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CYCLOSPORIN A. A POWERFUL IMMUNOSUPPRESSANT IN THE RAT

Ian F. Hutchinson, M.B.Ch.B., F.R.C.S.

A thesis submitted for the degree of Doctor
of Medicine in the University of Glasgow.

APRIL 1984

The work, described in this thesis, was carried out,
in the main, at the Laboratory for Surgical Research,
Peter Bent Brigham Hospital, Harvard Medical School,
Boston, Massachusetts.

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INDEX OF CONTENTS

INDEX OF CONTENTS

	<u>PAGE</u>
CYCLOSPORIN A. A POWERFUL IMMUNOSUPPRESSANT IN THE RAT.	1
INDEX OF CONTENTS	2
AUTHOR'S STATEMENT	7
ACKNOWLEDGEMENTS	10
SUMMARY	14
<u>CHAPTER 1</u> THE DEVELOPMENT OF IMMUNOSUPPRESSION IN ORGAN TRANSPLANTATION	19
1. Historical Aspects	20
2. Recent Developments	26
Aspects of Transplantation Biology	26
Immune Suppression	31
1. Agents and Procedures Affecting Predominantly Lymphoid Tissue	32
2. Chemical Immune Suppression	35
Other Methods of Prolonging Survival of an Organ Transplant	37
a) Tissue Typing	37
b) Donor Pre-treatment	41
c) Blood Transfusion	42
Cyclosporin A	46
Aims of the Studies	48

	<u>PAGE</u>
<u>CHAPTER 2 MATERIALS AND METHODS</u>	50
<u>The Animal Model</u>	51
1. General Considerations	51
2. Choosing the Rat Strains	52
3. Technique of Heterotopic Cardiac Allografting in Rats	54
(i) Anaesthesia	55
(ii) Instruments	56
(iii) The Operation	57
(a) Donor	57
(b) Recipient	59
(c) Transplantation	59
4. Post-operative Problems	61
5. Post-operative Monitoring	62
(a) The Heart	62
(b) Blood Analyses	63
6. Cyclosporin A	65
<u>Measurement of the Immune Response</u>	65
<u>Measurement of Humoral Activity</u>	67
a) Complement-dependent cytotoxicity	67
b) Antibody-dependent cell-mediated cytotoxicity	71
c) Reverse haemolytic plaque assay	74
<u>Measurement of Cell-mediated Activity</u>	77
a) Lymphocyte-mediated cytotoxicity	77
<u>Harvesting of Cellular Infiltrates</u>	81
<u>Thymectomy and Isolation of Thymocytes</u>	82
<u>Statistics</u>	82

	<u>PAGE</u>
<u>CHAPTER 3</u> RESULTS OF GRAFT SURVIVAL AND OF THE MEASUREMENT OF THE IMMUNE RESPONSE	83
1. Graft Survival in Untreated Recipients	84
2. Graft Survival in Recipients Treated with Cyclosporin A	84
3. Humoral Activity	89
a) Complement-dependent cytotoxicity	89
b) Antibody-dependent cell-mediated cytotoxicity	91
c) Reverse haemolytic plaque assay	92
4. Cell-mediated Activity	94
a) Lymphocyte-mediated cytotoxicity (spleen cells)	94
b) Lymphocyte-mediated cytotoxicity (graft infiltrating cells)	96
<u>CHAPTER 4</u> DOES CYCLOSPORIN A PRODUCE DONOR- SPECIFIC SUPPRESSION?	100
1. Are Suppressor Cells Present in Cyclosporin A-treated Recipients?	101
Cell Transfer Studies	102
Trypsinisation of Transferred Thymocytes	105
2. Specificity of Suppressor Effect on Test Allografts	107
3. Mixed Lymphocyte Response	111
<u>CHAPTER 5</u> RESPONSE OF LYMPHOID TISSUE TO CARDIAC ALLOGRAFTS IN RATS TREATED WITH	117

	<u>PAGE</u>
CYCLOSPORIN A	
1. Anatomy of Rat Spleen	119
2. Anatomy of Rat Thymus	119
3. Materials and Methods	121
a) Animal Model	121
b) Animal Groups	122
c) Cyclosporin A	122
d) Histological Methods	122
4. Results	123
a) Spleen	123
b) Thymus	125
c) Cardiac Allografts	131
<u>CHAPTER 6</u> DISCUSSION	133
REFERENCES	150

AUTHOR'S STATEMENT

Author's Statement

The majority of the work described in this thesis was carried out at the Surgical Research Laboratory, Harvard Medical School, Boston, Massachusetts, during the tenure of a Surgical Research Fellowship (1979 - 1980). A few of the experiments, assessing the histological changes in Cyclosporin A-treated rats, were performed, on return from the U.S.A., at the Department of Surgery, Western Infirmary, Glasgow.

The concept and the aims of the studies described were my own. The specific protocols of the experiments were designed following discussion with and advice of Dr. Nicholas L. Tilney, Director of Transplant Surgery, Peter Bent Brigham Hospital. Some of the techniques, e.g. cell transfer studies, complement-dependent cytotoxicity and lymphocyte-mediated cytotoxicity assays, had been previously used in Dr. Tilney's laboratory and accordingly equipment and facilities were already available.

All cardiac allograft operations (numbering over 290) and post-operative treatment and monitoring of the rats were carried out by myself. I was alone responsible for harvesting all organs and cells when the animals were sacrificed. The cell transfer studies, the complement-dependent cytotoxicity and the antibody-

dependent cell-mediated cytotoxicity experiments were carried out solely by myself. These studies were time-consuming and complex and enabled me to become fully acquainted with their principles and in vitro immunological methods.

I participated in all the studies of the reverse haemolytic plaque assay, the lymphocyte-mediated cytotoxicity and the mixed lymphocyte reaction, although these were under the supervision of Dr. Craig Shadur, Research Fellow in Nephrology, and Mrs. Ruth Kostick, Senior Technician in the Department of Renal Medicine, Peter Bent Brigham Hospital. Although most of these experiments were carried out by Mrs. Kostick and her staff, I was sufficiently well acquainted with the principles and techniques to enable me to perform a number on my own.

The histological examination of harvested organs was carried out by Dr. William Baldwin, Department of Nephrology, Leiden, the Netherlands.

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ACKNOWLEDGEMENTS

For the most part the work described in this thesis was carried out during the tenure of a Surgical Research Fellowship in the Department of Surgery (Laboratory for Surgical Research), Peter Bent Brigham Hospital, Boston, Massachusetts.

I wish to thank Sir Andrew Watt Kay, Emeritus Professor of Surgery at the University of Glasgow and Mr. S. G. MacPherson, Senior Lecturer in Surgery, Department of Surgery, Western Infirmary, Glasgow, both of whom proposed my appointment as Surgical Research Fellow.

In Boston my work was supervised by Dr. Nicholas L. Tilney, Director, Transplant Service, Department of Surgery, Peter Bent Brigham Hospital. His expertise in clinical and experimental organ transplantation is internationally known. At all times he was willing to give time to discuss the project and give advice on the work. His stimulation was ever present as also was his support. Without him this work could not have been completed. I am most grateful to him.

Dr. Terry B. Strom of the Department of Medicine, Peter Bent Brigham Hospital, willingly taught me his techniques for in vitro measurement of the immune response. Dr. William M. Baldwin, presently in the Department of Nephrology and Pathology, University

Medical Centre, Leiden, the Netherlands, advised on histological results.

Most of the figures and tables were prepared by members of staff from the Department of Photography and Graphics of the University of Newcastle upon Tyne and I would like to acknowledge their very evident expertise.

Mrs. Helen Durham has typed this thesis. This has required a great deal of patience on her part and I am most grateful.

Professor I.D.A. Johnston, Department of Surgery, Newcastle upon Tyne, has given me constant encouragement, and provided me with the necessary desk facilities. I wish to thank Mr. George Proud, Department of Surgery, Newcastle upon Tyne, and Mr. David N.H. Hamilton, Department of Surgery, Western Infirmary, Glasgow. Both are experienced in clinical and experimental organ transplantation and both advised in the presentation of this thesis.

I would like to acknowledge the stimulation, help and support of Mr. R.M.R. Taylor, Director of Transplant Surgery, Newcastle upon Tyne. He undertook the formidable task of reading the proofs of this thesis and his advice and criticism were particularly appreciated.

Finally I would like to acknowledge the support of my wife Lyn during our time in Boston and subsequently in the preparation of this thesis.

SUMMARY

SUMMARY

Organ transplantation is now an accepted method of treating end stage kidney failure. Other organs are also being transplanted and an increasing number of heart, liver, pancreas and bone marrow transplants are being carried out. Grafts may be unsuccessful for many reasons but the biggest problem is graft rejection e.g. only approximately 40% of all first cadaver kidney grafts are functioning after six years.

The first prolonged human cadaver renal allograft survival was reported from Boston in 1962, when a patient was treated with azathioprine, a purine analogue similar to 6-mercaptopurine, to prevent rejection. Since then, major advances in the understanding of the immune system, and the establishment of tissue typing laboratories have taken place. However, since the 1970s graft survival figures have not improved significantly. Indeed, azathioprine in combination with corticosteroids have remained unchallenged as the mainstay of immunosuppressive therapy during the past two decades.

Recently, the discovery of Cyclosporin A has raised hopes that a major advance in immunosuppression has been achieved. In 1976 at the Biological and Medical Research Division of Sandoz Limited, Basle, Switzerland, during a screening programme of fungus

extracts, a cyclic endecapeptide, Cyclosporin A (CyA), was identified as having marked immunosuppressive properties. This observation led to studies of CyA in different species with a variety of organ transplants, notably at Cambridge, England. Following these experiments a pilot human study was begun by Professor Calne in Cambridge using CyA in clinical organ grafting, initially as the sole immunosuppressant. The results were encouraging.

CyA had been shown to be a potent immunosuppressant yet its mode of action was unclear. Early workers had suggested that it acted primarily on lymphocytes, possibly on T lymphocytes. Further information has revealed that the mode of action may be mediated through a subpopulation of T cells, namely suppressor cells.

Using the cardiac allograft model in the rat, the aim of the study described in this thesis was to investigate the mechanisms by which CyA mediated its immunosuppressive activity.

(LEW x BN) F_1 and (LEW x WF) F_1 animals were used as organ donors and LEW animals as recipients. CyA was administered at 15 mg/kg/day for 7 days commencing immediately after transplantation. All grafts functioned normally for more than 100 days in healthy recipients. The humoral arm of the immune response of these grafted and CyA-treated recipients was assessed

by examining complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity and the reverse haemolytic plaque assay. The cell-mediated component was measured by examining lymphocyte-mediated cytotoxicity. Both the cell-mediated and the humoral components were profoundly suppressed throughout the 100 day period after transplantation. The nature of this unresponsiveness was examined in more detail by adoptively transferring thymocytes from CyA-treated animals bearing well-functioning grafts into unmodified animals and performing test heart grafts 24 hours later. Prolongation of these test heart grafts suggested that these transferred cells had suppressor characteristics. The failure of these cells to prolong third party test grafts suggested antigen-specific immunosuppression. Such donor-specific immunosuppression was supported in vitro by the results obtained in the mixed lymphocyte response in which there was significant suppression against donor cells but not third party cells. In both these in vivo and in vitro experiments the donor-specific suppressor activity appeared only after the cessation of CyA treatment.

The finding of cells with suppressor characteristics is supported by the results of studies of dynamic responses of certain lymphoid tissues to the cardiac allografts. Previous studies have suggested that T lymphocytes in bone marrow migrate first to the thymic

medulla and then to the splenic peri-arterial sheath (PAS) via the marginal zone (MZ). A second line of T lymphocytes may migrate first to thymic cortex and then to the splenic red pulp (RP). In these present experiments medullary thymocytes and T lymphocytes in the splenic PAS and MZ were strikingly reduced in size 1 to 2 weeks after the completion of CyA treatment. It has been postulated that helper and cytotoxic T lymphocytes may develop in the thymic medulla while suppressor T lymphocytes may originate in the thymic cortex. Thus CyA may be predicted to favour preferentially suppressor cell function. In the present experimental studies using the cardiac allograft in the rat it has been demonstrated that CyA is indeed a potent immunosuppressant, suppressing both cell-mediated and humoral immunity. It has also been shown that following the cessation of CyA treatment there is emergence of cells with antigen-specific suppressor characteristics. It is suspected that these suppressor cells act by abrogating or overriding the function of allospecific helper T cells necessary for initiating differentiation of cytotoxic T lymphocytes and antibody-producing B cells.

C H A P T E R 1

THE DEVELOPMENT OF IMMUNOSUPPRESSION
IN ORGAN TRANSPLANTATION

THE DEVELOPMENT OF IMMUNOSUPPRESSION
IN ORGAN TRANSPLANTATION

Organ transplantation is an increasingly used method of treatment. By far the organ most often transplanted is the kidney and renal transplantation is now the treatment of choice for most patients with end stage renal failure. Heart, lung, liver and bone marrow transplantations are now being carried out with some success. More recently vascularised pancreatic allograft transplantations have been performed, also with some degree of success. In all types of organ transplantation the host response to the transplant has been the commonest cause of failure. Even today after twenty years' experience in clinical transplantation rejection of the graft remains the single most important problem to be overcome.

1. Historical Aspects

The concept that deficient or diseased tissues or organs might be replaced by transplants from another individual has existed since antiquity. In Greek mythology, the Chimera, a mythical monster containing parts of many species who dwelt in Lycia, was the terror of the neighbourhood until she was killed by Bellerophon mounted on his winged steed Pegasus. The creature is described thus in the Iliad, VI, 180:

"A mingled monster of no mortal kind,
 Behind, a dragon's fiery tail was spread
 A goat's rough body bore a lion's head,
 Her pitchy nostrils flaky flames expire,
 Her gaping throat emits infernal fire."

The first generally cited legend of allografting* dates from the third century A.D. when twin brother surgeons St. Cosmas and St. Damian were alleged to have transplanted the whole leg of a dead Ethiopian Moor to a Caucasian man whose leg had been removed for tumour (Artelt, 1967). Perhaps the credulous public were unaware that the transplanted leg may have been black by reason of gangrene. Little was written thereafter until John Hunter's work, where he transplanted the spur of a cock to its comb, a human tooth to a cock's comb and a cock's testis to a hen (Hunter, 1771, 1778; Irvine, 1771). Not long afterwards Baronio of Italy

* Allograft (= allotransplant/homograft):

transplantation of an organ or tissues from one person or animal to another of the same species.

Autograft (= autotransplant): transplantation performed within the same individual i.e. as in transferring skin from one site to another.

Syngeneic transplant: transplantation between two animals of the same inbred strain i.e. genetically identical animals.

demonstrated that full thickness skin grafts, both in humans and in experimental animals, persisted indefinitely, while allografts sloughed in a few days (Baronio, 1804).

The modern era of whole organ transplantation might be said to have begun with the work of the ambitious French émigré, Carrel, and Guthrie at the beginning of the present century. This work was carried out initially in the Hull Physiological Laboratory of the University of Chicago and later at the Rockefeller Institute in New York City (Carrel, 1902). In brief, these workers, using dogs, were able to transplant kidneys and other organs and to achieve survival of autografts but not allografts, which were rejected by a process which was not then understood. Carrel recognised that the immune response of the recipient required modification to render the recipient tolerant of the organ allograft, but his talented mind was diverted to the study of tissue culture methods, which he continued from 1906 onwards.

Hektoen in 1916 first tried to modify the immune response in rabbits. He injected sheep blood cells intraperitoneally and reported reduced production of antibodies by the use of organic substances such as benzene. However, it was not until 1952 that Baker attempted to apply this to experimental renal transplantation when he used nitrogen mustard to reduce

antibody production in the animal recipient. The results were not encouraging. This is not surprising as very little was known of the biology of organ allograft rejection.

During the 1950s Dempster and Simonsen showed that canine kidneys grafted between individuals of the same species who were not identical twins were invariably destroyed or rejected. Between the years 1951 - 1953 Hume performed a small number of cadaver kidney transplants in the human (Hume et al, 1955). In some of these patients the function of the transplants enabled the patient to live for a limited length of time and one patient survived for six months. Some attempts were made to prolong the survival of these grafts by giving the recipients ACTH or steroids but inadequate doses were used.

In the 1950s ways were continually being sought to manipulate the immune response so that the body would accept foreign protein. Two important advances were made. First, Billingham, Brent and Medawar in 1953 demonstrated that if an animal still in utero or newly born was injected with foreign cells from another animal, it remained tolerant of that material throughout its life and would accept an allograft as if it were its own tissue provided that the graft was taken from the same inbred strain of animal as the cells that had been

originally injected. This "actively acquired immunological tolerance" of foreign cells was a demonstration that the immune response could be manipulated, though, obviously, when performed in this way it was unlikely to be of any practical clinical use. This finding, however, gave encouragement and confidence to clinicians that human allograft rejection might be avoided.

Then, in 1959, Schwartz and Dameshek in Boston reported that they were able to produce 'drug induced immunological tolerance'. Rabbits given the antimetabolite 6-mercaptopurine (6-MP) no longer produced antibodies when injected with human serum albumin. Indeed, in the following year they confirmed the importance of this observation by demonstrating prolonged skin allograft survival in rabbits treated with 6-MP (Schwartz et al, 1960). It is now known that the findings of Schwartz et al were not tolerance but a chemical suppression of part of the immune response. The demonstration of 'drug induced immunological tolerance' to foreign tissue in an animal opened up the opportunity to perform successful organ allografts in clinical practice. Calne in London recognised this and very soon had demonstrated prolonged renal allograft survival in dogs maintained post-operatively on 6-MP (Calne, 1960). This observation was also reported by Zukoski and colleagues in 1961. Calne then moved to Boston, where these experiments were

extended and Burroughs Wellcome provided derivatives of 6-MP for study, in particular, azathioprine, a purine analogue similar to 6-MP.

The first prolonged human cadaver renal allograft survival was reported from Boston in 1962, when a patient was treated with azathioprine. This case has been described in detail by F. D. Moore (Moore, 1964a). From 1962 onwards, successful renal allografts were reported from many centres using donor kidneys obtained from cadavers and giving the recipients azathioprine (or 6-MP) for post-operative immunosuppression.

About this time Dr. Willard Goodwin (1962) in Los Angeles had shown a beneficial effect of steroids in a patient with a renal allograft who had been treated with cyclophosphamide. Since azathioprine was more effective and less toxic than cyclophosphamide in animal experiments it was decided to embark on a programme of renal allografting in man using azathioprine as the basic immunosuppressive drug and to add steroids if rejection was not controlled (Murray et al, 1963). Since then azathioprine in combination with corticosteroids has become the mainstay of post-operative immunosuppression after renal transplantation. The reason why azathioprine is used in preference to 6-MP has never been made clear. No trials were carried out in man to compare the two drugs and the often repeated statement that azathioprine

is superior to 6-MP is not based on evidence that bears critical examination (Berenbaum, 1971). It is of great interest that azathioprine and steroids have not yet been replaced in clinical practice, at a time when so many advances have been made in the immunology of rejection.

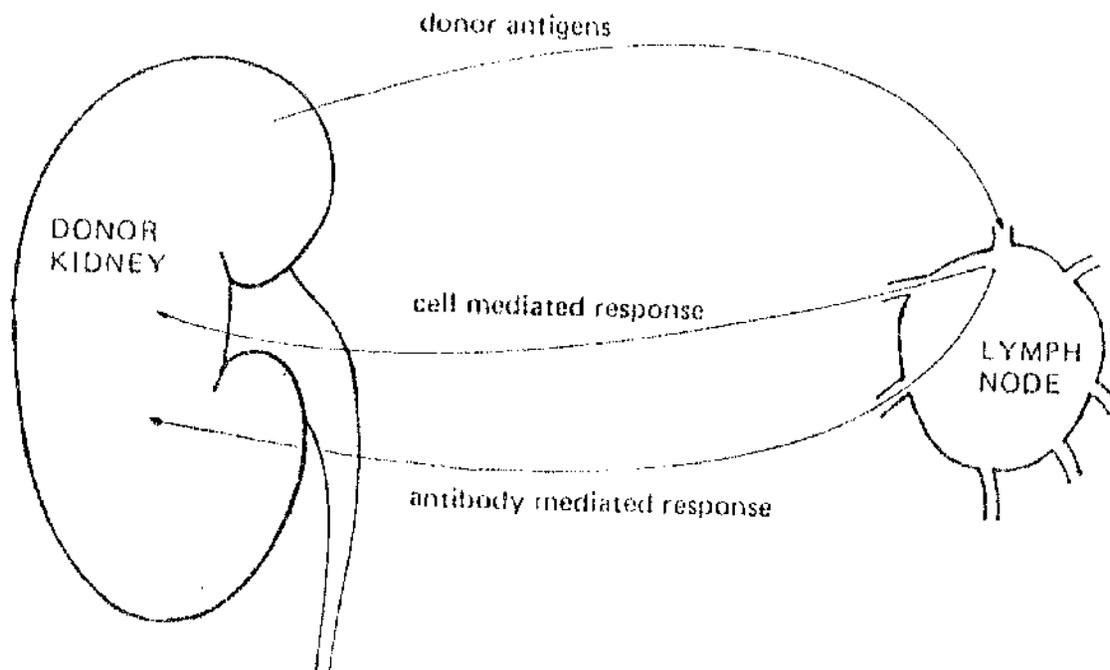
2. Recent Developments

During the last two decades attempts have been made to discover other techniques for suppressing the immune response of the recipient. During this time new information has been obtained about the rejection process and it can be anticipated that this greater knowledge will lead to improvements in the techniques of immune suppression and tolerance.

Aspects of Transplantation Biology

When an allograft is transplanted the body recognises it as foreign and attempts to eliminate it by the process of rejection. The initial response to the organ graft is mounted by the recipient lymphatic tissue. A very simple scheme for organ rejection is shown in Fig. 1.1. The allograft is detected by the immune reacting tissue (the 'afferent' arc) and, as a result, lymphocytes become activated and a response develops which causes rejection of the transplant. The response may be

FIGURE 1.1

Rejection Schema of a Renal Transplant

On transplantation of a kidney, donor antigens are recognised by the recipient lymphatic tissue as 'foreign' - the afferent arc. A response is mounted by the recipient lymphatic tissue against the transplant - the efferent arc. The response may be either cell-mediated by T lymphocytes, or antibody-mediated by B lymphocytes (see text).

cellular or humoral or a combination of both (the 'efferent' arc) but in either case the response is mediated by lymphocytes.

The cells involved in the cellular component of the immune response are under the influence of the thymus and these lymphocytes are referred to as thymus-dependent or T cells. When exposed to an allograft they proliferate. A number of T cell populations are described. Some are responsible for destroying the graft (cytotoxic cells), some are directed against the transplant (so-called helper cells because they "help" the immune response) and some protect the graft (suppressor cells).

The humoral response to an allograft is mediated through the B lymphocytes, which produce antibody to the graft. Experimental work in animals, for instance, by Good and his colleagues in Minnesota (Cooper et al, 1966) showed that the development of lymphocytes concerned with antibody production was dependent on the Bursa of Fabricius. No such structure has been found in humans but the term B lymphocyte (i.e. bursa-dependent lymphocyte) has become widely used for those lymphocytes which are found especially in the lymphoid follicles of lymph nodes that produce antibody in response to stimulation by antigen. The B lymphocytes proliferate to produce the plasma cell which produces the antibody.

The humoral antibody, which is circulating in the blood stream, modifies or destroys the allogeneic graft cells. It is also now recognised that B cell activity is dependent on T cell function.

Within these two broad mechanisms several types of rejection are recognised clinically:

- a) Hyperacute rejection: This occurs when the recipient is presensitized to the donor tissues, for instance ABO blood group incompatibility. Or it can occur as a result of blood transfusions which enable the recipient to become sensitized to donor antigens and develop circulating cytotoxic antibodies to the donor tissue. This is primarily an antibody-mediated response and may be apparent within minutes of connecting the grafted organ to the recipient. Histological examination of the transplanted kidney shows sludging of the red cells and microthrombi formation, particularly in the glomeruli.
- b) Acute rejection: This is either a cellular or humoral response. Early acute rejection, classically seen at 7 to 10 days after transplantation, is primarily a T lymphocyte response, the kidney showing a dense cellular infiltration with damage and rupture of the peritubular capillaries. Usually this type of response is seen in recipients not on immunosuppression. When acute rejection of renal transplants occurs, azathioprine and a corticosteroid

are normally being given, and then it is primarily a humoral, or antibody, response, at about 14 days after transplantation. Antibody binds to the walls of the glomerular capillaries. Usually in acute rejection different areas of the kidney will show varying histology suggestive of both a cell-mediated and humoral response.

- c) Late rejection: This occurs usually months or years after transplantation. Histology shows a sub-endothelial deposition of immunoglobulin and complement within the kidney vessels. This "vascular rejection", as it is sometimes called, can occur acutely or more insidiously over months or years. These changes are indistinguishable from those of malignant hypertension, and in less acute vascular rejection there may also be intimal proliferation in the arteries, leading to narrowing or obliteration of the lumen. These vascular changes result in renal ischaemia and failure, and are very difficult to reverse by immunotherapy.

This simple view of T and B lymphocytes responding to a transplant and initiating rejection has given way to mechanisms with much more complex pathways. Antibodies may be directed against the transplant, for instance cytotoxic antibodies - and yet others may protect the graft - the blocking, or enhancing, antibodies. Some antibodies seem to prime transplant tissue cells by

coating them, and thus make them more susceptible to attack by lymphocytes - this mechanism is termed antibody-dependent cell-mediated cytotoxicity.

In addition, increasing information is being obtained about ill-defined substances which were initially thought to be released by lymphocytes coming into contact with an antigen. Originally the name 'lymphokines' had been introduced to describe this group of non-antibody lymphocyte factors. More recently, the term 'interleukin' has been used to describe this group of peptide hormones. Two of this group of hormones are Interleukin 1 and Interleukin 2. In brief, they are involved in T cell activation and the essential features are as follows: Interleukin 1 (IL_1) is produced by macrophages on encountering the antigen. IL_1 acts on a subpopulation of T helper cells along with the antigen to evoke the production and release of Interleukin 2 (IL_2). This in turn acts on antigen-primed T helper cells and causes mitogenesis of these cells. Helper cells enhance the activity of cytotoxic cells and B cell antibody production directed against the antigen. This is further discussed on page 138.

Immune Suppression

Since the rejection of tissue or organ allografts is the culmination of interconnected cellular and humoral

events mediated by various lymphoid cell subgroups, agents or procedures acting directly against lymphoid tissue have been studied in the search for more efficient immunosuppression. Most of the techniques affect lymphocyte populations indiscriminately.

1. Agents and Procedures Affecting Predominantly Lymphoid Tissue

a) Whole Body Irradiation

Isolated successes were reported in kidney grafts between non-identical twins using total body X-irradiation to the recipient (Murray et al, 1960; Hamburger et al, 1962). The results overall were not encouraging due to a high incidence of sepsis in the irradiated recipients.

b) Thymectomy and Splenectomy

Starzl first advocated pre-transplant splenectomy in 1963 to reduce lymphoid mass and perhaps thereby to attenuate rejection. A few years later Zühlke (1967) and his associates observed that splenectomy and thymectomy in man significantly reduced the titres of IgM immunoglobulin after renal transplantation. Yet the role of splenectomy and thymectomy is uncertain. Although pre-transplant splenectomy is routinely practised by a few transplant surgeons, since 1970 most

have used splenectomy only for patients with pre- or post-transplant neutropaenia in an effort to improve azathioprine tolerance. Thymectomy is rarely performed in modern clinical practice.

c) Lymph Drainage Through a Thoracic Duct Fistula

The rationale for such an approach was provided by the investigations in rats of Gowans and his associates (Gowans, 1959; McGregor and Gowans, 1963). These workers demonstrated that the primary immune response to tetanus toxoid or sheep erythrocytes is severely depressed or abolished in animals whose lymphocyte population had been depleted by thoracic duct drainage. Drainage of lymphocytes by cannulation of the thoracic duct in humans was first tried in 1963 in St. Louis by Newton and later in Stockholm by Franksson (1967). A more recent review of the procedure in forty renal transplant recipients was given by Starzl in 1979. Despite some optimism the technique has not gained universal acceptance and is used only in a few centres.

d) Antilymphocyte and Antithymocyte Globulin

These agents are derived from the sera of animals injected with lymphocytes or thymocytes from another species. In the early 1960s Woodruff reported that antilymphocyte globulin, administered intraperitoneally, provided a major prolongation of skin allograft survival

in rodents. Unfortunately, the responses of the animals to injections of lymphocytes or thymocytes varies and it has been difficult to obtain preparations with standard biological effects. In addition, patients may develop a hypersensitivity to the injected animal protein. These globulins were first used in clinical transplantation in 1967 but controlled trials were not carried out until years later. During this time the results have varied but have been encouraging from only a few centres.

e) Total Lymphoid Irradiation

Total body irradiation has been administered as an immunosuppressive technique. Recently high dosage partially-selective lymphoid irradiation similar to that used in the treatment of Hodgkin's disease has been reported to be relatively non-toxic and permit long acceptance of experimental bone marrow, skin and organ allografts (Slavin et al, 1979). A few centres have begun to use lymphoid irradiation for preparing certain patients for kidney transplantation. The largest experience has been obtained in Minnesota and although the early results appear promising (Najarian et al, 1981) longer follow-up will be required before its usefulness can be properly evaluated.

2. Chemical Immune Suppression

Other investigators have studied agents with a general antiproliferative action. These agents, most of which are used as anti-cancer drugs, have a general cytotoxic action and render cells incapable of division. They include:

Antimetabolites

These compounds interfere with protein synthesis by competing for and blocking specific receptors. They include the purine antagonists 6-MP and azathioprine, the pyrimidine antagonist 5-fluouracil, and the folic acid antagonist methotrexate. Since these agents are only effective against proliferating cells, they are most effective when given after, rather than before, the exposure to antigen.

Azathioprine is a similar compound to 6-MP but with an imidazole group attached to a sulphur atom. It is rapidly converted back to 6-MP following ingestion and for this reason the activity of the two compounds is largely the same. As has been mentioned previously, the optimum time for administering these drugs is after exposure to antigen and it has been shown that antibody production in man is very little affected if they are given before (Hersch et al, 1966).

Alkylating Agents

These compounds possess an alkyl radical with active end groups, usually chlorine atoms, which can bind to two or more different molecules. With most alkylating agents DNA synthesis is inhibited to a greater extent than RNA synthesis. Although alkylating agents have been shown to be most useful in treating malignancies, they have been of little value on the whole as immunosuppressants. They include cyclophosphamide, mitomycin C, nitrogen mustard and sulpha mustard.

Cyclophosphamide interferes with the reproduction of immunologically competent cells and it is more effective in depressing antibody response in animals if given 24 to 48 hours after immunisation (Frisch and Davies, 1965). However, resting cells can also be damaged and small lymphocytes can be killed by a process unrelated to cell proliferation. In 1971 Starzl proposed that cyclophosphamide might be substituted for azathioprine with advantage in cadaveric renal and hepatic transplantation (Starzl et al, 1971). Patient follow-up was only two or three months, however, and there was no comparable control group. Toxicity (leucopaenia, thrombocytopaenia, haemorrhagic cystitis, testicular atrophy, nausea and vomiting) has probably been the reason why the drug has not been widely used in other transplant centres. Its main use has been in the

treatment of malignant disease rather than in transplantation.

Mitomycin C has also been used. This anti-cancer drug has been reported to enhance the immunosuppressant activity of 6-MP (Kenis et al, 1964), but the dose required has rendered it unsuitable as an immunosuppressive agent due to severe leucopaenia and thrombocytopaenia.

Other Methods of Prolonging Survival of an Organ Transplant

Immune suppression is imperfect and therefore attempts have been made to reduce the immune response mounted against the donor organ. The methods used include:

- a) Tissue typing
- b) Donor pre-treatment
- c) Blood transfusion.

a) Tissue Typing

Autografts do not reject and allografts between identical twins do not reject. Grafts between syngeneic animals do not reject. Therefore, could the rejection response be diminished if a close match between donor and recipient was achieved?

The first work was performed in mice (Gorer, 1937). A red blood cell antigen was identified, matching for which had a major effect on survival of tumour transplants. When the growth of a transplantable tumour was observed in inbred mice and in hybrid generations, it was found that the tumour would only grow in mice carrying certain genes which were also present in the stock of origin of the tumour. From this work, in 1948, Snell termed the antigens relevant for transplantation the 'histocompatibility antigens'. With the development of knowledge concerning histocompatibility or H_2 antigens in the mouse there was a search for similar antigens in humans. Gradually evidence accumulated that there was a system in man based on leucocytes. Van Rood, and Payne, working independently, demonstrated that leucocyte agglutinins were produced by foetal-maternal stimulation. Using these sera, Van Rood (1962) identified a two allele* system which he called 'group 4'. Payne described a similar but independent system of antigens and as more antigens became identified it became clear that there were two systems

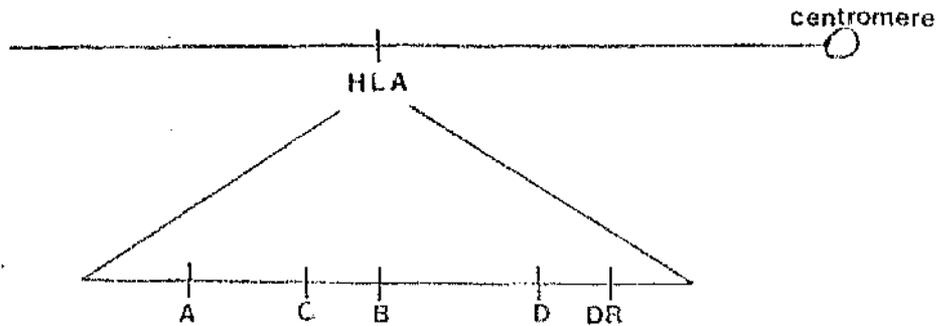
* Allele: genes forming a pair which may be alike, homozygous, or dissimilar, heterozygous - situated at a specific locus, or site, on one pair of chromosomes.

of serologically-determined antigens controlled by a set of alleles at two closely linked loci. This was initially known as the L.A. system (Leucocyte; A for the first locus), which is located on the short arm of chromosome 6. These antigens are now termed the H.L.A.-A and the H.L.A.-B antigens. A further locus H.L.A.-C has also been described. Antigens of the H.L.A.-A, B and C series are all serologically-determined using antisera of known specificity. A fuller account of these antigens is given by Bodmer, W.F. (1978).

Recently a further series of antigens has been described - the H.L.A.-D series (1975, Report of the Sixth International Histocompatibility Workshop). These antigens are defined by the way in which the lymphocytes respond to other lymphocytes of known type. Thus, typing for these antigens is based on the mixed lymphocyte reaction and not serological testing. More recently, a new serologically-determined series of antigens has been identified (1977, Report of the Seventh International Histocompatibility Workshop). This series is very closely related to the lymphocyte-determined H.L.A.-D and has become known as the H.L.A.-DR (i.e. D-related). A schematic demonstration of the H.L.A. system is given in Fig. 1.2.

FIGURE 1.2

Schematic Representation of the H.L.A. System
in the Short Arm of Chromosome 6



At least 5 loci are known to belong to the H.L.A. system, their designation (ordered from centromere) is DR, D, B, C, A.

The H.L.A. system is genetically coded, the genes being located on the surfaces of leucocytes, platelets, tissue cells and reticulocytes but are not demonstrable on mature erythrocytes. Each person has two antigens for each locus i.e. H.L.A.-A antigens, etc., and in clinical practice it is the antigens of the H.L.A.-A and B series that are typed routinely. H.L.A.-C and H.L.A.-D are not usually studied but increasingly H.L.A.-DR typing is also performed.

Clinical Relevance of H.L.A. in Transplantation

As details of the H.L.A. system have been discovered, national and international organ sharing schemes have

emerged to allow organ sharing on a "matched basis". Because of the rapidly increasing knowledge of these antigens it is hardly surprising that their role in clinical renal transplantation has been the subject of intense debate. Morris, Batchelor and Festenstein (1978) reviewed the role of H.L.A. in transplantation and their paper may be summarised:

- i) Matching for three or four H.L.A.-A and B antigens improves the clinical results by 10 to 30% over H.L.A.-A and B mismatches.
- ii) Matching for H.L.A.-C is probably of no benefit.
- iii) H.L.A.-D and DR matching is still being evaluated. Matching for H.L.A.-DR probably improves the prospects for renal graft survival.

Despite early optimism, the use of tissue typing and donor recipient matching has been only partially successful in improving graft survival. There are problems of the accuracy of typing and data collection in large series. Transplant registers do show that matching helps, although a few questions remain unanswered. What antigens are important? Which can be disregarded and which have yet to be discovered?

b) Donor Pre-treatment

Attempts have been made to improve graft survival by treating the donor organ to reduce its antigenicity.

Graft irradiation in vitro or pre-treatment of the donor with cytotoxic drugs before kidney retrieval have been the two main techniques used. Guttman and colleagues in 1969, after experimenting with many cytotoxic agents, found that high doses of cyclophosphamide and methyl prednisolone given to donor animals five hours before removal of a kidney, gave the best results. Accordingly, they used such a regime to treat human brain dead kidney donors (Guttman et al, 1975). The results were encouraging. However, at the present time the value of donor pre-treatment in cadaveric renal transplantation remains in doubt and is not widely used in clinical practice.

c) Blood Transfusion

It is now well established that the survival rate of renal allografts is higher in patients who have received than in those who have not received blood transfusions prior to transplantation. This observation was first made by Opelz in 1973, who found that patients who do not develop antibodies have an excellent prognosis following grafting. The first confirmation in Europe of the benefit of pre-transplant blood transfusion was made in Newcastle upon Tyne (Murray et al, 1974). Results from further work in Newcastle showed that the development of cytotoxic antibodies is no disadvantage provided a donor can be found with whom the recipient

has a negative cross-match. The presence of cytotoxic antibodies may delay transplantation but perhaps of more importance is the fact that their function and discovery may prevent a patient being transplanted with a kidney which will subsequently be uncontrollably rejected.

The factors responsible for the favourable influence of blood transfusion on renal allograft survival have not yet been identified. While the immunological effects of transfusion may be directed towards the impairment of cellular immune competence (Shenton et al, 1974), attention has been turned to the possible significance of lymphocyte-suppressing factors which emerge following blood transfusion (Shenton et al, 1979). It may be that more than one mechanism is responsible for the improvement of graft survival in transfused patients, including specific immunosuppression, graft selection and patient selection (Fabre et al, 1978).

Most centres will now deliberately give blood to patients who have never been previously transfused and who are on dialysis awaiting grafting. However, there is still controversy about the type of blood transfused, the optimal amount to give and the timing of the transfusion.

Regarding the type of blood transfused, some reports

indicate that the results of transplantation are better in patients transfused with frozen blood rather than in those transfused with other preparations (Polesky et al, 1977); other reports indicate that whole blood is better than frozen blood (Opelz and Terasaki, 1974), while another report shows a similar beneficial effect no matter whether whole or frozen blood is used (Briggs et al, 1978).

One report shows that even a single transfusion places the recipient in a more favourable category for graft survival (Persijn et al, 1977). Some show no difference in relationship to the number of transfusions (Festenstein et al, 1976) and others show that the more transfusions that have been received the higher the likelihood of prolonged graft survival (Perkins and Salvatierra, 1977). One centre reports that transfusions on the day of transplantation are associated with improved graft survival rates (Stiller et al, 1978) but other analyses do not confirm this (Salaman, 1978; Jeffrey et al, 1978).

Despite all these developments from the earliest days of kidney transplantation, rejection remains the most important cause of graft loss. Tissue matching and organ sharing schemes have improved results a little and deliberate transfusion policies have helped a lot. Azathioprine and corticosteroids remain the most widely

used drugs in human transplantation but these drugs have important side-effects and rejection may still occur when they are used. Steroids are associated with Cushingoid appearance, fluid retention, adrenal suppression, acne, psychosis, avascular necrosis of bone and gastro-intestinal bleeding. Azathioprine may cause profound leucopaenia which predisposes to widespread sepsis and sometimes death. The considerable problem of graft rejection remains. For instance, in the United Kingdom during the five year period 1977 to 1982 the survival of first cadaveric renal allografts at twelve months was 64%. Approximately 40% of all cadaver kidney grafts are functioning after six years: 60% are unsuccessful, the majority as a result of rejection.

Referring to azathioprine and corticosteroids nearly twenty years ago, Moore said "other drugs are being used to some extent; better drugs will be discovered and other chemical methods are certain to be effective" (Moore, 1964b). As yet the ideal immunosuppressant has not been found but the aim remains to find such a method which would not harm the donor organ or the recipient and which would prevent any rejection episodes being mounted against the graft. Such an ideal immunosuppressive agent should be easily obtainable at a reasonable cost, totally palatable and without side-effects for the patient. Recently the discovery of Cyclosporin A has raised hopes that such a drug has been

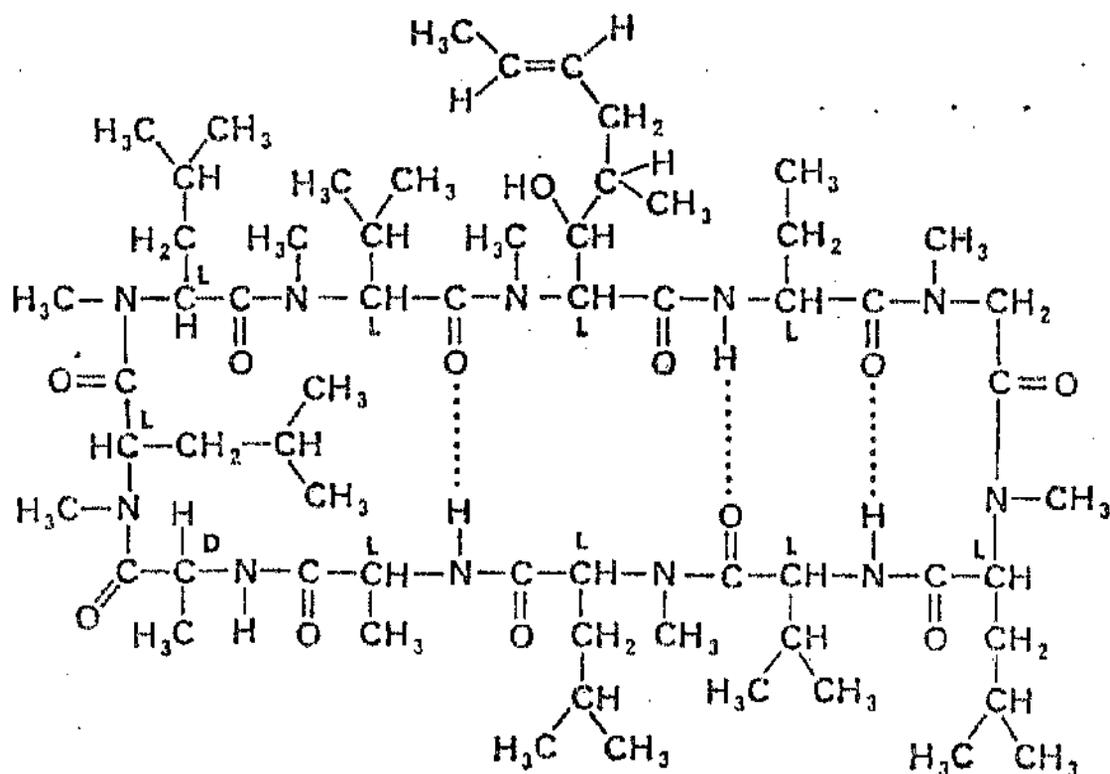
found.

Cyclosporin A

In 1976 at the Biological and Medical Research Division of Sandoz Limited, Basle, Switzerland, during a screening programme of fungus extracts, Borel and colleagues noted that "metabolites of the species Cylindrocarpon Lucidum Booth were found to depress antibody production in mice". Further investigations led to the isolation of the active principle, a cyclic peptide consisting of 11 amino acids with a molecular weight of 1,202.6, to which the name of Cyclosporin A (CyA) and experimental designation of OL-27-400 were given (Fig. 1.3). The compound, which is also found in cultures of Trichoderma polysporum (Link Ex Pers) Rifai, was a novel antilymphocytic agent containing 1 amino acid which had never previously been isolated or known in free form. Supplied as a white powder, the non-steroidal peptide is insoluble in water although it can be dissolved in alcohol or a fat emulsion (Petcher et al, 1976). It can be administered either orally or parenterally.

In 1976 Borel demonstrated that CyA depressed immune reactions both in vivo and in vitro and prolonged skin graft survival in mice. This observation led to studies of CyA in different species with a variety of organ transplants, notably at Cambridge, England. The

FIGURE 1.3

Cyclosporin A

Cyclosporin A (OL-27-400) is a metabolite derived from two species of fungi: Cylindrocarpum Lucidum Booth and Trichoderma polysporum (Link Ex Pers) Rifai. Its molecular weight and formula are 1202.6 and $C_{62}H_{111}N_{11}O_{12}$, respectively. It is a ring peptide consisting of 11 amino acids. The white powder is not water soluble.

rejection of heterotopic heart grafts in rats was delayed by CyA (Kostakis et al, 1977). CyA was more effective than azathioprine in prolonging canine renal grafts (Calne and White, 1977), orthotopic cardiac allografts in pigs (Calne et al, 1978a), renal allografts in rabbits (Green and Allison, 1978) and canine pancreatic grafts (McMaster et al, 1980).

Following these experiments a pilot human study was begun using CyA in clinical organ grafting initially as the sole immunosuppressant (Calne et al, 1978b). This study involved 50 patients receiving 56 cadaveric organ allografts, 45 kidneys, 6 pancreases and 5 livers. The results were encouraging and numerous other clinical investigations have subsequently been initiated in an attempt to identify such features as the ideal dosage, timing and duration of treatment and the side-effects of the drugs. Other studies were designed to investigate the pharmacodynamics of the drug. In spite of very active programmes of research there is yet much to be discovered about Cyclosporin A and particularly about its mode of action in a biological system.

Aims of the Studies

The aims of the studies described in this thesis were as follows:

- (1) To investigate CyA as an immunosuppressive agent in

the rat cardiac allograft model.

- (2) To study both the humoral and cellular arms of the host immune response when treated with CyA.
- (3) To discover whether donor-specific suppressor cells appear in recipient rats during or after treatment with CyA.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

The Animal Model

1. General Considerations

The experimental models in general use in organ transplantation research are either renal allografts in dogs or rats, where rejection of the kidney can be determined by the blood creatinine exceeding a chosen level, or cardiac allografts in rats, where rejection can be determined by the time when the transplanted heart ceases to beat.

It is very difficult to obtain the necessary numbers of inbred large animals e.g. dogs and therefore rats and mice were considered as suitable experimental animals. It is not easy to perform organ allografts in mice and it is technically easier to transplant the rat heart than the kidney. Therefore the model selected was that of the rat heterotopic cardiac allograft.

This rat heart model has several advantages. First, hearts grafted between inbred strains of rats that differ in major histocompatibility are rejected at an uniform and predictable time (6 - 8 days after transplantation). Second, this rejection process can be accurately monitored in individual hosts by palpation of the transplant through the abdominal wall.

Third, unlike renal allografts, the rejection of cardiac allografts is not preceded or accompanied by any major physiological abnormalities in the host, such as uraemia, that may alter immune responses. Fourth, rejection of the heterotopic cardiac allograft does not result in death of the recipient animal so that post-rejection recovery of the lymphoid organs can be studied in the recipient.

2. Choosing the Rat Strains

In selecting rats for the study the following requirements had to be met:

- a) The rats must be of different histocompatibility groupings to ensure they are allogeneic.
- b) All rats must be inbred and be readily obtainable from reliable commercial resources.
- c) They must share the same red cell antigens i.e. they must have the same blood groupings.
- d) They must be adult male rats weighing between 150 and 300 g (a weak sex antigen has been described in rats (Heslop, 1973) and therefore rats of one sex only were used; male rats were used as they are larger age for age than female rats).
- e) They should be hardy and resistant to infection, (rats are very susceptible to respiratory viral infections, which could interfere with immunological experiments) and be able to withstand surgical operations.

- f) The donor-recipient pairing should provide a model of rejection that is reliable and consistent with minimal variation of the time taken to reject the graft.

The following donor-recipient combinations fulfilled these criteria:

Heart donors - (Lewis x Brown Norway) F_1 Hybrids
 - (Lewis x Wistar Furth) F_1 Hybrids
 Heart recipients - (Lewis)

For many years in the Surgical Research Laboratory at the Harvard Medical School this combination of F_1 hybrid donor animals had been used in cardiac allograft experiments. Accordingly, there was a plentiful supply of these animals. This combination had been used in many earlier studies of immunological manipulation of the rejection process (Tilney et al, 1978; Hendry et al, 1979). The rats chosen are classified as follows, where AgB or H-1 is the histocompatibility type determined serologically and AgC is the red cell antigen.

RAT	AgB	H-1	AgC
LEW	1	1	2
(LEW x BN) F_1	3	n	2
(LEW x WF) F_1	4	w	2

The History of Each Rat

LEW (LEWIS) ORIGIN: LEWIS to APTEKMAN and BOGDEN, 1954 at 20 generations; to SILVERS, 1958 at 31 generations. 70+ generations. Albino appearance.

WF (WISTAR/FURTH) ORIGIN: J. FURTH, 1945 from Wistar Institute Stock. 30+ generations. Albino appearance.

BN (BROWN NORWAY) ORIGIN: SILVERS and BILLINGHAM, 1958, from a brown mutation maintained by H. D. KING and P. APTEKMAN. 40+ generations. Brown, non-agouti appearance.

3. Technique of Heterotopic Cardiac Allografting in Rats

The technique of heart transplantation is well described in the literature (Ono and Lindsey, 1969).

Essentially heterotopic cardiac allografting involves transplanting the donor heart to the abdomen of the recipient, the donor animal being sacrificed. The donor aorta is anastomosed to the recipient abdominal aorta and the donor pulmonary artery to the inferior vena cava of the recipient. The donor superior and inferior vena cavae are ligated, as also are the pulmonary veins. Blood flow to the transplanted heart

is through the donor aorta in retrograde fashion and thus into the coronary circulation to perfuse the myocardium with oxygenated blood. The blood collects in the coronary venous system and enters the right atrium and then the right ventricle from which it passes, via the pulmonary artery, to the inferior vena cava of the recipient. The myocardium is perfused and therefore the heart beats, though it is not functioning as a normal cardiac pump.

Whilst it is possible to use rats between 150 and 350 g in weight in this experimental model (below this weight the vessels are very small, and above it considerable fat deposition makes the transplant operation more difficult), it was desired to maintain uniformity throughout the studies as far as possible and therefore rats weighing 220 to 250 g were normally used.

Absence of a palpable beat, combined with an inability to record any electrical activity in the transplanted heart, was taken as clinical evidence of rejection. The technique of ECG recording is described later in this chapter.

(i) Anaesthesia

General anaesthesia was always used. Rats are somewhat unpredictable in their response to general anaesthesia and a number of drugs were tried. The best anaesthetic

was a combination of ether and a low dose barbiturate.

Induction was performed with ether by placing the rat in a large covered glass flask that contained an ether-soaked cottonwool base. After about 20 to 30 seconds the rat became unconscious, and it then received an injection of pentobarbitone sodium intra-peritoneally. The dose of pentobarbitone was 30 mg/kg body weight.

Anaesthesia was maintained with ether administered from a pad of cottonwool contained in a small cone. At all times the aim was to keep the anaesthesia as "light" as possible to facilitate rapid recovery at the end of the operation.

(ii) Instruments

The vascular anastomoses are of vessels approximately 1.5 mm in diameter and therefore microsurgical instruments and an operating microscope were used. The microscope was a binocular Carl Zeiss operating microscope, bench mounted, and with foot controls to control focussing and magnification. A magnification of approximately 6x was ideal.

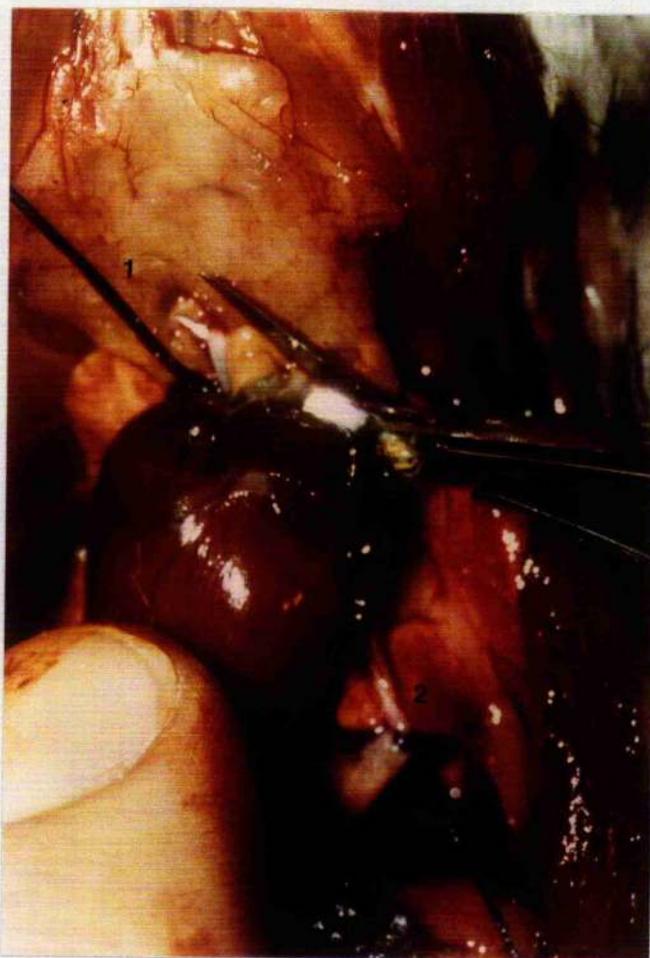
Beverage can 'pull tops' attached to elastic bands were ideal abdominal wall retractors during the implantation of the heart. Fine pointed forceps were required for handling the small vessels and jewellers' forceps, No. 5,

were used. A pair of Castroviejo iris scissors and a straight Castroviejo needle holder, the jaws of which were ground to a smaller size to suit this particular procedure, completed a small and uncomplicated set of instruments. It is essential when doing microsurgery to keep the instruments as simple as possible.

(iii) The Operation

(a) Donor (Fig. 2.1)

After the donor rat has been anaesthetised the abdomen is opened and 50 units of heparin injected intravenously through the inferior vena cava. The anterior chest wall is separated from the diaphragm and the anterior rib cage is divided with scissors on both sides of the sternum from lower rib margins to clavicles. A ligature (1) is placed around the superior vena cava and another around the inferior vena cava (2). One blade of the angled Castroviejo scissors is placed in the transverse sinus and the aorta and pulmonary artery are divided as far from the heart as possible to give as much length to these vessels which facilitates the anastomosis. The superior and inferior venae cavae are divided distal to the ligature. Pulmonary veins are ligated "en masse", divided and the heart removed. Once out of the body, the heart is immediately placed in a N/saline bath at 4°C. Then it is removed and irrigated with N/saline at

FIGURE 2.1Removal of the Donor Heart

A ligature (1) is placed around the superior vena cava and another around the inferior vena cava (2). One blade of the Castroviejo scissors is placed in the transverse sinus.

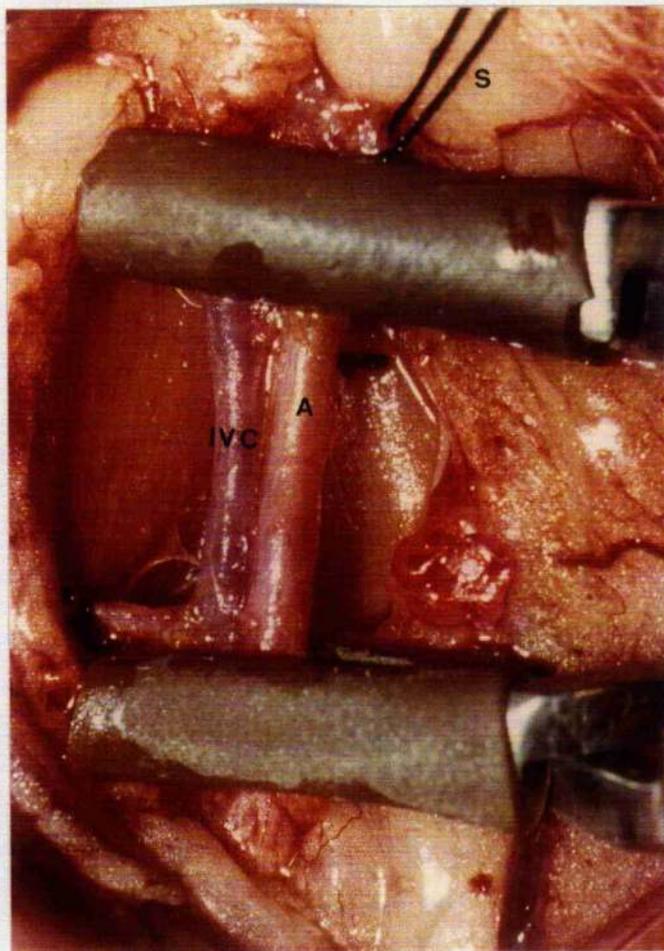
4°C, first through the aorta and then the pulmonary artery. The irrigation cools the heart, removes blood clot and by filling the ventricles prevents air embolism after the transplant.

(b) Recipient (Fig. 2.2)

Once the aorta (A) and the inferior vena cava (IVC) are adequately cleaned, and all branches and tributaries tied over a 1.5 cm length, a black silk sling (S) is placed around the aorta proximally (to control the aorta at the end of the operation when the clamps are released) and two small rubber guarded bulldog clamps are applied to the aorta and inferior vena cava. A longitudinal incision of 2.5 to 3 mm is made in the aorta (using a No. 11 scalpel blade) and the aorta is flushed free of blood and blood clot with saline using a 20 gauge flexible catheter.

(c) Transplantation

The aortic anastomosis is performed by starting at the rostral end, running a continuous suture (8/0 monofilament polyamide, Ethicon 8170) along the left side of the anastomosis and tying the suture at the caudal end of the anastomosis. The heart is then turned over to the left side of the rat's abdominal cavity and using the same suture, the other side of the anastomosis is completed. The inferior vena cava

FIGURE 2.2Preparation of the Recipient

The aorta (A) and the inferior vena cava (IVC) are cleaned. A black sling (S) is placed around the aorta proximally. Rubber-guarded bulldog clamps are placed across the aorta and inferior vena cava.

is opened longitudinally and blood and blood clot syringed out with normal saline. As with the aorta, sutures are placed at the end of the venotomy, and the pulmonary artery secured at each end to the inferior vena cava. The medial anastomosis is performed first using the rostrally placed suture. This has to be performed from within the vessel lumen. A continuous suture is used and, on reaching the caudal end of the incision, the suture is placed outside the vessel and is ligated to the stay suture at that end. Finally, the lateral side of the anastomosis is completed and the abdomen closed.

Using the light anaesthesia described earlier, the rats were normally conscious and walking around their cage thirty minutes after completion of the operation.

Typical times recorded for the operation were:

- a) Initial warm ischaemic time (i.e. the time taken to remove the heart from the chest and place in cold saline) 3 minutes
- b) Cold saline storage of heart 5 minutes
- c) Anastomosis time 15-18 minutes

4. Post-operative Problems

Very few were encountered. A small number of rats died after the operation without recovery and without obvious cause of death. Two rats developed paraplegia

and were sacrificed as soon as this was noticed. A small number of transplanted rats' hearts stopped beating within 24 to 48 hours. These were invariably infarcted and these rats were excluded from any study. Once the technique was mastered, the success rate in transplantation was over 95%.

5. Post-operative Monitoring

(a) The Heart

In the early days after transplantation simple digital palpation of the transplanted heart was sufficient to prove that the graft was functioning. When the heart-beat became difficult to palpate, ECG recordings were used. These were easily performed. The animals were given a light ether anaesthetic and then two electrodes were placed one on each side of the transplant, and the third, indifferent electrode was placed on the right thigh. Needle electrodes were used. The recording given was of the transplanted heart.

To record the rat's own heartbeat - which was always done to differentiate between transplant and native hearts - the electrodes on each side of the transplanted heart were placed to the right and to the left forelimbs respectively and a further recording taken.

When electrical activity from the transplanted heart was no longer recordable, a laparotomy was performed to ensure that the heart was not beating, and the heart was then removed for histological examination (to confirm rejection) and the animal was sacrificed.

An example of the ECG tracings obtained from the rat's own heart, and from the transplanted heart, at periods following transplantation until just before the cessation of the heartbeat are shown in Fig. 2.3.

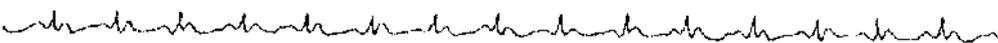
(b) Blood Analyses

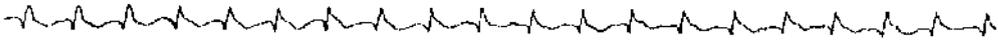
After transplantation, and during the period of rejection, several blood parameters were measured in recipient rats. The most suitable method for obtaining blood samples was as follows. Under a light anaesthetic, a small incision was made over the clavicle. The jugular vein and the upper part of the pectoralis major was exposed. Venepuncture was performed by introducing the needle to the vein through the pectoralis major muscle. This prevented subsequent bleeding when the needle was withdrawn. 1 ml samples were obtained and the wound was closed with black silk sutures. The total length of time for the procedure was 2 to 3 minutes. No rat was bled more than once if a transplant had been given and each experimental group of rats contained animals that were not bled to control any possible immunosuppressive

FIGURE 2.3

E.C.G. Recordings of the Rat's Own
Heart and the Transplanted Heart

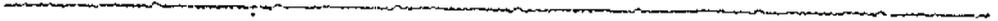
HETEROTOPIC CARDIAC ALLOGRAFT - CONTROL GROUP


Native heart


Transplanted heart - 2 days post-transplant


Transplanted heart - 4 days post-transplant


Transplanted heart - 6 days post transplant. Heartbeat not palpable.


Transplanted heart - 7 days post-transplant

The change in the electrical activity of the transplanted heart during the 7 days after transplantation is shown.

effects of the trauma of venepuncture.

6. Cyclosporin A

Cyclosporin A, supplied by Sandoz Limited, Basle, Switzerland, was dissolved in olive oil at 70°C by constant stirring for 30 minutes and administered for 7 days (15 mg/kg/day) by intramuscular injection beginning immediately after transplantation. Earlier studies in rats had shown that cardiac allografts could be maintained by an intramuscular dose of 15 mg/kg/day without toxic effects (White et al, 1980). Ryffel and colleagues described both hepatotoxicity and nephrotoxicity in rats receiving 45 mg/kg/day for 13 weeks and indeed a mortality rate of 30% was recorded in their study (1983).

Measurement of the Immune Response

In the past monitoring of B cell activity has been much more thoroughly studied than T cell activity, mainly because the product, antibody, could be so easily collected and characterised. However, the other arm of the immune response, T cell function, is equally important, although several features make it more difficult to study. In particular, T cells have diverse functions (e.g. cytotoxic cells, suppressor cells, helper cells) and the nature of their antigen-specific receptors has proved difficult to demonstrate.

Until the 1960s the most commonly used methods of measuring the immune response of a host involved the staining of antigen-bearing cells with dye. These antigen cells were incubated with serum or cells of the host. A strong host immune response would result in lysis of a large number of these cells. The number of intact cells following incubation was visually counted, thus measuring the humoral or cellular activity of the host. However, these techniques have associated pitfalls. Engelfriet and Britten (1966) have pointed out the problem of variable staining depending on dye concentration and the time of incubation with the dye. Some cells are only faintly stained and may not be counted. Furthermore, Walford et al (1965) have observed that stained cells enlarge and fragment with time, resulting in an underestimation of the percentage of cell damage.

These difficulties can be obviated by using radio-isotope techniques and studying not the cell but the supernatant radio-activity. The technique of labelling antigen cells with a radio-isotope was originally described by Goodman in 1961 studying mouse tumour cells. Radio-isotope release studies eliminate some sources of variability inherent in any technique which involves visually counting cells. The following tests of immune responsiveness were applied in our studies.

Measurement of Humoral Activity

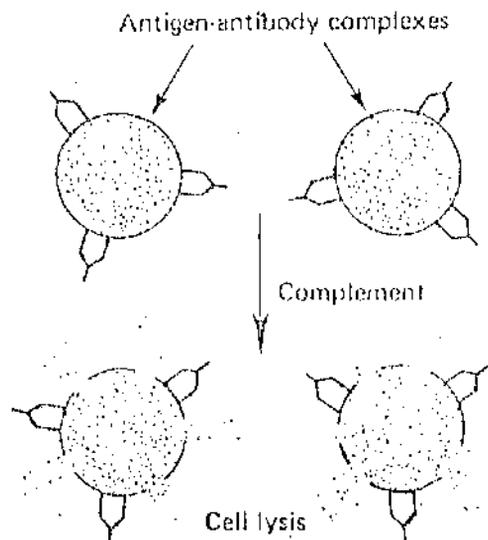
a) Complement-dependent cytotoxicity

Historically, complement activity was recognised by Bordet (1895), who showed that the lytic activity against red cells of freshly obtained rabbit anti-sheep erythrocyte serum was lost on ageing or heating to 56°C for half an hour but could be restored by the addition of fresh serum from an unimmunised rabbit. It is now known that heating serum to 56°C for half an hour inactivates complement.

The serum of normal animals of many species contains a group of at least eleven protein factors making up what is called the "complement system". This series of substances is activated by complexes of antigen with certain kinds of antibody. Combination of antigen and antibody leads to a structural change in the antibody molecule, a change which is recognised by one of the components of complement. A cascade reactions begins, activation of one member of the chain leading to the activation of many more of the next line. The end result is that the activity of one of the final activated components of complement "punches" a microscopic hole in the plasma membrane of the antigen cell (Fig. 2.4).

By labelling the antigen cell with radio-isotope the

FIGURE 2.4

COMPLEMENT DEPENDENT CYTOTOXICITY

Antigen-Antibody complexes in the presence of complement results in lysis of the antigen cell. By labelling the antigen cell with radio-isotope the amount of antibody present can be measured.

amount of antibody can be assessed by measuring the quantity of radio-isotope released.

Labelling of Cells

Antigen cells were Brown Norway thymocytes labelled with ^{51}Cr as described by Sanderson (1964). The thymus was disrupted with fine forceps in Roswell Park Memorial Institute (RPMI) 1640 and then filtered through cottonwool to remove debris. Cells were prepared by

washing twice in RPMI 1640, staining with trypan blue and counting using a light microscope. 1×10^7 cells in 0.2 ml of RPMI 1640 were incubated with 400 μ C of sodium chromate ($\text{Na}_2^{51}\text{CrO}$) for 90 minutes at 37°C . Following incubation the cells were washed twice with RPMI 1640 to reduce excess isotope and diluted to the desired concentrations.

Serum

Sera from grafted and untreated animals and animals grafted and CyA-treated were collected at varying intervals (5, 7, 14, 21, 50 and 100 days) after transplantation. Dilutions of 1:10 to 1:10,000 were tested in volumes of 25 μ L for complement-dependent cytotoxicity.

Assay

Serial dilutions of test sera (25 μ L) were incubated with ^{51}Cr -labelled Brown Norway thymocytes (1×10^4 in 25 μ L) for 60 minutes. 50 μ L of rabbit complement (1:10 dilution) were added to the target cells and serum in microtitre plates and incubated at 22°C in 5%

carbon dioxide* for 90 minutes. After incubation 150 μ L of RPMI 1640 was added to the plates, which were then centrifuged at 2,000 revolutions per minute (rpm) for 10 minutes.

Using an automatic pipette 100 μ L of the supernatant was removed and by counting in a γ counter for 1 minute the specific release of ^{51}Cr was measured.

Spontaneous Lysis and "Freeze Thaw"

Spontaneous lysis (i.e. cell lysis which would occur in the presence of cells, medium and complement without test sera) was determined by substituting test sera with tissue culture medium. The assay was carried out as above.

In order to determine the possible total release of ^{51}Cr , medium was substituted for serum in three wells of the microtitre plates. Prior to centrifugation the

* Carbon Dioxide: Both oxygen and carbon dioxide are probably essential for cell survival. Apart from its requirements in tissue metabolism, the bicarbonate ion is the most important buffering ion in most culture media and most systems require control of the carbon dioxide tension in the gas phase and in the medium.

mixture was alternately frozen and thawed using dry ice and acetone and water at 37°C. This procedure causes lysis of all cells and release of all the incorporated ^{51}Cr .

Percent Lysis

$$\% \text{ Lysis} = \frac{\text{Experimental } ^{51}\text{Cr Release (using test sera)} - \text{Spontaneous Lysis}}{\text{Freeze thaw lysis} - \text{Spontaneous Lysis}} \times 100$$

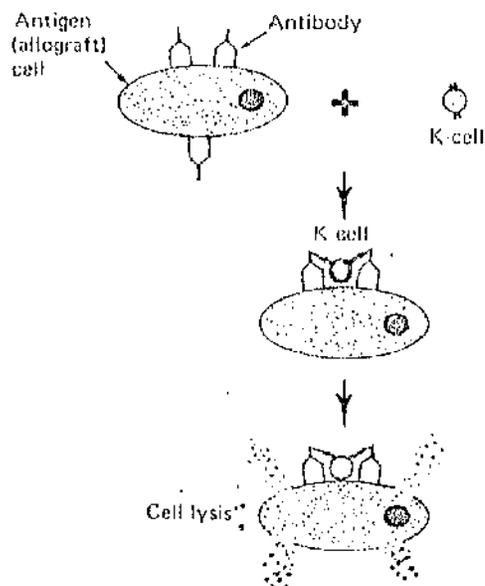
b) Antibody-dependent cell-mediated cytotoxicity

When a host is exposed to an antigen e.g. allograft cell, antibodies will be produced and antigen-antibody complexes formed. Combination with antibody will encourage the death of the allograft cell by promoting contact with phagocytes or by activation of the complement system producing direct membrane damage. A quite distinct cytotoxic mechanism was suggested by Perlmann and Perlmann in 1970. These workers described a class of host cells, known as K cells ("killer cells"), which could also attach to the antibody. These K cells are morphologically similar to small or medium size lymphocytes although without easily demonstrable immunoglobulin on their surface and are not phagocytic. After attachment, this K cell-antibody-complex can directly destroy the antigen cells i.e. the cells of the allograft. In other systems K cell activity

can be demonstrated against tumour cells or cells infected with micro-organisms. By labelling the antigen cell with radio-isotope, the amount of antibody can be assessed by measuring the liberated radio-activity (Fig. 2.5).

FIGURE 2.5

ANTIBODY DEPENDENT CELL-MEDIATED CYTOTOXICITY



Antigen-Antibody complexes in the presence of K cells results in lysis of the antigen cells. By labelling the antigen cell with radio-isotope, the amount of antibody can be readily assessed.

Target (Antigen) Cells

Brown Norway thymocytes were prepared by washing in RPMI 1640, staining with trypan blue and counting with a light microscope. 1×10^7 cells in 0.2 ml RPMI 1640 were incubated with 400 μ C of $\text{Na}_2^{51}\text{CrO}$ for 90 minutes at 37°C. Following incubation the cells were washed twice with RPMI 1640 to remove excess isotope and diluted to the desired concentration.

Serum

Sera from grafted untreated animals, and animals grafted and CyA-treated were collected at varying intervals (5, 7, 14, 21, 50 and 100 days) after transplantation. Dilutions of 1:10 to 1:10,000 were tested in volumes of 0.01 ml.

Effector Cells (containing K cells)

Splenocytes from unmodified LEW rats were used. The spleen was disrupted by gentle blunt dissection and the cells washed with RPMI 1640, stained with trypan blue and counted with a light microscope.

Assay

0.01 ml of serial dilutions of LEW anti-LBN sera was incubated with 0.5 ml of 1×10^4 ^{51}Cr -labelled Brown

Norway thymocytes for 30 minutes at 37°C in 5% CO₂. 0.1 ml effector cells in a ratio of 100:1 were added and the mixture was further incubated for another 3½ hours at 37°C in 5% CO₂. Following the incubation the microtitre plates were spun at 2,000 rpm for 10 minutes at 4°C. Using an automatic pipette 100 µL of the supernatant was removed and by counting in a γ counter for 1 minute the specific release of ⁵¹Cr was measured.

Spontaneous Lysis and "Freeze Thaw"

This technique was similar to that used in the complement-dependent cytotoxicity assay (Page 70) substituting medium for test sera in the above assay.

Percent Lysis

$$\% \text{ Lysis} = \frac{\text{Experimental } ^{51}\text{Cr Release (test sera)} - \text{Spontaneous Lysis}}{\text{Freeze thaw lysis} - \text{Spontaneous Lysis}} \times 100$$

c) Reverse Haemolytic Plaque Assay

The haemolytic plaque assay, first described by Jerne and Nordin in 1963, detected single cells which secreted immunoglobulin M (IgM) binding to surface determinants on red cells. Since then modifications have been made and the assay described below is based on that of Gronowicz and colleagues described in 1976.

This assay makes use of the fact that protein A produced by *Staphylococcus Aureus* binds to the F_c portion of the antibody IgG molecule (Gustafson et al, 1967). Sheep red blood cells (SRBC) are coated with Staphylococcal Protein A and, in the presence of complement and IgG, lysis of the SRBC's will occur.

By assessing the amount of red cell lysis which occurs then it is possible to measure the number of antibody-producing cells (in this study; splenocytes). (Fig. 2.6)

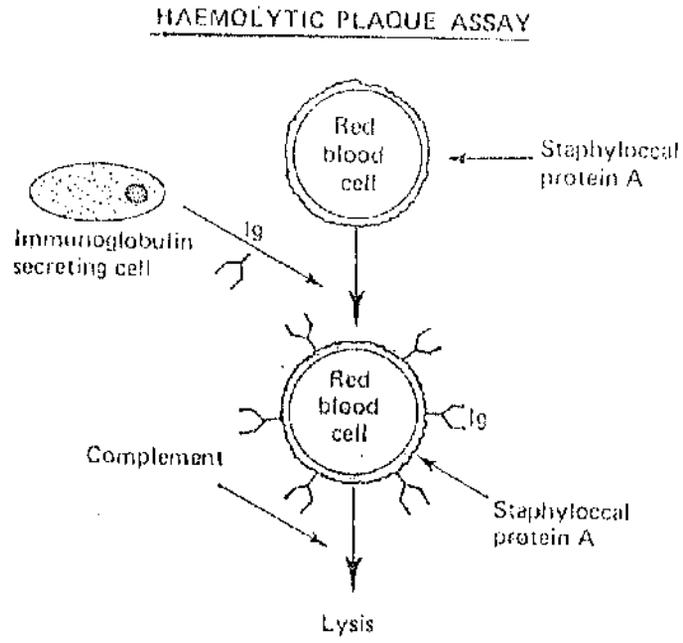
Coupling of Protein A to Sheep Red Cells

Protein A was coupled to sheep red cells with chromium chloride ($CrCl_3$) after the technique of Vyas and colleagues (1968). 0.1 ml of protein A (0.5 mg/ml) was mixed with 1 ml of $CrCl_3$ ($2.5 \times 10^{-4}M$) and 0.1 ml of packed, washed sheep red cells. All reagents were in 0.9% sodium chloride (NaCl). The mixture was incubated for 1 hour at $30^{\circ}C$. The red cells thereafter were washed once in 0.9% NaCl and twice in balanced salt solution.

Assay

The Staphylococcal Protein A red blood cells (20 μL SRBC) were mixed with 2×10^4 splenocytes, from LEW recipients of (LEW x BN) F_1 cardiac grafts, and guinea

FIGURE 2.6



Protein A produced by *Staphylococcus Aureus* binds to IgG. Sheep red blood cells are coated with Staphylococcal Protein A and, in the presence of complement and IgG, lysis will occur.

pig complement at 1:20 dilution in balanced salt solution. This mixture was warmed to 44°C prior to the addition of 150 µL of 50% Agarose and spread onto 5 cm Petri dishes. Following a 15 hour incubation at 37°C in 5% CO₂, complement-mediated lysis of SRBC produced clear zones of plaques around each individual antibody-secreting cell. These plaque-forming cells were visualised by direct light and their number expressed per 10⁶ splenocytes.

Measurement of Cell-mediated Activity

a) Lymphocyte-mediated Cytotoxicity

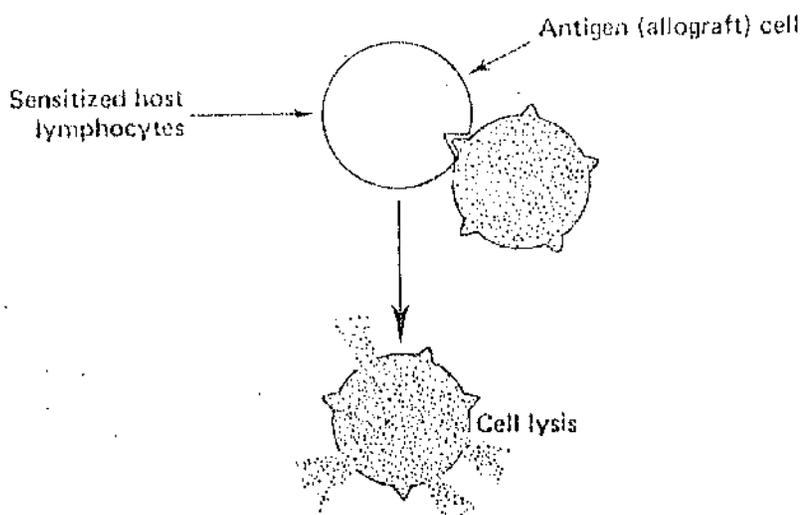
Govaerts, in 1960, first showed in vitro that living lymphocytes from renal grafted animals produced specific cytotoxic lesions in renal cultures from the corresponding donors. Since then numerous assays measuring the amount of lysis caused by direct contact of host lymphocytes ('effector cells') and antigen ('target cells') have been described. Cells acting as target cells must be easily recognisable as foreign by the host lymphocytes i.e. they must have numerous 'antigenic sites' on their surface. Those cells commonly used in lymphocyte-mediated cytotoxicity assays are peripheral blood cells and splenocytes. In the rodent peripheral blood lymphocytes have as many surface antigenic sites as thymocytes (Terasaki and McClelland, 1963) and hence both are effective. The

preparation of spleen cells is not easy since it is difficult to separate splenocytes from erythrocytes. Thymocytes were chosen in those studies because of their ease of preparation, their known high density of histocompatibility antigenic sites and their susceptibility to lysis under the conditions used (Hendry et al, 1979). The original assay was described by Vainio and colleagues in 1964. By labelling the donor strain cells with radio-isotope and measuring its release the level of cytotoxicity or "cellular immunity" in the host can be measured (Fig. 2.7). The following assay is based on that of Brunner and colleagues described in 1968.

Effector Cells

The attacking cell or effector cell population, from CyA-treated and untreated animals, were harvested at intervals (3, 7, 14, 21 and 100 days) after transplantation. These cells were recipient splenocytes or cells infiltrating the cardiac allografts. The cells infiltrating the cardiac allografts were, in the main, an heterogeneous population of mononuclear cells, including macrophages and T and B lymphocytes (Tilney et al, 1975).

FIGURE 2.7

LYMPHOCYTE MEDIATED CYTOTOXICITY

Antigen cells are lysed by direct contact with host lymphocytes. Radio-isotope release from antigen cells is a measure of the "cellular immunity".

Target Cells

Donor strain Brown Norway thymocytes labelled with ^{51}Cr (see page 68) acted as target cells.

Assay

Attacker to target cells were assayed in a ratio of

100:1 (2×10^7 /ml: 2×10^5 /ml). 100 μ L target cells and 100 μ L effector cells were incubated in microtitre plates for 3½ hours at 37°C in 5% CO₂. After the incubation 150 μ L of RPMI 1640 was added to the plates, which were then spun at 2,000 rpm for 10 minutes at 4°C. Using an automatic pipette 100 μ L of the supernatant was removed and by counting in a γ counter for 1 minute the specific release of ⁵¹Cr was measured.

Spontaneous Lysis and "Freeze Thaw"

In order to determine the spontaneous lysis of target cells which would occur thymocytes from unmodified Lewis animals were substituted for cells from grafted and treated animals.

The total ⁵¹Cr release possible was determined by adding 100 μ L ⁵¹Cr-labelled target cells and 100 μ L medium and alternately freezing and thawing this mixture, a technique which lyses all the cells causing release of all incorporated radio-isotope.

Percent Lysis

$$\% \text{ Lysis} = \frac{\% \text{ } ^{51}\text{Cr Release in Presence of Immune Lymphoid Cells} - \text{Total } ^{51}\text{Cr Incorporated (Freeze Thaw)}}{\% \text{ } ^{51}\text{Cr Release in Presence of Normal Lymphoid Cells} - \text{Total } ^{51}\text{Cr Incorporated (Freeze Thaw)}} \times 100$$

Harvesting of Cellular Infiltrates

The procedures used in isolating infiltrating cells from cardiac allografts were modifications of techniques previously described by Tilney et al (1975). Cardiac allografts were transplanted in groups of 4 animals and at serial intervals after grafting the donor hearts from each group were removed and cleaned of surrounding tissue and clot. Each myocardium was then diced in 5 ml of RPMI 1640 containing 0.005 M Hepes (hydroxyethylpiperazine ethane sulphonic) acid buffer and 4% (v/v) heat-inactivated foetal calf serum (inactivated by heating at 56°C for 30 minutes) with 0.1 ml of 0.2% EDTA (sodium ethylene diaminetetracetic acid). The tissue was then expressed through 60 gauge stainless steel mesh and filtered through cottonwool to remove remaining debris. The suspension of infiltrating cells was centrifuged twice at 200 g for 15 minutes, washed in RPMI 1640 and incubated for 20 minutes in 5% CO₂ at 25°C, then 20 minutes at 37°C in 10 ml of buffered RPMI 1640 containing 0.4 mg of deoxyribonuclease per ml. The cell suspension was then washed with culture medium, resuspended and layered over Ficoll-Hypaque. After centrifugation at 750 g for 15 minutes, the cells were collected from the interface layer, washed in RPMI 1640 and counted.

Thymectomy and Isolation of Thymocytes

In rats under ether anaesthesia, a midline cervico-thoracic incision was made and the sternum split in its upper third. The thymus was exposed, gently teased away from surrounding tissues and removed intact. Parathymic lymphnodes were always excluded from thymic tissue. The thymus was mechanically disrupted in RPMI 1640 with fine forceps and filtered through cottonwool to remove debris. The resultant suspension of thymocytes could then be stained with trypan blue and counted using a light microscope.

Statistics

Statistical significance was determined by the use of Student's two-tailed t-test. All graphs of results indicate the mean result and the standard error of the mean result.

CHAPTER 3

RESULTS OF GRAFT SURVIVAL AND OF THE
MEASUREMENT OF THE IMMUNE RESPONSE

RESULTS OF GRAFT SURVIVAL AND OF THE
MEASUREMENT OF THE IMMUNE RESPONSE

1. Graft Survival in Untreated Recipients

Cardiac allografts were performed in two groups of rats, seven animals in each group. (LEW x BN) F_1 and (LEW x WF) F_1 were used as donors, LEW animals being the recipients. No immunosuppressive agent was given.

Results

Cardiac Allograft Survival in Unmodified Recipients

When (LEW x BN) F_1 animals were used as donors the mean survival time \pm standard deviation (MST \pm SD) was 7 ± 2 days and using (LEW x WF) F_1 , 6 ± 1 day. These results are shown in Table 3.1.

2. Graft Survival in Recipients Treated with
Cyclosporin A

The same combination of donor-recipient pairs was used. In all experiments LEW rats were the recipients. In 14 allografts (LEW x WF) F_1 rats were the heart donors and in a second group of 14 allografts (LEW x BN) F_1 were used as donors (Fig. 3.1).

Table 3.1

Survival of Cardiac Allografts in Unmodified Recipients

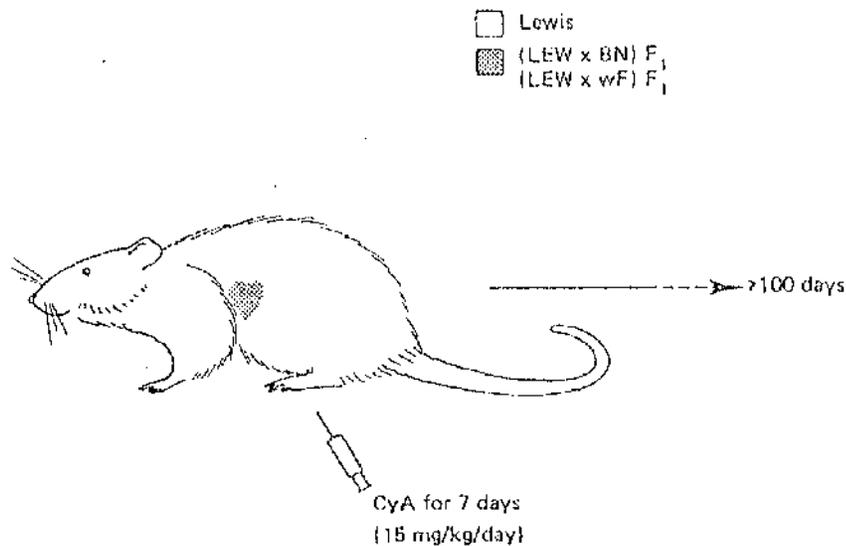
Donor	Recipient	No. in Group	Survival Times (days)	MST \pm SD (days)
(LEW x BN) F_1	LEW	7	5,6,6,6, 7,8,11	7 \pm 2
(LEW x WF) F_1	LEW	7	5,5,5,6, 6,7,9	6 \pm 1

The mean survival time \pm standard deviation using (LEW x BN) F_1 animals as donors was 7 \pm 2 days and using (LEW x WF) F_1 animals, 6 \pm 1 day.

Recipient rats received intramuscular CyA (15 mg/kg/day) for 7 days only commencing immediately after transplantation. During this time the animals were not used for any other experimental procedures which could alter the allograft survival time e.g. repeated anaesthesia for blood sampling may in itself be immunosuppressive (Humphrey et al, 1969).

FIGURE 3.1

Cardiac Allografts in CyA-treated Recipient Rats



The survival of all cardiac allografts was prolonged in recipients treated with CyA (15 mg/kg/day).

Results

Allograft Survival in Recipients Treated with Cyclosporin A

When recipient rats were treated with Cyclosporin A all grafts survived more than 100 days when both donor groups were used (Table 3.2).

It has therefore been clearly shown that in this donor-recipient combination Cyclosporin A significantly prolongs cardiac allograft survival.

Measurement of Humoral and Cell-mediated Activity in Untreated and Cyclosporin A-treated Grafted Animals

In all the following studies (LEW x BN)₁F₁ animals acted as organ donors and LEW animals recipients. The following studies were made:

Measurement of Humoral Activity

- a) Complement-dependent cytotoxicity
- b) Antibody-dependent cell-mediated cytotoxicity
- c) Reverse haemolytic plaque assay

Measurement of Cell-mediated Activity

- a) Lymphocyte-mediated cytotoxicity

Table 3.2

Survival of Cardiac Allografts
in Cyclosporin A Treated Recipients

Donor	Recipient	No. in Group	Survival Times
(LEW x BN) F_1	LEW	14	Each animal > 100 days
(LEW x WF) F_1	LEW	14	Each animal > 100 days

Using both (LEW x BN) F_1 and (LEW x WF) F_1 animals as donors, all cardiac allografts survived over 100 days.

3. Humoral Activity

a) Complement-dependent cytotoxicity

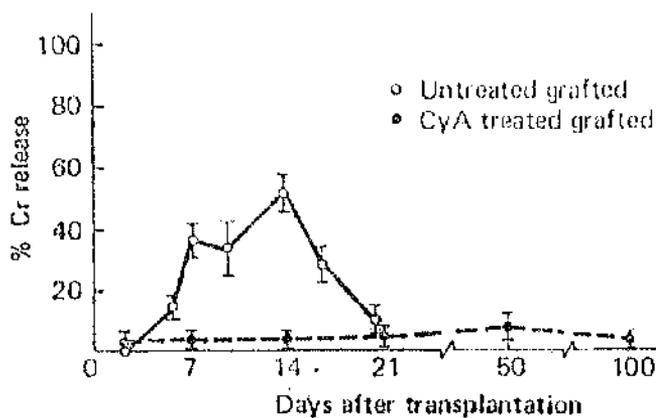
24 LEW rats received cardiac allografts from (LEW x BN) F_1 donors and were given no immune suppression. Blood was taken from four animals at each of 5, 7, 10, 14, 17 and 21 days after transplantation and tested for complement-dependent cytotoxicity.

24 LEW rats received cardiac allografts from (LEW x BN) F_1 donors and the recipient rats were treated with Cyclosporin A intramuscularly 15 mg/kg/day for 7 days. Blood samples were taken from recipient rats in groups of four at days 3, 7, 14, 21, 50 and 100 after transplantation and studied for complement-dependent cytotoxicity. No rat was used on more than one occasion for this study.

Results

Unmodified LEW recipients of (LEW x BN) F_1 cardiac allografts showed a rise in titre of antibody (35% - 42%) about the time of acute rejection at 5 to 7 days which persisted for one week thereafter. Negligible complement-dependent cytotoxicity (<5%) was demonstrated in CyA-treated recipients of well-functioning grafts as long as 100 days after transplantation ($P < 0.001$). In this sensitive assay less than 20% is a negative result (Fig. 3.2).

FIGURE 3.2

COMPLEMENT DEPENDENT CYTOTOXICITY

The lower line in the graph shows that there is negligible complement-dependent cytotoxicity at all points studied in the 24 rats treated with CyA. In untreated recipients complement-dependent cytotoxicity rises significantly at the time of acute rejection and persists for 7 to 10 days thereafter.

This experiment has produced evidence that the humoral arm of the immune response is significantly diminished in Cyclosporin A-treated recipients.

b) Antibody-dependent cell-mediated cytotoxicity

16 LEW rats received cardiac allografts from (LEW x BN) F_1 donors and were given no immune suppression. Blood was taken from four animals at each of 5, 7, 14 and 21 days after transplantation and tested for antibody-dependent cell-mediated cytotoxicity.

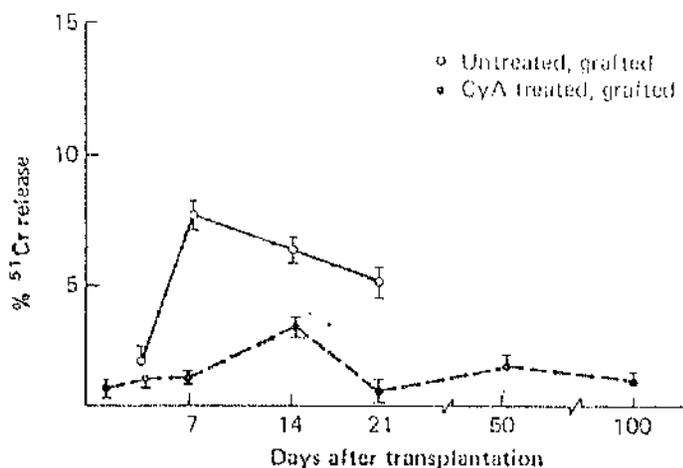
24 LEW rats received cardiac allografts from (LEW x BN) F_1 donors and the recipient rats were treated with intramuscular Cyclosporin A 15 mg/kg/day for 7 days. Blood samples were taken from these animals in groups of four at 5, 7, 14, 21, 50 and 100 days after transplantation and studied for antibody-dependent cell-mediated cytotoxicity. No rat was used on more than one occasion for this study.

Results

Antibody-dependent cell-mediated cytotoxicity peaked (7.5% - 8.5%) 7 days after transplantation in untreated allograft recipients, at the time of completed graft rejection. In CyA-treated graft recipients, antibody-dependent cell-mediated cytotoxicity did not rise above 3.5% during the 100 days period after transplantation ($P < 0.01$) (Fig. 3.3).

This experiment has produced further evidence that the humoral arm of the immune response is significantly diminished in CyA-treated recipients.

FIGURE 3.3

ANTIBODY DEPENDENT CELL MEDIATED CYTOTOXICITY

The lower line in the graph shows that there is negligible antibody-dependent cell-mediated cytotoxicity at all points studied in the 24 rats treated with CyA. In untreated recipients the antibody-dependent cell-mediated cytotoxicity rises significantly at the time of acute rejection and persists for 7 to 10 days thereafter.

c) Reverse haemolytic plaque assay

Protein A was coupled to sheep red blood cells as

described on page 75. Normal control values for this assay were determined by studying the spleen cells from 11 LEW rats which had received no graft or treatment. Splenectomy was performed and the spleens were mechanically disrupted with fine forceps. The suspension obtained was filtered through cottonwool to remove debris and the spleen cells appeared in the filtrate. Trypan blue was added to the filtrate to mark the spleen cells, which were then counted by light microscopy in a counting chamber. The assay was then performed as described on page 75.

In a similar way spleen cells were prepared from two test groups of rats. The first group comprised 19 LEW recipients grafted with (LEW x BN) F_1 hearts and given no immunosuppression. The spleens were removed 7 days after transplantation when rejection was established. The second group consisted of 21 LEW recipients grafted with (LEW x BN) F_1 hearts and treated for the first 7 days after transplantation with Cyclosporin A (15 mg/kg/day) intramuscularly. On day 7 the spleen cells from eleven recipients were studied, on day 14 four spleens, on day 21 four spleens and on day 100 two spleens.

Results

In the control group there were 1500 plaques per 10^6 spleen cells. In the group of rats grafted but

receiving no immune suppression the number of plaques was greatly increased to above 6000 per 10^6 spleen cells. No increase in plaque-forming cells was observed in any of the rats which were grafted and treated with Cyclosporin A (Fig. 3.4).

Thus each of the three tests of humoral activity in grafted recipients has shown that CyA produces a very significant decrease in the humoral component of the immune response when compared to animals grafted and not given CyA.

4. Cell-mediated Activity

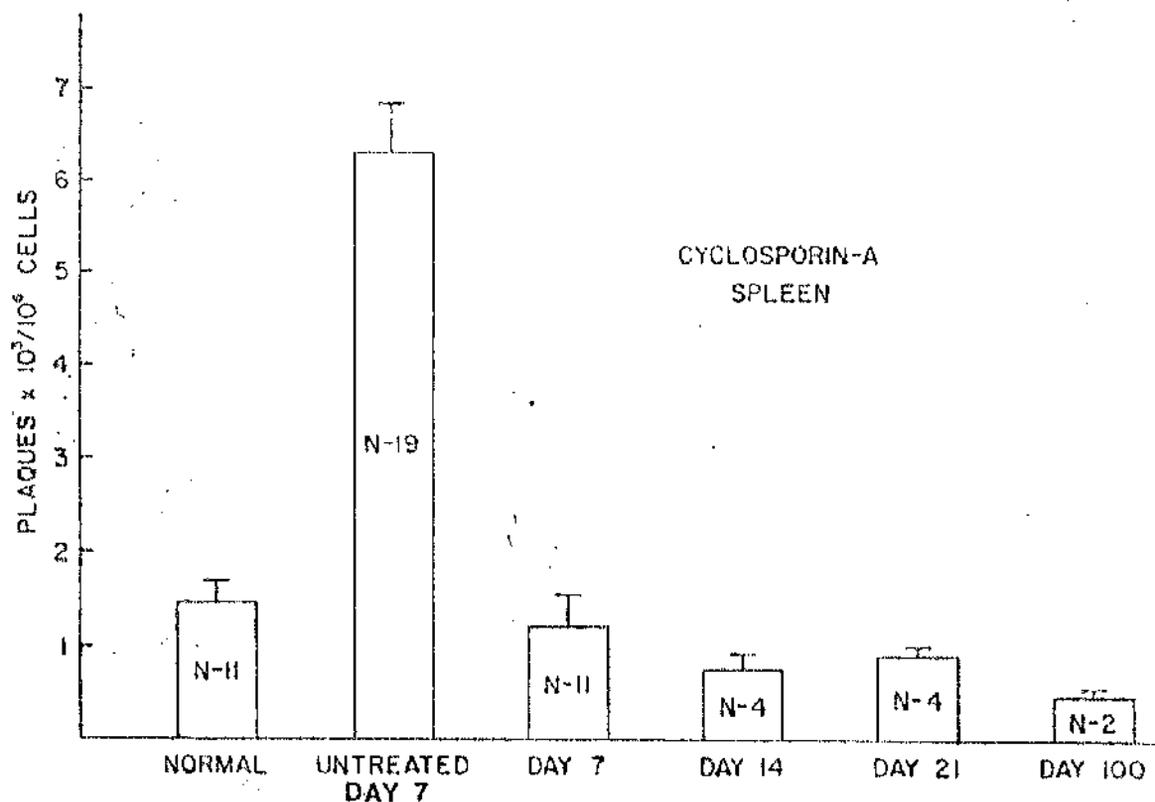
a) Lymphocyte-mediated cytotoxicity (spleen cells)

Target cells were prepared from the thymus of Brown Norway rats and labelled with ^{51}Cr as described on page 68.

Two groups each of 16 LEW recipients received cardiac allografts from $(\text{LEW} \times \text{BN})\text{F}_1$ donors. The first group of rats received no CyA and from this group the spleens of four animals were studied at each time interval of 3, 7, 14 and 21 days after transplantation. In the second group the recipient rats received CyA (15 mg/kg/day) intramuscularly for the first 7 days after transplantation and the spleens of four of these animals were studied at each time interval of 7, 14,

FIGURE 3.4

Plaque Formation in Control Rats and in
Grafted Rats with and without CyA



In the Reverse Haemolytic Plaque Assay, numbers of plaque-forming cells from spleens of CyA-treated, grafted animals were significantly ($P < 0.0001$) less than those from unmodified graft recipients throughout the entire follow-up period.

21 and 100 days. The lymphocyte-mediated cytotoxicity was assayed as described on page 77.

Results

A strongly positive lymphocyte-mediated cytotoxicity result was observed in the rats grafted and not given CyA. In the group given CyA a modest lymphocyte-mediated cytotoxicity result (15% - 18%) was seen at 7 days which was significantly less ($P < 0.0001$) than that noted in untreated animals (26% - 31%). From day 14 no significant lymphocyte-mediated cytotoxicity was shown in CyA-treated animals (Fig. 3.5).

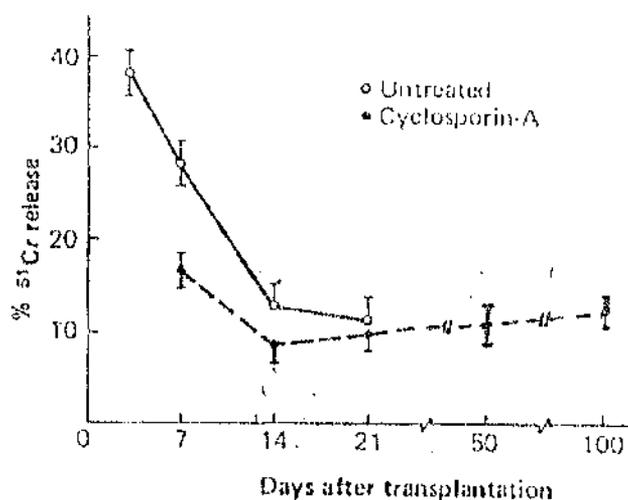
This experiment demonstrates that CyA significantly reduces the lymphocyte-mediated cytotoxicity when spleen cells from Cyclosporin A-treated animals are used as effector cells acting against target cells.

b) Lymphocyte-mediated cytotoxicity (graft infiltrating cells)

This experiment was performed in precisely the same manner as the previous one except that the effector cells were those lymphocytes invading the grafted hearts. The cells infiltrating the myocardium were extracted and prepared as described on page 81.

In a group of 12 LEW recipients grafted with (LEW x BN) F_1

FIGURE 3.5

LYMPHOCYTE MEDIATED CYTOTOXICITY
(SPLENOCYTES)

The upper line shows a strongly positive lymphocyte-mediated cytotoxicity in untreated recipients and the lower line shows a significantly reduced response in CyA-treated animals.

hearts and given no CyA, four animals were killed at day 7, four at day 14 and four at day 21 and the infiltrates of the transplanted hearts studied.

In a second group of 16 LEW recipients grafted with (LEW x BN) F_1 hearts and given CyA intramuscularly (15 mg/kg/day) for the first 7 days after transplantation, four of the heart transplants were examined at each of day 7, 14, 21 and 100 days after transplantation. The lymphocyte-mediated cytotoxicity between the target cells and the cells from the infiltrate was assayed.

Results

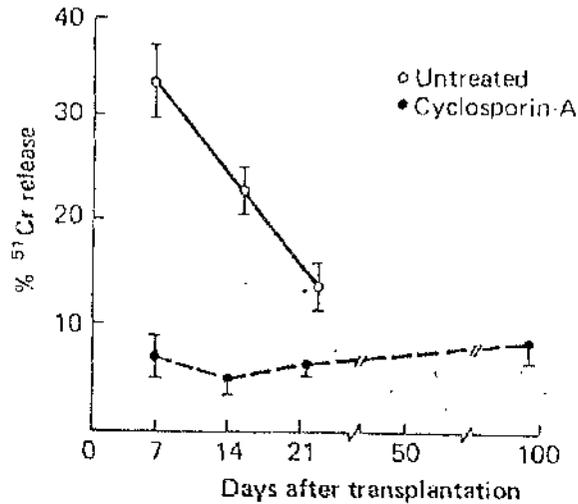
Fig. 3.6 shows a series of results for the infiltrating cells similar to that already shown for splenocytes.

Therefore CyA significantly reduces the lymphocyte-mediated cytotoxicity when both splenocytes and graft infiltrating cells are used as the effector cells against the same target cells.

The previous group of experiments has confirmed that Cyclosporin A is a potent immunosuppressant in the rat cardiac allograft model. (LEW x BN) F_1 and (LEW x WF) F_1 cardiac allografts functioned > 100 days without rejection episodes in recipients given intramuscular CyA (15 mg/kg/day) for 7 days only commencing immediately after transplantation. The drug causes a profound suppression of both humoral and cell-mediated immunity as measured by the significantly diminished complement-dependent

FIGURE 3.6

LYMPHOCYTE MEDIATED CYTOTOXICITY
(INFILTRATING CELLS)



The cytotoxicity mounted by lymphocytes infiltrating the heart grafts of CyA-treated recipients was abolished (3 to 8%) when compared with the lymphocyte-mediated cytotoxicity mounted by infiltrating cells from untreated controls which ranged from 30 to 39% ($P < 0.0001$) 7 days after transplantation.

cytotoxicity, antibody-dependent cell-mediated cytotoxicity, plaque-forming cells and lymphocyte-mediated cytotoxicity assays.

C H A P T E R 4

DOES CYCLOSPORIN A PRODUCE
DONOR-SPECIFIC IMMUNOSUPPRESSION?

DOES CYCLOSPORIN A PRODUCE
DONOR-SPECIFIC IMMUNOSUPPRESSION?

The experiments described in the previous chapter have confirmed CyA to be a potent immunosuppressant using the cardiac allograft model in the rat. Is this suppression antigen-specific or could antigens from a third party donor be "accepted" by CyA-treated recipients after the administration of CyA had been stopped? To answer this question an attempt was made to demonstrate in vivo the presence of cells with suppressor characteristics. If present, would these suppressor cells be antigen-specific? In addition, the mixed lymphocyte response (MLR) was studied to see whether any in vitro specificity was present.

1. Are Suppressor Cells Present in Cyclosporin A-treated Recipients?

As previously mentioned in Chapter 1, many immunosuppressive drugs act preferentially on dividing cells without any specificity and their immunosuppressive effects are a by-product of a general toxic effect on cells. The immune response of the host to the graft involves proliferation of lymphocytes against the graft and hence the highest degree of drug specificity possible would be for a drug to interact exclusively with lymphoid cells vital for graft rejection and to damage or destroy them.

The various lymphocyte subpopulations, their function and their intricate inter-relationships occurring throughout the immune system are gradually becoming better defined. For example, Gershon and colleagues demonstrated that lymphocytes can have a suppressive effect on the immune system. These suppressor cells can be defined as cells, usually subpopulations of lymphocytes, which suppress the reactivity of other lymphocytes involved in graft rejection.

The aim of the following study was to demonstrate whether these suppressor cells are present in CyA-treated cardiac allograft recipients. "Cell transfer" experiments were performed.

Cell Transfer Studies

'Test' heart grafts were performed between (LEW x BN) F_1 donors and LEW recipients. No pre- or post-operative immunosuppression was given. 24 hours before transplantation, these LEW recipients are injected intravenously with cells obtained from CyA-treated LEW recipients of (LEW x BN) F_1 cardiac allografts. Prolongation of survival of these 'test' heart grafts would identify the transferred cells as having suppressor characteristics.

Methods

Previous studies from the Surgical Research Laboratory, Harvard Medical School, had confirmed that both thymocytes and spleen cells transferred from enhanced recipients of cardiac allografts significantly increased the survival of test grafts in unmodified syngeneic animals (Tilney et al, 1978). Thymocytes produced optimal prolongation of test grafts and hence thymocytes were used in preference to spleen cells in the present cell transfer experiments. Additional experiments from the same laboratory, involving thymocyte transfer studies in rats, had identified the most effective "dose" of cells to be (1×10^8) (Hendry et al, 1979). No dose response experiments were undertaken in the present study.

(LEW x BN) F_1 cardiac allografts were transplanted into 20 LEW recipients and each rat treated with intramuscular CyA (15 mg/kg/day) for 7 days. Four animals were sacrificed at each of 7, 11, 21, 50 and 100 days. A further four (LEW x BN) F_1 cardiac allografts were transplanted into LEW recipients which received no CyA. In addition, four LEW animals were treated with CyA (15 mg/kg/day) for 7 days without any associated cardiac allograft. All these LEW animals acted as the sources of the cells to be transferred. Additionally, cells were transferred from unmodified LEW animals i.e. LEW animals without an allograft and

without CyA.

In each of the present series of experiments (1×10^8) thymocytes, prepared as described on page 82, were transferred. Prior to transfer these cells were "trypsinised" to remove any attached CyA. Following trypsinisation the cells were transferred by intravenous injection (under light ether anaesthesia) into groups of 4 unmodified LEW animals. 24 hours after being given the cells, each of these animals received a (LEW x BN) F_1 cardiac allograft.

Thus the sources of transferred cells were:

- Group 1) Untreated, ungrafted LEW animals
- Group 2) Untreated, grafted LEW animals
- Group 3) CyA-treated, ungrafted LEW animals
- Group 4) CyA-treated, grafted LEW animals 7 days after transplantation
- Group 5) CyA-treated, grafted LEW animals 11 days after transplantation
- Group 6) CyA-treated, grafted LEW animals 21 days after transplantation
- Group 7) CyA-treated, grafted LEW animals 50 days after transplantation
- Group 8) CyA-treated, grafted LEW animals 100 days after transplantation

Each group comprised four animals.

Trypsinisation of Transferred Thymocytes

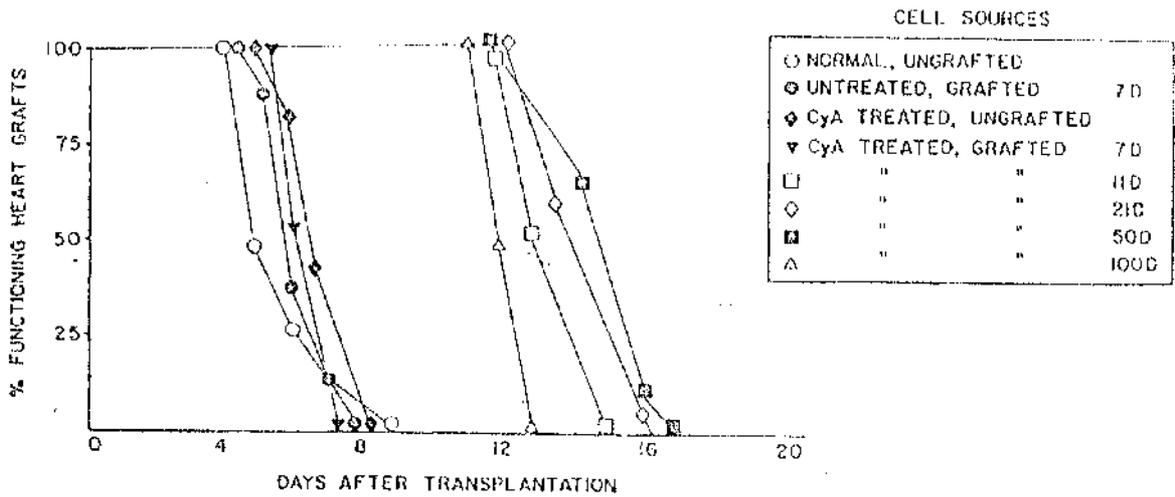
Single suspensions were prepared from the thymus by disrupting the organ with fine forceps in RPMI 1640 medium, filtering through cottonwool to remove debris and washing twice in RPMI 1640. The cells were then incubated in protein-free medium containing 10 μ L of trypsin per ml for 30 minutes at 37°C. These cells were then washed three times in RPMI 1640. Under these gentle conditions, the majority of surface proteins are removed from rat thymocytes and lymphocytes (Strom et al, 1977). The specific purpose was to ensure the removal of all surface CyA.

Results

The 'test' heart graft survival varied depending on the source of the transferred cells (Fig. 4.1). When cells had been taken from untreated and ungrafted animals, untreated and grafted, CyA-treated and ungrafted, CyA-treated and grafted and transferred 7 days after transplantation, all 16 associated test heart grafts rejected by 8 days. In contrast, cells transferred from LEW animals treated with CyA for 7 days and bearing well-functioning (LEW x BN) F_1 heart grafts for 11, 21, 50 and 100 days, prolonged the survival of the 16 associated test hearts significantly ($P < 0.001$).

These studies demonstrate that CyA-treated rats bearing

FIGURE 4.1

Cardiac Allograft Survival after Cell Transfer

Survival curves are noted for test (LEW x BN) F_1 cardiac allografts after thymocyte transfer. Four test heart grafts are in each group. Sources of thymocytes are shown.

well-functioning cardiac allografts develop a population of lymphoid cells with suppressor activity exerted in vivo against donor antigens. These cells have been shown to emerge after the cessation of CyA treatment. The inability of cells from CyA-treated but ungrafted and grafted and untreated animals to prolong test heart survival demonstrates that an antigenic stimulus as well as the effects of the drug is required for the development of these suppressor cells.

2. Specificity of Suppressor Effect on Test Allografts

The above experiment has demonstrated that suppressor cells do emerge in LEW recipients of (LEW x BN) F_1 hearts treated with CyA for the first 7 days after transplantation. Are these cells antigen-specific or could any donor antigen be "accepted" by these host animals? This question could be answered by implanting a third party heart graft alongside the (LEW x BN) F_1 heart after some time interval. This experimental technique has been carried out previously at the Surgical Research Laboratory (Tilney et al, 1978) but is both time-consuming and technically difficult. Alternatively, the (LEW x BN) F_1 heart could be removed from the LEW host and replaced by a third party heart. In both these experimental situations prolongation of third party heart graft survival would support the presence of antigen-specific suppressor cells.

In the present experiments to be described, specificity of these suppressor cells was examined by introducing a third party heart graft ((LEW x WF) F_1 hybrid) 24 hours after cell transfer and using this as the test heart.

Methods

16 LEW animals were grafted, 8 animals with (LEW x BN) F_1 cardiac allografts and 8 with (LEW x WF) F_1 cardiac allografts. Each LEW recipient was treated with CyA intramuscularly (15 mg/kg/day) for 7 days. 14 days following transplantation these animals were sacrificed and (1×10^8) thymocytes (obtained as described previously) were injected intravenously into 16 unmodified LEW rats. 24 hours following cell transfer these 16 LEW animals were each given a heart graft, 8 from (LEW x BN) F_1 animals and 8 from (LEW x WF) F_1 animals. Thus, of the 8 (LEW x BN) F_1 test heart grafts, 4 LEW hosts had received cells from LEW animals bearing well-functioning (LEW x BN) F_1 grafts and 4 from LEW animals bearing (LEW x WF) F_1 grafts. Similarly, of the 8 (LEW x WF) F_1 test heart grafts, 4 LEW hosts had received cells from LEW animals bearing well-functioning (LEW x WF) F_1 grafts and 4 LEW hosts had received cells from LEW animals bearing well-functioning (LEW x BN) F_1 grafts.

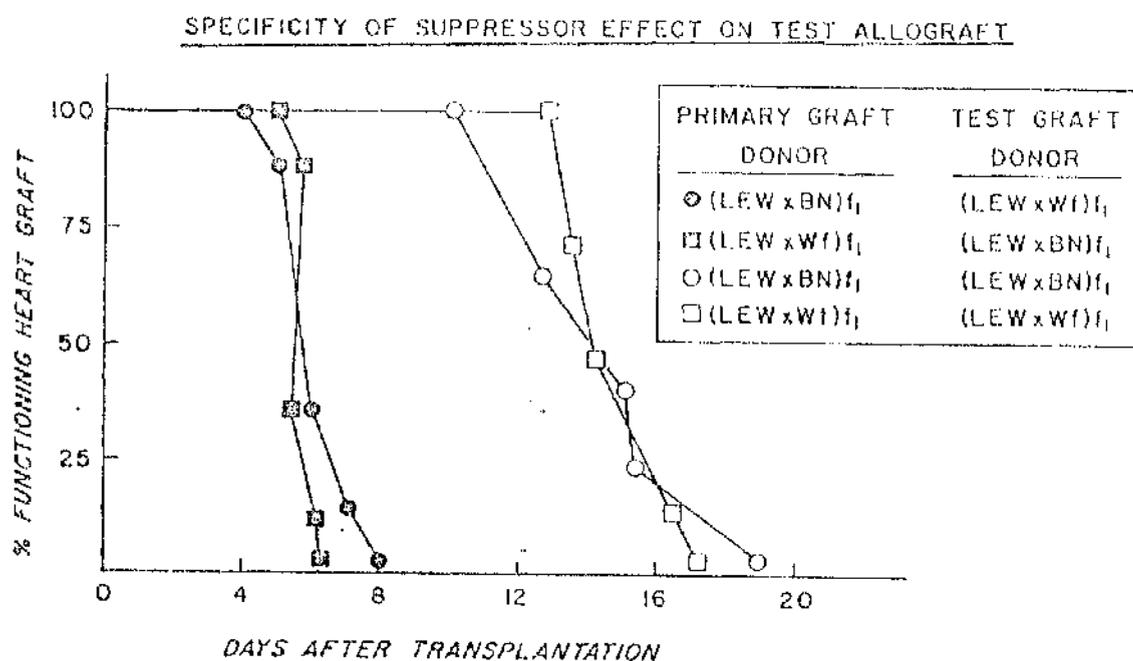
Results

The specificity of this suppressor effect is shown in Fig. 4.2. The survival of test (LEW x WF)₁ allografts was prolonged to 13 ± 2 days after transfer of (1×10^8) thymocytes from CyA-treated LEW recipients of (LEW x WF)₁ heart grafts 14 days after transplantation ($P = 0.002$). However, no prolongation was noted when thymocytes from CyA-treated LEW recipients of (LEW x BN)₁ heart grafts were transferred at 14 days into unmodified LEW recipients of (LEW x WF)₁ test grafts (MST \pm SD = 6 ± 1 day). Similarly, (LEW x BN)₁ test grafts in LEW recipients survived for only 6 ± 1 day after transfer of thymocytes from CyA-treated animals bearing (LEW x WF)₁ cardiac grafts. Hence specificity of unresponsiveness to the original donor strain, and not to a third party strain, was demonstrated.

These studies have demonstrated that CyA-treated rats bearing well-functioning cardiac allografts develop a population of lymphoid cells with specific suppressor activity exerted in vivo against donor antigens. These cells have been shown to emerge after the cessation of CyA treatment and do not prolong graft survival when the graft is taken from a third party donor.

Could the results of these in vivo experiments be

FIGURE 4.2



Prolongation of test cardiac allografts following thymocyte transfer from CyA-treated, grafted animals is a donor-specific phenomenon. Survival of appropriate test cardiac allografts is prolonged significantly ($P = 0.002$) in recipients bearing specific grafts but not with third party grafts.

supported by studies in vitro? Donor-specific suppression with CyA treatment was examined by studying the Mixed Lymphocyte Response (MLR).

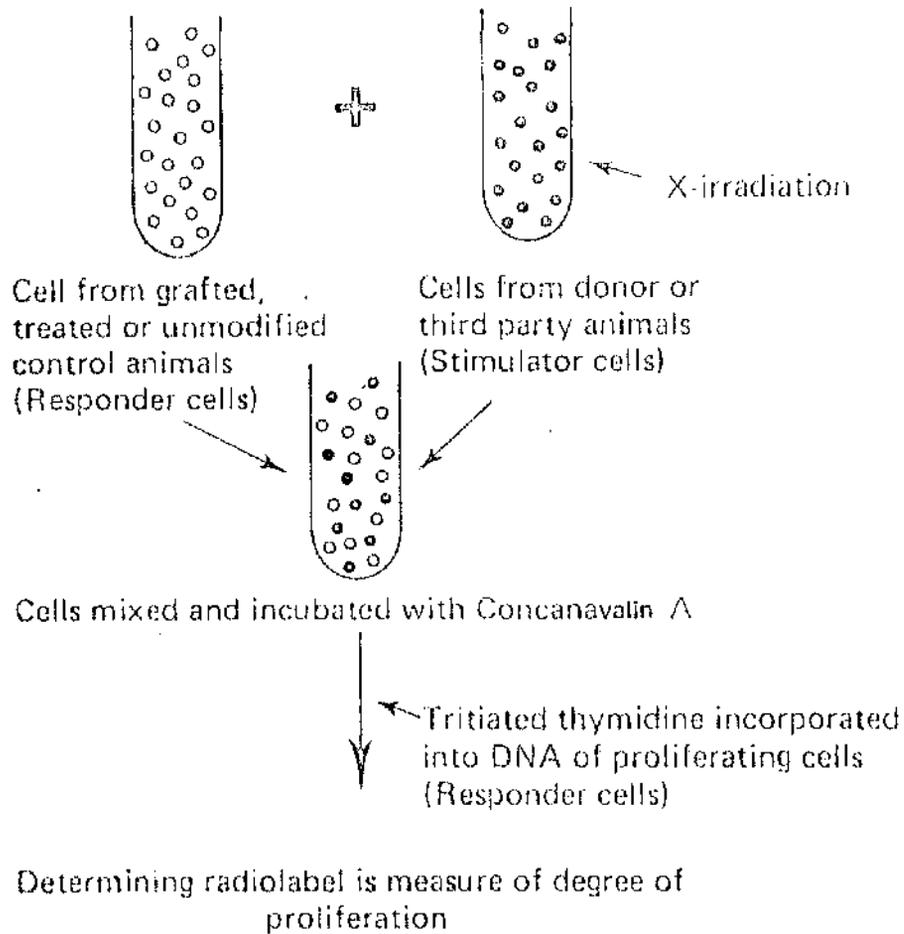
3. Mixed Lymphocyte Response

The initial description of this assay was provided by Bach and Hirschhorn in 1964. Cells from two individuals/strains which have different surface antigens are mixed together in vitro. If one cell population recognises the other as foreign it begins to proliferate. This proliferation can be measured by the incorporation of radio-labelled precursors into DNA (deoxyribonucleic acid) (Bain et al, 1964).

In practice the cells are allowed to react for 4 to 5 days and then tritiated thymidine (TdR) is added to the culture. If there has been any proliferation then TdR will be incorporated into DNA. By extracting the DNA and determining the amount of radio-label incorporated a measure of the degree of proliferation is obtained. Since both populations will recognise each other and respond by proliferating, one population can be treated with agents which prevent proliferation such as mitomycin C or X-ray so that it cannot proliferate but can only stimulate. This is called an one way MLR (Fig. 4.3).

In this study lymphocytes from donor animals or third

FIGURE 4.3

'ONE-WAY' MIXED LYMPHOCYTE RESPONSE

Lymphocytes from donor animals or third party animals (stimulator cells) are irradiated and mixed with lymphocytes from grafted, CyA-treated animals or control animals (responder cells) (see text).

party animals (stimulator cells) were irradiated and mixed with lymphocytes from grafted, CyA-treated animals or control animals (responder cells).

The result of this assay determines whether the lymphoid cells of grafted, CyA-treated animals react equally with donor cells and with third party cells.

Methods

Donor and recipient lymphocytes were obtained from the thymus and submandibular or cervical lymph nodes. They were removed under sterile conditions. These tissues were disrupted with fine forceps in RPMI 1640 medium and filtered through cottonwool to remove debris before washing twice in RPMI 1640. The cells were then purified by differential velocity sedimentation on a Ficoll-Hypaque (specific gravity = 1.080) gradient, based on the technique described by Harris and Ukaejiofo (1969). This technique involves layering the cell suspension onto 2 ml of a Ficoll-Hypaque mixture. A virtually pure suspension of lymphocytes appears as a thin white layer immediately below the Ficoll-Hypaque interface. These cells are collected using a fine pipette and are then washed twice and resuspended in RPMI 1640.

Cells were obtained from the submandibular and cervical lymph nodes of 4 unmodified (LEW x BN) F_1 rats and from

a group of 4 unmodified third party ((LEW x WF) F_1) animals. These lymph node cells were used as the stimulating populations in the one way MLR, and were irradiated with 3000 rads from a ^{137}C source. Responder lymph node or thymus cells (2×10^5) were collected from 4 control (unmodified) LEW animals and from groups of 4 LEW recipients of (LEW x BN) F_1 cardiac allografts treated with CyA (15 mg/kg/day) for 7 days and sacrificed at intervals of 7, 14, 50 and 100 days after transplantation. These responder cells were mixed with an equal number of stimulator irradiated lymph node lymphocytes from donor animals ((LEW x BN) F_1) or third party ((LEW x WF) F_1) animals in flat-bottomed microtitre plates. Three replicates were placed in wells of total volumes of 0.2 ml.

The cells were incubated at 37°C in 10% CO_2 for 96 hours with Concanavalin A in a concentration of 16 $\mu\text{g/ml}$. Concanavalin A is a glycoprotein prepared from Jack bean meal which can induce mitosis in T lymphocytes. 6 hours before harvesting 0.02 ml of normal saline containing 1.0 μc of $^3\text{HTdR}$ (specific gravity 6.7 c/mM) was added to each well. The cells were harvested onto fibreglass filters. The filters were then placed into vials and 4 ml of scintillation fluid was added for counting with a Beckmann beta counter.

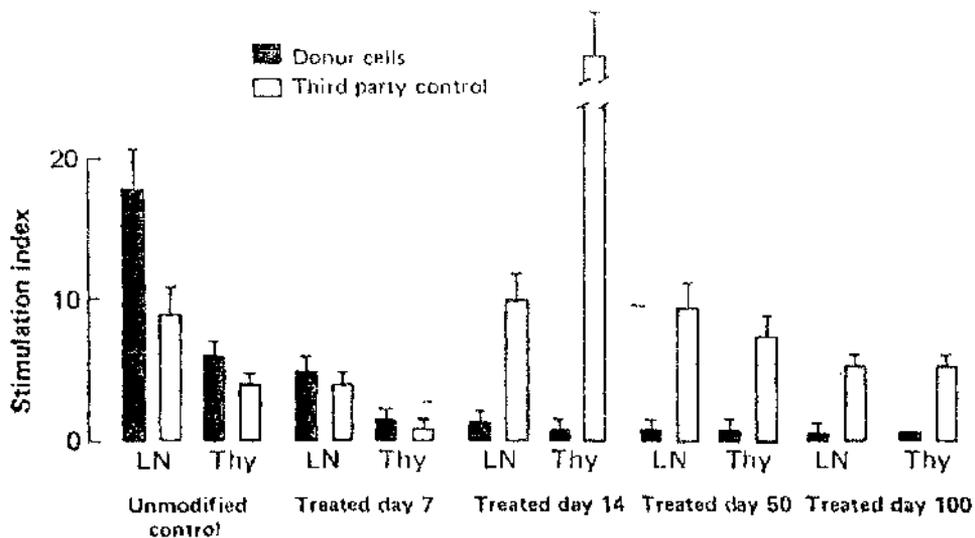
Results

The reactivity of lymph node lymphocytes and thymocytes from CyA-treated rats bearing well-functioning (LEW x BN) F_1 allografts, against X-irradiated donor lymphocytes was significantly less ($P < 0.0005$) than the reactivity when performed against X-irradiated third party lymphocytes, (LEW x WF) F_1 on days 14, 50 and 100 days following transplantation. On day 14 the reactivity of host thymocytes against the third party cells increased to an unexpectedly high level and no explanation can be given. However, at day 7 when CyA was still being administered to the recipient animals, the reactivity of host lymph node lymphocytes and thymocytes was generally suppressed against the cells from both the specific donor and the third party animals (Fig. 4.4).

Thus, in cardiac allografted and CyA-treated rats, the emergence of cells with antigen-specific suppressor characteristics has been demonstrated in vivo by thymocyte transfer experiments and these suppressor cells are most likely to be also responsible for the specificity detected in the one way MLR. It has also been demonstrated, both in vivo and in vitro, that these cells only appear when treatment with CyA was discontinued.

FIGURE 4.4

Reactivity of host lymph node lymphocytes and thymocytes
against donor and third party lymphocytes in the MLR



The activity of lymph node lymphocytes (LN) and thymocytes (Thy) from CyA-treated recipients was significantly ($P < 0.0005$) less in the MLR against donor lymphocytes (Stimulation index < 2) than against third party lymphocytes (Stimulation index 5-50) 14 to 100 days after grafting. However, when CyA was still being administered (day 7), MLR activity was suppressed against both specific and third party cells (Stimulation index < 5).

C H A P T E R 5

RESPONSE OF LYMPHOID TISSUE TO CARDIAC
ALLOGRAFTS IN RATS TREATED WITH CYCLOSPORIN A

RESPONSE OF LYMPHOID TISSUE TO CARDIAC
ALLOGRAFTS IN RATS TREATED WITH CYCLOSPORIN A

The experiments described previously have confirmed that CyA is a potent immunosuppressant in the rat cardiac allograft model and specific suppressor cells are produced in the host protecting the graft against rejection. The following study was designed to determine the dynamic responses of lymphoid tissues (spleen and thymus) to cardiac allografts, the hosts having been treated with CyA. These responses may give further information on the mechanism of action of the drug.

Organ allografts in rats elicit marked systemic responses, including temporary splenic enlargement and decrease in the size of the thymus (Baldwin et al, 1979a). In addition, it has been shown from the Surgical Research Laboratory, Harvard Medical School, that the lymphoid responses to allografts can be disrupted by various biological manipulations that increase graft survival. These include immunological enhancement, transfer of cells with suppressor properties or depletion of T lymphocytes (Baldwin et al, 1979b). Would the lymphoid tissues of recipient rats change when they were treated with CyA?

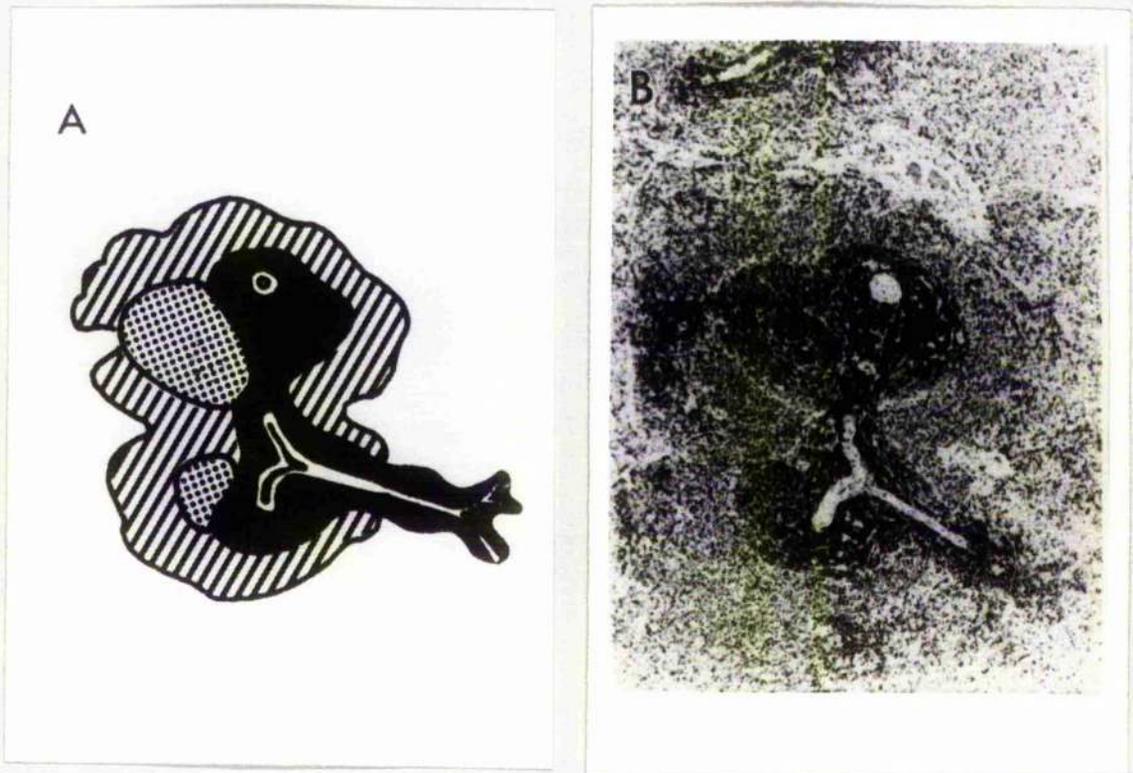
1. Anatomy of Rat Spleen

The parenchyma (splenic pulp) is of two distinct types. White pulp is typical lymphatic tissue which surrounds and follows the arteries. The red pulp (RP) is associated with numerous erythrocytes. On the cut surface the white pulp appears as scattered grey areas. It forms a peri-arterial sheath (PAS) of lymphocytes around the arteries. Cells present within this tissue are predominantly small lymphocytes but in addition there are medium size and large lymphocytes, monocytes and plasma cells. Between white and red pulp areas are poorly delineated marginal zones (MZ) of diffuse lymphatic tissue containing few lymphocytes and numerous macrophages. Areas of intense lymphocyte proliferation are known as germinal centres (GC). The positions of these compartments are shown in Fig. 5.1.

2. Anatomy of Rat Thymus

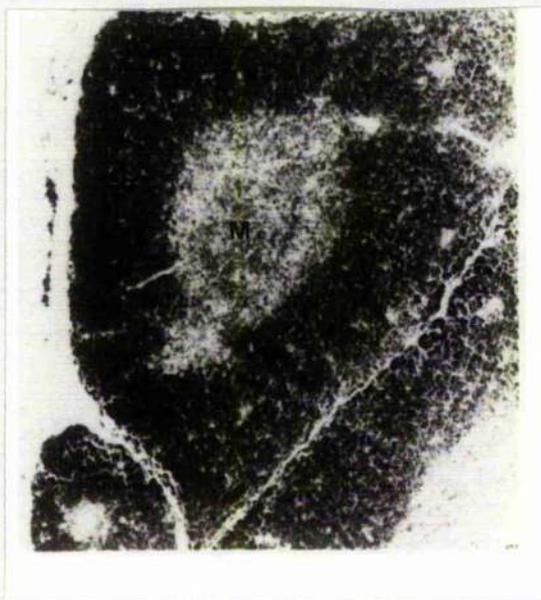
This organ consists of two lobes, each of which contains thousands of lobules, composed of cortical and medullary components. The cortex is densely populated with uniformly packed small lymphocytes whereas the medulla is more sparsely populated (Fig. 5.2).

FIGURE 5.1

White Pulp of Normal Rat Spleen(A) - Diagrammatically, (B) - Histologically

Arteries (shown in cross-section and partial longitudinal section) are surrounded by the compact small lymphoid cells of the PAS (black), and eccentrically located nodules of large lymphoid cells of the GC (stippled). Lymphoid cells within the macrophage network of the MZ (hatched) separate the PAS of larger arteries from the RP, but the PAS of terminal arterioles is in direct contact with the RP.

Figure 5.1A x50- B x40.

FIGURE 5.2Normal Rat Thymus

The normal thymus of a Lewis rat has a thick cortex (C) sharply demarcated from the medulla (M). x20

3. Materials and Methods

a) Animal Model

(LEW x BN) F_1 rats were the cardiac allograft donors and

LEW animals the recipients.

b) Animal Groups

- (1) 18 CyA-treated LEW rats bearing (LEW x BN) F_1 cardiac allografts were sacrificed in groups of 3 at 3, 7, 14, 21, 50 and 100 days.
- (2) 12 rats received a 7 day course of CyA but no graft. These animals were sacrificed in groups of 3 at days 3, 7, 14 and 21 after the commencement of treatment.
- (3) 12 rats received cardiac allografts but no CyA treatment. These were sacrificed in groups of 3 at days 3, 7, 14 and 21 after transplantation.

c) Cyclosporin A

CyA was administered intramuscularly to recipient rats in a dose of 15 mg/kg/day for 7 days commencing on the day of transplantation. No further immune suppression was given.

d) Histological Methods

The spleen, thymus and grafts of all rats were weighed and thin slices were fixed in cold (4°C) acidified formalin (5% glacial acetic acid and 10% formalin in water). Paraffin sections from each specimen were stained with Giemsa stain and Haematoxylin and

Eosin stains. The sizes of lymphoid compartments were qualitatively verified by planimetric measurements on projections of complete cross-sections through the thymus and spleen. These measurements were expressed as percentages of total cross-sectional area and multiplied by the weight of the individual organ in order to relate changes in compartment size to changes in organ size.

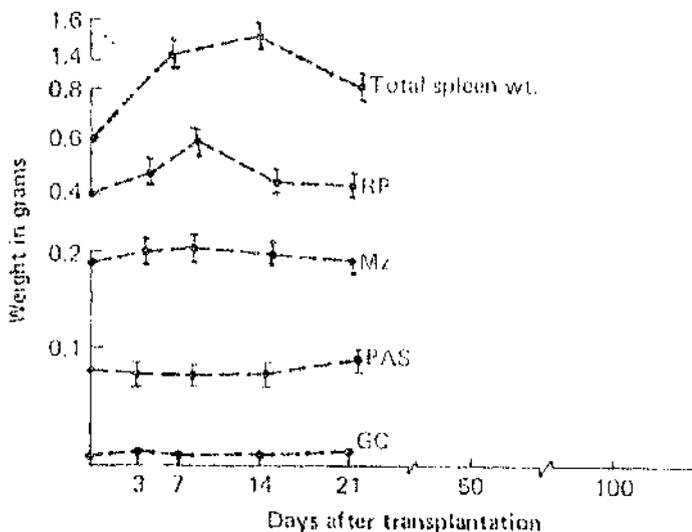
4. Results

a) Spleen

In untreated recipients marked splenomegaly occurred with transient increase from 0.63 g to 1.5 g in spleen weight. This was almost totally because of an expansion of the red pulp by immunoblasts and plasma cells (Fig. 5.3). In CyA-treated grafted animals not only did splenomegaly not occur during the entire 100 day period studied but splenic weight actually decreased slightly (from 0.63 g at day 0 to 0.48 g at 7 and 14 days). This was attributable to a 50% decrease in the size of the peri-arterial sheath area and a 67% decrease in the size of the marginal zone area (Fig. 5.4). The peri-arterial sheath and marginal zone areas are heavily populated by medullary thymus-derived lymphocytes (Goldschneider, 1976). The sizes of germinal centre and red pulp areas were less affected. These size changes were maximal from 7 days

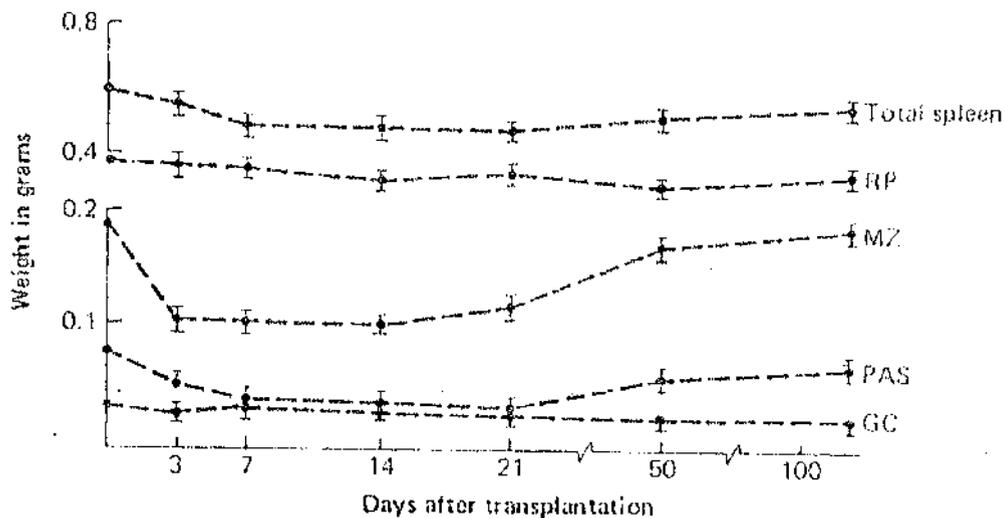
Spleen Weight- Untreated Recipients

FIGURE 5.3



Spleen Weight - Cyclosporin A-treated Recipients

FIGURE 5.4



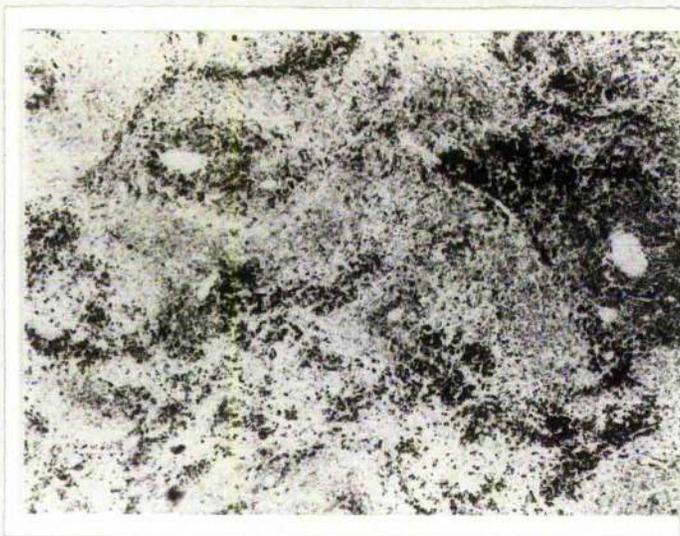
Weight changes occurring in the spleen of untreated (Fig. 5.3) and CyA-treated (Fig. 5.4) (LEW) recipients of (LEW x BN) F_1 cardiac allografts are shown. Relative weights of the splenic compartments are also shown (see text).

(when the daily CyA treatments were completed) to 21 days, whereas by 50 days all the splenic compartments had returned to their normal bulk.

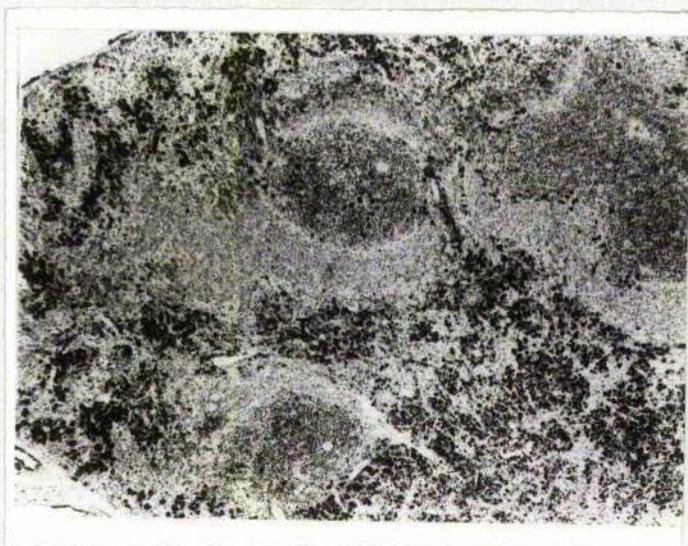
In the spleens of CyA-treated recipients, the PAS and MZ compartments populated by medullary thymus-derived lymphocytes decreased in size with progressive cell depletion. Some active germinal centres were formed and a moderate proliferation of large immunoblasts occurred in the peripheral PAS and RP at 3 days (Fig. 5.5). However, these immunoblasts did not expand the RP at 3 days and did not progress on to the considerable proliferation of immunoblasts and plasma cells in the RP observed at 7 days in untreated allograft recipients (Fig. 5.6). Instead, the RP and GC areas remained inactive 7, 14 and 21 days after grafting. However, in the CyA-treated animals, splenic morphology returned to normal by 50 and 100 days with well-demarcated cellular PAS and MZ areas and active germinal centres.

b) Thymus

Total thymic weight in untreated grafted recipients decreased by 40% during acute rejection (from 0.40 g to 0.24 g at 7 days), related primarily to a decrease in cortical thymocytes (Fig. 5.7). In CyA-treated rats thymic weight diminished further within the first few days after transplantation (0.12 g by 3

Spleen HistologyFIGURE 5.5

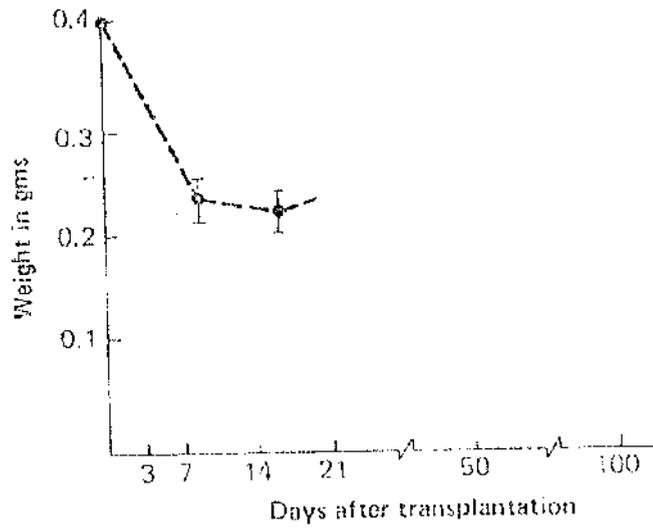
Spleen of CyA-treated (LEW) recipient of (LEW x BN) F_1 cardiac allograft at 3 days is shown. A moderate proliferation of large immunoblasts (I) is noted in the PAS and RP. x60

Spleen HistologyFIGURE 5.6

Spleen of untreated (LEW) recipient of (LEW x BN) F_1 cardiac allograft at 7 days is shown. Considerable proliferation of immunoblasts (I) in the RP is noted. x40

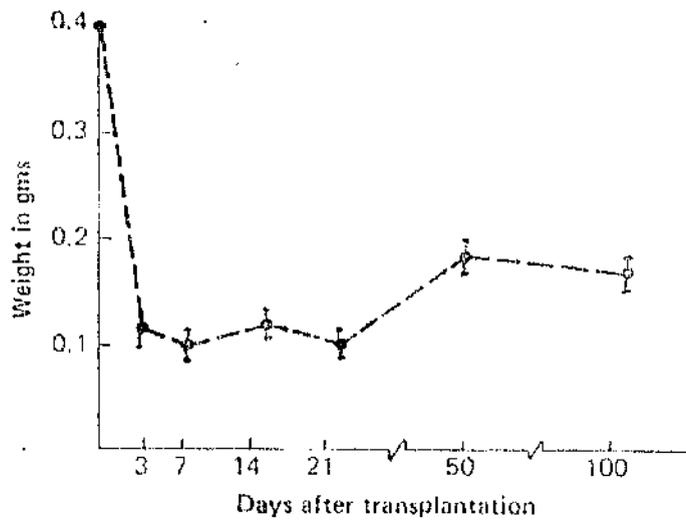
Weight Changes in Thymus of Untreated Recipients

FIGURE 5.7



Weight Changes in Thymus of Cyclosporin A-treated Recipients

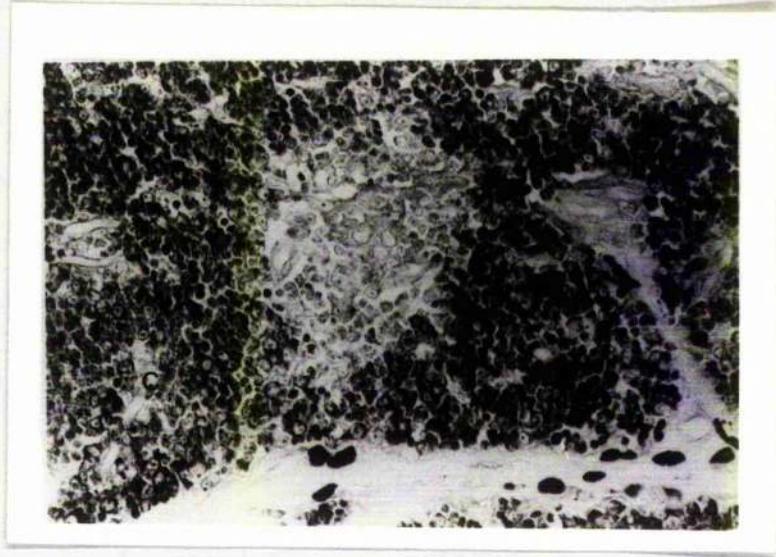
FIGURE 5.8



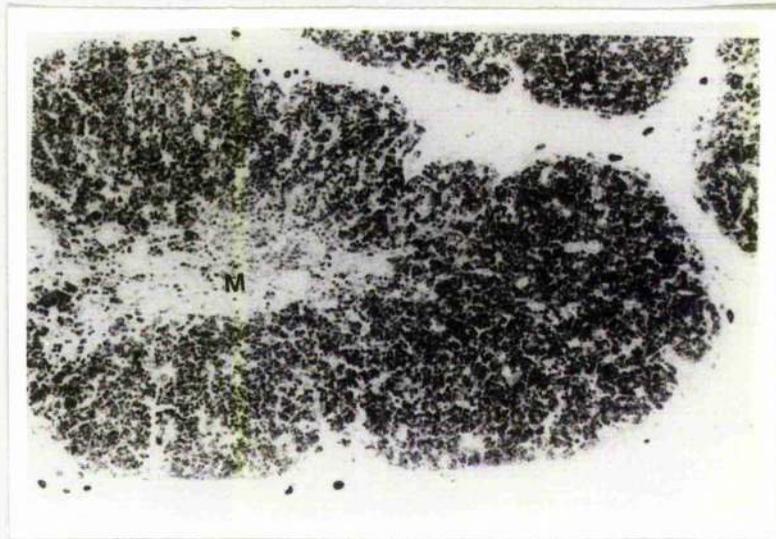
Weight changes occurring in the thymus of untreated and CyA-treated (LEW) recipients of (LEW x BN) F_1 cardiac allografts.

days), only partially recovering by 50 and 100 days (Fig. 5.8). Although both cortical and medullary compartments were decreased in size in the CyA-treated animals the medullary compartment was totally eliminated in all 6 rats with (LEW x BN) F_1 heart grafts killed at 14 and 21 days.

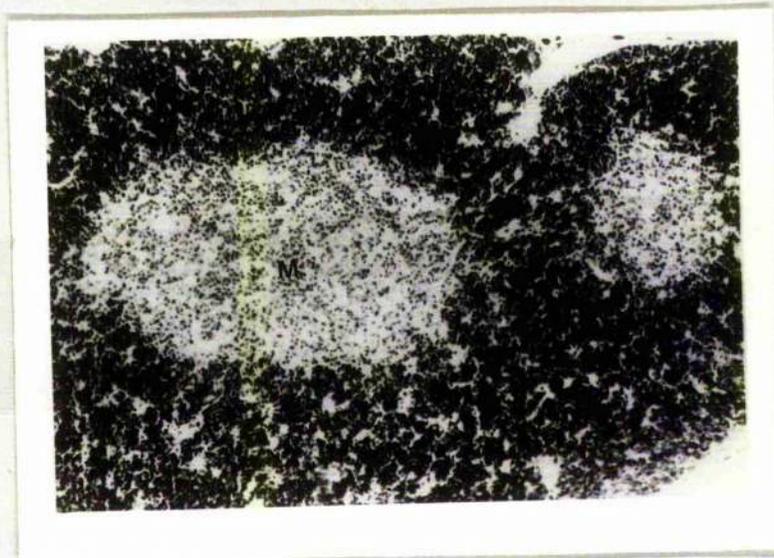
The most dramatic morphological changes after grafting and treatment with CyA occurred in the recipient thymus. At 3 days there was a marked increase in most cells in the thymic cortex (Fig. 5.9). Although increases in most cells were noted in untreated allograft recipients, proliferation was, at the most, focal. By 7 days most medullary thymocytes had been eliminated in the CyA-treated rats and vascular stroma and histiocytes containing phagocytosed cellular debris constituted the medulla. Similarly, at 14 and 21 days after grafting virtually all medullary thymocytes were eliminated and the medullary area was shrunken to 5% of its normal size (Fig. 5.10). At 50 days regeneration of the medullary compartment was manifested by the formation of multiple small medullary areas in each thymic lobule (Fig. 5.11) that expanded and coalesced to form normal medullary compartments by 100 days (Fig. 5.12). The CyA treatment alone, in animals who received no cardiac allograft, caused a similar, although slightly less extensive, decrease in medullary thymocytes when examined at 3, 7, 14 and 21 days.

Thymus HistologyFIGURE 5.9

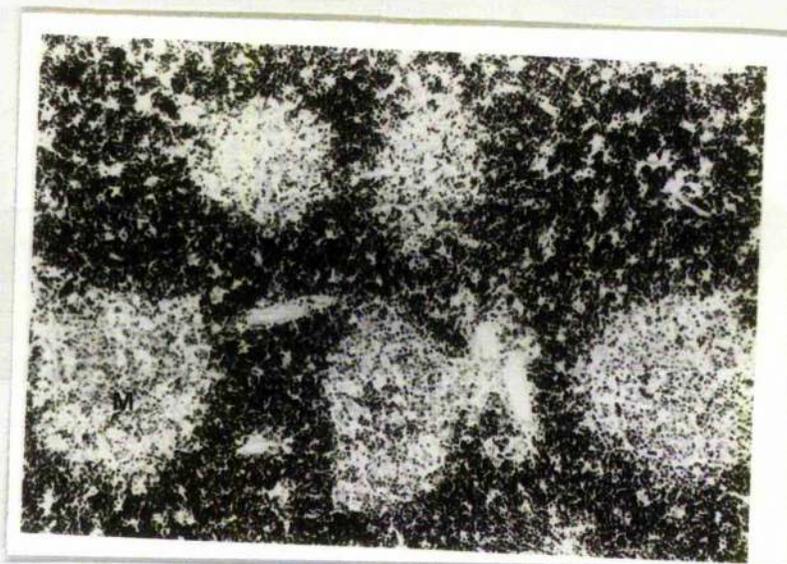
Thymus of CyA-treated (LEW) recipient of (LEW x BN) F_1 cardiac allograft at 3 days. A marked increase in most cells in the thymic cortex (C) is noted. x100

Thymus HistologyFIGURE 5.10

Thymus of CyA-treated (LEW) recipient of (LEW x BN) F_1 cardiac allograft at 21 days is shown. Virtually all medullary thymocytes (M) were eliminated and the medullary area was shrunken to 5% of its normal size. x40

Thymus HistologyFIGURE 5.11

Thymus of CyA-treated (LEW) recipient of (LEW x BN) F_1 cardiac allograft at 50 days is shown. The medullary compartment is regenerating as manifested by the formation of multiple small medullary areas (M) in each thymic lobule. x50

Thymus HistologyFIGURE 5.12

Thymus of CyA-treated (LEW) recipient of (LEW x BN) F_1 cardiac allograft at 100 days is shown. The medulla has been reconstituted (M).

x50

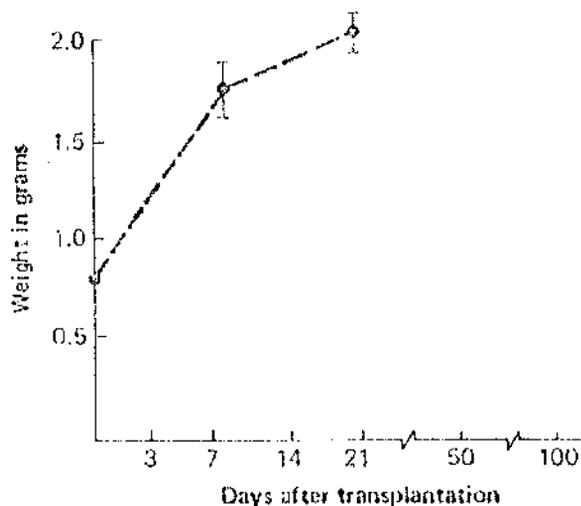
c) Cardiac Allografts

Acute rejection in untreated rats was accompanied by a progressive increase in graft weight (from 0.85 g at day 0 to 1.7 g at day 7) (Fig. 5.13). In CyA-treated rats cardiac grafts increase initially in weight to 1.25 g at 3 days but return to normal weight (0.88 g) by 7 days. Normal graft weight was then maintained until a slow progressive weight increase occurred after 50 days. These weight ranges were small (Fig. 5.14).

These experiments have demonstrated that CyA treatment results in the marked reduction in size of the thymic medulla, the splenic PAS and the splenic MZ. Previous studies have suggested that T lymphocytes which arise originally in the bone marrow and migrate to the thymic medulla then migrate to the PAS and MZ areas (Goldschneider, 1976). Because helper and cytotoxic T lymphocytes may develop in the thymic medulla while suppressor T lymphocytes may originate in the thymic cortex (Small, 1978), CyA may be predicted to favour suppressor cell function. These morphological changes in the spleen and thymus of CyA-treated animals support the result of the earlier cell transfer studies suggesting the presence of suppressor cells in rats receiving a cardiac allograft and treated with CyA.

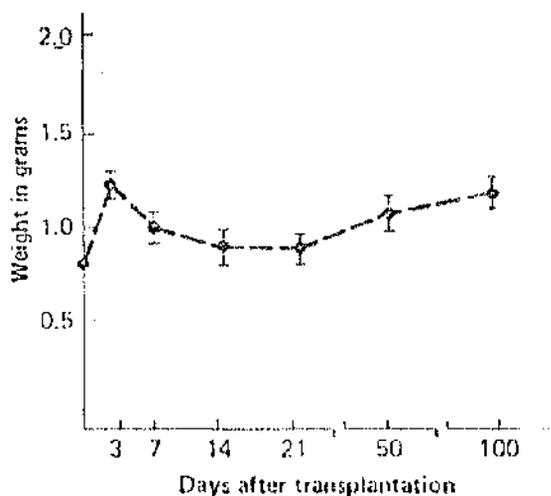
Cardiac Allograft Weights in Untreated Recipients

FIGURE 5.13



Cardiac Allograft Weights in Cyclosporin A-treated Recipients

FIGURE 5.14



Weight changes in $(LEW \times BN)F_1$ cardiac allografts in (LEW) hosts, untreated and CyA-treated.

CHAPTER 6DISCUSSION

DISCUSSION

The ideal immunosuppressive regimen would be both potent and safe, i.e. it would be effective in the vast majority of treated patients and be associated with negligible morbidity and mortality. The fact that a significant number of organ grafts fail, due largely to uncontrollable rejection, and that there are very serious and sometimes fatal complications of conventional immunosuppressive therapy is a clear indication that current therapy is far from ideal. The major problem with the drugs currently in use is that their immunosuppressive effects are a by-product of a general toxic effect on cells, with the result that potency and safety oppose one another.

The rejection of tissue or organ allografts is the culmination of interconnected cellular and humoral events mediated by various lymphoid cell subgroups. The lymphocyte populations, their function and their intricate inter-relationships occurring throughout the immune system are gradually becoming better defined. "Cytotoxic" cells are responsible for destroying the allograft and "helper" cells support the rejection process by promoting the activity of cell-mediated and humoral immunity. In addition, Gershon and colleagues (1972) demonstrated that lymphocytes can have a suppressive effect on the immune system. These suppressor cells can be defined as cells, usually

subpopulations of lymphocytes, which suppress the reactivity of other lymphocytes. Although B cells (Gill and Liew, 1979) or macrophages (Anaclerio et al, 1979) may act as suppressor cells, the latter usually non-specifically, most suppressor cells have proved to be T lymphocytes as shown by the loss of suppressor activity after treatment of the cell population with anti-T lymphocyte antibodies and complement.

In many experimental models as well as in clinical transplantation the predominant strategies available to inhibit host activity against foreign tissues have been various immunosuppressive agents, the majority of which affect lymphocyte populations indiscriminately. Probably the highest degree of drug specificity possible would be for a drug to interact exclusively with proliferating T cells known to be vital for graft rejection i.e. cytotoxic and helper T cells. However, even if this was the case, it is important to note that such a drug may still be associated with problems since it would also suppress the immune response to invading pathogens. Recent evidence suggests that CyA has a selective action on lymphoid cells and may be the first of a new generation of drugs capable of selective immunoregulation.

In 1976 Borel demonstrated that CyA depressed immune reactions both in vivo and in vitro and prolonged skin graft survival in mice. This observation led to

studies of CyA in different species with a variety of organ transplants. The rejection of heterotopic heart allografts in rats was delayed by CyA (Kostakis et al, 1977). CyA was more effective than azathioprine in prolonging dog renal grafts (Calne and White, 1977), renal allografts in rabbits (Green and Allison, 1978), orthotopic cardiac allografts in pigs (Calne et al, 1978a) and dog pancreatic grafts (McMaster et al, 1980).

One of the most important observations made in the small animal grafting experiments is that for many, although not all, of the organs transplanted e.g. heart, kidney and liver in the rat, a short course of CyA produces a very prolonged graft survival. This phenomenon suggests that CyA may suppress clones of responding cells or alter a balance between the T helper and T suppressor cells such that non-responsiveness is induced. Du Toit and colleagues in 1980 found however that canine renal and pancreatic allografts were invariably rejected 1 to 3 weeks after stopping CyA treatment 14, 21 or 100 days after transplantation, suggesting that clonal deletion is not a mechanism of action in the dog.

Following these early animal experiments a pilot study was begun using CyA in clinical organ grafting initially as the sole immunosuppressive agent (Calne et al, 1978b). This study involved 50 patients

receiving 56 cadaveric organ allografts, 45 kidneys, 6 pancreases and 5 livers. The results were encouraging and numerous other clinical investigations have subsequently been initiated. The initial concern that lymphomas would be common after the use of CyA has not been fulfilled.

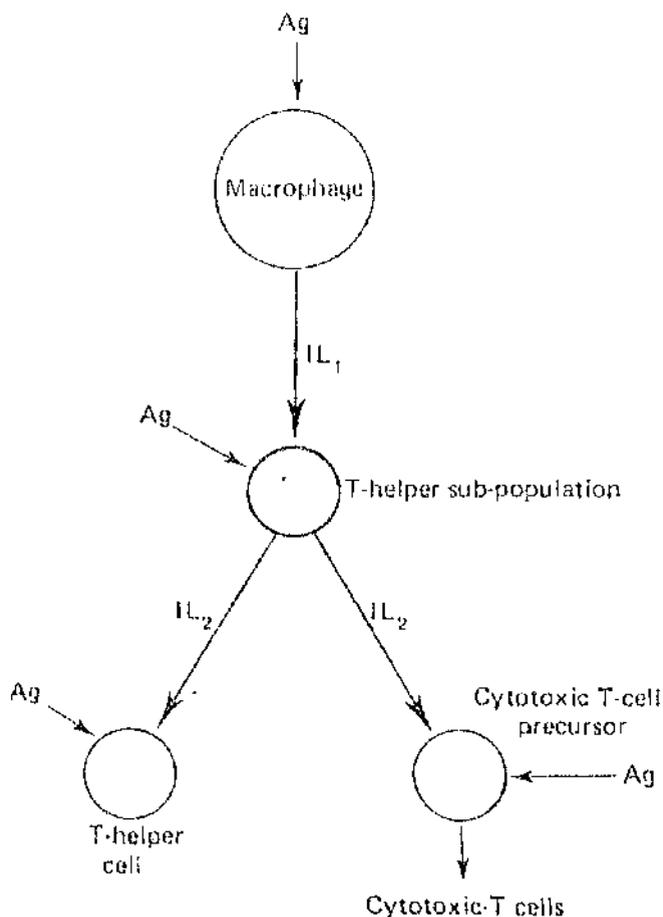
Although its pharmacodynamics are not well understood certain information is available. Borel and colleagues in 1976 reported that CyA inhibited humoral immunity in rodents as measured by the reduction of plaque-forming cells and haemagglutinin titres. It also suppressed cell-mediated immunity in such models as skin graft rejection, graft versus host disease, delayed hypersensitivity, skin reactions and experimental encephalomyelitis. Lymphocytes appear to be the primary target of CyA since granulocyte proliferation and macrophage function both appear to be unaffected by therapeutic doses (Borel et al, 1976; Borel and Weisinger, 1977; Paavonen and Hayry, 1980). In addition, it appears to act preferentially on T cells (Borel et al, 1976). Other early studies suggested that B cells in rats (Burckhardt and Guggenheim, 1979), and in humans (Gordon and Singer, 1979), were unaffected. However, more recently in the mouse, Kunkl and Klaus (1980) have suggested that CyA affects a subpopulation of B cells and that these are as sensitive to CyA as are certain subpopulations of T cells.

In studies on pig cells (White et al, 1979) and human lymphocytes (Weisinger and Borel, 1979) CyA was shown to inhibit an early stage of T cell activation and in addition recent studies, using human lymphocytes, have suggested a selective inhibiting effect on T helper cells (Leapman et al, 1981).

By considering the model (Fig. 6.1) proposed by Wagner and his colleagues in 1980 more information of T cell activation is obtained and possible sites of CyA activity are postulated.

Using this model of T cell activation Bunjes and colleagues presented evidence showing that, in the mouse, CyA selectively impairs the release of Interleukin 2 (IL₂) from activated T helper cells and that of Interleukin 1 (IL₁) from macrophages, thus inhibiting the generation of cytotoxic T lymphocytes and T helper cells. These workers did not however test suppressor cell activity although suppressor cells are apparently spared by CyA in this model. This mode of action explains why CyA is so effective in blocking mitogen-activated as well as allo-antigen-activated proliferative T cell responses in vitro (White et al, 1979; Horsburgh et al, 1980). Data from other in vitro investigations, using human lymphocytes, have suggested that CyA permits the generation of suppressor lymphocytes (Hess and Tutschka, 1980). Additional studies in vivo by Tutschka and colleagues in 1981

FIGURE 6.1

Method of T-cell Activation

Interleukin 1 (IL_1) is produced by macrophages on encountering the antigen (Ag). IL_1 acts on a sub-population of T helper cells along with the antigen to evoke the production and release of Interleukin 2 (IL_2). This in turn acts on antigen-primed T helper cells and cytotoxic T-cell precursors, inducing mitogenesis of these cells. Helper cells enhance the activity of cytotoxic cells and B cell antibody production directed against the antigen.

demonstrated suppressor cells in rats grafted with incompatible bone marrow and treated with CyA were able to prevent graft versus host disease. Thus it would appear from these experiments that CyA has a sparing effect on suppressor cells while inhibiting cytotoxic T lymphocyte generation in response to antigen.

In these present studies it has been shown that CyA is a potent immunosuppressive agent in the rat cardiac allograft model. Allografts in both combinations, (LEW x WF)₁ to LEW and (LEW x BN)₁ to LEW, function > 100 days without rejection episodes in consistently healthy recipients being given intramuscular CyA 15 mg/kg/day for 7 days only, commencing immediately after transplantation. Allografts in untreated animals survived only 7 days \pm 1 day.

In some experimental (Borel et al, 1976) and clinical (Keown et al, 1981) situations CyA suppresses both humoral and cell-mediated immunity. However, in CyA-treated rats receiving renal allografts (Homan et al, 1980a) and in recipients treated with a combination of CyA, antilymphocyte serum and enhancing antiserum (Homan et al, 1979; Homan et al, 1980b) antibody synthesis is diminished, although cell-mediated immune responses are only marginally suppressed. Homan and colleagues suggested that the suppression of the antibody response could be attributable to non-reactivity in the helper T lymphocyte or B lymphocyte

populations. A T helper effect seems more likely, both because CyA appears to act preferentially on T cells (Borel et al, 1976) and because T lymphocytes are more easily inactivated than B lymphocytes (Chiller et al, 1971).

In the studies described in this thesis lymphocyte-mediated cytotoxicity mounted by lymphocytes from various host lymphoid compartments, including cells infiltrating the grafts themselves, was reduced after transplantation and treatment with CyA. In addition, anti-donor antibody activity as measured by complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity and the reverse haemolytic plaque assay, was also diminished significantly when compared with control animals. Such suppression of cell-mediated and humoral immunity was noted throughout the 100 day period after transplantation.

Many of the experiments mentioned previously have proposed the sparing effect of CyA on suppressor cells and indeed the present cell transfer experiments would support this theory. In these investigations it has been demonstrated that CyA-treated rats bearing well-functioning cardiac allografts develop a population of lymphoid cells with suppressor activity exerted in vivo against donor allo-antigens. Thus, thymocytes from such animals can increase survival of test cardiac allografts following transfer into unmodified

syngeneic animals. In the present experiments lymphoid cells transferred from grafted animals at any time after cessation of CyA treatment increased survival of test allografts significantly. Survival is not prolonged if cells are transferred during the 7 days of treatment. The suppressor effect is fully sustained at least three months after transplantation by which time any remaining drug would have been metabolised. Such a state of specific unresponsiveness is not without precedent in organ graft systems: it has been noted that a similar result can be achieved in enhanced recipients of heart grafts (Tilney et al, 1978). Other investigators have also demonstrated prolonged test allograft survival following cell transfer but the immune responsiveness of the recipients was invariably diminished before transfer (Dorsch and Roser, 1977; Droege, 1975; Kilshaw and Brent, 1977). The work in this thesis shows the same phenomenon in recipients with normal immune systems prior to cell transfer.

Some authors (Calne et al, 1978b; White et al, 1979; Decg et al, 1980) have suggested that CyA itself or a metabolite is the suppressive principle which can be removed by washing the cells. The findings in these present experiments are in variance with this concept as stripping the surfaces of the transferred cells with trypsin did not affect their suppressor activity. Additionally, the inability of cells from CyA-treated but ungrafted animals to prolong test graft survival

also demonstrates that a continuing antigenic stimulus as well as effects of the drug are required for the development of suppressor cells.

These findings of cells with suppressor characteristics are supported by the results of the studies of dynamic responses of certain lymphoid tissues to the cardiac allografts. The medullary compartment of the thymus and the splenic peri-arterial sheath and marginal zone areas were strikingly reduced in size (by 97, 50 and 67% respectively) 1 to 2 weeks after the completion of CyA treatment. The elimination of medullary thymocytes with partial preservation of cortical thymocytes in the 2 weeks following only 7 days of CyA treatment was as consistent as it was dramatic. This selective effect of CyA on cells in the medulla of the rat thymus is unique. Other commonly used chemical immunosuppressants, such as 6-mercaptopurine, cyclophosphamide and prednisolone, preferentially affect cortical thymocytes (Miller and Cole, 1967; Weissman, 1973). Antilymphocyte serum, which also depletes T lymphocytes in the splenic peri-arterial sheath, has little or no effect on the thymus (Taub and Lance, 1968).

Studies using the rat masked thymocyte antigen suggest that T lymphocytes in these compartments may arise originally in bone marrow and migrate first to the thymic medulla and then to the splenic peri-arterial

sheath via the marginal zones (Goldschneider, 1976; Nicuwenhuis and Ford, 1976). A second line of T lymphocytes bearing the rat bone marrow lymphocyte antigen may migrate first to the thymic cortex and then to the splenic red pulp. These rat bone marrow antigen-bearing T lymphocytes may be less susceptible to CyA as the thymic cortex and the splenic red pulp were only reduced in size by 50% and 10% respectively. Because helper and cytotoxic T lymphocytes may develop in the thymic medulla while suppressor T lymphocytes may originate in the thymic cortex (Small, 1978; Durkin et al, 1978) CyA may be predicted to favour suppressor cell function.

The antigenic specificity of transferrable unresponsiveness using cells from LEW animals bearing (LEW x BN) F_1 and (LEW x WF) F_1 donor allografts was striking. These animals were chosen because they are incompatible for all known major histocompatibility antigens. The survival of test (LEW x WF) F_1 cardiac allografts was prolonged to 13 ± 2 days after thymocyte transfer from CyA-treated (LEW) recipients of (LEW x WF) F_1 heart grafts 14 days after transplantation. However, no prolongation was noted when thymocytes from CyA-treated (LEW) recipients of (LEW x BN) F_1 cardiac allografts were transferred at 14 days into unmodified (LEW) recipients of (LEW x WF) F_1 test grafts (MST \pm SD = 6 ± 1 day). Similarly (LEW x BN) F_1 test grafts in (LEW) recipients survived

for only 6 ± 1 day after transfer of thymocytes from CyA-treated animals bearing (LEW x WF) F_1 cardiac allografts. Hence the specificity of unresponsiveness to the original donor and not a third party animal was demonstrated.

Such striking specificity was also demonstrated in vitro, where reactivity by lymphocytes from CyA-treated, grafted rats in the mixed lymphocyte response was consistently suppressed against donor cells but no suppression of proliferation was noted against third party cells. However, as in the in vivo cell transfer experiments themselves, antigen-specific suppression occurred only several days following cessation of the drug.

Antigen-specific suppression has been suggested by Homan et al (1979) in studies of CyA-treated rats bearing renal allografts. These workers found that third party kidneys were rejected rapidly although the CyA-treated hosts lost almost completely their ability to mount an antibody response to these third party grafts. Green et al (1979) also supported antigen-specific suppression in studies of renal allografted rabbits, although Dunn et al (1978) in investigations into the effect of the drug in kidney grafts in outbred rabbits demonstrated powerful but totally non-specific immunosuppressive activity. White and his colleagues in 1980, using rat cardiac allografts followed by donor

or third party skin grafts at a later date, demonstrated that during CyA treatment and during the few days following cessation of treatment there was non-specific unresponsiveness to the skin grafts, whereas two weeks after stopping CyA administration specificity of response was shown.

The emergence of antigen-specific suppressor cells is not unique to CyA treatment. Dossetor and colleagues in 1981 studying 8 patients with renal transplants, receiving steroids and azathioprine, demonstrated that suppressor cells were not present in recipient blood before transplantation but that they were well established as early as 2 to 3 weeks after transplantation and were donor-specific. In this study the presence of these donor-specific suppressor cells was not however necessarily associated with a good clinical course. Jayavant and colleagues (1979) demonstrated, however, a significant correlation between a functioning, non-rejecting allograft and an allograft recipient's suppressor cell activity.

Suppressor cells have also been demonstrated with other immunosuppressive regimes. Askenase and colleagues in 1975 studying cyclophosphamide-treated mice, immunised with sheep red blood cells, suggested that suppressor cells are present during immunosuppressive treatment. In addition, it has also been shown from several laboratories that animals treated with antilymphocyte

serum possess suppressor T cells whereas comparable normal serum controls do not (Kelshaw et al, 1975; Wood and Monaco, 1979; Rudnicka, 1980). Thomas and fellow workers (1983) demonstrated that rabbit anti-thymocyte globulin, by inducing non-specific suppressor cells in the recipients, prolonged the survival of skin allografts in rhesus monkeys. Using total lymphoid irradiation when studying bone marrow grafting in mice (Slavin et al, 1979) and kidney allografts in baboons (Smit et al, 1981), suppressor cells have also been demonstrated.

Concluding Comments

The aims of the studies described in this thesis have been fulfilled. Cyclosporin A has been shown to be a very potent immunosuppressive agent in the rat cardiac allograft model. Both the humoral and cell-mediated components of the immune response are markedly suppressed. It has also been shown that antigen-specific suppressor cells emerge following the cessation of Cyclosporin A treatment. It is suspected that these suppressor cells act by abrogating or overriding the function of allo-specific helper T cells necessary for initiating differentiation of cytotoxic T lymphocytes and antibody-producing B cells.

The Future

The results of our experimental findings with CyA in the rat do not necessarily apply to other species. Although CyA is a powerful immunosuppressant in larger species, Du Toit and colleagues found that a short course of the drug failed to produce indefinite survival of kidney and pancreatic allografts in the dog. In the human, continuous CyA treatment is used to maintain organ graft survival. In these larger species, after many months of treatment, would suppressor cells emerge to allow the drug to be discontinued without additional treatment? If so, at what time could CyA be stopped without organ rejection? Would the dose of CyA need to be "tailed off"?

At present CyA is used by many transplant centres throughout the world. Some centres are extremely enthusiastic about its use (Calne et al, 1979; Starzl et al, 1981) whereas the experiences of the Royal Free Hospital, London (Sweny et al, 1981) and the Peter Bent Brigham Hospital, Boston (Carpenter et al, 1981) have been disappointing. It remains to be seen whether it will be more appropriate to use CyA alone, as Calne is doing, or CyA together with prednisolone in low dosage, as advocated by Starzl. Perhaps CyA and azathioprine would be a better combination?

The ideal immunosuppressive regimen would be without

side-effects. The initial concern, during the early clinical use of CyA, about the development of lymphomas (Calne et al, 1978b) has proved unfounded. Indeed, in the light of subsequent experience it is clear that the risk of developing a lymphoma is much the same in patients treated with CyA as in those treated with azathioprine. However, nephrotoxicity (Calne et al, 1979) and hepatotoxicity (Starzl et al, 1980) remain two toxic side-effects of the drug.

There remain many questions to be answered about CyA. It is a drug, however, that has made a significant advance in the search for the ideal immunosuppressant and it is probable that its full potential has yet to be realised.

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