymphocytotoxic titres at week: 1	10days	2	3	4	6	8	12	20	Comparisons with controls at 7days
1.	NIL	7	1/256	1/32	-	-	-	-	-	-	-	-
2.	Cyclo 5mg/kg day x 14days	5	TF	TF	TF	TF	TF	0	0	0	0	ND
3.	Cyclo 10mg/kg day x 14days	6	0	0	0	0	0	0	0	0	0	p<0.01
4.	OAS-55 500µl days 0,1	6	1/1	1/4-8	1/4	1/1	0	0	0	0	0	p<0.01
5.	OAS-55 500µl days 0,1 plus cyclophosphamide 5mg/kg/day x 14d	6	1/1	0	0	0	0	0	0	0	0	p<0.01
	OAS-55 500µl days 0,1 plus cyclophosphamide 5mg/kg/day x 28d	1	1/1	1/1	0	0	0	0	0	0	0	ND

TF = Technical failure

ND = Not done

Experiment (b)

TABLE 3: The effect of cyclophosphamide on enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts

(Appendix 11)

Treatment	No.of Rats	Median blood urea ± SD (mg/100ml) at week: 10d	2	3	4	6	8	12	Median survival and range(days)	Comparison of survival times with control
1. NIL	5	600±71	-	-	-	-	-	-	12(12-13)	-
Cyclophosphamide										
2. 3.5mg/kg/day x 28d	3	660±87	-	-	-	-	-	-	12(12)	ND
3. 5.3mg/kg/day x 28d	4	532±232	530±414	83±277	-	-	-	-	27(19-55)	$p < 0.01$
4. 7.0mg/kg/day x 14d	4	222±93	118±96	-	-	-	-	-	19(17-47)	$p < 0.01$
5.10.6mg/kg/day x 14d	5	78±24	108±47	-	-	-	-	-	15(10-22)'	NS
OAS-56										
6. 500µl days 0,1,3	6	125±69	682±124	218*	158*	153*	138*	152*	16(14- >140)	$p < 0.01$
7. plus cyclo 3.5mg/kg/day x 28d	7	83±14	95±115	97±55	87±55	65±208	76±102	62±75	>100 (17->140)	$p < 0.01$ NS different from enhancement alone
8. plus cyclo 5.3mg/kg/day x 28d	7	70±98	66±22	69±144	57±11	38±6	44±10	54±11	>100 (35->140)	$p < 0.01$ $p < 0.05$ vs enhancement

* Less than 3 values

' Deaths not due to rejection

ND = Not done

NS = Not significant

Experiment (b)

TABLE 4 (Appendix 12) The effect of cyclophosphamide on the lymphocytotoxic antibody responses of enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts.

	Treatment	No.of Rats	Median lymphocytotoxic antibody titres at week: 1	10d	2	3	4	6	8	12
1.	NIL	5	>1/512	1/16-32	-	-	-	-	-	-
	Cyclophosphamide									
2.	3.5mg/kg/day x 28 days	3	1/2	1/4	-	-	-	-	-	-
3.	5.3mg/kg/day x 28 days	4	0	0	0	0	-	-	-	-
4.	7.0mg/kg/day x 14 days	4	0	0	0	-	-	-	-	-
5.	10.6mg/kg/day x 14 days	5	0	0	0	-	-	-	-	-
	OAS-56									
6.	500µl days 0,1,3	5	1/1	1/4	1/2	-	-	-	-	-
7.	plus cyclo 3.5mg/kg/day x 28d	5	1/1	1/1	0	0	0	-	-	-
8.	plus cyclo 5.3mg/kg/day x 28d	5	1/1	1/1	1/1	0	0	0	0	0
	No Transplant	3	1/16	1/8	1/8	1/4	1/2	1/1	0	0

Experiment (b)

TABLE 5. Histological Results - Controls and cyclophosphamide treatment

Treatment	Day of Specimen	No. of Specimens	Median histological scores for each feature: INTERSTITIUM Mono	Poly	Oed.	Haem.	ARTERIES Fibrin	Sub.Mono	TUBULES Atro.	Nec.	GLOMERULI capillary necrosis	Median score for group
NIL	5 days (kidney)	3	+++		+							4
	7 day (biopsy)	4	0		+					++++	++++	10.5
	7 day (kidney)	6	++	++	+++	+++	+			+++	++++	16
ISOGRAFTS	7 days	5	+									1
Cyclophosphamide 3.5mg/kg	7 days	3	++		+++				++		++	10
Cyclophosphamide 5.3mg/kg	7 days	4	++		++				+		++	7.5
Cyclophosphamide 7.0mg/kg	7 days	4	++		++							4
Cyclophosphamide 10.6mg/kg	7 days	5	++		+							3

Experiment (b)

Table 5 (contd) Histological Results - enhanced groups

Treatment	Day of Specimen	No. of Specimens	Median histological score for each feature: INTERSTITIUM Mono	Poly	Oed.	Haem.	ARTERIES Fibrin	Sub.Mono	TUBULES Atro.	Nec.	GLOMERULI Capillary Nec.	Median score for Group
ISOGRAFTS	7	5	+									1
OAS-56 500ul days 0,1,3												
(a) Alone	7	5	+++		+							4
(b) plus cyclophosphamide 3.5mg/kg/day x 28d	7	7	++		+							3
(c) plus cyclophosphamide 5.3mg/kg/day x 28d	7	7	++		+							3.3
ISOGRAFTS	14	4	+/0									0.75
OAS-56												
(a) Alone	14	3	+++		++				++		+	7.7
(b) plus cyclophosphamide 3.5mg/kg	14	2	++		++				+			5.5
(c) plus cyclophosphamide 5.3mg/kg	14	2	++		+							3.5

Mono = Mononuclear cells
Poly = Polymorphs
Oed = Oedema
Haem = Haemorrhage
Fibrin = Fibrinoid necrosis
Sub.Mono = Subintimal mononuclear cell
Atro = Atrophy
Nec = Necrosis

TABLE 6

GVH Assay Results

Popliteal node weight assay in (DA X Lewis)F_1 males

Injected Cells	No. of nodes assayed	Mean lymph node weight (mg) and range
10^7 (DA X Lewis)F_1 spleen cells	6	6.7mg (4-9)
10^7 normal Lewis spleen cells	3	30.6mg (20-38)
10^7 spleen cells from rat 2415 at 8 weeks post-transplant	3	15mg (12-17)
10^7 spleen cells from rat 2417 at 7 weeks post-transplant	3	15.3mg (13-19)

Chapter 6 section (iii)

DOSE REQUIREMENTS OF ALS TO SUPPRESS REJECTION IN ENHANCED LEWIS RECIPIENTS OF (DA X LEWIS)F_1 RENAL ALLOGRAFTS

The original report of Batchelor et al. (1972) which demonstrated the "potentiation" of passive enhancement in the (DA X Lewis)$F_1 \longrightarrow$Lewis combination with rabbit anti-rat thymocyte serum (ATS) did not include a quantitation of the dose requirement to obtain the effect. The dose used was suboptimal but its relation to a fully effective dose of ATS was not determined. In a later report, Fabre and Morris (1974a) showed a similar potentiating effect of ALS in the stronger DA to Lewis combination. However the ALS was not titrated and the dose chosen appeared close to optimal. Because of the negative results obtained with azathioprine and prednisolone, and reports showing no advantage in the combined use of ALS and enhancing serum (Myburgh & Smit, 1973; Koene et al., 1975; Finch & Morris, 1977), a quantitative study of these two reagents was performed.

A range of doses of (rabbit anti-rat lymphocyte serum) ALS were tested to establish an optimal dose which alone suppressed rejection completely in the (DA X Lewis)$F_1 \longrightarrow$Lewis combination. This was then compared to the lowest dose which, when used with enhancing serum, would result in complete suppression of rejection.

MATERIALS AND METHODS

Rats, transplantation, lymphocytotoxic antibody titres and biopsies: as described in Chapter 5. Transplants were performed in the (DA X Lewis)$F_1 \longrightarrow$ Lewis model with right nephrectomy delayed to day 7.

Enhancing Serum

OAS-56 which had been raised without FCA (see Chapter 5 section 3) was used. 500µl was administered i.v. on day 0,1,3 after transplantation.

Rabbit anti-rat lymphocyte serum

The ALS used in these experiments was part of a large batch raised by Mr. D. Evans, F.R.C.S. For details of the method of preparation see Appendix 13. It was administered i.v. on days 0,1,3 and 5.

Experimental Groups

(a)	Dose response study of ALS(DE):	125µl, 250µl 375µl, 500µl, 750µl doses were compared.
(b)	ALS and enhancing serum	OAS-56 500µl alone
	OAS-56:	OAS-56 500µl plus 250µl doses of ALS.
		OAS-56 500µl plus 375µl doses of ALS.

RESULTS (For complete tabulated results see Appendices 14 & 15).

Survival and Blood Urea results

(a) ALS Dose Study (Table 1)

The optimal dose of ALS was found to be greater than 500µl but less than 750µl. Animals treated with 125µl or 250µl had severe acute rejection similar to that seen in controls, from which the majority died. At the 375µl and 500µl doses, rejection was delayed but not completely suppressed, whereas the 750µl dose resulted in normal renal function throughout.

(b) Combination of ALS and OAS-56 (Table 2)

The use of OAS-56 dose resulted in the pattern of rejection characteristic of this model i.e. a delay in rejection with significantly lower blood urea levels than controls at day 10, followed by a severe rejection reaction by day 14. In this experiment 4 of 6 animals died. This is the same group as in section ii of this chapter.

The addition of 250µl of ALS, which alone was almost ineffective, suppressed this delayed rejection markedly, but the effect was neither consistent nor complete. Survival times were not significantly prolonged when compared to enhancement alone ($p > 0.05$). At a dose of 375µl the effect was almost complete and 6 of 7 animals have survived for >100 days with normal blood ureas. Compared to enhancement alone survival was significantly prolonged ($p < 0.05$).

Lymphocytotoxic antibody titres

(a) ALS Dose Study (Table 3)

The results showed that only the highest dose of ALS (750µl) suppressed the lymphocytotoxin response completely. At lower doses, 250µl, 375µl, 500µl the rise was delayed. 125µl had no effect. At the 375µl dose the rise in titre coincided with the rejection seen at 2 weeks.

(b) Combination of ALS and OAS-56 (Table 4)

The combinations of ALS 375µl and OAS-56 500µl resulted in almost complete and indefinite suppression of the lymphocytotoxic antibody response. At the 250µl dose, which was associated with a low level of rejection, there was a persistent low level of antibody. Only a small part of the antibody detected in the enhanced animals resulted from the 1500µl passively administered after transplantation. This can be seen from the rise in titres after completion of the antiserum course. The three animals which received 1500µl of antiserum but no transplant showed a slow decline in antibody titres. By 8 weeks no antibody was detectable.

Histological Assessment (Table 5)

At 7 days control biopsies showed severe advanced acute rejection with severe damage to both tubules and glomeruli. The 125µl dose of ALS made no difference to this pattern. Although arterial fibrinoid necrosis was not seen in the control biopsies, it was seen in control whole kidneys at 7 days. This difference was probably due to the

sampling.With higher doses of ALS (250µl - 500µl) the histological pattern of rejection changed. Mononuclear cell infiltration was more obvious and glomerular and tubular damage less severe or absent. At the 750µl dose no evidence of rejection was seen.

Biopsies from animals treated with enhancing serum alone showed a 3+ cellular infiltrate and 1+ oedema at 7 days. By 14 days when renal function had deteriorated, the cellular infiltrate and oedema were still present and tubular and glomerular damage, and disruption of the tissue architecture were apparent. The addition of ALS 375µl which prevented the deterioration in renal function prevented the development of glomerular and tubular damage and the disruption tissue architecture. A 2+ mononuclear cell infiltrate was still present however.

DISCUSSION

These results confirm the previous report that ALS and enhancing serum act synergistically in the suppression of renal allograft rejection in this model. One may conclude that true synergism exists because the action of enhancing serum in this strain combination is limited. No increase in effect would be expected from increasing the dose.

The total dose requirement of ALS to secure prolonged suppression of rejection was reduced from 3000µl to 1500µl i.e. a reduction of 50%. This suggests that enhancing serum

caused not only a delay, but also a reduction, in the intensity of rejection. Burgos et al. (1974) and Strom et al. (1975a)have shown that passive enhancement results in a delay in the cell-mediated immense response to a renal allograft but that in vitro the amplitude of the response was not reduced. My results are not consistent with an unmodified cell-mediated immune response in this model. It is interesting that this dose reduction of 50% was the same as that found for cyclophosphamide and enhancing serum in the previous section of this chapter.

As I was unable to establish an in vitro test for CMI the explanation for the synergistic effect remains incomplete. However, the histological differences between the groups (OAS-56 alone and OAS-56 plus ALS) are consistent with the hypothesis that ALS suppressed a delayed and attenuated cell-mediated rejection reaction.

The lymphocytotoxic antibody results showed a clear difference between wholly effective and partly effective doses of ALS used alone or in conjunction with enhancing serum. Suppression of rejection correlated with suppression of lymphocytotoxic antibody responses suggesting that at the fully effective doses of ALS, helper T-cells were suppressed.

The relationship between ALS dose and immunosuppression is not linear (Berenbaum et al., 1971). Thus one cannot conclude that, because the dose requirement of ALS in this experimental situation was halved, the degree of overall

immunosuppression in the animals treated with 375µl ALS was half of those treated with 750µl. The true difference between the two doses could perhaps be quantitated by comparing the effects of each on the size of the recirculating pool of lymphocytes.

The use of ALS and enhancing serum in the suppression of mouse skin graft rejection has been carefully studied by Koene et al. (1975). They showed that synergism between these two agents could only be demonstrated with a weak ALS or small doses of a strong ALS. With an optimal dose of "strong" ALS the addition of enhancing serum provided no benefit. They suggested that because ALS could override the effects of enhancing serum their targets were the same. This could explain why Finch and Morris (1977) failed to demonstrate any synergistic effect of ALS and enhancing serum in the suppression of pancreatic islet rejection.

The final point to be made is that the prolonged survival of the renal grafts without rejection must depend on some maintenance mechanism. The short course of ALS could have caused only a temporary depression of immunity. The delayed rejection and lymphocytotoxic antibody responses in animals treated with 375µl ALS alone suggest that a gradual recovery of immunity took place. Such a recovery should also have taken place in the animals treated with enhancing serum and ALS and yet only 1 out of 7 animals rejected. This suggests that a controlling mechanism developed during the period of immunodepression which was able to contain any recovery of immune function directed against the graft.

There is no evidence that the use of ALS in short courses after transplantation deletes an antigen/reactive clone thereby allowing prolonged survival of the graft. Mottram and Fabre (unpublished) showed that Lewis rats bearing (DA X Lewis)F_1 renal allografts following enhancing serum and ALS treatment did not reject a freshly transplanted homozygous DA graft nor did the infusion of 3×10^8 TDL enable them to do so. Guttmann et al. (1969b) showed that long surviving ALS treated rats bearing (Lewis X BN)F_1 renal allografts had normal GVH reactivity. However, adoptive transfer of sensitised Lewis lymphocytes to ALS treated recipients early after transplantation resulted in graft rejection whereas later transfer did not. They suggested that early after transplantation the effect of ALS was to suppress cell-mediated immunity and that a different mechanism accounted for their prolonged survival. Because a long surviving (Lewis X BN)F_1 kidney transplanted to a fresh Lewis recipient was not rejected they concluded that graft adaptation was responsible.

Later work by Biesecker et al. (1973c) explored this model further. They found that a single dose of ALS to a virgin Lewis animal resulted in a prolonged ($\pm$50 days) depression in the ability of such an animal to mount a cell-mediated immune response to a subsequent kidney graft. By 90 days normal reactivity had returned.

If the ALS was administered at the time of transplantation, the depression in cell-mediated immune responses to donor antigens was permanent. Lie et al. (1977) found that ALS treated, long surviving rat renal allograft recipients were able to reject donor skin grafts, albeit more slowly than controls, and were able to mount normal GVH responses. They concluded that a state of enhancement had developed in these ALS treated animals which was explained by the presence of donor-specific blocking factors. These were detected by the inhibition by sera from these long-survivors of rosette formation by recipient strain lymph node cells and donor-strain erythrocytes.

Thus, one can conclude from these experiments that the administration of a suboptimal dose of ALS during the period of induction of enhancement results in a temporary but significant degree of non-specific immunosuppression at a critical period. Although immune function probably recovers, the maintenance state of enhancement develops allowing continual acceptance of the allograft.

TABLE 1: Dose Response Results for ALS(DE)* on Lewis recipients (DA X Lewis)F_1 renal allografts
(Appendix 14)

Treatment	No.of Rats	Median blood urea ± SD (mg/100ml) at week: 10d	2	3	4	6	8	12	20	Survival and (range) in days
NIL	5	600±71	-	-	-	-	-	-	-	12 (12 - 13)
125µl	3	>750'	-	-	-	-	-	-	-	11 (8 - 12)
250µl	5	740±226	-	-	-	-	-	-	-	12 (11 - >140)
375µl	4	126±13	460±207	267±57	213±58	110±35	130±28	110±22	111±71	>140
500µl	3	122±42	166±186	120'	118'	128'	227'	202'	-	112 (19 - >140)
750µl	3	66±3	57±7	68±19	43±26	74±8	45±9	45±3	51±5	>140

* Given i.v. on days 0, 1, 3, 5.

' Less than 3 values

TABLE 2: Dose Requirements of ALS(DE)[1] to suppress rejection completely in enhanced[2] Lewis recipients of (DA X Lewis)F_1 renal allografts.

(Appendix 14)

Treatment	No.of Rats	Median blood urea ± SD (mg/100ml) at week: 10d	2	3	4	6	8	12	20	Median survival range (days)	Comparison with enhancement alone
AS-56 500µl plus NO ALS	6	125 ±69	682 ±124	218*	158*	153*	138*	152*	-	16 (14 - >140)	-
AS-56 500µl plus 250µl ALS	5	76 ±20	125 ±146	135 ±60	184 ±132	185 ± 66	143 ± 59	87 ±67	-	112 (37 - >140)	NS
OAS-56 500µl plus 375µl ALS	7	64 ±32	56 ±26	70 ±29	87 ±163	80 ±16	71 ±17	70 ±15	53 ±10	>140 (36 - >140)	$p < 0.05$

* Two animals

[1] ALS(DE) given i.v. on days 0,1,3,5.

[2] OAS-56 given i.v. on days 0,1,3.

TABLE 3: Dose response results for ALS(DE) on the lymphocytotoxic antibody response in Lewis recipients of (DA X Lewis)F_1 renal allografts.
(Appendix 15)

Treatment	No. of Rats	Median lymphocytotoxic antibody titre[2] at week: 1	10d	2	3	4	6	8	12	20
NIL	5	>512	16	-	-	-	-	-	-	-
ALS 125µl	3	>512	-	-	-	-	-	-	-	-
ALS 250µl	5	1	8	ND	2'	16'	16'	32'	16'	0'
ALS 375µl	3	0	0	4	2	8	4	1	0	0
ALS 500µl	3	0	0	1	1	2/1	2/1	2/1	0	-
ALS 750µl	3	1	0	0	0	0	0	0	0	0

[1] One animal only

[2] Expressed as reciprocal of last dilution to give >20% specific cytotoxicity

TABLE 4: The effect of ALS(DE)[3] on the lymphocytotoxic antibody response in enhanced[2] Lewis recipients of (DA X Lewis)F_1 renal allografts.
(Appendix 15)

Treatment	No.of Rats	Median lymphocytotoxic antibody titre[1] at week: 1	10d	2	3	4	6	8
OAS-56 500µl (No Transplant)	3	16	8	8	4	2	1	0
Transplant Recipients								
OAS-56 500µl plus NO ALS	5	1	4	2	-	-	-	-
OAS-56 500µl plus 250µl ALS	4	2	2	4	2/4	4/8	2	1
OAS-56 500µl plus 375µl ALS	5	1	1	0	1	0	0	0

[1] Expressed as reciprocal of last dilution to give >20% specific cytotoxicity

[2] OAS-56 given i.v. days 0,1,3.

[3] ALS(DE) given i.v. days 0,1,3,5.

TABLE 5: Histological Appearances

Treatment	Time biopsy taken	No. of specimens	Median Histological Score for each feature: Interstitium Monos	Oedema	Haem	Arteries Fibrinoid	Tubules Necrosis/Atrophy	Glomeruli Capillary Necrosis	Median Total Score
NIL	7days	4	o	+	o	o	++++	++++	10.5
ALS 125µl x 4	7d	3	+	o	o	++++	++++	++++	13
250µl x 4	7d	5	++	+++	++	o	+	+++	11
375µl x 4	7d	3	++	+	o	o	o	o	3
500µl x 4	7d	3	+++	o	o	o	o	o	3
750µl x 4	7d	3	o	o	o	o	o	o	0
OAS-56 only	7d	5	+++	+	o	o	o	o	4
plus ALS 250µl x 4	7d	5	++	+	o	o	o	o	3
plus ALS 375µl x 4	7d	7	++	+	o	o	o	o	2
Isografts	7d	5	+	o	o	o	o	o	1
Isografts	14d	4	+/o	o	o	o	o	o	0.5
OAS-56 only	14d	3	+++	+	o	o	++	+	7
OAS-56 plus ALS 375µl	14d	3	++	+	o	o	o	o	4

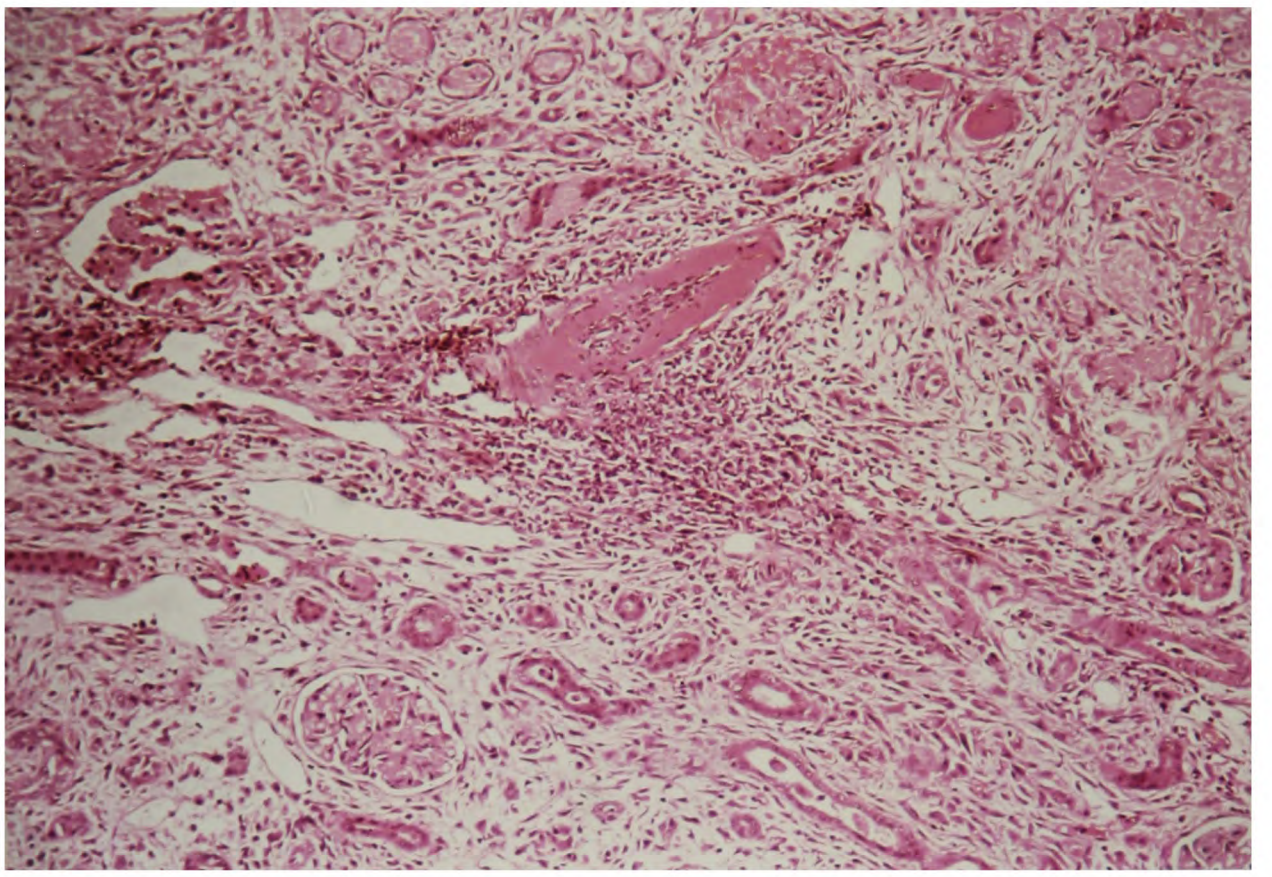


Fig. 2 (DA X Lewis)F_1 ⟶ Lewis renal allograft at 7 days after transplantation showing fibrinoid necrosis of an arteriole, glomerular capillary necrosis, tubular necrosis, interstitial oedema and cellular infiltrate. (H + E >140)

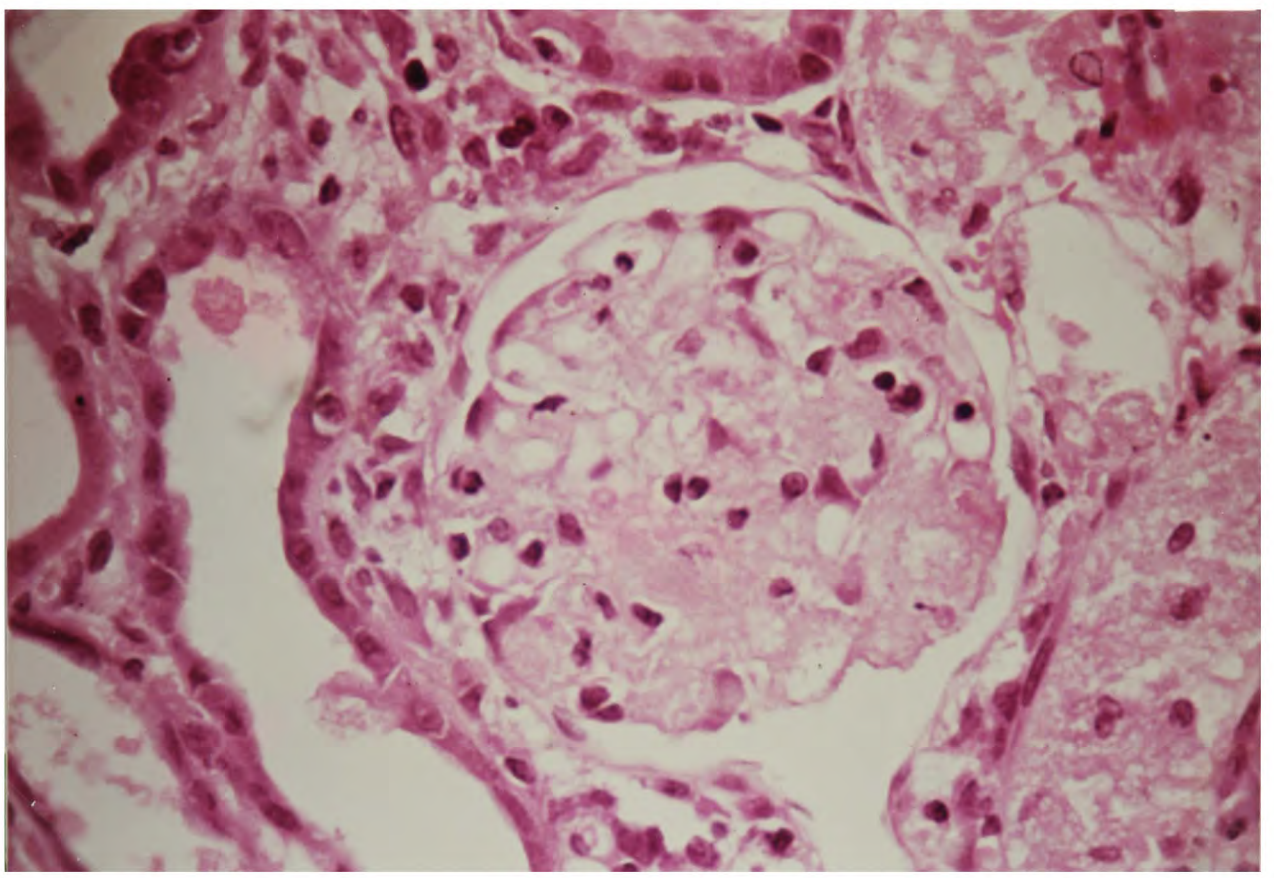


Fig. 3 (DA X Lewis)$F_1 \longrightarrow$ Lewis renal allograft at day 7 after transplantation showing a glomerulus with capillary necrosis and polymorphs in the lumina of the patent capillaries. (H + E x 420)

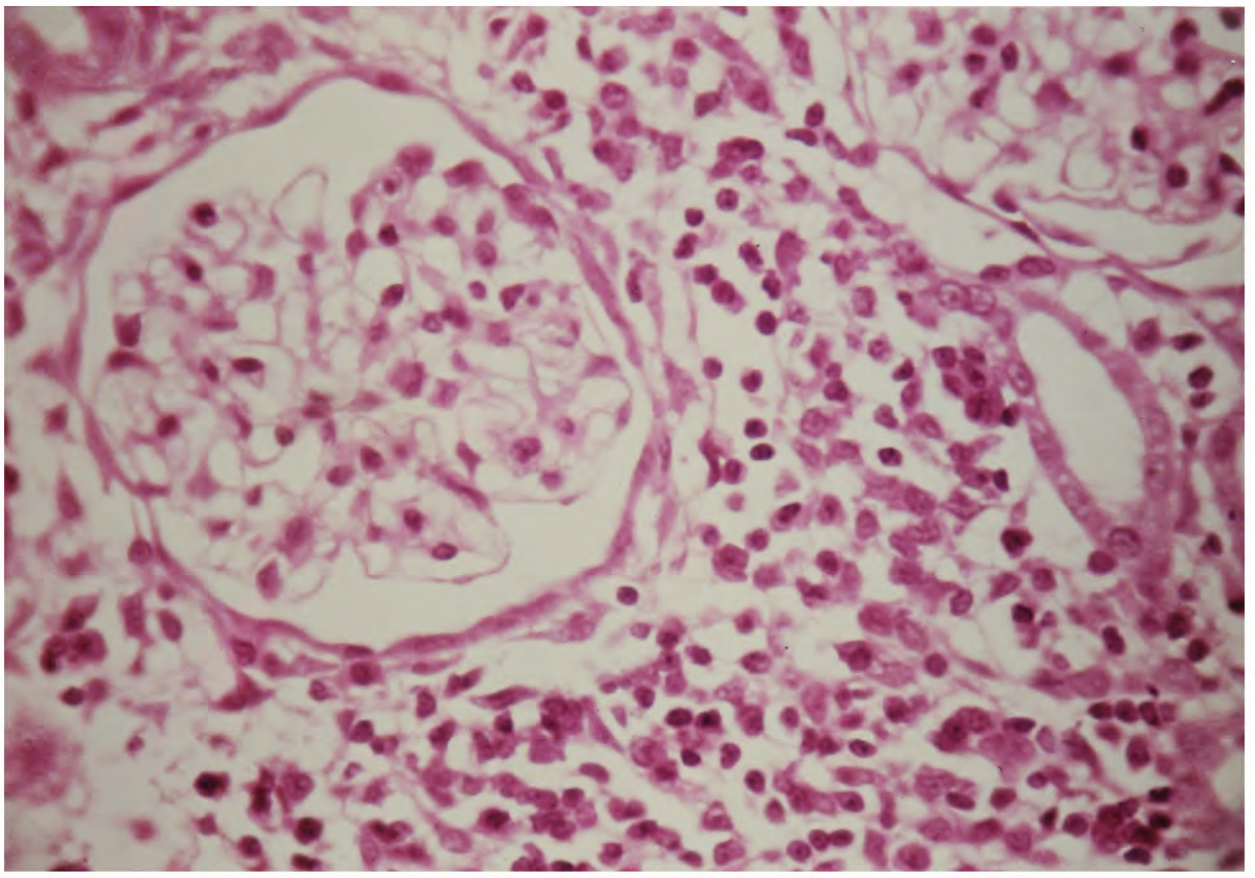


Fig. 4 Enhanced (DA X Lewis)$F_1 \rightarrow$Lewis renal allograft at day 7 after transplantation showing a normal glomerulus but marked interstitial oedema and a mononuclear cell infiltrate. (H + E x 420)

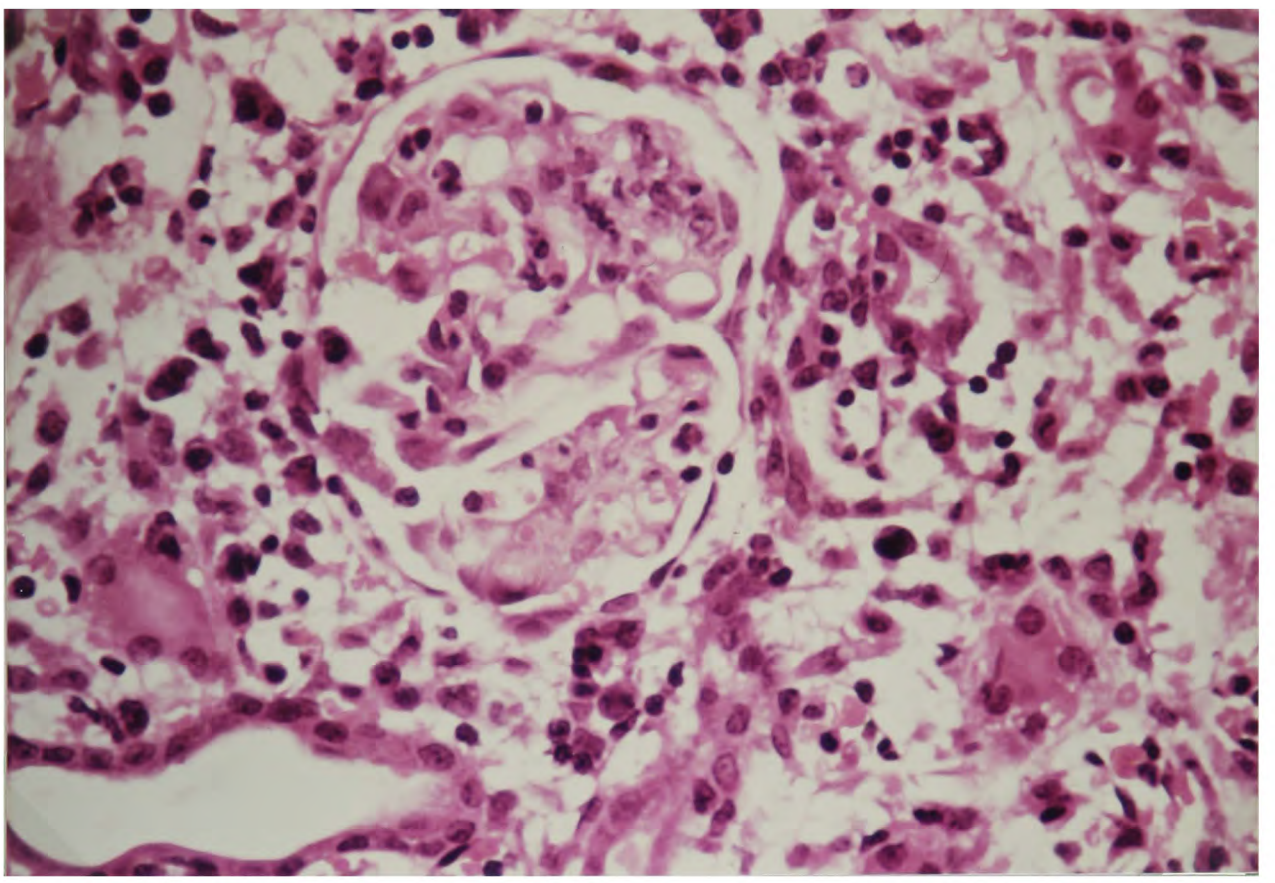


Fig. 5 Enhanced (DA X Lewis)F_1 → Lewis renal allograft 14 days after transplantation showing severe oedema, tubular disruption and focal necrosis of a glomerulus. (H + E x 420)

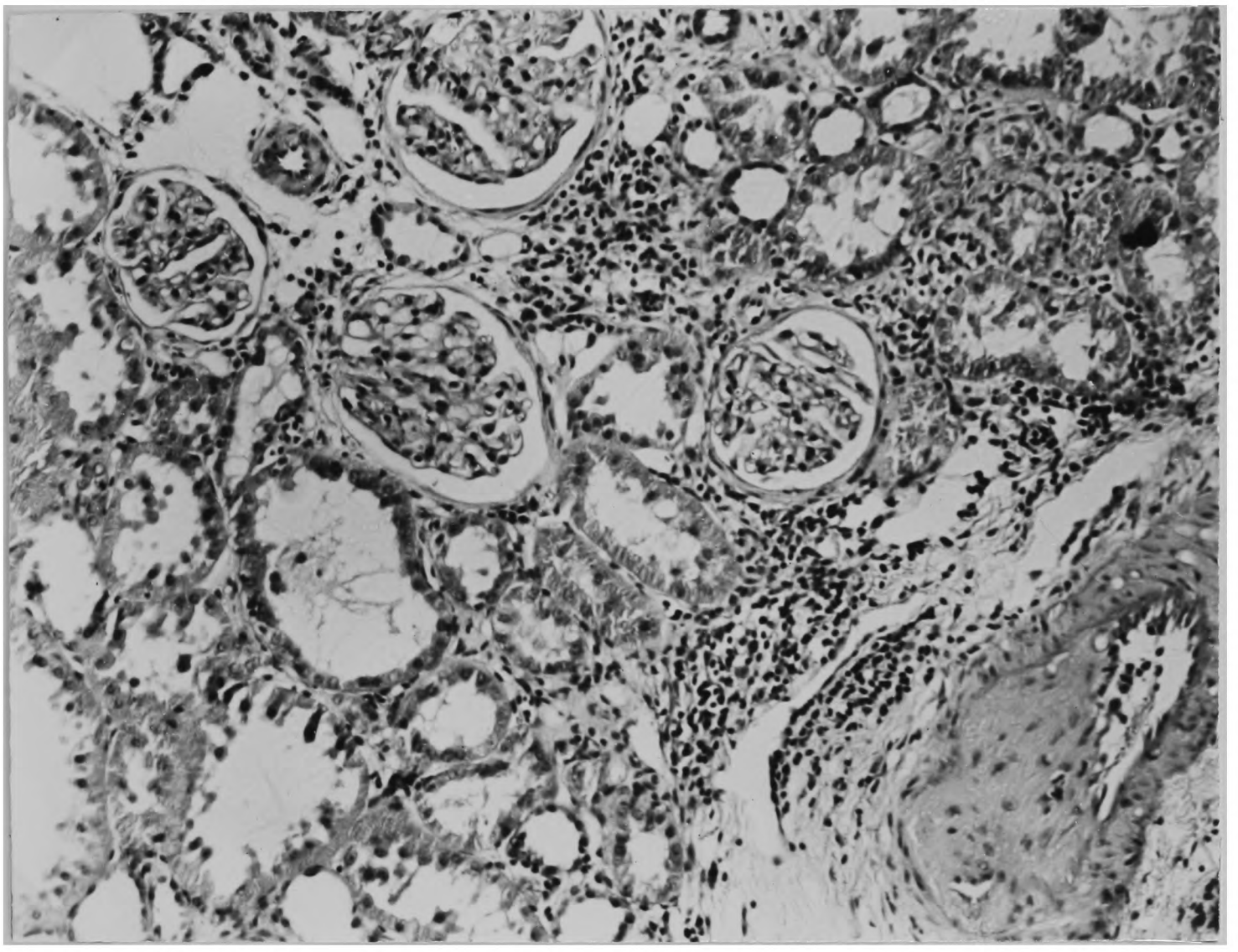


Fig. 6 Enhanced (DA X Lewis)$F_1 \rightarrow$ Lewis renal allograft $>$ 20 weeks after transplantation showing the features of chronic rejection i.e. interstitial fibrosis, scattered cellular infiltrates, intimal thickening of an artery and tubular atrophy.

(H + E x 190)

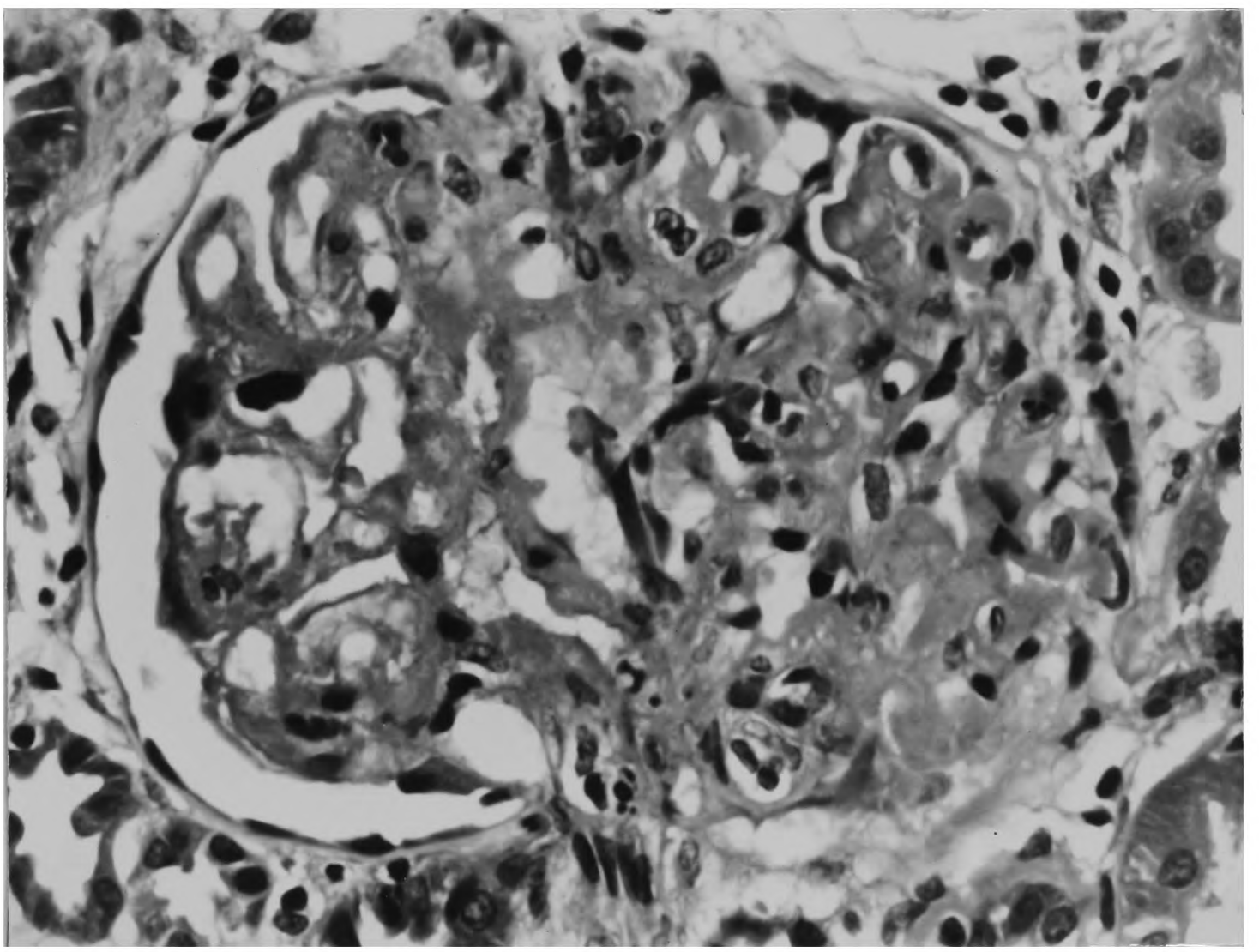


Fig. 7 Enhanced (DA X Lewis)$F_1 \rightarrow$ Lewis renal allograft > 20 weeks after transplantation showing a sclerosed glomerulus. (H + E x 770)

Legend to Fig. 8 - overleaf. (See Chapter 6 part ii)

(a) Lewis ⟶ Lewis renal isograft 14 days after transplantation showing normal appearances.

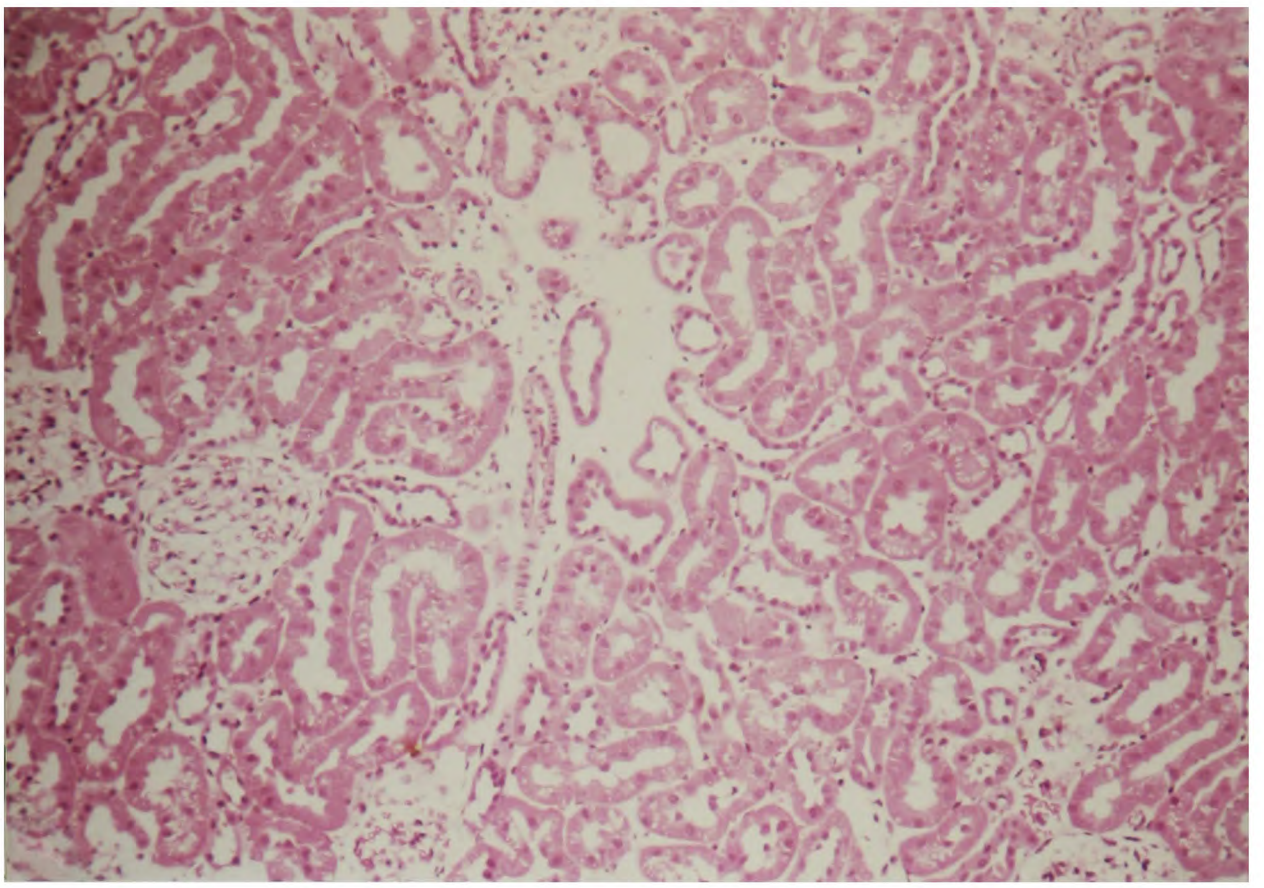
(b) (DA X Lewis)F_1 ⟶ Lewis renal allograft in a recipient treated with enhancing serum OAS-56 alone, 14 days after transplantation showing disruption of the tissue architecture, interstitial oedema, cellular infiltrate and damaged glomeruli.

(c) (DA X Lewis)F_1 ⟶ Lewis renal allograft in a recipient treated with enhancing serum OAS-56 <u>plus</u> cyclophosphamide 5.3mg/kg/day, at day 14 after transplantation. This shows preserved architecture, normal glomeruli but oedema and mononuclear cell infiltration are still present.

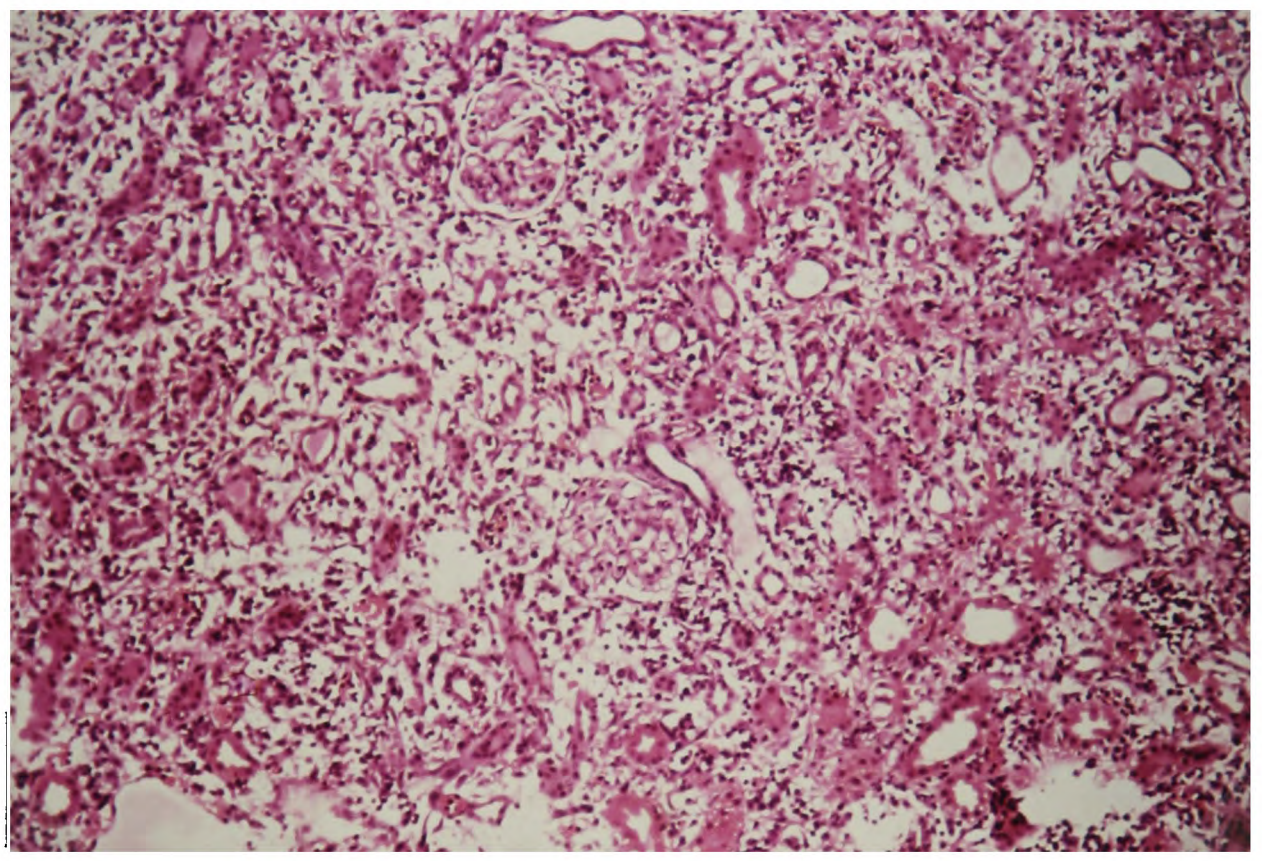
(H + E x 140)

Fig. 8

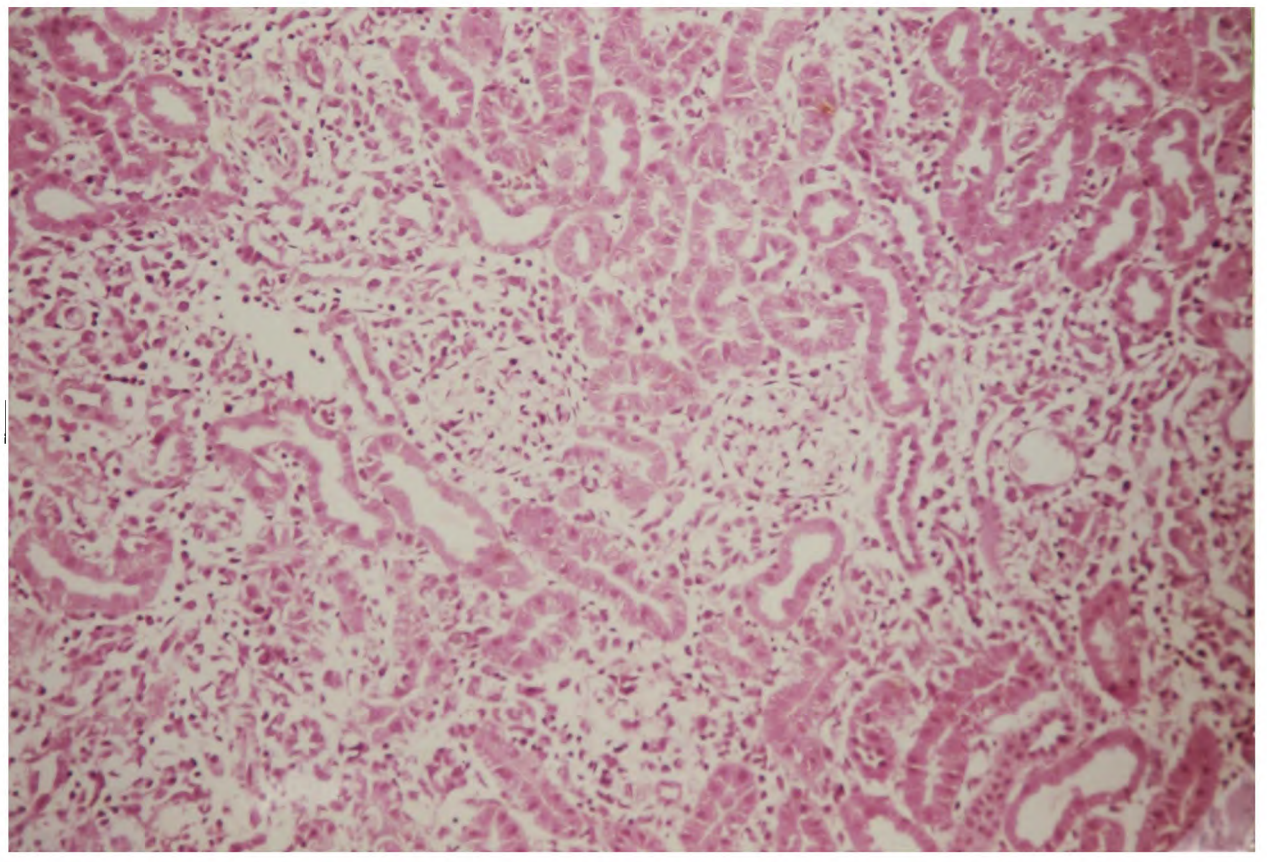
(a)



(b)



(c)



CHAPTER 7

EXPERIMENTS ON THE COMBINED USE OF CYCLOPHOSPHAMIDE AND ANTILYMPHOCYTE SERUM TO SUPPRESS RENAL ALLOGRAFT REJECTION

INTRODUCTION

Although the predominant role of T-cells in acute allograft rejection is well established, humoral mechanisms are also thought to contribute to the process (Carpenter, et al., 1976). In particular antibodies are thought to be responsible for vascular and glomerular lesions (Porter, 1976). In human renal transplantation the features of "acute humoral rejection" are found in patients on high doses of azathioprine and prednisolone in whom cell-mediated immunity should be suppressed. Moreover, this form of rejection has a particularly poor prognosis (Herbertson et al., 1977). Because primary humoral responses are relatively spared in patients receiving azathioprine and prednisolone (reviewed Morris, 1978a) a logical approach to the problem of the humoral component of rejection would be the use of immunosuppressants which act on both humoral and cellular immunity. Since ALS is directed at recirculating T-cells (its effect on humoral immunity is therefore dependent on suppression of helper T-cell function) its dominant effect is upon cell-mediated immunity. Cyclophosphamide has been shown to be relatively selective for resting B-cells, though if given after antigen can depress both T and B-cell immune responses (reviewed Turk et al., 1976). ALS and cyclophosphamide would seem an ideal combination for suppressing allograft rejection, and this combination has in fact previously been tested.

(Floersheim, 1969; Starzl et al., 1971; Polak et al., 1972; Caves et al., 1973). However, the design of these experiments was such that one could draw no valid conclusions about synergism (Berenbaum, 1977).

In the following experiments various doses of cyclophosphamide and antilymphocyte serum were used alone and together in the strong DA to Lewis combination. The pattern of rejection includes glomerular and arterial lesions thought to be characteristic of the humoral component of rejection in the rat (Abbas et al., 1974a and b).

MATERIALS AND METHODS

Rats, renal transplantation, lymphocytotoxic antibody titres: as before. DA kidneys were transplanted into Lewis recipients using the delayed nephrectomy model.

Cyclophosphamide Cyclophosphamide for injection, Endoxana® was used throughout. This was aliquotted as the powder which contains 450mg of sodium chloride/ 1069mg of cyclophosphamide B.P. The doses quoted are for cyclophosphamide B.P. The solution was made up fresh with water for injection immediately before use. The first injection was given i.v. but all subsequent doses were given i.p. In all experiments cyclophosphamide was given for 14 days from the time of transplantation (see Fig. 1).

Antilymphocyte serum: ALS 5, 6., a rabbit anti-rat lymphocyte serum was a gift from Dr. John Fabre. It had been prepared using a standard technique (see Appendix 13). The ALS was administered i.v. on days 0, 1, 3, 5, post-transplant (see Fig. 1).

Histological Assessment: It was in these experiments that the histological assessment technique was developed. The scoring was performed as described in Chapter 5 section 4. Both autopsy and biopsy specimens were examined.

Statistics and Assessment of Synergy: Comparisons between groups were performed using a two-tailed Wilcoxon rank sum test. The presence of synergy was assessed using the guidelines proposed by Berenbaum (1977). Synergy, additivism, or antagonism were deemed to have been demonstrated if the combination of the two agents produced an effect which was respectively, greater than, equal to, or less than that expected from the sum of its two constituents.

i.e. if $\frac{x}{y} + \frac{a}{b} < 1$ the combination was synergistic

$= 1$ the combination was additive

> 1 the combination was antagonistic

x = dose of cyclophosphamide used in the combination.

y = dose of cyclophosphamide needed to produce the same effect as the combination.

a = dose of ALS used in the combination.

b = dose of ALS needed to produce the same effect as the combination.

Experimental Groups: The treatment protocol is shown in Figure 1.

(1) Controls

No treatment given.

(2) ALS Titration

(a) 100µl x 4 (days 0,1,3,5) Total dose = 400µl

(b) 200µl x 4 (" ") " = 800µl

(c) 350µl x 4 (" ") " =1400µl

(d) 500µl x 4 (" ") " =2000µl

(3) Cyclophosphamide Titration

(a) 3.5mg/kg/day x 14 days Total dose = 49mg/kg

(b) 4.9mg/kg/day x 14 days " = 69mg/kg

(c) 7.7mg/kg/day x 14 days " = 108mg/kg

(d) 10.6mg/kg/day x 14 days " = 148mg/kg

(4) Combination Groups

(a) ALS 200µl x 4, plus cyclophosphamide 4.9mg/kg/day x 14 days

(b) ALS 200µl x 4, plus cyclophosphamide 3.5mg/kg/day x 14 days

(c) ALS 100µl x 4, plus cyclophosphamide 3.5mg/kg/day x 14 days

RESULTS

Renal Function and Survival (Tables 1, 2, 3 & 4 and Appendix 16)

The dose response results for ALS (Table 1) showed that only the highest dose tested (500µl) suppressed early acute rejection (median blood urea ± SD at day 10 = 70 ± 8mg/100ml) but the effect was short-lived for 4 of the 5 animals had had rejection episodes by 3 weeks (median blood urea ± SD at day 21 = 200 ± 61mg/100ml).

The results for cyclophosphamide (Table 2) showed that the 10.6mg/kg/day dose suppressed rejection completely but this dose appeared toxic since 3 of the 4 animals died with normal renal function. At the lower doses of cyclophosphamide 3.5mg/kg/day and 4.9mg/kg/day the blood urea levels at day 10 were not significantly lower than controls ($p > 0.05$) but survival was significantly prolonged in the group treated with 4.9mg/kg/day ($p < 0.02$).

The combination of ALS 200µl and cyclophosphamide 4.9mg/kg/day suppressed acute rejection completely (Table 3). The median blood urea ± SD at 10 days = 55 ± 17mg/100ml which was the effect obtained with 10.6mg/kg/day of cyclophosphamide and a greater effect than that obtained with 500µl of ALS. In contrast to the ALS 500µl group, 4 of 5 of which had rejected by 3 weeks, 7 of 8 of the combination group had normal blood urea levels at this time. It is arguable whether this dose combination was synergistic because 10.6mg/kg/day of cyclophosphamide may be a greater

dose than that actually required to achieve complete suppression of rejection. If the effect of the combination of ALS and cyclophosphamide is assumed to be equal to the effect of cyclophosphamide alone at 10.6mg/kg/day, the calculation to detect synergism is as follows:-

$$\text{Sum} = \frac{4.9}{10.6} + \frac{200}{>500} = \angle\, 0.86$$ suggesting the presence of synergism

If one uses the results obtained with 7.7mg/kg/day of cyclophosphamide which were <u>worse</u> than the combination

$$\text{Sum} = \frac{4.9}{>7.7} + \frac{200\mu l}{>500\mu l}$$

$$= <0.64 + <0.4 = \angle 1.04$$

Therefore this combination is definitely additive and possibly synergistic. However, despite impressive suppression of rejection the animals appeared unwell and 5 of 9 died in the first month with minimal evidence of rejection in the allografts at autopsy.

Calculations for the other two combinations also suggested that the combinations were additive rather than synergistic in their effect on acute rejection as measured by 10 day blood urea levels (see Table 4). Analysis for a synergistic effect on graft survival was not performed because survival was adversely affected by under-immunosuppression (rejection) and by over-immunosuppression (infections/toxicity).

Histological studies of 7 day biopsies and autopsy specimens (Tables 5 & 6).

Biopsies from untreated controls showed severe rejection changes including glomerular capillary necrosis, interstitial haemorrhage, arterial fibrinoid necrosis in half of the specimens, but only a few infiltrating mononuclear cells.

The 100µl dose of ALS made no difference to the histological pattern. At the 200µl dose, which had only a slight effect on rejection as measured by graft function, the histological score was significantly lower than controls. Glomerular capillary necrosis was less severe and only one of four autopsy specimens showed arterial fibrinoid necrosis.

The 3.5mg/kg dose of cyclophosphamide had no significant effect on the histological pattern apart from a lower incidence of arterial fibrinoid necrosis at autopsy, 2/5 vs 4/4 in controls. At the 4.9mg/kg dose of cyclophosphamide which like the 200µl dose of ALS had only a trivial effect on rejection as measured by graft function, the mean histological score for the group was significantly lower than controls.

At the higher doses of both agents the histological features rejection became progressively milder and consisted of a mild to moderate cellular infiltrate and interstitial oedema.

There was no discernible difference between the two agents on the type of histological lesion prevented i.e. cyclophosphamide showed no selectivity for the lesions thought to be humorally-mediated and ALS was no more effective than cyclophosphamide in reducing the degree of mononuclear cellular infiltrate.

Synergy was not detected in the combinations (Table 4). In the highest dose combination group (ALS 200µl plus cyclophosphamide 4.9mg/kg) there was little evidence of rejection and the mean score for this group was not significantly different from those in the ALS (500µl) and cyclophosphamide (10.6 mg/kg) groups.

Lymphocytotoxic Antibody Titres (Table 7 and Appendix 17)

Untreated controls produced a strong lymphocytotoxic antibody response. The response was significantly suppressed by both ALS and cyclophosphamide at all doses tested. Cyclophosphamide may have been marginally superior to ALS in that the response was completely abolished at doses $\geqslant$4.9mg/kg/day. ALS treated animals, even at the highest dose, 500µl, showed a delayed but definite lymphocytotoxic antibody response. There was, however, no striking difference between ALS and cyclophosphamide in suppressing the 7 day lymphocytotoxic antibody titres. Lymphocytotoxins were not detected in the groups treated with the combinations of agents.

DISCUSSION

These results show no important differences between the action of antilymphocyte serum and cyclophosphamide on the suppression of renal allograft rejection, lymphocytotoxic antibody responses or the histological pattern of rejection.

Although there was no striking synergy when the two agents were used together, there was clearly an additive effect in that the combinations suppressed rejection as effectively as large doses of antilymphocyte serum or cyclophosphamide alone. However, the high dose combination (ALS 200µl plus cyclophosphamide 4.9mg/kg) appeared to be more toxic than the 500µl dose of ALS. The median survival in the ALS (500µl) group was 80 days compared to 30 days in the combination group. Whereas death in the ALS group appeared to follow rejection, toxicity accounted for the early deaths in the combination group.

These results also showed that there was no correlation between suppression of the humoral response and suppression of rejection. The doses of ALS and cyclophosphamide which suppressed the humoral response were substantially lower than those required to suppress rejection of the graft. For example at a dose of 3.5mg/kg of cyclophosphamide the median 7 day lymphocytotoxic titre was 1:1 compared to $>$ 1:512 for controls and yet there were no differences in 10 day blood urea levels, survival times or

histological scores. Despite the depression in antibody levels, this group showed glomerular necrosis in 7 day biopsies and arterial fibrinoid necrosis at autopsy in 2/5 cases.

There did appear to be some correlation (Table 6) between the incidence of arterial fibrinoid necrosis found at autopsy and the level of the lymphocytotoxic antibody response. Glomerular necrosis on the other hand was seen in animals with no detectable lymphocytotoxins.

The suppression of the humoral response by ALS alone is consistent with the fact that the primary response to MHC antigens is T-dependent (Rolstad et al., 1974). These results showed that ALS suppressed the lymphocytotoxic antibody response at lower doses than those required to suppress rejection. This is supported by the finding of a marked mononuclear cell infiltrate in grafts of animals treated with 200µl doses of ALS which almost completely suppressed the humoral response (Tables 5 & 7).

This finding contrasted with that of Lance and Batchelor (1968) who showed ALS treated mice bearing healthy skin allografts had produced anti-donor haemaglutinins. It also suggests that adequate doses of ALS should suppress the "humoral" component of rejection.

The combination of ALS and cyclophosphamide was investigated in a dog heart transplant model by Caves et al. (1973). Although the best results were obtained with

ALS and cyclophosphamide and prednisone, there was a high incidence of death due to toxicity. No dose studies were performed which precluded any assessment of synergism. It is significant that the combination of ALS and cyclophosphamide was no better than the combination of azathioprine and antilymphocyte serum.

Cyclophosphamide has been substituted for azathioprine in human renal transplantation and although no formal trial was conducted the result was similar for both agents (Starzl et al., 1971).

Putnam et al. (1975) investigating the use of cyclophosphamide in dog renal transplants and found that although it suppressed the response to SRBC, and reduced the incidence of arterial lesions it did not prolong graft survival. It was moreover no more effective than azathioprine in the prevention of vascular lesions and did not augment the effect of azathioprine in prolonging graft survival.

Friedman et al. (1973) used azathioprine,cyclophosphamide and prednisolone in a rabbit renal transplant model and found the use of all three drugs together significantly prolonged survival whereas single and double drug regimens were ineffective. The essential dose response studies were not performed making it impossible to assess whether the better results obtained with the combination were any more than a reflection of the larger total amount

of immunosuppression in the triple drug regimen.

Beatty et al (1978) failed to show any advantage in conjugating ALS to an alkylating agent Trenimon, in the suppression of skin allograft rejection in the mouse. They did find that the combined use of both agents unconjugated gave better results than those obtained with either agent alone. This too was misconstrued as an example of synergism.

Polak et al. (1972) showed that the use of ALS and an ineffective dose of cyclophosphamide gave greater prolongation of skin graft survival in guinea-pigs than ALS alone. They attributed this difference to the prevention by cyclophosphamide of the development of antibodies to ALS thus prolonging its effect. This is unlikely to be the explanation for the superiority of the combination. ALS acts immediately and is rapidly cleared from the circulation. The duration of the immunosuppressive effect depends not on the persistence of the agent in the circulation but on the time taken for the depleted recirculating lymphocyte pool to recover. A more likely explanation is that cyclophosphamide delayed this recovery.

It would be interesting to test whether small doses of cyclophosphamide given continually after ALS could maintain the state of immunosuppression by delaying recovery. This possibility is suggested by the fact that animals treated with the high dose combination (200µl of ALS and 4.9mg/kg of cyclophosphamide) took longer to recover

from the course of immunosuppression than those treated with the high dose of ALS(500µl). Whereas the latter all showed rejection by 3 weeks the former did not. Simmons et al. (1968) have shown that it is possible to prolong the effect of ALS by the administration of either corticosteroids or methotrexate.

These results show that cyclophosphamide in adequate doses can suppress both the humoral and cellular immune response to a renal allograft, although an effect on graft survival required much larger doses than those which suppress the humoral response. The well established B-cell selectivity of cyclophosphamide is only apparent if given before antigenic challenge and is likely to be due to a difference in turnover rates of resting B- and T-cells. (Turk and Poulter, 1972). If cyclophosphamide is given after antigenic challenge, a time when both B- and T-cells are proliferating, no differential effect is likely to be seen. (Turk and Stone, 1963)

I concluded from these experiments that although cyclophosphamide and ALS act additively in the suppression of renal allograft rejection there is no particular advantage in the use of them together.

Fig 1

Treatment Schedule

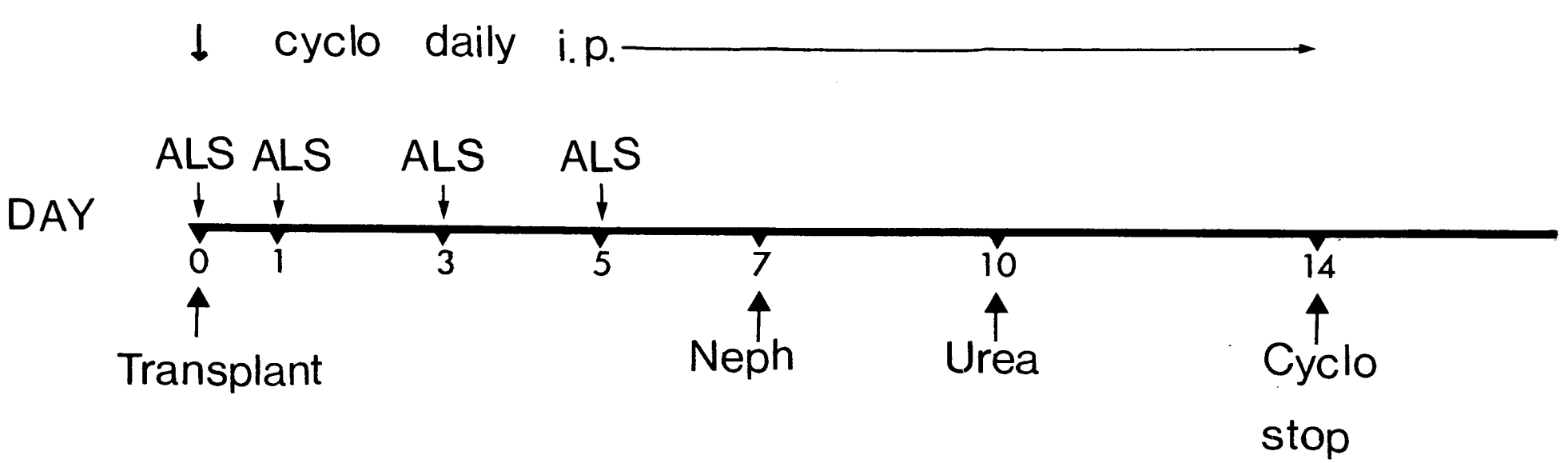


TABLE 1 (Appendix 16) Titration of ALS (5,6) - Effect on blood ureas and survival times of Lewis recipients of DA renal allografts

Treatment	No.of Rats	Median blood urea (mg/100ml) ± SD at weeks: 10d	2	3	4	6	8	12	20	Median survival and range (days)
NIL	10	587±52	-	-	-	-	-	-	-	11 (10 - 14)
100µl x 4	5	620±90	-	-	-	-	-	-	-	11 (9 - 13)
200µl x 4	5	550±172	-	-	-	-	-	-	-	13 (10 - >140)
350µl x 4	3	125*±185	510*±164	-	-	-	-	-	-	16 (13 - 19)
500µl x 4	5	70±8	110±17	200±61	220±53	140±190	115±54	110*	164*	80 (60 - >140)

* Less than 4 values

TABLE 2 Titration of Cyclophosphamide - Effect on blood ureas and survival times of Lewis recipients of DA renal allografts.
Appendix 16)

Treatment	No.of Rats	Median blood urea (mg/100ml) ± SD at weeks: 10d	2	3	4	6	8	12	20	Median survival and range (days)
NIL	10	587±52	-	-	-	-	-	-	-	11 (10 - 14)
3.5mg/kg	5	580±132	-	-	-	-	-	-	-	11 (10 - 12)
4.9mg/kg	9	510±146	170±46	195±267	-	-	-	-	-	21 (10 - 100)
7.7mg/kg	3	165*±95	113*	-	-	-	-	-	-	17 (13 - 18)
10.6mg/kg	4	57±9	51±7	50*±46	-	-	-	-	-	23 (20 - 80)

* Less than 4 values

TABLE 3 (Appendix 16) Combination of ALS and Cyclophosphamide - Effect on blood ureas and survival times of Lewis recipients of DA renal allografts

Treatment	No.of Rats	Median blood urea (mg/100ml) ± SD at weeks: 10d	2	3	4	6	8	12	20	Median survival and range (days)
ALS 100µl^{x4} plus Cyclo 3.5mg/kg	5	160±92	415±333	466*	-	-	-	-	-	23 (15- >140)
ALS 200µl^{x4} plus Cyclo 3.5mg/kg	5	88±30	103±249	75*	68*	-	-	-	-	30 (17 - 95)
ALS 200µl^{x4} plus Cyclo 4.9mg/kg	9	55±17	57±6	59±26	59*	115*	230±181	305*	250*	30 (16 - >140)

* Less than 4 values

TABLE 4 **Results of the Analysis of the Combinations**

Combination	Sum of ALS and Cyclophosphamide Fractions	
	Blood urea Day 10	Histological Score at day 7
1. ALS 100µl Cyclo 3.5mg	>0.62 <0.95 synergism not proven	>0.74 <1.2 synergism not proven
2. ALS 200µl Cyclo 3.5mg	>0.73 <1.02 synergism not proven	>0.9 <1.71 synergism not proven
3. ALS 200µl Cyclo 4.9mg	<1.04 synergism not proven	>0.86 <1.2 synergism not proven

TABLE 5 Histological Features - 7 day biopsies of DA allografts in Lewis recipients

Treatment	No. of Specimens	Intestitium				Arteries		Tubules		Glomerular Capillary Necrosis	Mean Score ± S.D.
		Mono	Poly	Oed	Haem	Fibrin	Subint Monos	Atrophy	Necrosis		
NIL	4	+	++	+	+++	±	o	o	+++	+++	13.2±2
ALS 100µl x 4	5	+	+	+	+++	±	o	+	+++	+++	14.6±2
200µl x 4	5	+++	o	++	o	o	o	++	o	+	8±1
350µl x 4	3	++	o	+	o	o	o	o	o	o	3.3±0.6
500µl x 4	5	+	o	+	o	o	o	o	o	o	2.4±0.5
Cyclo 3.5mg/kg	5	++	o	+++	o	o	o	++	+	+++	11.8±3
4.9mg/kg	7	+++	o	+++	o	o	o	++	o	+	9.6±2.7
7.7mg/kg	3	++	o	++	o	o	o	o	o	o	4.3±0.6
10.6mg/kg	4	++	o	+	o	o	o	o	o	o	3±0
Combination ALS 100µl plus Cyclo 3.5mg/kg	5	++	o	++	o	o	o	+	o	+	7±1.9
ALS 200µl plus Cyclo 3.5mg/kg	5	++	o	+	o	o	o	+	o	o	4.2±0.4
ALS 200µl plus Cyclo 4.9mg/kg	9	+	o	+	o	o	o	+	o	o	2.3±1.4

Key: mono = mononuclear cell infiltrate; poly = polymorph cell infiltrate; oed = oedema; haem = haemorrhage; fibrin = fibrinoid necrosis; subint monos = subintimal mononuclear cell

TABLE 6 Incidence of arterial fibrinoid necrosis found at autopsy

(Appendix 17)

Treatment	Incidence	Median lymphocytotoxic titre at day 7
NIL	4/4	>1/512
ALS 100µl x 4	5/5	1/16
ALS 200µl x 4	1/4	1/1
Cyclophosphamide 3.5mg/kg/day	2/5	1/1
Cyclophosphamide 4.9mg/kg/day	0/5	0

TABLE 7

(Appendix 17)

The effect of ALS (5,6) and Cyclophosphamide on lymphocytotoxic antibody responses of Lewis recipients of DA allografts

Treatment	Rat No.	Median lymphocytotoxic antibody titre (reciprocal) at week: 1	10d	2	3	4	6	8	12	20
NIL	10	>512	>512	-	-	-	-	-	-	-
ALS (5,6)										
100µl x 4	5	16	32	-	-	-	-	-	-	-
200µl x 4	5	1	2	1	-	-	-	-	-	-
350µl x 4	3	0	0	4	-	-	-	-	-	-
500µl x 4	5	0	0	0	0	2	0	0	0	0
Cyclophosphamide										
3.5mg/kg/day x 14	5	1	4	-	-	-	-	-	-	-
4.9mg/kg/day x 14	9	0	0	1	0	0	0	0	-	-
7.6mg/kg/day x 14	3	0	0	0	-	-	-	-	-	-
10.3mg/kg/day x 14	4	0	0	0	0	-	-	-	-	-
Combinations										
100µl ALS plus cyclo 3.5mg/kg	5	0	0	0	0	-	-	-	-	-
200µl ALS plus cyclo 3.5mg/kg	5	0	0	0	0	0	-	-	-	-
200µl ALS plus cyclo 4.9mg/kg	9	0	0	0	0	0	-	-	-	-

CHAPTER 8

EXPERIMENTS ON THE PROBLEM OF IMMEDIATE ANTIBODY-MEDIATED GRAFT DAMAGE

I(a) The use of $F(ab')_2$ fragments of enhancing antibodies in the Lewis $\rightarrow$ DA combination.

I(b) Attempts to block enhancement with $F(ab')_2$.

II Antibody-mediated damage in the DA $\longrightarrow$ Lewis model - two approaches to its prevention:

(a) Blocking by Lewis anti-DA $F(ab')_2$.

(b) Use of a red cell absorbed (anti-Ia-like) serum.

INTRODUCTION

One of the major barriers to the clinical application of passive enhancement is the problem of immediate antibody mediated damage to the allograft. The experiments described in this section were designed to investigate this problem.

I(a) The use of $F(ab')_2$ in the Lewis $\longrightarrow$ DA model.

Because antibody-mediated damage to a graft occurs after interaction of the alloantibody with graft antigen followed by complement fixation, the feasibility of using the non-complement-fixing $F(ab')_2$ fraction of the antibody molecule for enhancement has been studied by several groups. The prevailing view is that $F(ab')_2$ can induce enhancement but that it is less effective than whole IgG. However, in most of these studies the difference in enhancing capacity of whole IgG and $F(ab')_2$ was not adequately compared. Moreover, in many cases neither the purity of the $F(ab')_2$ nor the antigen binding capacity after pepsin degradation were adequately established. The purity of the $F(ab')_2$ is of particular importance, since minute quantities of whole antibody can cause enhancement (Fabre and Morris, 1973).

The Lewis to DA renal allograft model was chosen because of the consistent enhancement of kidney allografts that can be obtained with low doses of enhancing serum in this strain combination (Fabre and Morris, 1974a). The basic experimental design was as follows. A large dose of hyperimmune DA anti-Lewis serum was raised and shown to enhance Lewis renal allografts in DA recipients. $F(ab')_2$ was prepared from the IgG of this serum, purified and the IgG contamination quantitated. The antigen binding capacity, complement-dependent lymphocytotoxicity, and in vivo enhancing capacity of the $F(ab')_2$ preparation were then compared with those shown by unmodified serum and IgG.

MATERIALS AND METHODS

Rats, renal transplantation, histological assessment, lymphocytotoxic antibody titrations, and the antigen binding assay have been described in the chapter on materials and methods.

Antiserum

OAS-58 was raised in 18 rats by serial spleen cell immunizations (see Chapter 5 section 3) and harvested by several cardiac punctures and a final bleed by aortic puncture. The blood was allowed to clot, incubated at 37°C for 1 hour, the clot separated with an orange-stick and left at 4°C overnight. The serum was then

centrifuged at 900G for 30 minutes and stored at -80°C. The batches of serum obtained from the cardiac punctures and the final bleed were pooled yielding a volume of 111 mls.

Preparation of IgG and $F(ab')_2$

The pooled serum was centrifuged at 35,000g for 20 minutes at 20°C on a Beckman L5-65 Ultracentrifuge to remove debris. Ten mls of serum were retained for in vivo use.

IgG was precipitated using 18% w/v sodium sulphate. When the sodium sulphate was fully dissolved the solution was incubated at 37°C for 30 minutes in a water bath and then centrifuged at 35,000g for 20 minutes. The precipitate was then redissolved in distilled water at 40% of the original serum volume. This precipitation procedure was then repeated twice. The final IgG precipitate was dissolved in 20mls of distilled water and dialysed twice against 1 litre of sodium acetate buffer adjusted to pH 4.2 with glacial acetic acid. After dialysis the sample was centrifuged at 1000g at 20°C for ten minutes.

The protein concentrations were measured on an SP 500 spectrophotometer assuming an E^{280} of 14.0 for 1% solutions for both IgG and $F(ab')_2$ (Little and Donahue, 1968).

After adjusting the protein concentration to 10mg/ml, the IgG was digested with pepsin (Sigma Chemical Company, 35,000 units/mg) at an enzyme:substrate ratio of 4:100 for 20 hours at 37°C, pH 4.2 (Williams, 1975). After centrifugation at 900g for 10 minutes the digestion was stopped by adjusting the pH to 7.0 using 2M TRIS. To control for experimental manipulation during the $F(ab')_2$ preparation, a portion of the sodium sulphate precipitated IgG was incubated at pH 4.2 for 20 hours without pepsin, and the pH then adjusted to 7.0.

Both samples were then dialysed against Tris-buffered saline (with 0.02% sodium azide and pH adjusted to 7.2 with 5M HCl). The volumes were then reduced to a convenient size for gel filtration using an Amicon Ultrafiltration cell Model 52 with a 5 UM10 Diaflo filter.

The samples were then run on a Sephadex G-200 column and 5ml samples collected with a fraction collector. The elution profile for IgG is shown in fig.1 and for the $F(ab')_2$ preparation in fig. 2. The IgG specimen showed 2 peaks. The first was in the same position as blue dextran (mw 2,000,000) for this column and was assumed to be aggregates. The second peak was in the expected position for IgG and was pooled and dialysed against normal saline to remove sodium azide before in vivo use. The protein concentration of the IgG sample was 0.76mgs/ml. It was aliquotted, assayed and used at this concentration which was assumed to be approximately

a 1/10 dilution of serum IgG. To have used the IgG at 7-10mg/ml would have required concentration to an unmanageably small volume. The $F(ab')_2$ preparation showed 4 peaks: (a) a small peak of undigested aggregates, (b) the $F(ab')_2$ and undigested IgG, (c) pFc' and (d) peptides. To minimise the amount of IgG included in the $F(ab')_2$ preparation, only the latter half of the second peak was pooled (the shaded area in fig. 2). After pooling, the $F(ab')_2$ preparation was dialysed against normal saline to remove the sodium azide and the concentration adjusted to 6mg/ml by ultrafiltration.

The purity of the $F(ab')_2$ preparation was examined (see Chapter 5 section 7) using SDS polyacrylamide gel electrophoresis. The SDS gels showed that an obvious IgG contaminant was present in the $F(ab')_2$ preparation (see Fig. 3). The $F(ab')_2$ was therefore further purified by passage through a Sepharose CL-4B-protein A column (Pharmacia Sweden). Protein A is a constituent of staphylococcal cell walls and has affinity for the Fc portion of certain IgG subclasses; it will therefore bind IgG but not $F(ab')_2$. The gel was prepared by soaking the beads in Tris-buffered saline with 0.02% azide and then pouring them into a 10ml sealed glass syringe. The gel was then allowed to pack to a volume of 5mls. The binding capacity of the gel was 25mg IgG/ml of gel. The $F(ab')_2$ was passed slowly through the column, dialysed against normal saline to remove the sodium azide,

reduced to a concentration of 6mg/ml and aliquotted for in vivo use.

RESULTS

(i) Characteristics of the $F(ab')_2$ preparation

The antigen binding capacity of the enhancing serum OAS-58, IgG, $F(ab')_2$ and normal DA serum were compared using the binding assay, and were found to be essentially similar (see fig. 4). The comparison of the preparations in the two-stage complement-dependent ^{51}Cr lymphocytotoxicity assay are shown in fig. 5. As expected the $F(ab')_2$ had lost its complement-dependent lympho-cytotoxicity whereas the IgG and OAS-58 gave similar titration curves.

The purity of the $F(ab')_2$ preparation was then quantitated on SDS gels, the sample being compared with a standard IgG preparation (obtained from Dr. John Fabre). The standard IgG had been purified by DEAE-50 cellulose ion exchange chromatography and had been shown to be $>95\%$ pure. Two µg, 1µg, 0.5µg, 0.25µg, 0.125µg and 0.0625µg samples of the standard IgG were loaded to separate gels and compared with the IgG band on a gel to which 100µg of the $F(ab')_2$ preparation had been loaded (see fig. 6). The gels were scanned using a Gilford Gel Scanner and a Unicam SP 500 spectrophotometer at a wavelength of 560mµ and a scan rate of 2cms/minute.

The height of the peaks for the known amounts of IgG were compared with those of the $F(ab')_2$ preparation. From these readings the $F(ab')_2$ was shown to be $>99\%$ pure.

(ii) Transplant experiments

The results of the transplantation experiment are shown in Table 1 and Appendix 18.

Control (untreated animals) all underwent acute fatal rejection episodes. Both 25µl and 50µl doses of enhancing serum OAS-58 gave excellent enhancement. The 500µl dose of the IgG preparation (which in terms of binding capacity was equivalent to the 50µl dose of enhancing serum) also enhanced but the effect was less consistent than with the unmodified serum. Although blood urea levels at 10 days were low, only 1 of the 5 animals had a rejection-free course. The animals treated with the 500µl dose of $F(ab')_2$ all had severe acute rejection (median blood urea $\pm$ s.d. at day 10 = 632 $\pm$ 83mg/ml). This rejection was fatal in 4 of the 6 recipients. Rats 2323 and 2325 died on day 8 i.e. one day after nephrectomy. Autopsy revealed severe rejection and pulmonary congestion. Two animals 2317 and 2342 survived the acute rejection episodes whereas no animals in the control group survived. This suggested that the $F(ab')_2$ preparation had a small immunosuppressive effect. Assuming the $F(ab')_2$ preparation contained 1% IgG, each 500µl dose of $F(ab')_2$ would have

included the equivalent of 5ul of whole serum or 50µl of the IgG preparation. To test whether this contamination could account for the slight effect of the $F(ab')_2$ preparation, four animals were treated with 50µl of IgG on days 0 and 1. Although all 4 had rejection episodes their blood urea levels at 10 days were lower than the $F(ab')_2$ treated group ($p < 0.02$) and their survival was prolonged, although not significantly differently from the $F(ab')_2$ treated group. Thus the $F(ab')_2$ preparation was significantly less effective than a dose of IgG which in terms of binding capacity was 100 times smaller than that of $F(ab')_2$.

Histology

The 7 day biopsies were assessed blind and scored for various features of rejection. The results for the group are shown in Table 2.

Untreated animals showed only a scanty mononuclear cell infiltrate and the prominent features were tubular necrosis, glomerular capillary necrosis and interstitial haemorrhage. The paucity of mononuclear cells may well have been due to the late stage of rejection.

$F(ab')_2$ treated animals also showed severe rejection but the mean score for the group was significantly lower than controls ($p < 0.01$). The most obvious difference was the absence of glomerular capillary necrosis in this group at 7 days. The importance of this difference is open to

question because histological comparison of the kidneys removed at autopsy showed no difference in either score, or type of lesion between the control and the $F(ab')_2$ treated groups. Indeed both groups showed the sinister feature of arterial fibrinoid necrosis.

Animals treated with enhancing serum or 500µl IgG showed essentially a similar degree and pattern of rejection. The histological scores were lower than in either controls or the $F(ab')_2$ treated group, and the pattern was markedly different. There was a moderate mononuclear cell infiltrate and some interstitial oedema but no haemorrhage, tubular or glomerular necrosis. Biopsies of the allografts of animals treated with 50µl of IgG showed a similar degree of rejection to the $F(ab')_2$ treated group. Thus, the histological results confirmed the trivial immunosuppressive effect of the $F(ab')_2$ preparation.

Lymphocytotoxic antibody response at day 7 (see Table 3)

Whereas treatment with both IgG and hyperimmune serum resulted in a partial suppression of the lymphocytotoxic antibody response, treatment with $F(ab')_2$ did not. Results in the animals treated with $F(ab')_2$ were similar to controls.

DISCUSSION

These results show that $F(ab')_2$ is approximately 100 fold less effective than whole antibody in the induction

of enhancement of renal allografts. The $F(ab')_2$ preparation did have some slight immunosuppressive effect in that 2 of 6 rats recovered from the rejection reaction and survived indefinitely. One cannot say whether this was due to a slight enhancing effect of $F(ab')_2$ itself or due to the tiny amounts of IgG within the preparation, but the fact that 50µl of IgG was significantly immunosuppressive suggests the effect of the $F(ab')_2$ preparation was due to contamination.

These results imply that the Fc part of the antibody molecule is necessary for the induction of enhancement. It is unlikely that the digestion of the IgG resulted in any selective loss of the anti-Ia-like activity thought to be crucial for enhancement by some workers, because exhaustive red blood cell absorption could remove no more than 20% of the binding capacity of this $F(ab')_2$ preparation to Lewis lymph node lymphocytes (Hart and Fabre, 1978). One possibility which was not explored was that the diminished effectiveness of $F(ab')_2$ was due to the much shorter half-life of $F(ab')_2$ compared to whole IgG (Spiegelberg and Weigle, 1965). However, Sinclair et al. (1970) have shown that the diminished effectiveness of $F(ab')_2$ in suppressing the anti-sheep erythrocyte antibody response cannot be attributed to the more rapid metabolism of $F(ab')_2$. Hoffman and Kappler (1978) have recently suggested that specific immunosuppression by antibody can occur by two mechanisms. The first is

Fc-independent and requires the masking of antigenic sites by antibody (or $F(ab')_2$) preventing antigenic recognition; large doses of antibody are needed for the effect. The second is Fc-dependent and involves the prevention of T and B-cell co-operation by the nonspecific attachment of antigen-antibody complex to the Fc receptors of macrophages and B-cells, thus taking antigen which could stimulate helper cell activity, out of circulation.

The role of the Fc part of the IgG molecule in enhancement is unknown. Recently, Hutchinson and Zola (1977) have postulated a mechanism which involves opsonisation of antigen reactive cells by antigen-antibody complexes bound to their receptors. In this model, the Fc part of the antibody would attach the whole complex to the Fc receptors on the macrophage.

Whatever the explanation, this large difference in potency between $F(ab')_2$ and whole antibody makes the clinical use of $F(ab')_2$ impractical and so alternative strategies were investigated.

I(b) $F(ab')_2$ blocking experiments

Having established that $F(ab')_2$ alone was virtually ineffective in the induction of enhancement, a subsidiary question was proposed. Could $F(ab')_2$ be used to block antibody-mediated damage by whole IgG without interfering

with the latter's enhancing action? This approach had been suggested previously by the experiments of Holter et al. (1973).

Effect of $F(ab')_2$ on enhancement by IgG

Using the same reagents, the following experiment was performed. Five hundred µl of $F(ab')_2$ DA anti-Lewis (3mg) was administered after the release of the vascular clamps. Thirty minutes later 500µl (0.38mg) of DA anti-Lewis IgG was administered. This dose of IgG had been shown to be close to the minimum effective dose to produce enhancement. These doses were repeated in the same order and time sequence, on the day after transplantation.

The results are shown in Table 4 and demonstrate that $F(ab')_2$ did not block the enhancing effect of IgG. Indeed, graft function appeared rather better in the animals treated with $F(ab')_2$ and IgG than those treated with IgG alone. The lymphocytotoxic antibody responses at day 7 (Table 3) and the histological scores (Table 2) were similar to those of animals treated with 500µl of IgG alone.

The effect of $F(ab')_2$ on complement-dependent lymphocytotoxicity.

Before attempting to set up a model of hyperacute rejection in vivo, the effect of $F(ab')_2$ on another complement-dependent process, complement-dependent

lymphocytotoxicity, was examined.

A comparison of the lymphocytotoxic antibody titration curves of enhancing serum OAS-58 was made after diluting the serum (a) in normal saline and (b) in non-cytotoxic $F(ab')_2$.

The result,(fig. 7) showed that the presence of $F(ab')_2$ had a markedly inhibitory effect on the lympho-cytotoxic action of whole serum, reducing the titre from 1:32 to 1:2. This result illustrates the point that comparison of cytotoxicity is an inappropriate method of quantitating the contamination of a $F(ab')_2$ preparation with IgG. At a dilution of 1:16 (antiserum:$F(ab')_2$) no lymphocytotoxicity was demonstrated. Thus a $F(ab')_2$ preparation that was 6.25% contaminated with IgG would still be non-cytotoxic.

In the final experiment I attempted to block the lymphocytotoxicity of the alloantiserum by pre-incubating the target cells with $F(ab')_2$.

Four x 10^5 ^{51}Cr-labelled Lewis PBL were incubated in 300µl of neat $F(ab')_2$ for 60 minutes at 4^oC. The cells were then washed twice in medium (Hanks' balanced salt solution with 10% FCS) and adjusted to a concentration of 2 x 10^6/ml. These cells were then used as targets for the DA anti-Lewis serum, OAS-58, and, to test for

non-specific blocking, against ALS (DE). A separate control aliquot of cells was similarly incubated in normal saline without $F(ab')_2$.

The results,(fig. 8) showed that pre-incubation of cells in $F(ab')_2$ reduced the lymphocytotoxic antibody titre of the alloantiserum OAS-58 but not of the ALS. The results are expressed as specific cytotoxicity to facilitate comparison between the two cell preparations.

These blocking experiments confirmed the ability of $F(ab')_2$ to block complement-dependent lymphocytotoxicity in vitro and also showed that $F(ab')_2$ did not inhibit the enhancing capacity of whole antibody. The next experiments were designed to test whether $F(ab')_2$ could be used to block hyperacute rejection by whole antibody without interfering with enhancement.

Fig.1

ELUTION PROFILE OF IgG SAMPLE ON SEPHADEX G-200

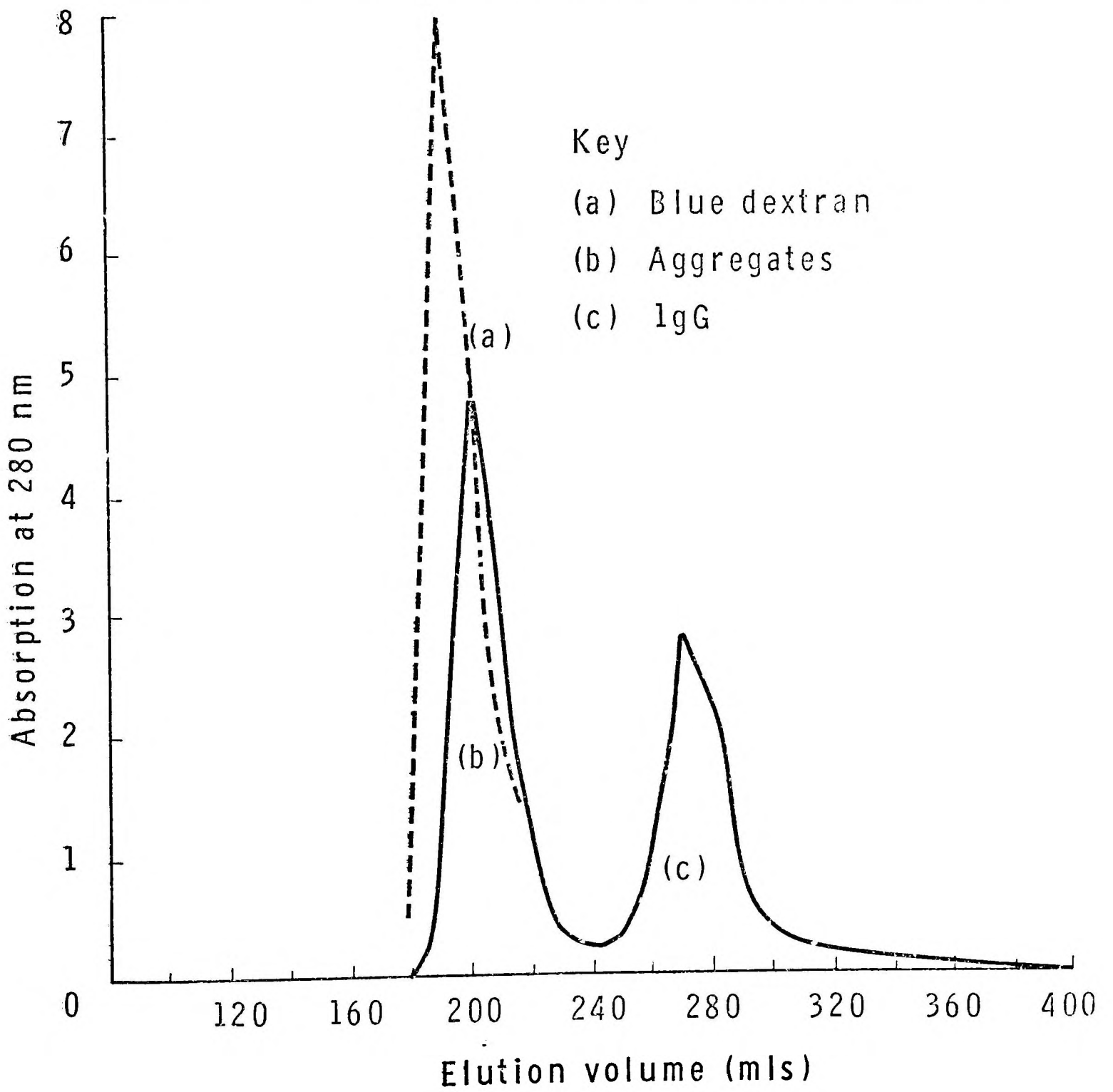
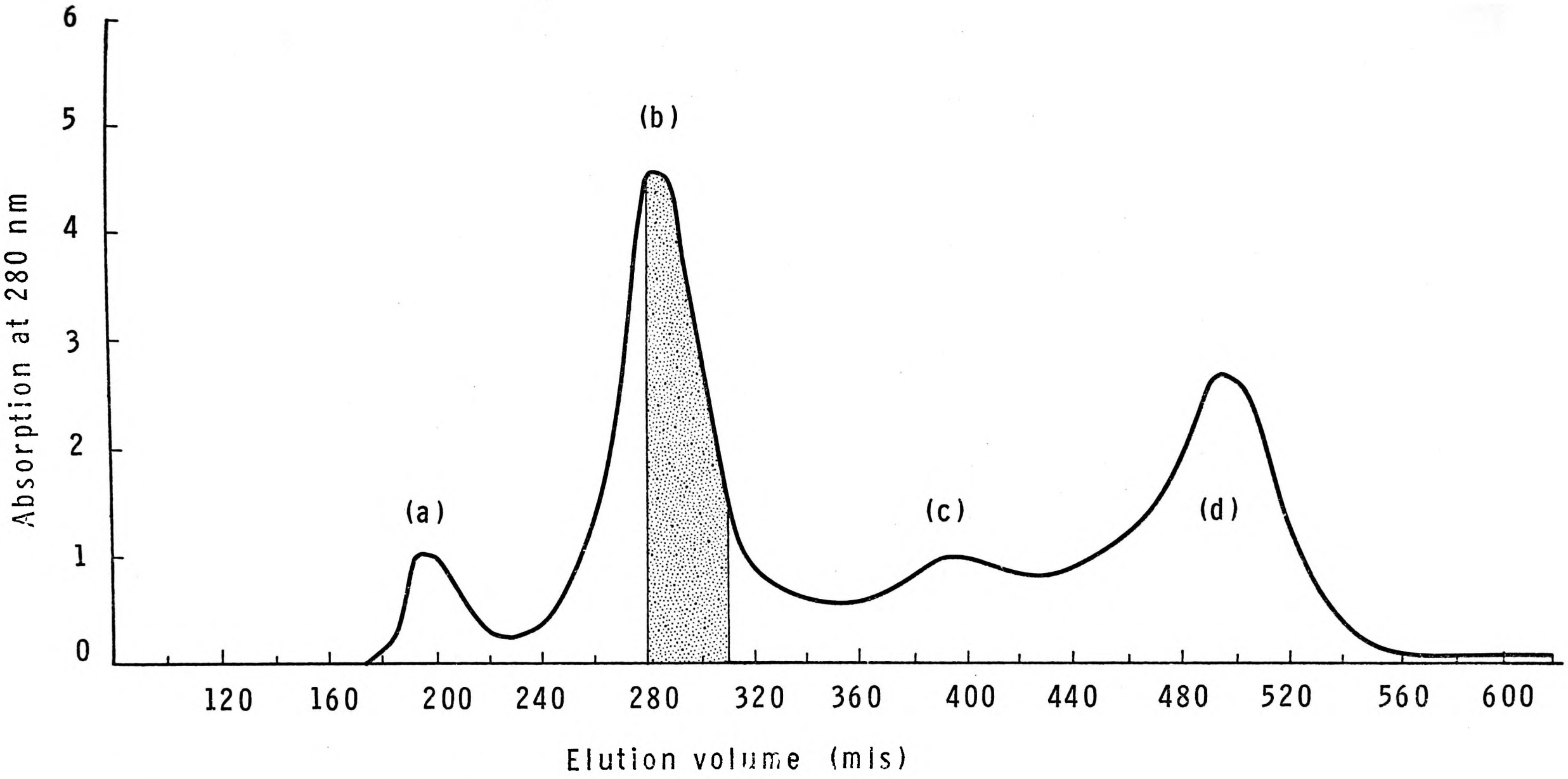


Fig. 2

ELUTION PROFILE OF $F(ab')_2$ ON SEPHADEX G-200



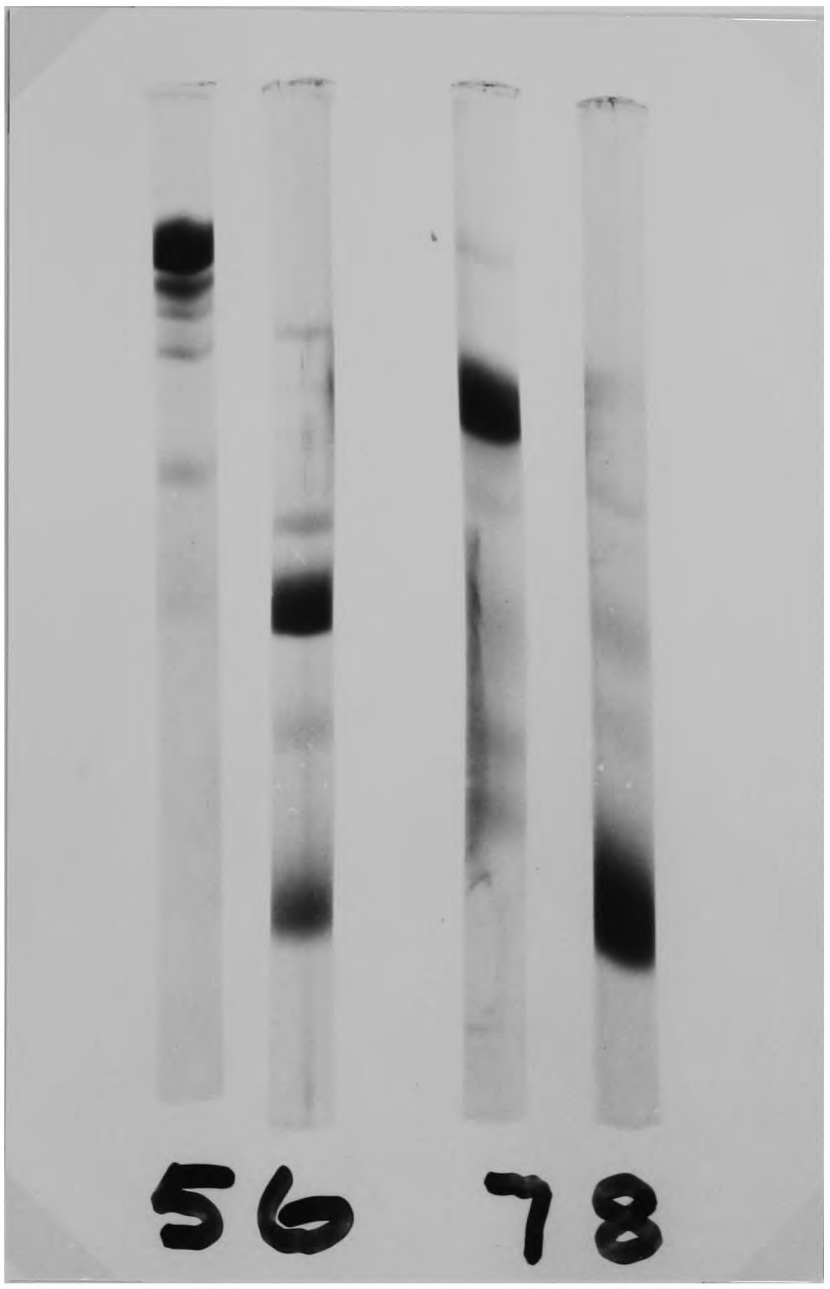
Key (a) Undigested high molecular weight aggregates

(b) Undigested IgG and $F(ab')_2$. Shaded area shows tubes pooled for pure $F(ab')_2$

(c) PFC

(d) Peptides

Fig. 3 SDS Gels of IgG and $F(ab')_2$ OAS-58 (before purification on Protein-A.



5 = Unreduced IgG

6 = Reduced IgG

7 = Unreduced $F(ab')_2$

8 = Reduced $F(ab')_2$

Note band above main $F(ab')_2$ band in Gel 7 corresponding to the position of IgG in Gel 5.

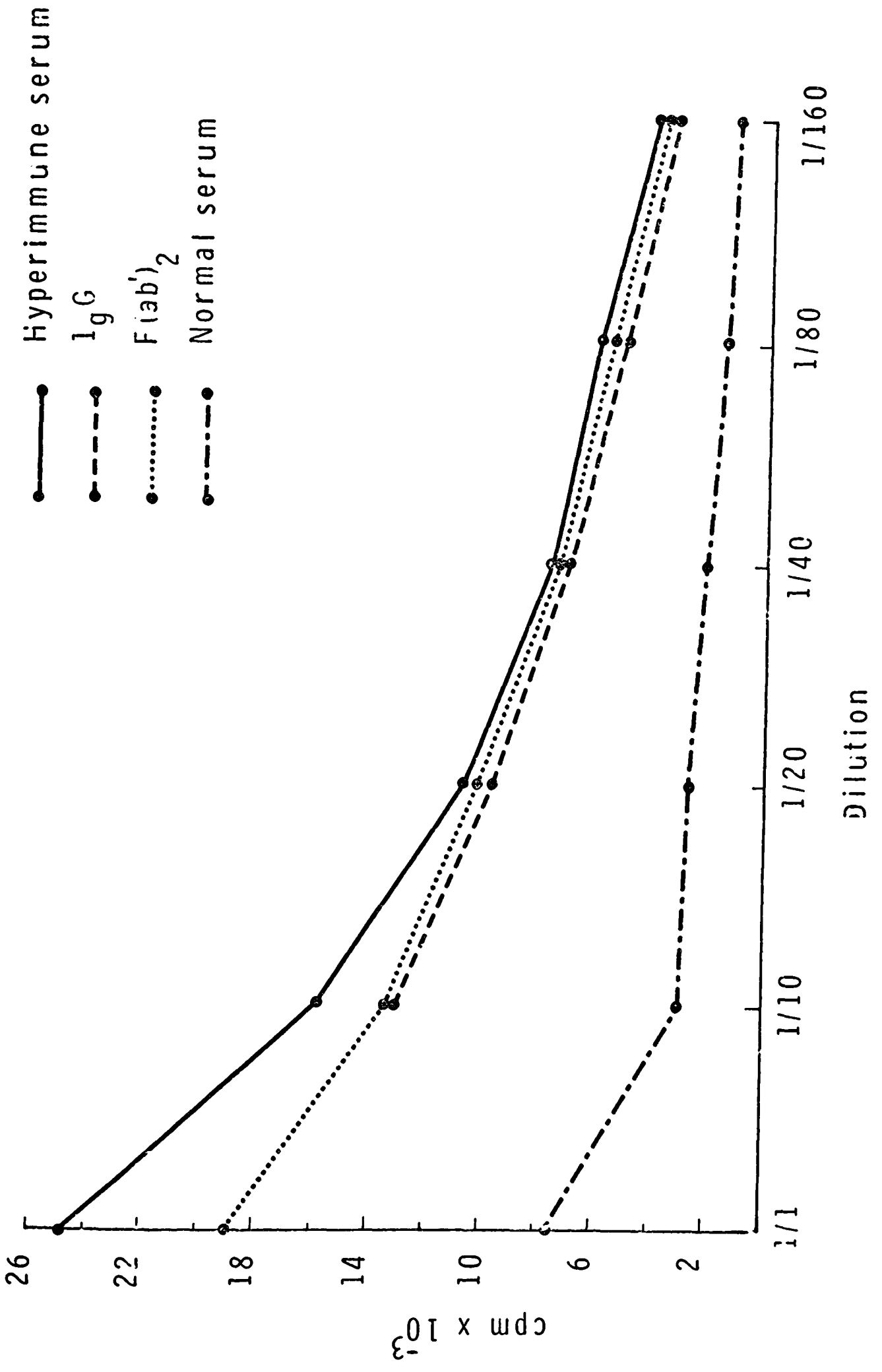


Fig.4 BINDING ASSAY VS LEWIS LYMPH NODE CELLS

Fig. 5

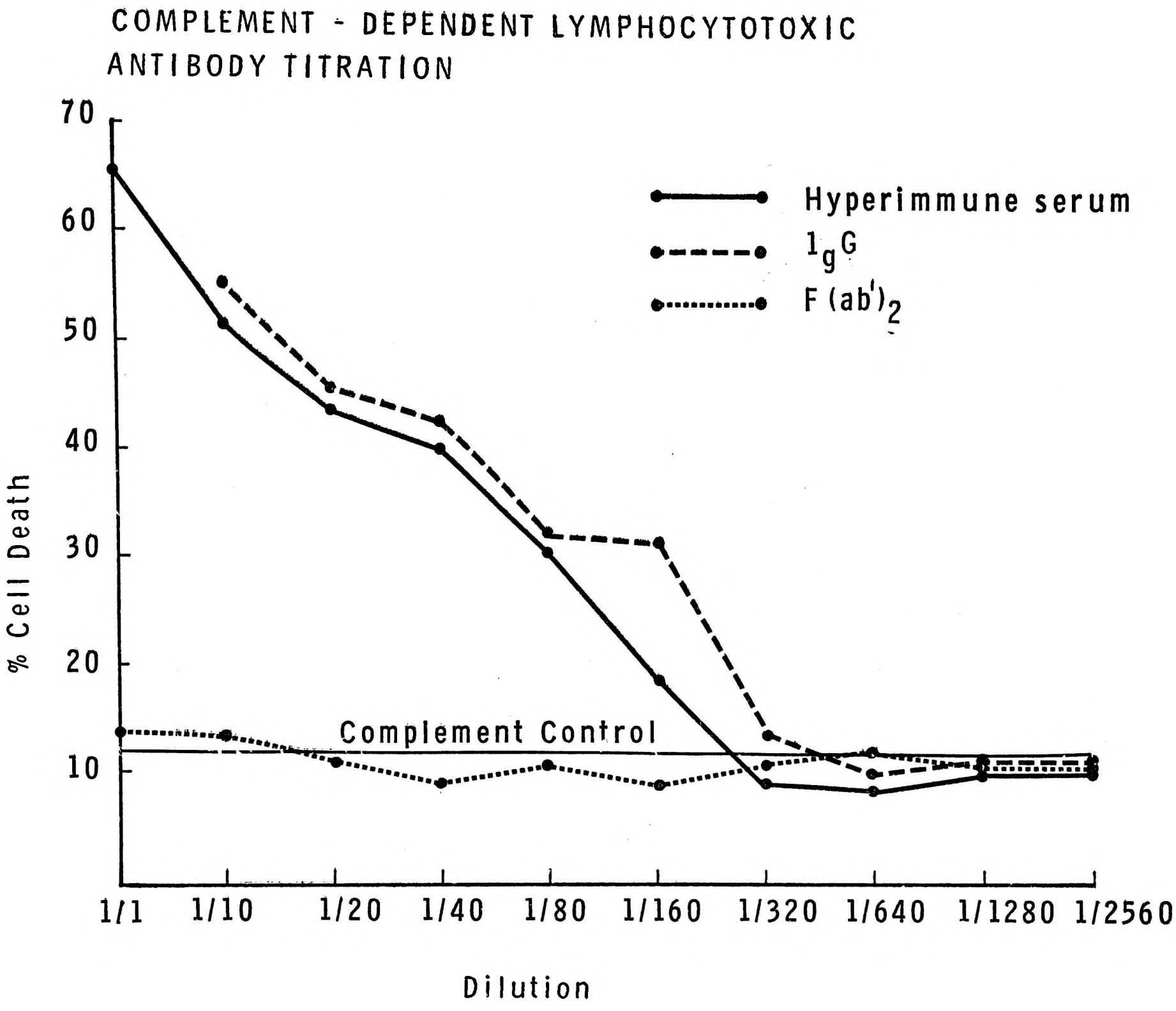
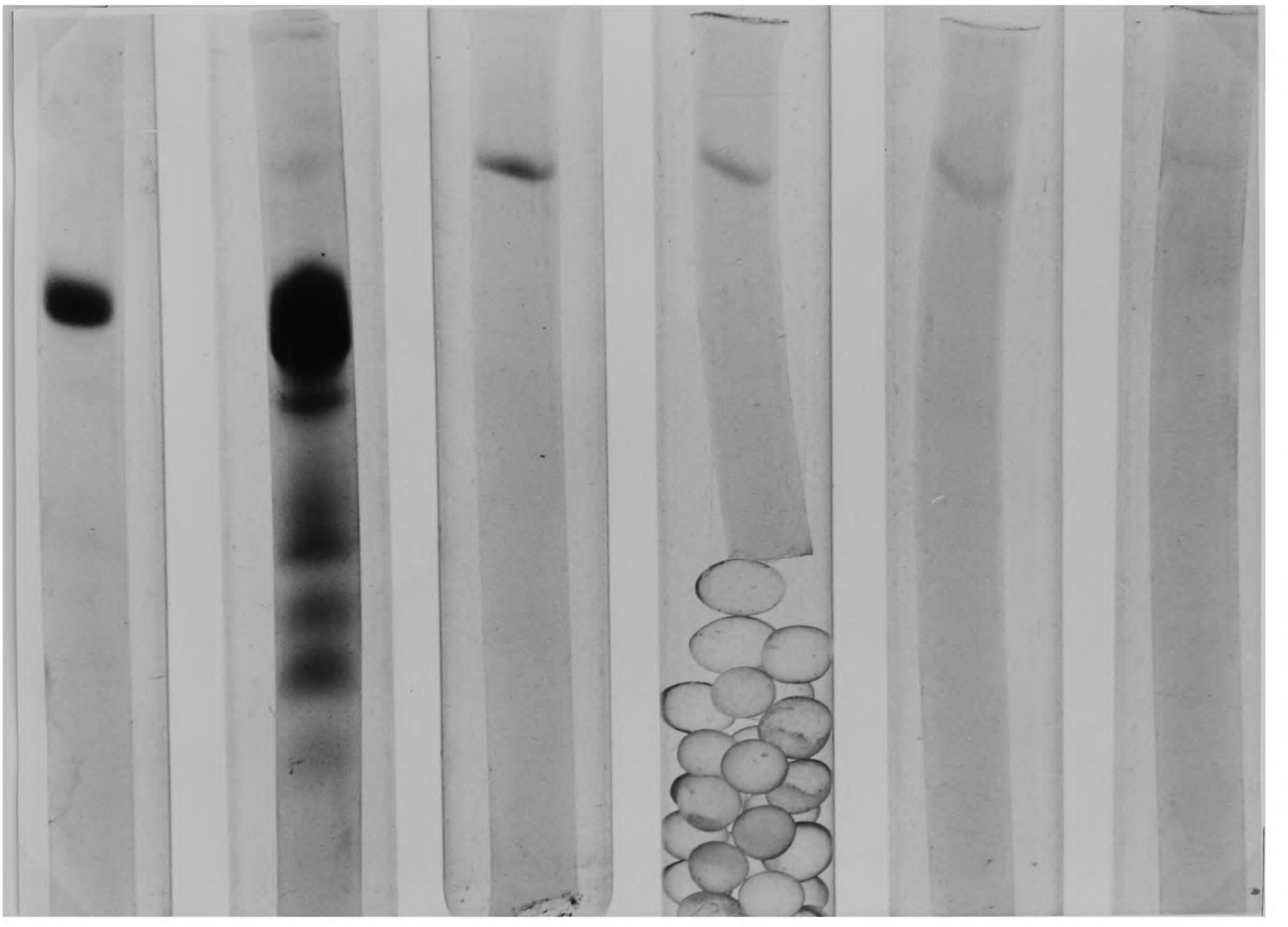

COMPLEMENT - DEPENDENT LYMPHOCYTOTOXIC ANTIBODY TITRATION
70
60
50
40
30
20
10
% Cell Death
Hyperimmune serum
1_gG
$F(ab')_2$
Complement Control
1/1
1/10
1/20
1/40
1/80
1/160
1/320
1/640
1/1280
1/2560
Dilution

Fig. 6 SDS Gels of $F(ab')_2$ after passage through Protein-A



From left to right

1. 25µg $F(ab')_2$ - No IgG contaminant seen
2. 100µg $F(ab')_2$ - Faint IgG contaminant seen
3. 2µg IgG Standard
4. 1µg IgG "
5. 0.5µg IgG "
6. 0.25µg IgG "

Gel 2 contains $\lesssim$ 1µg IgG.

Fig.7

REDUCTION IN COMPLEMENT - DEPENDENT CYTOTOXICITY BY $F(ab')_2$

^{51}Cr release assay vs Lewis PBL

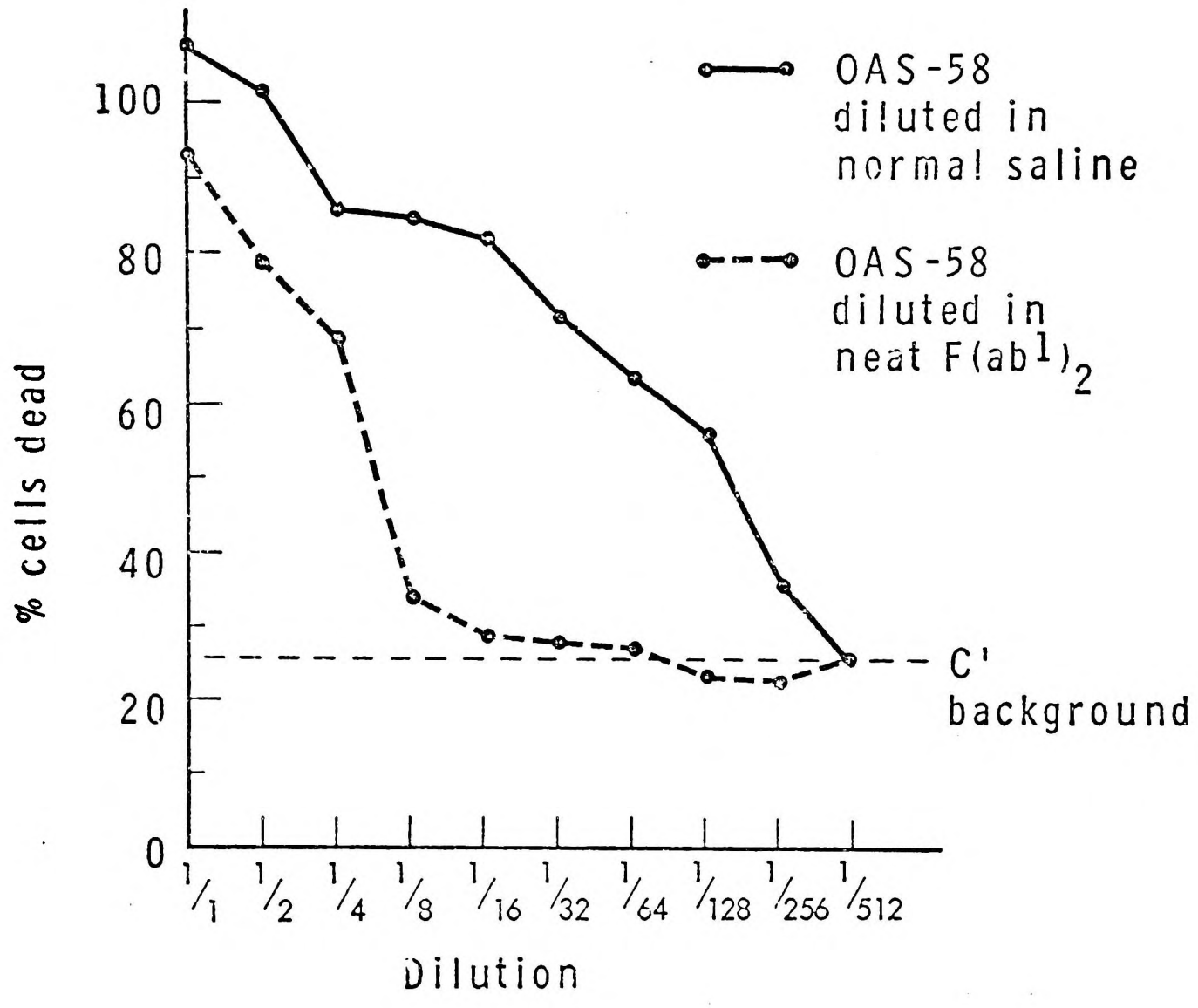


Fig.8

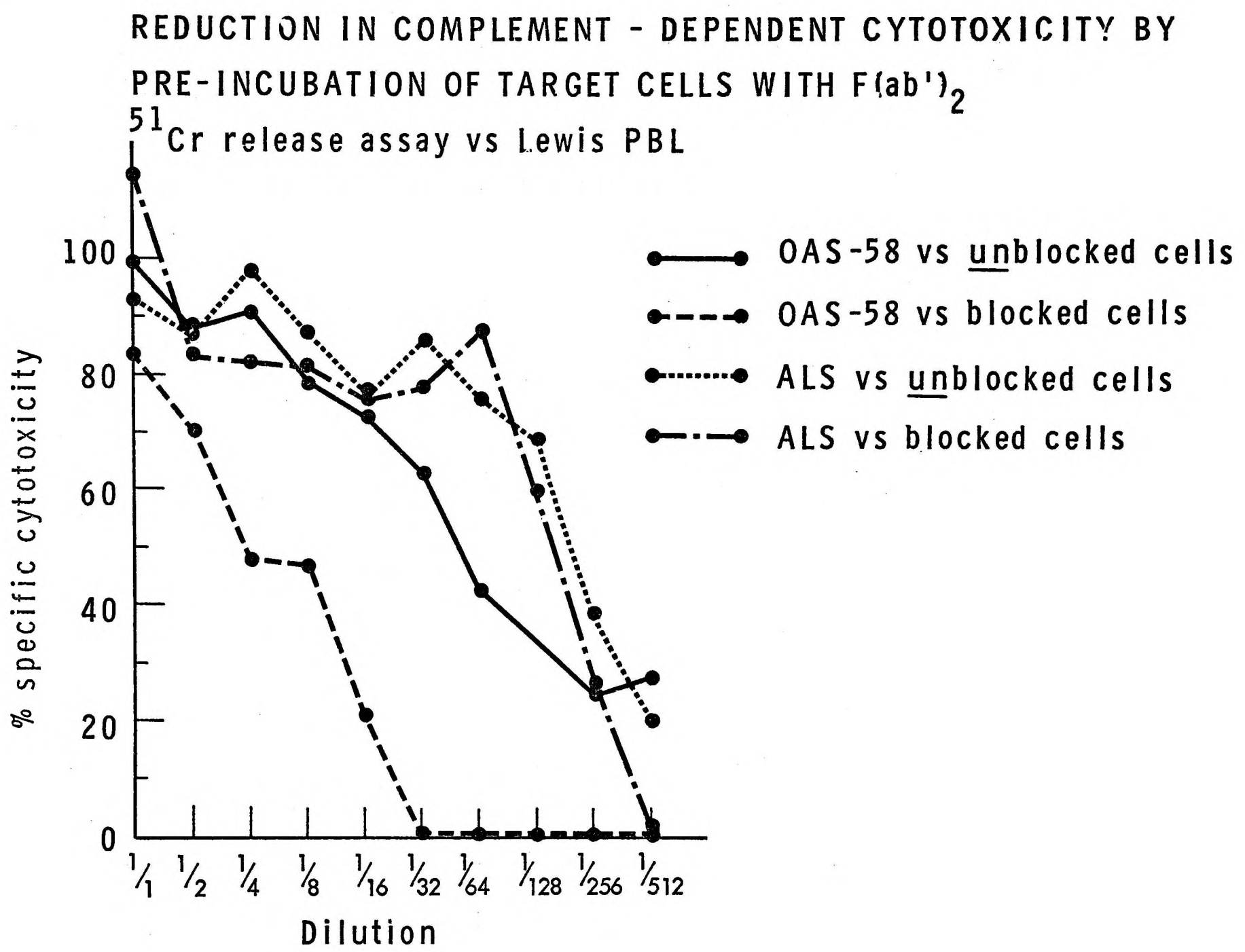

REDUCTION IN COMPLEMENT - DEPENDENT CYTOTOXICITY BY
PRE-INCUBATION OF TARGET CELLS WITH F(ab')2
51Cr release assay vs Lewis PBL
% specific cytotoxicity
100
80
60
40
20
0
1/1 1/2 1/4 1/8 1/16 1/32 1/64 1/128 1/256 1/512
Dilution
OAS-58 vs unblocked cells
OAS-58 vs blocked cells
ALS vs unblocked cells
ALS vs blocked cells

TABLE 1. Results of Lewis to DA renal allografts in untreated recipients and recipients treated with OAS-58, IgG, $F(ab')_2$

Treatment	No. of rats	Median blood urea ± SD mg/100ml at weeks: 10d	2	3	4	6	8	12	20	Median Survival (range) in days
NIL	5	760±62	-	-	-	-	-	-	-	11(10-11)
25µl OAS-58 i.v. days 0,1	5	148±163	95±41	83±28	68±18	54±9	84±19	58±9 *	62±3 *	>140(17->140)
50µl OAS-58 i.v. days 0,1	5	101±11	113±28	65±17	66±17	65±17	66±7	66±46	64±6	>140(52->140)
500µl IgG prepared from OAS-58 (0.38mg) i.v. days 0, 1	5	121±71	199±162	117±108	106±64	124±66	94±26	90±37	66±48	>140(37->140)
50µl IgG of OAS-58 (0.038mg) i.v. on days 0,1	4	323±127	220±82	125±21	109±18	89±10	99*	59*	88*	>47(28->140)
500µl $F(ab')_2$ of OAS-58 (3.0mg) i.v. on days 0,1	6	632±83	178*	100*	73+	94*	92*	85*	93*	10.5(8->140)

* less than 4 values

TABLE 2. Features of 7 day Biopsies

Treatment	No. of Biopsies	Median Score for each feature: Interstitial Tissue			Arteries		Tubular Necrosis	Glomerular Capillary Necrosis	Mean Score ± SD
		Mononuclear cells	Oedema	Haemorrhage	Fibrinoid Necrosis	Subintimal mononuclear cells			
Nil	5	+	o	+	o	o	++++	++++	10.2± 0.8
Serum 25µl	5	+++	++	o	o	o	o	o	3.8± 1.6
Serum 50µl	5	++	o	o	o	o	o	o	2.6± 0.8
IgG (500µl)	5	++	+	o	o	o	o	o	4±1.8
IgG (50µl)	4	+++	++	o	o	o	o	+	7±1.4
$F(ab')_2$ 500µl	6	++	+	+	o	o	++	o	6.6± 1.3
Blocking Experiment									
500µl IgG plus 500µl $F(ab')_2$	4	+++	++	o	o	o	o	o	4.2± 0.05
Autopsy Specimens									
Nil	5	++	+	o	++	o	++++	+++	11.8± 1.6
$F(ab')_2$ 500µl	4	++	+	+++	++	o	+++	++	11.3± 4.3

TABLE 3 Lymphocytotoxic antibody titrations at day 7 post graft. DA recipients of Lewis renal allografts

Treatment given i.v. days 0, 1	No. of rats	Mean specific cytotoxicity ± SD at dilution: 1/1	1/2	1/4	1/8	1/16	1/32
Nil	5	89±16	30+13	12±3	8±1	4±3	1±1
50µl OAS-58	5	50±17	4±6	0	0	0	0
25µl OAS-58	5	42±24	5±7	0	0	0	0
500µl IgG	5	39±23	8±8	4±2	0	0	0
50µl IgG	4	43±19	16±3	7±3	4±2	2±1	0
500µl $F(ab')_2$	6	93±25	31±9	14±6	5±2	6±4	1±1
500µl $F(ab')_2$ plus 500µl IgG	4	60±17	15±7	9±5	5±4	5±5	0

Table 4. Blocking Experiment. Lewis ⟶ DA renal grafts

Treatment	Rat No.	Blood Urea mg/100ml at week: 10d	2	3	4	6	8	12	20	Survival (days)
500µl $F(ab')_2$	2333	107	104	85	75	65	73	77	64	> 140
followed 30'	2345	140	117	94	60	90	69	64	69	> 140
later by 500µl	2346	77	110	82	61	79	84	76	80	>140
IgG (days 0,1)	2349	82	106	111	83	87	71	80	81	>140

	No.of Rats	Median Blood Urea ± S.D. mg/100ml at week: 10	2	3	4	6	8	12	20	Median Survival (range) in days
NIL	5	760±62	-	-	-	-	-	-	-	11(10-11)
500µl IgG days 0, 1	5	121±75	199±162	117±108	106±64	124±66	94±26	90±37	66±48	> 140(37- >140)
500µl $F(ab')_2$ days 0, 1	6	632±83	-	-	-	-	-	-	-	10.5(8- >140)

II ANTIBODY-MEDIATED DAMAGE IN THE DA ⟶ LEWIS MODEL - TWO APPROACHES TO ITS PREVENTION.

Introduction

The DA ⟶ Lewis model was chosen because large doses of Lewis anti-DA serum have been shown to cause antibody-mediated damage to DA renal grafts (Fabre and Morris, 1974a). Because this effect was reported to be inconsistent and dose-dependent, 2ml of guinea pig complement were administered two minutes after the antiserum to increase the chance of producing antibody-mediated damage (French, 1972).

(a) Blocking by Lewis anti-DA $F(ab')_2$

In the experiments using $F(ab')_2$ in the Lewis ⟶ DA model it was established that $F(ab')_2$ alone was at least 100 fold less effective than whole antibody in the induction of enhancement and did not therefore represent a realistic solution to the problem of antibody-mediated damage. However, it was clearly shown that a large dose of $F(ab')_2$ did not inhibit the enhancing effect of a near minimum effective dose of IgG but did inhibit complement-dependent lymphocytotoxicity. I therefore re-examined the suggestion of Holter et al. (1973) that $F(ab')_2$ could be used to block hyperacute rejection if it was administered before whole antibody. In my experiments the $F(ab')_2$ was administered 15 minutes before the antiserum and complement.

(b) The use of red cell absorbed (anti-Ia-like) serum

Although anti-Ia-like antibodies have been shown to enhance both rat kidney and heart allografts (Soulillou et al., 1976; Davies and Alkins, 1974) they were shown to be ineffective in enhancing rat kidney allografts by Jeekel et al. (1977). Because of this uncertainty, and the demonstration by Davies & Butcher (1978) and Hart and Fabre (1978) that Ia-like antigens are expressed in large amounts on rat kidneys, the ability of an anti-Ia-like serum to enhance or damage renal allografts was examined in the DA——→Lewis combination.

MATERIALS AND METHODS

Rats: Male DA donors and male Lewis recipients were used throughout.

Renal transplantation: Because it was important to be able to assess early graft function an immediate bilateral nephrectomy was performed on all recipients. This required a modified ureteric anastomosis (see Chapter 5 Section 2). The day after operation the animals were placed in metabolic cages and 24-hour urine volumes measured for the next 6 days. Blood urea estimations were performed on days 3 and 7 post transplant and weekly thereafter.

Enhancing sera and $F(ab')_2$

Two sera were used (for details of their preparation see Chapter 5, Section 3). OAS-59 was used for inducing hyperacute rejection and for the absorption experiments. $F(ab')_2$ was prepared from OAS-57 and had been intended for a different set of experiments which were abandoned. OAS-57 was not significantly different from OAS-59 in terms of antigen-binding capacity and lymphocytotoxicity titre.

Preparation of $F(ab')_2$

The method used for the preparation of Lewis anti-DA $F(ab')_2$ from OAS-57 was similar to that used for the preparation of $F(ab')_2$ from OAS-58 (see page 223). Because purity was not critical the latter two thirds of the $IgG/F(ab')_2$ peak which was eluted from the Sephadex G-200 column was pooled and it was not passed through the Protein-A Sepharose CL-4B column. It was dialysed against normal saline, adjusted to a protein concentration of 6mg/ml and aliquotted for in vivo use. Quantitative SDS gel studies on this $F(ab')_2$ preparation revealed $2\frac{1}{2}\%$ contaminating IgG.

Red Cell Absorption

Using a similar Lewis anti-DA anti-serum to OAS-59, Hart and Fabre (1978) had found that the anti-SD component of 1ml of a $F(ab')_2$ preparation at a concentration of 1mg/ml could be removed by absorption with 3×10^9 DA red cells.

Because the concentration of IgG in undigested serum would be ±10mg/ml, a test absorption of OAS-59 was performed using 3×10^{10} red cells for absorption of 1ml of serum. A binding assay of this test aliquot showed that all red cell antibodies had been removed, leaving approximately a quarter the lymph node lymphocyte binding capacity in the serum. The absorption of 20mls of pooled heat inactivated serum OAS-59 for in vivo use was based on this quantitative test absorption.

Red cells were purified as follows. Twenty DA male rats were bled out by aortic puncture into heparinised syringes and the blood diluted in twice its own volume with DAB in lithium heparin tubes. After centrifugation at 350g at 20°C for 10 minutes, the buffy coat and diluted plasma were removed. The red cells were then resuspended in three times their own volume in DAB and underlaid with Triosil-Ficoll(S.G. 1.090)and centrifuged at 1400g for 20 minutes at room temperature. After removing the white cell layer the red cells were washed with DAB (350g for 10 minutes at 4°C). The washed red cells were resuspended in cold DAB, counted using a haemocytometer and centrifuged at 350g at 4°C for 10 minutes and resuspended in 0.5% BSA DAB at a concentration of 3×10^9 red cells/ml. No white cells were seen during counting.

Because each ml of serum had to be absorbed with a total of 3×10^{10} red cells each ml was absorbed against 10^{10} packed cells for 60 minutes at 4°C three times. For packing and after each absorption, the cell/serum

mixture was centrifuged at 1000g for ten minutes. A small aliquot was removed for assay after each absorption. Because a red cell cytotoxicity assay of the serum after these three absorptions revealed residual activity, a further 60 minutes absorption at $4^{o}C$ against 10^{10} red cells was performed. This absorption did not remove the residual activity and so a further absorption against 10^{10} red cells was performed at $37^{o}C$. Two aliquots of the 4 times absorbed serum had been test absorbed at $4^{o}C$ and $37^{o}C$. The $4^{o}C$ absorption had not removed the residual activity whereas the $37^{o}C$ absorption had. The five times absorbed serum was then aliquotted for in vivo use, assayed for binding to red cells and lymph node lymphocyte targets, and tested for cytotoxicity against red cells, peripheral blood lymphocytes, thymocytes and spleen cells.

Binding Assays (see Chapter 5, Section 6).

Cytotoxicity Assays (see Chapter 5, Section 5).

The medium used in these experiments was 199 with 10% foetal calf serum. Red cell absorbed guinea-pig complement was used in all assays. For the spleen cell and red cell target assays the results were adjusted so that % cytotoxicity with neat serum = 100%.

Examination of sera using the fluorescence activated cell sorter (FACS)

This was performed with Dr. Derek Hart in an attempt to show that red cell absorption of the Lewis anti-DA anti-serum would result in a serum with activity against Ig^{+ve} cells only. Thoracic duct lymphocytes were obtained by a standard technique. The cells were collected on ice into normal saline with 200units/ml of heparin. The cells were then washed in 0.5% BSA/DAB and red cell contamination reduced by incubation in ACT at 37°C for 10 minutes. The cells were then washed and after checking the viability with trypan blue the concentration was adjusted to 1×10^8/ml. Twenty-five µl of a 1/20 dilution of normal serum, unabsorbed OAS-59 and absorbed OAS-59 were incubated with 25µl of cells for 60 minutes at 4°C. After washing, the cells were incubated with 100µl of FITC labelled rabbit $F(ab')_2$ anti-rat $F(ab')_2$ at 50µg/ml for 60 minutes at 4°C. The cells were then washed twice and resuspended in 1ml of 0.5% BSA/DAB with 0.02% Sodium azide.

Analysis was performed on the FACS Mark II at the Dunn School of Pathology.

RESULTS

(1) $F(ab')_2$ preparation

When compared to unmodified serum the $F(ab')_2$ preparation of OAS-57 had a markedly reduced lympho-cytotoxic antibody titre (Fig. 1) but virtually identical antigen binding capacity as undigested serum to both red cell targets (Fig. 2) and lymph node lymphocyte targets (Fig. 3). The unmodified serum OAS-57 had similar binding and cytotoxic characteristics to OAS-59.

(2) Characteristics of the red cell absorbed serum OAS-59

Absorption reduced the binding to red cells to negligible levels (Fig. 2). Examination of the binding to lymph node lymphocyte targets showed that the amount of antibody had been reduced to just under a quarter of the pre-absorption levels (Fig. 3).

The complement dependent lymphocytotoxic assays showed that the absorbed serum had no activity against red cells (Fig. 4), a plateau at 25-35% above background against PBL (Fig. 5), 56% above background against spleen cells (Fig. 6) and 10-15% above background against thymocytes (Fig. 7).

However, when the absorbed serum was tested against thoracic duct lymphocytes using the FACS, residual binding to 89% of cells was detected. The fluorescence profile

showed two populations of cells - a small densely staining and a large weakly staining population.

(3) Transplant Results (see Table 1)

Hyperacute rejection occurred in all 5 animals treated with 1ml of OAS-59 and 2mls of guinea-pig complement. All became anuric post-operatively and blood urea levels were extremely high 2-3 days post transplant. Similar results were obtained in the three animals treated with 0.5mls of OAS-59 and 2mls GPC'.

Control animals, treated with 1ml of normal Lewis serum and 2mls of guinea pig complement did not undergo hyperacute rejection. All 5 produced urine on the first postoperative day, but two did have raised blood urea levels at day 3. All 5 had typical acute rejection with high blood ureas at day 7 and 4 of 5 animals were anuric by day 7. When the administration of 1ml of unmodified OAS-59 was preceeded by 1ml of $F(ab')_2$, hyperacute rejection was prevented in 4 of 5 animals. This dose of $F(ab')_2$ (6mgs) represented the same antigen binding capacity as 1ml of unmodified serum. In the four animals which did not undergo hyperacute rejection graft survival was prolonged and the blood urea levels at day 7 were lower than controls and animal survival was prolonged.

When the red cell absorbed serum was used, all 5 animals had good early graft function with low blood urea levels at day 3. Rejection was delayed in that 7 day blood

urea levels were lower than controls ($p < 0.02$) and survival prolonged ($p < 0.05$). Seven day blood urea levels were, however, higher than in the group treated with whole unabsorbed serum and $F(ab')_2$ ($p < 0.05$).

(4) Histological Appearances (see Table 2)

(a) OAS-59 and GPC' The hyperacute rejection reaction was characterised by an interstitial polymorph but not mononuclear cell infiltrate, interstitial haemorrhage, tubular and glomerular necrosis (see Fig. 8).

(b) Normal Lewis serum and GPC' In this group the features of acute rejection were seen i.e. an interstitial mononuclear but not polymorph cellular infiltrate, interstitial oedema and haemorrhage, tubular and glomerular necrosis.

(c) $F(ab')_2$ plus OAS-59 and GPC'. In the four animals that had prolonged survival the features were of acute rejection and 3 had developed arterial fibrinoid necrosis. This may be a reflection of their longer survival compared to the animal treated with normal Lewis serum and complement. In the single animal in which the graft did not function only mild changes were seen suggesting that hyperacute rejection may not have been the cause of failure.

(d) Absorbed OAS-59 and GPC' These animals showed the features of acute rejection.

DISCUSSION

The results of these experiments suggest two possible ways in which the deleterious consequences of the administration of anti-donor serum can be avoided without loss of the protective effect. They are firstly, blocking by $F(ab')_2$ and secondly the use of anti-Ia-like sera.

However, the model has an important flaw which limits the force of any conclusions. The rat is peculiarly insensitive to antibody-mediated damage and the administration of xenogeneic complement represents only transitory compensation for this deficiency.

The $F(ab')_2$ blocking experiment confirmed the findings of Holter et al. (1973). In my experiments blocking of antibody-mediated damage occurred in 4/5 recipients at a ratio of $F(ab')_2$:IgG of 1:1. The chances of antibody-mediated damage occurring could be reduced by increasing the amount of $F(ab')_2$ administered. $F(ab')_2$ could be administered without blocking enhancement at a ratio of at least 10:1 as shown in the experiments in the previous section. Certain important questions still remain. Firstly, how long would the blocking effect of $F(ab')_2$ persist? Its half-life is only about 6 hours which would mean that the ratio of IgG:$F(ab')_2$ would rapidly increase with the possibility that hyperacute damage might be triggered, not immediately, but a day or two after transplantation. It could also be argued that

a delayed onset of antibody mediated damage could be prevented by further infusion of $F(ab')_2$ but this would become costly in terms of serum requirements. A delayed hyperacute rejection reaction might not be detected in this model because the xenogeneic complement would have been consumed. On the other hand, hyperacute damage may require an immediate critical high concentration of antibody. By the time the $F(ab')_2$ had been metabolised the antibody level may have fallen below this critical point. French and Batchelor (1972) have shown that passively administered antibody is rapidly lost from the circulation. My own results comparing the antibody titres of transplanted immunosuppressed Lewis recipients with untransplanted Lewis recipients of 1500µl of antiserum confirmed this finding (see Chapter 6). This reinforces the need for some means of estimating the minimum effective dose of enhancing antibody. Indeed it is possible that a minimum effective dose may be insufficient to trigger antibody-mediated damage. Fabre and Morris (1974) demonstrated this dose-dependency of antibody-mediated damage in their experiments in the DA ⟶ Lewis model. High doses of serum (2-3mls) without xenogeneic complement commonly caused antibody-mediated damage whereas the lower dose (0.5ml) did so infrequently. McDowell et al. (1973) who were attempting to induce enhancement in rabbits also found that antibody-mediated damage was dose-dependent.

The second question is whether it is actually necessary to infuse the $F(ab')_2$ before the antibody. Would hyperacute rejection follow simultaneous administration of antibody and $F(ab')_2$? If it did not, then enhancing antisera would only need to be partially digested until the ratio of IgG:$F(ab')_2$ was appropriate. This approach was adopted in the human trials of $F(ab')_2$ (French and Batchelor, 1972) and the results were disappointing. Several of the living related donor kidney transplant recipients became oliguric after the $F(ab')_2$ infusions suggesting the possibility of antibody-mediated damage.

Finally, what is the mechanism by which $F(ab')_2$ blocks antibody-mediated damage? $F(ab')_2$ probably blocks complement-dependent antibody-mediated graft damage by competing for antigenic sites thereby spacing the intact IgG molecules on the cell membrane. Möller and Möller (1961) demonstrated the relationship between cytotoxic sensitivity and concentration of antigen on the cell surface. Limiting the concentration of antibody on the cell surface would therefore alter complement activation and reduce cytotoxic sensitivity (Winn, 1962).

Several points arising from the absorbed serum experiment require discussion. Firstly, the description of a red cell absorbed rat alloantiserum as an anti-Ia serum needs to be justified. A region (or SD) antigens but not B region (or Ia-like) antigens can be absorbed by

red cells (Davies & Alkins, 1974; Davies & Butcher, 1978). After absorption no binding or cytotoxicity to red cells was detected and one quarter of the lymph node lymphocyte binding capacity remained. On cytotoxicity this serum had a plateau against peripheral blood lymphocytes, a similar but higher plateau against a B-cell enriched population (spleen cells) and a low plateau against thymocytes. This is good circumstantial evidence that the antibody was directed against Ia-like antigens which are expressed on B-cells, a minority of T-cells and thymocytes (Hammerling, 1976). That the major antigen recognised is probably Ia-like has been confirmed in this strain combination by Hart and Fabre (1978) who demonstrated in a backcross experiment that the antigen detected was linked to the MHC. The experiments using the FACS demonstrated that the absorbed serum still contained an antibody directed against non-Ig bearing cells. This is likely to be an antibody to the alloantigen PTA-peripheral T-cell antigen (Butcher and Howard, 1977b).

If one accepts that the serum was anti-Ia-like then these experiments confirm that such sera can enhance, and that despite the presence of Ia-like antigens on kidney parenchyma their administration does not result in hyper-acute rejection. The dose of absorbed antiserum (2mls) had an equivalent amount of antibody to the 0.5ml of unabsorbed serum which did cause hyperacute rejection. Thus there is a qualitative difference between the sera.

Why antibodies directed at the Ia-like antigens do not cause hyperacute antibody-mediated damage is not known. The absorption had not preferentially excluded complement fixing antibody because the absorbed serum was still cytotoxic. Which cells in the kidney express the Ia-antigens has not been demonstrated and so the question of accessibility of antibody to antigen remains open. Ia-antigens are thought to be present on vascular endothelium (Hirschberg et al., 1975) the site where hyperacute rejection occurs. It is possible that the antigen density on endothelium is insufficient to allow triggering of the hyperacute rejection reaction.

These experiments do not allow any comparison of the enhancing efficacy of absorbed and non-absorbed sera. This would require careful dose studies preferably in the (DA X Lewis)F_1 → Lewis model in which passive enhancement alone has a more obvious protective effect.

In summary, these experiments suggest two promising methods of avoiding the complication of antibody-mediated damage. Their relevance to the clinical situation will be discussed in the final chapter.

Fig.1

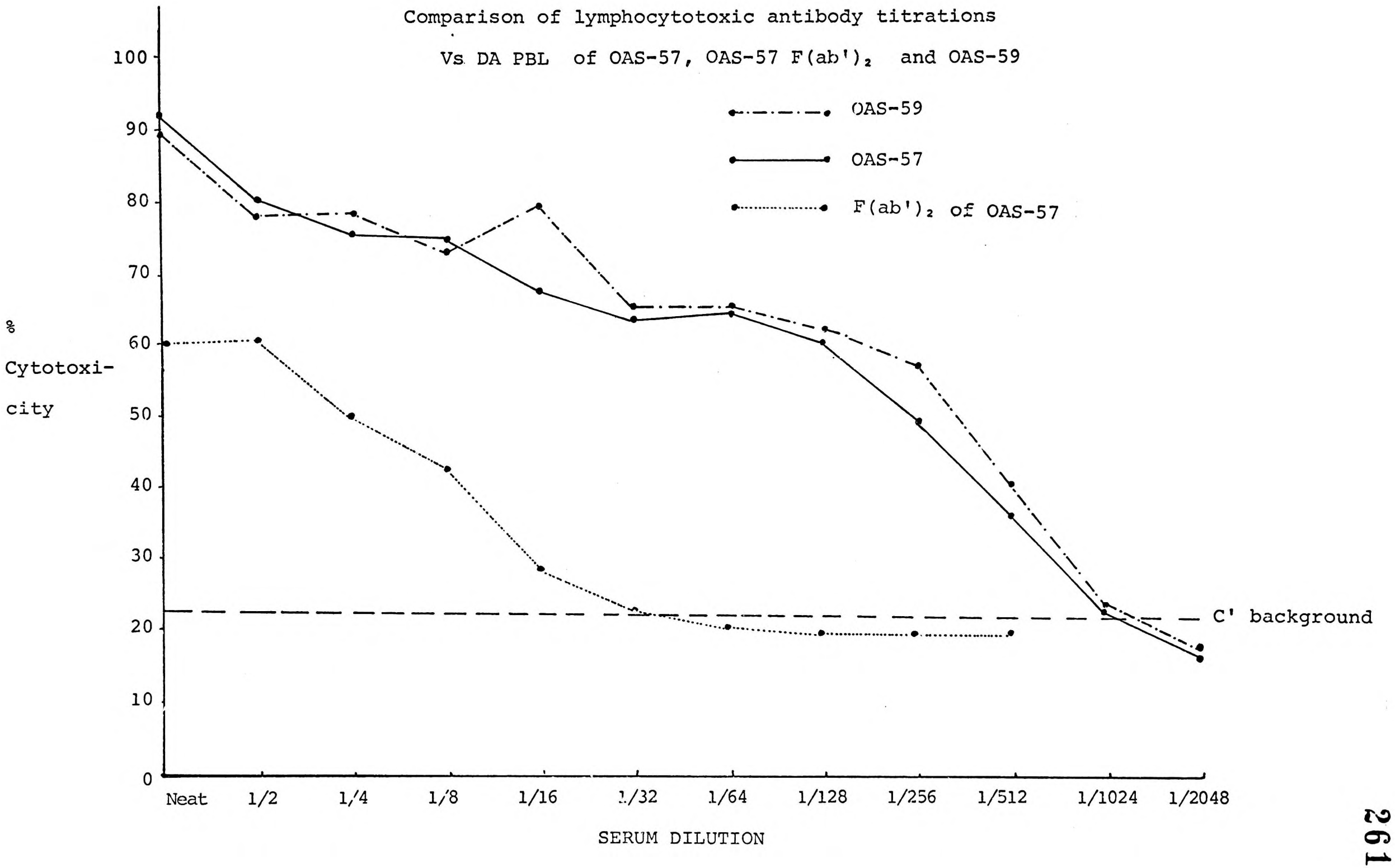
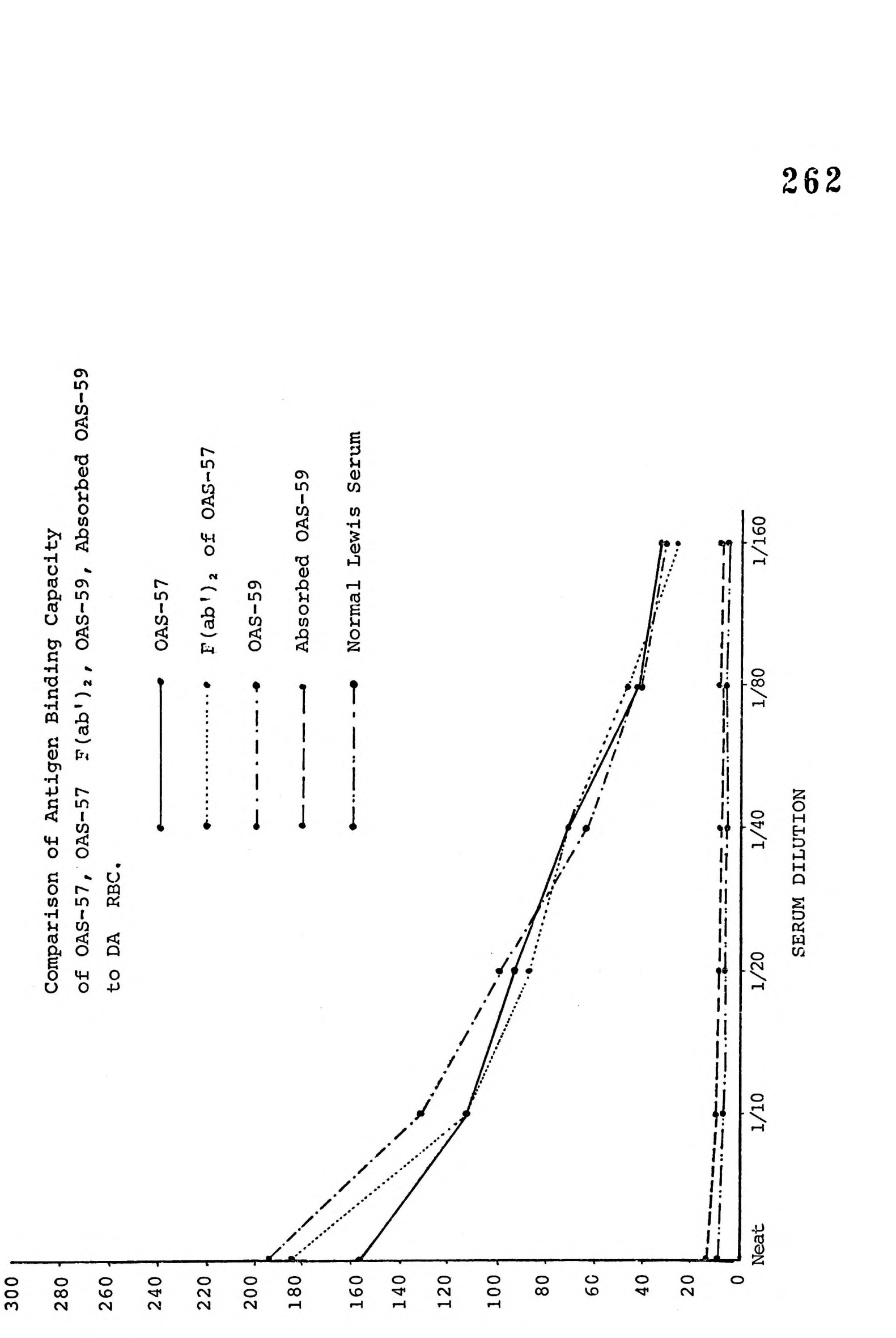

Comparison of lymphocytotoxic antibody titrations
Vs DA PBL of OAS-57, OAS-57 F(ab')2 and OAS-59
OAS-59
OAS-57
F(ab')2 of OAS-57
% Cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
C' background
Neat
1/2
1/4
1/8
1/16
1/32
1/64
1/128
1/256
1/512
1/1024
1/2048
SERUM DILUTION

Fig.2


Comparison of Antigen Binding Capacity
of OAS-57, OAS-57 F(ab')2, OAS-59, Absorbed OAS-59
to DA RBC.
OAS-57
F(ab')2 of OAS-57
OAS-59
Absorbed OAS-59
Normal Lewis Serum
ng
RAR
bound
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
Neat
1/10
1/20
1/40
1/80
1/160
SERUM DILUTION

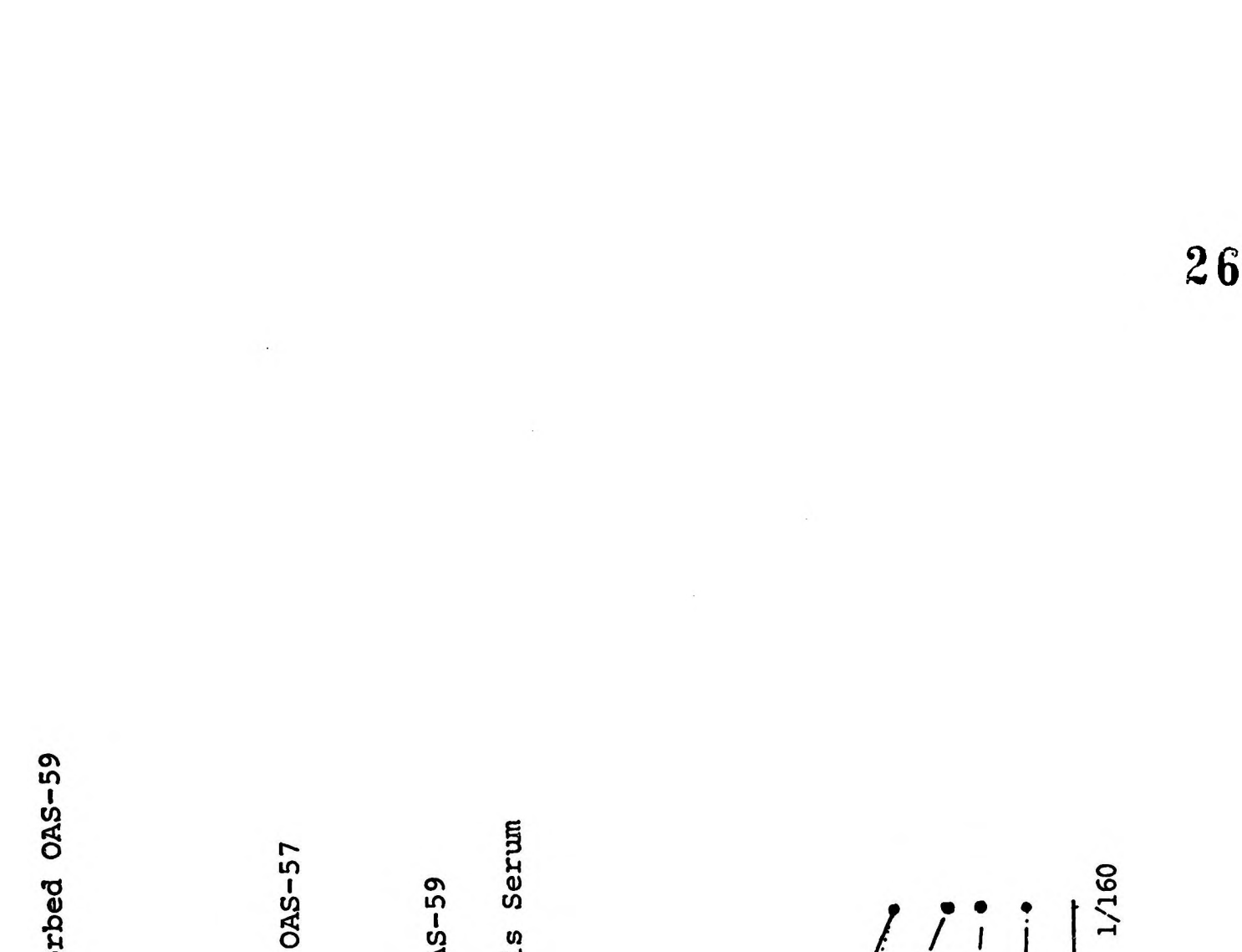
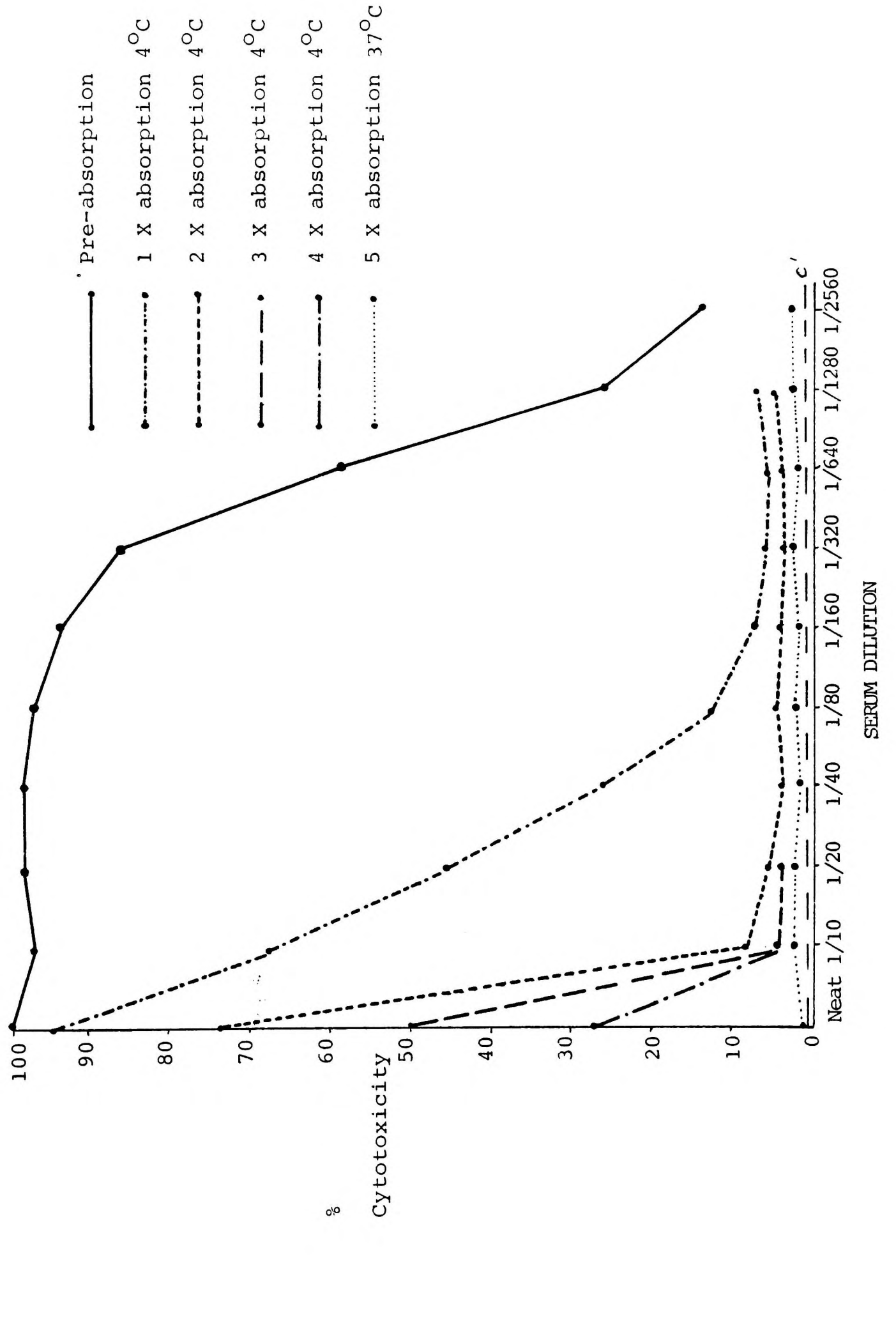

Comparison of Antigen Binding Capacity
of OAS-57, OAS-57 F(ab')2, OAS-59, Absorbed OAS-59
to DA L.N. Lymphocytes
OAS-57
F(ab')2 of OAS-57
OAS-59
Absorbed OAS-59
Normal Lewis Serum
ng RAR bound
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
Neat
1/10
1/20
1/40
1/80
1/160
SERUM DILUTION

Fig.4


OAS-59 TITRATION VS DA RBC
Pre-absorption
1 X absorption 4°C
2 X absorption 4°C
3 X absorption 4°C
4 X absorption 4°C
5 X absorption 37°C
% Cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
Neat 1/10 1/20 1/40 1/80 1/160 1/320 1/640 1/1280 1/2560
SERUM DILUTION

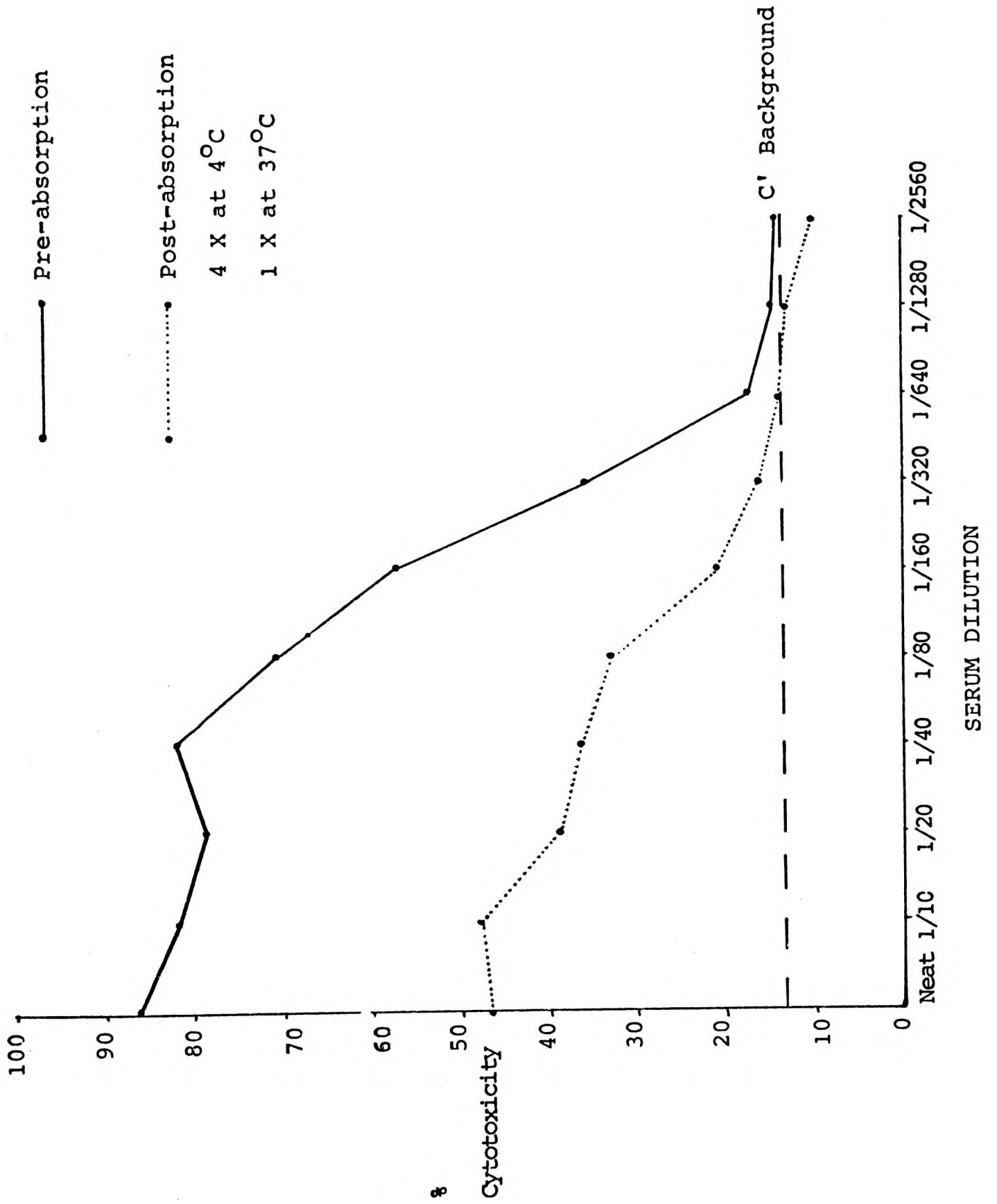

Pre-absorption
Post-absorption
4 X at 4°C
1 X at 37°C
OAS-59 TITRATION vs. PBL
C' Background
% Cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
Neat 1/10 1/20 1/40 1/80 1/160 1/320 1/640 1/1280 1/2560
SERUM DILUTION

Fig.5

Fig.6

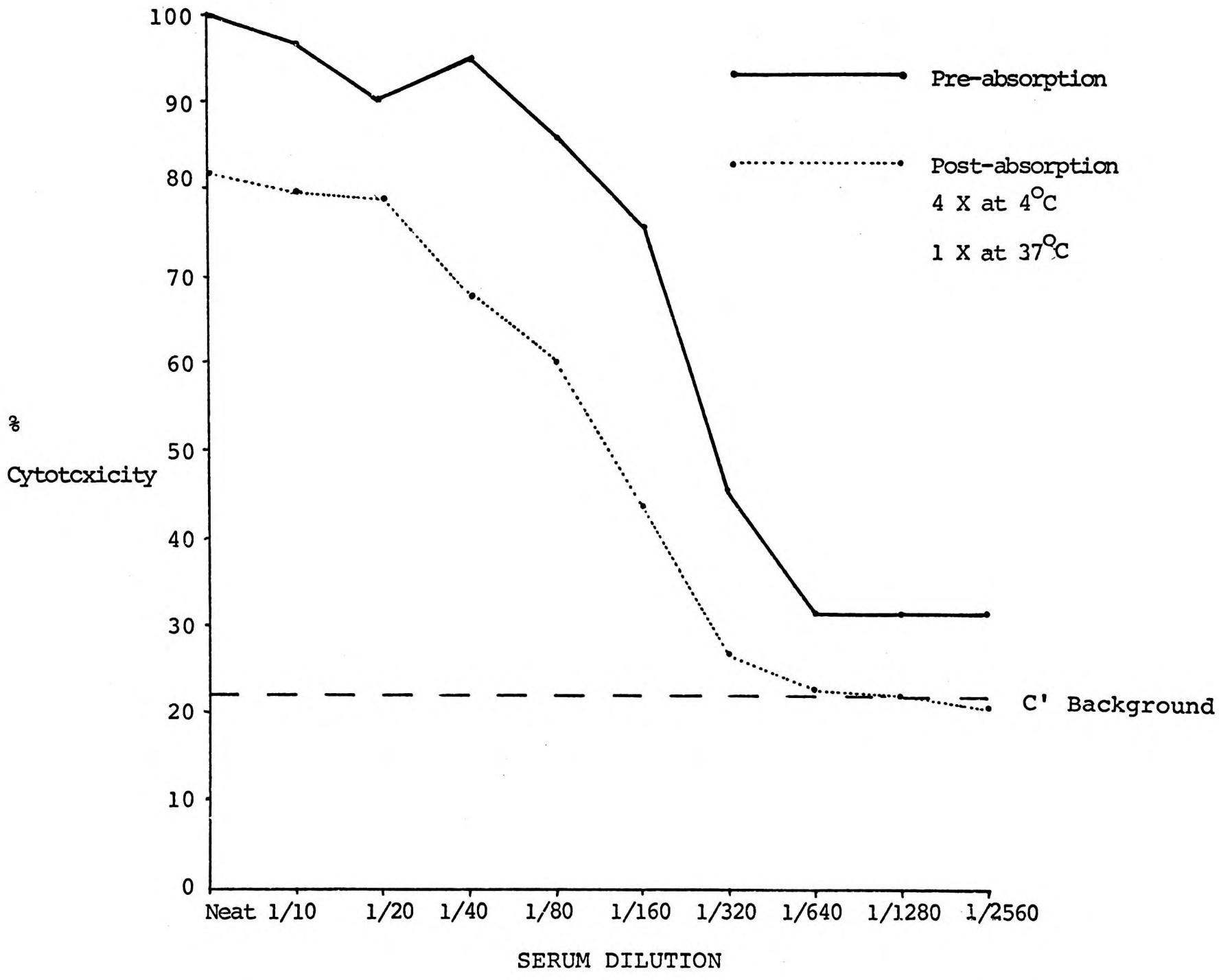
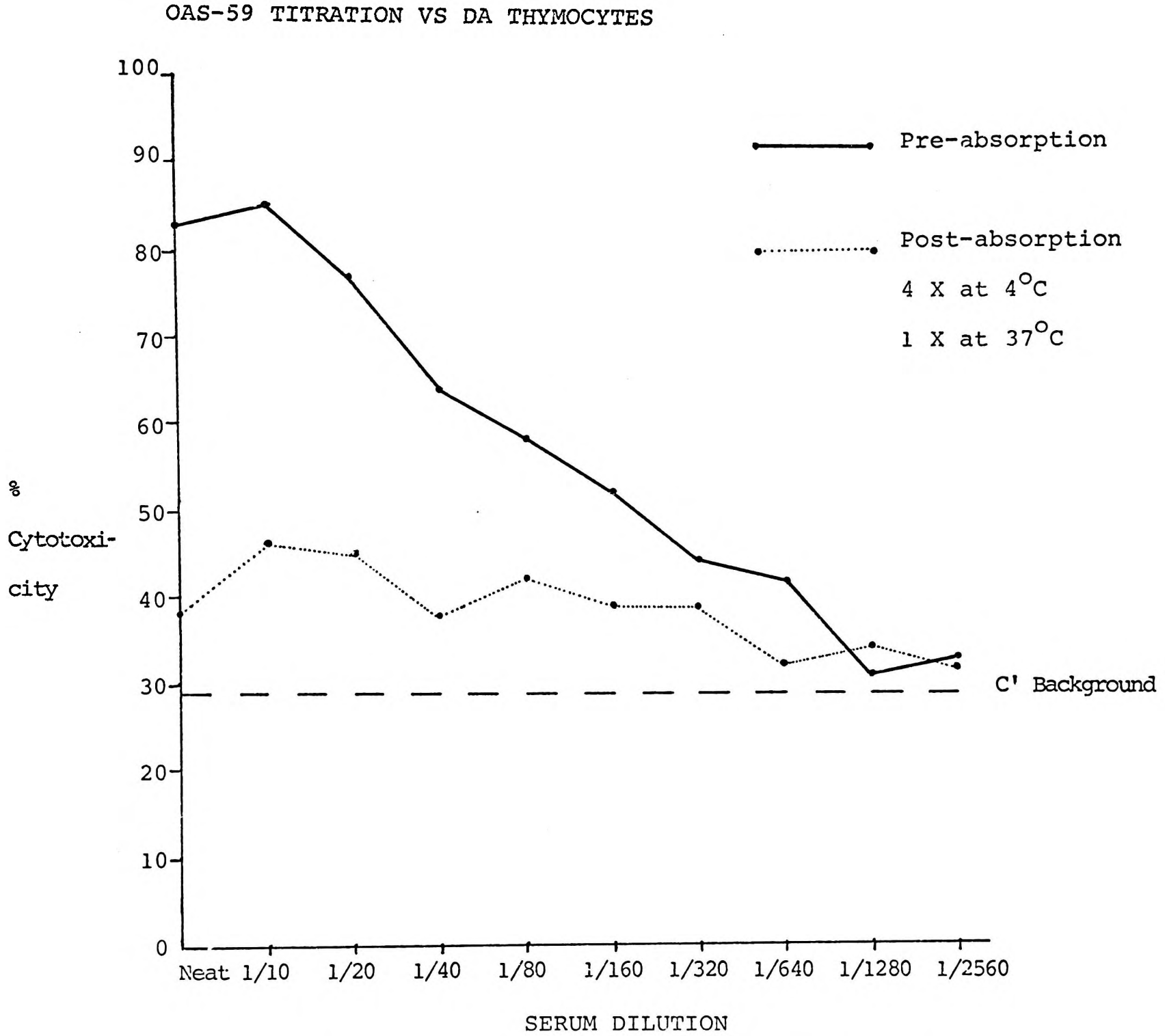

OAS-59 TITRATION VS DA SPLEEN CELLS
100
90
80
70
60
50
40
30
20
10
0
%
Cytotcxicity
Pre-absorption
Post-absorption
4 X at 4°C
1 X at 37°C
C' Background
Neat 1/10 1/20 1/40 1/80 1/160 1/320 1/640 1/1280 1/2560
SERUM DILUTION

Fig.7


OAS-59 TITRATION VS DA THYMOCYTES
100
90
80
70
60
50
40
30
20
10
0
%
Cytotoxi-
city
Pre-absorption
Post-absorption
4 X at 4°C
1 X at 37°C
C' Background
Neat 1/10 1/20 1/40 1/80 1/160 1/320 1/640 1/1280 1/2560
SERUM DILUTION

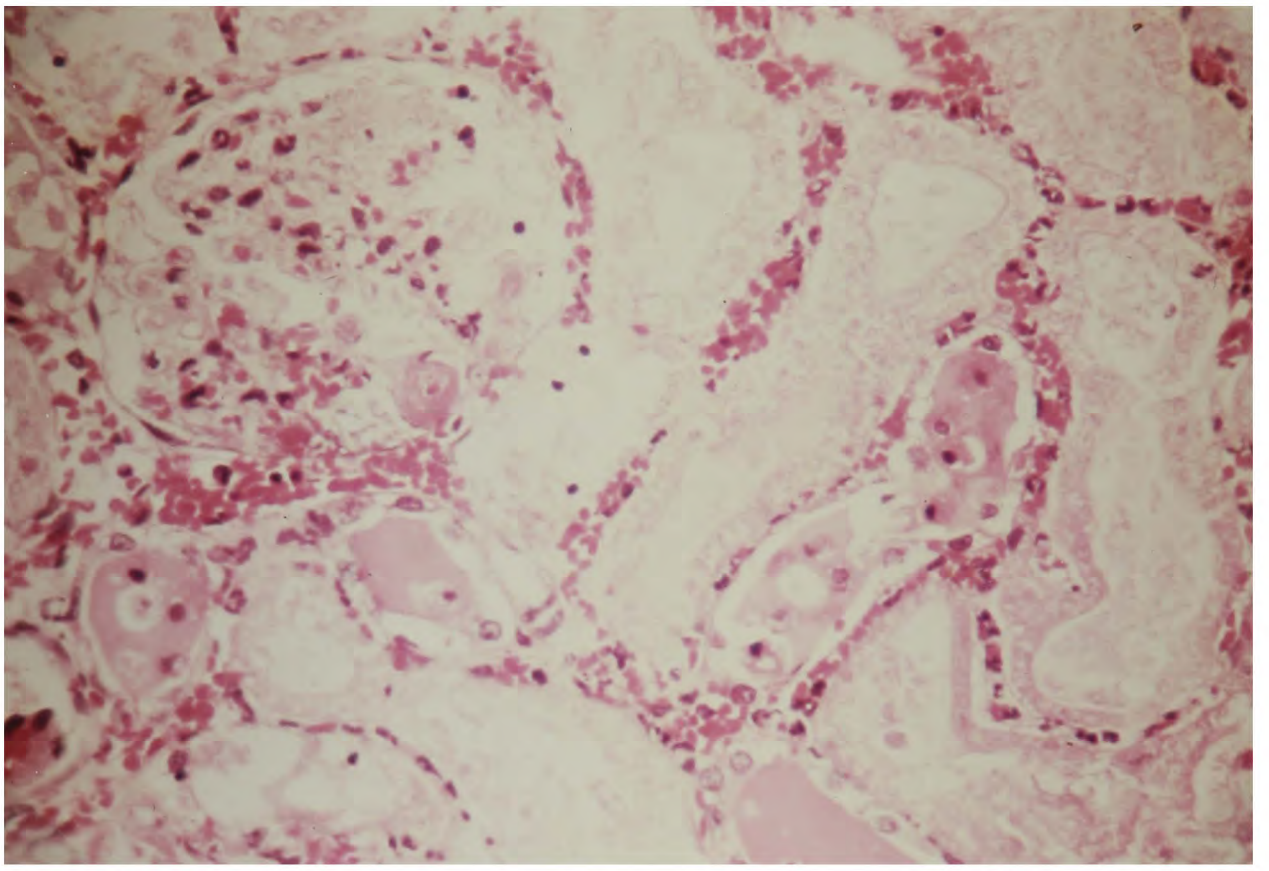


Fig. 8 Histological features of a DA renal allograft on day 3 after transplantation and administration of Lewis anti DA serum (1ml) and 2mls of GPC'. Note the glomerular and tubular necrosis and the infiltration with polymorphs. (x 360, H and E).

TABLE 1. TRANSPLANT RESULTS DA⟶LEWIS Renal transplant with immediate bilateral nephrectomy

Treatment	Rat No.	Urine Volume (mls/24hrs) at day 2	3	4	5	6	7	Blood Urea mg/100ml at day: 3	7	14	Survival (days)
1ml normal	2378	5	67	37	47	14	13	385	350	-	8
Lewis serum	2379	48	22	14	11	2	0	149	715	-	7
followed by	2382	64	20	14	10	4	0	105	510	-	7
2mls GPC'	2392	47	40	21	15	18	0	300	560	-	9
	2393	38	23	ND	20	3	0	71	410	-	7
1ml OAS-59	2371	0	-	-	-	-	-	770*	-	-	2
followed by	2372	0	0	0	-	-	-	970	-	-	4
2mls GPC'	2373	0	-	-	-	-	-	820*	-	-	2
	2374	0	0	0	-	-	-	910	-	-	4
	2375	0	-	-	-	-	-	495*	-	-	2
0.5mls OAS-59	2461	0	0	0	-	-	-	750	-	-	3
followed by	2462	0	0	0	-	-	-	720	-	-	3
2mls GPC'	2463	0	0	0	-	-	-	515	-	-	3
1ml $F(ab')_2$	2376	22	9	9	10	19	11	89	77	-	11
followed by	2381	17	14	14	17	18	10	61	76	-	13
1ml OAS-59	2388	29	35	33	31	30	20	285	103	-	11
followed by	2389	25	25	15	13	15	15	110	50	-	14
2mls GPC'	2377	0	-	-	-	-	-	685*	-	-	2
2mls absorbed	2421	23	29	27	22	24	30	79	170	-	10
OAS-59	2422	40	10	30	15	12	23	83	230	-	10
followed by	2423	48	34	16	20	25	23	165	205	-	12
2mls GPC'	2424	12	27	21	19	28	0	74	375	-	9
	2425	25	17	13	13	14	20	83	180	120	27

* Day 2 post-mortem specimen

TABLE 2: Histological Features (Median Score for Group)

Treatment	No.of specimens	Days Post-TX	Interstitium				Arterial Fibrinoid Necrosis	Tubular Necrosis	Glomerular Necrosis
			Monos	Polys	Oed	Haem			
1ml Normal Lewis serum plus 2mls GPC'	5	7-9	++	o	+	++	o	+++	++
1ml OAS-59 plus 2mls GPC'	5	2-4	o	++	o	++	o	+++	++
0.5mls OAS-59 plus 2mls GPC'	3	3	o	++	o	+	+	+++	++
1ml $F(ab')_2$ plus 1ml OAS-59 plus 2mls GPC'	1*	2	o	+	o	o	o	+	o
	4	11-14	+++	o	+	o	++	++	++
2mls absorbed OAS-59 plus 2mls GPC'	5	9-27	+++	o	+	o	o	++	++

* see text

CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

The aim of this thesis was directed towards the improvement of clinical immunosuppressive regimens, and in particular to the application of passive enhancement to human renal transplant practice. It is fair to ask whether the results of the experiments have brought these goals any closer and I shall deal with each of the groups of experiments separately.

1. <u>The use of non-specific immunosuppressive agents with passive enhancement</u>.

It was Russell (1971) who first showed that passive enhancement and non-specific immunosupprressive measures when used together resulted in greater survival of (mouse skin) allografts than could be achieved with either method alone. In his experiments it was ALS and irradiation which were the non-specific measures. Both French and Batchelor (1972) using azathioprine and prednisolone, and Batchelor et al. (1972) using ALS, confirmed that potentiation of enhancement was possible with non-specific measures.

The results of my experiments did not support the conclusions of these workers completely. I was able to show that enhancement could be potentiated by cyclophosphamide at a dose which was 50% below that which alone was fully effective. This effect depended on continuing treatment for at least 4 weeks. No disadvantage occurred despite the profound suppression of the enhanced animals own humoral responses. This result conflicted with that of Koene et al. (1975) who used a mouse skin graft model.

I also confirmed the potentiating effect of ALS on passive enhancement (Batchelor et al., 1972) and showed that like cyclophosphamide the potentiating dose was 50% of the fully effective dose. However, no significant potentiation was demonstrated with azathioprine and prednisolone, the standard agents used for human transplant recipients. Indeed, azathioprine may actually have antagonised the effect of enhancement. This result also conflicted with Koene et al. (1975).

What is the relevance of these findings?

In retrospect, I feel that the experimental approach was unlikely to provide an answer to the question of how non-specific immunosuppressive agents interact with passive enhancement. Firstly, the immunosuppressive effects and mechanisms of neither passive enhancement nor the non-specific agents were adequately defined. The addition of two unknowns could not give an interpretable result. Secondly, the effects of drugs vary so much between species that, whatever the result of any animal experiment, one would hesitate to apply the findings to man.

The demonstration of only a trivial effect with azathioprine and prednisolone in the suppression of rat renal allograft rejection compared to its potent effect in chronically uraemic man, illustrates this point. Because these drugs are effective in dogs they are being used in current experiments in the application of passive enhancement to this species (Fabre, 1978 unpublished) and would be the first choice in enhancement studies in man.

Another observation which must modify any claims about the efficacy of the combination of passive enhancement and non-specific immunosuppressive drugs is the ability of these latter agents to induce a state of "enhancement", quite independent of the administration of enhancing serum. Both ALS alone and cyclophosphamide alone, in high doses, completely suppressed the allograft response with no sign of rejection 4 to 5 months after stopping the treatment. Although there are some reports that it is possible to discontinue azathioprine in long surviving human kidney graft recipients, (Sheriff et al., 1978) withdrawal of immunosuppressive therapy in man usually results in rejection (Uehling et al., 1976).

These criticisms of the section notwithstanding certain important points emerged.

(a) Passive enhancement allowed a reduction in the intensity of non-specific immunosuppression required to achieve prolonged graft survival. One must also be cautious with the significance of this conclusion because the only measure of the levels of immunosuppression achieved by the sub-optimal doses of ALS and cyclophosphamide was that they could not alone suppress rejection. There is no way of knowing the extent to which the overall immuno-competence of the animal was impaired by these "suboptimal" doses. The work of Hall et al. (1978), showing that only a small fraction of the total recirculating lymphocyte pool is required to effect rejection at a normal tempo, suggests the "suboptimal" doses must have caused a substantial

depletion of the recirculating lymphocyte pool. Moreover, the potentiating dose of cyclophosphamide, 5.3mg/kg/day, was able to abolish the humoral response to the histocompatibility antigens of the graft/donor completely which suggests that the degree of immunosuppression achieved although suboptimal in terms of graft survival, was not trivial.

(b) The duration of administration of non-specific immunosuppressive agents with enhancing serum was critical. Although ALS could be given for only 5 days, its effects are likely to have lasted for several weeks. Cyclophosphamide needed to be given for 4 weeks to achieve complete and permanent suppression of rejection. Two weeks treatment allowed delayed but severe rejection to occur. This is an important point because in several experiments investigating this question, treatment was confined to short courses immediately after transplantation, e.g. Koene et al. (1975).

Ideally, the problem of selecting the appropriate non-specific immunosuppressive agent to be used with specific immunosuppressive measures should be approached by meticulous dissection of the immune responses in enhanced animals as has been performed by Burgos et al. (1974), Strom et al. (1975a),Abbas et al. (1974a & b). This would allow identification of those components of the allograft response which were either unimpaired or only partially suppressed in enhanced animals. A similar approach to the non-specific immunosuppressive agents would allow a selection of the appropriate agent, dose, and time of administration.

If the (DA X Lewis)F_1 $\longrightarrow$ Lewis model is analogous to the models examined by Burgos et al. (1974) and Strom et al. (1975a) then it is a delayed, and in part attenuated, cell-mediated rejection reaction which must be suppressed in enhanced animals. The synergistic action of ALS and passive enhancement support this hypothesis. The hypothesis could not be tested because I was unable to establish an in-vitro assay for cell-mediated cytotoxicity to DA antigens in Lewis recipients of (DA X Lewis)F_1 renal allografts.

2. The combined use of ALS and cyclophosphamide.

Experiments with this theoretically attractive combination showed that although they acted additively in the suppression of rejection they were not synergistic.

The hypothesis that they would act synergistically was based on two assumptions:- (1) that acute renal allograft rejection is mediated by both T-cell and B-cell dependent immune responses, and (2) that ALS and cyclophosphamide have selective actions on these two types of response.

In fact no selectivity of the two agents could be demonstrated when they were compared for their efficacy in suppressing rejection and alloantibody production in response to an allograft.

Because adequate suppression of T-cells should be sufficient to suppress rejection (Hall et al., 1978) the additional use of cyclophosphamide is unlikely to provide anything more than an increase in overall immunosuppression.

The addition of cyclophosphamide will have the disadvantage of depleting the host of rapidly dividing cells such as polymorphs and B-cell precursors which may be required for the defence against infection but not be essential for graft rejection.

3. Passive enhancement and the problem of hyperacute rejection.

It was in these experiments that the clearest answers were obtained. The $F(ab')_2$ fraction of enhancing antibody was found to be, for practical purposes, ineffective in inducing renal allograft enhancement. This result was in agreement with the unpublished data of Stuart (1973) and Batchelor (1978). It conflicted with the findings of Shaipanich et al. (1971) and (1973). It was consistent with the observations of Marquet (1979) who showed that antigen-antibody complexes could enhance rat cardiac allografts but antigen-$F(ab')_2$ complexes could not. On the other hand, it was found that $F(ab')_2$ did not inhibit the enhancing effect of whole antibody and that if administered before whole antibody could prevent complement-dependent antibody-mediated damage to the graft. This latter finding confirmed that of Holter et al. (1973) in rabbits. Their experiments were a more stringent test of this blocking effect because the rabbit is sensitive to antibody-mediated damage without the addition of xenogeneic complement.

A blocking role for $F(ab')_2$ would appear therefore to be the limit of its use in the context of enhancement.

The use of "anti-Ia-like" sera was also shown to be a possible means of avoiding antibody-mediated damage to the graft without forfeiting the specific immunosuppressive effect. This finding was consistent with the observations made for skin grafts in mice by Jansen et al. (1975b) and McKenzie and Henning (1978). The practicalities of preparing anti-Ia-like sera in man are formidable. Conventional anti-HLA sera could be platelet absorbed to remove antibodies directed against A,B, and C locus antigens. Alternatively sera which react with donor B lymphocytes and not T lymphocytes would have to be selected at the time of cross-match. The first approach has been adopted by Marquet et al. (1978) in the rhesus monkey and the results suggest that passive enhancement in primates does not have the dramatic effect on rejection which is seen in rodents. Nevertheless, a slight effect should not be ignored for it may be sufficient to reduce steroid requirements or reduce the number of irreversible rejection episodes.

The problem of the source of enhancing antibody may be overcome by using xenosera. Hutchinson et al. (1976) have demonstrated enhancement of rat renal allografts with rabbit antisera specific for rat alloantigens.

It is still possible that these precautions to avoid antibody-mediated damage in man may not be necessary.

Antibody-mediated damage to grafts by passively administered sera seems to be very dose-dependent (McDowall et al., 1973; Fabre & Morris, 1974a;Berden et al., 1977) and so, if small doses of serum could be given, antibody-mediated damage might not occur.

The goal of controlling allograft rejection without compromising host defences may be reached not by a single step but by a series of improvements in patient management, for example:DRw matching (Ting and Morris, 1978); exploiting the as yet unexplained effect of pre-transplant blood transfusion (Editorial, Lancet, 1978);finding the minimum doses of corticosteroids required (Kauffman et al., 1979);and eventually, by the application of enhancement.

APPENDICES

1. Results of Lewis→Lewis renal isografts in the delayed nephrectomy model.

2. Histological assessment of Lewis→Lewis isografts.

3. The effect of low doses of azathioprine and prednisolone on enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts with immediate nephrectomy. (Chapter 6 section i Tables 1 & 3)

4. The effect of low doses of azathioprine and prednisolone on the lymphocytotoxic antibody responses of Lewis recipients of (DA X Lewis)F_1 renal allografts. (Chapter 6 section i Tables 2 & 4).

5. The effect of azathioprine 30mg/kg/day x 14 days on enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts with immediate nephrectomy. (Chapter 6 section i Table 6).

6. The effect of azathioprine 30mg/kg/day x 14 days on the lymphocytotoxic antibody responses of Lewis recipients of (DA X Lewis)F_1 renal allografts with immediate nephrectomy. (Chapter 6 section i Table 7).

7. The effect of high doses of azathioprine and prednisolone on enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts with delayed nephrectomy. (Chapter 6 section i Table 8).

8. The effect of high doses of azathioprine and prednisolone on the lymphocytotoxic antibody responses of Lewis recipients of (DA X Lewis)F_1 renal allografts with delayed nephrectomy (Chapter 6 section i Table 9).

9. (Experiment A) The effect of cyclophosphamide on enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts.
(Chapter 6 section ii Table 1).

10. (Experiment A) The effect of cyclophosphamide on the lymphocytotoxic antibody responses of enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts. (Chapter 6 section ii Table 2).

11. (Experiment B) The effect of cyclophosphamide on enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts.
(Chapter 6 section ii Table 3).

12. (Experiment B) The effect of cyclophosphamide on the lymphocytotoxic antibody responses of enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts. (Chapter 6 section ii Table 4).

13. Preparation of ALS(DE) and ALS(5,6) Rabbit anti-rat lymphocyte sera.
(Chapter 6 section iii and Chapter 7).

14. Dose response results for ALS(DE) on enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts (Chapter 6 section iii Tables 1 & 2).

15. Dose response results for ALS(DE) on the lympho-cytotoxic antibody responses of enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts. (Chapter 6 section iii Tables 3 & 4).

16. The effect of ALS(5,6) and cyclophosphamide on Lewis recipients of DA renal allografts.Renal function and survival.
(Chapter 7 Tables 1,2,3).

17. The effect of ALS (5,6) and cyclophosphamide on Lewis recipients of DA renal allografts.(Antibody titres)
(Chapter 7 Tables 6,7).

18. Results of Lewis DA renal allografts in untreated recipients and those treated with DA anti Lewis (OAS-58), serum, IgG, $F(ab')_2$.
(Chapter 8 section 1a Table 1).

APPENDIX 1 — Results of Lewis ⟶ Lewis renal isografts with delayed nephrectomy

(Chapter 5 section 2)

Rat No.	Blood urea (mg/100ml) at week: 10d	2	3	4	6	8	12	20	Survival (days)
2400	43	43	50	49	56	59	55	61	>140
2403	60	55	56	64	67	63	86	73	>140
2404	62	60	73	52	59	54	90	55	>140
2407	49	37	53	62	60	64	69	84	>140
2411	48	48	52	62	60	45	47	57	>140

APPENDIX 2

(Chapter 5 sections 2 and 4)

Histological assessment of Lewis→Lewis isografts

Rat No.	Day Post Transplant	HISTOLOGICAL SCORE									
		INTERSTITIUM				ARTERIES		TUBULES		GLOMERULI	Histo score
		Monos	Polys	Oed	Haem	Fib.	Subint.Mono	Necrosis	Atrophy	Capillary necrosis	
2400	7	+	0	0	0	0	0	0	0	0	1
2403	7	++	0	+	0	0	0	0	+	0	4
2404	7	+	0	0	0	0	0	0	0	0	1
2407	7	++	0	+	0	0	0	0	0	0	3
2411	7	+	0	0	0	0	0	0	0	0	1
2403	14	0	0	0	0	0	0	0	0	0	0
2404	14	0	0	0	0	0	0	0	0	0	0
2407	14	+	0	0	0	0	0	0	+	0	2
2411	14	+	0	0	0	0	0	0	0	0	1

Monos = Mononuclear cell infiltrate
Polys = Polymorph infiltrate
Oed = Oedema
Haem = Haemorrhage

Fib = Fibrinoid necrosis
Subint.Mono = Subintimal Mononuclear cells

Experiment (a)

APPENDIX 3

(Chapter 6 section 1
Tables 1 and 3)

The effect of low doses of azathioprine and prednisolone on enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts with immediate nephrectomy.

Treatment	Rat No.	Blood urea (mg/100ml) at weeks: 1	2	3	4	6	8	12	20	Survival (days)
1. NIL	1105	ND*	-	-	-	-	-	-	-	7
	1108	ND*	-	-	-	-	-	-	-	7
	1152	ND*	-	-	-	-	-	-	-	7
	1107	705	-	-	-	-	-	-	-	7
	1106	413	-	-	-	-	-	-	-	9
	1109	663	-	-	-	-	-	-	-	10
	1225	520	-	-	-	-	-	-	-	7)Sacrificed
	1226	605	-	-	-	-	-	-	-	7) for
	1227	690	-	-	-	-	-	-	-	7)histology
2. Azathioprine 4mg/kg day x 6 weeks	1144	ND*	-	-	-	-	-	-	-	7
	1140	525	-	-	-	-	-	-	-	9
	1145	450	-	-	-	-	-	-	-	9
	1138	250	-	-	-	-	-	-	-	10
	1142	455	-	-	-	-	-	-	-	10
	1143	438	-	-	-	-	-	-	-	10
3. Azathioprine 8mg/kg day x 6 weeks	1151	ND	-	-	-	-	-	-	-	8
	1150	390	-	-	-	-	-	-	-	8
	1160	435	-	-	-	-	-	-	-	10
	1146	350	-	-	-	-	-	-	-	11
	1158	270	-	-	-	-	-	-	-	11
	1159	460	188	206	207	165	160	280	-	111
4. Methylprednisolone 4mg/kg/day x 3 weeks then 2mg/kg/day x 3 weeks	1195	265	-	-	-	-	-	-	-	9
	1213	430	-	-	-	-	-	-	-	9
	1196	135	190	185	116	106	92	-	-	62'
	1187	640	170	149	123	102	104	90	71	>140
	1188	370	67	79	74	59	68	63	52	>140
	1192	425	80	137	158	120	75	90	69	>140
5. Methylprednisolone 8mg/kg/day x 3 weeks then 4mg/kg/day x 3 weeks	1162	263	-	-	-	-	-	-	-	9
	1165	350	-	-	-	-	-	-	-	9
	1167	118	-	-	-	-	-	-	-	9
	1161	195	-	-	-	-	-	-	-	10
	1166	280	ND	-	-	-	-	-	-	17
	1163	141	76	112	106	86	81	66	50	>140
6. Azathioprine 8mg/kg/day x 6 weeks plus prednisolone 8mg/kg/day x 3 weeks then 4mg/kg/day x 3 weeks	1179	440	-	-	-	-	-	-	-	8
	1175	405	-	-	-	-	-	-	-	11
	1173	240	-	-	-	-	-	-	-	12
	1172	228	-	-	-	-	-	-	-	13
	1177	205	-	-	-	-	-	-	-	13
	1176	160	154	106	82	85	87	85	90	>140
7. OAS-53 500µl days 0, 1, 3, 5.	1268	355	135	510	-	-	-	-	-	23
	1265	122	280	210	395	-	-	-	-	39
	1264	138	ND	290	ND	ND	145	160	123	>140
	1266	74	100	101	129	142	110	105	106	>140
	1267	85	115	184	290	175	168	148	130	>140
	1269	245	195	235	250	240	ND	115	440	>140
8. OAS-53 500µl days 0, 1, 3, 5 plus azathioprine 8mg/kg/day x 6 weeks	1274	610	-	-	-	-	-	-	-	9
	1281	113	164	510	-	-	-	-	-	23
	1271	91	59	70	-	-	-	-	-	26
	1273	76	119	100	155	113	95	80	65	>140
	1277	285	145	78	400	162	109	88	48	>140
	1283	135	180	365	176	182	162	121	105	>140
9. OAS-53 500µl days 0, 1, 3, 5 plus prednisolone 4mg/kg/day x 3 weeks then 2mg/kg/day x 3 weeks	1276	415	-	-	-	-	-	-	-	9
	1270	405	-	-	-	-	-	-	-	10
	1272	93	190	138	ND	-	-	-	-	30
	1275	97	ND	101	ND	ND	23	96	95	>140
	1278	220	78	79	78	89	ND	85	77	>140
	1282	106	270	300	500	170	235	330	405	>140
	1284	64	65	70	44	46	46	56	57	>140

ND = Not done

* = dead

' Died with abscess of abdominal wall

Experiment (a)

APPENDIX 4

(Chapter 6 section 1 Tables 2 and 4)

The effect of low doses of azathioprine and prednisolone on the lymphocytotoxic antibody responses of enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts.

Treatment	Rat No.	Titre at weeks 1	2	3	4	6	8	12	20
1. NIL	1105	ND	-	-	-	-	-	-	-
	1108	ND	-	-	-	-	-	-	-
	1152	ND	-	-	-	-	-	-	-
	1107	1/32	-	-	-	-	-	-	-
	1106	1/4	-	-	-	-	-	-	-
	1109	1/32	-	-	-	-	-	-	-
	1225	ND	-	-	-	-	-	-	-
	1226	1/2	-	-	-	-	-	-	-
	1227	1/2	-	-	-	-	-	-	-
2. Azathioprine 4mg/kg/day x 6 weeks	1144	ND	-	-	-	-	-	-	-
	1140	1/32	-	-	-	-	-	-	-
	1145	ND	-	-	-	-	-	-	-
	1138	1/32	-	-	-	-	-	-	-
	1142	1/32	-	-	-	-	-	-	-
	1143	1/32	-	-	-	-	-	-	-
3. Azathioprine 8mg/kg/day x 6 weeks	1151	ND	-	-	-	-	-	-	-
	1150	1/32	-	-	-	-	-	-	-
	1160	1/4	-	-	-	-	-	-	-
	1146	1/8	-	-	-	-	-	-	-
	1158	1/2	-	-	-	-	-	-	-
	1159	1/32	1/1	0	0	1/1	1/1	0	-
4. Methylprednisolone 4mg/kg/day x 21 days 2mg/kg/day x 21 days	1195	0	-	-	-	-	-	-	-
	1213	1/16	-	-	-	-	-	-	-
	1196	1/1	1/2	1/2	1/1	0	0	ND	ND
	1187	1/32	1/4	0	0	0	0	ND	ND
	1188	1/4	1/1	1/1	1/1	0	0	ND	ND
	1192	1/4	0	1/2	0	0	0	ND	ND
5. Methylprednisolone 8mg/kg/day x 21 days then 4mg/kg/day x 21 days	1162	1/32	-	-	-	-	-	-	-
	1165	1/4	-	-	-	-	-	-	-
	1167	1/4	-	-	-	-	-	-	-
	1161	1/32	-	-	-	-	-	-	-
	1166	ND	1/1	-	-	-	-	-	-
	1163	0	1/1	0	0	0	0	0	0
6. Azathioprine 8mg/kg/day x 6 weeks plus prednisolone 8mg/kg/day x 3 weeks then 4mg/kg/day x 3 weeks	1179	1/8	-	-	-	-	-	-	-
	1175	0	-	-	-	-	-	-	-
	1173	1/2	-	-	-	-	-	-	-
	1172	1/8	-	-	-	-	-	-	-
	1177	1/2	-	-	-	-	-	-	-
	1176	1/2	1/1	0	0	0	0	0	ND
7. OAS-53 500µl days 0,1, 3, 5.	1268	0	0	0	-	-	-	-	-
	1265	0	0	0	0	-	-	-	-
	1264	0	ND	0	ND	ND	ND	ND	ND
	1266	1/1	0	0	0	ND	ND	ND	ND
	1267	1/1	0	0	0	ND	ND	ND	ND
	1269	0	0	0	1/1	ND	ND	ND	ND
8. OAS-53 500µl days 0,1, 3, 5 plus azathioprine 8mg/kg/day x 6 weeks	1274	0	-	-	-	-	-	-	-
	1281	1:1	0	0	0	0	0	0	0
	1271	1:1	0	0	0	0	0	0	0
	1273	1:1	0	0	0	0	0	0	0
	1277	1:1	0	0	0	0	0	0	0
	1283	1:1	1:1	0	1:1	0	0	0	0
9. OAS-53 500µl days 0, 1,3,5 plus methylprednisolone 4mg/kg/day x 21 days 2mg/kg/day x 21 days	1276	0	-	-	-	-	-	-	-
	1270	0	-	-	-	-	-	-	-
	1272	1:1	0	0	ND	-	-	-	-
	1275	0	ND	0	ND	ND	0	0	0
	1278	1:1	1:1	0	0	0	ND	0	0
	1282	0	0	0	0	0	0	0	0
	1284	1:1	0	1:1	0	0	0	ND	0

ND = Not done

Experiment (b)

APPENDIX 5: The effect of azathioprine 30mg/kg/day x 14days on enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts with immediate nephrectomy.

(Chapter 6 section i Table 6)

	Treatment	Rat No.	Blood urea (mg/100ml) at week: 1	2	3	4	6	8	12	20	Survival (days)
1.	NIL	(see Appendix 3 for individual results)									
		n=6	634±114	-	-	-	-	-	-	-	7 (7-10)
2.	Azathioprine 30mg/kg/day x 14 days	1348	365	171	-	-	-	-	-	-	17
		1347	400	215	-	-	-	-	-	-	19
		1350	385	ND	240	360	360	200	-	-	77
		1344	140	265	520	490	210	185	165	245	>140
		1346	335	165	195	260	230	146	104	126	>140
3.	OAS-55 500µl days 0,1	1330	108	160	-	-	-	-	-	-	16
		1332	103	340	420	340	245	210	145	-	98
		1338	67	430	128	170	177	192	165	-	133
		1337	58	205	285	185	133	119	75	95	>140
		1340	49	310	300	212	148	118	83	97	>140
		1341	185	550	275	330	280	235	200	250	>140
4.	OAS-55 500µl days 0,1 plus azathioprine 30mg/kg/day x 14 days	1365	66	114	-	-	-	-	-	-	18
		1360	51	163	-	-	-	-	-	-	19
		1364	59	79	-	-	-	-	-	-	20
		1412	39	57	710	-	-	-	-	-	20
		1363	43	82	565	-	-	-	-	-	24
		1370	52	87	340	315	320	260	280	-	91

Experiment (b)

APPENDIX 6: (Chapter 6 section i Table 7)

The effect of azathioprine 30mg/kg/day x 14days on the lymphocytotoxic antibody response of Lewis recipients of (DA X Lewis)F_1 renal allografts with immediate nephrectomy.

	Treatment	Rat No.	Lymphocytotoxic antibody titre at week: 1	2	3	4	6	8	12	20
1.	NIL*	1821	1/4	-	-	-	-	-	-	-
		1822	1/2	-	-	-	-	-	-	-
		1839	1/4	-	-	-	-	-	-	-
		2437	1/4	-	-	-	-	-	-	-
		2438	1/8	-	-	-	-	-	-	-
2.	Azathioprine 30mg/kg/day x 14days	1348	1/2	0	-	-	-	-	-	-
		1347	ND	1/1	-	-	-	-	-	-
		1350	1/4	ND	1/1	ND	1/1	0	-	-
		1344	0	0	0	ND	0	1/1	0	0
		1346	1/4	1/1	1/1	1/1	1/2	1/2	0	0
3.	OAS-55 500µl i.v. days 0,1.	1330	1/1	1/2	-	-	-	-	-	-
		1332	1/2	1/1	1/1	0	0	0	0	-
		1338	1/1	1/2	1/1	0	1/2	1/1	0	-
		1337	0	1/2	0	1/1	0	0	0	0
		1340	1/1	1/1	0	0	0	0	0	0
		1341	1/1	1/2	0	0	0	0	0	ND
4.	OAS-55 500µl i.v. days 0,1 plus azathioprine 30mg/kg/day x 14days	1365	1/2	1/2	-	-	-	-	-	-
		1360	1/1	1/1	-	-	-	-	-	-
		1364	1/1	1/1	-	-	-	-	-	-
		1412	1/1	1/2	0'	-	-	-	-	-
		1363	1/1	1/2	ND	-	-	-	-	-
		1370	1/1	1/1	0	0	0	0	0	-

' 20 days ND = Not done * Extra controls

Experiment (c)

APPENDIX 7

(Chapter 6 section 1 Table 8)

The effect of high doses of azathioprine and prednisolone on enhanced and non-enhanced Lewis recipients of (LA X Lewis)F_1 renal allografts with delayed nephrectomy.

Treatment	Rat No.	Blood urea (mg/100ml) at weeks: 10d	2	3	4	6	8	12	20	Survival (days)
1. NIL	1393	560	-	-	-	-	-	-	-	11
	1398	530	-	-	-	-	-	-	-	11
	1400	640	-	-	-	-	-	-	-	11
	1435	505	-	-	-	-	-	-	-	11
	1439	670	-	-	-	-	-	-	-	11
	1397	460	-	-	-	-	-	-	-	12
	1403	330	-	-	-	-	-	-	-	13
2. Azathioprine 30mg/kg/day x 21 days	1531	ND	-	-	-	-	-	-	-	10
	1524	650	-	-	-	-	-	-	-	10
	1528	610	-	-	-	-	-	-	-	10
	1532	575	-	-	-	-	-	-	-	11
	1529	425	-	-	-	-	-	-	-	12
3. Methylprednisolone 16mg/kg/day x 21 days	1508	ND	-	-	-	-	-	-	-	10
	1509	ND	-	-	-	-	-	-	-	10
	1504	600	-	-	-	-	-	-	-	10
	1511	550	-	-	-	-	-	-	-	10
	1503	475	-	-	-	-	-	-	-	11
4. Azathioprine 30mg/kg/day x 3 weeks plus methylprednisolone 16mg/kg/day x 3 weeks	1571	ND	-	-	-	-	-	-	-	10
	1564	575	-	-	-	-	-	-	-	10
	1566	500	-	-	-	-	-	-	-	10
	1574	665	-	-	-	-	-	-	-	11
	1589	550	-	-	-	-	-	-	-	13
	1569	400	120	74	82	82	105	80	65	>140
5. OAS-55 500µl days 0,1	1465	ND	385	-	-	-	-	-	-	16
	1452	55	305	320	250	150	175	245	-	100
	1449	44	129	270	210	215	135	41	-	113
	1454	56	190	360	155	165	148	112	105	>140
	1457	50	99	135	150	145	155	100	135	>140
	1553	45	275	155	155	165	130	140	150	>140
6. OAS-55 500µl days 0,1 plus azathioprine 30mg/kg/day x 3 weeks	1490	36	51	ND	-	-	-	-	-	21
	1488	46	39	435	-	-	-	-	-	24
	1492	68	71	230	-	-	-	-	-	28
	1499	340	95	89	-	-	-	-	-	28
	1480	47	47	150	270	205	-	-	-	49
7. OAS-55 500µl days 0,1 plus prednisolone 16mg/kg/day x 3 weeks	1475	36	185	-	-	-	-	-	-	18
	1461	41	109	-	-	-	-	-	-	19
	1474	31	105	195	250	-	-	-	-	36
	1463	33	85	485	435	475	225	350	270	>140
	1477	75	102	175	500	225	175	150	120	>140
	1587	40	175	175	88	85	60	75	65	>140
8. OAS-55 500µl days 0,1 plus azathioprine 30mg/kg/day plus prednisolone 16mg/kg/day x 3 weeks	1581	50	60	435	-	-	-	-	-	21'
	1519	47	57	59	550	-	-	-	-	28
	1527	51	55	260	475	-	-	-	-	32
	1521	ND	50	66	170	325	325	350	-	104
	1520	ND	57	61	138	200	240	155	138	>140

ND = Not done

' Died with multiple abscesses.

Experiment (c)

APPENDIX 8

(Chapter 6 section 1) Table 9

The effect of high doses of azathioprine and prednisolone on the lymphocytotoxic antibody responses of enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts with delayed nephrectomy.

Treatment	Rat No.	Lymphocytotoxic antibody titre at week: 1	10d	2	3	4	6	8	12	20
1. NIL	1393	1/256	1/32	-	-	-	-	-	-	-
	1398	1/128	ND	-	-	-	-	-	-	-
	1400	1/128	1/256	-	-	-	-	-	-	-
	1435	>1/512	1/128	-	-	-	-	-	-	-
	1439	1/256	1/32	-	-	-	-	-	-	-
	1397	1/256	ND	-	-	-	-	-	-	-
	1403	1/128	1/32	-	-	-	-	-	-	-
2. Azathioprine 30mg/kg/day x 21 days	1531	>1/512	ND	-	-	-	-	-	-	-
	1524	1/256	1/256	-	-	-	-	-	-	-
	1528	1/64	1/16	-	-	-	-	-	-	-
	1532	1/512	1/256	-	-	-	-	-	-	-
	1529	1/256	1/32	-	-	-	-	-	-	-
3. Methylprednisolone 16mg/kg/day x 21 days	1508	>1/512	ND	-	-	-	-	-	-	-
	1509	1/512	ND	-	-	-	-	-	-	-
	1504	>1/512	1/256	-	-	-	-	-	-	-
	1511	>1/512	1/512	-	-	-	-	-	-	-
	1503	1/256	1/256	-	-	-	-	-	-	-
4. Azathioprine 30mg/kg/day plus methylprednisolone 16mg/kg/day x 21 days	1571	1/32	ND	-	-	-	-	-	-	-
	1564	1/8	0	-	-	-	-	-	-	-
	1566	1/4	0	-	-	-	-	-	-	-
	1574	1/256	1/256	-	-	-	-	-	-	-
	1589	1/16	1/2	-	-	-	-	-	-	-
	1569	1/32	0	0	0	0	0	0	0	0
5. OAS-55 500µl days 0,1.	1465	1/1	1/16	1/64	-	-	-	-	-	-
	1452	1/1	1/4	1/4	0	0	0	0	0	-
	1449	1/2	1/2	1/4	1/2	1/2	0	0	0	-
	1454	1/1	1/8	1/4	1/1	1/4	1/1	1/1	1/1	0
	1457	0	1/1	1/4	1/1	0	0	0	0	0
	1553	1/1	1/8	1/4	0	0	0	1/1	0	0
6. OAS-55 500µl days 0,1 plus azathioprine 30mg/kg/day x 21 days	1490	TF	1/2	1/4	-	-	-	-	-	-
	1488		1/2	ND	0	-	-	-	-	-
	1492		1/2	1/4	1/1	-	-	-	-	-
	1499		1/2	1/4	0	-	-	-	-	-
	1480		1/2	1/4	1	1	1	-	-	-
7. OAS-55 500µl days 0,1. plus methylprednisolone 16mg/kg/day x 21 days	1475	TF	ND	1/4	-	-	-	-	-	-
	1461		1/8	ND	-	-	-	-	-	-
	1474		1/8	1/4	1/2	1/2	-	-	-	-
	1463		1/16	1/8	1/2	1/1	0	0	0	ND
	1477		1/4	1/1	1/4	1/1	0	0	0	0
	1587		1/8	1/8	1/4	1/1	0	0	0	0
8. OAS-55 500µl days 0,1. plus azathioprine 30mg/kg/day x 21 days plus methylprednisolone 16mg/kg/day x 21 days	1581	2	0	0	0	-	-	-	-	-
	1519	1	0	0	0	0	-	-	-	-
	1527	1	1	1	0	0	-	-	-	-
	1521	1	ND	1	0	0	0	0	0	-
	1520	1	ND	1	0	0	ND	0	0	0

TF = Technical failure ND = Not done

Experiment (a)

APPENDIX 9

(Chapter 6 section 11 Table 1).

The effect of cyclophosphamide on enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts.

Treatment	Rat No.	Blood urea (mg/100ml) at weeks: 10d	2	3	4	6	8	12	20	Survival (days)
1. NIL	1393	560	-	-	-	-	-	-	-	11
	1397	460	-	-	-	-	-	-	-	12
	1398	530	-	-	-	-	-	-	-	11
	1400	640	-	-	-	-	-	-	-	11
	1403	330	-	-	-	-	-	-	-	13
	1435	505	-	-	-	-	-	-	-	11
	1439	670	-	-	-	-	-	-	-	11
2. Cyclophosphamide 5mg/kg/day x 14 days	1541	490	-	-	-	-	-	-	-	13
	1546	475	99	395	-	-	-	-	-	25
	1539	275	80	65	67	67	85	70	67	>140
	1548	550	200	115	90	126	125	100	130	>140
	1559	300	85	75	75	97	87	75	58	>140
3. 10mg/kg/day x 14 days	1537	75	57	-	-	-	-	-	-	15[1]
	1536	65	40	60	50	-	-	-	-	30[2]
	1535	75	50	55	50	45	65	60	ND	>140
	1544	66	45	65	48	42	52	60	55	>140
	1555	105	55	55	58	65	70	60	83	>140
	1558	90	55	55	64	65	63	55	41	>140
4. OAS-55 500µl days 0,1.	1465	ND	385	-	-	-	-	-	-	16
	1452	55	305	320	250	150	175	245	-	100
	1449	44	129	270	210	215	135	41	-	113
	1454	56	190	360	155	165	148	112	105	>140
	1457	50	99	135	150	145	155	100	135	>140
	1553	45	275	155	155	165	130	140	150	>140
5. OAS-55 500µl days 0,1. plus Cyclophosphamide 5mg/kg/day x 14 days	1578	50	48	-	-	-	-	-	-	20
	1575	155	210	66	535	-	-	-	-	29
	1586	39	47	325	275	-	-	-	-	32
	1612	42	47	275	145	>600	-	-	-	42
	1576	50	58	155	335	130	180	140	165	>140
	1592	125	55	108	160	260	165	195	150	>140
OAS-55 500µl days 0, 1. plus Cyclophosphamide 5mg/kg/day x 28 days	1622	60	55	50	37	50	33	47	34	>140

[1] Died under biopsy

[2] Died lung abscess

ND = Not done

Experiment (a)

APPENDIX 10

(Chapter 6 section 11 Table 2)

The effect of cyclophosphamide on the lymphocytotoxic antibody responses of enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts

	Treatment	Rat No.	Lymphocytotoxic antibody titre at week: 1	10d	2	3	4	6	8	12	20
1.	NIL	1393	1/256	1/32	-	-	-	-	-	-	-
		1397	1/256	ND	-	-	-	-	-	-	-
		1398	1/128	ND	-	-	-	-	-	-	-
		1400	1/128	1/256	-	-	-	-	-	-	-
		1403	1/128	1/32	-	-	-	-	-	-	-
		1435	1/512	1/128	-	-	-	-	-	-	-
		1439	1/256	1/32	-	-	-	-	-	-	-
2.	Cyclophosphamide 5mg/kg/day x 14days	1541	TF	TF	-	-	-	-	-	-	-
		1546	TF	TF	TF	TF	-	-	-	-	-
		1539	TF	TF	TF	TF	TF	0	0	0	0
		1548	TF	TF	TF	TF	TF	0	0	0	0
		1559	TF	TF	TF	TF	TF	0	0	0	0
3.	10mg/kg/day x 14days	1537	0	0	0	-	-	-	-	-	-
		1536	0	0	0	0	0	-	-	-	-
		1535	0	0	0	0	0	0	0	ND	ND
		1544	0	0	0	0	0	0	0	0	0
		1555	0	0	0	0	0	0	0	0	0
		1558	0	0	0	0	TF	0	0	0	ND
4.	OAS-55 500µl i.v. on days 0,1	1465	1/1	1/16	1/64	-	-	-	-	-	-
		1452	1/1	1/4	1/4	0	0	0	0	0	-
		1449	1/2	1/2	1/4	1/2	1/2	0	0	0	-
		1454	1/1	1/8	1/4	1/1	1/4	1/1	1/1	1/1	0
		1457	0	1/1	1/4	1/1	0	0	0	0	0
		1553	1/1	1/8	1/4	0	0	0	1/1	0	0
5.	OAS-55 500µl i.v. on days 0,1 plus cyclophosphamide 5mg/kg/day x 14days	1578	1/1	0	0	-	-	-	-	-	-
		1575	1/1	1/1	0	0	0	-	-	-	-
		1586	1/1	1/1	0	0	0	-	-	-	-
		1612	0	0	0	0	0	0	-	-	-
		1576	1/1	0	0	0	0	ND	0	0	0
		1592	1/1	0	0	0	0	0	0	0	0
	plus cyclophosphamide 5mg/kg/day x 28 days.	1622	1/1	1/1	0	0	0	0	0	0	0

TF = Technical failure ND = Not done

Experiment (b)

APPENDIX 11

(Chapter 6 section iii Table 3)

The effect of cyclophosphamide on enhanced and non-enhanced Lewis recipients of (DA x Lewis)F_1 renal allografts.

Treatment	Rat No.	Blood urea (mg/100ml) at week: 10d	2	3	4	6	8	12	20	Survival (days)
NIL	2110	486	-	-	-	-	-	-	-	13
	2114	635	-	-	-	-	-	-	-	12
	2121	535	-	-	-	-	-	-	-	12
	2128	655	-	-	-	-	-	-	-	12
	2145	600	-	-	-	-	-	-	-	12
Cyclophosphamide										
3.5mg/kg/day x 28days	1989	660	-	-	-	-	-	-	-	12
	2029	660	-	-	-	-	-	-	-	12
	2054	510	-	-	-	-	-	-	-	12
5.3mg/kg/day x 28days	1937	579	725	-	-	-	-	-	-	19
	1953	485	235	83	-	-	-	-	-	23
	1928	680	975	550	225	-	-	-	-	31
	1977	146	88	58	65	51	-	-	-	55
7.0mg/kg/day x 14days	2046	260	135	-	-	-	-	-	-	17
	2093	185	102	-	-	-	-	-	-	19
	2101	275	285	-	-	-	-	-	-	19
	1986	71	66	36	55	48	-	-	-	47
10.6mg/kg/day x 14days	2063	36	-	-	-	-	-	-	-	10[3]
	2052	80	-	-	-	-	-	-	-	14[4]
	2103	59	48	-	-	-	-	-	-	15[4]
	2056	78	140	-	-	-	-	-	-	16[3]
	2106	99	108	99	-	-	-	-	-	22[3]
OAS-56 500µl days 0,1,3 alone	1854	170	690	-	-	-	-	-	-	16
	1857	88	690	-	-	-	-	-	-	16
	1866	102	675	-	-	-	-	-	-	14
	1868	97	750	-	-	-	-	-	-	14
	1861	270	610	175	125	145	76	74	99	>140
	2418*	147	400	260	190	160	200	230	-	>100
OAS-56 500µl days 0,1,3 plus Cyclophosphamide 3.5mg/kg/day x 28days	1865	62	325	-	-	-	-	-	-	17
	1855	84	195	-	-	-	-	-	-	20
	1856	78	95	160	143	-	-	-	-	40
	1860	67	82	90	66	65	75	47	59	>140
	1867	96	340	200	195	475	275	205	185	>140
	2419*	102	91	66	75	49	77	77	-	>100
	2420*	83	93	97	87	64	64	47	-	>100
OAS-56 500µl days 0,1,3 plus Cyclophosphamide 5.3mg/kg/day x 28days	2058	80	62	50	57	-	-	-	-	35[1]
	1942	325	66	75	57	37	37	-	-	60[2]
	1936	75	110	117	83	38	40	60	-	130
	1952	70	85	450	57	50	46	71	ND	>140
	1931	70	85	68	65	35	41	45	52	>140
	2415*	54	58	62	48	43	59	54	-	>100
	2417*	48	44	69	58	ND	60	45	-	>100

[1] Unknown [2] Lung abscess [3] Infection [4] Punctured bowel

ND = Not done

* Extra transplants for histology.

Experiment (b)

APPENDIX 12 (Chapter 6 section 11 Table 4)

The effect of cyclophosphamide on the lymphocytotoxic antibody responses of enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts

Treatment	Rat No.	Lymphocytotoxic antibody titre at week: 1	10d	2	3	4	6	8	12
NIL	2110	1/512	ND	-	-	-	-	-	-
	2114	1/512	8	-	-	-	-	-	-
	2121	1/512	1/32	-	-	-	-	-	-
	2128	1/512	1/64	-	-	-	-	-	-
	2145	1/512	1/16	-	-	-	-	-	-
Cyclophosphamide									
3.5mg/kg/day x 28 days	1989	1/2	1/4	-	-	-	-	-	-
	2029	1/2	1/16	-	-	-	-	-	-
	2054	0	0	-	-	-	-	-	-
5.3mg/kg/day x 28 days	1937	1/1	0	ND	-	-	-	-	-
	1953	0	0	0	0	-	-	-	-
	1928	0	0	0	0	0	-	-	-
	1977	0	0	0	0	0	0	-	-
7.0mg/kg/day x 14 days	2046	ND	0	0	-	-	-	-	-
	2093	0	0	ND	-	-	-	-	-
	2101	0	0	ND	-	-	-	-	-
	1986	0	0	0	0	0	0	-	-
10.6mg/kg/day x 14 days	2063	ND	0	-	-	-	-	-	-
	2052	0	0	-	-	-	-	-	-
	2103	0	0	0	-	-	-	-	-
	2056	0	ND	ND	-	-	-	-	-
	2106	0	0	0	0	-	-	-	-
OAS-56 500ul days 0,1,3	1854	1/1	1/4	1/2	-	-	-	-	-
	1857	1/1	1/4	1/2	-	-	-	-	-
	1866	1/1	1/4	1/2	-	-	-	-	-
	1868	1/1	1/4	1/2	-	-	-	-	-
	1861	1/1	1/4	1/2	1/1	0	1/1	0	0
OAS-56 500µl days 0,1,3 plus cyclophosphamide 3.5mg/kg/day x 28 days	1865	1/1	1/1	1/1	-	-	-	-	-
	1855	1/2	1/1	0	-	-	-	-	-
	1856	1/4	1/1	0	0	0	-	-	-
	1860	0	1/1	0	0	0	0	0	0
	1867	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
OAS-56 500µl days 0,1,3 plus cyclophosphamide 5.3mg/kg/day x 28 days	2058	1/2	1/2	1/1	0	0	-	-	-
	1942	1/1	1/1	1/1	0	0	0	ND	0
	1936	1/1	1/1	1/1	1/1	1/1	0	0	0
	1952	1/2	1/1	1/1	0	0	0	0	ND
	1931	1/1	1/1	1/1	1/1	0	0	0	0
OAS-56 500µl days 0,1,3 NO TRANSPLANT	CW-1	1/16	1/8	1/8	1/4	1/2	1/1	0	0
	CW-2	1/16	1/8	1/8	1/4	1/2	1/1	0	0
	CW-3	1/16	1/16	1/8	1/4	1/2	1/1	0	0

ND = Not done

Appendix 13.

Preparation of ALS(DE) and ALS(5,6) Rabbit anti-rat lymphocyte sera.

ALS(DE)

This was a gift from Mr D.Evans F.R.C.S.

Thoracic duct lymph was collected from 3 DA male rats into normal saline at 4°C. The thoracic duct lymphocytes (TDL) were washed in DAB and then incubated in ACT for 10 minutes at 37°C to lyse contaminating red cells. Viability of 90% was confirmed using trypan blue exclusion. A total of 2.7×10^8 cells were obtained. Six rabbits (New Zealand White) were injected i.p. with 4.5×10^7 cells each. Three weeks later each rabbit was boosted with an intravenous injection of 4×10^7 TDL and three weeks later a final intravenous boost with 3.6×10^7 TDL was performed. Ten days, and 17 days, after the final boost each rabbit was bled from an ear vein. Three days later all six were bled out by cardiac puncture. The serum obtained from these bleeds was separated, pooled and heat-inactivated at 56°C for 30 minutes. The lymphocyto-toxin titre against PBL was 1:320.

ALS (5,6)

This was a gift from Dr John Fabre.

It was part of a pool from six New Zealand White rabbits immunised with rat thoracic duct lymphocytes carefully depleted of contaminating erythrocytes by incubation at 37° for 10 minutes in tris ammonium chloride. The rabbits

were immunised with 10^8 TDL at weeks 0,2,4 and 9, the initial injection being given intraperitoneally and the others intravenously. They were bled three times during weeks 5 and 6 and again during weeks 10 and 11 and exsanguinated at week 12. All bleeds were pooled. The ALS was heat-inactivated before use.

APPENDIX 14

(Chapter 6 section iii
Tables 1 and 2)

Dose Response results for ALS(DE) on enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts.

Treatment	No.of Rat	Blood urea (mg/100ml) at week: 10d	2	3	4	6	8	12	20	Survival (days)
NIL	2110	486	-	-	-	-	-	-	-	13
	2114	635	-	-	-	-	-	-	-	12
	2121	535	-	-	-	-	-	-	-	12
	2128	655	-	-	-	-	-	-	-	12
	2145	600	-	-	-	-	-	-	-	12
ALS(DE) on days 0,1, 2,5.										
125µl	2197	1000[1]	-	-	-	-	-	-	-	11
	2206	ND	-	-	-	-	-	-	-	8
	2207	905	-	-	-	-	-	-	-	12
250µl	2191	890	-	-	-	-	-	-	-	12
	2192	970	-	-	-	-	-	-	-	11
	2196	875[1]	-	-	-	-	-	-	-	12
	2275	605	-	-	-	-	-	-	-	11
	2278	435	245	385	300	225	175	160	186	>140
375µl	2236	140	635	320	105	91	76	92	86	>140
	2237	120	171	184	230	160	130	120	220	>140
	2242	131	555	275	205	86	134	140	111	>140
	2431*	110	365	260	220	130	130	99	-	>100
500µl	2180	145	435	-	-	-	-	-	-	19
	2175	122	166	155	146	180	410	350	-	112
	2176	64	77	84	90	75	43	54	42	>140
750µl	2160	66	54	92	87	67	54	49	51	>140
	2169	66	57	68	40	82	45	43	43	>140
	2170	60	667	55	43	74	36	45	51	>140
OAS-56 500µl plus NO ALS	1854	170	690	-	-	-	-	-	-	16
	1857	88	690	-	-	-	-	-	-	16
	1866	102	675	-	-	-	-	-	-	14
	1868	97	750	-	-	-	-	-	-	15
	2418*	147	400	260	190	160	200	230	-	>100
	1861	270	610	175	125	145	76	74	99	>140
OAS-56 500µl plus 250ul ALS	2362	76	365	245	310	-	-	-	-	37
	2363	63	125	135	140	225	180	-	-	70
	2347	113	400	205	430	270	220	200	-	112
	2413*	67	111	106	114	130	99	87	-	>100
	2364	83	114	120	184	145	106	80	87	>140
OAS-56 500µl plus 375ul ALS	2366	49	52	135	500	-	-	-	-	36
	2365	145	56	60	44	76	52	51	44	>140
	2367	60	49	54	55	54	52	55	53	>140
	2359	64	66	62	93	99	75	81	61	>140
	2290	75	72	76	87	87	88	69	70	>140
	2409*	73	123	104	89	94	93	90	52	>140
	2412	56	48	70	61	74	67	70	ND	>140

[1] 11 days

* Biopsy experiment

ND = Not done

APPENDIX 15

(Chapter 6 section iii
Tables 3 and 4)

Dose response results for ALS(DE) on the lymphocytotoxic antibody responses of enhanced and non-enhanced Lewis recipients of (DA X Lewis)F_1 renal allografts.

Treatment	Rat No.	Lymphocytotoxic antibody titre at week: 1	10d	2	3	4	6	8	12	20
NIL	2110	>1/512	ND	-	-	-	-	-	-	-
	2114	>1/512	1/8	-	-	-	-	-	-	-
	2121	>1/512	1/32	-	-	-	-	-	-	-
	2128	>1/512	1/64	-	-	-	-	-	-	-
	2145	>1/512	1/16	-	-	-	-	-	-	-
ALS 125µl	2197	1/256	1/32	-	-	-	-	-	-	-
	2206	>1/512	-	-	-	-	-	-	-	-
	2207	>1/512	>1/512	-	-	-	-	-	-	-
250µl	2191	1/4	1/32	-	-	-	-	-	-	-
	2192	1/1	1/8	-	-	-	-	-	-	-
	2196	1/2	1/1	-	-	-	-	-	-	-
	2275	1/1	1/2	-	-	-	-	-	-	-
	2278	0	1/8	ND	1/2	1/16	1/16	>1/32	1/16	0
375µl	2236	1/1	1/1	1/4.	1/2	1/2	1/4	0	0	0
	2237	0	0	1/2	1/2	1/8	1/8	1/1	0	0
	2242	0	0	1/4	1/2	1/8	1/4	1/4	0	0
500µl	2175	1/2	0	1/1	1/1	1/1	1/2	1/2	1/2	-
	2176	0	0	0	1/1	1/2	1/1	1/1	0	0
	2180	0	0	1/2	-	-	-	-	-	-
750µl	2160	0	0	0	1/1	ND	0	0	0	1/1
	2169	1/1	0	0	0	0	0	0	0	0
	2170	1/1	0	0	0	0	0	0	0	0
OAS-56 500µl on days 0,1,3 NO TRANSPLANT	CW-1	1/16	1/8	1/8	1/4	1/2	1/1	0	0	ND
	CW-2	1/16	1/8	1/8	1/4	1/2	1/1	0	0	ND
	CW-3	1/16	1/16	1/8	1/4	1/2	1/1	0	0	ND
OAS-56 500µl on days 0,1,3	1854	1/1	1/4	1/2	-	-	-	-	-	-
	1857	1/1	1/4	1/2	-	-	-	-	-	-
	1866	1/1	1/4	1/2	-	-	-	-	-	-
	1868	1/1	1/4	1/2	-	-	-	-	-	-
	1861	1/1	1/4	1/2	1/1	0	1/1	0	0	ND
OAS-56 500µl on days 0,1,3 plus ALS(DE) 250µl on days 0,1,3,5	2362	1/2	1/2	1/4	1/2	1/4	-	-	-	ND
	2347	1/1	1/2	1/4	1/16	>1/32	1/8	1/8	0	ND
	2363	1/2	1/2	1/4	1/4	1/8	1/2	1/1	ND	ND
	2364	1/2	1/1	1/4	1/2	1/4	0	1/1	ND	ND
OAS-56 500µl on days 0,1,3 plus ALS(DE) 375µl on days 0,1,3,5	2366	1	1	1	2	1	-	-	-	-
	2290	1	1	0	0	1	0	0	ND	ND
	2365	1	1	0	1	0	0	0	ND	ND
	2367	0	0	0	0	0	0	0	ND	ND
	2369	0	0	0	1	0	0	0	ND	ND

ND = Not done

APPENDIX 16

(Chapter 7 Tables 1,2,3)

The effect of ALS (5,6) and cyclophosphamide on Lewis recipient of DA renal allografts.

Treatment	Rat No.	10d	2	3	4	6	8	12	20	Survival (days)
NIL	1526	ND	-	-	-	-	-	-	-	10
	2034	ND	-	-	-	-	-	-	-	10
	1543	525	-	-	-	-	-	-	-	11
	1568	595	-	-	-	-	-	-	-	11
	1728	670	-	-	-	-	-	-	-	11
	2031	535	-	-	-	-	-	-	-	11
	2037	625	-	-	-	-	-	-	-	12
	1540	650	-	-	-	-	-	-	-	12
	1570	580	-	-	-	-	-	-	-	12
	1448	575	-	-	-	-	-	-	-	14
ALS (5,6) 100µl days 0,1,3,5.	1762	400	-	-	-	-	-	-	-	9
	1744	730	-	-	-	-	-	-	-	10
	1748	690	-	-	-	-	-	-	-	11
	1759	620	-	-	-	-	-	-	-	11
	1761	525	-	-	-	-	-	-	-	13
200µl days 0,1,3,5	1607	ND	-	-	-	-	-	-	-	10
	1616	550	-	-	-	-	-	-	-	10
	1673	645	785	-	-	-	-	-	-	13
	1657	320	505	-	-	-	-	-	-	15
	1603	295	325	275	62	140	185	31	260	>140
350µl days 0,1,3,5	2080	445	640	-	-	-	-	-	-	13
	2094	125	510	-	-	-	-	-	-	16
	2082	125	315	-	-	-	-	-	-	19
500µl days 0,1,3,5	1750	70	89	77	225	ND	ND	-	-	60
	1764	65	110	190	260	485	115	-	-	80
	1765	70	92	210	220	140	200	-	-	80
	1753	83	118	200	160	105	99	112	250	>140
Cyclophosphamide										
3.5mg/kg/day x 14days	1776	445	-	-	-	-	-	-	-	10
	1780	555	-	-	-	-	-	-	-	11
	1782	780	-	-	-	-	-	-	-	11
	1795	710	-	-	-	-	-	-	-	11
	1777	580	-	-	-	-	-	-	-	12
4.9mg/kg/day x 14days	1797	250	-	-	-	-	-	-	-	10
	2015	630	-	-	-	-	-	-	-	13
	1664	765	185	-	-	-	-	-	-	18
	2004	500	230	-	-	-	-	-	-	18
	1662	490	170	740	-	-	-	-	-	21
	2006	535	205	350	440	-	-	-	-	32
	1660	560	32	195	450	190	225	-	-	64
	1669	510	125	117	165	150	205	170	-	97
	2009	370	112	89	71	52	72	73	-	108
7.7mg/kg/day x 14days	2069	75	-	-	-	-	-	-	-	13
	2077	165	106	-	-	-	-	-	-	17
	2065	265	120	-	-	-	-	-	-	18
10.6mg/kg/day x 14days	1999	54	52	-	-	-	-	-	-	20*
	1998	67	50	44	-	-	-	-	-	23*
	2002	47	45	127	-	-	-	-	-	23*
	2000	61	61	50	54	71	56	118	66	>140
ALS plus cyclophosphamide										
ALS 100µl x 4 plus cyclophosphamide 3.5mg/kg/day x 14days	1726	170	750	-	-	-	-	-	-	15
	1739	112	750	-	-	-	-	-	-	16
	1720	160	415	750	-	-	-	-	-	23
	1740	80	86	102	73	89	125	165	-	105
	1719	320	135	466	500	265	240	215	95	>140
ALS 200µl x 4 plus cyclophosphamide 3.5mg/kg/day x 14days	1733	137	565	-	-	-	-	-	-	17
	1736	112	525	-	-	-	-	-	-	20
	1737	63	85	75	90	-	-	-	-	30
	1738	72	103	79	68	240	300	-	-	61
	1734	88	85	67	68	95	475	390	-	95
ALS 200µl x 4 plus cyclophosphamide 4.9mg/kg/day x 14days	2016	61	46	-	-	-	-	-	-	16*
	1666	52	49	46	-	-	-	-	-	22*
	2003	55	52	63	-	-	-	-	-	22*
	1672	57	54	110	-	-	-	-	-	23*
	1667	47	59	34	ND	-	-	-	-	30*
	1658	43	65	70	74	ND	515	-	-	59
	2025	62	57	63	ND	115	185	305	-	115
	1671	44	64	55	59	200	230	375	400	>140
	2007	98	63	50	54	57	95	112	100	>140

* Death not due to rejection

ND = Not done

APPENDIX 17. The effect of ALS (5,6) and cyclophosphamide on the lymphocytotoxic antibody responses of Lewis recipients of DA renal allografts.

(Chapter 7 Tables 6,7)

Treatment	Rat No.	Lymphocytotoxic antibody titre at week: 1	10d	2	3	4	6	8	12	20
NIL	1526	>1/512	ND	-	-	-	-	-	-	-
	2034	1/256	ND	-	-	-	-	-	-	-
	1543	>1/512	>1/512	-	-	-	-	-	-	-
	1568	>1/512	>1/512	-	-	-	-	-	-	-
	1728	>1/512	>1/512	-	-	-	-	-	-	-
	2031	1/512	>1/512	-	-	-	-	-	-	-
	2037	1/512	ND	-	-	-	-	-	-	-
	1540	ND	1/512	-	-	-	-	-	-	-
	1448	>1/512	ND	-	-	-	-	-	-	-
ALS (5,6)										
100µl days 0,1,3,5	1762	1/2	1/2	-	-	-	-	-	-	-
	1744	1/16	1/16	-	-	-	-	-	-	-
	1748	1/8	1/32	-	-	-	-	-	-	-
	1759	1/16	1/32	-	-	-	-	-	-	-
	1761	1/128	1/32	-	-	-	-	-	-	-
200µl days 0,1,3,5	1607	1/1	ND	-	-	-	-	-	-	-
	1616	1/1	1/1	-	-	-	-	-	-	-
	1673	1/1	1/2	0	-	-	-	-	-	-
	1657	0	1/2	1/1	-	-	-	-	-	-
	1603	1/2	1/8	1/4	1/1	0	ND	ND	ND	ND
350µl days 0,1,3,5	2080	0	1/8	1/16	-	-	-	-	-	-
	2094	0	0	1/4	-	-	-	-	-	-
	2082	0	0	1/4	-	-	-	-	-	-
500µl days 0,1,3,5	1750	0	0	1/1	0	1/2	ND	ND	-	-
	1764	0	0	0	1/1	1/1	0	0	-	-
	1765	0	0	0	0	1/1	1/2	0	-	-
	1753	0	0	0	0	1/2	0	0	0	0
	1769	0	0	1/1	0	1/2	0	0	0	0
Cyclophosphamide										
3.5mg/kg/day x 14days	1776	1/1	1/4	0	0	0	0	0	0	0
	1780	1/1	1/4	0	0	0	0	0	0	0
	1782	1/1	1/4	0	0	0	0	0	0	0
	1795	1/2	1/32	-	-	-	-	-	-	-
	1777	1/4	1/32	-	-	-	-	-	-	-
4.9mg/kg/day x 14days	1797	0	0	-	-	-	-	-	-	-
	2015	0	0	-	-	-	-	-	-	-
	1664	0	0	ND	-	-	-	-	-	-
	2004	0	0	1/1	-	-	-	-	-	-
	1662	0	0	0	0	-	-	-	-	-
	2006	0	0	1/1	0	0	-	-	-	-
	1660	0	0	0	0	0	0	0	-	-
	1669	0	0	0	0	0	0	0	0	-
	2009	0	0	1/1	0	1	1	0	0	-
7.7mg/kg/day x 14days	2069	0	0	-	-	-	-	-	-	-
	2077	0	1/1	0	-	-	-	-	-	-
	2065	1/1	0	1/1	-	-	-	-	-	-
10.6mg/kg/day	1999	0	0	0	-	-	-	-	-	-
	1998	0	0	0	0	-	-	-	-	-
	2002	0	0	0	ND	-	-	-	-	-
	2000	0	0	0	0	0	0	0	0	ND
ALS (5,6) plus cyclophosphamide										
ALS 100µl x 4 plus cyclophosphamide 3.5mg/kg/day x 14days	1726	0	0	0	-	-	-	-	-	-
	1739	0	0	0	-	-	-	-	-	-
	1720	0	1/1	0	0	-	-	-	-	-
	1740	0	0	0	1/1	0	0	0	0	-
	1719	0	1/1	0	0	0	0	0	0	-
ALS 200µl x 4 plus cyclophosphamide 3.5mg/kg/day x 14days	1733	1/1	0	0	-	-	-	-	-	-
	1736	0	0	0	-	-	-	-	-	-
	1737	0	0	0	0	0	-	-	-	-
	1738	0	0	0	0	1/1	0	0	-	-
	1734	0	0	0	0	0	0	0	0	-
ALS 200µl x 4 plus cyclophosphamide 4.9mg/mg/day x 14days	2016	0	0	0	-	-	-	-	-	-
	1666	0	0	0	0	-	-	-	-	-
	2003	0	0	0	1/2	-	-	-	-	-
	1672	0	0	0	0	-	-	-	-	-
	1667	0	0	0	0	ND	-	-	-	-
	1658	0	0	0	0	0	ND	0	-	-
	2025	0	1/1	0	1/1	ND	0	0	0	-
	1671	0	0	0	0	1/1	1/1	0	0	0
	2007	0	1/1	0	0	0	0	0	0	0

APPENDIX 18

(Chapter 8 section 1a Table 1)

Results of Lewis → DA renal allografts in untreated recipients and those treated with OAS-58, IgG, $F(ab')_2$.

Treatment	Rat No.	Blood urea (mg/100ml) at week: 10d	2	3	4	6	8	12	20	Survival (days)
NIL	2218	705	-	-	-	-	-	-	-	10
	2223	670	-	-	-	-	-	-	-	10
	2224	805	-	-	-	-	-	-	-	11
	2225	760	-	-	-	-	-	-	-	11
	2233	810	-	-	-	-	-	-	-	11
25µl OAS-58 i.v. days 0,1	2232	117	91	-	-	-	-	-	-	17[1]
	2211	162	109	77	65	46	98	-	-	70[1]
	2217	480	175	122	96	60	68	57	60	>140
	2259	148	95	88	71	65	103	73	84	>140
	2261	72	66	54	54	48	69	58	62	>140
50µl OAS-58 i.v. days 0,1	2256	122	114	102	89	93	-	-	-	52
	2250	102	98	59	54	65	67	51	68	>140
	2251	101	113	76	77	67	76	79	69	>140
	2253	101	128	64	66	59	58	52	59	>140
	2255	94	55	65	48	46	65	150	58	>140
500µl IgG prepared from OAS-58 (0.38mg) i.v. days 0,1	2319	250	199	97	79	-	-	-	-	37[2]
	2312	65	58	66	51	48	45	46	43	>140
	2324	160	186	117	106	82	100	67	78	>140
	2348	121	460	300	195	185	88	113	150	>140
	2350	74	380	275	185	165	101	123	55	>140
50µl IgG prepared from OAS-58 (0.038mg) i.v. days 0,1	2440	168	106	83	164	-	-	-	-	28[4]
	2432	430	295	120	109	106	-	-	-	47[1]
	2436	405	190	130	100	87	78	52	90	>140
	2430	240	250	152	109	89	119	66	85	>140
500µl $F(ab')_2$ of OAS-58 (3.0mg) i.v. days 0,1	2323	-	-	-	-	-	-	-	-	8[3]
	2325	-	-	-	-	-	-	-	-	8[3]
	2337	660	-	-	-	-	-	-	-	10
	2310	695	-	-	-	-	-	-	-	11
	2317	605	215	105	97	96	103	74	115	>140
	2342	505	140	94	48	91	81	96	71	>140

[1] Died unknown cause

[2] Multiple lung abscesses

[3] Autopsy revealed severe rejection and pulmonary congestion

[4] Pneumonia

PUBLICATIONS ARISING FROM THIS THESIS

1. Winearls C.G., Millard P.R. and Morris P.J. (1978) Effect of azathioprine and prednisolone on passive enhancement of rat renal allografts. Transplantation 25: 229-234.

2. Winearls C.G., Fabre J.W., Millard P.R. and Morris P.J. (1979) The combined use of antilymphocyte serum and cyclophosphamide to suppress renal allograft rejection in the rat. Clin. Exp. Immunol. (in press).

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EFFECT OF AZATHIOPRINE AND PREDNISOLONE ON PASSIVE ENHANCEMENT OF RAT RENAL ALLOGRAFTS[1]

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SUMMARY

Passive enhancement provides only partial suppression of rejection in the (DA × Lewis)F, to Lewis renal allograft model. Suboptimal (8 mg/kg/day) and supraoptimal (30 mg/kg/day) doses of azathioprine administered with enhancing serum failed to suppress the rejection reaction in enhanced animals. Similarly, suboptimal (4 mg/kg/day) and optimal (16 mg/kg/day) doses of methylprednisolone were ineffective. However, the onset of rejection in enhanced animals was delayed by the use of both azathioprine (30 mg/kg/day) and methylprednisolone (16 mg/kg/day). The survival times of enhanced animals treated with azathioprine were significantly shorter than those of animals treated with enhancing serum alone, suggesting that this agent may prevent the development of autoenhancement. Although suboptimal doses of antilymphocyte serum suppress rejection in this enhancement model, the dose requirements of conventional immunosuppressive agents appear to be maximal rather than minimal.

Indefinite survival of renal allografts in the rat can be produced by passive enhancement in certain strain combinations across the major histocompatibility system, Ag-B. Across a particularly strong barrier (DA to Lewis) passive enhancement was only partially effective, in that rejection was delayed but not suppressed indefinitely. In this situation antithymocyte serum was shown to have a synergistic effect (*3*), leading to almost complete suppression of rejection and indefinite survival of five of six animals. This suggested a possible clinical application of this type of immunosuppression.

In human renal transplantation, the standard immunosuppressive agents are azathioprine and prednisolone. There has not been a careful study of the effect of these agents on passive enhancement, although French and Batchelor (*14*) have suggested that suboptimal doses of the drugs act synergistically with enhancing serum. Since any clinical trial of passive enhancement is unlikely to be attempted without the concurrent use of azathioprine and prednisolone, these experiments were designed to show either a synergistic or an antagonistic effect of these drugs with passive enhancement.

MATERIALS AND METHODS

Rats. Inbred DA(Ag-B^4), Lewis (Ag-B^1), and (DA × Lewis)F$_1$ hybrid male rats between 10 and 20 weeks old were used in the experiments. Animals were bred and maintained in the Nuffield Department of Surgery Animal House. Renal transplantation was performed in the strong (Da × Lewis) F$_1$ to Lewis renal allograft model, in which passive enhancement provides incomplete suppression (*11*).

Renal transplantation. The technique has been published in detail (*10*) but certain modifications have been made. In the initial experiments, immediate bilateral nephrectomy was performed and in these animals the ureter was anastomosed over a polythene cannula (external diameter 0.5 mm, internal diameter 0.2 mm; Dural Plastics, Australia). In later experiments right nephrectomy was delayed to day 7, at which time the rat was reopened, the integrity of the transplant ureteric anastomosis assessed, and the right kidney removed. In these experiments the ureters were anastomosed end to end using 4 10/0 nylon sutures only. The latter technique reduced the incidence of urine leakage and hydronephrosis. The function of the transplants was assessed by weekly blood urea estimations. The initial estimation was at day 10 if the right nephrectomy was at day 7. Autopsies were performed on most rats that died.

Histology. Biopsy and autopsy specimens were fixed in 10% formalin and stained with haematoxylin and eosin. The sections were examined blind by PRM and scored from 0 to ++++ for each of the following features: mononuclear cell infiltration, arterial fibrinoid necrosis, and glomerular necrosis.

Enhancing sera. Lewis male rats were immunised with DA spleen cells as previously described (*12*). Two batches of enhancing serum were produced, OAS-53 with a lymphocytotoxin titre of 1/64 and OAS-55 with a titre of 1/512. Titres were determined in a one-stage microlymphocytotoxicity assay using ^{51}Cr-labelled peripheral blood lymphocytes as target cells. The titre was the last dilution to give 50% lysis above background. All injections of enhancing serum were given i.v.

Lymphocytotoxin titres. Serum samples obtained at day 7 after transplantation were tested in one- or two-stage complement-dependent microlymphocytotoxicity assays using ^{51}Cr-labelled peripheral blood lymphocytes as targets.

Drugs. Azathioprine for injection (Burroughs Wellcome) and methylprednisolone (methylprednisolone sodium succinate; Upjohn, Crawley, Sussex, England) were prepared each day in sterile water and normal saline, respectively. The first injection was given i.v. at the time of transplantation, but all subsequent injections were given by the i.p. route. The toxicity of azathioprine in Lewis rats was tested by administering 15, 30, 60, and 75 mg/kg daily to four animals in each group. After 2 weeks the animals were killed, weighed, and bone-marrow

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smears prepared from their femoral marrows. These were stained with Wright-Romanowsky and examined blind by Dr. A. Sharp, a haematologist.

Statistical analysis. Comparisons between groups were made using the Wilcoxon rank sum test or a one-tailed Fisher's exact test.

Experimental groups. Five experimental groups were designed to test both low and high doses of the agents. Doses were designated as high or low on the basis of comparison with levels used in humans, adjusted in the case of azathioprine for surface area. On this basis the human dose of azathioprine of 3 mg/kg is equivalent to 15 mg/kg/day in the rat.

RESULTS

Azathioprine toxicity experiment (Table 1). Weight loss over the 2-week period of administration was only apparent in the animals treated with 60 and 75 mg/kg.

The bone marrows showed a range of features from mild depression of the myeloid series to frank aplasia. Three of four animals treated with 30 mg/kg showed depression of myelopoiesis. At the 15-mg/kg dose, there was little effect on the bone marrow.

Low-dose azathioprine (Table 2). Azathioprine at 4 mg/kg/day did not improve renal function at day 7 nor did it increase survival. At a dose of 8 mg/kg/day, the rejection was slightly less severe, in that the blood urea levels at 7 days were lower ($P < 0.02$) and the rats survived longer ($P < 0.05$).

Enhancing serum (OAS-53) modified rejection markedly. The mean blood urea level at 7 days was considerably lower ($P < 0.01$) and survival times were increased, with most animals surviving for over 100 days ($P < 0.01$). However, the continuing elevation of blood urea levels and serial histology showed that all grafts had been damaged by rejection.

The concurrent administration of azathioprine to enhanced animals did not influence survival, nor was there any modification of the low-grade rejection seen in the enhanced animals.

Low-dose methylprednisolone (Table 3). At a dose of 8 mg/kg/day, there was a significant reduction in the blood urea level at 7 days ($P < 0.01$) and graft survival was significantly prolonged ($P < 0.05$). At a dose of 4 mg/kg/day, the reduction in blood urea at 7 days appeared to be less marked but was still significantly lower than that of controls ($P < 0.05$), and graft survival was prolonged significantly ($P < 0.05$). Enhancing serum was given with the 4-mg/kg dose schedule. There was no evidence of either a synergistic or an inhibitory effect either in terms of renal function or survival with the combined therapy.

High-dose azathioprine (Table 4). In the previous experiments, the effect of the enhancing serum (OAS-53) had been variable (mean blood urea level ± SD at 7 days was 170 ± 109 mg/100 ml, range 74 to 355 mg/100 ml). It was possible that some of the transplanted kidneys suffered serum-mediated damage. Thus, OAS-55 was given in a smaller dosage, 500 μl on days 0 and 1. At this lower dose, there was less variability of response. The mean blood urea level at 7 days was 95 ± 50 mg/100 ml (range 49 to 185 mg/100 ml). Thus, there was clearly a significant protective effect, but by 14 days all animals showed a marked deterioration in renal function (mean blood urea level ± SD = 333 ± 144 mg/100 ml).

The high dose of azathioprine modified acute rejection considerably. All grafts underwent acute rejection, but the blood urea levels at 7 days were significantly lower than those of controls ($P < 0.01$) and animal survival was prolonged ($P < 0.01$).

TABLE 1. Azathioprine toxicity after 14 days of treatment

Group	No. of rats	Weight change ± SD (%)	Bone marrow
Controls (1 ml of normal saline i.p. daily)	4	+5.8 SD = 1.9	All four normal
Azathioprine (15 mg/kg)	4	+3.7 SD = 2.4	One animal only had mild myeloid depression
Azathioprine (30 mg/kg)	4	+2.0 SD = 1.1	Three animals had moderate myeloid depression
Azathioprine (60 mg/kg)	4	−3.3 SD = 2.2	Three animals had severe myeloid depression and megaloblastic erythropoiesis; one animal was aplastic
Azathioprine (75 mg/kg)	4	−5.2 SD = 0.7	Two animals showed severe myeloid depression and megaloblastic erythropoeisis; two were aplastic

TABLE 2. The effect of the low doses of azathioprine on enhanced and nonenhanced Lewis recipients of (DA × Lewis)F_1 renal allografts with immediate nephrectomy

Treatment	No. of rats	Blood urea in mg/100 ml (mean ± SD) at weeks				Survival times (days)	Comparison of survival times
		1	2	4	20		
None	6	599 ± 114	–	–		7, 7, 7, 7, 9, 10	–
Azathioprine (4 mg/kg/day for 6 weeks)	6	424 ± 103	–	–	–	7, 9, 9, 10, 10, 10	Not significant versus no treatment
Azathioprine (8 mg/kg/day for 6 weeks)	6	381 ± 75	–	–	–	8, 8, 10,11, 11, 111	$P < 0.05$ versus no treatment
OAS-53 (500 μl i.v. on days 0, 1, 3, 5)	6	170 ± 109	165 ± 74	266 ± 110	200 ± 160	23, 39, >140, >140, >140, >140	$P < 0.01$ versus no treatment
OAS-53 + azathioprine (8 mg/kg/day for 6 weeks)	6	218 ± 206	133 ± 47	244 ± 136	73 ± 29	29, 23, 26, >140, >140, >140	$P < 0.01$ versus no treatment but not significantly different from enhancement alone

TABLE 3. The effect of low doses of methylprednisolone on enhanced and nonenhanced Lewis recipients of (DA × Lewis)F_1 renal allografts with immediate nephrectomy

Treatment	No. of rats	Blood urea in mg/100 ml (mean ± SD) at week				Survival (days)	Comparisons of survival times
		1	2	4	20		
None	6	599 ± 114	–	–	–	7, 7, 7, 7, 9, 10	–
Methylprednisolone (4 mg/kg for 21 days, then 2 mg/kg for 21 days)	6	378 ± 170	127 ± 63	118 ± 34	64 ± 10	9, 9, 62, >140, >140, >140	$P < 0.05$ versus no treatment
Methylprednisolone (8 mg/kg for 21 days, then 4 mg/kg for 21 days)	6	225 ± 89	–	–	–	9, 9, 9, 10, 17, >140	$P < 0.05$ versus no treatment
OAS-53 (500 μl i.v. on days 0, 1, 3, 5)	6	170 ± 109	165 ± 74	266 ± 110	200 ± 160	23, 39, >140, >140, >140, >140	$P < 0.01$ versus no treatment
OAS-53 + methylprednisolone (4 mg/kg for 21 days, then 2 mg/kg for 21 days)	7	200 ± 152	151 ± 97	Not done	159 ± 165	9, 10, 30, >140, >140, >140, >140	$P < 0.01$ versus no treatment; not significant from enhancement alone

TABLE 4. The effect of azathioprine (30 mg/kg/day for 14 days) on nonenhanced and enhanced Lewis recipients of (DA × Lewis)F_1 renal allografts with immediate nephrectomy

Treatment	No. of rats	Blood urea in mg/100 ml (mean ± SD) at week				Survival (days)	Comparisons of survival times
		1	2	3	12		
None	6	599 ± 114	–	–	–	7, 7, 7, 7, 9, 10	–
Azathioprine (30 mg/kg/day for 14 days)	5	325 ± 106	204 ± 46	318 ± [a]	–	17, 19, 77, >140, >140	$P < 0.01$ versus no treatment
OAS-55 (500 μl on days 0, 1)	6	95 ± 50	333 ± 144	282 ± 103	134 ± 54	16, 98, 133, >140, >140, >140	$P < 0.01$ versus no treatment
OAS-55 + azathioprine (30 mg/kg/day)	6	52 ± 10	97 ± 37	538[a]	–	18, 19, 20, 20, 24, 91	$P < 0.01$ versus no treatment $P < 0.05$[b] versus enhancement alone

[a] Less than four values.
[b] One-tailed Fisher's exact test.

TABLE 5. The effect of azathioprine (30 mg/kg/day for 21 days) on enhanced and nonenhanced Lewis recipients of (DA × Lewis)F_1 renal allografts with 7-day nephrectomy

Treatment	No. of rats	Blood urea in mg/100 ml (mean ± SD) at				Survival (days)	Comparisons of survival times
		10 days	2 weeks	3 weeks	12 weeks		
None	7	528 ± 114	–	–	–	11, 11, 11, 11, 11, 12, 13	–
Azathioprine (30 mg/kg for 21 days)	5	565 ± 98	–	–	–	10, 10, 10, 11, 12	Not significant versus control
OAS-55 (500 μl i.v. on days 0, 1)	6	48 ± 7	231 ± 110	248 ± 100	128 ± 75	16, 100, 113, >140, >140, >140	$P < 0.01$ versus control
OAS-55 + azathioprine (30 mg/kg for 21 days)	5	107 ± 130	61 ± 23	226 ± 150	–	21, 24, 28, 28, 49	$P < 0.02$ versus enhancement alone[a]

[a] One-tailed Fisher's exact test.

The combined use of azathioprine (30 mg/kg/day) with enhancing serum prevented the deterioration in renal function seen in the animals treated with enhancing serum alone (see Table 4). However, when the drug was withdrawn after 14 days, all animals experienced severe acute rejection resulting in the death of five of six animals, in contrast to the enhanced group in which only one of six animals died.

High-dose azathioprine for 21 days (Table 5). This rapid deterioration in renal function after withdrawal of azathioprine led to the next experiment in which azathioprine was administered for 3 weeks. In addition, delayed nephrectomy at 7 days was introduced to decrease the technical failures from urine leaks. All other conditions of the experiment were the same, except that the first blood urea measurement was at 10 days, 3 days after nephrectomy.

The immunosuppressive effect of azathioprine alone noted in the immediately nephrectomised animals was not seen when nephrectomy was delayed. Survival times and blood urea levels at 10 days were similar to those of untreated animals.

Despite this difference, the drug still prevented the deterioration in renal function seen at 14 days in the enhanced group. The mean blood urea ± SD at this stage for the combination group was 61 ± 23 mg/100 ml compared with 231 ± 110 mg/100 ml for the enhanced group ($P < 0.01$). However, continuing the drug for another week had no further protective effect. Indeed as in the previous experiment, rejection was progressive and fatal in most of the animals.

High-dose methylprednisolone (Table 6). No immunosuppressive effect was apparent when methylprednisolone at 16

TABLE 6. The effect of high-dose methylprednisolone on enhanced and nonenhanced Lewis recipients of (DA × Lewis)F_1 renal allografts with 7-day nephrectomy

Treatment	No. of rats	Blood urea in mg/100 ml (mean ± SD) at				Survival (days)	Comparisons of survival times
		10 days	2 weeks	3 weeks	12 weeks		
None	7	528 ± 114	–	–	–	11, 11, 11, 11, 11, 12, 13	–
Methylprednisolone (16 mg/kg for 21 days)	5	524 ± 63	–	–	–	10, 10, 10, 10, 11	Not significant versus controls
OAS-55 (500 μl i.v. on days 0, 1)	6	48 ± 7	231 ± 110	248 ± 100	128 ± 75	16, 100, 113, >140, >140, >140	$P < 0.01$ versus controls
OAS-55 + methylprednisolone (16 mg/kg for 21 days)	6	43 ± 16	115 ± 58	258 ± 152	191[a]	18, 19, 36, >140, >140, >140	$P < 0.01$ versus controls; not significant versus enhancement alone

[a] Less than four values.

TABLE 7. The effect of high-dose azathioprine and methylprednisolone for 21 days on enhanced and nonenhanced Lewis recipients of (DA × Lewis)F_1 recipients

Treatment	No. of rats	Blood urea in mg/100 ml (mean ± SD) at					Survival (days)	Comparisons of survival times
		10 days	2 weeks	3 weeks	4 weeks	12 weeks		
None	7	528 ± 114	–	–	–	–	11, 11, 11, 11, 11, 12, 13	–
OAS-55 (500 μl i.v. days 0, 1)	6	48 ± 7	231 ± 110	248 ± 100	184 ± 44	128 ± 75	16, 100, 113, >140, >140, >140	$P < 0.01$ versus control
Azathioprine (30 mg/kg) + prednisolone (16 mg/kg for 21 days)	6	538 ± 98	–	–	–	–	10, 10, 10, 11, 13, >140	Not significant versus controls
	Rat no.							
OAS-55 + azathioprine and prednisolone for 21 days	1519	47	57	59	550	–	28	Survival not significantly different from enhancement alone
	1520	ND[a]	57	61	200[b]	155	>140	
	1521	ND	50	66	325	350	104	
	1527	51	55	260	–	–	32	
	1581	50	60	–[c]	–	–	21	

[a] ND, not done.
[b] 6 weeks.
[c] Died of infection.

mg/kg/day was used alone, nor did it alter the course of chronic rejection when used with enhancing serum.

High-dose methylprednisolone and high-dose azathioprine (Table 7). The two drugs together were ineffective in suppressing rejection, but when used in combination with enhancing serum OAS-55, excellent renal function was maintained in all animals for 2 weeks and in three of the five for 3 weeks. However, all suffered severe rejection episodes at week 4. Furthermore, this drug combination seemed to be toxic, the animals looking unwell during treatment and one died at day 21 with a severe wound infection.

Histology. The grafts from untreated animals at 7 days showed patchy fibrinoid necrosis of arterioles and glomeruli, with obliteration of the capillary lumina, patchy tubular necrosis and interstitial oedema, haemorrhage, and a cellular infiltration. These cells included lymphocytes, plasma cells, and neutrophil polymorphonuclear cells and were seen in the interstitium and the walls of vessels. These features appeared in both the delayed and immediately nephrectomised groups.

The grafts of animals treated with enhancing serum showed markedly different features. At 7 days the glomeruli and tubules were normal, but there was an unmodified cellular infiltrate of lymphocytes and plasma cells. At day 14 there was disorganisation of the tissue architecture, with disruption of the tubules and focal necrosis of glomeruli. At 20 weeks the glomeruli had become sclerosed, the interstitium fibrotic, the tubules atrophic, and a mild cellular infiltrate persisted.

Azathioprine and prednisolone when used alone or together did not result in any modification of the histological picture of rejection. In particular it was noted that the classic glomerular lesion seen in controls was unmodified, even by the use of both drugs together in optimal doses. However, when both drugs were used with enhancing serum, there was an attenuation of the rejection reaction seen in enhanced animals. Although the degree of cellular infiltrate was unaltered, the tissue architecture was better preserved and in the three animals studied the glomerular lesion seen in enhanced animals at 14 days onward was absent. When these animals died they showed a severe rejection reaction similar to that seen in untreated enhanced animals.

Lymphocytotoxic antibody. Neither low nor high doses of the drugs altered the lymphocytotoxic antibody response at day 7. Animals treated with enhancing serum had significantly reduced antibody activity.

DISCUSSION

These experiments have failed to show any significant synergistic effect between azathioprine and prednisolone and

passive enhancement, and indeed suggest an antagonistic effect of azathioprine in certain situations.

In our model untreated recipients experience an early irreversible acute rejection reaction which is invariably fatal. The histological features are those of acute humoral rejection (*1, 2, 20*). In contrast enhanced animals do not develop these early lesions. Renal function remains good for 10 days, after which all animals show marked functional and morphological deterioration (see Table 8). Most survive indefinitely but with elevated blood urea levels and small scarred kidneys showing the features of chronic rejection.

Both Burgos et al. (*5*) and Strom et al. (*22*) have shown that enhanced rats have a delayed but otherwise unmodified cell-mediated immune reaction to their grafts. Abbas et al. (*1, 2*) have demonstrated a cellular rejection reaction within the enhanced kidneys. Both groups concluded that passive enhancement suppresses an early humoral rejection and that the cell-mediated immune response, after an initial unmodified peak, wanes. These findings could explain the deterioration in our model, although a continuing humoral response would have to be postulated to explain the lesions of chronic rejection.

The functional and morphological deterioration seen in our enhancement model is suppressed by antithymocyte serum (*3*), a finding which is entirely consistent with the data on cell-mediated responses cited above. Antilymphocyte sera are thought to act by depleting the long-lived pool of recirculating lymphocytes that are responsible for T cell-mediated immune processes such as killer and helper cell activity (*18*).

Azathioprine was found to have little effect on acute rejection. This finding is consistent with the hypothesis that the early lesion is primarily antibody mediated, since there was no difference in antibody response between control animals and animals treated with azathioprine.

This minimal immunosuppressive action of azathioprine should be seen in the light of the relative resistance of the rat to the immunosuppressive actions of the drug. Husberg (*15*) has shown that azathioprine at a dose of 30 mg/kg made no difference to the cell-mediated cytotoxicity of thoracic duct lymphocytes measured in BN recipients of Wistar-Furth renal grafts, nor was the number of thoracic duct lymphocytes reduced. There was however a slight reduction in antibody levels and "blocking factor" production. In the same model antilymphocyte serum was found to cause a profound reduction in the cell-mediated cytotoxicity. Shehadeh et al. (*21*) found that Lewis recipients of (L × BN)F_1 renal allografts had little significant renal function, at day 7 after transplantation, when treated with azathioprine in doses ranging from 8 to 40 mg/kg/day. In contrast to these findings are those of Tinbergen (*24*), who showed that 4 mg/kg/day of azathioprine prolonged the survival of Wag recipients of BN kidneys. However, no functional or morphological data was presented. Currey (*8*) has shown that doses of azathioprine that exceed the LD_{50} are required to obtain suppression of primary antibody responses, tuberculin skin testing, and adjuvant arthritis. Thus, it would appear that to suppress the in vivo manifestations of the immune responses in the rat with azathioprine may require doses exceeding the acceptable levels of toxicity. The drug is not a placebo in rats, because our own toxicity studies have shown that at a dose of 30 mg/kg/day Lewis rats show some retardation of growth, and their bone marrows show depressed myelopoiesis. On the basis of surface area ratios (5:1), this dose is equivalent to 6 mg/kg/day in humans. This ratio is consistent with the findings of Frankel et al. (*13*), who found that the maximum dose which the rat is capable of tolerating in long-term experiments is about 15 mg/kg/day. From this we have concluded that the toxic effects of azathioprine in man and the rat run in parallel but that this is not reflected in comparable immunosuppressive effects.

Despite the trivial immunosuppressive effects that were obtained with azathioprine alone, some effect was seen when the large dose was used with passive enhancement (Tables 4 and 5). However, rejection was rapid and irreversible when it commenced, so that the number of long-term survivors was less in the group given both azathioprine and passive enhancement, when compared with passive enhancement alone. Thus, in this case the effect of azathioprine inhibited the long-term effects of passive enhancement, perhaps by preventing the development of autoenhancement. The mechanisms responsible for the maintenance state of continuing survival of enhanced renal allografts have not been established, although the evidence of Bowen et al. (*4*) suggests that it is an active mechanism. Strom et al. (*22*) and Stuart et al. (*23*) have suggested that it depends on the presence of blocking factors in the serum. Hutchinson and Zola (*16*) have proposed that the host antigen-reactive T cells become complexed with donor antigen and enhancing antibody, which are then opsonised and removed by host macrophages, thus depleting the host of antigen reactive cells. Azathioprine might suppress the production of the critical opsonic antibody required for this mechanism, but we have no evidence to support this. Although suppressor T cells have not been demonstrated to play a role in the maintenance state of passive enhancement, it is possible that they do so. In which case azathioprine might selectively suppress these cells while allowing clonal expansion of killer T cells, resulting in delayed but irreversible rejection.

An unexpected finding was the difference in survival times between the two groups of animals treated with azathioprine alone, depending on the timing of nephrectomy. Immediately nephrectomised animals survived significantly longer than those undergoing 7-day nephrectomy. We have attributed this difference to the nonspecific immunosuppressive effect of uraemia, although the drug effect could have been increased by poor excretion during the early rejection crisis. The pharmacokinetics of azathioprine in the rat has not been fully studied, but the mouse has been shown to excrete in the urine a

TABLE 8. The difference between control and enhanced Lewis recipients of (DA × Lewis)F_1 kidneys

	Day 7–10		Day 14	
	Control	Enhanced	Control	Enhanced
Mean blood urea	500 mg/100 ml	100 mg/100 ml		200 mg/100 ml
Cellular Infiltrate	++	++		++
Glomerular Necrosis	+++	0	dead	+
Fibrinoid Necrosis	++	0		0
Lymphocytotoxic antibody	1:4	<1:1	–	1:1
Survival	–	–	0%	100%

significant fraction of azathioprine both as a free drug and as 6-mercaptopurine (*9*).

Rats are considered steroid-sensitive animals (*7*) and yet high doses of methylprednisolone had little effect on acute rejection, or on the rejection experienced by enhanced animals. Although the small dose of 4 mg/kg/day prolonged survival significantly, all of the animals experienced a severe acute rejection, and the improved survival may have been attributable to the nonspecific effects of uraemia superimposed on a marginal effect of the drug.

If the early rejection crisis is antibody mediated, then the failure of methylprednisolone to prevent this lesion is understandable. The antibody response at 7 days was unaltered. Currey (*8*) has demonstrated that doses of 100 mg of hydrocortisone per kg per day (equivalent to 20 mg of prednisolone per kg per day) suppressed the haemolysin response to sheep red blood cells to only 70% of control levels. This same dose did however suppress tuberculin sensitivity. Neifield et al. (*19*) showed that 10 mg of methylprednisolone hemisuccinate per kg per day prolonged the survival of Lewis skin grafts on Sprague-Dawley recipients from 9 to 15 days. Husberg (*15*) showed that 12 mg/kg/day caused a significant reduction of thoracic duct lymphocyte cell-mediated cytotoxicity and cytotoxic antibody production in BN recipients of WF kidneys. Thus, the dose used in these experiments should have been in the effective range for suppressing cell-mediated immunity. The fact that it did not alter the course of enhanced animals suggests that either the reaction in our model is particularly intense, or that there are other effector mechanisms responsible for the deterioration in function. This result differs from the findings of Chutná (*6*), who demonstrated an additive effect if hydrocortisone was given with passive enhancement in a mouse skin graft model. Koene et al. (*17*) however showed no synergistic effect of prednisolone when used with enhancing serum in their skin graft model, although they did demonstrate synergism with antilymphocyte serum.

When both azathioprine and prednisolone were given in conjunction with enhancing serum, normal renal function was maintained for 3 weeks in three of the five animals. This suppression was not permanent because, just as in the azathioprine experiments, the withdrawal of the drug was followed by severe acute rejection.

Thus, these experiments fail to show any significant synergistic effect of azathioprine and prednisolone with passive enhancement, and indeed suggest that if an augmentation of the effects of passive enhancement is to be obtained with these agents, then the normal dose schedules would have to be used in clinical practice. Furthermore, the antagonistic effect of azathioprine on passive enhancement needs further study, since this might represent a contraindication to their combined use. Further definition of the mechanisms by which passive enhancement both induces suppression of rejection as well as maintains the state of suppression should allow more precise planning of adjuvant drug therapy.

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