LYMPHOCYTE ACTIVITY AND FLOW IN

RENAL ALLOTRANSPLANTATION

BY

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SUMMARY

The lymphocyte is the principle cell responsible for the main events which occur in the transplantation reaction. The activity of the lymphocyte, has been studied in two ways. First, effector lymphocytes, sensitised by allografting in rats, have been tested for their ability to kill antigenic cells in vitro. Secondly, lymphocytes injected into allogeneic rats have been studied in their ability to proliferate in presence of antigenic tissues.

The flow of lymphocytes after renal allotransplantation has been studied in sheep by measuring the total body lymphocyte flow after thoracic duct cannulation and drainage, and the renal lymphocyte flow by cannulation of the lymphatics leaving a transplanted kidney.

Lymphocyte cytotoxicity measured by in vitro cytotoxicity tests has been achieved with sensitised spleen cells against allografts of tumour and kidney but not skin grafts. Lymph node lymphocytes are not effective as cytotoxic cells.

The ability of sensitised lymphocytes to proliferate in the presence of antigenic tissue measured by the graft-versus-host popliteal node weight assay has not been proved. This assay does not show the immune competence of lymphocytes injected into either allogeneic or xenogeneic animals.

The flow of lymphocytes is markedly increased in the area of a renal allograft and flowing from the graft. This effect is localised to the graft itself and is not reflected in an alteration in the flow of lymphocytes through the whole body. The action of immunosuppressive drugs on lymphocyte flow is also localised to the area of the allografted organ.

To my father and mother

Dr. Ernest Evans and Mrs. Margaret Evans in gratitude for my genetic endowment and for their help and encouragement

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The lymphocyte is responsible for the main events which occur in the transplantation reaction. The activity of the lymphocyte, sensitised by allografting, has been studied in rats in its capacity to kill antigenic cells and to proliferate in the presence of antigenic tissues. The flow of lymphocytes after renal allotransplantation has been studied in sheep by measuring the total body lymphocyte flow after thoracic duct cannulation and drainage, and the renal lymphocyte flow by cannulation of the lymphatics leaving a transplanted kidney.

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INTRODUCTION

SECTION I - Lymphocyte Cytotoxicity

SECTION II - Graft-versus-Host Response

SECTION III - Lymphocyte Flow

CONCLUSION

GLOSSARY

EQUIPMENT LIST

BIBLIOGRAPHY

INTRODUCTION

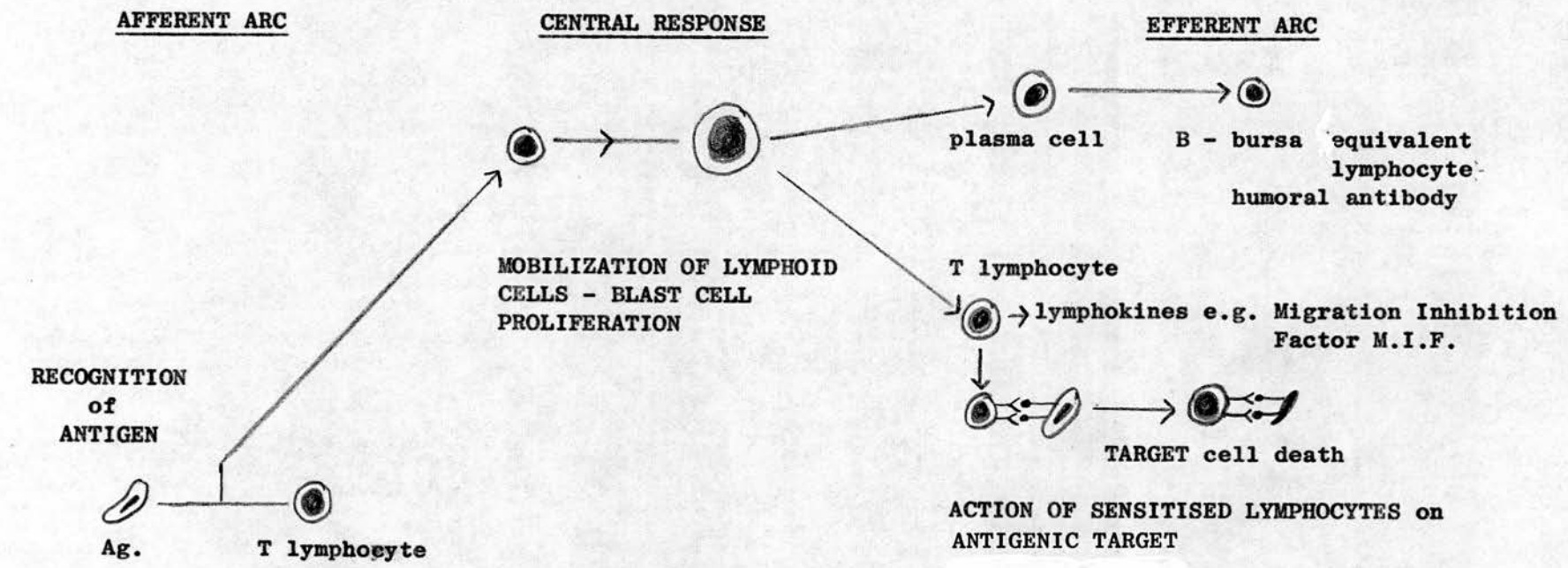
It is generally held that the immune response to an allograft is an immune reaction of the class known as cell mediated. The mechanism of rejection following organ or tissue transplantation is not completely understood. The response is divided for descriptive purposes into the afferent arc, the central response and the efferent arc (diagram 1). Recognition of the cells of an allograft as antigenic occurs in the afferent arc. This information is transferred centrally where lymphocytes are mobilised and large lymphoblasts are produced. Death of a transplanted cell is thought to be the result of direct action of specifically sensitised effector (killer) lymphocytes and represents the efferent arc of the response. In the primary immune response production of sensitised killer lymphocytes occurs in about 5 days.

Recognition of antigen

The first step is contact of the recipient's circulating lymphocytes with the cell surfaces of the graft which carry the histocompatibility antigens. The small T (thymus derived) lymphocyte alone is capable of recognition of the antigen and there is no need for phagocytosis of the antigen by macrophages.

Proliferation of lymphoid cells

Stimulated by antigen, the immunologically competent lymphocyte starts the process which ends in the production of sensitised effector cells which act directly against the cells bearing the antigen. During this part of the response there is a production of a large lymphoblasts which are also termed transformed cells. These



Mixed Lymphocyte Reaction (M.L.R.)	M.L.R.	Lymphocyte Aggregation
Sheep Cell Rosettes	Incorporation of nuceosides	Lymphocyte Cytotoxicity
Rosette Inhibition Assay	(^{3}H thymidine)	Rosette Inhibition Assay
Graft - versus - host (G-v-H)	(^{3}H uridine)	Peripheral lymphocyte count
P.H.A. responsiveness	G-v-H	Lymphocyte Migration Inhibition
		G-v-H

SCHEME of the IMMUNE RESPONSE (CELL MEDIATED) to ALLOGRAFT and TUMOUR

The various tests to measure the immune response, the appropriate parts of the immune response and the parts at which the test apply are shown.

Diagram 1

blast cells in turn produce the sensitised effector cells.

Action of sensitised cells on the antigenic target cell

The onslaught of sensitised T lymphocytes on the grafted organ now occurs. The method of attack by the lymphocytes may be either by a direct action of one cell on the other, or by the production of some mediator by the lymphocyte.

Although allograft rejection depends on cell-mediated immunity the role of circulating (humoral) antibodies is not negligible. The role of the circulating antibody is not defined, but there appear to be some antibodies which exert a harmful effect (cytotoxic antibodies) and some which exert a protective effect (blocking antibodies).

The work presented in this thesis will study the activity of the lymphocyte only, in transplant rejection. The work will be divided into three sections:

In the first section the role of the T-lymphocyte will be studied in the cell-mediated-immune response by in vitro methods. The in vitro tests will be performed to study the individual killing capacity of effector lymphocytes which have been sensitised against an antigenic allograft in vivo. The killing capacity will be tested against tissues bearing the same antigen as the allografts. These in vitro lymphocyte cytotoxicity tests represent the efferent arc of the cell mediated immune response (diagram 1).

In the second section a bio-assay will look at the ability of the T-lymphocyte to proliferate in the presence of antigenic tissue, by a graft-versus-host node weight assay. This assay represents the whole of the immune response.

The third section will look at the flow of lymphocytes in the

whole body and in the grafted organ after renal allotransplantation. The introduction of immunosuppressive drugs has altered the pattern of organ transplantation. These drugs have an anti-lymphocyte effect and prolong the function of an allograft. Studies will be performed to look at the effect of immunosuppressive drugs on the flow of lymphocytes flowing through a renal allograft and flowing through the body, in order to determine the specificity of changes in flow through a renal allograft.

Before embarking on the work of the thesis proper, a short examination of the in vitro tests of lymphocyte function relating to the transplant rejection response, will be made. The various tests of lymphocyte function will be described below and the supposed areas of the cell-mediated immune response which these tests measure are shown in diagram 1. Many of these tests show alterations in allograft rejection confirming that the lymphocyte plays an important role. The value in the diagnosis of clinical rejection episodes will be mentioned.

Peripheral blood T lymphocyte

The ability to form spontaneous rosettes with sheep red cells is a function of T cells in man. Human T lymphocytes have the capacity to adhere to sheep erythrocytes, and the sheep red blood cells form rosettes around the lymphocyte without any prior sensitization; this is called spontaneous cyto-adherence. Like the cells that recognise antigen, the cells forming spontaneous rosettes are small lymphocytes, they require no preliminary action by macrophages, and this ability to form rosettes would appear to reflect the afferent arc of the immune response.

The number of rosette forming cells in the peripheral blood in patients after transplantation are not increased (Wood and Gray, 1973).

Phytohaemagglutinin (PHA) responsiveness

Certain plant mitogens such as PHA can react with T cells and these cells then proliferate to blast cells. This reaction is similar to that seen after antigenic recognition, but with PHA it is entirely non-specific. The ability of the T cell to transform with PHA reflects the T cell activity and is routinely used in many laboratories in the assessment of immune status of the body. PHA responsiveness has been used in man as a predictive test prior to transplantation (Rubin, Stenzal, Hirschhorn and Bach, 1964). Human patients were found to have an increased PHA responsiveness after renal transplantation (Tennenbaum, St. Pierre and Cerilli, 1968) which can be decreased by increased doses of immunosuppressive drugs. In clinical work the results have too wide a range to be a useful measurement.

Rosette Inhibition Assay

The phenomenon of sheep cell rosetting has been used to follow, in the human transplant patients, the metabolism of immunosuppressive drugs, and to determine at a specific time what degree of immunosuppressive activity has been obtained by the drugs (Hamburger, 1970). The minimal inhibitory concentration of anti-lymphocyte globulin (A.L.G.) which is required to diminish rosetting by human peripheral blood lymphocytes in vitro, has been studied by Wood and Gray (1973) and Bewick, Ogg et. al., (1972). Wood and Gray (1973) found that the measurement of the rosette inhibitory titre of A.L.G. did not give a

predictive test of rejection episodes, and was not an indication of the amount of immunosuppression used, Bewick et. al., (1972) found that the lower the amount of A.L.G. required to diminish rosetting in vitro, the more stable the course of the transplant recipient. Those patients who were rejecting became exposed to a very great risk of infection if the dose of routine immunosuppression was increased to the levels suggested by this test.

Nucleoside Incorporation

The uptake of ^{3}H thymidine and ^{3}H uridine by peripheral blood lymphocytes is a measurement of DNA and RNA synthesis. The production of rapidly dividing cells during the central part of the cell-mediated immune response requires an increase in DNA and RNA Activity, therefore the uptake of ^{3}H thymidine and ^{3}H uridine reflects to the rate of division of lymphocytes in the presence of antigen. ^{3}H thymidine incorporation by peripheral blood lymphocytes has been shown to be increased in allograft rejection in rats (Blamey, Nicol, Baxter and Deane, 1974) and to predict clinical rejection episodes in man (Blamey, Bennett, Singer, Nicol and Harrison, 1975) but the increased uptake is not specific to rejection as there is also an increase in the presence of infection. ^{3}H uridine uptake has been shown to be of less value by the same workers.

Mixed Lymphocyte Culture (M.L.C.)

The in vitro recognition of antigen and the stimulation of production of transformed cells has been reproduced almost exactly by an in vitro test - mixed lymphocyte culture. Two sets of allogeneic lymphocytes are cultured together, one set of lymphocyte is treated

with either mitomycin or irradiation to prevent division of these cells, which act as the antigen and are termed the stimulator cells. The other set of lymphocytes, termed the responder cells, after 5 days will have transformed to blast cells that have synthesised increased amounts of DNA and this can be measured by an increased uptake of ^{3}H thymidine. The amount of transformation will be dependent on the degree of antigenicity of the stimulator cells (Hamburger and Bach, 1973).

This test has great value for prediction of donor and recipient compatability before transplantation. As a measurement of the degree of transplant rejection in following the course of transplantation the length of duration of the test would make it of little value clinically, and in practice it has been found that there is no oo correlation between the degree of rejection and transformation in M.L.C. of recipient lymphocytes against donor cells (Bach, 1974). When the recipient lymphocytes did not transform before transplantation they did not acquire the capacity to do so afterwards, and recipient lymphocytes kept their ability to respond to donor lymphocytes in vitro if they had done so prior to transplantation.

Leucocyte Migration Inhibition

Sensitised lymphocytes release chemical mediators which are termed lymphokines. One of these causes the inhibition of cell migration and is named migration inhibition factor (M.I.F.). Lymphocytes (usually mixed with macrophages) introduced into a capillary tube will tend to migrate through the open end of the tube, but if the cells have been previously sensitised to an antigen, the presence of that antigen in the medium inhibits the migration. This

is due to the release of M.I.F. This in vitro test has been shown by Wood, Gray, Briggs and Bell (1973) to be a useful monitor of the strength of the immune response. Using donor specific antigen from spleen, lymph node and liver, but not kidney, they tested human transplant peripheral blood lymphocytes for migration inhibition, and found a good correlation between impending rejection and the production of M.I.F. This test would appear at present to be the most useful clinical test. It is not however entirely dependent on specificity against HL-A histocompatibility antigens; Weeke, Weeke and Bendixen (1970) obtained no inhibition of migration if tissue extracts from the donor (other than parenchymal renal extract) were used as antigen. Presumably these tissues have the same major transplantation antigens as the kidney.

Leucocyte Aggregation

The sensitised effector lymphocytes come in contact with the antigenic target cell in order to kill it. The aggregation of the lymphocytes around the target cell appears to be the earliest stage of lymphocytes cytotoxicity (Kahan, Tom, Mittal and Bergan, 1974). They performed an in vitro test observing leucocyte aggregation using donor skin target cells and peripheral blood lymphocytes from human transplant patients. Although the test could predict rejection episodes, when clinical rejection was established the lymphocytes became unresponsive towards the donor cells. They suggested that this might be due to the accumulation of immune cells within the graft, rather than circulating in the peripheral blood.

SECTION I

LYMPHOCYTE ACTIVITY IN ALLOGRAFT REJECTION

LYMPHOCYTE CYTOTOXICITY

A. BACKGROUND

B. AIMS

C. METHODS

D. RESULTS

E. DISCUSSION

F. CONCLUSION

G. APPENDICES

LYMPHOCYTE CYTOTOXICITY

A. BACKGROUND

Introduction

Previous work on the allograft response:-

1. Methods
   a. Destruction of monolayers
   b. Colony inhibition
   c. Cell counting
   d. Chromium release
2. Cell populations used in in vitro cytotoxicity tests
3. The effect of antibody on cytotoxicity in the allograft response

Cytotoxicity in the tumour response

Conclusion on published work from other centres

Introduction

The capacity of the sensitised effector lymphocyte to kill antigenic target cells of an organ has been demonstrated in vitro but has been difficult to achieve. In vitro cell mediated cytotoxicity represents the effector phase of the allograft response, because both have target cell destruction in common. In studying the background of lymphocyte cytotoxicity, work with tumour and tissue grafts will both be considered. Many of the in vitro methods used in attempts to show cytotoxicity in the transplant situation were originally used to show cytotoxicity against tumour specific antigen. The methods of these tests will now be discussed.

Previous work on the allograft response

1. Methods

a. Destruction of monolayers

The actions of lymphocytes and sera from dogs which had received an allografted kidney were studied by Govaerts (1960). Using the second kidney of the donor he obtained tissue cultured epithelial target cells. He observed under the microscope the effect of recipient thoracic duct lymphocytes and peritoneal macrophages on the target cells, before, during and after immunization. Target cells in the presence of recipient lymphocytes and serum, taken at the time of rejection which occurred between the seventh and twelfth day, showed cell retraction, agglutination and the target cells were surrounded by lymphocytes. The method was slow and inaccurate due to subjective evaluation of the cell damage.

b. Colony inhibition

A colony inhibition test was described by Hellström (1967). Tumour target cells (Moloney virus induced mouse leukaemia) were plated in petri dishes. After twenty-four hours incubation lymphocytes, sensitised by allogeneic transfer of tumour from the donor mouse to an H-2 incompatible recipient,were added for 4 days. The ability of the lymphocytes to prevent the target cells from growing into colonies was considered evidence of killing. This is a very sensitive test and the effector lymphocyte to target cell ratio may be low. The disadvantage for clinical purposes is that the test takes 5 days to perform and can only be applied to certain types of cell as targets, those which grow in monolayers.

c. Cell counting (Microcytotoxicity Tests)

In the methods described as microcytotoxicity tests, target cells are allowed to adhere to glass or plastic. The detachment of these target cells after the addition of lymphocytes denotes cell destruction.

In animal experiments a method of microscopic counting of targets after staining with trypan blue has been compared by Brunner, Mauel and Schindler (1966) with a cloning[1] efficiency assay equivalent to colony inhibition. They studied the effect of allogeneic sensitised spleen lymphocytes from C57BL mice, which were immunised by injection of mastocytoma cells from $DBA/_2$ mice, on mastocytoma target cells. The lymphocytes produced a 67% to 96% reduction in the $DBA/_2$ mastocytoma cells after incubation together in tubes for 48 hours, and a 95% decrease in the cloning efficiency at 12 hours. This effect

[1] Cloning - the ability of target cells to form colonies.

was achieved with a very small ratio of effector to target cells 3 : 1. Comparing the two methods the maximum reduction in cloning efficiency occurred by 12 hours and in the cell counting at 48 hours. Brunner et. al., (1966) presumed that the loss of cloning ability was an earlier stage of cell death.

A method of cell counting has also been described by Biesecker, Fitch, Rowley, Scollard and Stuart (1973) using Brown Norway (B.N.) ascites tumours as targets plated as monolayers in wells. Lewis (L.) rats were immunised by intraperitoneal injection of B.N. spleen cells or unilateral renal transplantation with Lewis x Brown Norway F_1 hybrid ($LBNF_1$) kidneys, and killing was obtained with the immunised lymphocytes from 5 to 50 days after the primary immunization.

Lucas and Walker (1974) described a similar cell counting microcytotoxicity test using effector cells from rats sensitised by skin and renal allografting. The target cells plated in the wells of microtest plates were both lung fibroblasts and tumour cells. This work was done in conjunction with a chromium release assay and the results will be mentioned later (see below).

For experiments designed to measure lymphocyte cytotoxicity obtained by organ grafting against normal non tumour tissue, a microcytotoxicity test (M.C.T.) has been devised by Pierce, Quadracci et. al., (1971). This M.C.T. has been applied to the clinical situation. They studied 26 recipients who had received living related donor allografts and were on routine immunosuppressive therapy with azathioprine, prednisolone and anti-lymphocyte globulin (A.L.G.). Donor skin fibroblasts were tissue cultured and used as target cells and plated in microtest plates containing 96 wells of 0.3 ml. capacity. Peripheral blood lymphocytes from the

recipient were added to pre-incubated target cells at a ratio 3,000 : 1 and incubated for a further 36 hours and stained. Control lymphocytes were obtained from normal human volunteers. Cytotoxicity was demonstrated by the reduction in target cells by 20% in the wells containing recipient lymphocytes as compared with control. Out of 42 tests in 26 patients, lymphocytes in 85% of the tests were cytotoxic in the first 12 months, but then only 13% of the lymphocytes were cytotoxic after this. In the first month there was poor correlation, but from the second to twelfth month excellent correlation between the amount of cytotoxicity and the number of rejection episodes, and the amount of prednisolone required to reverse these episodes. This test only gives information about the immune status of the recipient at the time of the experiment therefore frequent observations are required and are only possible if live donor target cells are available.

Pudifin et. al., 1973 have also reported a specific cytotoxic effect on the part of recipient lymphocytes in humans. Target cells were obtained fresh from the liver of human cadaver donors and tissue cultured. The lymphocytes from one recipient were found to be cytotoxic and from a second not cytotoxic, three weeks after transplantation. The first recipient had a transplant nephrectomy two weeks later for rejection. The second recipient has a functioning allograft.

d. Chromium release

Incubation, in vitro, of target cells with ^{51}Chromium (^{51}Cr.) allows the uptake of ^{51}Cr. into the cell, and binding of this isotope to cytoplasmic protein (Pees, 1975). Death of the cell allows release

of the ^{51}Cr. into the surrounding media. The ^{51}Chromium release test was considered by Perlmann and Holm (1969) to be the most reliable method of determining cytotoxicity: the measurement of the ^{51}Cr. is quantitative and not dependent on subjective evidence such as cell counting. Also they stated there is no reutilization of the ^{51}Cr. at cell death, and as cytotoxicity occurs within a few hours after incubation with immune lymphocytes, longer culture conditions which allow for promotion (feeder effect) or inhibition of growth of target cells are not present.

The ^{51}Chromium test has been used by Biesecker (1973) in a rat model (see cell counting). The target cells chosen were B.N. fibrosarcoma ascites forming tumour cells which gave a spontaneous release (when targets were incubated with media alone) of 15 to 30% at 6 hours. Having immunised Lewis rats with ($LBNF_1$) kidneys or B.N. fibrosarcoma allografts the sensitised spleen cells were incubated with the target cells. The primary response was first detected by an increase in ^{51}Cr. release on the third day with a peak at day 5, disappearing on day 12. The secondary immune response was also detected on the third day, was maximum on the fourth day and disappeared on the sixth day. The results of the ^{51}Cr. release corresponded closely to the appearance of clinical rejection in allografted rats. On day 4 and 5 a rise in blood urea nitrogen was detected in those rats receiving an allograft whose contralateral normal kidney was removed. Biesecker et. al., (1973) also compared their ^{51}Cr. release results with the M.C.T. The cell mediated immunity demonstrable on ^{51}Cr. release was transient, whereas with the M.C.T. cytotoxicity could be demonstrated from 5 to 50 days. They could give no reasons for the difference between these two tests.

Brunner, Mauel, Cerottini and Chapus (1968) also described a $^{51}Cr.$ release assay in the animal model described in cell counting (see above). They again compared the efficiency of the assay with the inhibition of cloning efficiency. The percentage release of chromium from the target cells on addition of sensitised lymphoid cells in 12 tests was:- 19% to 50% at three hours, 41% to 90% at six hours, indicating that most of the target killing in this test had occurred within six hours. These workers obtained a reduction (21% to 64%) in cytotoxicity with prior incubation of the sensitised lymphocytes with drugs, actinomycin D and cyclohexamide which block protein synthesis.

In the allograft situation in which tissue rather than tumour sensitization was used, Husberg (1972) described a similar technique of $^{51}Cr.$ release. Using donor kidney cultured fibroblasts from rats as target cells and immune thoracic duct lymphocytes as effector cells he found immune lymphocytes alone destroy allogeneic kidney cells. The addition of immune sera and complement made the destruction more aggressive. In this work Husberg found no alteration in the killing ability of the sensitised lymphocytes from rats receiving immunosuppression with cyclophosphamide. There was a quantitative effect on the lymphocytes, with a reduction from $6,600/cm^3$ to $3,000/cm^3$ in the peripheral blood lymphocytes in the rats who had received cyclophosphamide.

In man, Wolf, Fawley and Hume (1971), used kidney cells obtained from a rejected kidney at the time of transplant nephrectomy as target cells. Lymphocytes were obtained from recipient peripheral blood. With a chromium release assay they showed, in 16 patients, destruction of the renal cell monolayer when reacted with recipient

lymphocytes, in the absence of human serum or complement. The reaction was complete in 12 hours when an average of 47% (range 30% to 70%) release was obtained as against a 20% release from target cells alone.

A chromium release assay was used by Lucas et. al., (1974) comparing the results with a cell counting method and ^{86}Rb incorporation into target cells (at cell death K^{+} leaves the target cells and ^{86}Rb is taken up into the dead cell). The study demonstrated that both short (^{51}Cr. release) and long term (M.C.T.) assays measured the same actions. However the chromium release assay, with low ratios of lymphocytes to target cells and high spontaneous release, was thought to present such formidable conditions that only a cell counting method would have sufficient sensitivity to detect cytotoxicity in the primary response to both skin and renal allografts.

2. Cell populations used in in vitro cytotoxicity tests

Targets

The target cells are obtained from the donor animal or donor inbred strain. In the tests already described the targets have been either tumour or normal tissue. Tumour cells in animals are easy to obtain and culture. Established cell lines are an advantage as they are easy to culture as monolayers and consist of one cell type. Fibroblasts are also easy to culture and are commonly used as normal tissue targets, but these cells are difficult to kill in comparison with tumour cells. Skin fibroblasts have been used in cytotoxicity test for human lymphocyte activity, and this is satisfactory when live donor kidneys are being transplanted. There is a problem in obtaining target cells when human cadaver kidneys are grafted, although tissue cultured cells of organs removed at death can be used for a short time.

Effector cells

The cells from sensitised recipients, in the experiments already described and in this work are obtained by in vivo sensitization. The effector cells used are lymphocytes, cytotoxicity is a function of live lymphocytes and therefore it is essential that 90 to 100% of the lymphocytes are viable (Perlmann and Holm, 1969). The efficiency of various types of effector cells has been compared by Takasugi and Klein (1970). Spleen cells and peritoneal cells were the most effective; followed by peripheral blood lymphocytes, thymocytes; lymph node lymphocytes were the least effective as killer cells.

In vitro cytotoxicity is due to immune T lymphocytes and is largely independent of B lymphocytes, alloantibody producing cells

or macrophages. This was shown by Brunner and Cerottini (1971). In vitro cytotoxicity was abolished in mice by treatment of immune spleen cells or peritoneal cells with anti θ serum and complement, b but was unaffected by the removal of macrophages using adherence columns, or by pre-treatment with anti-macrophage serum and complement. Cytotoxicity by immune T cells was also confirmed by transfer of thymus cells into irradiated mice. Other workers, Golstein, Blomgren, Svedmyr and Wigzell (1973) have shown that T cells are necessary for anti-allogeneic cell mediated immunity and they alone are sufficient without the participation of other cells.

Although T cells have been shown to destroy target cells in vitro it has recently become apparent that lymphoid cells other than T cells can exert a cytotoxic effect on target cells. A cytotoxic cell has been found which is a non T cell, removed through an anti-immunoglobulin coated column, and is called a K cell (Allison, 1974).

Perlmann and Holm (1960) felt that thoracic duct cells should be used for cytotoxicity experiments as the lymphocyte populations should be free of monocytes (macrophages) and polymorphs which can themselves be cytotoxic. This has been confirmed by Lucas and Walker (1974), who found that both sensitised and control (non sensitised) spleen cells had a cytotoxic effect on allogeneic target cells although the sensitised spleen cells had a greater added specific cytotoxicity; the absorption of spleen cells to remove the macrophages eliminated the non specific cytotoxicity and only the sensitised cells then demonstrated cytotoxicity.

Other work has been performed to identify the killer cells by extracting cells from a rejecting graft. Strom, Tilney, Carpenter and Busch (1975) extracted the cells from failed (rejected) human

allografts. Using a ^{51}Cr. release method described by Brunner et. al., (1968) and using donor peripheral blood lymphocytes as target cells they found good correlation between the extent of in vitro cytotoxicity by the extracted lymphocytes and the histological grading. Methods were described using E rosettes, EA rosettes and EAC rosettes[1] to determine which mononuclear cells appeared to be doing the damage. In early graft failure the T cell was the prominent killer cell, but later in rejection a mixture of T and non T cells were cytotoxic. Also the recipient graft cells had a greater capacity for killing than the recipient peripheral blood lymphocytes taken and tested at the same time.

Perlmann and Holm (1969) have shown that lymphoid cells which are from non sensitised (normal) donors can be induced to kill in vitro by activation into blast cells with PHA.

1 see glossary

3. The effect of antibody on cytotoxicity in the allograft response

Blocking factors directed against cytotoxic lymphocytes have been demonstrated by Hellström and Hellström (1972).in the colony inhibition test, and by Pierce et. al., (1971) with a M.C.T. Pierce et. al., (1971) found that in humans the addition of post transplant recipient serum decreased in vitro lymphocyte cytotoxicity. This in vitro effect was reflected in vivo in a group of patients, in whom M.C.T. showed cytotoxic lymphocytes the effect of which was blocked by serum: clinically these patients had a smooth course. Hellstrom and Hellstrom also monitored human recipients of kidney grafts; they found that the lymphocytes of patients who were clinically without signs of graft rejection, behaved in one of two ways. One third of the patients lymphocytes were not cytotoxic, two thirds had cytotoxic lymphocytes, but the cytotoxic effect was blocked by serum from the graft recipient. Patients in whom there were one or several rejection crises showed cytotoxic lymphocytes with no blocking serum.

Cytotoxicity in the tumour response

Experimental animal tumours express antigens capable of eliciting tumour immune rejection responses. This has been clearly established in *in vivo* studies where exposure to syngeneic tumour results in the development of immunity. Lymphoid cells of mice carrying graft of methylcholanthrene induced (M.C.) sarcomas were transferred to syngeneic mice (Rosenau and Morton, 1966). Direct challenge of tumour to the recipient mice did not lead to growth of the tumour. Also lymphoid cells from immune mice were incubated with a suspension of tumour and transferred into irradiated syngeneic mice and no growth of the tumour occurred. For obvious ethical reasons evaluation in vivo of immunity in human cancer patients against transplanted cancer cells is not possible.

Tumour specific antigens on most chemically-induced tumours are characteristic for the individual tumour, whilst most virus-induced tumours show specificities related to the oncogenic virus. In vitro tests indicate that most human tumours express antigens which are characteristic of the histological type of tumour (Baldwin and Robins, 1976).

Considerable evidence from *in vitro* studies has been held to confirm the immune response to experimental animal tumours. A number of methods have been used to study lymphocyte cytotoxicity against tumour specific antigen.

Rosenau and Morton (1966) used a cell counting technique; they found that the lymphoid cells from mice bearing tumour grafts did inhibit the growth of tumour target cells by 71%, the in vitro test was not as sensitive as the in vivo test in which the suppression of tumour growth was 100%. The colony inhibition assay was first

described by Hellstrom (1967) to allow measurement of the effects of immune lymphoid cells on target tumour cells in culture. The target cells in this test were seeded at low density on to petri dishes and after attachment the medium was replaced by lymphoid cells. The dishes were incubated for 3 to 7 days, then the colonies stained and counted. Target killing was said to have occurred when there was a reduction in colonies after exposure to sensitised lymphocytes compared with those in the appropriate controls. Another micro-cytotoxicity assay has been developed in which the tumour cells are seeded into wells of microtest plate, and after incubation with effector cells for 36 to 72 hours the numbers of individual tumour cells surviving on each well are counted. This micro-assay (M.C.T.) was developed by Takasugi and Klein (1970) where sensitised spleen cells were obtained from mice hyperimmunised to M.C. sarcoma. The mice received weekly intraperitoneal (i.p.) injections of irradiated sarcoma cells. The immunised spleen were cytotoxic against the tumour; moreover the sensitised cells were specifically cytotoxic as they showed no effect against other tumour target cells.

This M.C.T. has been extensively used by Baldwin, Embleton and Robins (1973), and the techniques of these workers have been closely followed in the experimental work of this section. They sensitised syngeneic rats either by multiple injections of irradiated chemically induced hepatomas (4-dimethylaminoazobenzene, DAB) or by primary transplanted tumour grafts. The lymphocytes from the rats bearing primary grafts were cytotoxic in 5 out of 7 tests, and the lymphocytes from hyperimmunised animals in 13 out of 17 tests. No cytotoxic antibody was found in the animals with primary grafts until the grafts were excised. Repeated immunization of the rats gave high

levels of cytotoxic antibody.

Further modifications of M.C.T. have been introducted using isotopically labelled ^{125}1-iododeoxyuridine (^{125}IUdR) cells (Seeger and Owen, 1973). The ^{125}IUdR may be incorporated into the target cells prior to their incubation with lymphocytes. The ^{125}IUdR can al also be used in 'post labelling' techniques; the targets and lymphocytes are incubated together for at least 48 hours and washed. The remaining target cells are labelled with ^{125}IUdR for a further 24 hours and counted on a γ counter. This post labelling technique avoids the problems of visual counting and the problem of reutilization of isotope (Ghaffer, Cullen, Dunbar and Woodruff, 1974) and at present appears to be the most satisfactory of the micro-cytotoxicity tests.

The release of ^{51}chromium from labelled tumour cells has also been used as an index of lymphocyte cytotoxicity in animal tumours (Pearson, Hodis and Friberg, 1969), and also the release of ^{3}H proline as a method of studying cytotoxicity in human bladder tumours, (Bean, Pees, Rosen and Oettgen, 1973).

The results of in vitro studies with human tumours must however be considered more critically. The early enthusiastic reports of in vitro cytotoxicity in human tumours are now doubted (Takasugi, Mickey and Terasaki, 1973) as much of the cytotoxicity appears to be non-specific. Many of the technical problems related to the in vitro tests in the tumour immune response have been discussed by Pees (1975) mainly in relation to human tumours. He suggests that primary cultures of target cells are better than long term cell lines. The tumour cells after at least one passage in vitro are usually free of blocking factors. He reiterates the problem of target cell

proliferation in M.C.T. and therefore favours the use of isotope release assays, where growth and cell division have only an insignificant influence.

The T lymphocyte has been shown to be the cytotoxic effector cell in allograft rejection (see above). The role of T lymphocytes in tumour immunity is less clear. Pees (1975) maintained that for cytotoxicity against tumour associated antigen, T cells are required in association with non T cells. Macrophages may become effector cells either by specific immunization, or non immune macrophages may become effector cells by virtue of reaction with factors (specific macrophage arming factor, S.M.A.F.) liberated by the sensitised lymphocytes (Evans and Alexander, 1971). Tumour cell killing by normal lymphoid cells in the presence of antibody has been implicated in a number of tumour systems, and it has been suggested that the antibody dependent cytotoxic cell is a "null" or "K" cell (Greenberg and Shein, 1973). Serum from tumour bearing individual has been shown to interfere with cellular immunity (Hellström and Hellström, 1969); this blocking activity is thought to be due to antigen : antibody complexes (Baldwin, Price and Robins, 1972) which protects tumour cells from attack by sensitised lymphocytes.

Conclusion on the published work from other centres

Cytotoxicity has been demonstrated with ease against tumour specific antigen in both in vivo and in vitro animal experiments although not with human tumours. Lymphocyte cytotoxicity has been obtained in vitro in the allograft response, in both animals and humans, although much of the work reported has been with tumour allografts or using tumour cells as target cells. The cell counting techniques and the isotope release assays do not, in the same systems, necessarily correlate well and most workers appear to favour isotope release methods as being more accurate due to lack of subjective observation, also the tests are short term and therefore cell growth and division do not influence the results.

The immune response in vivo to an allograft whether an organ or tumour graft is much stronger than the response to syngeneic tumour, yet the virulence of the in vivo response to an allograft is not mirrored by the in vitro cytotoxic response. The in vivo immune reaction against tumour is weak with relatively little or no infiltration of tumour with lymphoid cells, whereas in vitro lymphocyte cytotoxicity has been demonstrated by sensitised lymphocytes against tumour target cells in both laboratory animals and man with greater ease.

B. AIMS

The efferent arc of the immune response to an allograft is represented by the direct cytotoxic action of specifically sensitised effector T lymphocytes on the cells of the graft.

The present work has been performed to make full investigation into the cytotoxic properties of the individual lymphocyte. The recipient animal has been sensitised *in vivo* by either allograft or tumour. The *in vitro* tests measure the capacity of the recipient lymphoid cells primed in this way to kill either donor tissue cells or tumour cells. Direct comparison of the *in vivo* effect, with *in vitro* cytotoxicity may then be made.

In vitro cytotoxicity has been demonstrated by several previous workers either using hyperimmunised cells as killer cells, or by using easily lysed cultured tumour cell lines as target cells. The intention of the experiments which follow is to reproduce cytotoxicity in these situations - and then by changing one parameter at a time (either killer cells or target cells) to move towards a reproduction of target cell killing in the allograft response (killing of target cells derived from the allograft by cells from the animal rejecting the allograft). (Diagram 2)

C. METHODS

All the experiments have been performed in rats: two in vitro methods of evaluating cytotoxicity, cell counting and a chromium release assay have been used. In Appendix Ia a full description of the methods is given.

Experiment 1 (Diagram 2)

Wistar rats were immunised with syngeneic tumour - hepatoma or methylcholanthrene induced sarcoma - from the same strain of line inbred Wistar rats (Appendix Ib). The effector lymphocytes were removed from the recipient (Appendix Id) and tested for cytotoxicity against tissue cultured tumour target cells (Appendix Ib).

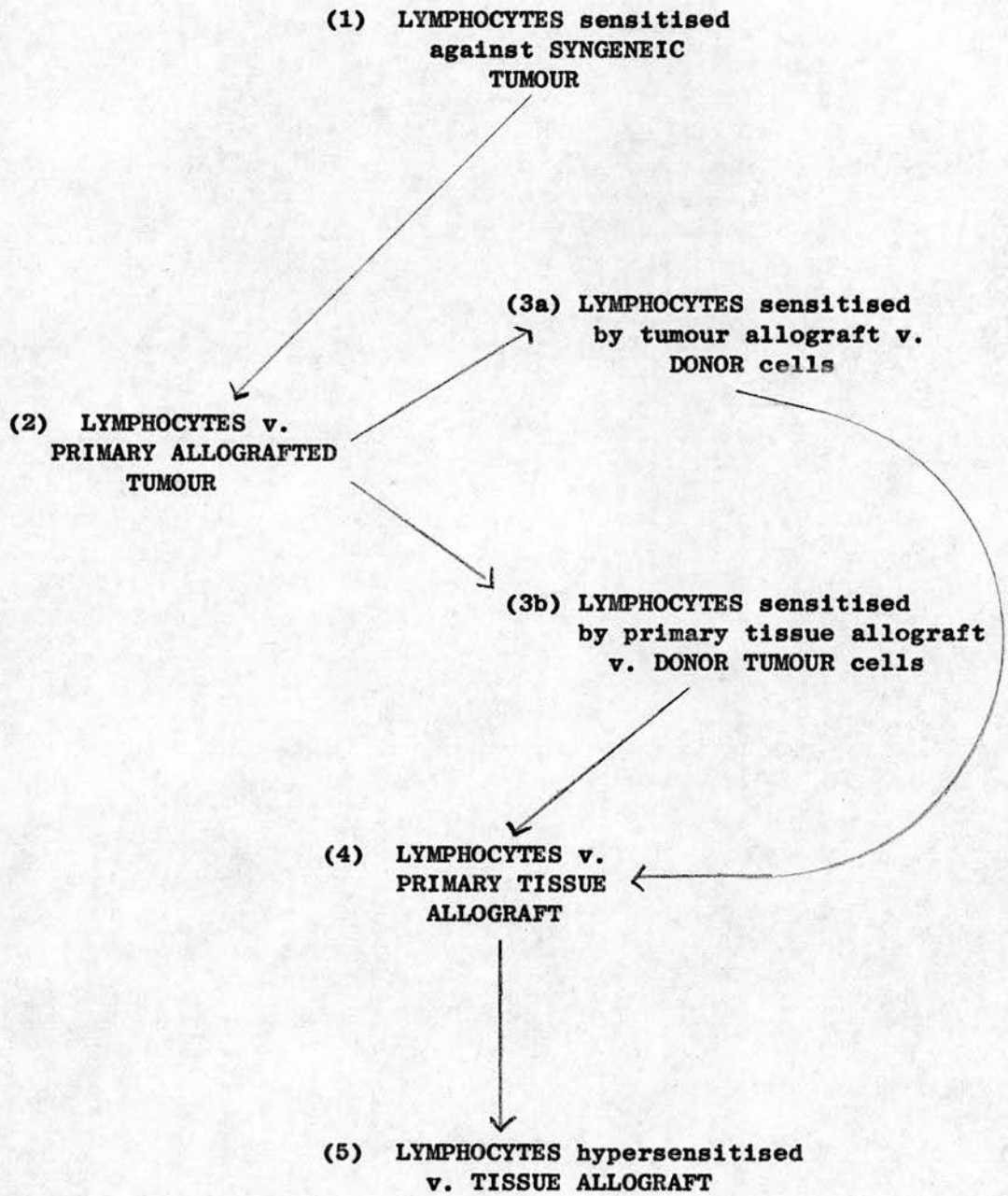
Experiment 2

The same hepatoma and methylcholanthrene induced tumours from inbred Wistar rats were excised and transferred as a primary tumour graft (Appendix Ic) into either allogeneic outbred Sprague-Dawley (S.D.) rats or inbred A.S. rats thus altering the sensitising antigens from tumour specific antigens to transplantation antigens. The recipient lymphocytes were tested for cytotoxicity against the same tumour target cells as experiment 1.

Experiment 3

a) The target cells were altered to tissue cultured fibroblasts of the donor Wistar strain (Appendix Ic). The effector cells remain the same as in experiment 2 and are from allogeneic recipients sensitised by transfer of tumour from the donor strain.

b) The target cells were tumour cells of the donor strain



METHODS OF SENSITIZATION IN LYMPHOCYTE CYTOTOXICITY TESTS

Numbers in brackets indicates the numbers of the experiments.

Diagram 2

(experiment 1 and 2). The effector cells were obtained by sensitization of allogeneic A.S. rats by the transfer of a skin graft from the donor Wistar strain and the recipient lymphocytes were tested for cytotoxicity against the tumour target cells.

Experiment 4

The target cells were again tissue cultured fibroblasts of the donor strain. The effector cells were obtained from S.D. rats who had received primary kidney or skin grafts (Appendix Ic) from the inbred Wistar donor strain. This experiment again measures the cytotoxicity in the primary response against transplantation antigen.

Experiment 5

Cytotoxicity in the hyperimmune situation was also compared with that of the primary response. The target cells were again tissue cultured fibroblasts of the donor strain. The effector cells were obtained from either outbred S.D. or inbred A.S. rats, who had received three skin grafts, at intervals of two weeks, and two intraperitoneal injections of lymphocytes from the donor Wistar strain.

Techniques of tissue culturing, immunization by syngeneic and allogeneic tumour, skin and kidney grafting and preparation of lymphocytes are described in Appendices Ib, Ic and Id.

Target Cells

All the target cells in these experiments were from short term tissue cultures of tumour or fibroblasts. The tumour cells were found to grow optimally in Eagles Hepes medium plus 10% foetal calf serum (FCS) and the fibroblasts in Waymouths plus 10% FCS. The

target cells were used for the cytotoxicity tests after about two weeks from the start of culturing, when the division of the cells had settled down to a slow but steady rate. If the target cells were used before two weeks, overcrowding in the test plates occurred.

Effector cells

The effector cells in these experiments were either lymph node lymphocytes, obtained from mesenteric, cervical and axillary lymph nodes; spleen cells, thoracic duct lymphocytes or blood lymphocytes. The preparation of these cells is described in Appendix Id. The cells obtained were mostly lymphocytes, although the lymph node and spleen preparations contained macrophages. The red cells were separated from the spleen preparations. In most of the experiments no effort was made to identify the different T, B and macrophage populations and their capacity to kill target cells. Some experiments were performed with T cell and B cell enriched populations obtained by fractionation on a nylon wool column (see Appendix Id). These separate fractions were tested for cytotoxicity.

Controls

In these experiments lymphocytes of non sensitised animals were used as effector cell controls. In the syngeneic tumour experiments (1) the control cells were obtained from non immunised syngeneic Wistar rats. In the experiments involving allogeneic transfer of either tumour or grafted tissue (2, 3, 4, 5) the control lymphocytes were obtained initially from animals syngeneic to the donor.

In later experiments the controls were changed to the use of

lymphocytes from unsensitised animals syngeneic to the immunised recipient.

D. RESULTS

1. Cell counting

Experiment 1 Syngeneic tumour

Experiment 2 Allogeneic tumour

Experiment 3

Experiment 4 Primary allogeneic tissue grafts

Experiment 5 Allogeneic hyperimmunization

Summary of results

2. ^{51}Chromium Release Assay

1. Cell counting

The results of the microcytotoxicity test using a direct cell counting method are considered first in each experimental group. A complete microcytotoxicity experiment is shown in Appendix Ie with the method of immunization, plating of microtest plates and the calculation of cytotoxicity. Significant killing is considered to have occurred when there is a reduction in the remaining target cells after incubation with immune lymphoid cells, compared with the number of target cells after incubation with non immune control lymphoid cells: a Student's T test for unpaired data is applied to each experiment as the test of significance. Cytotoxicity is thus considered to occur only when there is a reduction of viable cells in the test wells of approximately 20%.

Experiment 1 Syngeneic Tumour

Wistar rats were immunised with syngeneic tumour and the effector cells from these rats were tested against target cells of the tumour which had been raised in tissue culture.

Rats were immunised with D23 hepatoma and their lymphocytes were tested against cells of this tumour. The results are shown in Table I. Using unfractionated lymph node lymphocytes (LNL) as killer cells cytotoxicity was obtained in only 4 out of 16 experiments. Against this in 2 experiments there was apparent killing by control cells in that the number of target cells in the wells at the end of the experiment were reduced in comparison with the wells to which test lymphocytes had been added. One experiment was carried out using spleen cells as the effector lymphocytes and cytotoxicity was obtained in this experiment. When fractionation of the lymph node lymphocytes was used, killing was obtained in 3 out of 8 experiments by T (eluted) lymphocytes. It was noteworthy that in 5 of the 8 experiments where B (retained) lymphocytes were used, "control killing" was obtained. Two other tumours (MC7 and MC57) were used to hyperimmunise rats and the lymphocytes from the rats were tested for cytotoxicity against the cells of these tumours which had been tissue cultured. The results are shown in Table II. Using lymph node lymphocytes as the effector cells in only 3 out of 17 experiments was killing of tumour cells demonstrated and against this killing by the control unsensitised lymphocytes was obtained in 4 out of 17. When spleen cells were used as the effector cells, killing was obtained in 1 out of 2 tests. Fractionation of the cells gave no killing and in fact 5 out of 6 tests with retained cells (B cells) showed control killing, 2 out of 6 tests with eluted cells (T cells) also showed control killing.

Tables I and II

Cytotoxicity achieved with lymphocytes and spleen cells from rats hyperimmunised with various syngeneic tumours. Some cytotoxicity was achieved.

TARGETS	EFFECTORS	SENSITIZATION	RATIO EFFECTORS : TARGETS	KILLING 1	2 (a)	2 (b)	3 (a)	3 (b)	4 (a)	4 (b)	5
D23	L.N.L.	Syngeneic Hyperimmunised	4,000 : 1	-			+	-	-	(-)	-
			2,000 : 1	(-)	-	+	+	-	-	-	-
			1,000 : 1		-	+					
	L.N.L. fractionated	"	Eluted								
			4,000 : 1				-	-	-	+	
			2,000 : 1				+	-	-	+	
			1,000 : 1								
			Retained								
			4,000 : 1				(-)	-	-	(-)	
			2,000 : 1				(-)	(-)	-	(-)	
			1,000 : 1								
	Spleen cells	"	4,000 : 1								
			2,000 : 1								+
			1,000 : 1								+

+ denotes significant reduction in target cells by the addition of immunised lymphocytes as compared with controls. (-) denotes significant reduction in control wells as compared with test wells. Numbers denote the number of the experiments using syngeneic tumour.

Table I Experiment 1

TARGETS	EFFECTORS	SENSITIZATION	RATIO EFFECTORS : TARGETS	KILLING 6	7	8	9 (a)	9 (b)	10	11
MC7	L.N.L.	Syngeneic Hyperimmunised	4,000 : 1				-			
			2,000 : 1		-	(-)	-	(-)	-	+
			1,000 : 1		-	(-)	-	(-)	-	+
	L.N.L. fractionated	"	Eluted							
			2,000 : 1	-		(-)				
			1,000 : 1	-		(-)				
			Retained							
			2,000 : 1	-		(-)				
			1,000 : 1	(-)		(-)				
	Spleen cells	"	2,000 : 1						-	
			1,000 : 1						+	

TARGETS	EFFECTORS	SENSITIZATION	RATIO EFFECTORS : TARGETS	KILLING 12	13
MC57	L.N.L.	Syngeneic Hyperimmunised	2,000 : 1	-	-
			1,000 : 1	-	+
	L.N.L. fractionated	"	Eluted		
			2,000 : 1	-	
			1,000 : 1	-	
			Retained		
			2,000 : 1	(-)	
			1,000 : 1	(-)	

Table II Experiment 1 continued

Experiment 2 Allogeneic Tumour

In these experiments a primary grafted tumour was transferred to an allogeneic rat. The lymphoid cells were removed at various times after grafting and used to test for cytotoxicity against the tissue cultured tumour cells. The results are shown in Tables III, IV and V. Killing was not consistently achieved with immune LNL but was achieved in 50% of the tests carried out between the fourth and eighth day after sensitization. Thereafter the number of tests showing any cytotoxicity fell away and no cytotoxicity was demonstrated on the part of cells taken later than 12 days after sensitization. In only 4 of the tests was there "control killing" and it is perhaps noteworthy that 2 of these were on the fourth day after sensitization. When spleen cells were used as the effector cells killing was only occasionally obtained and appeared to require a higher ratio of effector to target cells. There was a difference between the results dependent upon the tumour used - very little cytotoxicity was obtained against the MC57 and D192 tumour cells.

Tables III and IV

Cytotoxicity achieved with lymphocytes and spleen cells from rats immunised by a primary allogeneic tumour graft. Cytotoxicity achieved between the 5th and 12th day after grafting.

TARGETS	EFFECTORS	SENSITIZATION	RATIO EFFECTORS : TARGETS	KILLING 1	2	3	4	5	6	
D23	L.N.L.	Primary graft D23 tumour Allogeneic	4,000 : 1	+	(-)	(-)		+	-	
			2,000 : 1	-	(-)	-	-	+	-	
			1,000 : 1				-	+	-	
			100:: 1					-	-	
				8	4	14	22	6	13	Days after immunization
				7	8					
MC57	L.N.L.	Allogeneic	4,000 : 1	-	(-)					
		Primary MC57 tumour graft	2,000 : 1	-	-					
				5	15					Days after immunization

Table III Experiment 2

TARGETS	EFFECTORS	SENSITIZATION	RATIO EFFECTORS : TARGETS	KILLING				
				9	10	11	12	
D202	L.N.L.	Allogeneic Primary graft D202 tumour	4,000 : 1			+		
			2,000 : 1	+	-	-	-	
			1,000 : 1	+	-		-	
	Spleen cells	"	4,000 : 1			+		
			2,000 : 1	-	+	-	-	
			1,000 : 1	-	-		-	
				5	8	12	22	Days after immunization
				13	14	15	16	
D192	L.N.L.	Allogeneic Primary graft D192 tumour	4,000 : 1					
			2,000 : 1	-	-	-	-	
			1,000 : 1	+	+	-	-	
	Spleen cells	"	4,000 : 1					
			2,000 : 1	-	-	-	(-)	
			1,000 : 1	+	-	-	-	
				4	7	11	14	Days after immunization

Table IV Experiment 2 continued

Days	4	5	6	7	8	11	12	13	14	15	22
Test killing (number)/(total)	1/4	2/4	3/4	1/2	1/4	0/2	1/2	0/4	0/4	0/2	0/4
Control killing	2/4	–	–	–	–	–	–	–	1/4	1/2	–

Cytotoxicity using effector lymph nodellymphocytes against primary allografted D23, D192, D202 and MC57 tumours. The numerator is the number of tests in which killing occurred over the number of tests performed.

Table V

Experiment 3

This series of experiments was divided into 2 parts.

a. Rats were sensitised by primary tumour allograft and lymphocytes from these rats were used as effector cells against fibroblasts cultured from embryos of the tumour bearer strain.

b. Rats were sensitised by primary skin allograft and lymphocytes from these rats were used as effector cells against tumour cells from a tumour which had been induced in the donor strain.

Experiment 3a (Table VI)

In 3 out of 8 experiments carried out with lymph node lymphocytes between the fifth and the twelfth day after sensitization, killing was obtained against fibroblasts of the tumour bearer strain. In 1 out of 8 tests using spleen cells killing was obtained.

Experiment 3b (Table VI)

No cytotoxicity whatever was obtained in 8 tests using both lymphocytes and spleen cells sensitised by primary skin grafting against cells from a tumour which had been induced in the donor strain.

Table VI

Cytotoxicity with lymphocytes and spleen cells from rats in 'cross-over' experiments.

a) The rats were immunised by an allogeneic tumour graft and the effectors were tested against fibroblasts of the donor strain. Some cytotoxicity was achieved.

b) Immunisation was by primary skin grafting and the effectors were tested against tumour cells of the donor strain. No cytotoxicity was achieved.

TARGETS	EFFECTORS	SENSITIZATION	RATIO EFFECTORS : TARGETS	KILLING 1	2	3	4	
a) FIBROBLASTS (from embryos of tumour bearer strain)	L.N.L.	Allogeneic Primary graft D202 tumour	4,000 : 1			-		
			2,000 : 1	-	+	+	-	
			1,000 : 1	+	(-)		-	
	Spleen	"	4,000 : 1			-		
			2,000 : 1	-	-	+	-	
			1,000 : 1	-	-		-	
				5	8	12	22	Days after immunization
b) D192	L.N.L.	Allogeneic Primary skin graft	2,000 : 1	-	-			
			1,000 : 1	-	-			
	Spleen	"	2,000 : 1	-	-			
			1,000 : 1	-	-			
				6	10			Days after immunization

Table VI Experiment 3

Experiment 4 Primary allogeneic tissue grafts

In these experiments either skin or renal grafts were transferred to allogeneic rats (Table VII).

No cytotoxicity against donor strain fibroblasts was achieved after skin grafting using either lymph node lymphocytes, thoracic duct lymphocytes (T.D.L.) or spleen cells except in one experiment 4(2), where a reduction of 16% of the target cells occurred after the addition of LNL at a ratio of 2,000 : 1.

After renal allografting however there was definite cytotoxicity achieved using spleen cells in 3 out of 4 experiments, but not LNL (0 out of 4 experiments), at 7-8 days post grafting - experiment 4(4 and 6). It was also noted that the spleens of these rats on removal were considerably enlarged in comparison with the spleen of the rat which had received a skin graft at the same time. The control effector cells in these experiments were obtained from non immunised rats of the same strain as the recipient rat.

Table VII

Cytotoxicity with lymphocytes and spleen cells from rats immunised by primary skin and renal allografts. Cytotoxicity was achieved after renal allografting.

TARGETS	EFFECTORS	SENSITIZATION	RATIO EFFECTORS : TARGETS	KILLING 1	2	3	4	5	6
FIBROBLASTS	L.N.L.	Allogeneic Primary skin graft	range 200 : 1 - 3,000 : 1	-					
			4,000 : 1		-	-			
			2,000 : 1		+	-	-	-	
			1,000 : 1			-	-	-	
	"	Allogeneic primary renal graft	4,000 : 1						
			2,000 : 1				-		-
			1,000 : 1				-		(-)
	T.D.L.	Allogeneic primary skin graft	4,000 : 1		-				
			2,000 : 1		-				
	Spleen cells	Allogeneic primary skin graft	4,000 : 1			-			
			2,000 : 1			-	-	-	
			1,000 : 1			-	-	-	
	"	Allogeneic primary renal graft	4,000 : 1						
			2,000 : 1				+		-
			1,000 : 1				+		+
				7	5	7	7	8	8

Days after immunization

Table VII Experiment 4

Experiment 5 Allogeneic Hyperimmunization

In these experiments the recipient rats were immunised with 3 skin grafts and 2 i.p. injections of donor lymphocytes (Table VIIIa and b). Very little cytotoxicity was achieved using LNL either unfractionated or fractionated. However using spleen cells there was consistent cytotoxicity and cytotoxicity with peripheral blood lymphocytes was also achieved. In many of the experiments using spleen cells there was a reduction in the target cells after the addition of control spleen cells (non specific cytotoxicity), but there was a greater reduction of the remaining targets after the addition of immune spleen cells (specific cytotoxicity).

Two further experiments were performed in this group:

1. Hyperimmune serum was used as the effector in place of the hyperimmune lymphocytes. No cytotoxicity was demonstrated.

2. Hyperimmune serum plus hyperimmune lymphocytes was used as the effector. There was no alteration in the cytotoxicity achieved in comparison with that achieved by hyperimmune lymphocytes alone. Hyperimmune serum, plus control lymphocytes were not tested.

The final table in the results section (Table IX) is shown rather to demonstrate in greater detail the results obtained within each experiment. This is experiment 5 (13). Further reference to this table will be made in the discussion section.

Table VIII

Cytotoxicity achieved with lymphocytes and spleen cells from rats hyperimmunised by allogeneic tissue grafts or donor fibroblasts. Cytotoxicity was consistently achieved when spleen cells were effectors.

TARGETS	EFFECTORS	SENSITIZATION	RATIO EFFECTORS : TARGETS	KILLING 1	2	3	4	5	6	7	8	9	10	11	12	13
FIBRO-BLASTS	L.N.L.	Allogeneic Hyperimmunised	4,000 : 1	-	-	-	-	-								
			2,000 : 1	-	-	-	-	-		-	-	-	-	-	-	+
			1,000 : 1							+	-	-	-	-	-	-
	L.N.L. fractionated	"	Eluted													
			4,000 : 1		-											
			2,000 : 1		-				-							
			1,000 : 1						-							
			Retained													
			4,000 : 1		+											
			2,000 : 1		-				-							
			1,000 : 1						-							
Days after immunization				4	4	4	11	10	18	11	15	9	5	4	11	14

Table VIIIa Experiment 5

TARGETS	EFFECTORS	SENSITIZATION	RATIO EFFECTORS : TARGETS	KILLING 1	2	3	4	5	6	7	8	9	10	11	12	13
FIBRO-BLASTS	Spleen cells	Allogeneic Hyperimmunised	4,000 : 1				+									
			2,000 : 1				-				-	+	+	+	+	+
			1,000 : 1								+	+	+	+	+	-
	Blood Lymphocytes	"	4,000 : 1													
			2,000 : 1						+							
			1,000 : 1						+	-						
			Days after immunization	4	4	4	11	10	18	11	15	9	5	4	11	14

Table VIIIb Experiment 5

TARGETS	EFFECTORS	KILLING Immune v. Control cells TARGETS REMAINING		%	KILLING Immune cells v. media alone TARGETS REMAINING	%	KILLING Control cells v. media alone TARGETS REMAINING	%
FIBRO-BLASTS	L.N.L. 1,000 : 1	Test 45.8 ± 2.0 Control 47.1 ± 1.8	N.S.	+ 2	Test 45.8 ± 2.0 Media 33.9 ± 1.2	-35	Control 47.1 Media 33.9	-38
	2,000 : 1	Test 42.6 ± 2.6 Control 50.6 ± 2.9	p<0.02	+15	Test 42.6 ± 2.6 Media 39.1 ± 1.9	- 8	Control 50.6 Media 39.1	-29
	Spleen cells 1,000 : 1	Test 42.1 ± 2.5 Control 40.6 ± 2.4	N.S.	- 3	Test 42.1 ± 2.5 Media 33.9 ± 1.2	-24	Control 40.6 Media 33.9	-19
	2,000 : 1	Test 33.7 ± 2.1 Control 42 ± 2.6	p<.001	+19	Test 33.7 ± 2.1 Media 39.1 ± 1.9	+13	Control 42.0 Media 39.1	- 7

Sensitization of lymphocytes by allogeneic hyperimmunization

Table IX Experiment 5(13)

Summary of Results in Microcytotoxicity Tests (Diagram 2a)

1 a) Unfractionated lymph node lymphocytes sensitised against syngeneic tumour in ratios varying from 1,000 to 4,000 : 1 effector to target cells were cytotoxic in 4 out of 16 tests using D23 hepatoma, 3 out of 17 tests using M C - sarcomas.

b) Control lymphocytes especially B cells and macrophages were able to produce a non specific reduction in target cells.

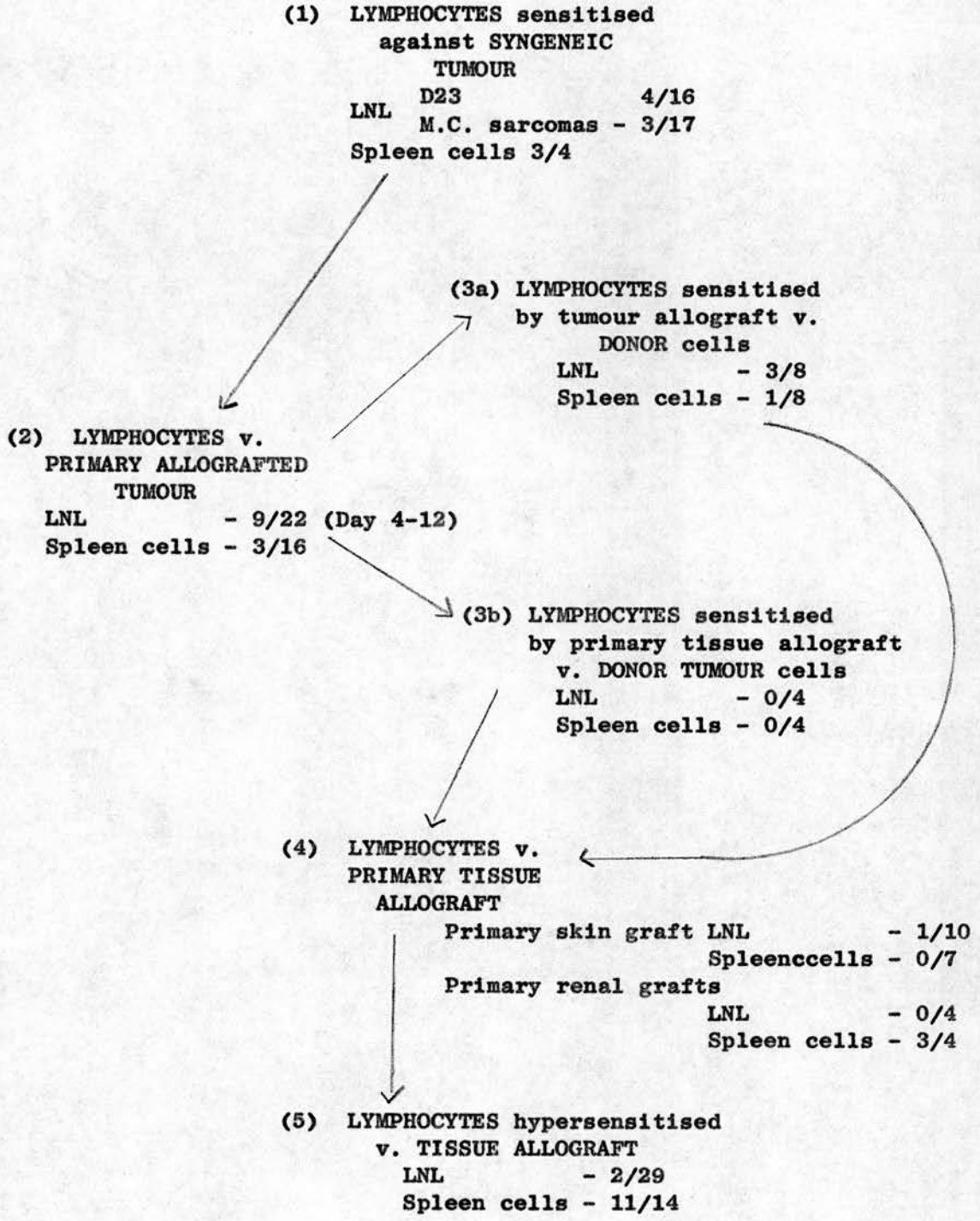
2. Lymph node lymphocytes, sensitised against primary tumour allografts were cytotoxic, especially between the fourth and twelfth day after sensitization.

3. Cytotoxicity was achieved with cross over grafting (experiment 3) when tumour grafts and not skin grafts were transferred.

4. Lymphocytes, both spleen and lymph node, sensitised against primary skin allografts are not cytotoxic. Spleen cells sensitised against primary renal allograft are cytotoxic, but not lymph node lymphocytes.

5. Cytotoxicity was easily achieved with spleen cells from hyper-immunised animals, but no cytotoxicity was obtained with lymph node lymphocytes.

6. Throughout all the experiments the addition of lymph node lymphocytes to the target cells usually caused promotion of growth of the target cells (feeder effect).



RESULTS IN LYMPHOCYTE CYTOTOXICITY TESTS

The fractions denote the number of tests where killing was obtained over the number of tests performed.

Diagram 2a

7. In many experiments the addition of non immunised spleen cells gave a non specific reduction in target cells. There was however a greater reduction after the addition of immune spleen cells which we consider is the specific cytotoxic effect.

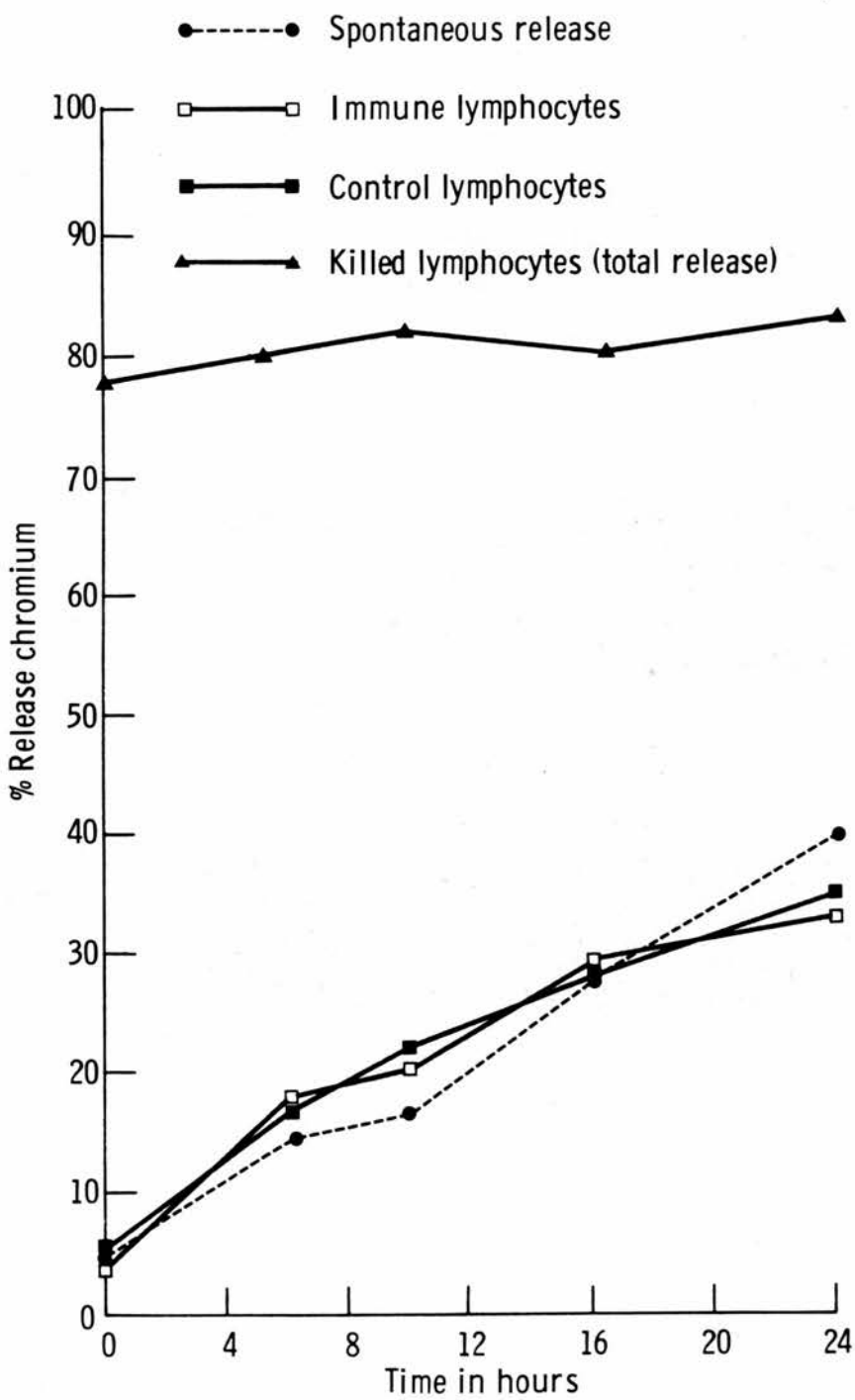
2. ^{51}Chromium release assay

A chromium release assay was performed concurrently with many of the cell counting M.C.T. The results of the ^{51}Cr. assays are shown in Table X. A complete assay is shown in Appendix Ie of the experiment 2(1), the rats being immunised by transfer of allogeneic D23 tumour. No cytotoxicity on the part of immune killer lymphocytes was achieved in this experiment which is also shown in figure 1. The spontaneous release of chromium from the labelled target cells ranged from 4.3% at the start of the experiment to 40.5% at 24 hours. There was no increase in the release of chromium with the addition of immune lymphocytes over a 24 hour period. In the concurrent M.C.T., 24% cytotoxicity was achieved with immune lymphocytes at a ratio of 4,000 : 1, the ratio of effector to target cells in the ^{51}Cr. assay was much lower at 200 : 1.

The results in Table X show that cytotoxicity was not demonstrated with this assay except in one experiment. The spontaneous release in all the experiments varied from 25% to 75% at 24 hours, the amount of release in each of the triplicate tubes over a 24 hour period was remarkably consistent. It was possible to obtain a high release on killing the cells by heating.

Figure 1

CHROMIUM RELEASE ASSAY EXPERIMENT 2 (i)



CELLS	SENSITIZATION	No. of days post sensitization	Ratio of effectors : target	KILLING
TARGET D23 EFFECTOR L.N.L.	Allogeneic 1^{o} tumour graft D23	4	200 : 1	-
		7	200 : 1	-
		11	200 : 1	-
		14	200 : 1	-
TARGET MC57 EFFECTOR L.N.L.	Allogeneic 1^{o} tumour graft MC57	5	200 : 1	70% at 8 hrs.
		13	200 : 1	-
TARGET FIBRO-BLASTS EFFECTOR L.N.L.	Allogeneic 1^{o} skin graft	5	200 : 1	-
TARGET FIBRO-BLASTS EFFECTOR L.N.L.	Allogeneic hyper-immunised	2	200 : 1	-
		3	1,000 : 1	-
		4	200 : 1	-
		4	200 : 1	-
		5	250 : 1	-
		5	200 : 1	-
		8	200 : 1	-
		8	200 : 1	-
		9	100 : 1	-
		10	200 : 1	-
		11	200 : 1	-
		15	100 : 1	-
TARGET FIBRO-BLASTS EFFECTOR Spleen cells	Allogeneic hyper-immunised	4	200 : 1	-
		11	200 : 1	-

Chromium Release Assay Results - indicates no significant killing of target cells.

Table X

E. DISCUSSION

Microcytotoxicity Test

1. Cytotoxicity against syngeneic tumour
2. Cytotoxicity against allogeneic tumour
3. Cytotoxicity in cross over grafting experiments
4. Cytotoxicity against allogeneic tissue grafts

Conclusion regarding microcytotoxicity tests

Chromium release assay

1. Allogeneic tumour
2. Allogeneic tissue grafts

DISCUSSION

Microcytotoxicity Test

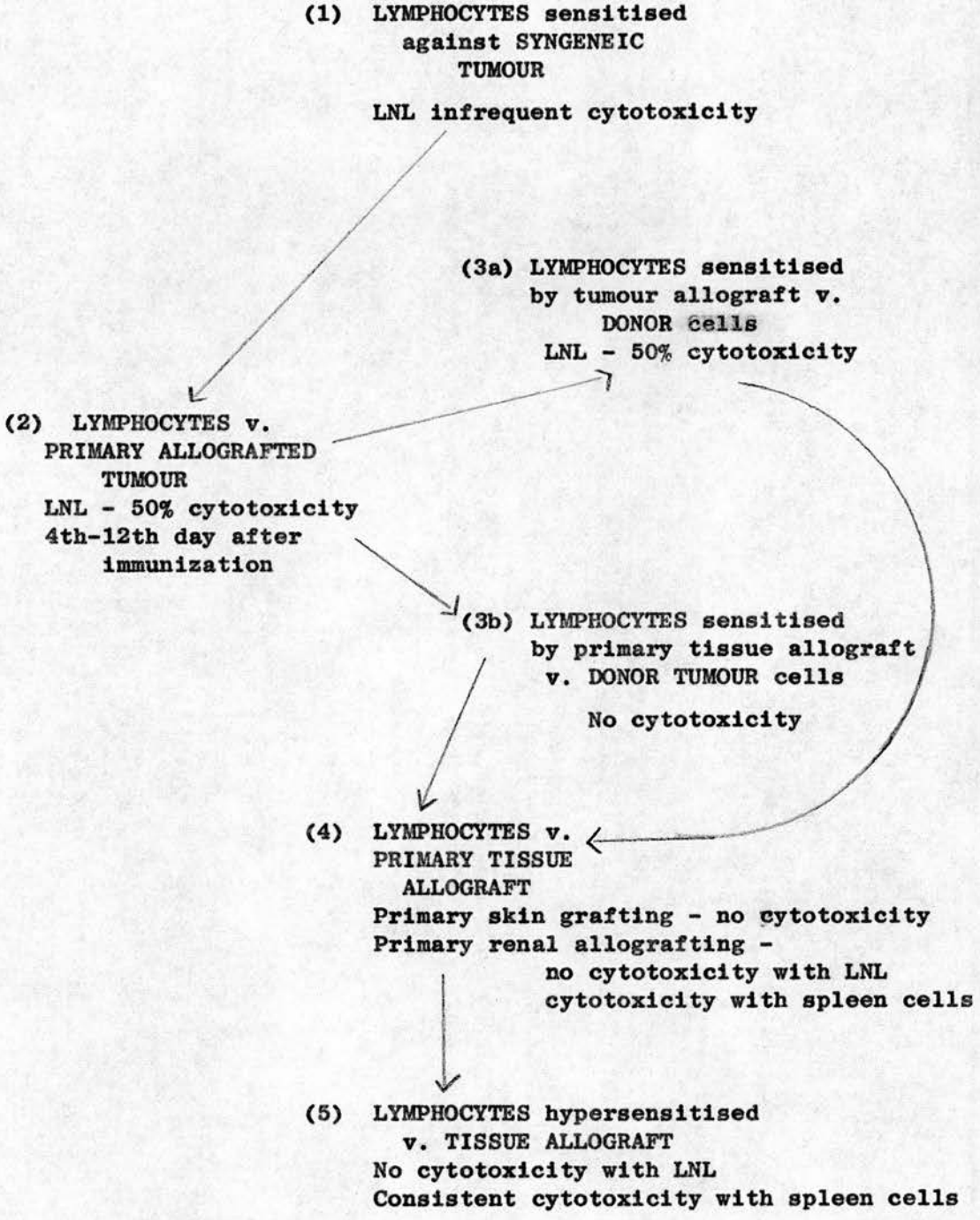
The results obtained in experiment 5 show clearly that the system used is satisfactory for the demonstration of cytotoxicity and that cytotoxicity can be achieved against fibroblasts using spleen lymphocytes as effectors. With this point established the whole experimental plan proceeded as set out in diagram 2. The results have been given in a previous section and are summarised in diagram 2b and will be considered experiment by experiment.

Experiment 1 Cytotoxicity against syngeneic tumour

The technique has been followed measuring cytotoxicity against syngeneic tumour used by Professor Baldwin and his team of the Cancer Research Campaign Laboratories, Nottingham. The method has given consistent results in the past (Baldwin et. al., 1973, Zoller, Price and Baldwin, 1975). This cytotoxicity test was studied by the present experimenters in Professor Baldwin's laboratory and was followed precisely in all respects - the tissue culture conditions, the choice of media, the effector to target cell ratios and the length of time incubation of the cells. In addition to the lymph node lymphocytes which were used as effector cells by Baldwin et. al., experiments here have also been performed using spleen cells as the effector cells.

The experiments performed here have only infrequently demonstrated cytotoxicity using LNL and in fact where 7 out of 33 tests showed killing on the part of immune cells against the tumour cells, 6 out of 33 experiments showed apparent killing by control cells.

This "control killing" which has been referred to several times in the results section merits examination in greater detail and



CYTOTOXICITY OBTAINED IN MICROCYTOTOXICITY TEST

Diagram 2b

includes 2 separate phenomenon. The first of these is that sometimes control (unsensitised) cells do demonstrate killing. As there is considerable reduction in the number of target cells remaining at the end of the experiment in the wells to which control lymphocytes have been added in comparison with wells which only hold target cells and media. The second phenomenon is that of the feeder effect. This occurs when there is an increase in the number of target cells found at the end of the experiment in those wells to which lymphocytes have been added (Table IX). Either immune or control lymphocytes can demonstrate this effect. It is clear from this that a variety of effects can be obtained which leads to difficulty in the final interpretation of the experiments. Thus what has been referred to as "control killing" in the results section, can either be true killing by control cells or on occasions can be a greater stimulatory effector on the part of immune cells than on the part of control cells. It is noteworthy that the retained cells (B cells plus macrophage fraction) consistently demonstrated this stimulatory effect.

Experiment 2 Cytotoxicity against allogeneic tumour

It might be expected that the greatest ease in obtaining cytotoxicity would be by the transfer of tumour from one animal to a non identical animal across a major histocompatibility barrier (Ag-B in the rats). The inbred Wistar donor strain (WAG) used has known genes a,c,H-1^{W},HBA; the recipient inbred A.S. strain has c,H-1^{1} (Testing and Staats, 1973). The antigenic makeup of the outbred S.D. strain is not known. Even though M.L.R. performed between WAG and AS lymphocytes by Dr. Colin Brooks of the Cancer Research Campaign Laboratories, Nottingham (Appendix 2b) showed

very little incompatibility, the tumour grafts, skin grafts and kidney grafts made across these strains all rejected acutely.

The first experiments in this group were performed using D23 and MC7 tumours. These tumours have been passaged for 11 and 5 years respectively. Therefore 2 newly induced hepatomas, D192 and D202 have also been used as it was felt that newly induced tumours were more likely to have retained antigenic makeup. Symes (1965) has shown that immunological stimulation in spontaneous mouse tumours decreases with an increasing number of passages of the tumour.

The method of immunization used in this experiment (transfer of tumour into the buttocks plus intraperitoneal injection of tumour) has proven to be a satisfactory immunization procedure in many other experiments.

In this experiment cytotoxicity was demonstrated with reasonable consistency using cells taken between 4 and 12 days after sensitization. This confirms the results of Biesecker et. al., 1973 who found cytotoxicity against ascites tumour cells in the third to the seventh day only after primary sensitization and the results of Zoller et. al., 1975 who found cytotoxicity against DAB hepatomas was only shown by lymphoid cells taken between 2 and 12 days after sensitization.

This experiment has then clearly demonstrated cytotoxicity on the part of primarily sensitised cells against allogeneic tumour cells. It would seem likely that the cytotoxic effect is being exhibited against the transplantation antigens as cytotoxicity has been obtained consistently in this experiment whereas cytotoxicity was not consistently obtained in experiment 1 where any cell killing was directed against tumour associated antigen.

Cytotoxicity in cross over grafting experiments

Experiment 3a

The only difference between this experiment and experiment 2 was in the target cells used. The method of sensitization (allogeneic tumour) was the same as that in experiment 2 but the target cells in the wells were fibroblasts rather than tumour cells. Both fibroblasts and tumour cells bear the same transplantation antigens, however, cytotoxicity was again obtained in 50% of the tests carried out with lymphocytes taken between 5 and 12 days after primary sensitization, agreeing with the previous experiment.

Experiment 3b

The difference between experiment 3b and experiment 2 is in the method of sensitization of the lymphoid cells. The target cells used are the same in both experiments (allogeneic tumour). In experiment 2 the lymphocytes were sensitised by grafting the tumour itself. In experiment 3b the lymphocytes have been sensitised by skin grafting from a strain of origin of the tumour. Again it is stressed that both the skin graft and the tumour graft bear the same transplantation antigens. In experiment 3b however there was no killing of the tumour cells in any of the tests made.

The discussion now anticipates the results of experiment 4 which will be discussed in the next section. Experiment 4 in fact confirms that cytotoxicity against fibroblasts of the donor strain could not be obtained by cells primarily sensitised by skin grafting. This series of experiments was set up to show where this breakdown in cytotoxicity occurred and it is clear from experiments 3a and 3b that the breakdown is not in the failure of lymphoid cells to kill fibroblasts but

rather the failure of lymphoid cells sensitised by skin grafting to kill target cells of the donor strain in vitro.

Experiment 4 Cytotoxicity against allogeneic tissue grafts

No cytotoxicity was obtained with LNL nor with spleen cells which had been immunised by primary skin allografting. No cytotoxicity was obtained by lymph node lymphocytes after primary renal allografting. Cytotoxicity was obtained in 3 out of 4 tests using spleen cells sensitised by primary renal allografting. As has been discussed under experiment 3, it is clear that the breakdown in cytotoxicity has occurred because of the method of sensitization. Further work is required using allogeneic organ grafts but allogeneic skin grafting does not appear to sensitise the lymphocytes sufficiently for them to kill target cells of the donor strain in vitro. Blamey et. al., (1973) found a previous skin grafting did not speed up the rejection of a renal allograft in rats although second set rejection did occur in further skin grafts and it would appear from a variety of other experiments that skin graft rejection may occur in situations where organ grafts are not rejected (Calne, Sells, Pena, Davis, Millard, Herbertson, Binns and Davies, 1969, Salaman et. al., 1971). The difference here may well lie in the different methods of vascular connection in an organ graft and in a tissue graft.

Overall conclusion regarding microcytotoxicity testing experiments

The consistent demonstration of cytotoxicity on the part of spleen lymphocytes which had been hypersensitised by allogeneic grafts confirmed that there was nothing wrong with the overall methodology.

The aim of this whole section of the thesis has been to investigate whether cytotoxicity could be demonstrated on the part of lymphocytes, sensitised by primary allografting, against cells of the same histocompatibility antigenic makeup as those of the allograft. Experiment 4 clearly showed that no such demonstration was possible when the lymphocytes had been sensitised by a primary skin allograft. However cytotoxicity could be demonstrated on the part of splenic lymphocytes when the cells had been sensitised by primary organ; graft. Here then may lie an important difference in the sensitization produced by these two different kinds of grafts and further work on this subject is required.

The experiments have examined why cytotoxicity could not be demonstrated by cells sensitised by primary skin allografting against tissue cells bearing the same antigens as those of the skin allograft. At the outset of the experiments there appeared to be two possibilities: firstly, in the method of sensitization of the lymphocytes and secondly, that cells tissue cultured from the cells of normal tissues were too difficult to kill. The cells tissue cultured from tumour are much easier to kill. Satisfactory cytotoxicity has been demonstrated on the part of cells primarily sensitised by allogeneic tumour tested in vitro against those tumour cells. In experiment 3 however an important difference emerged. When the method of sensitization remained the same as in experiment 2, that is cells were sensitised against allogeneic tumour, but

were tested for cytotoxicity against fibroblasts of the same makeup as the allogeneic tumour - experiment 3a - cytotoxicity was demonstrated. This shows that the reason for the failure to demonstrate cytotoxicity does not lie in the target cell itself. Experiment 3b showed that the failure to demonstrate cytotoxicity lay in the method of sensitization for whilst the target cells were the same as those used (and killed) in experiment 2, no cytotoxicity could be demonstrated on the part of lymphocytes which had been primarily sensitised by skin grafting rather than tumour grafting. This brings us back to the discussion in experiment 4 revolving around the degree of sensitization produced by various types of grafting. Skin grafting appears to produce the least satisfactory sensitization, tumour grafting very good sensitization, organ grafting may well lie between these two. The results in cytotoxicity experiments appear to mirror the degree of sensitization produced by these various types of graft.

There is one further point that should be added and that revolves around the type of effector used. In all groups of experiments we have shown that lymph node lymphocytes are not as effective as killer cells. Removal of inappropriate nodes and damage during preparation might be the reasons but Takasugi and Klein (1970) also found these lymphocytes to be the least effective. Spleen cells and peripheral blood lymphocytes, when used, showed a greater capacity for killing in the cell counting method. Non immunised spleen cells also showed a non specific killing effect, killing was increased with immunised spleen cells. A single experiment 5(14) in which spleen lymphocytes were fractionated did not show whether the B cell plus macrophage or the T cell fraction were responsible for either

the specific or non specific effect. Lucas and Walker (1974) reduced the non specific effect by the removal of macrophages. The reasons for the effectiveness of spleen cells in this cell counting method are not clear. There are different proportions of each type of cell in the spleen and other lymphoid organs and there may be greater numbers of cytotoxic lymphocytes within the spleen.

Chromium release assay

1. Allogeneic tumour

The chromium release assay used probably measures the total breakdown of a cell with the contents of the cytoplasm escaping - this is the very end point of cell to cell cytotoxicity.

No cytotoxicity was obtained in the chromium release assay, using lymphocytes sensitised against primary allografts of tumour. The failure to achieve any cytotoxicity may be due to many reasons. The ratio of lymphocytes to target cells was 200 : 1 which was much less than in the cell counting techniques and there may not have been a sufficient number to cause killing. More important however is the contact between the lymphocytes and target cells in 0.4 mls. of media. Attempts to increase the contact of the cells was made by placing the tubes containing lymphocytes and target cells on a shaker during incubation. This shaking only served to increase the spontaneous release of ^{51}Cr. from the cells and was therefore abandoned. The other method to make definite contact between the lymphocyte and target cell, by spinning the cells down together prior to incubation, was not tried.

A fairly high spontaneous release from the human cells was observed during incubation, indicating that these cells are readily damaged under the conditions of culture. The spontaneous release at 0 hrs. at the beginning of the experiment was 4% to 33% increasing to 41% to 64% at 24 hours. The total release after intentional killing of the target cells by heating ranged from 84% at the beginning of the experiment to 97% at 24 hours, therefore additional lymphocyte cytotoxicity over and above spontaneous release should have been demonstrable if present.

With the addition of either immune or control lymphocytes there was usually a decrease in the release obtained at any one time as compared with the target cells alone. This may be due to a protective effect on the target cell by the lymphocyte. Or there may be some reutilization of the $^{51}Cr.$, after release from the target cell; a small amount of the chromium may possible be taken up by the lymphocytes, which sediment with the target cells on spinning.

2. Allogeneic tissue grafts

No cytotoxicity was demonstrable by this assay using lymphocytes immunised by skin grafting, or hyperimmunised lymphocytes. Even the addition of immunised spleen cells at a ratio of 200 : 1, which had proved cytotoxic in the cell counting method, caused no increase in the release of chromium. The average spontaneous release (60% at 24 hours) from the tissue cultured fibroblasts was high. The reasons why this assay may not have demonstrated cytotoxicity even in the hyperimmune experiments are the same as mentioned above.

F. CONCLUSION

As cytotoxicity has been difficult to achieve in these experiments - with some exceptions such as marked cytotoxic effect of hyper-immunised spleen cells - it leads us to believe that the in vitro methods cannot reproduce killing by lymphocytes in vivo.

Is in vitro cytotoxicity a good representer of in vivo cytotoxicity? Fortner, Kuperman and Lucas (1975) showed that in vivo rejection of tumour did not correlate with in vitro splenic lymphocyte cytotoxicity. There may be several reasons for this In in vitro tests there is a limited time and space in which the sensitised lymphocyte can work. Clearly in vitro there is a lack of additional help from non specific cells which may be recruited in vivo to assist in graft rejection. Also any effect of tissue localisation is lost in vitro. Another method which has been advanced as measuring lymphocyte activity in allograft rejection in the graft-versus-host assay. This has therefore been chosen in order to incorporate these missing features from the in vitro experiment. This graft-versus-host study will be presented in Section II.

Another explantation might be that each individual lymphocyte has a small cytotoxic effect which is not evident in the in vitro tests. This effect may be multiplied many times by an enormous increase in the number of lymphocytes flowing through a graft at the time of rejection. Studies on lymphocyte flow through a rejecting kidney will be shown in Section III.

G. APPENDICES I

a. Methods of in vitro cytotoxicity tests

1. Cell counting
2. Chromium release assay

b. Method of immunization with syngeneic tumour

c. Methods of immunization with allogeneic tumour or graft

d. Preparation of lymphocytes

e. Result of Microcytotoxicity experiment 5(9) and chromium release assay experiment 2(1)

Cell counting method comparing control cells

APPENDIX I(e)

Two methods of measuring in vitro lymphocyte cytotoxicity are used.

1. Cell counting
2. Chromium release assay

1. Method of cell counting

Tissue cultured target cells were obtained either as DAB or MC - sarcoma tumour cells (Appendix Ib) or fibroblasts (Appendix Ic). The cells were removed from tissue culture bottles with trypsin and washed twice and were plated in microtest plates containing 96 wells of 0.3 ml. size. A hundred viable cells were plated into each well in 0.2 mls. media in which they were tissue cultured (either Eagles Hepes or Waymouths) containing 20% heat inactivated foetal calf serum (FCS). The plates were placed in sealed boxes which had been gassed for five minutes with 5% carbon dioxide in air and incubated at 37° C. between 24 and 48 hours. The target cells settled and adhered to the base of the well.

After incubation the media was removed from all the wells. Lymphocytes were added to the wells in 0.2 mls. of appropriate media in varying ratios from 1,000/1 effector cells to target cells, up to 4,000/1. Media was readded to the wells containing targets cells only. The end wells were not used for counting.

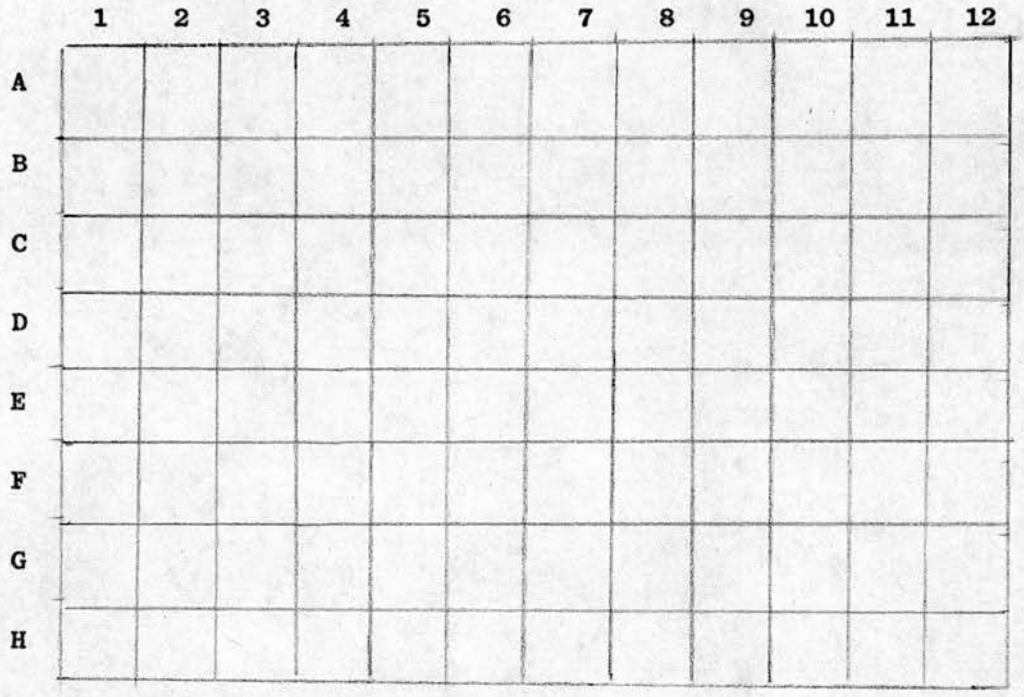
Control and immune lymphocytes were added in corresponding columns from the ends of the plate. Two columns of target cells and media alone were also plated (diagram 3).

The plating of the lymphocytes varied with each plate in order that observer bias at counting was reduced. The person who plated the lymphocytes was not the same as the one who counted the cells.

One hour after adding the lymphocytes, a drop of FCS was inserted

Diagram 3

A scheme of plating of Lymphocytes and Target cells for Direct Counting



Microtest plate II 96 wells 12 across x 8 down

Column 1 and 12	not used
Column 2 and 11	Target cells + media
Column 3 and 4	Non immunised (control) lymph node lymphocytes (LNL) and Target cells
Column 5 and 6	Control spleen cells and Target cells
Column 7 and 8	Immunised (test) spleen cells and Target cells
Column 9 and 10	Test LNL and Target cells

100 Target cells are plated in each well

A different plate is used for the different ratios of effector lymphocytes for example 1×10^5 lymphocytes added to these target cells gives a ratio of 1,000 : 1 effector to target cells.

in each of the wells and the plate was gassed in 5% CO_2 in air at 37° C. for 48 hours.

Staining plates

The plates were washed by a gentle flow of 0.9% saline in order to remove debris and dead cells but leaving the viable cells attached to the well surface. The saline was drained with a gentle shake and the cells fixed with pure methanol for 15 minutes. The methanol was then shaken off and 0.5% crystal violet added to the wells for five minutes. The plates were then rinsed and dried. The number of cells remaining in each well are counted under an Olympus cell counting microscope.

Significant cytotoxicity was achieved when there was a statistically significant reduction of the mean of immune as compared with control wells. This corresponded to a reduction in mean counts of 20% (Appendix Ie).

2. Chromium release assay

Tissue cultured target cells were removed from tissue culture bottles with trypsin and washed three times. The cells were resuspended in 0.2 mls. media. 50 μ Ci. of ^{51}Chromium in 0.3 ml. of media was added to give a final volume of 0.5 mls. The cells were incubated with the ^{51}Chromium for three hours, then washed three times with media, resuspended in 0.2 mls. of media and counted. The number of viable cells were counted by trypan blue exclusion. The cells were kept at 4° C. for half an hour to allow the less viable cells to die and then the cells were recounted.

Five x 10^3 viable labelled cells in 0.2 mls. of media were pipetted into small tubes. There were four groups of tubes.

Group 1 Target cells + media

Group 2 Target cells * immune (test) lymphocytes 1 x 10^6

Group 3 Target cells + non immunised (control) lymphocytes 1 x 10^6

Group 4 Target cells (killed by heating) + media

The lymphocytes were added in 0.2 mls. of media and incubated at 37° C. The first set of tubes demonstrated the spontaneous release of ^{51}Chromium from viable cells over the period of the test. The fourth set demonstrated the amount of ^{51}Chromium released when the cells were destroyed (total release).

The small tubes (in triplicate) were spun at 350 g. for 5 minutes at 0 hr., 2 hrs., 4 hrs., 8 hrs.,and 24 hours. Half the supernatant and then removed and put in separate ignition tubes (which were marked). The tubes were then placed in γ counter (Wallac 80,000 Gamma sample counter) and counted for 10 minutes.

The percentage of chromium release was calculated for each tube as

$$\frac{\text{counts in supernatant} \times 2}{\text{counts in supernatant + pellet counts}} \times 100$$

In both lymphocyte cytotoxicity tests the tissue culture media used (either Eagles Hepes or Waymouths) contained 20% FCS and gentamycin 0.05 mgs/ml.

APPENDIX I(b)

Methods of Immunization with Syngeneic Tumour

Animals

Inbred Wistar rats (Cancer Research Department, Nottingham) have been used for the tumour experiments. These have been brother sister mated since 1952, and were syngeneic by routine skin grafting. Rats were fed on Oxo cube diet 41B with tap water ad libitum.

Tumour induction

Hepatomas

Hepatomas were induced by feeding rats for three months on a low protein rice diet containing dimethylaminoazobenzene, made up as follows:

890 g ground unpolished rice

60 g dried carrot powder

20 g corn oil

0.06 g 4-dimethylaminoazobenzene

Animals were then transferred to a basal rice diet and most rats deveoped hepatomas within five months of the initial exposure to the carcinogen.

Fibrosarcomas

Fibrosarcomas were induced by the injection into the right flank of 5 mgm. of 3-methylcholanthrene (Koch Light and Co.) dissolved in 0.5 mls. trioctanoin (Eastmann Organic Chemicals Ltd.) or by the subcutaneous implantation of a solid pellet of carcinogen in cholesterol. Tumours arose five to eight months after initial exposure.

Carriage of tumour transplantation lines

Tumour bearing animals were killed by cervical dislocation and the skin sterilized by immersing the rats in 1% savlon. The tumours were removed aseptically and transplanted subcutaneously (s.c.) into rats of the same sex as the donor. The recipient rats were chosen so that they were no more than four generations removed from common ancestry with the tumour donor. Transplants were made under ether anaesthesia by subcutaneous implantation of small tumour fragments using a number 4 or 5 guage trocar through a small incision made in the skin. The skin was closed with Michel clips.

Immunization by surgical excision of subcutaneously growing tumour transplantations

Primary s.c. tumours or transplant grafts were allowed to grow to a mean diameter 1 to 2 cms. Under ether anaesthesia the skin around the tumour was incised and the tumour mass freed from the body well and removed. The skin was closed.

Immunization by implantation of irradiated tumour grafts

Ice cooled fragments of tumour tissue were exposed to ^{60}Co irradiation (10,000 to 15,000 rads). A 1,000 curie source in the Department of Chemistry was used for this irradiation. The tumour tissue was placed in McCartney bottles surrounded by ice, clamped 10 cms. from the irradiation source and irradiated for 15 minutes. The tissues were subsequently implanted s.c. into anaesthetised rats. Grafts were given at 2 to 4 week intervals, each animal receiving up to 5 grafts. Further challenge with viable tumour cells was made at intervals to the rats, starting at 5×10^4 cells up to 5×10^5 tumour cells. Viable tumour was injected into untreated controls at

the same time. The test animals were known to be immune as the viable tumour did not grow.

Preparation of tumour cell suspensions

Freshly removed tumour was minced and washed with Hank's balanced salt solution containing penicillin 100 units/ml. and streptomycin 100 ug/ml. Trypsin 0.25% solution in Hank's was added to the tumour and incubated for 15 minutes at 37° C. The tumour was washed and further trypsin plus 0.02% deoxyribonuclease was added and incubated with a plastic coated magnet and agitated on a 600 r.p.m. stirrer for 30 minutes. The supernatant was decanted and tumour cells removed from the supernatant trypsin by centrifugation 30xg for 3 minutes then resuspended in Eagles medium with Dnase and washed four times. The tumour cells were filtered through a 120 mesh stainless steel screen. Cell viability was assessed by trypan blue dye exclusion (1 vol. 4.2% NaCl to 4 vols. 0.2% aqueous dye). The number of unstained cells were counted in a haemocytometer and dilutions for culture or injection were made in Eagles Hepes medium.

APPENDIX I(c)

Methods of Immunization with allogeneic tumour or graft

Animals

Inbred Wistar rats (W.A.G. Bantin and Kingman Ltd., Grunston, Nr. Alborough, Hull), inbred A.S. (Charles Salt Laboratories, Agnes Jones/Robert Hunt Hospital, Oswestry) and outbred Spraque Dawley rats (S.D.) (Joint animal breeding unit, J.A.B.U., University of Nottingham School of Agriculture, Sutton Bonington) have been used for these experiments. These line inbred strains have been brother/sister mated for at least 20 generations. The inbred Wistar rats are syngeneic by routine skin grafting. The animals were fed on 41B diet with tap water ad libitum.

Transfer of allogeneic tumour

Primary subuctaneous tumour D23, D192 and D202 hepatomas or MC57 methylcholanthrene induced sarcoma were removed from Wistar rats obtained from Cancer Research Department, Nottingham (see Appendix Ib). The tumour was removed aseptically and divided into eight small pieces, 5 mm. in diameter, and 1 larger piece. The larger piece was disrupted by pressing with a glass rod through 120 stainless steel mesh into a petri dish containing Eagles Hepes medium. The cells which had passed through the mesh were drawn up into a sterile container and media was added to make up 20 ml. The cells were not counted but divided arbitarily into four 5 ml. aliquots. Four A.S. or S.D. rats for each experiment were anaesthetised with ether, both buttocks of each rat were cleaned with savlon. A small incision was made in the skin and a piece of tumour was tucked under the skin which was closed with 2/0 silk. Each rat then received an intraperitoneal injection of

tumour cells. The four rats in each experiment were killed on different days following the transfer of the tumour, ranging from 4 to 21 days, for removal of lymph nodes and spleen.

Method of skin grafting

Full thickness skin grafting was performed between inbred female Wistar donor rats to recipient female S.D. or A.S. rats. The recipient rat was anaesthetised with ether and the skin on its back close clipped and cleaned with savlon. A 3 cm. square patch of skin was removed. The remaining skin was then either sutured to cover the defect or the animal sacrificed. The panniculus carnosus was scraped of the underside of the graft. The recipient rat was anaesthetised and the skin again clipped and cleaned. An area of skin comparable in area to the graft was then removed from its back, taking care to dissect the panniculus carnosus off the skin and leaving it intact on the recipient. The skin graft was applied to the recipient and sutured in place with 4/0 silk (plate 1a). A dressing (circumferential) wasaapplied to the rat to prevent it rubbing off the graft. The dressing was removed on the seventh day. Rejection was judged to have occurred if the graft became hard and impliable (plate 1b). Skin grafting between syngeneic inbred Wistar rats was performed to see if the technique was correct and the skin remained soft and eventually grew hairs (plate 1c).

Method of intraperitoneal injection of lymphocytes

Injection of intraperitoneal lymphocytes was used in the hyperimmune experiments, the S.D. or A.S. rats had previously been sensitised with three skin grafts from inbred Wistar rats. The lymphocytes taken from the Wistar rats were prepared in the manner

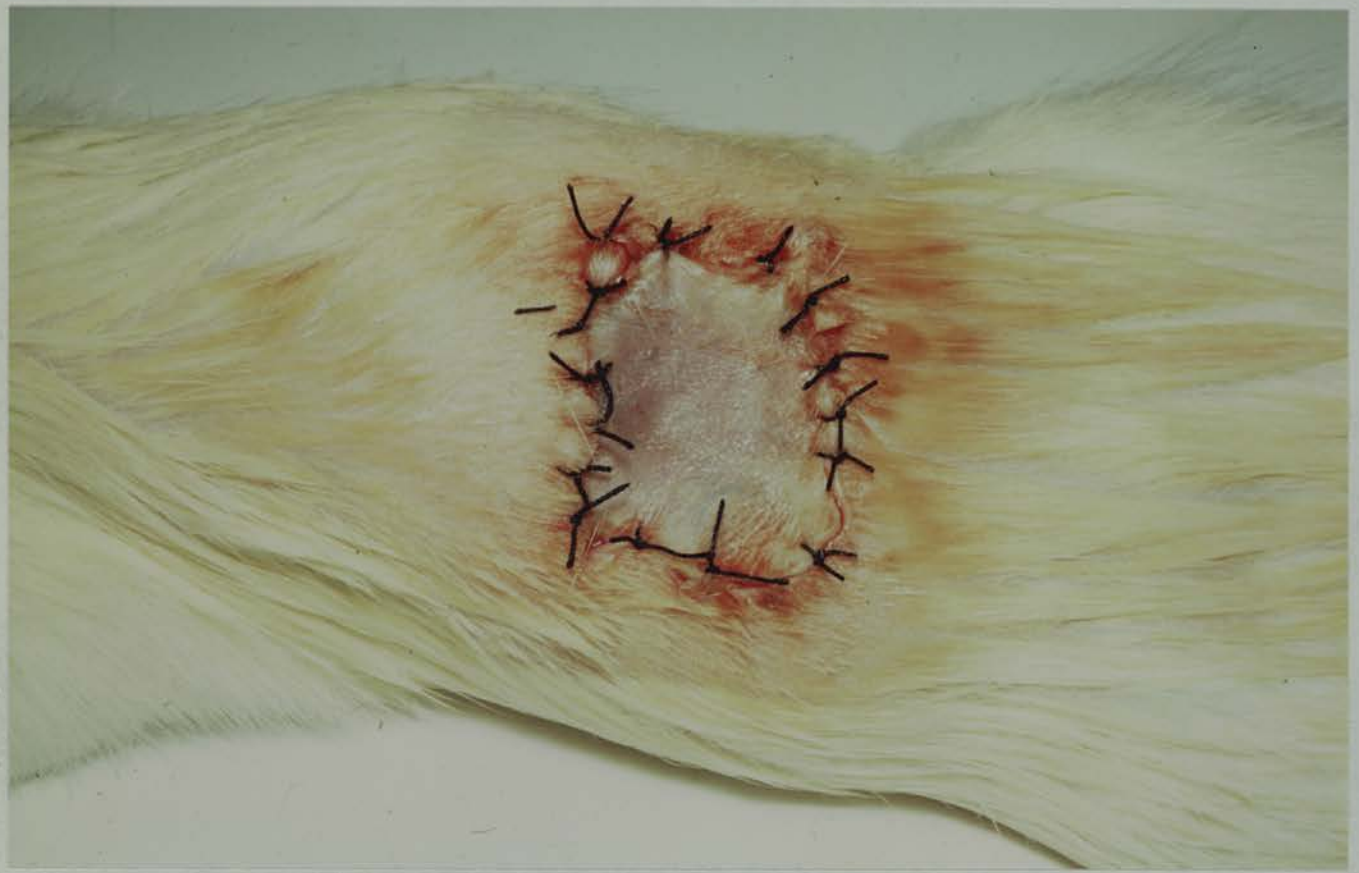


Plate 1a) SKIN GRAFT IN THE RAT AFTER OPERATION

Plate 1 b) REJECTING SKIN GRAFT 7th DAY

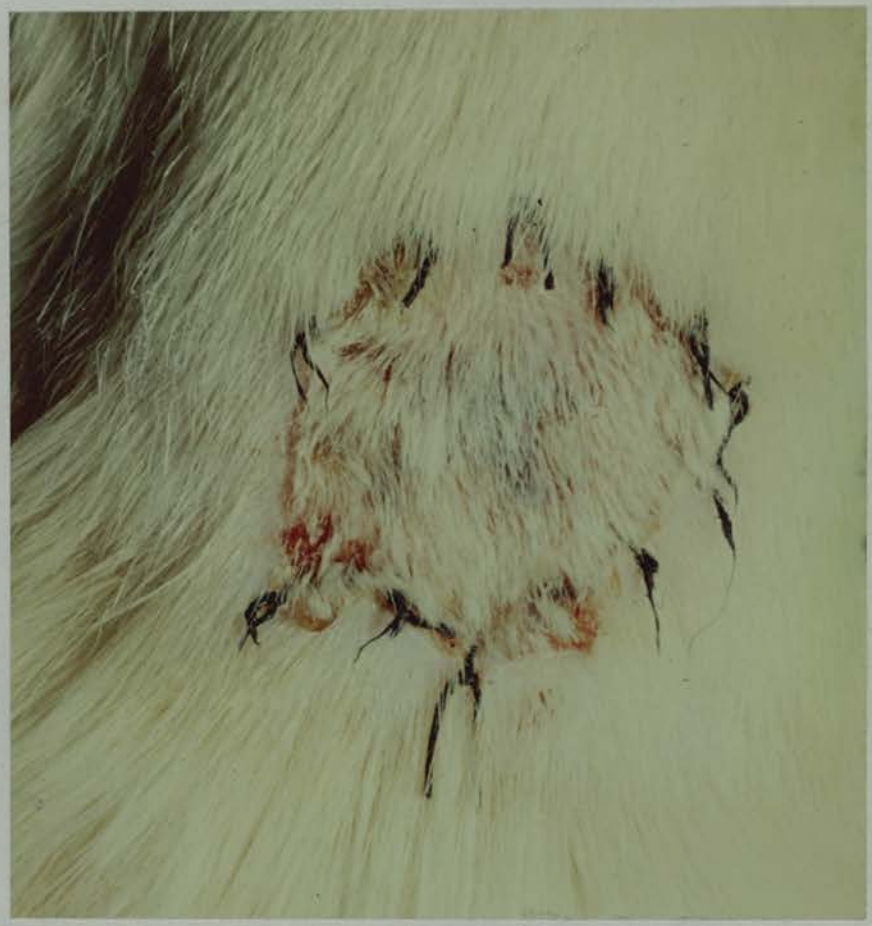


Plate 1 c) NON REJECTING SKIN 10th DAY

described below; 1×10^7 lymph node lymphocytes were injected in 5 mls. of Waymouths through a size 19 guage (G) needle into the peritoneal cavity.

Method of renal transplantation

The technique used has been described by Blamey, Baxter, O'Brien and Bennett (1974).

Female rats around 200 gms. were used for this procedure. Inbred Wistar donor kidneys were transplanted into S.D. recipients.

The donor rat was anaesthetised with ether. The animal was laid out on its back on a cork board with the limbs stretched. The skin of the abdominal wall was shaved and cleaned. A midline laparotomy incision from above the pubis to the sternum was made. The gut was moved to the right side of the abdomen and packed away. A Nikon double operating microscope was now used.

The left kidney was then exposed (plate 2). The ureter was identified and divided at the junction of the middle and upper third. The fat was cleared from the kidney and the renal vessels. The ovarian and adrenal vessels were coagulated and divided. The renal vein was divided at the inferior vena cava, the renal artery was ligated as it arose from the aorta, a small incision was made in the artery just distal to the ligature and a fine silastic tubing 0.6 mms external diameter was inserted and tied into the artery. The renal artery was then divided close to the aorta and removed. Approximately 5 mls. of ice cold dextran 40 in saline, containing 4 units of heparin/ml. and 3 mgs. of papaverine/ml. was then perfused into the kidney via the tubing. The ureter was identified and a short length of silastic tubing 0.6 mms. external diameter was inserted up the lumen. (plate 3a)

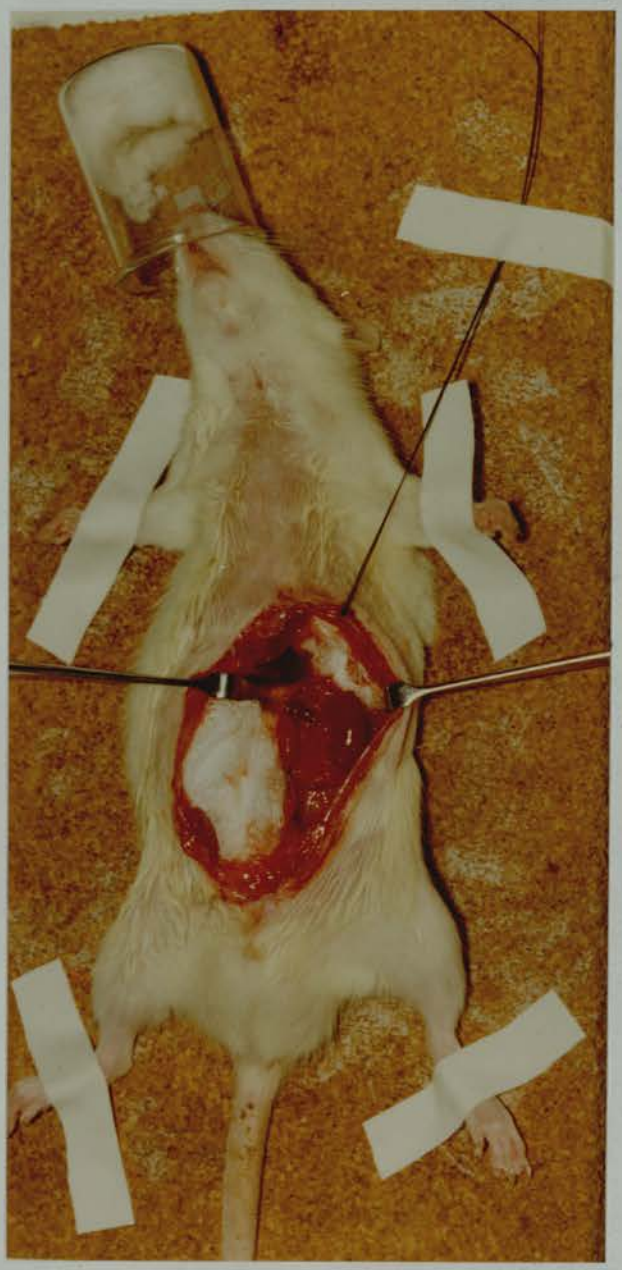


Plate 2 RENAL TRANSPLANTATION IN THE RAT
SHOWING EXPOSURE OF LEFT KIDNEY

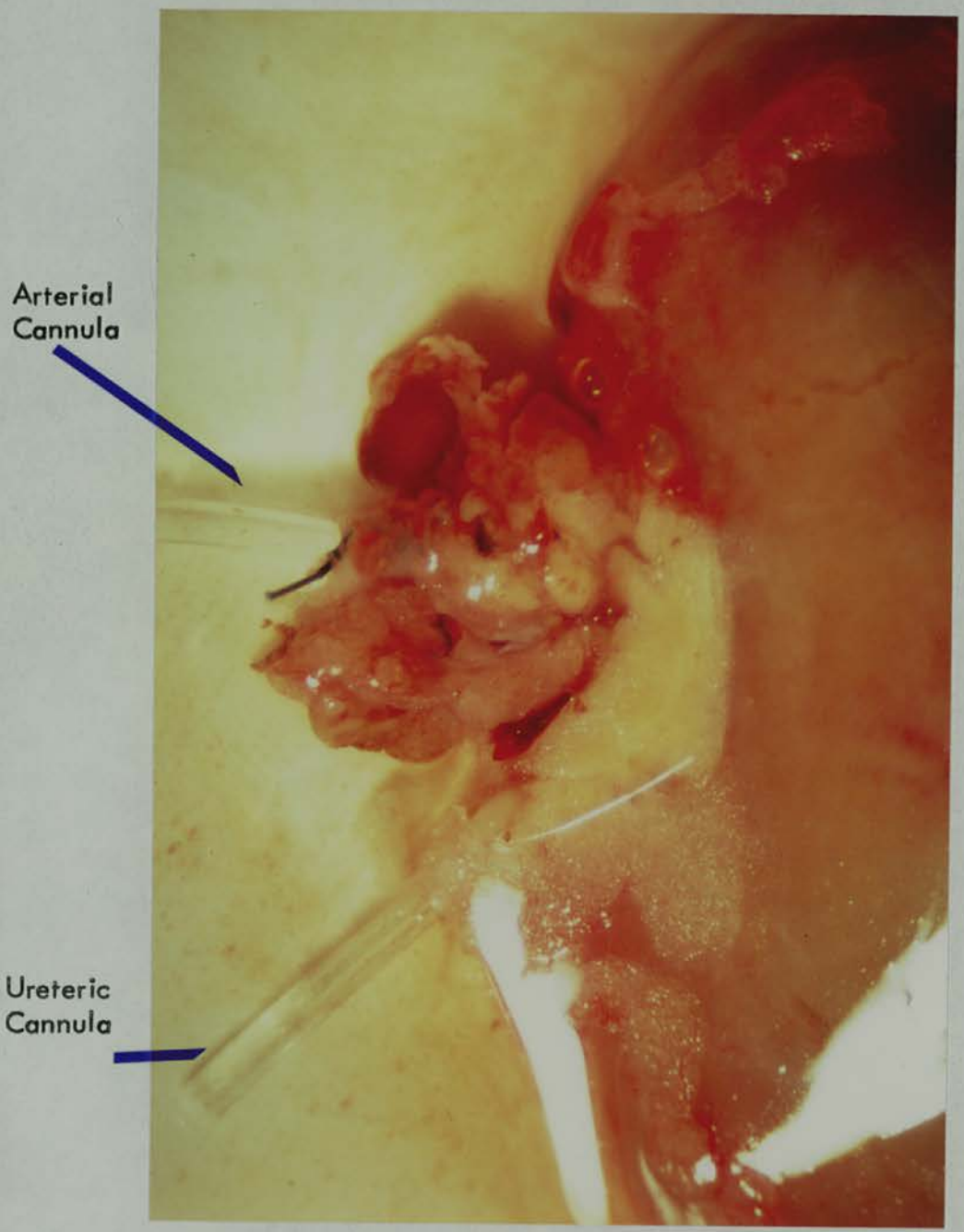


Plate 3a) RENAL TRANSPLANTATION IN THE RAT

DONOR KIDNEY PERFUSED

Magnification x 30

The recipient rat was now prepared. A tail vein was cannulated with a 25 guage needle attached to polythene tubing for the infusion of normal saline containing 1 unit heparin/ml. to replace blood loss during the operation. The left kidney was dissected in the same manner. The ureter was divided at the renal pelvis. Microvascular clamps (Scoville-Lewis) were placed at the origins of the renal artery and vein and the vessels divided at the hilum.

The donor kidney was placed orthotopically. The ureteric anastomosis was performed by placing part of the silastic tubing in the donor ureter into the recipient ureter and oversewing the loose connective tissue of each ureter together with 7/0 silk, so that both lumens came together over the splint.

The renal veins of the donor and recipient were sutured together through the full thickness of the wall with 10 interrupted 10/0 nylon sutures on an atraumatic needle. Suturing front and back walls of the renal vein together was a possible hazard at this point, and was prevented by blowing up the donor vein by further perfusion of the donor renal artery. Similar end to end anastomosis was performed between the two arteries using 6 interrupted 10/0 sutures. When the anastomosis was completed first the venous and then the arterial clamps were removed. Bleeding was controlled by packing. (Plate 3b)

The kidney was well perfused with arterial blood. The wound was closed with 2/0 silk and the anaesthesia discontinued.

The method of preparation of fibroblasts for tissue culture

The fibroblasts for these experiments were obtained from Wistar embryos. A twenty day pregnant rat was anaesthetised with ether and killed by cervical dislocation. The abdominal skin was cleaned with

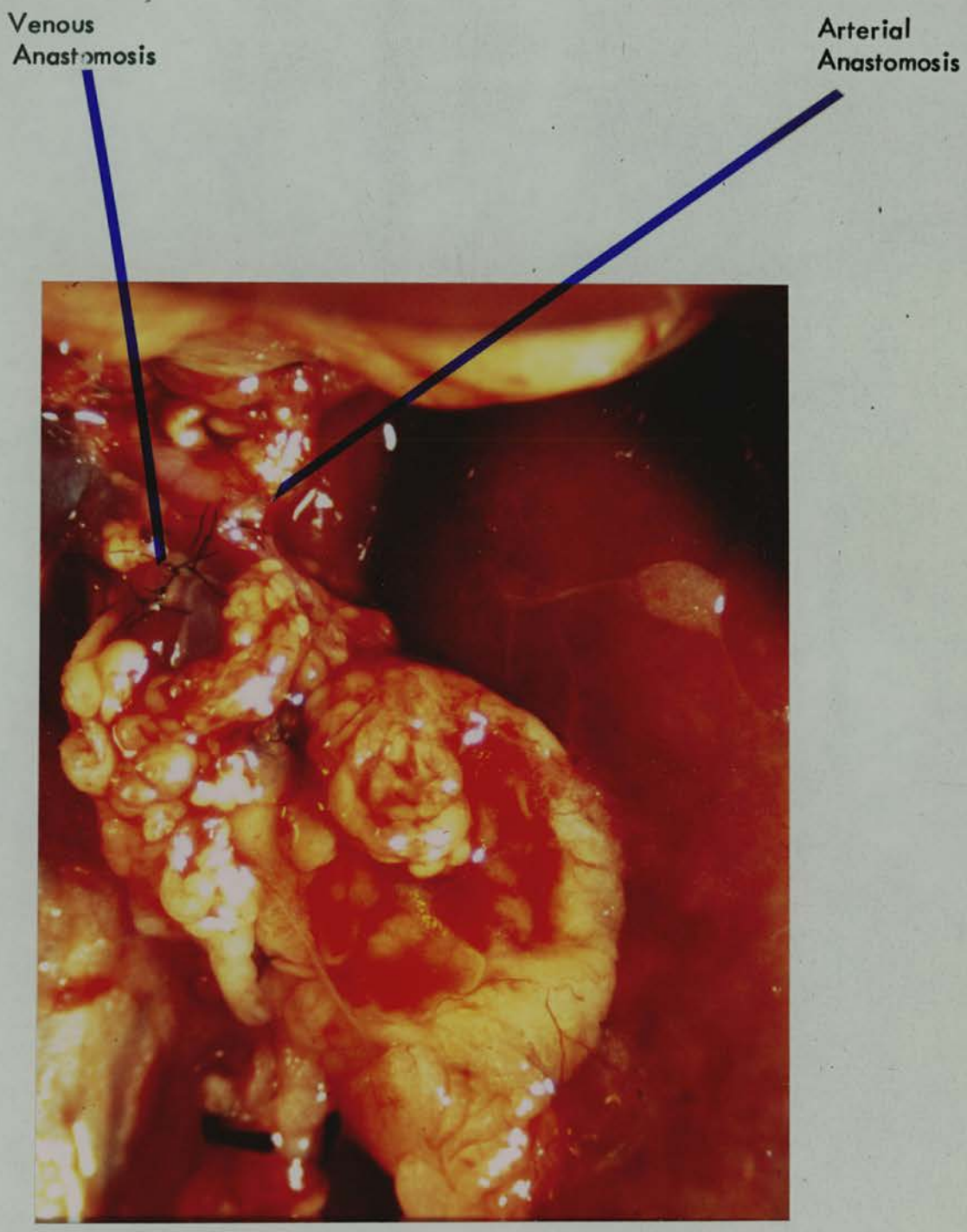


Plate 3 b) RENAL TRANSPLANTATION IN THE RAT.
TRANSPLANTED KIDNEY IN RECIPIENT AFTER VASCULAR
ANASTOMOSIS COMPLETED

Magnification x 30

savlon. In an aseptic manner the peritoneal cavity was opened and the embryos removed. The placenta and amniotic sacs were discarded and the embryos chopped finely and washed in Hanks to remove blood. The chopped embryos were incubated in trypsin with a plastic coated magnet and agitated on a 600 r.p.m. stirrer for 30 minutes at 37° C. The supernatant was decanted and spun at 160 g. for 15 minutes. The pellet was washed three times in Waymouths media to remove the trypsin. The cells plus fragments were passed through 120 steel mesh. The cells obtained were placed with Waymouths with 10% FCS and gentamycin 0.05 mgs/ml. in tissue culture bottles and incubated.

When the cells required subculturing, they were removed from the tissue culture bottle with trypsin. The cells were washed three times and aliquots added to new tissue culture bottles with Waymouths plus 10% foetal calf serum, and gentamycin.

APPENDIX I(d)

Method of preparation of lymphocytes

1. *Removal of lymph nodes and spleen*

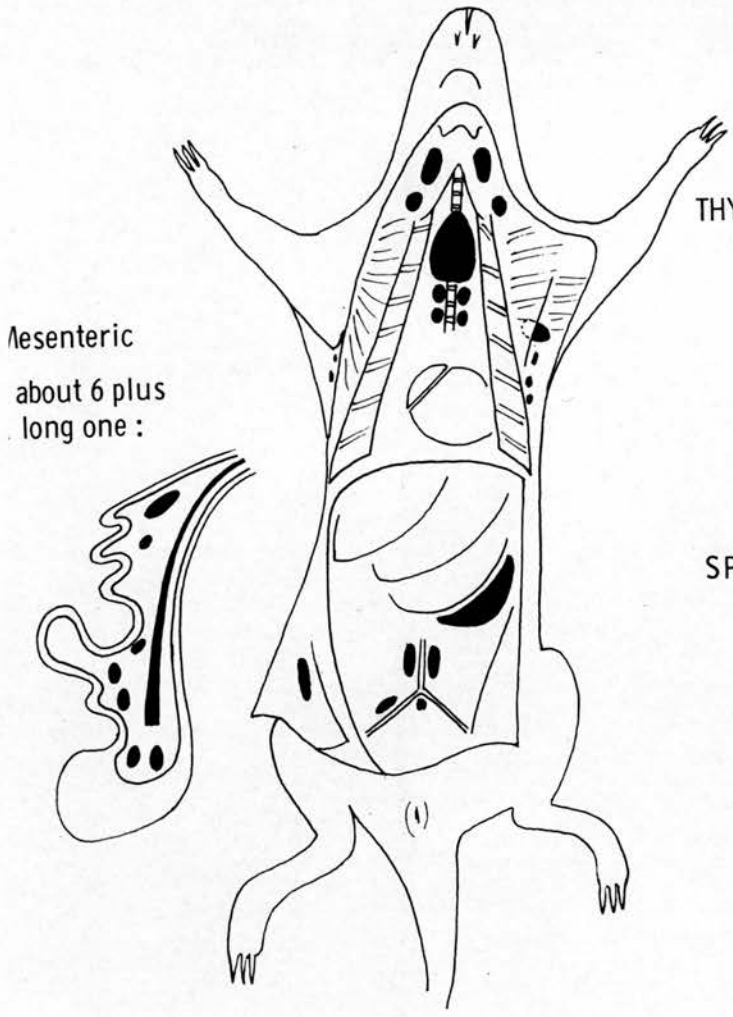
The immunised and non immunised control rats were anaesthetised with ether. The animals were killed by cervical dislocation and pinned out on a board, lying supine. The skin was clipped and cleaned. In an aseptic manner the skin was opened in the midline from pubis to neck. The skin on the neck and thorax was dissected off. The cervical and axillary lymph nodes were removed (see diagram 4) and cleared of fat. The peritoneal cavity was opened and the mesenteric lymph node chain removed and also cleared of fat. The lymph nodes were placed on 120 stainless steel mesh in a petri dish and chopped finely with scissors. A small amount of media was placed in the petri dish and the cells of the lymph nodes squashed through the mesh with a glass rod. The cells were then drawn up from the petri dish, the viable lymph node lymphocytes were counted in a haemocytometer by trypan blue exclusion.

The spleen was removed and spleen cells obtained in a similar fashion. The spleen cells were placed on Ficoll Triosil and spun at 700 g. for 20 minutes to separate red cells from the white cells. The lymphocytes were then removed from the layer in between the Ficoll Triosil and the medium, and washed three times in medium. The viable cells were counted.

2. *Preparation of blood lymphocytes (Diagram 5)*

The rats were anaesthetised with ether. A 21 gauge needle was inserted directly into the heart of the rat and up to 10 mls. of blood was withdrawn into a heparinised syringe (1,000 units

Diagram 4 DISTRIBUTION OF LYMPHOID TISSUE IN THE RAT



Bilateral :

Cervical (1)
Apical (1-2)

THYMUS

Paratracheal (3-4)
Retroscapula (1-2)
Axillary (2-3)

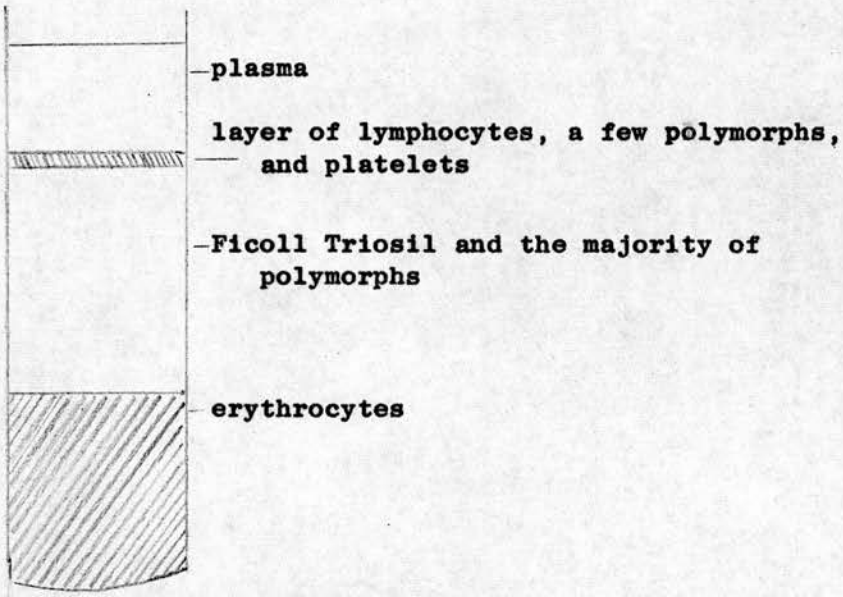
Mesenteric

about 6 plus
long one :

SPLEEN

Paraortic (1)
Superficial
inguinal (1)
Iliac (1-2)

Diagram 5



Separation of lymphocytes by Ficoll Triosil gradient

Ficoll Triosil is made up:

Solution A Triosil 20 mls. plus 24 mls. water

Solution B Ficoll 4.5 G. in 50 mls. water

Add 20 mls. solution A to 48 mls. Solution B

preservative free heparin). The blood was then placed on a Ficoll Triosil gradient and spun at 700 g. for 20 minutes. The lymphocytes were removed from the interface between the Ficoll Triosil and the serum. They were washed three times in medium and the viable cells were counted.

3. Method of thoracic duct cannulation for collection of thoracic duct lymphocytes

The method used for cannulation of the thoracic duct in the rat was described by Bollmann et. al., (1948). The immune and control rats were anaesthetised with ether. The rat was pinned out supine with lower limbs spread out and the left upper limb on the right side of the head. The operation was performed under aseptic conditions. A left subcostal incision was made from the midline anteriorly to the level of the mid-axillary line. A 12 ins. piece of polythene tubing (external diameter 0.75 mm.) was fashioned with an umbrella handle tip. This was placed in the abdomen with the tip inside and the rest of the cannula brought out on the left posteriorly.

The left kidney was mobilised and placed over the right hand side of the abdomen. The abdominal contents were then packed away from the site of operation, the upper part of the abdominal aorta. The thoracic duct was exposed as it lay on the left side of the aorta and 6/0 ethilon ligature placed around the thoracic duct but left untied. The cannula was filled with heparinised saline (1 unit/ml.) an incision made across half the lumen of the thoracic duct proximal to the ligature and the cannula inserted and pushed distally. The ligature was tied around the duct and cannula. The curve of the umbrella handle lay neatly under the diaphragm and was fixed to the posterior abdominal wall with a 6/0 suture.

The wound was closed with 4/0 silk. A tail vein was cannulated for infusion of heparinised saline.

The animal was placed in a restraining cage. The thoracic duct cannula was placed in a cooled sterile flask containing 1,000 units of heparin. The intravenous infusion of saline was given at the rate of 40 mls./day with a constant infusion pump.

The thoracic duct lymphocytes when collected were washed three times in media and counted.

4. Method of fractionation of lymphocytes

The method used here for separating T cells from macrophages and B cells has been described by Julius, Simpson and Herzenberg (1973). Those cells which are adherent are B cells and macrophages.

Sterile nylon wool (Leuco-pak) was used for separation of the lymphocytes. The nylon wool had to be washed to remove a toxic product which otherwise decreased the survival of the lymphocytes. The wool was washed in 0.2 N HCL and rinsed five times in tap water, deionised and distilled water. The wool was dried and 0.6 gram aliquots packed into the barrel of 10 ml. plastic syringes. The syringes were then autoclaved.

The syringes were placed in a vertical position and washed through with 25 mls. tissue culture media plus 5% FCS. When washed the syringes were filled with media till the wool was covered and incubated at 37^{o} C. for one hour.

The lymphocyte cell suspension were counted and approximately 1×10^{8} viable cells in a volume of 1 ml. was added to the columns, and an additional 1 ml. of media added after. The columns were incubated at 37^{o} C. for 45 minutes. The column effluent was

collected at a rate of 1 ml./minute. The cells which came through with the effluent (the eluted cells) were recounted with trypan blue. To obtain the adherent cells (retained cells) the wool was removed from the column and the cells squeezed off the wool. These cells were also counted with trypan blue.

Appendix (Ie)

Microcytotoxicity Test

Experiment 5(9)

TARGETS

Inbred Wistar (I.W.) fibroblasts (tissue cultured)
plated 31.7.75 100 cells per well

IMMUNE CELLS

From S.D. ♀ , received skin grafts from I.W. 2.5.75), 20.5.75), 2.6.75) Added
received I.P. L.N.L. from I.W. 23.6.75), 23.7.75) 1.8.75

CONTROL CELLS

From I.W. ♀ Added 1.8.75

	1	2	3	4	5	6	7	8	9	10	11	12
A		76	78	72	70	50	24	58	81	71	58	
B		74	68	102	85	66	19	50	96	76	62	
C		73	79	93	72	71	25	62	57	80	90	
D		67	102	112	88	64	26	55	92	102	63	
E		87	97	99	77	55	9	42	61	95	89	
F		89	67	90	69	29	10	55	70	70	74	
G		68	66	74	104	72	41	66	79	79	66	
H		77	70	86	61	61	32	64	89	89	46	

Row 2 + 11 Targets + media

Row 3 Targets + non immune L.N.L. 1 x 10^5 cells

Row 4 Targets + non immune L.N.L. 2 x 10^5 cells

Row 5 Targets + non immune spleen cells 1 x 10^5

Row 6 Targets + non immune spleen cells 2 x 10^5

Row 7 Targets + immune spleen cells 2 x 10^5

Row 8 Targets + immune spleen cells 1 x 10^5

Row 9 Targets + immune L.N.L. 2 x 10^5 cells

Row 10 Targets + immune L.N.L. 1 x 10^5 cells

All wells contained FCS

RESULTS

Experiment 5(9)		remaining target cells after 48 hrs. incubation	
Test	L.N.L. (1×10^5) mean $\pm$ S.E.	= 82.6 $\pm$ 4.1	N.S.
Control		= 78.4 $\pm$ 4.9	
Test	L.N.L. (2×10^5)	= 80.9 $\pm$ 5.3	N.S.
Control		= 91.0 $\pm$ 4.8	
Test	Spleen (1×10^5)	= 56.5 $\pm$ 2.8	$p < 0.001$
Control		= 80.6 $\pm$ 4.1	
Test	Spleen (2×10^5)	= 23.3 $\pm$ 3.8	$p < 0.001$
Control		= 58.5 $\pm$ 5	
Media alone		= 72.4 $\pm$ 3.1	

$$\text{Cytotoxicity Percentage} \quad \frac{(\text{Control} - \text{Test})}{\text{Control}} \times 100$$

Cells (Lymphocyte : Target)	Immune v. Control Lymphocytes	Immune cell v. media alone	Control cells v. media
L.N.L. 1,000 : 1	- 5%	-14%	- 8%
L.N.L. 2,000 : 1	+11%	-11%	-25%
Spleen 1,000 : 1 cells	+30%	+22%	-11%
Spleen 2,000 : 1 cells	+60%	+68%	+19%

N.S. = not significant.

The p has been calculated by Student T test for unpaired data.

A - in the percentage cytotoxicity of cells v. media indicates a feeder effect on addition of lymphocytes to the target cells.

Cytotoxicity achieved with immune spleen cells at ratios 1,000 and 2,000 : 1.

Appendix Ie

Chromium release assay

Experiment 2(1)

TARGETS

D23 tissue cultured tumour cells

IMMUNE CELLS

From S.D. ♀ received D23 tumour graft 24.2.75 from Wistar (I.W.)

CONTROL CELLS

Wistar (I.W.) ♀

} test performed 3.3.75 7 days after immunization

Target cells 5,000/tube incubated with ^{51}Cr.

1. Target + media (spontaneous release)

2. Targets + control L.N.L. 1 x 10^6/tube (ratio 1 : 200)

3. Targets + test L.N.L. 1 x 10^6/tube (ratio 1 : 200)

4. Killed target cells (total release)

Chromium Counts

	0 hrs.			6 hrs.		10 hrs.		16 hrs.		24 hrs.	
	pellet	supernatant	% release	% release							
1.	19	1169		12.8		14.0		30.8		41.7	
	32	1189	4.3%	15.9	14.9%	18.7	17%	25.1	28.4%	41.9	40.5%
	25	1072		16.1		18.1		29.2		37.8	
2.	27	1102		17.6		21.7		26.2		30.6	
	35	1124	5.6%	15.6	17.3%	24.9	22.8%	29.8	28.3%	38.5	35.5%
	35	1145		18.7		21.7		28.8		37.4	
3.	25	1105		23.2		23.0		30.8		33.3	
	30	1177	4.2%	17.1	18.6 %	20.7	21.1%	30.6	29.9%	33.7	33.9%
	19	1185		18.4		19.8		28.3		34.8	
	433	676	77.9%	81.2%		85.8%		81.2%		86%	

Counts are taken over 2 minutes

$$\% \text{ release} = \frac{\text{counts supernatant} \times 2}{\text{counts pellet + supernatant}} \times 100$$

Percentage of cytotoxicity is estimated from

$$\frac{(\% \text{ release from immune cells} - \% \text{ release from control cells})}{(\% \text{ total release} - \% \text{ release from control cells})} \times 100$$

e.g. at <u>16 hours</u> $\frac{29.9 - 28.3}{81.2 - 28.3} \times 100 = 3\%$ cytotoxicity

There was no cytotoxicity achieved in this experiment although cytotoxicity was achieved in a concurrent M.C.T. at a ratio of 4,000 : 1 effectors to targets.

Appendix I(e)

Cell counting comparing controls cells

Controls

TARGETS- 1) D23 tissue cultured tumour from inbred Wistar strain
2) Inbred Wistar tissue cultured fibroblasts

CONTROL CELLS Non immunised ♀ inbred Wistar (I.W.)
Non immunised ♀ outbred S.D.

CONTROL CELLS		TARGETS 1 TARGETS + MEDIA	105.6	TARGETS 2 TARGETS + MEDIA	23.6
L.N.L. 1,000 : 1	I.W.	142.1	N.S.	40.9	N.S.
	S.D.	136.1		37.8	
2,000 : 1	I.W.	127.5	N.S.	48.8	N.S.
	S.D.	149.9		51.4	
Spleen cells 1,000 : 1	I.W.	68.0	$p<0.001$*	46.1	N.S.
	S.D.	40.5		41.9	
2,000 : 1	I.W.	24.9	N.S.	41.6	$p<0.001$*
	S.D.	17.3		26.3	

N.S. = not significant

The numbers shown are the mean of the number of cells remaining in 16 wells.

A reduction in targets cells is obtained in non-immunised Spraque Dawley rats with spleen cells, as compared with inbred Wistar spleen cells. Against Targets 1 (D23 tumour) both non immunised syngeneic and allogeneic spleen cells cause a reduction in the remaining target cells as compared with targets and media alone.

This comparison of control cells was performed as the initial

choice of control effector cells (I.W. syngeneic to the donor) was unsatisfactory. The control cells during the experiments were changed to lymphocytes to animals syngeneic to the recipient. The lymph node lymphocyte LNL gave similar results when tested against the same targets. Non immunised allogeneic spleen cells had a non specific 'cytotoxic' effect - this was likely to make cytotoxicity with immunised spleen cells more difficult to achieve. Results in the experiments have therefore all been accepted as valid.

SECTION II

LYMPHOCYTE ACTIVITY IN ALLOGRAFT REJECTION

GRAFT-versus-HOST RESPONSE

A. BACKGROUND

B. G-v-H reaction to measure immune responsiveness.

C. G-v-H response as an index of activity of the injected cells.

D. Preliminary experiments

E. Rat experimental system

- Methods
- Results

F. Xenogeneic cell injections

- Methods
- Results

G. DISCUSSION

- Theory of graft-versus-host response
- G-v-H activity in rat allogeneic systems
- G-v-H activity in xenogeneic systems

H. APPENDICES

a) Method of g-v-h popliteal node weight assay

b) Method and results of mixed lymphocyte culture

A. BACKGROUND

A graft-versus-host reaction is a reaction of lymphoid cells injected into the host against the host. This occurs when the host has antigens present which are different from the injected graft cells and the host is unable to react against the donor inoculum. Examples of these reactions occur when lymphoid cells from an allogeneic donor are injected into new-born rats, and irradiated rats (Gowans, McGregor and Cowen, 1962) and when lymphoid cells from a parent rat are injected into the F_1 hybrid. The reaction occurs because the host fails to reject the graft cells and therefore the latter can react against the host antigen. The range of reaction varies from a local proliferation of cells to a total wasting disease.

The small lymphocyte is held responsible in initiating this reaction since in experiments large lymphocytes were eliminated before injection (Gowans, 1972). The identity of the initiator cell has been more clearly defined since then as a T-lymphocyte. B cells were removed from a rat thoracic duct cell population by passing the lymphocytes through a glass bead column, without diminishing the g-v-h activity (Hunt, 1973). The degree of g-v-h activity is directly proportional to the number of reactive cells in the inoculum of lymphocytes (Atkins and Ford, 1972). The donor cells after injection pass to the local draining node and this is the basis of the spleen weight and popliteal node weight assays (see below).

The fate of the donor cells has been studied by Ford, Burr and Simonsen (1970) by injecting ^{3}H uridine labelled lymphocytes in the footpad. All the recipients were killed 48 hours after injection and the distribution of radioactivity determined : 20% of the cells injected into the footpad had entered the recirculating lymphocyte

pool of the spleen and lymph nodes: 6% were retained in the draining popliteal node. Several hours after administration of these lymphocytes there is an increased number of transformed cells in the lymphoid tissue (Ford, 1973).

Most of the work on g-v-h activity has been performed from parental strain cells injected into the F_1 hybrid, e.g. DA -> $DALF_1$. In this system there is no host-versus-graft reaction involved. There are several methods of demonstrating a g-v-h reaction:-

1. Spleen weight assay

The spleen weight assay was developed in the mouse (Simonsen, 1962). An intravenous injection of parental spleen cells at a dose of 10 million cells was given to the F_1 hybrid. The spleen weight was measured 8 to 10 days later and was considerably enlarged compared with non injected controls of the same age. Microscopically the spleens showed an accumulation of pyroninophilic lymphoid cells and large collections of disintegrated polymorphs. The increased number of cells in the spleen was found by chromosome studies to be almost entirely due to dividing donor cells (Gowans, 1962).

2. Elkins method

Elkins (1964) found that local inoculation of lymphoid cells from inbred parental strain rats under the capsule of the kidney of F_1 hybrid rats was followed by local infiltration and destruction of the renal parenchyma. The maximum response was observed seven days after inoculation. Histological examination of the kidney revealed marked lymphocyte infiltration together with numerous primitive looking cells and frequent mitoses. The appearance in this localised g-v-h

reaction was histologically very similar to the appearance of a rejecting primary renal allograft.

3. Popliteal node weight assay

This assay was developed by Ford, Burr and Simonsen (1970). Injection of parental strain (A) or (B) thoracic duct lymphocytes into the footpad of (A x B) F_1 hybrid recipients was found to cause a marked increase in the weight of the popliteal node on the side of injection as compared with the opposite popliteal node. The control footpad was injected with cells syngeneic to the recipient. The weights of the popliteal nodes were measured 7 days after inoculation. The test popliteal nodes weighed up to 70 mgs., in comparison with the control nodes of 2.2 mgs.

B. G-v-H REACTION TO MEASURE IMMUNE RESPONSIVENESS

The popliteal node weight assay was modified by Salaman, Millar and Brown (1975) and claimed to measure the immune responsiveness in normal and immuno-depressed patients. Injecting human peripheral blood lymphocytes into the footpads of rats they were able to obtain a definite increase in the enlargement of the popliteal nodes, but there was very little difference between the response obtained with normal and irradiated cells, so it was felt that this response was largely the product of a host-versus-graft reaction. Irradiation of the recipient rats with 300 rads was found to abolish this host-versus-graft element, but still allowed for a threefold increase in the node weight with injected live xenogeneic cells in comparison with formalised dead cell controls. This latter increase in node weight was therefore thought to represent the g-v-h component of the

reaction on the part of the injected cells.

C. G-v-H RESPONSE AS AN INDEX OF ACTIVITY OF THE INJECTED CELLS

Extending the argument of Salaman et. al. (above):- if the degree of reactivity of the injected cells is reflected by the degree of g-v-h measured as weight of the local lymph node, then cells from an immunosuppressed subject should give a lower lymph node weight than do normal subjects.

Also cells from an individual with heightened reactivity should show increased g-v-h activity.

These postulates have been examined in this thesis in a rat experimental system, and with some observations using sheep cells and human cells.

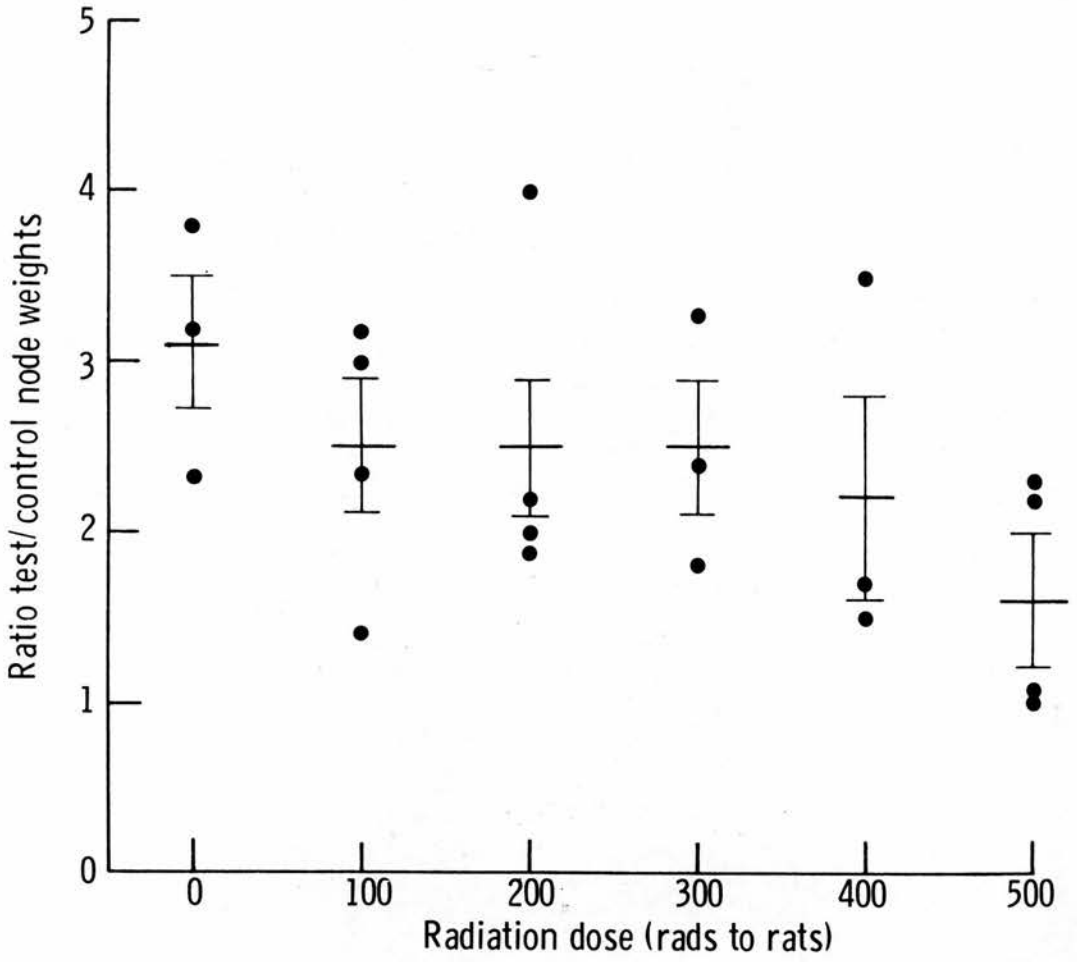
D. PRELIMINARY EXPERIMENTS

The technique of popliteal node weight assay is described in Appendix 2(a). In order to measure graft-versus-host activity of injected cells in an allogeneic or xenogeneic system it is preferable to diminish the host response in the recipient rat. Figure 2 shows the effect of host irradiation on the g-v-h response. A dose of 2×10^6 human blood lymphocytes was injected into rats receiving radiation from 100 rads to 500 rads and also rats receiving no irradiation. The test node weights were considerably larger in those animals which either were not irradiated or received a small dose. The control nodes however were also larger in these unirradiated animals: (table XIa) possibly due to a generalised lymphatic response after injection of foreign cells into a rat still capable of host-versus-graft, or due to the reduction of node size in rats which were irradiated. A dose of 200 to 400 rads appeared to give the maximum ratio between test/control cells (Figure 2). A dose of 300 rads was therefore selected as the dose to abolish host-versus-graft activity in recipient rats. Table XIa also confirms that the injection of 0.1 ml. of media in the footpad as a control does not alter the size of the recipient node weight either with or without irradiation.

Figure 2 **The effect of host irradiation on the G-v-H response**

2×10^6 peripheral blood human lymphocytes were injected into the left footpad of Wistar rats. The right footpad was injected with media. The popliteal nodes were weighed seven days later. Each dot • represents the ratio of the test : control node weight in one rat.

THE EFFECT OF HOST IRRADIATION ON THE GvH RESPONSE



Footpad Injection	Recipient irradiation (300 rads)	Weights of popliteal nodes (mgs)
Nil	+	0.9, 1.2, 1.0
	-	2.4, 1.4, 2.0
R.P.M.I.	+	1.0, 1.0, 1.5
	-	3.0, 1.5, 2.0

Weights of popliteal nodes after injection of 0.1 ml. media into the footpad of rats. The nodes were removed on the seventh day after injection.

Table XIa

The usual duration of time between injection of cells and removal of lymph nodes is seven days. The suggestion by Dorsch and Roser (1974) that four days would be a more satisfactory time for removal in allogeneic systems was considered and figure 3 shows the ratio of test : control node weights in rats either irradiated with 300 rads or non irradiated, receiving either xenogeneic or allogeneic cell inoculation. The recipient rats receiving either human or rat cells (on day 4 or day 7) were identical in age and weight. In the unirradiated rats the ratios were higher after xenogeneic injection on the fourth day and after allogeneic injection on the seventh day. In the irradiated rats the maximum increase in ratio was on the seventh day. The nodes in the rest of these experiments were therefore removed on the seventh day after inoculation.

Figure 3 also shows that lack of irradiation of the recipient did not alter the popliteal node weight ratio when allogeneic rat donor cells were injected. Non irradiated recipients in the first series of experiments (see rat experimental system) were therefore not used.

THE RATIOS OF NODE WEIGHTS ON THE 4th and 7th DAY AFTER INJECTION

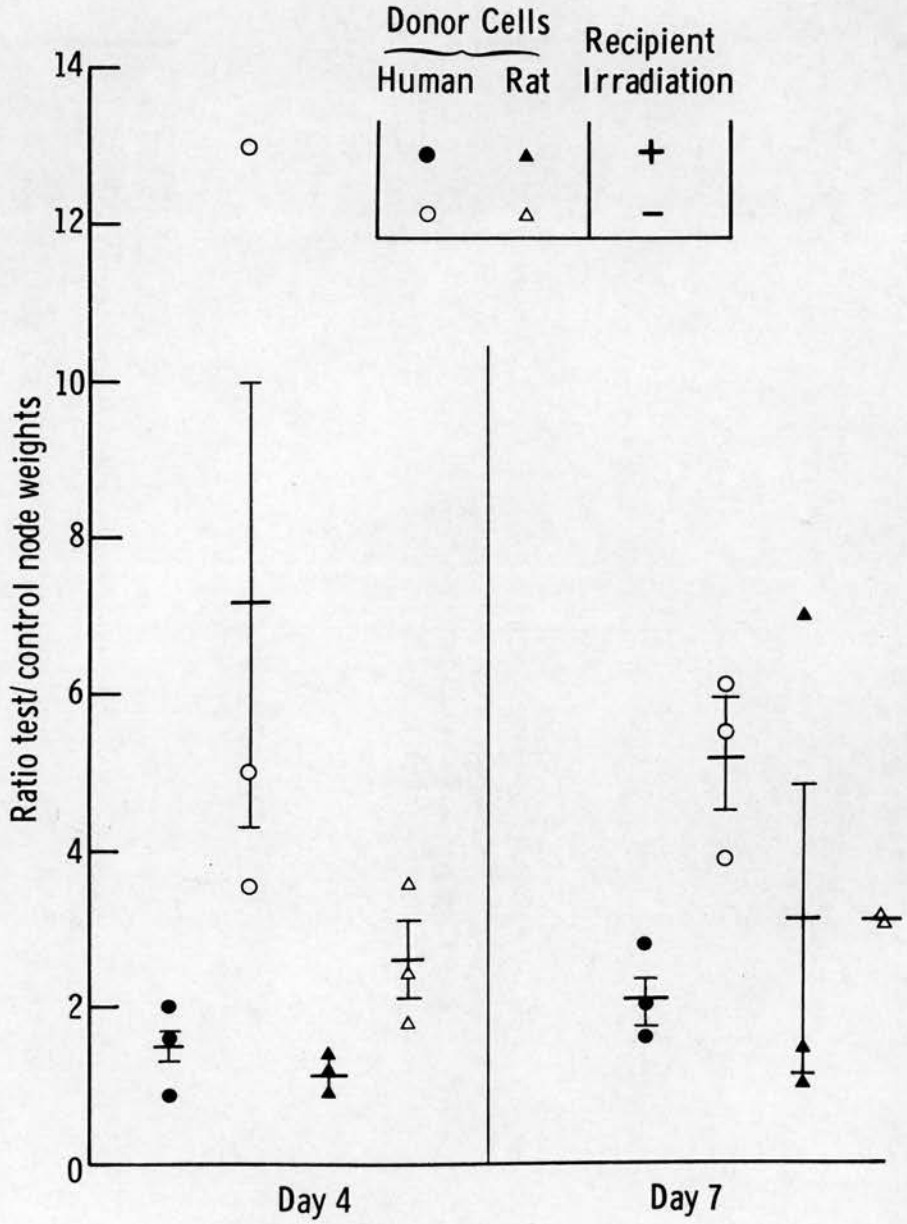


Figure 3 The ratio of popliteal node weights on the 4th and 7th day after the injection of lymphocytes into the recipient rat footpad. Media controls used. Each ● represents the ratio in one rat.

The amount of g-v-h activity is related to the number of cells in the donor inoculum. A few experiments were performed to compare the node weights in both irradiated and non irradiated rats using xenogeneic human peripheral blood lymphocytes as the donor inoculum. Figure 4 confirms that a dose of 2×10^6 cells increased the node weight ratio in both irradiated and non irradiated recipients. This dose was selected wherever possible depending on the availability of lymphocytes. Table XIb shows the node weight ratios using the parent to F_1 hybrid combination in unirradiated recipients. The dose of 2×10^6 donor cells gave the greatest increase in node weight.

RATIO OF NODE WEIGHTS IN RATS AFTER INJECTION OF XENOGENEIC HUMAN CELLS

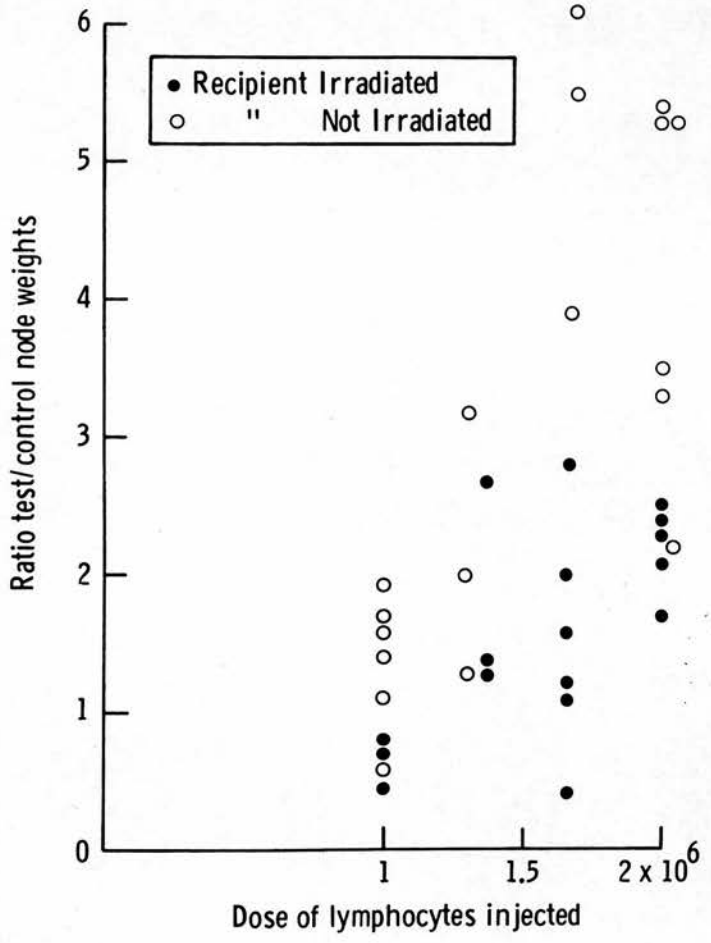


Figure 4

Each ● represents the node weight ratio in one rat. Media controls used.

XRT	Dose of cells in donor inoculum		
	1×10^6	2×10^6	4×10^6
+	2.8	3.1	6.8
-	2.4	6.7	3.4

Popliteal node weight ratios in response to increasing numbers of donor lymphocytes from I.W. rats into $(IWAS)F_1$ recipients. Each number represents 1 rat. Media controls used.

Table XIb

Some experiments were also performed using irradiated donor cells. The node weights after test and control cell injection are shown in Table XIc.

Human donor cells were injected after irradiation with 20,000 rads; in non irradiated rats the node weights were the same - 1.2 mgs. to 3.0 mgs. Using a dose of 2,400 rads to obtain control cells, there was some increase in node weight in the non irradiated recipients.

The use of large doses of irradiation 20,000 rads to the donor cells would appear to not only prevent the lymphocytes from dividing but also destroy their antigenicity as compared with a dose of 2,400 rads where the cells may have lost their ability to divide but were still antigenic (Hodis and Terry, 1974, Kasakura and Lowenstein, 1968). The cells after a larger dose of irradiation could be used as adequate control cells. This effect however is only seen in unirradiated recipients in which the host-versus-graft (h-v-g) element has not been abolished. In the irradiated recipients there appears to be very little difference between the node weights when media or irradiated cells were used as controls.

In view of this throughout the rest of the experiments, where host-versus-graft had been abolished by recipient irradiation, media controls were used.

POPLITEAL NODE WEIGHTS (MGS.)

FOOTPAD INJECTION	RECIPIENT IRRADIATION 300 rads	RECIPIENT NO IRRADIATION	Comment
xenogeneic cells	3.3 3.7	10.7 17.6 11.6	Only slight lymph node weight increase attributable to G-v-H. Most of the response is H-v-G
media control	1.4 1.5	2.0 3.3 2.2	
xenogeneic cells	1.8 1.9 2.2	9.4 5.1 8.7	
media control	0.8 0.9 1.3	2.7 2.3 2.3	
xenogeneic cells	1.4 1.2 1.4	8.6 5.8 9.3	
media control	0.5 0.6 0.9	1.4 1.5 1.7	
xenogeneic cells	3.8 1.5 0.5	7.0 10.5 7.6	20,000 rads to lymphocytes obliterates antigenicity also H-v-G to donor cells
irradiated xenogeneic cells 20,000 rads	1.6 0.6 0.9	1.4 3.0 1.2	
xenogeneic cells	1.3 2.6 1.6	4.5 6.0 7.1	
irradiated xenogeneic cells 20,000 rads	1.0 2.0 0.6	1.4 3.0 2.2	
xenogeneic cells	1.1 1.3 0.7	4.4 2.6 2.6	2,400 rads still allows smme H-v-G
irradiated xenogeneic cells 2,400 rads	0.8 0.7 0.3	3.6 3.1 4.1	
xenogeneic cells	2.0 2.8 3.8		
irradiated xenogeneic cells 2,400 rads	4.6 2.6 3.2		
irradiated xenogeneic cells 20,000 rads	0.4 0.8 1.0	2.7 2.6 3.5	
media control	0.9 0.5 1.0	1.6 2.0 1.6	

Effect of irradiation of the recipient on the popliteal node weight using human peripheral blood lymphocytes as donor cells. The mode weights after test and different control cells injections are shown.

Table XIc

The effect of immunosuppression of the donor rat by irradiation has also been looked at in an allogeneic system. Donor rats received 300 rads of radiotherapy thirty minutes prior to the removal of lymphocytes. These lymphocytes were injected into the footpad of allogeneic rats which were either irradiated or not irradiated.

The results of donor rat irradiation are shown in Table XId. Donor irradiation served to obliterate all g-v-h response. The node weights were slightly increased without recipient irradiation, but were the same as controls when any host-versus-graft response had been obliterated with recipient irradiation.

Donor XRT		Recipient XRT		Ratios Test : Control		Mean
I.W. or A.S.	-	A.S. or I.W.	-	2.9	2.4	2.7 ± 0.2
			+	1.7	1.3	1.5 ± 0.2
I.W. or A.S.	+	A.S. or I.W.	-	1.8	1.3	1.6 ± 0.2
			+	1.1	0.9	1.0 ± 0.1

The effect of donor irradiation on popliteal node weights. Each ratio is the mean ratio of 3 rats.

Table XId

In the experiments in which donor parent lymphocytes are injected into F_1 hybrid, IW - (IWAS)F_1, there should be no recognition by the recipient that the donor cells are antigenic, i.e. no h-v-g reaction. Table XIe shows the results where either parent I.W. or A.S. lymphocytes were injected into (IWAS)F_1 footpads, and also (IWAS)F_1 lymphocytes were injected into I.W. footpads for comparison. The increase in node weight ratio after the injection of F_1 hybrid cells into the non irradiated parent is a pure host-versus-graft reaction which is almost obliterated by host irradiation. This confirms that 300 rads host irradiation removes the h-v-g component in our experiments. The greater increase in the ratios after injection of parental cells in the F_1 system is pure g-v-h which will be discussed later (section 11E).

Donor LNL	Recipient	Recipient XRT	No. of Tests	Ratio Test : Control	Mean Ratios
A.S. or I.W.	(IWAS)F_1	-	3	10.5, 2.9, 2.5	5.3 ± 2.6
		+	2	12.5, 3.3	7.9 ± 4.6
(IWAS)F_1	I.W. or A.S.	-	2	3.0, 3.6	3.3 ± 0.3
		+	2	1.3, 2.6	1.9 ± 0.6

The ratio of popliteal lnnode weights in the parent to F_1 hybrid system to determine the degree of host-versus-graft activity. Each ratio represents the mean of 3 rats. Media controls used.

Table XIe

Conclusion

From the results of the preliminary experiments and from other papers quoted, certain parameters have been taken for the experiments which follow:-

1. The recipient popliteal nodes have been removed on the seventh day after injection of donor inoculum into the recipient footpad.

2. The number of lymphocytes in the donor inoculum was 2×10^6 cells in 0.1 ml. of media.

3. Media (0.1 ml.) was injected into the opposite recipient footpad as the control.

4. The dose of irradiation of 300 rads to the recipient rat was used to remove the host-versus-graft reactivity in the allogeneic and xenogeneic systems.

E. RAT EXPERIMENTAL SYSTEM

When lymphocytes from a donor (graft) animal are injected into an other non identical recipient (host) animal, the lymphocytes recognise the host as foreign and proliferation of the cells occurs. In the assay used in these experiments the proliferation of cells occurs in the popliteal node after injection of donor lymphocytes into the footpad. The present work has been performed to study the T cell activity (i.e. proliferation) in relation to the allograft response. In an allogeneic system the effect of presensitization of donor lymphocytes by prior skin and organ grafting will be studied by observing the weight of the popliteal node after injection of these presensitised cells. The alteration of this effect by the use of immunosuppressive agents in the donor animal will also be studied.

Methods

The technique of the popliteal node weight assay is described in Appendix 2(a).

1. The first set of experiments were performed by injecting lymph node lymphocytes from a donor strain of rats into allogeneic rats. The recipient rats received 300 rads irradiation prior to injection to abolish any h-v-g- reactivity present.

There were 5 groups of cell donors. Diagram 6

a) NORMAL RATS - lymph node lymphocytes from an allogeneic donor strain were injected into the footpad of the recipient rats.

b) IMMUNOSUPPRESSED RATS - the rats of an allogeneic donor strain were immunosuppressed with cyclophosphamide 5 mgs/100 g. and cortisone 5 mgs/100 g. intra-muscularly (i.m.) on alternate days for two weeks prior to removal of lymph nodes. The lymphocytes were then injected into the recipient rat footpad.

c) PRIMARY ALLOGRAFTED RATS - a skin allograft was transferred from an inbred rat of the recipient strain to the donor rat. Seven days later the 'specifically' presensitised lymph node lymphocytes of the rat bearing the graft were injected into the footpad of the recipient rat.

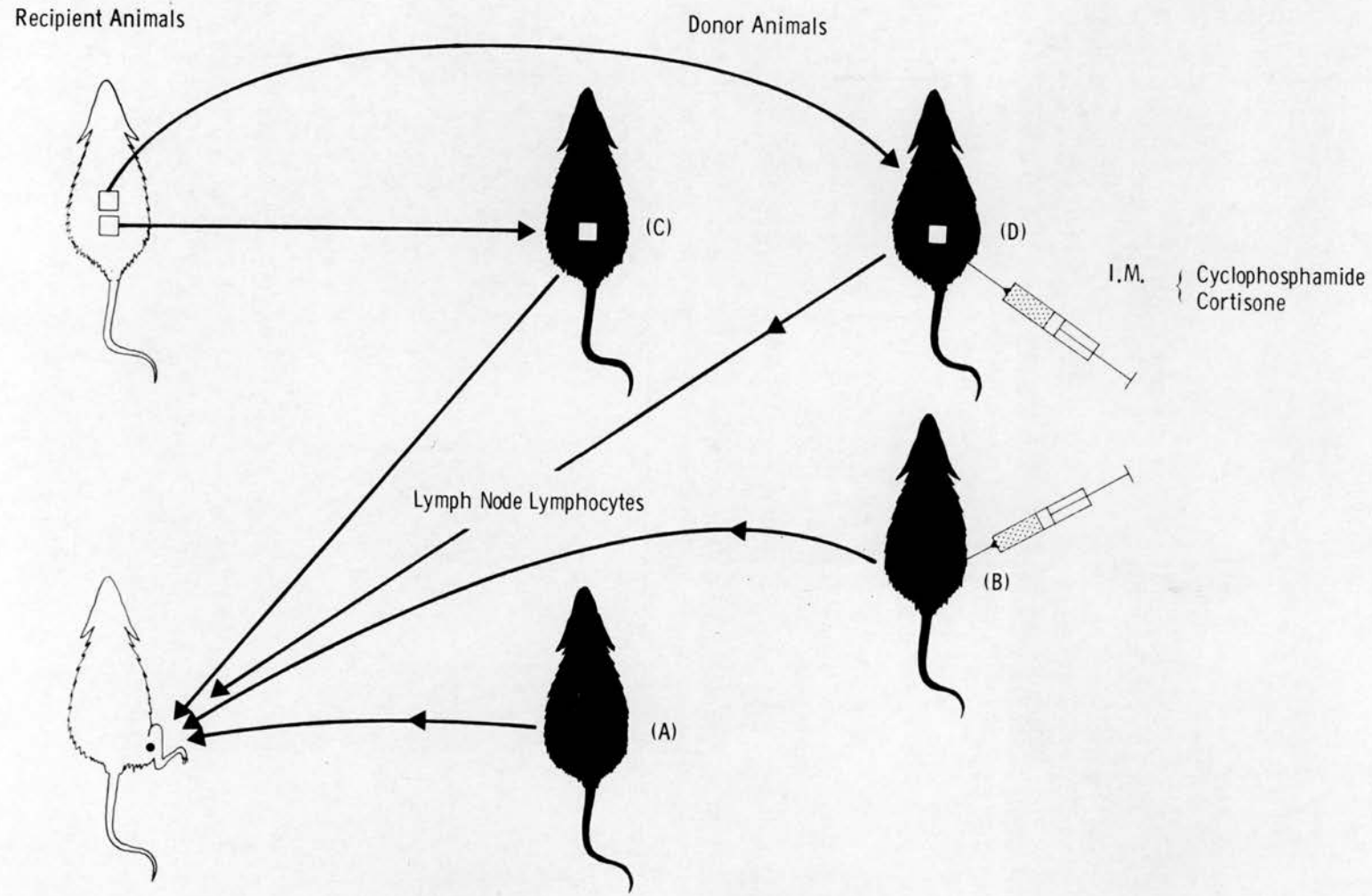
Also cells were used from animals grafted with skin from rats allogeneic to both donor and recipient strain. The donor cells were then considered to be 'non specifically' presensitised.

d) ALLOGRAFTED AND IMMUNOSUPRRESSED RATS - skin was allografted from an inbred rat of the recipient strain to a donor rat also receiving immunosuppressive drugs during the period of the graft. Seven days later the lymph node lymphocytes of the immunosuppressed

Diagram 6 **Scheme of Host-versus-Graft experiments in allogeneic rats.**

The donor rats lymphocytes were injected into the footpad of the recipient rats. Four groups of donor rats are shown: (A) NORMAL, (B) IMMUNOSUPPRESSED, (C) PRIMARY ALLOGRAFTED. The skin graft being taken from the recipient strain. (D) PRIMARY ALLOGRAFTED AND IMMUNOSUPPRESSED.

Diagram 6



SCHEME OF GRAFT VERSUS HOST EXPERIMENTS IN ALLOGENEIC RATS (GROUP 1)

rat bearing the graft were injected into the footpad of a rat of the recipient strain.

e) Further experiments were performed whereby the donor of the cells was specifically hypersensitised by transferring three skin grafts and two intraperitoneal injections of lymphocytes at intervals from a rat of the recipient strain to the donor strain.

2. The second set of experiments eliminated any host-versus-graft activity which might be manifest in the popliteal nodes, by using cells from inbred Wistar or A.S. parent into (inbred Wistar x A.S.)F_1 hybrid - (IWAS)F_1.

There were two groups of cell donors.

a) NORMAL RATS where either parent (I.W. or A.S.) lymphocytes were injected into (IWAS)F_1 footpads.

b) PRIMARY ALLOGRAFTED RATS

i. (IWAS)F_1 skin was grafted onto I.W. or A.S. rat. The lymphocytes from this rat were injected into the footpad of (IWAS)F_1 recipient either <u>seven</u> or <u>28</u> days after grafting.

ii. Skin from an A.S. rat was grafted onto I.W. and then the lymphocytes from the I.W. were injected into the footpad of (IWAS)F_1 at <u>7</u> days.

Results

First series of experiments

The results of the g-v-h experiments after allogeneic lymphocyte inoculation from A.S. to Wistar rats are shown in figure 5 and table XIIa - the recipient rats all received 300 rads irradiation, each experiment was performed in 3 rats.

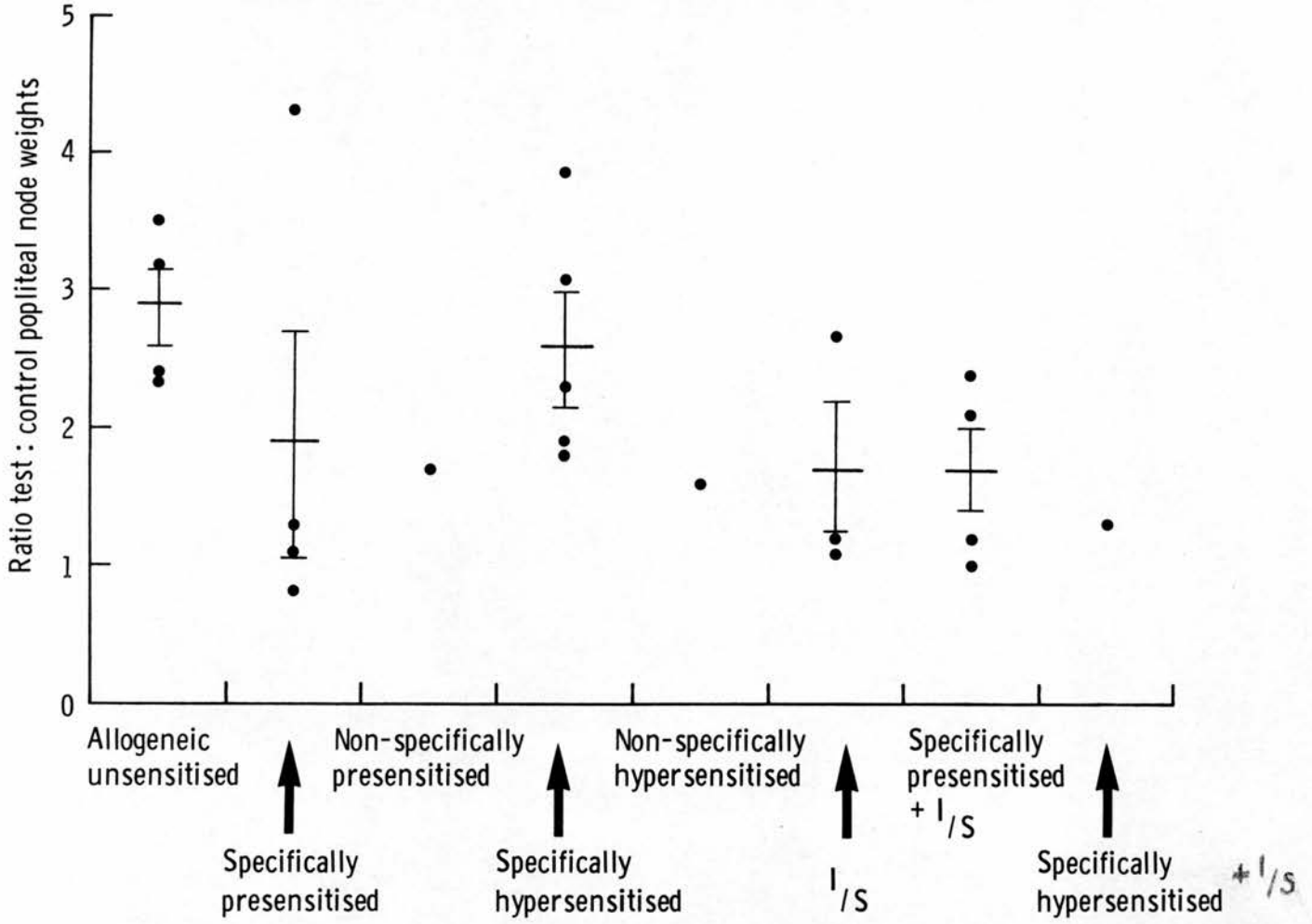
The popliteal node weight ratios were increased to a mean of 2.9 after injection of allogeneic cells. This increase was not maintained after specific presensitization of the donor cells with a skin graft, the ratios of test : control popliteal nodes were 0.8 to 1.3 (except one ratio of 4.3 which was only achieved because of the abnormally small size of a control node which weighed 0.2 mgs.).

When the donor cells were from animals specifically hypersensitised, against the recipient, the ratios were again increased to a mean of 2.6.

The results of the ratios when the donor cells were from immunosuppressed animals, are a little lower than the ratios with cells from normal donors. This may be due to the smaller number of cells in the donor inoculum.

Figure 5

The Ratio of Popliteal Node Weights (Test : control). The donor inoculum from Rats into Irradiated Recipient Allogeneic Rats. (Each • Represents the mean of 3 Rats in each experiment).



Donor	No. of rats	Ratio of node weights Test : Control
Allogeneic unsensitised	11	2.8 ± 0.6
Specifically presensitised	11	1.7 ± 0.5
Specifically hypersensitised	15	2.6 ± 0.3
Allogeneic immunosuppressed	9	1.7 ± 0.4

The ratio of popliteal node weights from donor rats into <u>irradiated</u> allogeneic recipients. The ratio is estimated from the separate weights of each rat, rather than from each test of three rats. There is no significant difference between the mean ratios in each group.

Table XIIa

Second series of experiments

The results of the g-v-h experiments after allogeneic lymphocyte inoculation from parent to F_1 rats are shown in table XIIb.

There was a considerable increase in node weight in both those recipient rats which received irradiation, and those which received no irradiation.

Presensitization by skin grafting served to increase the node ratios even more and there was a greater increase in the irradiated rats who received lymphocytes from the donor presensitised 28 days prior. There are two reasons for this, firstly, the control nodes (media control) were much smaller in the irradiated recipients. Secondly, the 'test' node weights from the irradiated rats often were truly greater after presensitization especially after 28 days (see discussion).

Donor LNL	Recipient	Recipient XRT	No. of Tests	Ratios Test : Control	Mean ratios
Allogeneic unsensitised					
A.S. or I.W.	(IWAS)F_1	-	3	10.5 2.9 2.5	5.3 $\pm$ 2.6
		+	2	12.5 3.3	7.9 $\pm$ 4.6
Primary allografted presensitised by (IWAS)F_1 skin					
7 days A.S. or I.W.	(IWAS)F_1	-	4	2.6 4.3 3.8 5.3	4.0 $\pm$ 0.56
28 days A.S. or I.W.	(IWAS)F_1	-	3	1.6 2.3 3.8	2.6 $\pm$ 0.6
7 days A.S. or I.W.	(IWAS)F_1	+	4	7.7 2.4 6.1 4.1	5.1 $\pm$ 1.2
28 days A.S. or I.W.	(IWAS)F_1	+	3	12.1 8.0 7.7	9.3 $\pm$ 1.4
Primary allografted I.W. presensitised by A.S. graft					
I.W. 7 days	(IWAS)F_1	-	1	5.0	5.0
		+	1	6.7	6.7

The ratio of popliteal node weights. The donor inoculum is from parent rats into F_1 hybrid. Each ratio represents the mean of 3 rats.

Table XIIb

F. XENOGENEIC CELL INJECTIONS

The hypothesis has been advanced that the popliteal node weight assay in the rat may be used to determine the degree of immune competence of the cell donor in the assay (Salaman et. al., 1975). Peripheral blood lymphocytes from patients with renal failure and controls were injected into the footpads of irradiated mice. Those patients whose lymphocytes initiated a large response were called 'responders' and those with a small response 'non responders'. They suggested that by this assay it could be determined which patients waiting transplantation might be most suited as allograft recipients.

The follow up of these patients after transplantation has not yet been long enough to have sufficient evidence as to whether this hypothesis is correct.

As there appears to be a paradoxical inverse relationship between antigenic strength and g-v-h activity (Ford and Simonsen, 1971). this particular xenogeneic system might be considered of limited value as a measurement of T cell activity.

The next experiments were set up using lymphocytes from xenogeneic animals and injecting the cells into host rats in order to see whether animals either after immune stimulation or immunosuppression had an altered g-v-h reactivity. In other words to see whether this assay could be used as a measurement of immune competence of the donor.

Methods

The technique of popliteal node weight assay is described in Appendix 2(a). The experiments were performed by injecting donor lymphocytes into both irradiated and non irradiated rats.

1. Sheep cells

The thoracic duct lymph was obtained from sheep used in the experiments in Section III of this thesis:-

a) Lymphocytes from sheep in which the thoracic duct alone was cannulated were injected into the rat footpad ('normal sheep').

b) Lymphocytes from the thoracic duct of sheep receiving i.v. prednisolone 100 mgs/day and azathioprine 400 mgs/day were injected in the rat ('immunosuppressed sheep').

c) Thoracic duct lymphocytes were obtained from sheep, who had received a renal allograft, and injected into the rat ('rejecting allografted sheep').

d) Thoracic duct lymphocytes were obtained from sheep, who had received both a renal allograft and immunosuppressive drugs, and injected into the rat ('immunosuppressed allografted sheep')½

2. Human cells

a) Peripheral blood lymphocytes were obtained from healthy adults and used as the donor cells ('normal human').

b) Blood lymphocytes from a patient with a functioning and non rejecting renal transplant on routine immunosuppression were used as the donor cells ('non rejecting allografted human').

c) Blood lymphocytes from a patient with a renal transplant during a rejection crisis were used as the donor cells ('rejecting allografted human').

d) Blood lymphocytes from patients receiving multiple combined chemotherapy for widespread malignant disease (carcinoma of breast and stomach) were used as donor cells. The dose of chemotherapeutic agents given to these patients is methotrexate 0.3 mgs/Kg., 5 fluoro-uracil 15 mgs/Kg., vincristine 0.015 mgs/Kg. i.v. weekly, cyclophosphamide 2.5 mgs/Kg. orally daily. The blood was removed 7 days after i.v. injection ('immunosuppressed human').

Results

Xenogeneic sheep cells

The results of g-v-h activity using xenogeneic sheep as donors are shown in figure 6. Very little increase in node weight ratio was obtained in irradiated recipients, which contrasts fairly strongly with the increased ratios in the non irradiated recipients. There appeared to be very little difference in g-v-h activity whether lymphocytes came from a sheep with a rejecting renal allograft or from a normal sheep.

Xenogeneic human cells

The g-v-h activity was usually increased twofold after the injection of xenogeneic cells (Figure 7). There was no alteration in the activity whether the lymphocytes from a patient with a rejecting allograft, or from a patient who was not rejecting his graft. Surprisingly the lymphocytes in the peripheral blood from the patients on combined chemotherapy gave considerable g-v-h activity.

Figure 6 The Ratio of Popliteal Node Weights (Test : Control). The Donor Inoculum from Sheep into ● Irradiated Rats and ○ Non-Irradiated Rats. Each ● Represents the Mean of 3 Rats.

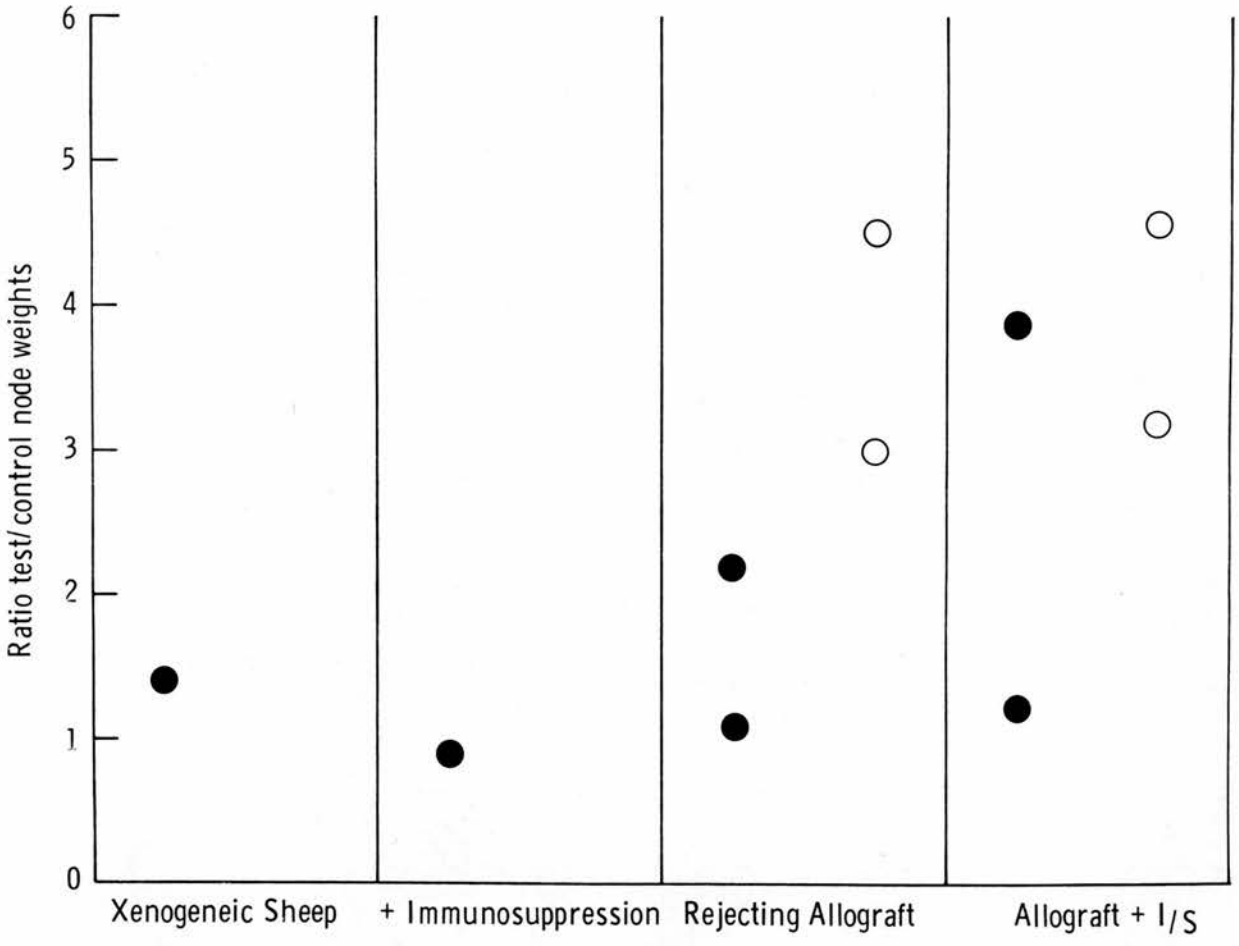
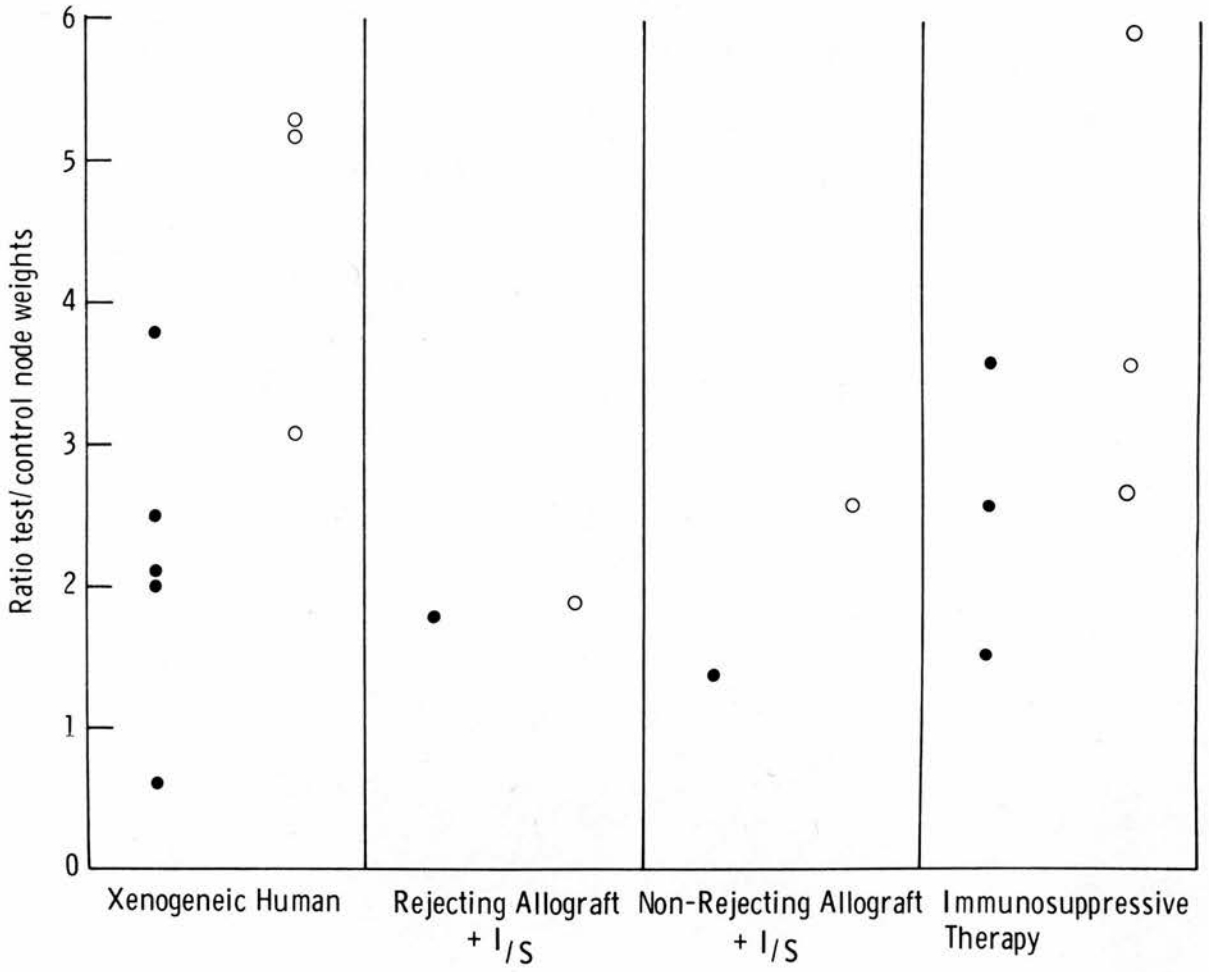


Figure 7 The Ratio of Popliteal Node Weights (Test : Control). The Donor Inoculum from Humans into • Irradiated and ○ Non-Irradiated Rats. Each • Represents the Mean of 3 Rats



G. DISCUSSION

The theory of graft-versus-host response

In the graft-versus-host assay the immuno-competent cells are injected, the target cells are fixed and there is an unlimited supply of non specific cells, which can be recruited from the host circulating pool, to assist in the reaction. The reaction is proliferation and recruitment of cells in response to the recognition by the injected cells that the host cells are foreign. The assay thus should be a reconstruction of what happens in vivo during allograft rejection.

The effect of presensitization on G-v-H response

There appears to be a paradoxical inverse relationship between the antigenic strength of the animal and the g-v-h activity. In rats the augmentation of g-v-h activity increases with the weakness of the antigenic barrier (Ford and Simonsen, 1971). With weak strain combination of rats (AgB identical strains) the donor rats were immunised with two skin grafts of the recipient strain. The injection of cells from immunised donors gave a g-v-h activity that was double that of the non-immune donors. Using AgB different strain (strong strain combination) the immunization of the donor considerably reduced the g-v-h activity as compared with non-immune donors.

A decrease in g-v-h activity between allogeneic strains of rats in comparison with parent $\rightarrow F_1$ hybrid has <u>also</u> been shown by Dorsch and Roser (1974). They did however have definite lymph node weight enlargement which was maximum on the fourth and not seventh day after inoculation (figure 8). They believed that the allogeneic cells

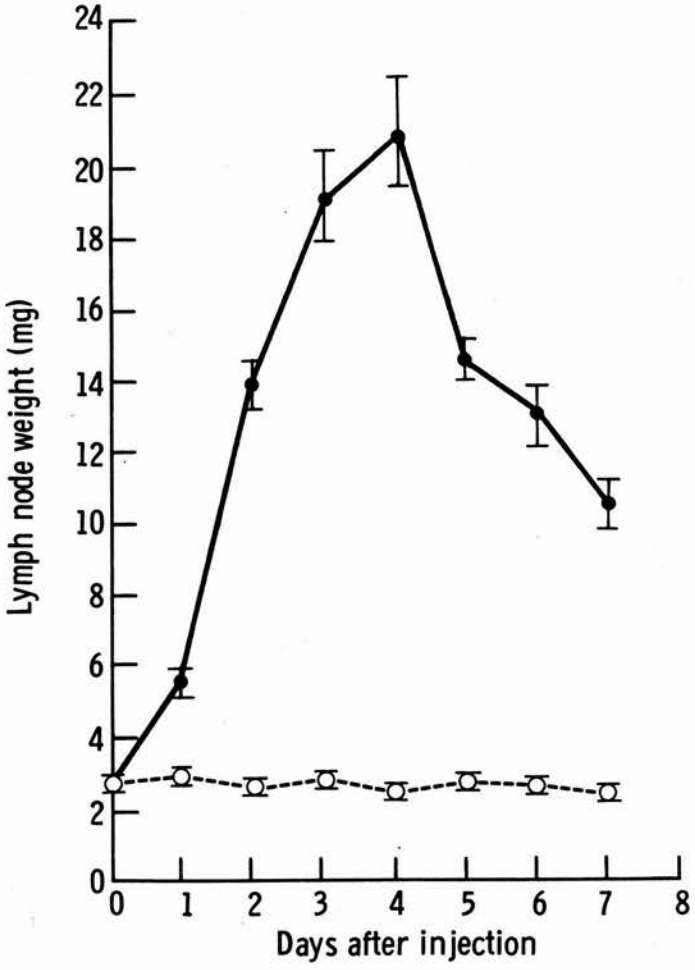


Figure 8 Lymph node weight response to allogeneic cells: ●—● allogeneic cells, o---o control syngeneic cells. Dorsch and Roser (1974)

were ultimately rejected by the host. Presensitization of the donor cells reduced the weight of the popliteal node, the peak of the weight increase being even earlier, on the second day. They suggested that the reduction of the response in the sensitised animals was mediated by a circulating antibody produced by the host.

In contrast to these findings Bain and Alton (1966) found that injection of C57L spleen cells, sensitised against C_3H tissue, into C_3H mice caused splenomegaly at five days. In comparison injection of non-sensitised C57L spleen cells caused less splenomegaly, and the increase in size of the spleen took longer.

The reduction of G-v-H by suppression of the host

If steroids are given to the host (F_1 recipient) within 24 hours of inoculation with parental cells the g-v-h activity is decreased (Cohen and Cloman, 1971). Cyclophosphamide was also found to decrease g-v-h activity when given to the host

The reduction of G-v-H by suppression of donor cells

Dorsch and Roser (1974) determined the contribution made by g-v-h to enlargement of popliteal nodes by allogeneic transfer of cells. They compared the response to normal allogeneic cells with mitomycin C - treated allogeneic cells. Normal cells gave a lymph node weight of 18 mgs. and cells treated with mitomycin C in vitro, a weight of 9.0 mgs., concluding that the g-v-h element was responsible for half the increase in node weights. Ford and Simonsen (1971) irradiated donors with 300 rads and found no alteration in g-v-h activity.

The host component in G-v-H activity

In the parent to F_1 hybrid combinations, there will be no host-versus-graft element. When allogeneic cells are used as we have seen there is a marked host-versus-graft reaction which allows for considerable generalised lymph node enlargement (Salaman et. al.,

1975) which can be abolished by host irradiation.

There has been recent evidence in the parent to F_1 hybrid strains that there is a considerable host component in the enlargement of the popliteal node. The grafted cells may release factors which stimulate the host cells to be trapped into the node, rather than the increase in node weight being due to donor cell division. This has been shown by Rolstad (1976) whereby using allo antisera with specificity for the donor it was found that about 90% of the live cells suspended from the node were of host origin. Also by labelling host cells with $^{51}Cr.$, re-injection of these cells into the g-v-h recipient gave a considerable accumulation of $^{51}Cr.$ in the stimulated node as compared with the unstimulated control node in the same rat.

G-v-H reactions have proved useful for the study of the reaction of lymphoid cells to transplantation antigens in vivo. The g-v-h measures lymphoid cell proliferation and not host cell damage and in this is comparable to the M.L.C. It is thought that although g-v-h and target cell destruction are both functions of T cells there are probably two types of cell involved. The cell involved in the g-v-h has been called an initiator lymphocyte and in cell mediated immunity a cytotoxic lymphocyte (Ford, 1973). The fact that immunization against strong AgB antigen by skin grafting in the rat gives no change in the g-v-h response (see above) suggests that, in this situation, there is no increase in initiator cells produced by allografting; or any effect may have been reduced by the effect of suppressor cells from the host; or the effector lymphocyte whether an initiator or cytotoxic lymphocyte may be trapped within the skin graft itself. It is probable however that cytotoxic lymphocytes are derived from these initiator lymphocytes.

In the light of previous work on the popliteal node weight g-v-h assay the results from the present work will be considered.

G-v-H activity in rat allogeneic systems

Unsensitised rats

When allogeneic cells were injected there was a definite g-v-h response. This is however much smaller than that when parental cells were injected to the F_1 hybrids.

When host rats were not irradiated in the first series of experiments, there was no additional activity attributable to a host versus graft reaction. No host-versus-host reactivity was expected in the second series of experiments between parents to F_1 hybrids, and additional host irradiation indeed made no alteration in node weight ratios, although there was a decrease in the control node weights.

In M.L.C. there was very little reactivity between lymphocytes of the Wistar and A.S. strains used. As it is known there is an increase in g-v-h activity the more compatible the strains (Ford and Simonsen, 1971) greater g-v-h activity might have been expected in the present experiments (Appendix 2b).

Presensitized rats

By prior skin grafting from the host to donor strain, the transferred cells were considered to be presensitised on removal after 7 days. It is possible at seven days that many of the immune lymphocytes are still in the graft or adjacent nodes, therefore in the second series of experiments the lymphocytes were removed from the presensitised donor at 4 weeks.

In the allogeneic system A.S. to I.W. there was no increase in g-v-h activity with presensitization of the cells. The node weight ratios was even decreased after 1 skin graft, although after

hyperimmunization the ratios were the same as the normal rate. This agrees with the findings of Ford and Simonsen (1971), who transferred the cells from specifically hypersensitised animals 10 to 21 days after the second skin graft. This lack of reactivity might be due to lymphocyte accumulation in the graft after primary grafting but is unlikely to be so after hyperimmunization with 2 skin grafts and one intraperitoneal injection of cells.

The results of presensitization in the parent to F_1 hybrid also show that there is no increase in the node weight ratios as compared with the unsensitised rats except in the irradiated rats receiving presensitised lymphocytes at 28 days. These experiments do clarify the situation as to the timing of removal of lymphocytes. There was no difference in the node weight ratios in non irradiated rats when lymphocytes were removed at either seven or twenty-eight days after grafting indicating that the lymphocytes removed from the general lymphoid tissues at both times were 'immunised' in an identical fashion.

The interesting feature of these experiments is the considerable increase in the node weight ratios after irradiation of the host which was an actual increase in weight as well as a decrease in control node weight. These F_1 rats would not be expected to have a host-versus-graft element in the response, therefore irradiation presumably should have no effect except to any host component (i.e. recruitment) of the popliteal node weight increase. This particular experiment therefore refutes strongly the work of Rolstad (1976) that there is a considerable host component in the enlargement of the popliteal node.

The enlargement of the popliteal nodes in irradiated (IWAS)F_1

rats, after both 7 and 28 days prior sensitization of the A.S. rat with (IWAS)F_1 skin grafts, deserves further thought. The A.S. rat is immunised against the I.W. component of the hybrid graft, and when the immunised cells are injected into the F_1 hybrid presumably the A.B. cells are reacting as a second set reaction against the I.W. component in the recipient rat.

There is also another inexplicable point raised in these experiments. All other workers have stated that there is no host-versus-graft in the parent to F_1 situation. This may not be true as with transfer of F_1 cells to parent we have shown some increase in node weight ratio.

Immunosuppressed rats

The decrease in the node weights after immunosuppression may be an artefact due to the lesser number of cells used, which ranged from 0.05×10^6 to 0.6×10^6 per injection. The small number of cells was due to marked lymphoid atrophy after cortisone and cyclophosphamide but Ford et. al., (1970) obtained a maximum response of g-v-h activity with 0.3×10^6 cells, an increase in the number of cells did not increase the response thereafter. Irradiation of the donor, prior to removal of lymphocytes, also decreased the popliteal node weight ratio in irradiated recipients as compared with unirradiated recipients but not in comparison with normal rats.

It is likely that immunosuppression of the donor may decrease the ability of the lymphocytes to initiate a g-v-h response in this allogeneic system, confirming the work of Cohen and Claman (1971) in the parent $\rightarrow F_1$ system. No work has been done in this study using immunosuppression or donor suppression in the parent $\rightarrow F_1$ experiments.

G-v-H activity in xenogeneic systems

Most work in g-v-h activity has been performed in either parent to F_1 hybrid or allogeneic systems. The reason for considering xenogeneic systems is to find a method of measuring the immune competence of lymphocytes injected into irradiated hosts in order to determine whether the animals from whom the donor lymphocytes are obtained are 'responders'. In order to confirm this hypothesis we have injected lymphocytes from both sheep and humans, in whom there was a rejecting allograft, with no increase in the popliteal node weight ratios as compared with normal sheep and human controls. This refutes this hypothesis as presensitised animals are presumably 'responders' and more likely to reject a further allograft.

The other part of this theory is that lymphocytes from animals with decreased immune competence are less likely to respond in the g-v-h assay. Using irradiated recipient rats (Salaman et. al.,) found that injection of antihuman ALG to the recipient reduced the g-v-h response with human peripheral blood lymphocytes. Again we have shown by removing lymphocytes from sheep on azathioprine and prednisolone, and from patients on multiple chemotherapy, and injecting them into xenogeneic irradiated rats, that a decrease in immune competence does not result in a decrease in g-v-h activity.

The effect of host irradiation

In the allogeneic rat experiments very little alteration was obtained by host irradiation to obliterate any h-v-g except in the parent to F_1 hybrid presensitised animals which has been discussed.

When xenogeneic human and sheep cells were injected there was

a large increase in the weight of the popliteal node which was reduced by host irradiation. The radiation either blocks the host-versus-graft response or may abolish the non specific recruiting effect of the g-v-h response or possibly a combination of both.

Conclusion

As a measurement of T cell activity in the allograft situation this assay has limited value. Although g-v-h can be obtained between allogeneic rats which can be reduced by immunosuppression, there has been no augmentation of the reaction when grafted cells were presensitised as one would expect in a second set rejection of a graft.

The hypothesis advanced that the popliteal node weight in the rat might be used to determine the degree of immune competence of the cell donor of the assay is also doubted. As a predictive test for renal transplantation in humans the full use of this assay has yet to be determined.

Two additional findings of interest have come from the experiments when parental rats and F_1 hybrids were used. Firstly, we have shown there is a host-versus-graft reactivity when F_1 hybrid cells are injected into parental rats, which was obliterated by host irradiation. Secondly, we have shown that irradiation of the host increases reactivity when parent cells are injected into F_1 hybrids and therefore it would appear that it is less likely that there is any host component participating in the g-v-h response.

APPENDIX 2(a)

Method of graft-versus-host popliteal node weight assay

The methods used for the graft-versus-host (G-v-H) assay have been described by Ford et. al., (1970) with modifications described by Salaman et. al., (1975).

Animals

The recipient rats used in these experiments were inbred Wistar (I.W.) A.S. and (IWAS)F_1 hybrids. The donor lymphocytes were also from the same rats and were also from sheep and humans.

Cell preparation

The donor lymphocytes were prepared from lymph nodes of rats (Appendix Id). The strains of rats from which the cells for injection were obtained were all allogeneic or semi - allogeneic to the recipient. The donor cells were also obtained from the thoracic duct lymph of sheep (Section III Methods), these cells were washed three times in R.P.M.I. to remove the lymph. Lymphocytes were also prepared from human heparinised peripheral blood. The blood was layered on Ficoll Triosil and spun at 700 g. for 20 minutes. The lymphocytes were collected from the interface between the serum and Ficoll Triosil and washed three times in R.P.M.I.

The cells were all counted with trypan blue to obtain viable cell counts and the appropriate number of cells for injection were resuspended in 0.1 ml. of media. The dose of cells in the inoculum was variable but usually 2×10^6 cells in 0.1 mls. were injected.

In some experiments irradiated cells were used as controls. After the cells were washed they were exposed to a dose of γ rays from a

^{60}Co. source of either 2,400 rads or 20,000 rads.

After completion of irradiation the cells were again counted.

Injection of cells and removal of popliteal nodes

In each experiment age, weight, and sex matched inbred rats were used. The recipient rats were anaesthetised with ether.

The 0.1 mls. test cell suspension were injected into the left foot of the rat. A 25 gauge needle was inserted into the heel on the ventral surface of the footpad. The needle was advanced subcutaneously into the loose tissue of the sole of the foot and the inoculum injected. Pressure was applied to the injection site for one minute to prevent bleeding. Either 0.1 mls. of media alone or control cells were injected similarly into the right footpad.

The recipients were killed seven days later with ether. Both popliteal nodes were found (Diagram 7) cleaned of surrounding fat and placed in saline in a microtest plate. The nodes were then blotted immediately prior to weighing to an accuracy of 0.1 mg.

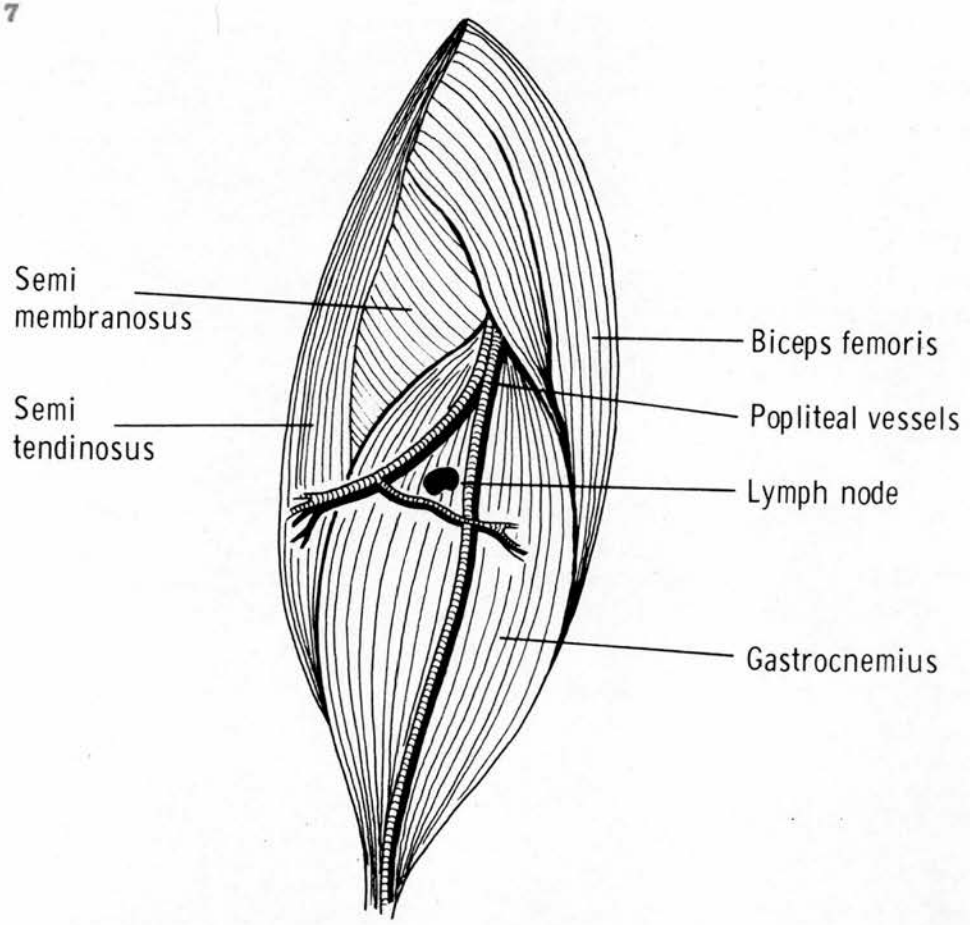
Suppression of Host-versus-Graft response

In these experiments half of the rats (three) were irradiated with rays from ^{60}Co. source (T.E.M. Mobiltron) at a source to target distance of 60 cms. A dose of 300 rads was given to the midline of the body in order to suppress the recipient host-versus-graft res response.

Presensitization and immunosuppression of allogeneic rat donors

Presensitization of the donor rats was performed by skin grafting from the recipient inbred strain to the donor rat (Appendix Ic). In

Diagram 7



POSTERIOR VIEW OF LOWER LEG IN THE RAT
SHOWING THE POPLITEAL FOSSA AND POPLITEAL NODE

addition in some experiments the rats were hypersensitised with three skin grafts and two intraperitoneal lymph node lymphocyte injections from the recipient strain.

Some of these presensitised rats were given immunosuppressive drugs. Cyclophosphamide 5 mgs/100 g. and cortisone acetate 5 mgs/ 100 g. i.m. wasggiven alternate days for the period of grafting.

Assay method

The weight of the popliteal nodes in each animal was obtained. The ratio of the test node/control node weight was calculated for each animal and the mean of the three rats in each experiment was calculated.

APPENDIX 2(b)

Mixed Lymphocyte Culture (M.L.C.)

The M.L.C. experiments have been performed by C. Brooks of the Cancer Research Campaign Laboratories, Nottingham. The full method has been described by Brooks (1975).

Rat lymph node cells (LNC) were cultured in flat bottomed microplate wells for 48 hours, 72 or 96 hours. In the two way reactions each culture contained 1×10^6 LNC, mixed cultures having two cell types at a 1 : 1 ratio. The one way reactions cultures contained 1×10^6 responder LNC plus 0.5×10^6 irradiated (2,000 rads) stimulating allogeneic cells. Control cells were unstimulated autologous cells. After culture the cells were labelled with ^3H thymidine for approximately 16 hours and counted in a B scintillometer.

The results are shown in table XIII. The numbers in columns 3 and 4 are counts per minute. The counts in column 4 are the additional counts on stimulation in M.L.R. over the unstimulated control. The index of transformation is obtained by dividing the counts on stimulation by the control counts. The greater the index of transformation the stronger the reactivity between the 2 sets of lymphocytes. From these results one can conclude that Wistar and A.S. strains are weakly incompatible for M.L.C. antigens compared with A.S. and A.S.2. However since there is some reactivity the Wistar and A.S. strains are probably Ag B incompatible.

Combination	Assayed at	Unstimulated Control	Stimulation in M.L.R.	Index of transformation
AS ⟷ AS_2	48 hours	1,179 ± 66	3,855 ± 156***	4.27
AS_2 v AS_x	72 hours	66 ± 39	381 ± 79***	6.77
WAG ⟷ AS	48 hours	901 ± 149	479 ± 198*	1.53
WAG v AS_x	48 hours	2,349 ± 279	-318 ± 327^{NS}	0.86
WAG ⟷ AS	72 hours	1,273 ± 173	686 ± 246*	1.53
WAG v AS_x	72 hours	3,582 ± 390	609 ± 421*	1.17
WAG ⟷ AS	96 hours	3,616 ± 504	1,079 ± 594^{NS}	1.29
WAG v AS_x	96 hours	5,277 ± 228	105 ± 475^{NS}	1.01

⟷ Two way reaction

x Stimulator cells

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

Index of transformation 1.0 = Zero reactivity

1-2 = Weak reactivity

2 = Strong reactivity

M.L.C. results between A.S. and $A.S._2$, WAG and A.S. rats.

Table XIII

SECTION III

LYMPHOCYTE FLOW IN ALLOGRAFT REJECTION

A. INTRODUCTION

B. BACKGROUND

1. Technique of thoracic duct cannulation in various species.
2. Flow rates and cellularity of lymph.
3. Differences in the composition of lymph from the thoracic duct and other sites.
4. Route of circulating lymphocytes.
5. The effect of lymph lymphocyte depletion on blood lymphocytes.
6. The response of the body lymphocyte flow to immune-stimulation.
7. Thoracic duct depletion as a means of immunosuppression.
8. The mode of action of immunosuppressive drugs. Azathioprine and steroids.

C. AIMS OF THE PRESENT WORK

D. MATERIALS AND METHODS

1. Groups studied.
2. Method of thoracic duct cannulation.
3. Method of renal transplantation.
4. Investigations performed on lymph and venous blood.

E. RESULTS

F. DISCUSSION

1. Renal transplantation in sheep.
2. Total body lymphocyte and renal lymphocyte flow. Peripheral blood counts.
3. Transformed cells.

G. CONCLUSION

H. APPENDIX

A. INTRODUCTION

In allograft rejection, as previously described in the introduction to this thesis, there is a massive *in vivo* response against the graft. There is a large inflex of white blood cells, mainly lymphoid and plasma cells, into the graft from the blood, these cells causing damage to the transplanted organ.

In the first part of this thesis we have seen that an *in vitro* killing effect of each individual lymphocyte is difficult to achieve and does not mirror the marked *in vivo* response. Perhaps the small cytotoxic effect of each individual lymphocyte is multiplied by a massive increase in the number of lymphocytes in the graft and flowing through a graft.

In the next section experiments have been performed to measure the number of lymphocytes flowing through a renal allograft, by cannulation of the lymphatics of that graft. Also the number of lymphocytes flowing through body have been measured, by thoracic duct drainage, during the period of allograft rejection.

The effect of immunosuppressive drugs on this flow of lymphocytes, both through the grafted kidney and through the whole body, has also been studied in order to determine where the drugs act.

B. BACKGROUND

Lymph has been collected in various animals for different experiments and studies, and also for therapeutic reasons. This work covers many aspects; here a selection of the work is reviewed which is relevant to the flow and cellularity of lymph.

1. Techniques of thoracic duct cannulation in various species

Surgical techniques for the collection of lymph from sheep have been described by Lascelles and Morris (1961). They described the technique of cannulating the thoracic duct through a right thoracotomy. The right thoracotomy approach was chosen in preference to the left neck exploration as many sheep have accessory lymph ducts and aberrant communications between the thoracic and right lymphatic dcuts; also Lascells and Morris maintained that fixation of cannulae was difficult in the neck. They described a method of setting up a lymphovenous shunt in order to prevent loss of weight from protein depletion which would result from the loss of lymph from the body. Their work was a study of the flow of lymph from certain organs (i.e. collection from hepatic, intestinal and mammary lymph ducts as well as the thoracic duct). The technique used in the present work for cannulation of the thoracic duct is that described by Shnain, Kassai and Jabbin (1973), and entails exploration of the lower left neck for cannulation of the thoracic duct.

The technique for collection of lymph in the calf was described by Fish, Mattingley, Ritzmann, Sarles and Remmers (1969c), who cannulated the thoracic duct in the left side of the neck, this method was later modified for use in humans (Fish, Sarles, Mattingley., Ross and Remmers, 1969d). The purpose of their work was not only the

study of cell populations in lymph, but also the effect of depletion of lymphocytes on the body as a means of immunosuppression. Their work gives insight into the difficulties of maintaining a thoracic duct fistula. Initially they had a high failure rate (Fish et. al., 1969c): of 23 cannulations 10 failed, either at operation owing to inability to cannulate the thoracic duct, or subsequently from blockage of the cannula. The use of heparin infusion into a side wing of the inserted cannula reduced this complication in later work, and Lascelles and Morris (1961) too stressed the need to use heparin intravenously to prevent clotting of the cannula. Fish and his co-workers also had a large initial complication rate in their 13 remaining calves in the above experiments, 8 died, 2 after re-operation for malfunction, 2 of thrombotic occlusion of the superior vena cava, 4 of infection, and 5 calves had wound infections. However in following papers 1969a, 1969d, 1970a, 1970b and 1972 they showed that chronic thoracic duct fistulae could be maintained in both calves and humans for up to periods of 150 days. The reinfusion of lymphocyte free lymph, by continuous flow centrifugation was essential for the maintenance of health (Fish et. al., 1969b), and they stressed that the loss of protein would otherwise lead to an abnormal physiological state.

The collection of lymph in rats has been described by Bollman, Cain and Grindley (1948): the thoracic duct is cannulated below the diaphragm and continuous reinfusion of lymph is given through the femoral vein. This technique has been modified by Gowans (1957).

2. Lymph flow rates and cellularity of lymph

Flow rates

The flow of lymph varies considerably according to the animal, the technique, the prevention of clotting and the replacement of extracellular fluid which has been drained.

The flow of lymph in sheep from the thoracic duct was shown by Lascelles and Morris (1961) to vary from 60 to 170 mls/hr. (1,440 to 4,080 mls/day). They were replacing the lymph through a lymph venous fistula with the addition of 1 ml./hr. of saline containing one unit of heparin in each ml. The flow of lymph was also increased by oral fluids and feeding. The flow obtained from sheep by Shnain et. al., (1973) was of the same order 1,380 to 3,440 mls/day without intravenous replacement of fluid.

In man, with intravenous reinfusion of lymphocyte free lymph, Fish et. al., (1969d) obtained an initial flow rate of 3,200 to 9,800 mls/day; after 6 weeks removal of the lymphocytes the number of lymphocytes collected each day was 0.6 to 1.2 x 10^9. These workers achieved a reduction of 90% of the number of lymphocytes obtained at the time of cannulation by the twenty-first day following drainage.

In the rat (Gowans, 1957) the flow rate of lymph, with reinfusion of either intravenous heparinised fluid or cell-free lymph into the femoral vein, was 24 to 60 mls. on the first day and this flow remained steady through the 7 days of collection. The number of lymphocytes dropped from 24 to 32 x 10^6/hr. to 4 to 6 x 10^6/hr. (approximately 5.8 to 0.96 x 10^8/day) on the 7th day. The maximum depletion occurring in the first 48 hours when a reduction of 70% had occurred (see Figure 9).

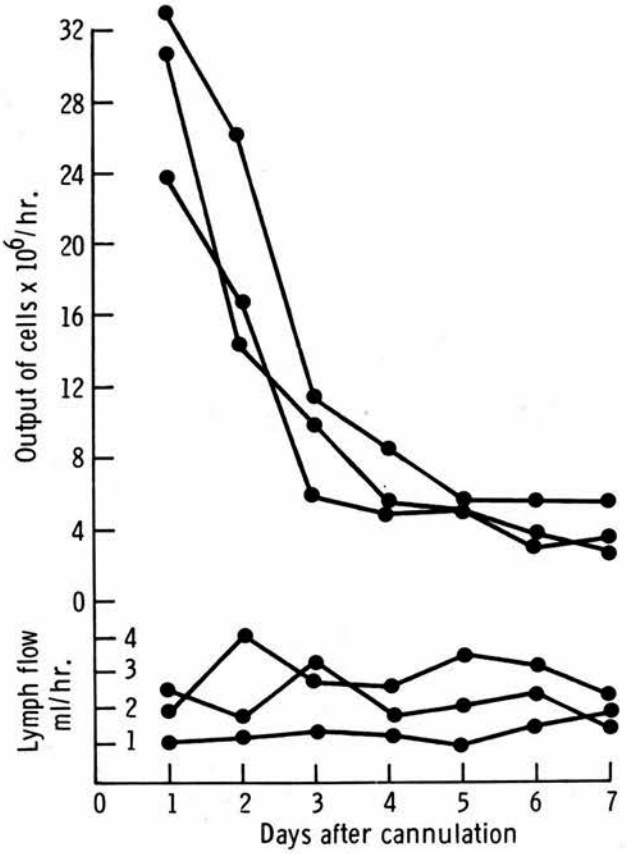


Figure 9 - Thoracic duct lymphocyte flow in the rat.

Gowans, 1957.

The daily output of thoracic duct lymphocytes was sufficient to replace those in the peripheral blood eleven times. A profound fall in lymphocytes could be prevented by reinfusing lymph and live cells back into the femoral vein of the rat which suggests that continuous entry of lymphocytes from the blood into the lymph is essential for maintaining the number of thoracic duct lymphocytes (Gowans, 1957).

Cells in thoracic duct lymph

The main cell in thoracic duct lymph is the lymphocyte. Polymorphs are not present, but there is usually a very small number of monocytes (macrophages) (Roser, 1970). Red cells are also present

in the thoracic duct lymph (Heath, Lascelles and Morris, 1962) although usually in small numbers, and are thought to enter the lymph through lymphovenous channels.

Lymphocytes

The main lymphocyte in rats (Gowans, 1957) and sheep (Hall and Morris, 1963) is the small lymphocyte. The medium lymphocyte in sheep corresponds to the small lymphocyte in other animals (Hall et. al., 1963). The proportions of T and B cells in thoracic duct lymph in the mouse have been studied by Sprent (1973); By using T and B cell markers in the mouse he found 82% T cells and 16% B cells. The ratio altered to 40% T cells and 50% B cells after 3 days depletion of thoracic duct lymph. This indicates that T cells are mobilised more rapidly by thoracic duct drainage, and this was confirmed by rapid depletion of the T cell dependent area (paracortical) in the lymph nodes.

The thoracic duct lymph also contains a number of large basophilic cells. This cell has various names, "transformed cell, plasmoid cell, large pyroninophilic lymphocyte, immunoblasts". The percentage of large basophilic cells in the lymph varies in different animals: sheep 2% (Hall, et. al., 1962), in rats 5% (Gowans, 1957) and in calves 10% (Townsend, Fish, Vyvial, Kianar and Ritzmann, 1970). In calves Townsend et. al. found the lymph contained small lymphocytes, large basophilic cells and large pale lymphocytes. On the first day of thoracic duct drainage most of the lymphocytes were small, but by the seventh day of drainage the large pale lymphocytes were more plentiful. The large basophilic cells stayed at 10% of the cell count even though the total lymphocyte count fell from 3.3×10^7 to $0.4 \times$

10^7/ml. over the seven days.

Large basophilic cells do not recirculate in the lymph to any significant extent. This has been shown by Delorme, Hodgett, Hall and Alexander (1969) who obtained 'immunoblasts' from the thoracic duct lymph of rats, labelled the cells with ^{3}H-thymidine, and reinjected them intravenously into a syngeneic recipient. During a 48 hour collection of thoracic duct lymph of this recipient none of the labelled cells appeared in the lymph, whereas on labelling small lymphocytes with ^{3}H-uridine in a similar experiment 20% reappeared in the lymph.

3. The differences of composition of lymph from the thoracic duct and from other sites

There is a difference between the rate of flow, cell population and composition of lymph draining different sites of the body.

There is a difference in the composition of lymph going to a lymph node and draining from the same lymph node - afferent and efferent lymphatic vessels respectively. The afferent lymph contains red cells and white cells, the number of polymorphs is 10%, macrophages 10% as well as lymphocytes 80%. The number of lymphocytes - 500 to 3,000/cu.mm. - in the afferent lymph (Hall and Morris, 1963) is less than in the efferent lymphatic of that node - 5,000 to 20,000/cu.mm. (Hall and Morris, 1962). Gowans (1962) suggested that the large cellular output from a single node was due to removal of lymphocytes from the blood.

There are also differences in the lymphocyte population found in lymph from different regions in the body. In thoracic duct and intestinal lymph dividing cells were common, both immature and mature plasma cells were present in the lymph. The output of cells in the hepatic, lumbar and mammary lymph duct was 2 to 4 times greater than the output from the intestinal duct alone (Heath, Lascelles and Morris, 1962). Simultaneous cannulation of the thoracic duct and right lymphatic duct in dogs, drained for 5 hours, showed marked differences in rate of flow, lymphocyte concentration and morphological types suggesting that there is a definite functional as well as an anatomical difference between these two lymph channels (Leeds, Unley, Busch, Rosenbaum and Yoffey, 1971).

4. The route of circulating lymphocytes

The lymphocytes in the thoracic duct come from the peripheral blood. This was shown by Gowans (1959) who obtained lymphocytes from the thoracic duct, labelled them with ^{3}H-thymidine, and infused them back into the rat intravenously. After a few hours 97% of the small lymphocytes in the thoracic duct lymph were shown to have taken up the ^{3}H label, indicating that lymphocyte circulating was from lymph, to blood, to lymph. This work did not indicate which route was taken by the lymphocytes passing from the blood back into the lymph. A route through the tissue spaces would increase the time taken for the removal of lymphocytes from the blood. This was confirmed by Gowans (1964). Some lymphocytes may pass into the lymph from the blood via the tissue spaces, as the number of lymphocytes is increased, after immune stimulation, in the afferent lymphatic draining from the tissue spaces into a lymph node. This is discussed in more detail in (6) (see below).

The recirculating lymphocyte pool in normal adult rats contains 2×10^{9} small lymphocytes (Gowans and Knight, 1964). This was determined by labelling the RNA of thoracic duct lymphocytes with tritiated adenosine. These cells were reinfused intravenously, the number of labelled cells in the thoracic duct was counted, and the number of labelled cells in the lymphoid tissue was estimated by autoradiography after 2 days. The pool of the animal's own cells with which the labelled cells had mixed was identified as the recirculating pool. The average lifespan of circulating thoracic duct lymphocytes has been studied in mice by Sprent and Basten (1973). They found that the lifespan of B lymphocytes was 5 to 7 weeks, and that of T lymphocytes was much longer, 4 to 6 months; the B

lymphocytes were identified by labelling with radioactive immune complexes, and the T cells by radioactive anti-θ sera.

5. The effect of thoracic duct lymph depletion on the circulating blood lymphocytes

As the lymph circulates back to the blood, then depletion of the thoracic duct lymphocytes will reduce the number of circulating blood lymphocytes. A reduction of lymphocytes by 94% in calves (Fish et. al., 1969c) led to corresponding reduction in blood lymphocytes by 57%. This also showed that the blood lymphocyte population was not entirely dependent on lymphocytes entering through the lymphatic ducts. These workers assumed that the bone marrow contributed to the maintenance of the lymphocytes in the blood. Other workers have shown that thoracic duct depletion reduces blood lymphocytes: Fitts, Majeski, Sharbaugh, Hargest, Graber and Henniger (1972) showed that 85% reduction of thoracic duct lymphocytes in calves over 11 days, reduced the blood lymphocytes from 10,000 mm^3 to 4,400 mm^3. Townsend, Fish, Vyvial, Kianar and Ritzmann (1970) also showed in calves a reduction from 3,000 to 1,800 mm^3 over 7 days depletion. Sprent (1972) showed that T cells recirculate rapidly from blood to lymph in mice. Seven days depletion of thoracic duct lymphocytes decreased the number of T cells in the body and therefore in the blood to a greater extent than B cells.

The flow of lymphocytes is therefore a circulation from blood to lymph, to blood. If this is altered by massive thoracic duct depletion, the bone marrow forms a large number of new lymphocytes to replace those lost to the body, possibly with the additional help by the mobilization of lymphocytes from other lymphoid tissues.

6. The response of the lymphocyte flow to immune stimulation

Lymphocyte flow from an allograft

The lymphocyte is an essential part of the immune response. We are concerned with cell-mediated immunity in which the T lymphocyte is considered of importance. The meeting of antigenic allografted cells and the lymphocyte may take place in the graft, i.e. the lymphocytes may migrate from the blood into the graft and be activated peripherally, or antigenic material from the graft may pass via afferent lymphocytes to the local draining node, and the lymphocytes be this activated centrally. In renal allografts there is evidence of peripheral activation: study of lymph from renal transplants has been made by Pederson and Morris (1970), who cannulated the main lymphatic leaving a kidney and draining towards the renal node (renal afferent lymphatic), in both autotransplanted and allotransplanted kidneys. From the autograft the lymph flow increased over the first 48 hours to a maximum of 3.3 mls. per hour; this compared with the increase from the allograft, which reached a maximum of 60 mls. per hour a few days later. The initial increase in the lymph flow was probably due to oedema from non-immune tissue damage, and after 48 hours the flow subsided in the autograft, whereas in the allograft the continuing marked increase in lymph flow was accompanied by a corresponding increase in the cell count (Figure 10). Most of the cells were lymphocytes and transformed cells. There were usually about 3 to 10% macrophages, identified on Leischman stained smears, in the lymph throughout the response. At the peak of the response up to 60% of the total number of cells were transformed, with a corresponding increase in these cells on histology of the renal cortex after the fourth day. If the cells were diverted from the

body there was a decrease in reaction in the draining nodes, with a coincident but slight increase in the length of survival of the graft. Also if the cells, leaving a rejecting kidney, were injected into a distant site, they evoked a significant cellular and antibody response in previously unstimulated nodes.

After skin grafting in sheep there was little or no increase in the number of lymphocytes passing from the graft up the afferent lymphatic to the draining node (Hall, 1967). This difference is perhaps due to the method of vascular connection established by grafted skin.

The collection of lymph from human allografts (Hamburger, Dimitrui, Bankir, Debray-Sachs and Auvert, 1971) with rejection held in check by immunosuppressive drugs, has shown a rise in the number

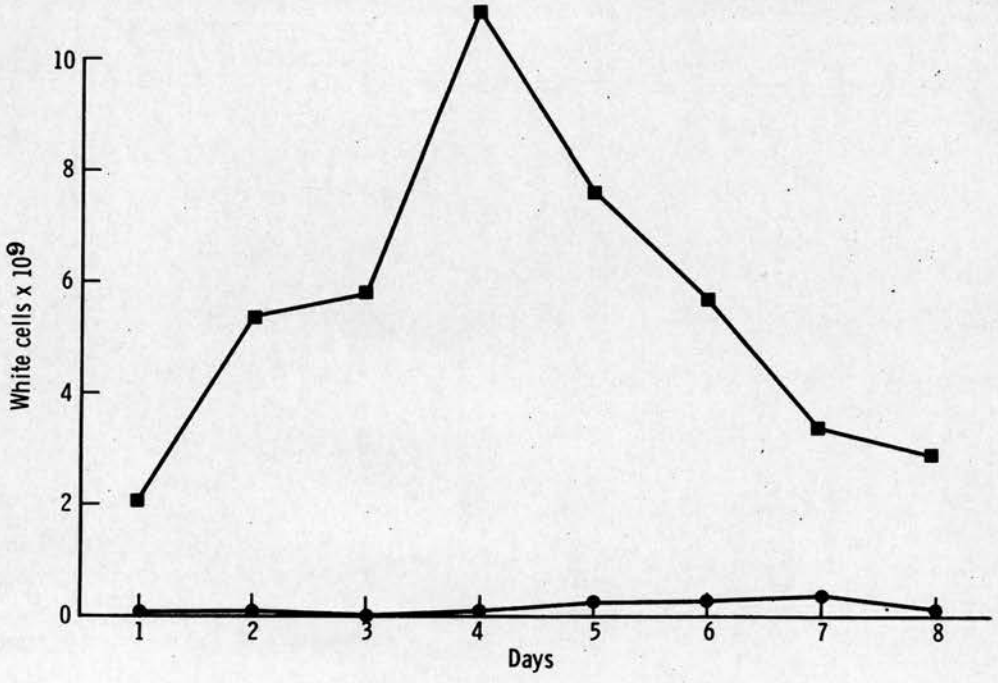


Figure 10 Renal lymphocyte flow in sheep receiving an autograft or allograft

■—■ allograft

●—● autograft

(Pederson and Morris, 1970.)

of lymphocytes flowing from the kidney to a maximum of 19 x 10^7 on the sixth day. There was a corresponding increase in the percentage of transformed cells, which was maximum on the sixth and remained steady at 15% for the period of cannulation of seven days.

Second set rejection of renal allografts was studied by Pederson and Morris (1974) who showed that rejection was accelerated with an increase in the numbers of red blood cells and polymorphs, but a decrease in the lymph flow and lymphocyte count.

Lymphocyte flow from the draining node

There is an increase in lymphocyte activity in the node draining an allograft (Brent, 1958). In the skin graft the initial response is in the primary follicles of the node immediately proximal to an allograft, although other distant nodes were affected (Hall, 1967). Hall showed that the flow of small lymphocytes was increased, and there was an even greater increase in the transformed cells which reached 40% as compared with 5 - 10% in the resting node; of these large cells 60% took up thymidine on in vitro incubation. This response began on the eighth post-transplant day and was maximum on the fifteenth. After a renal allograft, transformed cells appeared in the draining nodes at about 4 days (Pederson et. al., 1970). This is two days after their appearance in the afferent lymph, but in these experiments the flow of this lymph had been diverted from the body; the transformed cell response may either be from direct absorption of the cells from the area around the graft, or some factor may be transmitted to the node by the blood.

There is other evidence for the increased activity in the node following stimulation with non-transplantation antigen. Hall et. al.,

(1962) showed that the efferent lymph node drainage after antigenic stimulation with human serum globulin increased the number of transformed cells at 50 hours. The same authors (1963) showed a difference between the primary and secondary response. In the secondary response the transformed cells appeared earlier, coinciding with the production of antibody.

Lymphocyte flow in the thoracic duct

The lymph draining from a stimulated node will pass to the thoracic duct. Delorme, Hodgett, Hall and Alexander (1969) were unable to cannulate the popliteal efferents in rats, but did cannulate the thoracic duct below the diaphragm. They injected a number of antigenic materials into the lower half of the body: allogeneic tumour, syngeneic tumour, B.C.G. and fluorodinitrobenzene (F.D.N.B.) to initiate T cell responses. The flow of lymphocytes was not mentioned but the number of transformed cells was studied instead. The experiments were performed in germ-free rats. The numbers of transformed cells in control germ-free rats was 2%. this increases to 3 - 8% in rats which have not been reared in a germ-free unit and the authors stressed the importance of using germ-free animals if the increase in transformed cells was expected to reflect evidence of an immune reaction. There was a slight increase in the percentage of transformed cells from 2% in controls to a 5% peak on the fifth day after challenge with B.C.G. Irradiated syngeneic tumour (the antigenic stimulus here being tumour specific antigen) gave a greater increase of 6% on the fifth day. The greatest increase was after irradiated allogeneic tumour which was 17%, the maximum increase coming earlier - on the second day. The response to transplantation

antigen was therefore brisker than with other antigenic stimuli.

It is postulated that, rather than by the transfer of antigen, the immune response is carried around the body in the lymph by transformed cells (Hall,,1967). The immune response is therefore activated centrally by information provided by the transformed cells, and intact lymphatic pathways, in addition to other vascular connections, are necessary for the movement of transformed cells.

7. Thoracic duct depletion as a means of immunosuppression

A reduction of the effector cells (lymphocytes) by thoracic duct drainage has been used as a means of suppressing allograft rejection. Chronic thoracic duct fistulae have been performed on 14 patients receiving cadaver kidney allografts (Fish et. al., 1969a). The fistulae were made 10 to 108 days before transplantation. A significant lymphocyte depletion of 90% and a loss of thymic areas of lymph nodes was shown in these patients. Routine immunosuppressive drugs were withheld for 19 to 50 days, when maintenance immunosuppression was given. Eight patients have survived with functioning kidneys. Similar work on calves (Fish et. al., 1970a) showed that 14 days depletion prior to transplantation as compared with 7 days, gave a much greater reduction in the rejection process. There was no difference in these two groups in the number of cells removed prior to transplantation, the single determinent being the longer period of depletion.

Tilney and Murray (1967) also used lymphocyte depletion as a means of immunosuppression, although the patients were also on routine immunosuppressive drugs after transplantation: the doses of the drugs were azathioprine 2-3 mgs/Kg/day and prednisolone 2 mgs/Kg/day. They suggested that the addition of lymphocyte depletion to conventional immunosuppressive regime would allow a maintenance dose of immunosuppression to be used sooner after transplantation.

8. The mode of action of immunosuppressive drugs

Azathioprine[1]

Immunosuppressive drugs are synthetic products which are able to suppress immune responses. At present azathioprine is the most commonly used agent and has so far proved the most effective in human transplantation, and has been used for suppression of allograft rejection since 1961. Azathioprine is an anti-metabolite, it interferes with purine metabolism and therefore DNA, RNA and protein synthesis. In man the usual dose is 3 mgs/Kg/day. The activity of azathioprine depends on the production of metabolites release on breakdown by the liver. Both azathioprine and its active metabolites are degraded rapidly in less than 4 hours and therefore the drug is safe to use in renal failure as there is no summation of effect. In clinical practice azathioprine reduces the number of white cells in the peripheral blood, and may produce bone marrow asplasia in a few patients.

The effect of azathioprine on the lymphocyte

The method of action of azathioprine is not completely understood. The main effect of azathioprine is on the T lymphocyte. The inhibition of formation of sheep cell rosettes in humans - rosette inhibition activity - (see Main Introduction) has been a method of measuring the immunosuppressive activity of the drug (Bach and Dardenne, 1970). Azathioprine proved, in vitro, to inhibit the formation of spontaneous rosettes at a minimal inhibitory concentration of 0.5 ms/ml. The peak of inhibitory activity in vivo occurred 1 hour after injection of azathioprine intravenously. They

1. Imuran (Wellcome, Beckenham)

also found that the in vivo activation of azathioprine is more efficient in man than in mouse, thus immunosuppressive doses in mouse require to be much higher.

Azathioprine has been shown in vitro to reduce the transformation of lymphocytes in mixed lymphocyte culture (Laborde and Bach, 1971). They showed that the incorporation of thymidine in M.L.C. was inhibited at non cytotoxic concentration (10 mg/ml.). At weaker concentrations (0.001 mg/ml. to 0.1 mg/ml.) azathioprine exercised a stimulatory effect. When azathioprine was introduced 24 hours after the beginning of the culture there was a reduction in the inhibitory effect. These workers concluded that the activity of azathioprine is exercised in the course of the first 48 hours, that is to say at the beginning of the immune response before an increase in DNA synthesis (i.e. cell proliferation).

It has been postulated that azathioprine may act on all three limbs of the immune response in vivo (Hamburger, Crosnier, Dormant and Bach, 1972). Azathioprine may prevent recognition of the antigen by the T lymphocytes (the efferent arc). This is supported by the inhibition of sheep rosetting and M.L.C. described above. Secondly azathioprine may inhibit DNA and RNA synthesis and therefore reduce the number of transformed sensitised cells (central response). Thirdly there may be a direct effect on the efferent mechanism, on the sensitised lymphocytes. The effect of azathioprine in the afferent arc is considered the most likely and most important as in vitro the effect of azathioprine is lost if not given immediately after contact between antigen and lymphocyte.

Steroids

Steroids are anti-inflammatory drugs and prolong graft survival without having a true immunosuppressive effect. Steroids when given alone to prevent renal allograft rejection have little success.

Cortisol is a naturally occurring glucocorticoid and prednisolone, prednisolone and methylprednisolone are synthetic glucocorticoids. Prednisolone is commonly used for immunosuppressive therapy, initially prednisolone is used in high doses orally, 3 mgs/Kg/day, reducing to a maintenance dose of 0.25 mgs/Kg/day. Large doses (one gram) of methylprednisolone[1] given intravenously at the time of rejection have been found to be effective at abolishing rejection episodes (Bell, Briggs, Colman, Quin, Wood, Paton and Macpherson, 1973).

The effect of steroids on the lymphocyte

Steroids have been shown in vitro to inhibit, DNA, RNA and protein synthesis. If added to M.L.R. at the beginning of culture steroids inhibit lymphocyte transformation (Bach, 1975). Rosenau and Moon (1962) showed that in vitro lymphocyte cytotoxicity against tumour was also reduced. Steroids have also been shown in vivo to cause lympholysis, repeated injections at high doses causes atrophy of most lymphoid organs. Schnappauf and Schnappauf (1968) showed that prednisolone decreased the flow of lymphocyte in the thoracic duct but they did not know whether this was due to a generalised lymphoid depletion, or a specific inhibition of lymphocyte migration. In allotransplantation of skin grafts steroids prolong graft survival.

Corticosteroids appear to exert a suppressive effect on the immune response. There is no evidence that recognition of antigen is

1. Solumedrone (Upjohn, Crawley)

prevented, but the inflammatory response initiated after recognition, is reduced (Melby, 1974).

C. AIMS OF THE PRESENT WORK

The aims of the work are twofold:

1. To study the quantitative change in the lymphocyte mass at the time of immune stimulation with a renal allograft. The work will be divided into two parts:

a) To confirm the increase in the number of lymphocytes leaving the lymphatic of a rejecting kidney - renal lymphocyte flow.

b) To see if this increase in the number of lymphocytes leaving the kidney is specific to the kidney or whether there is an increase in the total body lymphocyte flow.

The total body lymphocyte flow has been measured by thoracic duct cannulation and drainage.

2. To study the effect of immunosuppressive drugs on total body lymphocyte and renal lymphocyte flow; and to determine whether immunosuppressive agents alter either the total number of lymphocytes, or the number of transformed lymphocytes (dividing cells) in the thoracic duct and renal lymph. The effect of immunosuppressive drugs will be studied alone, and also in combination with a renal allograft with rejection abrogated.

D. MATERIALS AND METHODS

1. All the experiments were performed in male sheep, Suffolk cross or Hereford cross. The sheep were wethers of approximately 30 kilograms in weight. The sheep were brought from the field to individual pens one week prior to operation in order to become acclimatised to their surroundings. They were fed on pregnant ewe nut. They were also allowed water ad libitum. During the week of acclimatization they were sheared.

a) Thoracic duct cannulation to measure total body lymphocyte flow

For the experiments five different groups of sheep were used:

1. Normal
2. Immunosuppression
3. Post renal transplantation - autograft
4. Post renal transplantation - allograft
5. Post renal transplantation - allograft and immuno-suppression

Group 1

These sheep had thoracic duct cannulation alone performed.

Group 2

These sheep received immunosuppressive drugs: prednisolone 200 mgs/day and azathioprine[1] 3 mgs/Kg/day either orally or intravenously. The thoracic duct was cannulated two weeks after the start of the drugs.

Group 3

These sheep had a left nephrectomy performed, and the kidney was transplanted into the neck of the same sheep. The thoracic duct was cannulated during the same operation, or one week later.

[1] Imuran (Wellcome, Beckenham)

Group 4

A nephrectomy was performed on a sheep and the kidney transplanted into an unrelated sheep. The thoracic duct was cannulated in the latter either at this operation or one week later.

Group 5

These sheep also received an allografted kidney. During the operation they received one gram of methylprednisolone[2]; thereafter they received prednisolone commencing at 200 mgs/day and reducing by 10 mgs. each day, and azathioprine 3 mgs/Kg of body weight/day. The thoracic duct was cannulated either in the first or the second week after transplantation.

b) Renal lymphatic cannulation to measure renal lymphocyte flow

Four groups of sheep were used for these experiments.

1. Renal autograft
2. Renal allograft
3. Allograft and immunosuppression
4. Allograft and prednisolone

Group 1

A nephrectomy was performed and the kidney transplanted into the neck of the same sheep. The renal lymphatic was cannulated at the same operation.

Group 2

A kidney from one sheep was transplanted into the neck of an unrelated sheep. The renal lymphatic was cannulated at the same operation.

[2]Solumedrone (Upjohn, Crawley)

Group 3

These sheep received a renal allograft and also one gram of methylprednisolone during the operation, thereafter prednisolone commencing 200 mgs/day and reducing by 10 mgs each day and azathioprine 10 mgs/Kg of body weight/day.

Group 4

These sheep received a renal allograft and prednisolone. The dose of prednisolone was the same as group 3.

2. Method of Thoracic Duct Cannulation

Preparation

The sheep were starved of food but not water for the 24 hours prior to operation.

Anaesthesia

The sheep were anaesthetised with veterinary nembutal (60 mgs/ml) the dose varying according to the size of the sheep, average dose being 900 mgs. for a wether. The animal was intubated with a cuffed endotracheal tube, size 9, and ½ litre/min. of oxygen, 250 mls/min. nitrous oxide with halothane ½% were administered through a Boyles machine on closed circuit. The animals breathered spontaneously.

Position

The animal was placed on its right side in a left lateral position, the fore limbs drawn towards the lower limbs and tied securely, and the neck extended to give the maximum exposure of the lower left side of the neck. (Plate 4)

Preparation of the skin

The skin on the left prescapular region was close shaved. The skin was first cleaned with a savlon 1% with a soft scrubbing brush, and then cleaned with chlorhexidine 0.5% in 70% spirit. The animal was then towelled up with sterile drapes.

Technique of operation (Diagram 8)

A longitudinal incision approximately 12 centimetres long was made into the left side of the neck from the level of the first rib along the jugular furrow. The subcutaneous fascia was divided in the same line. Lying dorsally in the line of the incision was the ventral edge of the brachiocephalic muscle. In the more caudal end of the wound two vessels (omocervicalis artery and vein) ran at right

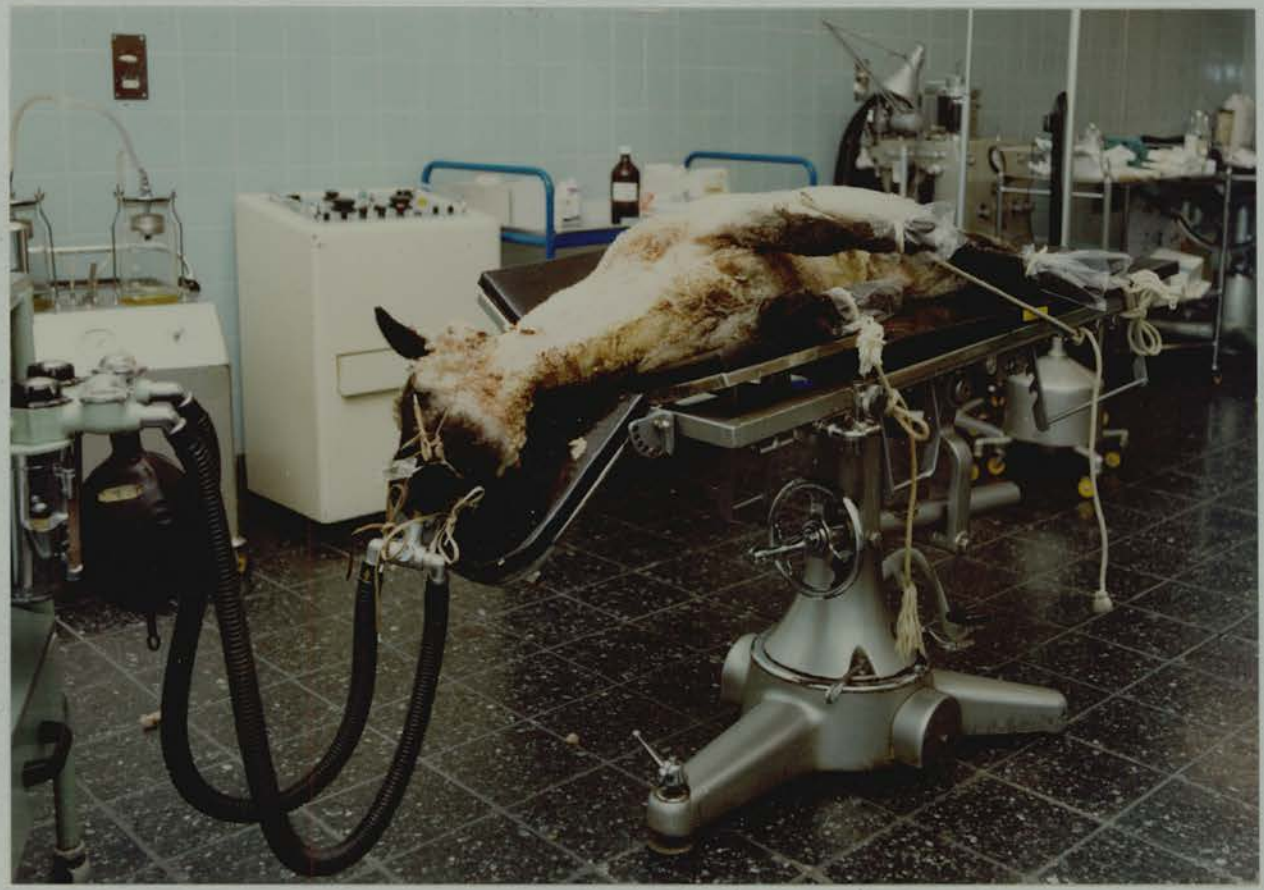
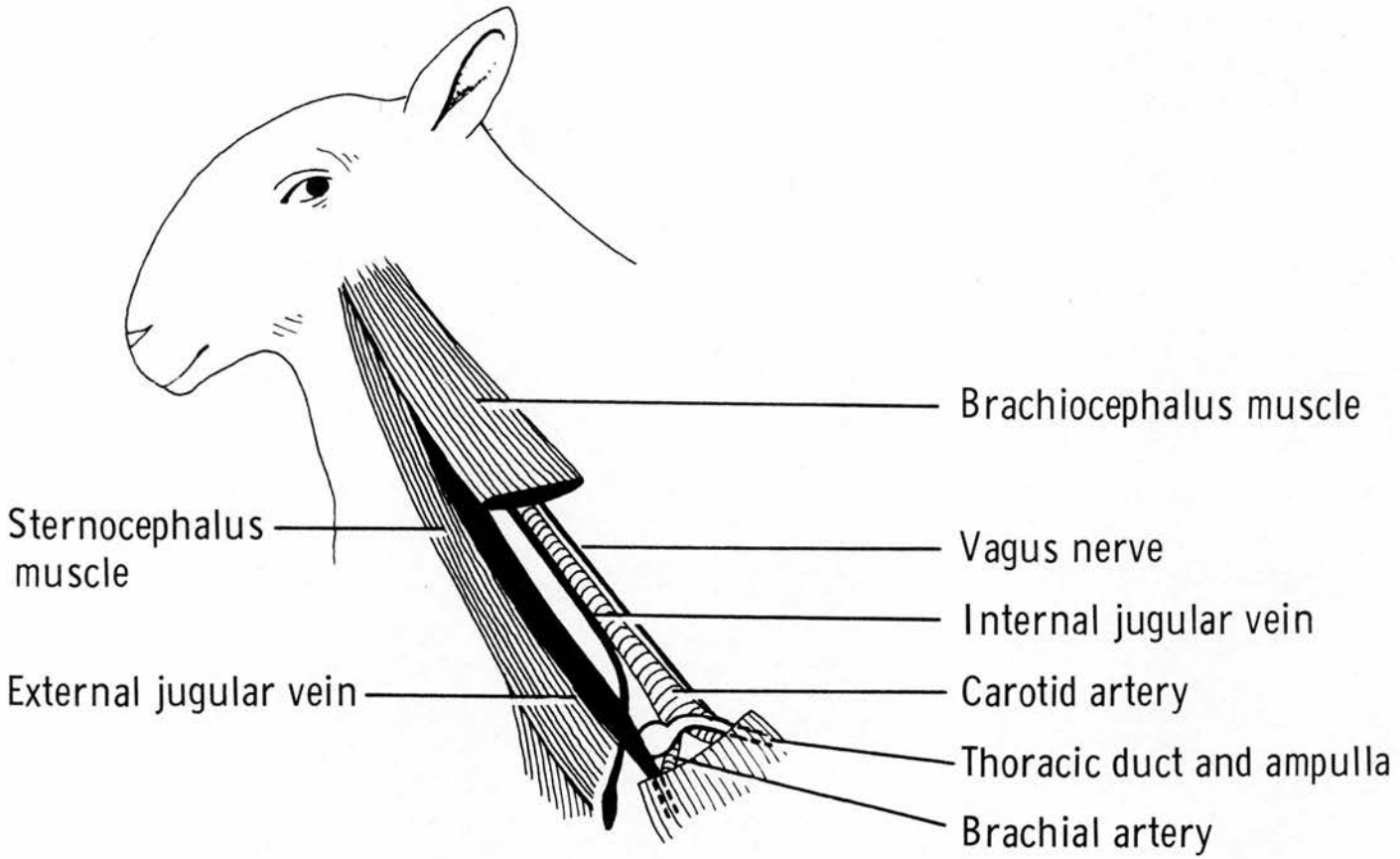


Plate 4 POSITION OF SHEEP FOR THORACIC DUCT EXPLORATION
IN THE LEFT SIDE OF THE NECK

Diagram 8

ANATOMY OF THE THORACIC DUCT (LEFT SIDE OF NECK) OF THE SHEEP



angles to the incision, and these vessels were divided between clamps and tied. Deep to the divided vessels the external jugular vein was exposed as it joins the brachial vein. The brachiocephalic muscle was dissected carefully off the surrounding loose areolar tissue and retracted. Careful dissection under the muscle in this area anterior to the first rib, bounded medially by the external jugular vein, with the carotid artery running posteriorly, revealed the ampulla of the thoracic duct as it enters the external jugular vein. A ligature was then tied round the ampulla, and the thoracic duct immediately became distended and could be seen passing caudally down the posterior mediastinum. A trocaflex cannula, 1.2 millimetres external diameter and 30 centimetres long, with a sharp stilette was then inserted into the thoracic duct. The stilette was then withdrawn as the cannula was gently pushed down the main branch at least four centimetres (occasionally there was more than one branch to the thoracic duct as it enters the ampulla) (plate 5a). The cannula was fixed with another ligature distal to the site of entry to prevent leakage of lymph, and was fixed to subcutaneous tissues by coiling it around in a circle and enclosing the two lengths of tubing in spiral cable wrapping. This was stitched to the subcutaneous fascia in order to prevent the cannula being pulled out (plate 5b). Already the flow of lymph was brisk and collection was started. An intravenous cannula 1.65 mm. external diameter was inserted into the external jugular vein about two centimetres cephalad from the entrance to the thoracic duct and fed in at least three centimetres and fixed by tying it in the vessel. An infusion of heparinised saline (2,000 units of heparin to 500 mls. of saline) was commenced. The subcutaneous tissues were closed with 2/0 chromic catgut, the skin

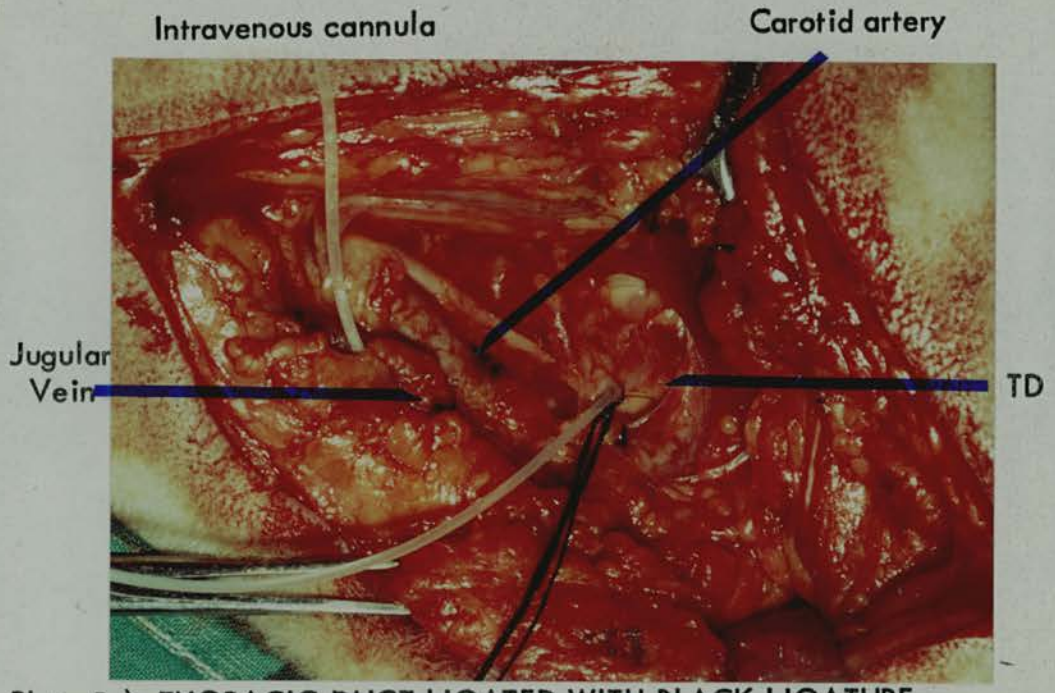


Plate 5a) THORACIC DUCT LIGATED WITH BLACK LIGATURE CANNULA INSERTED

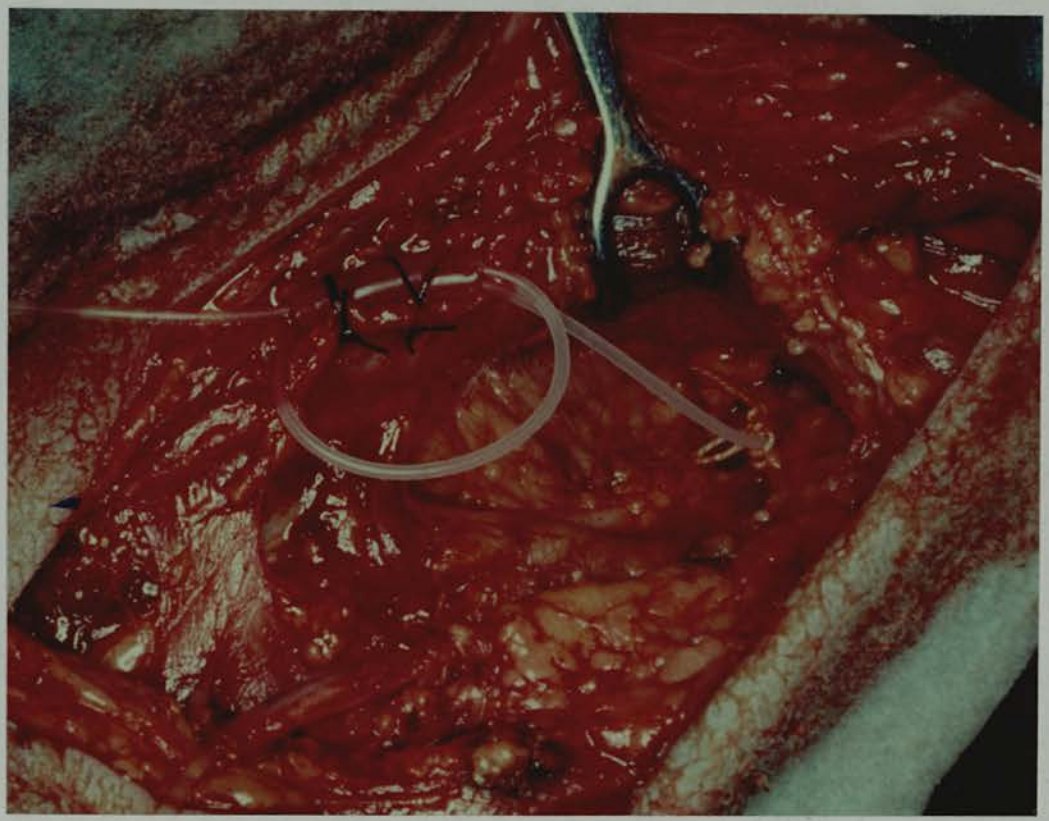


Plate 5b) THORACIC DUCT CANNULA SECURED WITH SPIRAL CABLE WRAPPING

was closed with michel clips and sprayed with nobecutane. A netelast bandage was applied encircling the neck and upper chest and the cannulae were protected under this. The thoracic duct cannula was placed with added connection tubing into the collection bag. The venous cannula was connected to an infusion bottle on a drip stand. The sheep was brought around from the anaesthetic and extubated.

Postoperative Management and Lymph Collection

The sheep was placed in a restraining pen (plate 6). It was encouraged to stand up almost immediately and when fully conscious a loose halter was placed around its neck to prevent it chewing the cannulae.

The lymph was collected into six litre sterile bottles which contained 5,000 units of heparin, bottles being changed daily.

The sheep was given postoperative sedation (pentazocine 30 mgs. intravenously) immediately after the operation. All the animals were given penicillin 600,000 units and streptomycin 500 mgs. daily. Intravenous infusion of normal saline was given, at least three litres per day, varying according to the amount of lymph drained from the sheep the previous day. The saline contained three grammes potassium chloride per day. Also two bottles (500 mls. each) of Aminosol vitrum was infused each day in an attempt to replace some of the large quantity of protein lost in the lymph. Many of the sheep lost a considerable amount of weight owing to protein loss. Heparin 2,000 units a day was also infused continuously.

At the end of seven days the animal was sacrificed, an intravenous injection of a large dose of nembutal 1.5 G. being given.

Plate 6 THORACIC DUCT CANNULATION AND DRAINAGE.
SHEEP IN A RESTRAINING PEN

A number of immunosuppressed sheep died before the end of the thoracic duct drainage probably owing to sepsis.

3. Method of Renal Transplantation

Removal of Donor Kidney

Initially the donor sheep were wethers, and this was satisfactory when the thoracic duct cannulation experiments alone were performed. When experiments for cannulation of the renal lymphatics were begun it was found that the lymphatics of kidneys from these sheep were extremely small, therefore larger older rams were used.

Anaesthesia

The same anaesthetic was given as for thoracic cannulation (see above).

Preparation and Position

The sheep was laid supine with all four limbs tied separately to the side of the table. The abdominal skin was clipped closely and scrubbed with savlon and then cleaned with chlorhexidine and draped with sterile towels. (Plate 7)

Technique of Operation

A left paramedian incision was made from the costal margin down below the glans penis midway between this and the pubis. The tissues were divided in the line of the incision and the peritoneum opened. The kidney was found, and the ureter was also freed about ten centimetres below the renal pelvis, divided between clamps and the lower end tied. The upper end of the ureter was mobilised with it blood supply. The renal vein was found and a ligature tied round as close to the inferior vena cava as possible, tying any branches. The renal artery was located and ligated near its origin and the kidney removed; extra renal fat was cleared from the kidney. In the experiments involving the cannulation of the renal lymphatics care

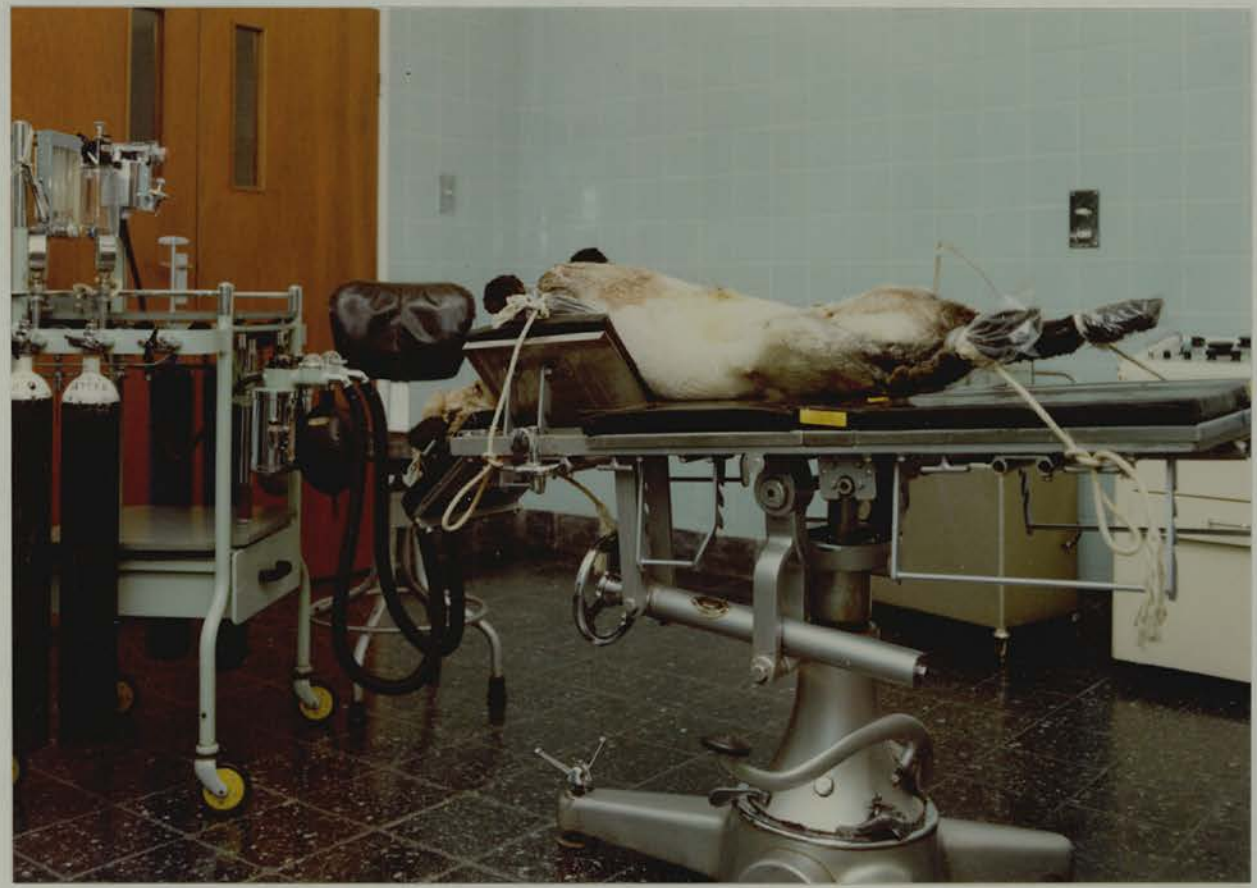


Plate 7 DONOR SHEEP - SUPINE POSITION ON THE OPERATING TABLE

was needed to avoid dissection around the renal vein and artery. Also the renal lymph node which lies at the origin of the renal artery was removed in continuity with the specimen. In no case was there duplication of the renal arteries, although occasionally there was a double renal vein.

After securing haemostasis the wound was then closed with a prolene suture to all layers, except the skin which was closed with michel clips, and sprayed with nobecutane. The anaesthetic was stopped and the animal woken up.

Perfusion of the Donor Kidney

Immediately after removal, the donor kidney was perfused with low molecular dextran 40 5% in electrolyte solution (Perfudex) cooled to 4^{o} centigrade, one litre containing 1,000 units of heparin and 2 mls. of lignocaine 2%. One hundred millilitres was run into the artery through a Tibbs cannula until the perfusate from the renal vein was clear. (Plate 8a)

The kidney was placed in a sterile bag containing cool perfusate and then placed in crushed ice.

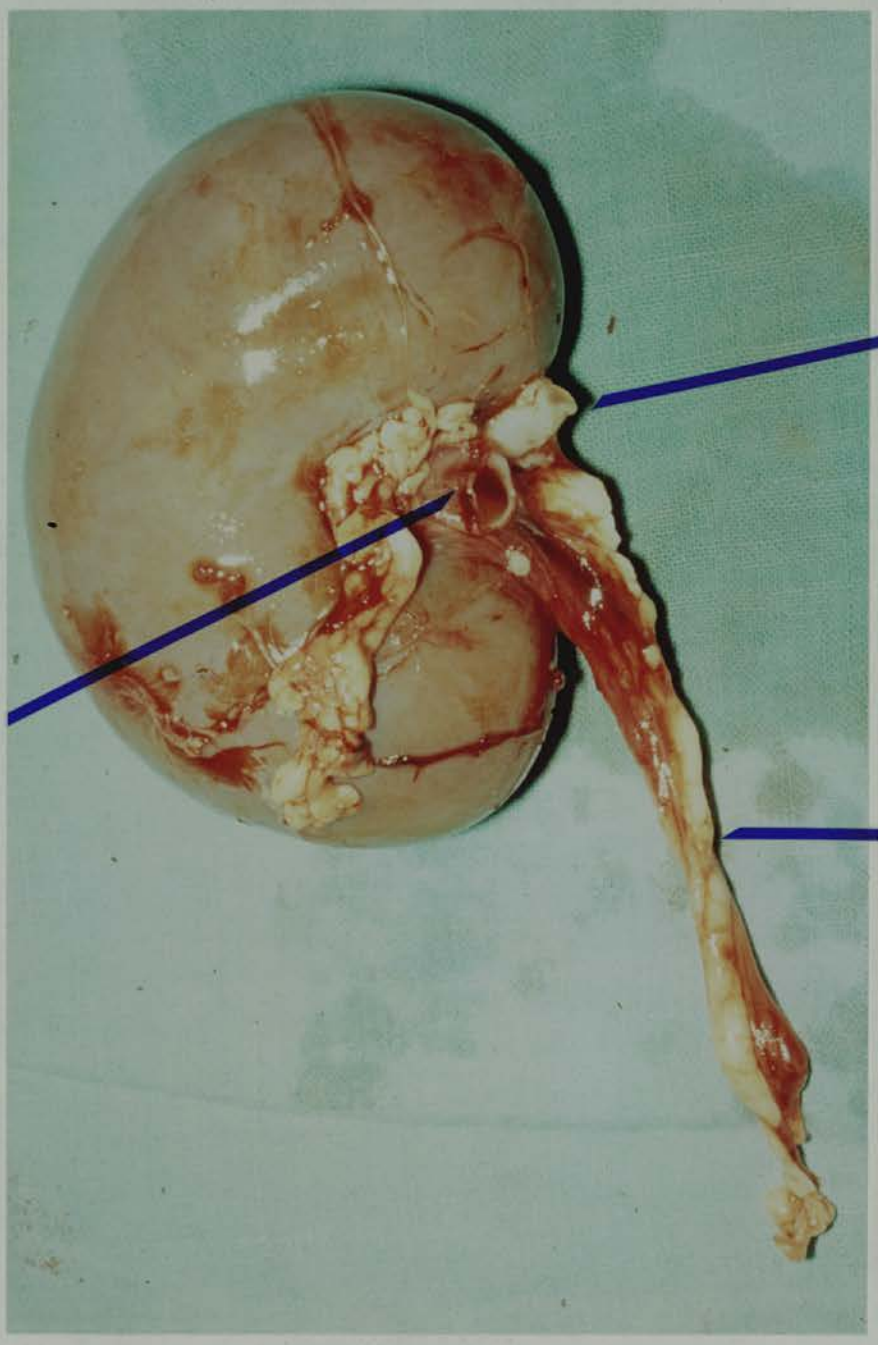


Plate 8 a) DONOR KIDNEY PERFUSED

Magnification x $1\frac{1}{2}$

Transplantation of the donor kidney into the recipient

Anaesthetic

The anaesthetic (see above).

Position

In those sheep where the thoracic duct was cannulated at the same operation, for collection of lymph during the first week after transplantation, the kidney was transplanted into the left side of the neck. The right side of the neck was used for those sheep whose thoracic duct was cannulated one week later.

The sheep were positioned as for thoracic duct exploration and the skin prepared in the same manner.

Technique of operation (plate 8b)

An incision was made from the ear caudally along the distal half of the jugular furrow. In the upper part of the wound the external jugular vein lay immediately under the skin, and was cleared. The common carotid was then found just deep and lateral to the vein lying under the brachiocephalic muscle, and was also cleared.

An intravenous cannula was inserted into the external jugular using an available small branch proximal to the anastomosis as the site of entry; a ligature was tied round the branch to fix the cannula. An infusion of saline containing 10,000 units of heparin was begun.

A length of the external jugular vein above this was occluded between bulldog clamps, and the vein was opened in the line of the vessel anteriorly, the length of opening matching the diameter of the donor vein. The recipient vein was flushed with a few millilitres of heparinised saline (1,000 units of heparin in 100 mls. of saline).

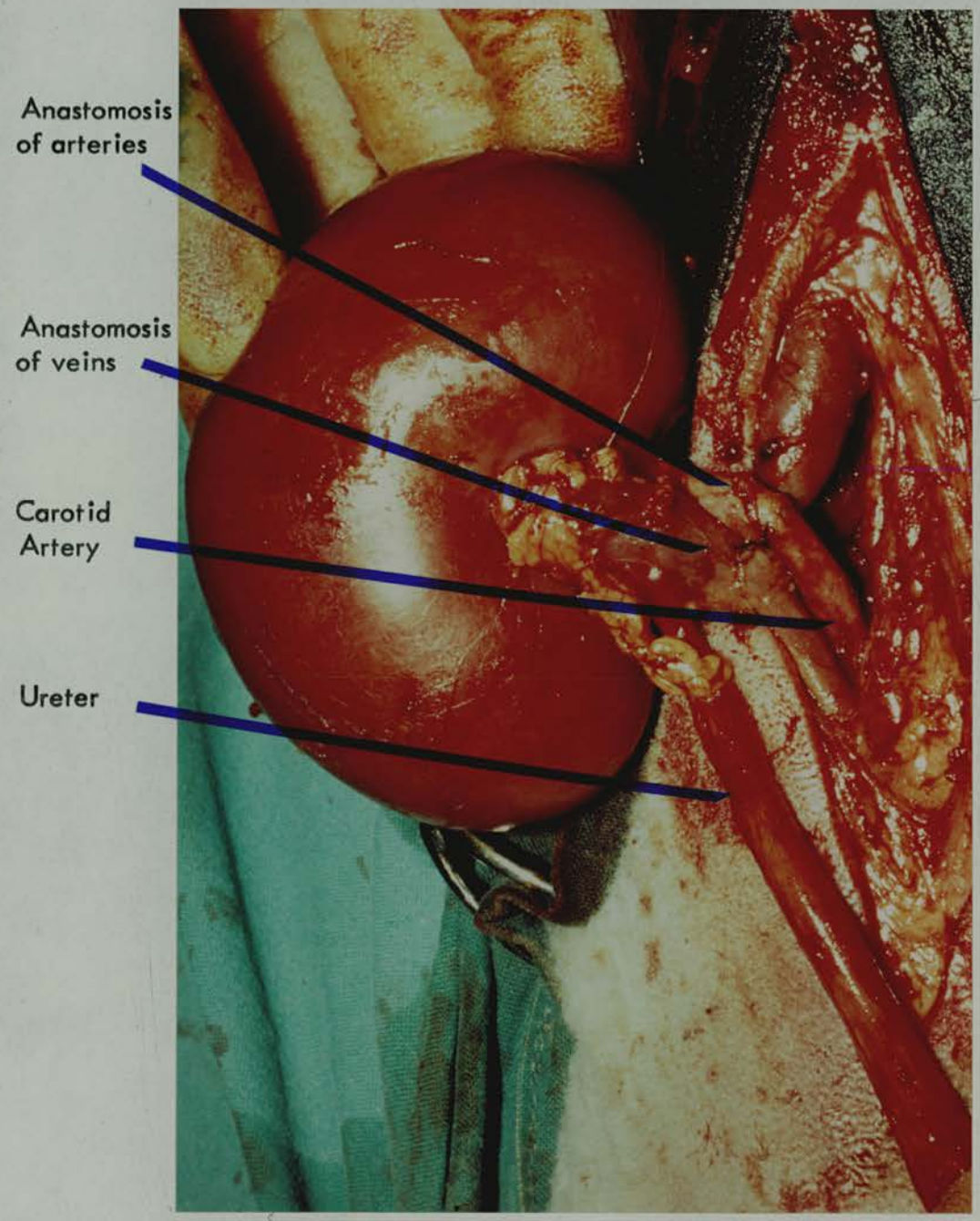


Plate 8 b) RENAL ALLOGRAFT AFTER ANASTOMOSIS INTO RECIPIENT

Magnification x 1½

The artery was then tied distally and the proximal part of the cleared area was clamped with a bulldog clamp, the vessel cut across and the lumen flushed with heparinised saline.

The donor kidney was then removed from the ice. The vein was anastomosed first, the end of the donor vein being sutured to the side of the recipient vein with continuous 6/0 silk. The renal artery was then sewn end to end with the carotid artery with 6/0 continuous suture. Just prior to the last suture being inserted the vessels were flushed with heparinised saline. The venous clamps were then removed, the proximal first. If the bleeding was not marked the arterial clamp was then removed. The kidney was then seen to be perfused with blood. After haemostasis was satisfactory a small biopsy was taken from the renal cortex. The subcutaneous tissues were then divided laterally so that the kidney could lie comfortably under the skin.

The ureter was brought out through the skin through a separate opening with a cannula of external diameter 1.65 millimetres inserted and fixed inside with a catgut suture. The edges of the ureter were then sutured to the skin. The subcutaneous tissues were closed with catgut and the skin with continuous prolene suture.

Cannulation of the renal lymphatics

Those kidneys which were used for experiments cannulating the lymphatics leaving the kidney, were perfused with low molecular dextran 40 (see above). After perfusion a ligature was tied round the renal efferents distal to and leaving the renal node, care also being taken not to distrub the tissues around the renal artery and vein when clearing the extrarenal fat. The kidney was transplanted into the recipient.

After the kidney was perfused with arterial blood the lymphatics leaving the kidneys were seen to become distended with lymph: usually there were at least two small lymphatics. The longest and largest was chosen and cut in half near the renal node. A fine cannula, external diameter 0.75 millimetres, with an obliquely cut tip was inserted and gently pushed up the lymphatic towards the kidney. Two sutures of 4/0 silk were tied round to keep the cannula in place. The cannula was then brought out through the skin through a separate opening and lymph collected in a small capped bottle with a fine hole made in the lid. The bottle was attached to the skin by tying around two nylon tapes which were sewn to the skin. The bottles contained 5,000 units of heparin to prevent the collected lymph from clotting.

Post-operative care

All the sheep received penicillin and streptomycin, the dose being doubled initially in the sheep receiving immunosuppressive drugs. The antibiotic was changed to gentamycin 80 mgs. daily for the immunosuppressed sheep during the course of the experiments.

Renal transplantation with immediate thoracic duct cannulation

The post-operative management was the same as for animals which

had received thoracic duct cannulation alone (see above) except that the dose of heparin was increased to 20,000 units daily in two divided doses.

Renal transplantation awaiting thoracic duct cannulation

The sheep were placed in ordinary pens. The venous and ureteric cannula were protected by netelast dressing. No intravenous infusion was given during the first week, though heparin was given into the cannula, 20,000 units daily.

Renal lymphatic cannulation

Sheep with renal lymphatic cannulation were kept in a restraining pen. A very slow intravenous infusion of saline and mannitol 10% was given at a rate of 500 mls. daily. The animal was again anti-coagulated with heparin.

Immunosuppression

The sheep on immunosuppressive durgs had received 1 G. of methylprednisolone and 200 mgs. of azathioprine intravenously before the completion of the operation. They then received either orally or intravenously prednisolone 200 mgs. on the first post-operative day, decreasing by 10 mgs. a day, and azathioprine, 3 mgs. per Kg. of body weight per day (mg/Kg/day). As this dose of immunosuppressive drugs was found not to alter rejection on serial renal biopsies, the dose was increased to 5 mgs/Kg/day (approximately 200 mgs. a day). The dose was further increased to 10 mgs/Kg/day.

Renal biopsies

Initially there were a number of both allografted and autografted kidneys which were found to be ischaemic at postmortem. Therefore

serial renal biopsies were taken on day two, four, and seven, also the kidney was sent for histology after it was removed at postmortem either on the eighth or fifteenth day. The biopsies were taken using a disposable Trucut biopsy needle. The incidence of thrombosis in the renal vessels leading to renal ischaemia decreased after the sheep were anticoagulated with heparin 20,000 units daily, although occasionally a kidney still became ischaemic.

4. INVESTIGATIONS PERFORMED

On the Thoracic Duct and Renal Lymph

1. Total daily flow of lymph
2. Daily white cell count
3. Percentage of transformed cell on the first and eighth day of drainage
4. Differential white cell counts on renal lymph
5. Cell populations in the thoracic duct lymph, macrophages, B cells and T cells
6. Graft versus Host popliteal node weight assay (section 2)

On Venous Blood

1. Total white cell counts and differential counts
2. Coombs Test

Histology

Histological examination of serial renal biopsies taken during operation, on the second, fourth and seventh post-operative day, plus macroscopic and microscopic examination of the post mortem kidney.

Investigation of Lymph

1. Total daily flow of lymph

The total quantity of lymph collected either from the thoracic duct or renal lymphatic was measured every 24 hours starting on the day of operation (day 0). The daily speciments were collected from 09.00 hours to 09.00 hours.

2. Daily white cell count

An aliquot of each days lymph from the thoracic duct, which had been collected as one specimen in a six litre bottle, was taken, making certain that the whole specimen was mixed well. Heparin had been added to the collecting bottle to prevent clotting of the lymph. The aliquot was diluted 1:20 in a white cell counting pipette (thoma); lymph was drawn up to the 0.5 mark, then trypan blue to the 11 mark, The trypan blue had been diluted 1 aliquot to 4 of 4.25% saline. The pipette was agitated and a small amount placed under the cover slip of a haemocytometer (Neubauer Improved). The cells in the 4 large outer corner squares (each with 16 small squares) were counted. The total and the viable cells were counted, the non-viable cells taking up the trypan blue; care was taken not to count the small number of red cells which were sometimes present. The number counted was divided by 20 and this result was then equivalent to the number x 10^6 per cm^3 (ml). The much lower lymph counts at the end of the seven days drainage was diluted 1:10 with trypan blue, and the number was then divided by 40 to get the cell count x 10^6 per ml. The viable cells were expressed as a percentage of the total.

An aliquot of lymph from the renal lymphatic was taken. Owing to the large numbers of red cells in the renal lymph, counting

visually in a haemocytometer was difficult. Therefore 20 µl. specimen was diluted in 10 mls. saline (1:500 dilution). Zaponin, 2 drops, was added to lyse the red cells, and the white cells counted in a Coulter counter at the following settings attentuation 1, aperture 16, threshold 23.

3. Percentage of transformed cells

An aliquot of lymph collected in sterile bottles was dropped on a slide and a smear made, and allowed to dry in the air. The smear was fixed in methanol for five minutes. May Grunwald stain (diluted 50:50 in Sørensen buffer was applied for one minute, then Giemsa (diluted 1:50 with Sørensen buffer) was added for half a minute. The slide was washed and left in buffer for one minute and dried in air. The slides were examined under the oil immersion lens of a light microscope. The larger cells with deep basophilic cytoplasm were recognised as transformed cells and the number of these cells out of a hundred lymphocytes was counted.

4. Differntial white cell counts

An aliquot of lymph was dropped on to a slide and a smear made and dried in air. The slide was then flooded with Leishmans stain for two minutes and two volumes of 6.8 buffer was added, mixed and left for seven minutes. The slide was washed for two minutes with continuous gentle flow of buffer, and dried. The slide was counted under the microscope, using the oil immersion lens. A hundred cells were counted and the percentage of polymorphs and of mononuclear cells obtained.

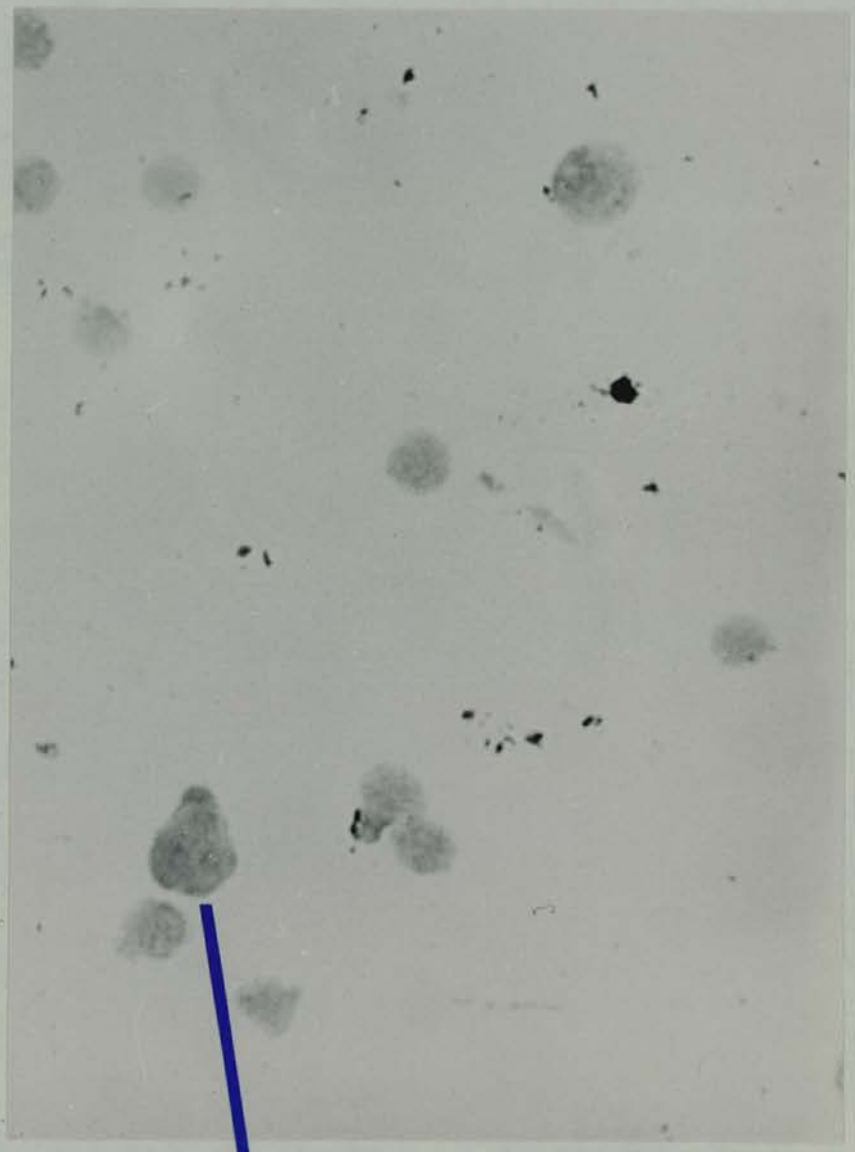


Plate 9. Lymph smear showing transformed cells, Magnification X800

5. Cell populations in the thoracic duct lymph

Macrophages

The method used has been described by Golstein and Blomgren (1973). An 0.1% solution of acridine orange was diluted 1:10 with Waymouth plus 10% foetal calf serum (FCS). Five million viable cells were counted and added to 2 mls. of this solution and incubated for half an hour at 37^{o} C.

The cells were then washed twice, resuspended in media and looked at under ultraviolet (U.V.) light (see below). The macrophages appear as cells with the cytoplasm stained orange, and the nucleus green. The lymphocytes owing to their small content of cytoplasm appear green only.

B and T cells

A B cell marker was used to identify the B cells, and the remainder of the mononuclear cells in the thoracic duct lymph (excluding the macrophages) were considered to be T cells. Fluorescent antiglobulin (RAsh/FITC)[1] was used as the B cell marker. This was diluted 1:10 with isotonic saline. Four drops of the solution was added to 5 x 10^{6} viable cells in 0.1 ml. Hanks medium and the cells were put in ice for 30 minutes. The cells were washed once with Hanks plus azide 0.02% and a few drops of glycerol P.B.S. were then added to the pellet. The cells were then examined under a Reichart "Zetopan" fluorescent microscope using as a light source an H.B.O. 200 mercury lamp employing a U.G.I./1.5 mm. exciter filter. Dark ground illumination was used in conjunction with a colourless Sp. 2 barrier filter, and the cells were examined under a x 100 objective. Glycerol was used as immersion medium for both

[1] (RAsh/FITC) Sera Service, Maidenhead

condenser and objective, owing to its non fluorescent properties.

Investigations on blood

1. Total white cell and differential count

The blood was collected in a sequestrene bottle. An aliquot of blood was diluted 1:20 in a blood diluting pipette with white cell diluting fluid (2% acetic acid with a very small amount of crystal violet stain) which lyses the red cells. The white cells were then counted in a haemocytometer (see 2 above). The differential count on the blood smears was performed as for the lymph (see 4 above).

2. Coombs' Test

Donor sheep red cells were washed three times in isotonic saline and made up to 10 to 15% suspension in isotonic saline. One volume of this suspension was incubated with one volume of recipient sheep serum for one hour at 37° C. The controls used were: Positive control Human blood group B cells incubated with sheep serum, Negative control (a) Human group B cells incubated with human neutral AB serum, (b) Sheep cells washed but untreated. The cells were then washed three times in isotonic saline. Two drops of anti-sheep globulin reagent (RAsh/FITC) were added to each tube, mixed and spun at 1,000 r.p.m. for 1½ minutes. The tests were examined microscopically, agglutination indicating a positive result. Two drops of sensitised cells were added to each negative test and the tubes spun again, agglutination indicating that the anti-sheep globulin had not been neutralised i.e. that the negative result was not due to experimental error.

Histological examination of the kidneys

Biopsy specimens of the kidneys were taken with a biopsy needle (Trucut) at operation after revascularization of the kidney. Further biopsies were also taken on the second, fourth and seventh post-operative days. The whole kidney was removed at postmortem on either the eighth or fifteenth day. All specimens were fixed in 10 per cent formalin phosphate buffered at pH 7. Blocks were embedded in paraffin wax and cut on a sledge microtome at 4 μ. Sections were stained with Ehrlichs haematoxylin and 2 per cent aqueous eosin Y. Some sections were stained by the methyl green - pyronin method to show the presence of pyroninophilic, RNA rich, large lymphoid cells.

E. RESULTS

1. Thoracic duct cannulation - total lymphocyte flow.

2. Renal lymphatic cannulation - renal lymphocyte flow.

3. White cell counts in peripheral blood.

4. Renal transplants and histology.

E. RESULTS

1. Thoracic duct cannulation (TOTAL LYMPHOCYTE FLOW)

There are 34 sheep in which the thoracic duct flow was measured for 7 whole days.

Group 1	Normal sheep	7
Group 2	Immunosuppression	6
Group 3	Renal autograft	3
Group 4	Renal allograft	10
Group 5	Renal allograft plus immunosuppression	8

There were also 13 sheep in which the thoracic duct was cannulated but did not drain for 7 whole days but drained for 4-6 days.

Group 1	Normal sheep	1
Group 2	Immunosuppression	1
Group 3	Renal autograft	2
Group 4	Renal allograft	5
Group 5	Renal allograft plus immunosuppression	4

Flow rate and cellularity of lymph

The flow rates and cellularity of lymph from the thoracic duct are shown in a normal (Figure 13) sheep and an immunosuppressed sheep (Figure 14). The lymph flow rates varied each day but the range was 2,000 to 6,400 mls. in all groups of sheep. The flow rate remained fairly steady through the whole period of thoracic duct drainage and in the successful cannulations no blocking of the cannulae occurred. The cellularity of lymph (i.e. the lymphocyte count) was always initially higher at approximately 1×10^7 lymphocytes per ml. and the number decreasing over the 7 days period. The product of the total flow x cellularity was calculated for each 24 hour period, giving the total daily lymphocyte count.

Total daily lymphocyte count

In all groups of sheep the total daily lymphocyte count was initially high, the peak collection occurring either on the day of operation, or on the first or second day. Thereafter the count dropped to a much lower level and remained steady (see Figures 13 and 14). The level of the peak varied for each sheep but the range was 12 to 40 $\times 10^9$ lymphocytes a day.

In the allografted sheep there was very little difference in the total daily count during the first and second week of collection (Figure 15). The lymph was collected during the first 7 days after transplantation during the period of developing rejection, and the second 7 days during the period of advanced rejection and necrosis of the graft (see below Histology).

In those sheep which received both an allograft and immunosuppression there was also no difference between the total daily count when collection of lymph was in the first and the second week after

transplantation (Figure 16). This figure also shows in two sheep that the dose of azathioprine, either 100 mgs. daily intravenously or 400 mgs. daily intravenously, did not alter the lymphocyte flow. The variations of daily lymph counts were however considerable and Figure 17 shows a marked reduction in the cellularity, but not the volume, of lymph in a sheep receiving 400 mgs. azathioprine daily, during the second week after allotransplantation and the commencement of immunosuppressive agents.

FIGURE 13 THE FLOW RATE AND CELLULARITY OF LYMPH T. D.

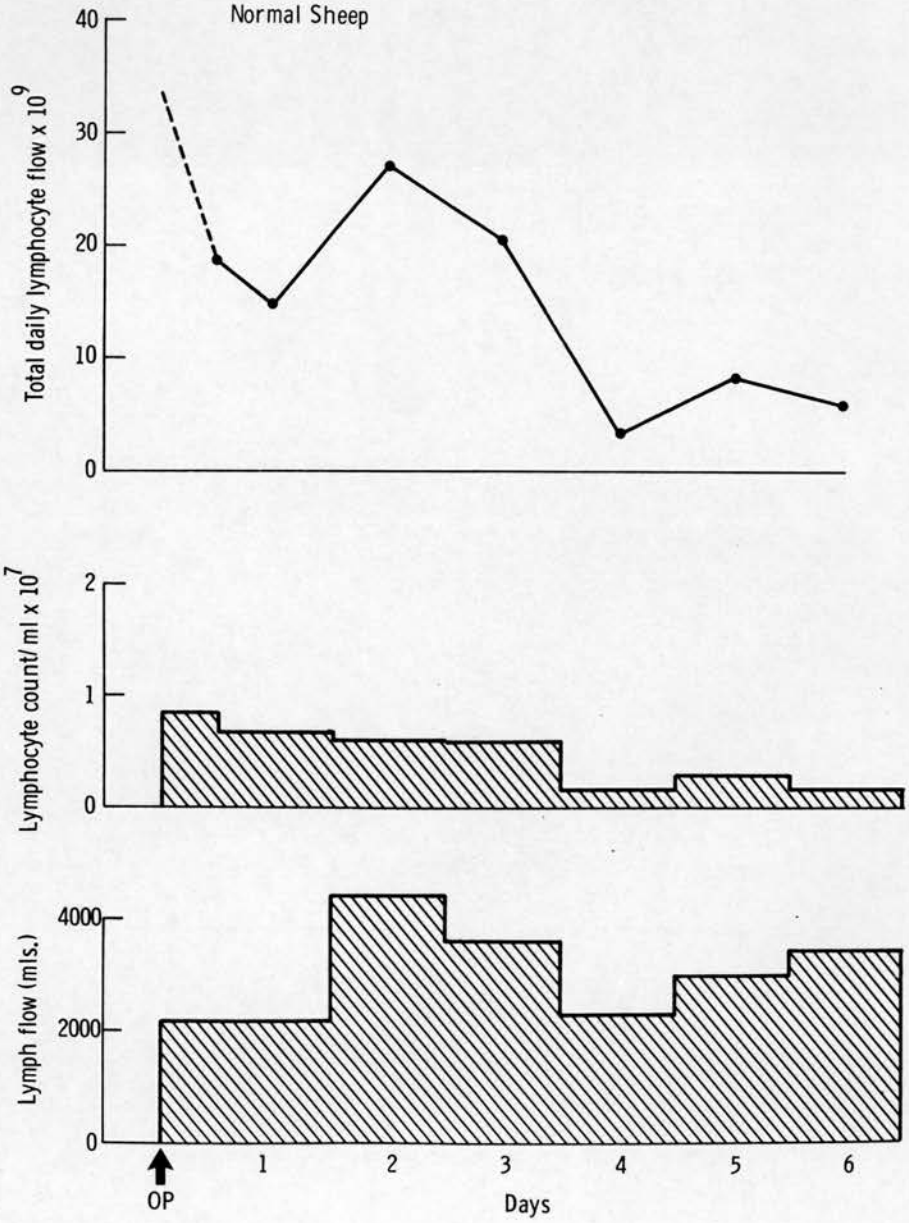


FIGURE 14

THE FLOW RATE AND CELLULARITY OF LYMPH T.D.

Immunosuppressed Sheep

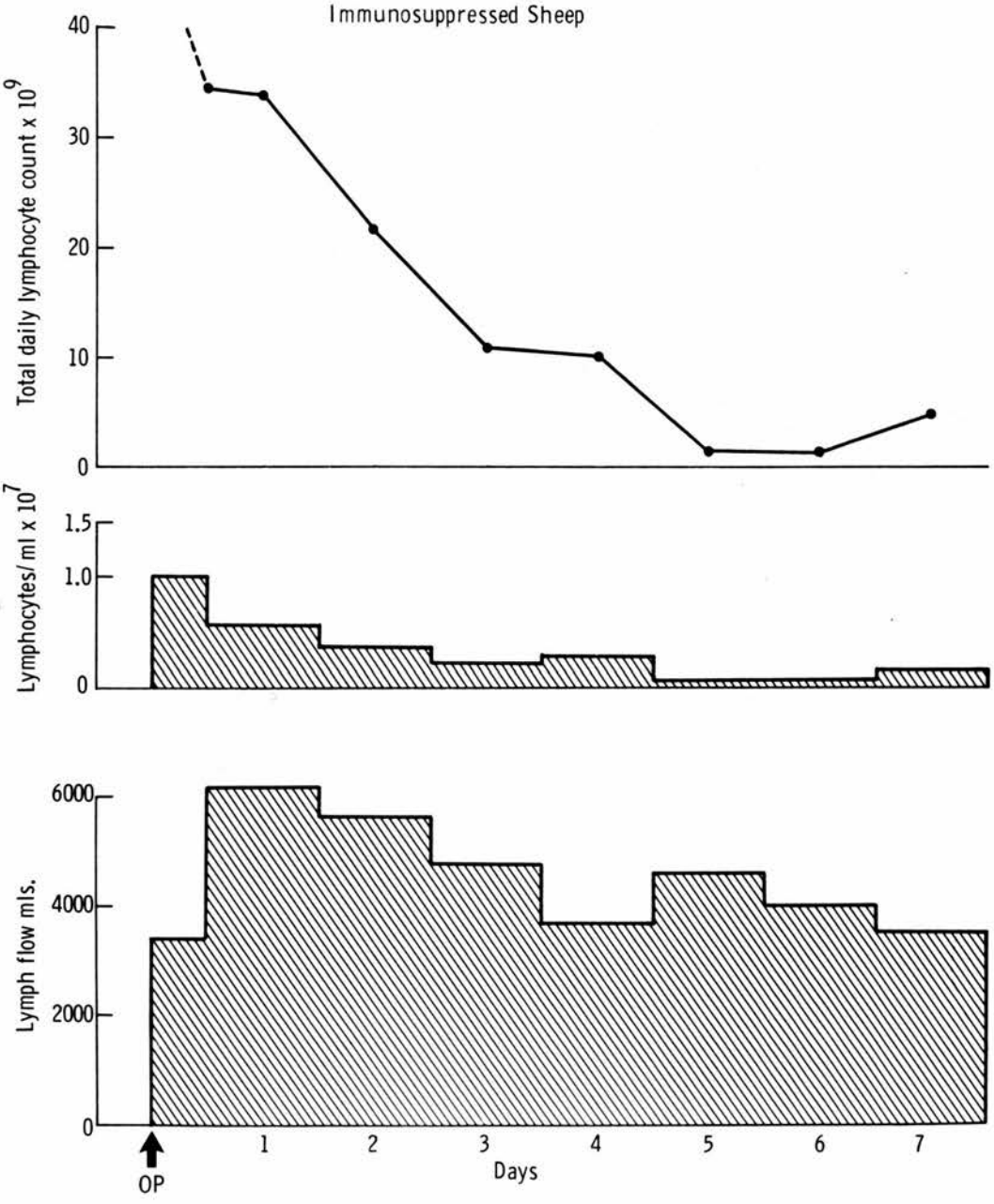


Figure 15 and 16

The total daily lymphocyte counts in sheep receiving an allograft (Figure 15) and an allograft and immunosuppression (Figure 16). The daily lymphocyte flow rates are compared during the first ●—● and second week ■—■ after transplantation.

FIGURE 15 ALLOGRAFT

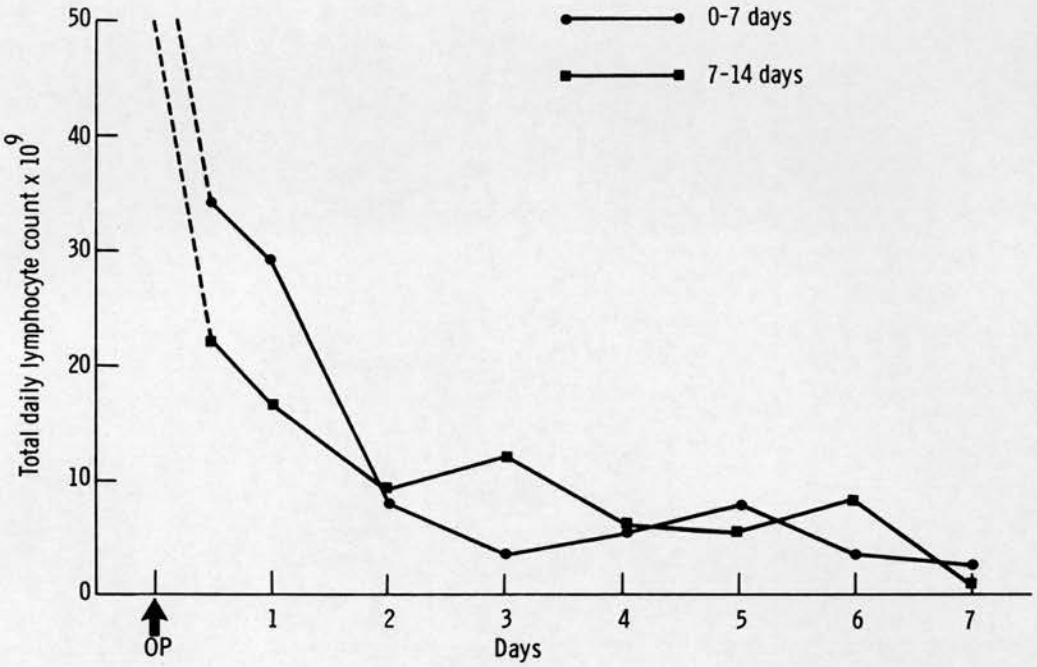
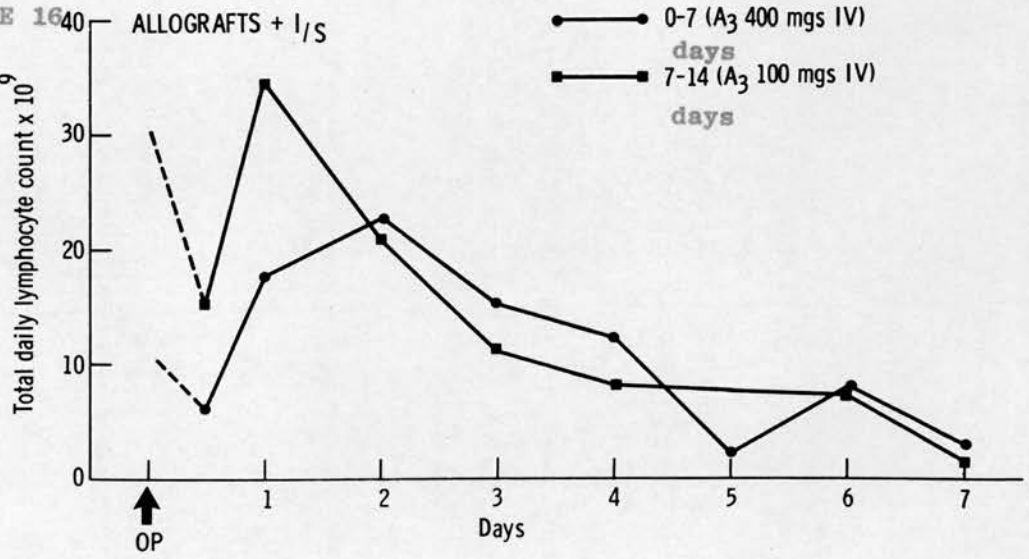
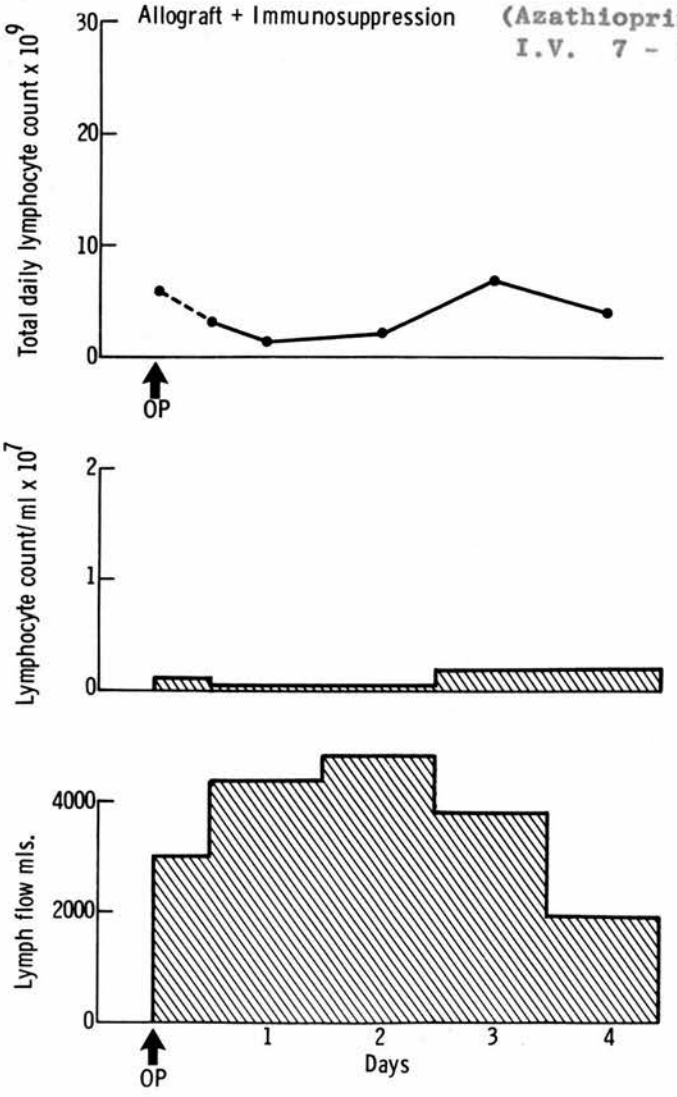


FIGURE 16



Az = daily dose of Azathioprine

FIGURE 17 THE FLOW RATE AND CELLULARITY OF LYMPH T. D.



Thoracic duct cannulations for 7 days (Total lymphocyte flow)

The total daily lymphocyte flow for seven days should reflect the total body lymphocyte flow. After an initial high number, the lymphocytes remain steady at a much lower level (Figures 13 and 14).

Sheep not receiving renal transplantation (Group 1 and 2)

There were 7 normal sheep, in which the thoracic duct alone was cannulated for seven days, acting as controls for group 2. The total lymphocyte flow in the seven days for each of the normal sheep is shown in (Figure 18) and the mean is 140.6×10^9 lymphocytes (Table XIV). There was also one normal sheep in which the thoracic duct was drained for 4 days (Table XV).

There were 6 sheep which received immunosuppressive drugs for 2 weeks prior to thoracic duct drainage. In 5 of these sheep the dose of immunosuppression was prednisolone 100 mgs. daily and azathioprine 100 mgs. daily either intravenously or orally. The individual total lymphocyte flow is shown in Figure 18 and the mean is 126.6×10^9 (Table XIV). There is no obvious difference in the total lymphocyte flow between sheep receiving the immunosuppression orally or intravenously. There is also no statistically significant difference between the mean of either the normal sheep or the immunosuppressed sheep (low dose) as measured by Student T test for unpaired data. Subsequently the dose of immunosuppression was increased to prednisolone 100 mgs. and azathioprine 400 mgs. daily. There were 2 sheep receiving this higher dose of immunosuppressive drugs. The thoracic duct of one of these drained for 7 days (Table XIV) and the other for 5 days (Table XV). There was no obvious reduction in the total lymphocyte flow with the increased dose of azathioprine.

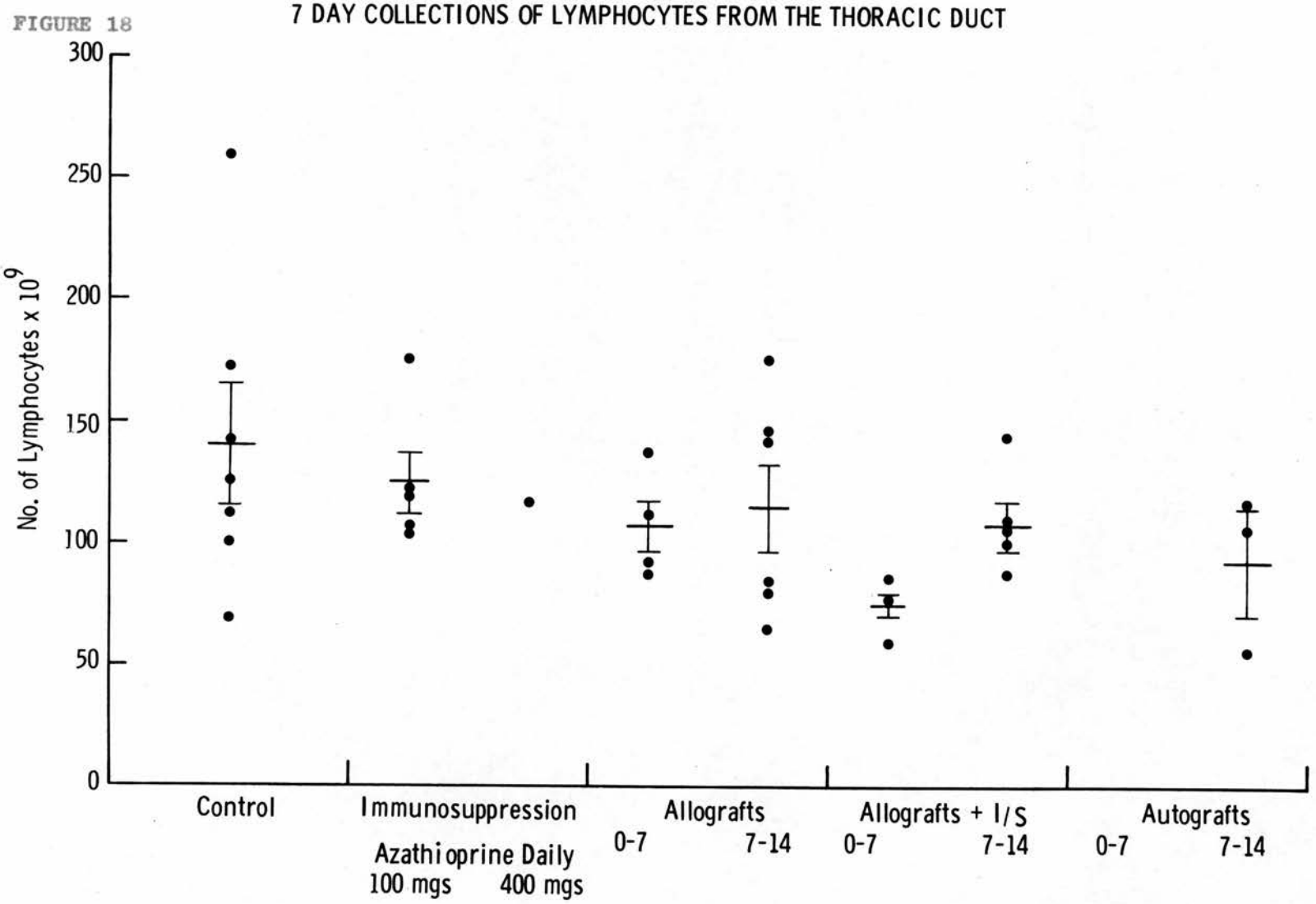
Sheep receiving a renal transplant (Groups 3, 4 and 5)

The sheep receiving a renal autotransplant (Group 3) were the controls for groups 4 and 5. The total lymphocyte flow in each of the 3 sheep is shown in Table XIV, and there are 2 sheep in addition whose thoracic duct drained for 5 days (Table XV). There was no autografted sheep whose thoracic duct drained for 7 whole days during the first week after transplantation. There were 3 sheep which had the thoracic duct drained during the second week after transplantation and the mean of their total lymphocyte flow is 92.8×10^9.

Ten sheep received allotransplants alone (Group 4) - 4 had their thoracic duct drained during the first week, and 6 during the second week after transplantation. The total lymphocyte flow of each group are shown in Tables XIV and XV. There is no difference in the results from the sheep receiving either a renal allograft or autograft.

Eight sheep received both a renal allograft and immunosuppression. The doses of immunosuppression varied. Although there appears to be a reduction of the total lymphocyte flow in the first week, the mean is little different from the lymphocyte flow in the autografted group; the number of sheep in this group however is too small (and there is no autograft control for the first week), for the results to be analysed statistically. It should be noted however that the total lymphocyte flow for one sheep receiving an allograft and immunosuppression (intravenously 400 mgs. azathioprine) was very low 16.8×10^9 (Table XV, Figure 17) the thoracic duct in this sheep was cannulated during the second week after transplantation and the animal had already received one week's immunosuppressive drugs at the higher dose.

FIGURE 18


7 DAY COLLECTIONS OF LYMPHOCYTES FROM THE THORACIC DUCT
No. of Lymphocytes x 10^9
300
250
200
150
100
50
0
Control
Immunosuppression
Azathioprine Daily
100 mgs
400 mgs
Allografts
0-7
7-14
Allografts + I/S
0-7
7-14
Autografts
0-7
7-14

NORMAL	IMMUNOSUPPRESSION		ALLOGRAFT		ALLOGRAFT PLUS IMMUNOSUPPRESSION		AUTOGRAFT	
	Azathioprine 100 mgs daily	Azathioprine 400 mgs daily	Day 0 - 7	Day 7 - 14	Day 0 - 7	Day 7 - 14	Day 0 - 7	Day 7 - 14
(7)	(6)	(1)	(4)	(6)	(2)	(5)	(0)	(3)
259.9 x 10^9	119.5 o.	117.9 i.v.	138.2	117.2	86.3† i.v.	145.6** o.		106.2
172.0	175.1 o.		92.2	146.9	64.4† i.v.	107.5** i.v.		117.4
143.7	108.8 o.		90.0	143.3	76.2† i.v.	80.7***i.v.+o.		54.7*
99.5	121.1 i.v.		110.3	80.9		109.2† i.v.+o.		
69.4	107.7 i.v.			84.4		101.6† i.v.		
113.4				64.8				
126.4								
mean ± S.E.								
140.6 ± 23.3	126.6 ± 12.4	117.9	107.7 ± 11.1	116.3 ± 18.5	75.6 ± 6.3	108.9 ± 10.5		92.8 ± 19.3
Group 1	Group 2		Group 4		Group 5		Group 3	

Thoracic duct cannulations for 7 days The figures in brackets show the total number in each group. The numbers in each group are written in the order in which the operation was performed. * Indicates collection made from 14 to 21 days post transplant. In the immunosuppressed groups the abbreviations o. and i.v. indicate whether the drugs were given orally or intravenously. Doses of azathioprine daily, ** 100 mgs., *** 200 mgs., † 400 mgs. All the figures are x 10^9.

Table XIV

NORMAL	IMMUNO-SUPPRESSION	ALLOGRAFT		ALLOGRAFT PLUS IMMUNOSUPPRESSION		AUTOGRAFT	
	Azathioprine 400 mgs.	Day 0 - 7	Day 7 - 14	Day 0 - 7	Day 7 - 14	Day 0 - 7	Day 7 - 14
56.0 (4) $\times 10^9$	89.3 i.v. (5)		48.0 (6)		57.4 i.v. (6)**	54.7 (5)	38.1 (5)
			72.1 (5)		51.6 o. (4)***		
			89.4 (4)		37.1 i.v. (4)†		
			52.3 (4)*		16.8 i.v. (4)†		
			31.7 (4)				

Thoracic duct cannulations for less than seven days These collections were from 4 to 6 days. The figure in brackets indicates the number of days collection. Doses of azathioprine daily ** 100 mgs., *** 200 mgs., † 400 mgs. * Indicates collection 14 - 21 days.

All the figures are $\times 10^9$.

Table XV

Transformed cells on the thoracic duct lymph

The percentage of large basophilic cells (transformed cells) have been counted in the thoracic duct lymph on the first and seventh day of drainage. In 3 normal sheep the percentages of transformed cells on the first day were 3, 4, and 7; these percentages were only slightly altered on the seventh day (Figure 19). Thoracic duct lymph from sheep on immunosuppression only, and after a renal allograft, usually showed an increase in the percentage of transformed cells by the seventh day of drainage. The percentage of transformed cells in lymph of sheep receiving an allograft and immunosuppression, remained unaltered by the end of the first week but again was increased by the seventh day if the thoracic duct was cannulated during the second post transplant week (Figure 19). The means of the percentage of transformed cells on the seventh day of thoracic duct drainage are shown in Figure 20. These again show that the percentage of transformed cells is increased on the seventh day after allotransplantation. There is however an even greater increase in the percentage of transformed cells in sheep after immunosuppression alone on the seventh day.

FIGURE 19

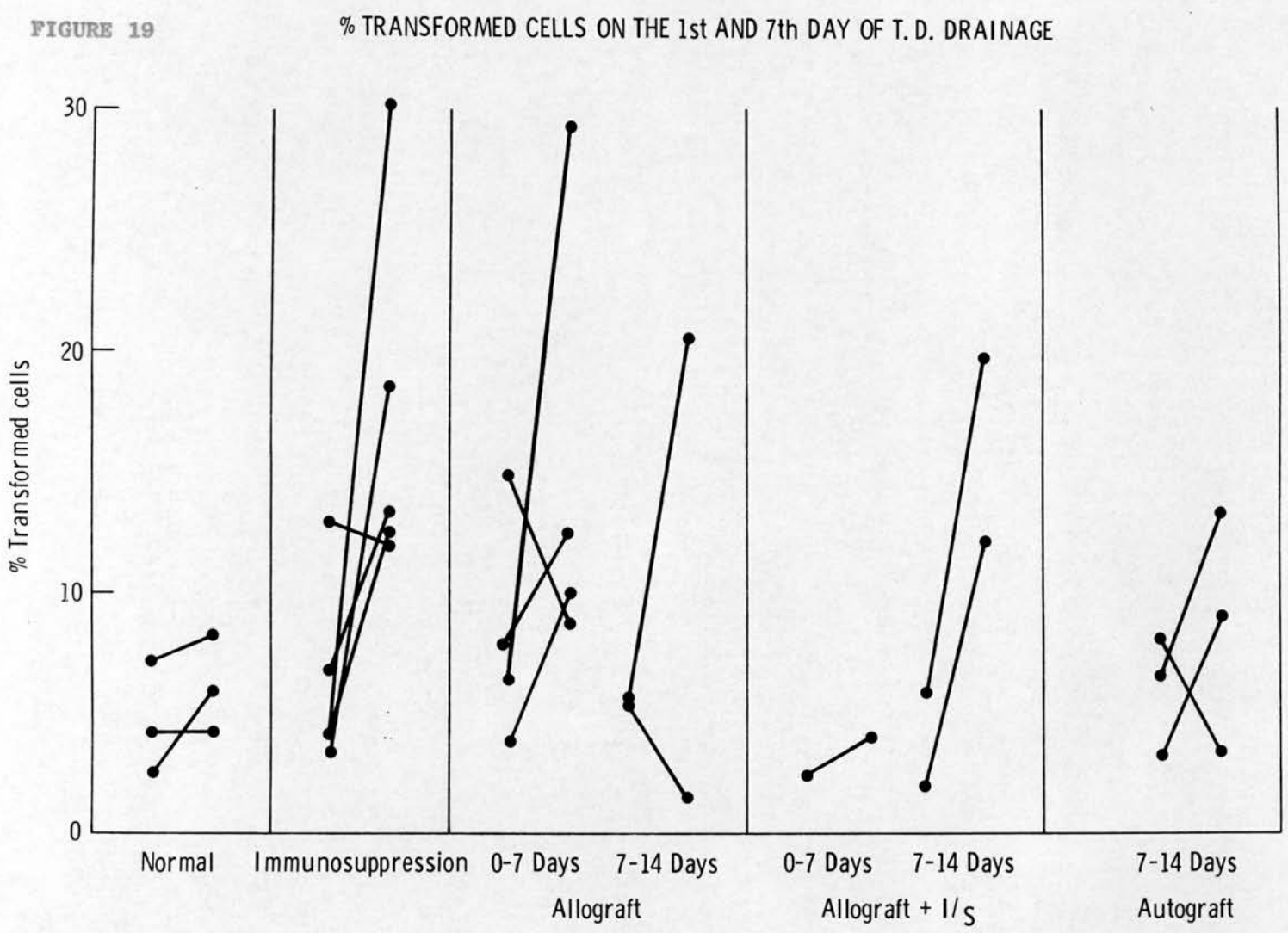
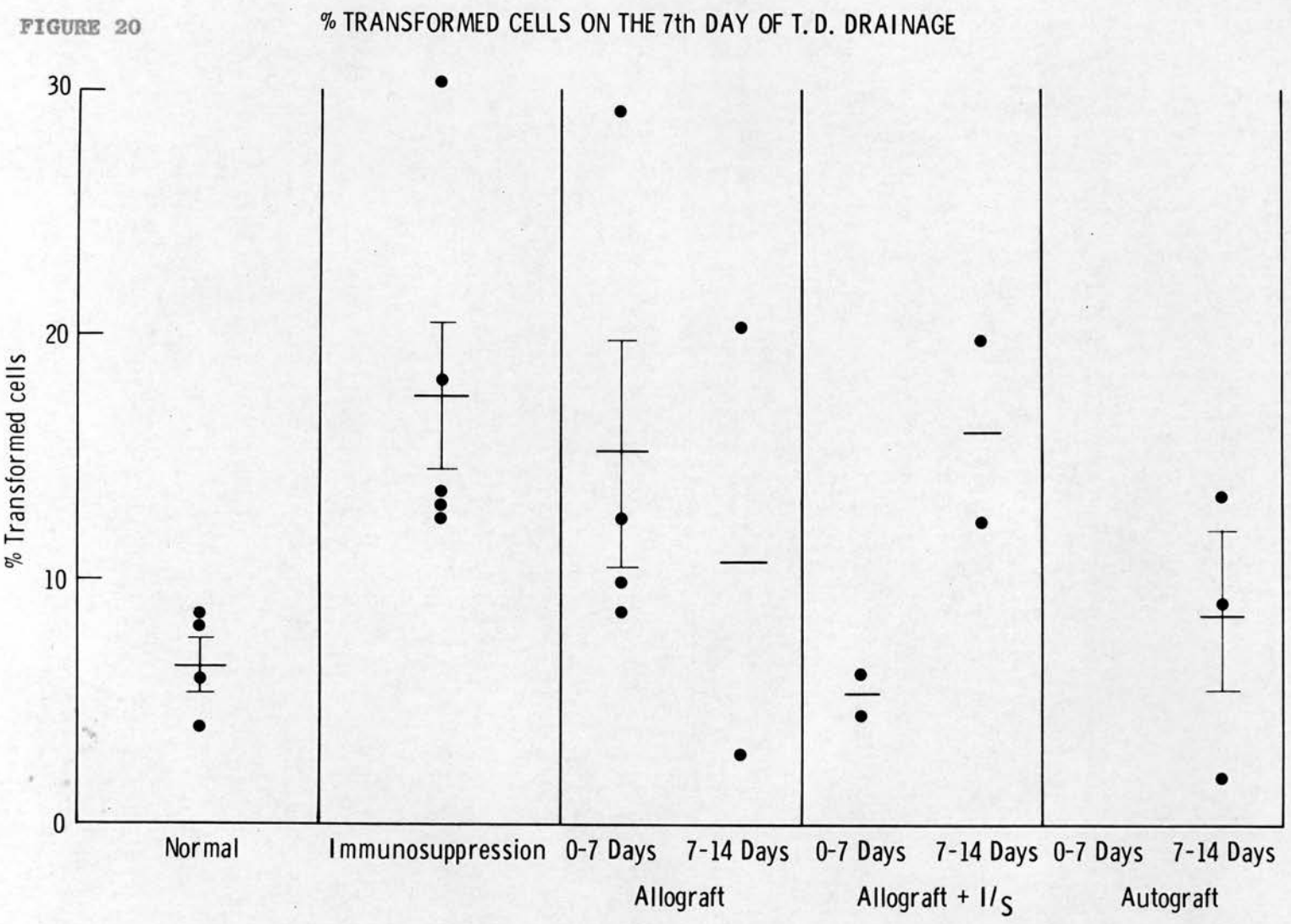

% TRANSFORMED CELLS ON THE 1st AND 7th DAY OF T. D. DRAINAGE
% Transformed cells
30
20
10
0
Normal
Immunosuppression
0-7 Days
7-14 Days
Allograft
0-7 Days
7-14 Days
Allograft + I/S
7-14 Days
Autograft

FIGURE 20


% TRANSFORMED CELLS ON THE 7th DAY OF T. D. DRAINAGE
% Transformed cells
30
20
10
0
Normal
Immunosuppression
0-7 Days
7-14 Days
Allograft
0-7 Days
7-14 Days
Allograft + I/S
0-7 Days
7-14 Days
Autograft

Cell populations in the thoracic duct lymph

In three sheep the proportion of B lymphocytes and macrophages were counted in the thoracic duct lymph. On the first day of drainage the percentage of macrophages ranged from 17 to 23% and the B cells from 16 to 21%. One of the sheep was on immunosuppressive drugs and the other two received an allograft. The proportion of these cells were not counted on the seventh day of drainage. Thoracic duct drainage over seven days removed both T and B cells from the body, histological sections of mesenteric lymph nodes on the eighth day showed a reduction in the number of lymphoid follicles and fewer lymphocytes in the paracortical areas.

2. Renal lymphatic cannulation (RENAL LYMPHOCYTE FLOW)

The lymphatics draining the kidney have been cannulated successfully in 9 sheep. A successful cannulation was one in which the lymph flowed continuously for 5 or more days. Three of these were allografted kidneys, and 3 were in sheep which received both an allograft and immunosuppression, azathioprine plus prednisolone, and three in sheep receiving an allograft and prednisolone only. The total lymphocyte flow and the number of days drainage, with the day of maximum flow rate of while cells is shown in Table XVI.

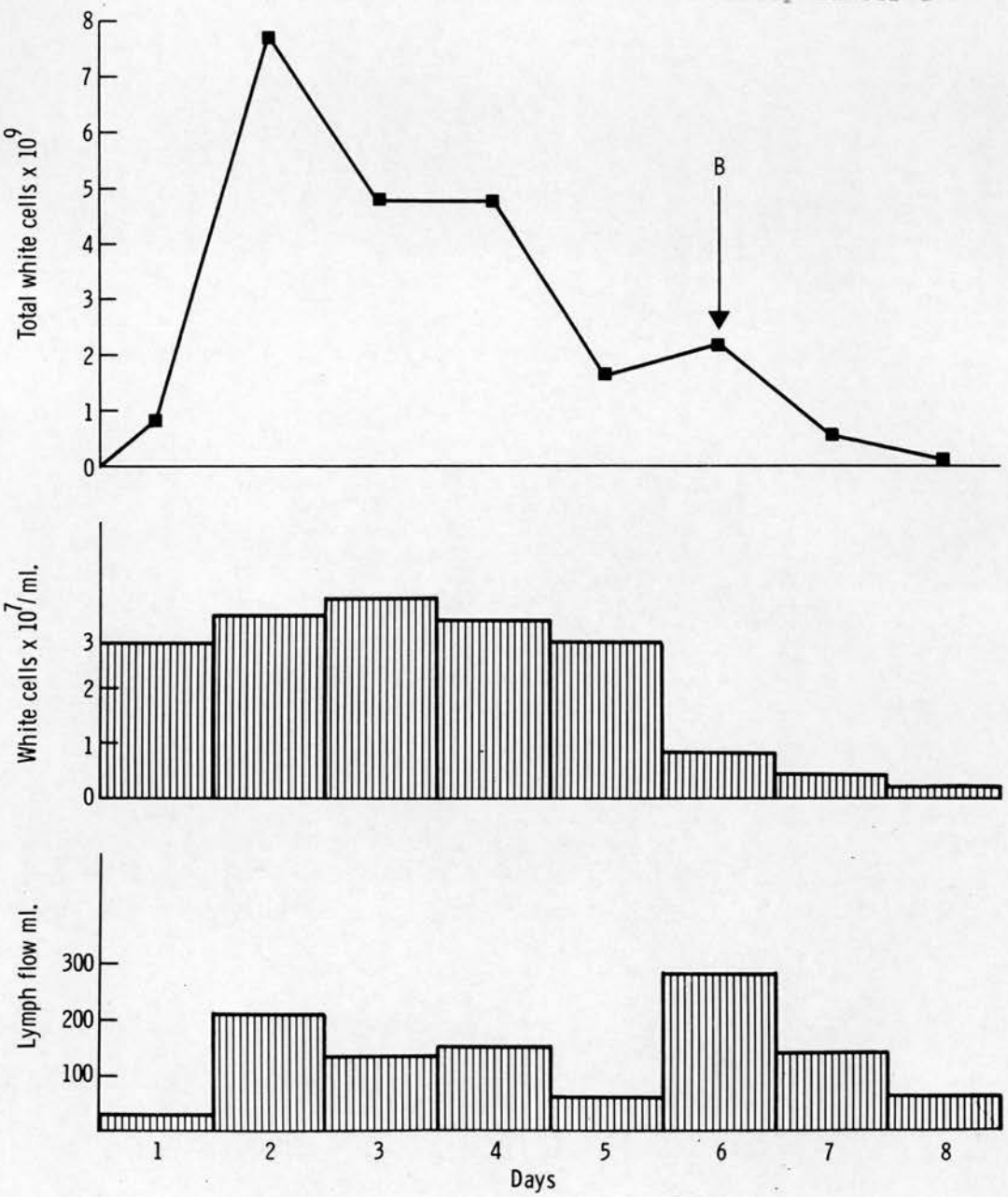
The drainage in the allografted sheep number 3, 6 and 7 was the most successful, continuing for 9 days. Figure 21 shows the daily flow and cellularity of lymph from sheep 3. The peak total flow of lymphocytes was on the second day when 7.8×10^9 white cells were collected. The maximum lymph flow occurred on the sixth day when 277 mls. of lymph drained, but the number of white cells had decreased considerably to 0.8×10^7/ml. Renal biopsy taken on this day showed that an advanced degree of rejection had taken place within the kidney.

A comparison of the daily renal lymphocyte flow in sheep 2 with an allograft only and sheep 5 with an allograft and immunosuppression is shown in Figure 22.

The mean of the total flow of white cells over the first 5 days in the allografted sheep is 12.3×10^9, and in the sheep receiving both an allograft and immunosuppression there is a decrease 3.1×10^9 (although the figures are not statistically significant). The mean total flow of white cells over 5 days in sheep receiving an allograft and prednisolone lay between these two means at 6.3×10^9 (Table XVI).

FIGURE 21

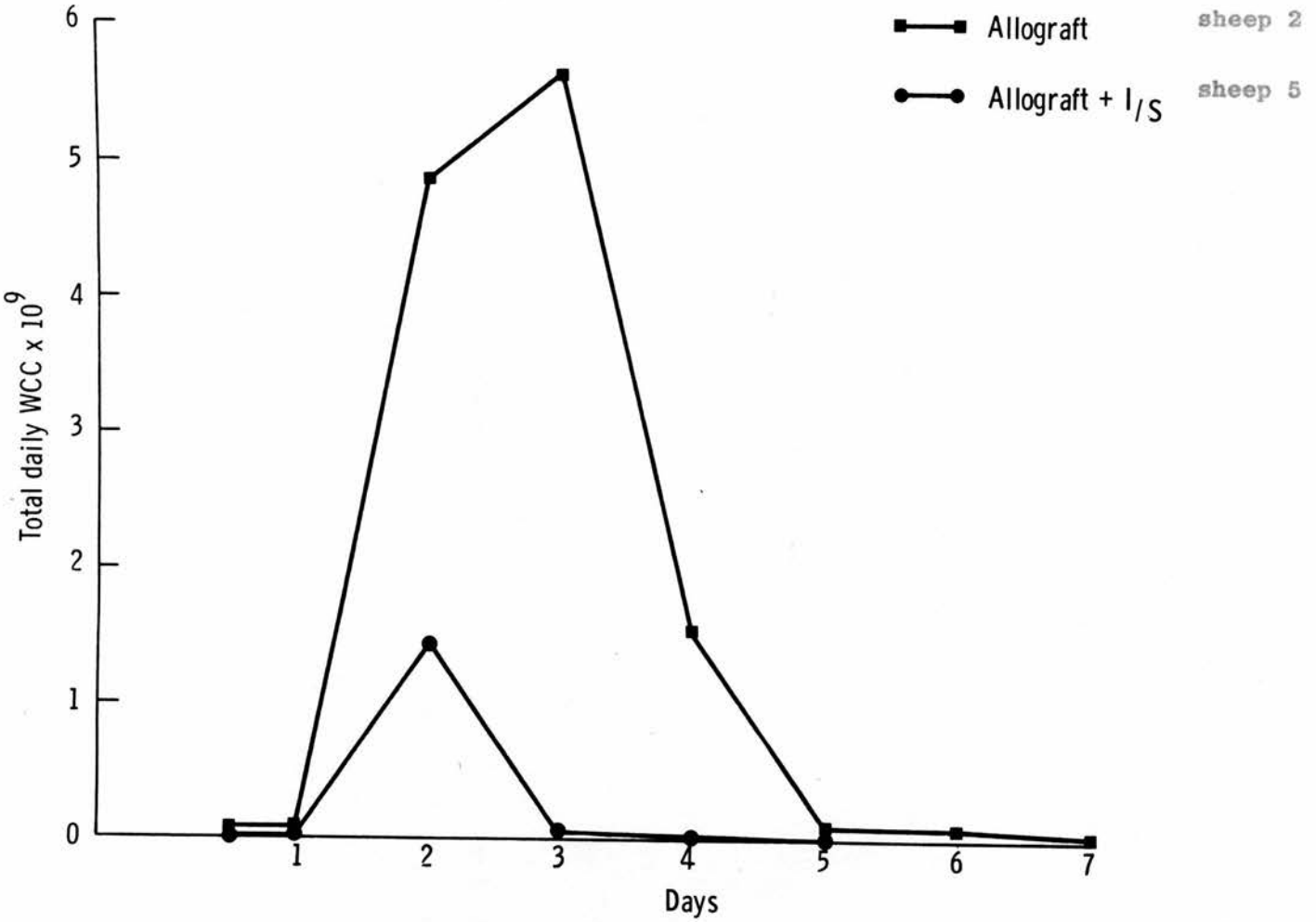
RENAL LYMPHATIC DRAINAGE sheep number 3



The daily flow of lymph and white cell drainage from an allografted kidney.

B = transplant biopsy.

FIGURE 22 RENAL LYMPHOCYTE FLOW IN AN ALLOGRAFTED AND ALLOGRAFTED PLUS IMMUNOSUPPRESSED SHEEP



SHEEP	No. of days drainage	Maximum total W.C.C./day x 10^9	DAY of MAXIMUM white cell flow: LYMPH FLOW in mls	W.C.C./ml x 10^7	DAY	TOTAL DRAINAGE of white cells for 5 days x 10^9	
1. Allograft	6	1.6	63	2.54	4	4.8	
2. "	7	5.7	210	2.7	3	12.3	= 12.3 ± 4.3
3. "	9	7.8	211	3.69	2	19.8	
4. Allograft + immunosuppression	5	0.4	150	0.26	3	1.12	
5. "	5	1.5	140	1.04	2	1.58	= 3.1 ± 1.7
6. "	9	2.0	153	1.29	1	6.55	
7. Allograft + prednisolone	10	2.6	145	1.81	3	8.2	
8. "	5	3.8	42	9.1	2	9.3	= 6.3 ± 2.5
9. "	5	0.9	57	1.5	4	1.4	

Renal lymphocyte flow in sheep. Those sheep receiving immunosuppression were given azathioprine 400 mgs. i.v. + prednisolone 200 mgs. i.v. daily. Those sheep receiving prednisolone were given 200 mgs. i.v. daily.

Table XVI

Differential white cell count and transformed cells in renal lymph

The white cell counts performed on the renal lymph were total counts. The cells were mostly lymphocytes, the range of polymorphs in sheep 3, which received a renal allograft was 2% on the first post-operative day to 10% on the seventh day of drainage. The percentage of transformed cells in the renal lymph of the same sheep was 3.3% on the first, 9.8% on the fourth and 16.5% on the sixth day of drainage of lymph, when advanced rejection was demonstrated on renal biopsy of the allografted kidney.

3. White cell total and differential count in peripheral blood

The peripheral blood total and differential white cell counts were performed in all the groups of sheep. There was considerable variation in the number of white blood cells, the range in normal sheep 4,000 to 11,500/mm^3 with a mean in 6 sheep of 8,150 $\pm$ 1,007 mm^3. The percentage of mononuclear cells was higher than the polymorphs, the range being 56 - 71% (mean 62.3 $\pm$ 3.4%).

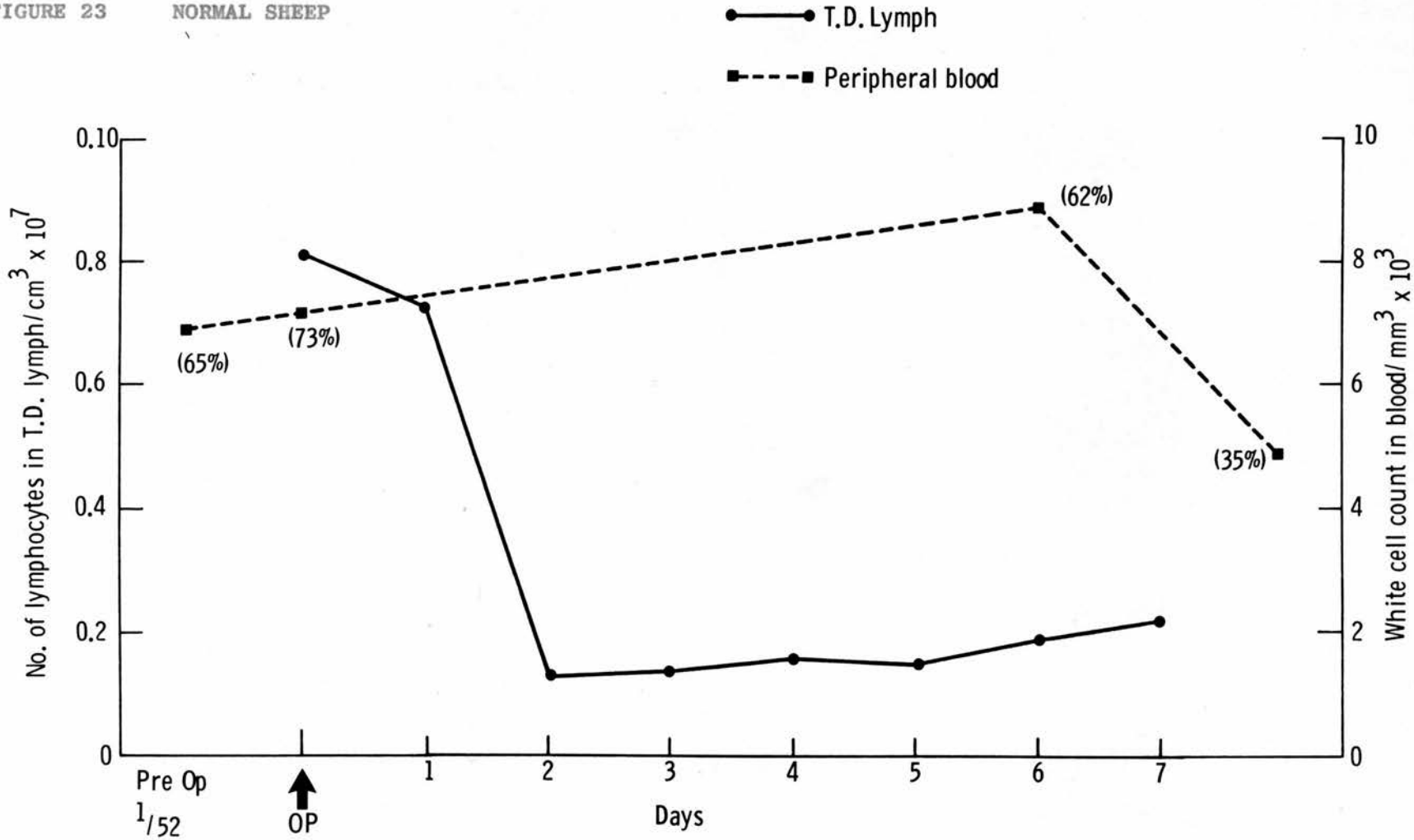
The means counts of the peripheral blood white cells and the percentage of mononuclear cells in all groups of sheep is shown in Table XVII. In the normal sheep the total number of white cells in the peripheral blood was reduced by 7 days depletion of thoracic duct lymphocytes, 4,000 cu.mm. The percentage of mononuclear cells remained steady. In the immunosuppressed sheep the white cell count 2 weeks prior to thoracic duct cannulation at the time of starting immunosuppressive drugs was 9,000/mm^3 with 81% mononuclear cells. There was very little alteration in this figure on immunosuppression alone but after 7 days lymph drainage both the number of white cells and the percentage of mononuclear cells were reduced.

With the exception of the allografted sheep during the first week and the sheep receiving autografts, the peripheral white cell count was reduced after 7 days lymphocyte depletion. There was very little alteration in the percentage of mononuclear cells in these groups.

The comparison of the numbers of white cells in the peripheral blood and the thoracic duct lymph from 1 normal sheep is shown in Figure 23. Although the thoracic duct white cells dropped rapidly on the second day, the white cell population in the blood was maintained until the sixth day of drainage, and thereafter dropped considerably.

Figure 23 The effect of lymphocyte depletion by thoracic duct drainage on the peripheral white cell count. The figure in brackets is the percentage of mononuclear cells counted.

FIGURE 23 NORMAL SHEEP



Day			Day 0 - 7	Day 7 - 14	Day 0.- 7	Day 7 - 14	Day 0 - 7	Day 7 - 14
2 weeks preop		9,000 (81)		9,750 (76)				
1 week preop		7,650 (82)		10,600 (71)		6,700 (70)		
Operation	8,150 (62)	8,013 (72)	5,400 (60)	8,200 (69)	4,500 (70)	12,100 (61)	8,300 (73)	12,800 (63)
Day 1								
Day 6		6,960 (69)						
Day 7	4,000 (61)		9,500 (52)	700 (52)	1,800 (68)	7,100 (54)		13,600 (59)
Day 8		6,044 (55)						
	NORMAL	IMMUNO-SUPPRESSION	ALLOGRAFT		ALLOGRAFT PLUS IMMUNOSUPPRESSION		AUTOGRAFT	

Peripheral blood white cell counts during thoracic duct drainage The means only in each group are shown. The figures in brackets are the percentage of mononuclear cells. The numbers are the number of white cells per mm^3.

Table XVII

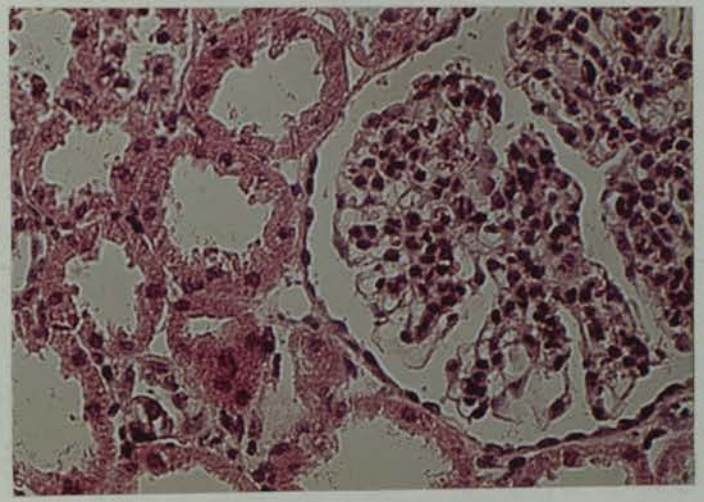
4. Renal Transplants

Immediately after renal transplantation the kidneys all passed urine, which varied in amount. The urine was usually heavily blood stained and the flow ceased after 48 hours, except in those kidneys which were autotransplanted. The recipient sheep had their own kidneys in situ. There were no biochemical tests performed for the diagnosis of rejection, which was assessed solely on histological grounds.

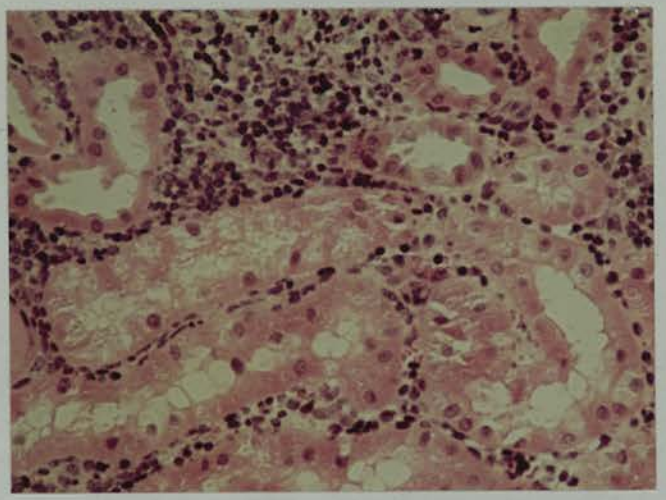
Histological findings in transplanted kidneys

Allografts

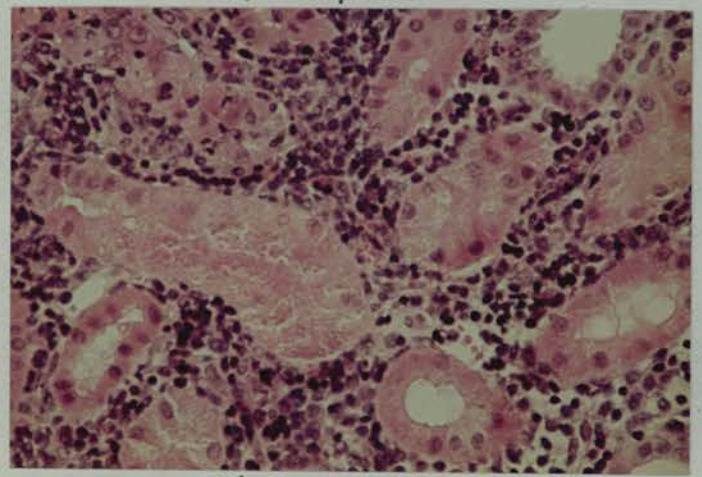
The diagnosis of rejection was made on histological grounds. Since in the late stage of rejection the histological picture may be predominantly ischaemic and undistinguishable from thrombotic infarction, serial biopsies were taken from the transplant throughout the period of each experiment. Only if a definite cellular infiltrate was seen in each biopsy was a diagnosis of rejection made. The kidneys which showed ischaemic changes without cellular infiltration were deemed ischaemic due to early occlusion of the major vessels. As an example of severe rejection developing in a grafted kidney plates 10 to 11 are shown. The operative renal biopsy in plate 10a shows mild tubular damage only, the glomeruli are normal and there is no cellular infiltrate. The biopsy from the same kidney on the second day shows preservation of the glomeruli, continuing mild tubular damage and a scanty infiltrate of inflammatory cells in the interstitial tissues (plate 10b). By day 7 there is more severe tubular damage with a heavier cellular infiltrate (plate 10c). This infiltrate is composed of small and large lymphoid cells, plasma cells and histiocytes. Many of the large lymphoid cells, and the plasma cells have intensely



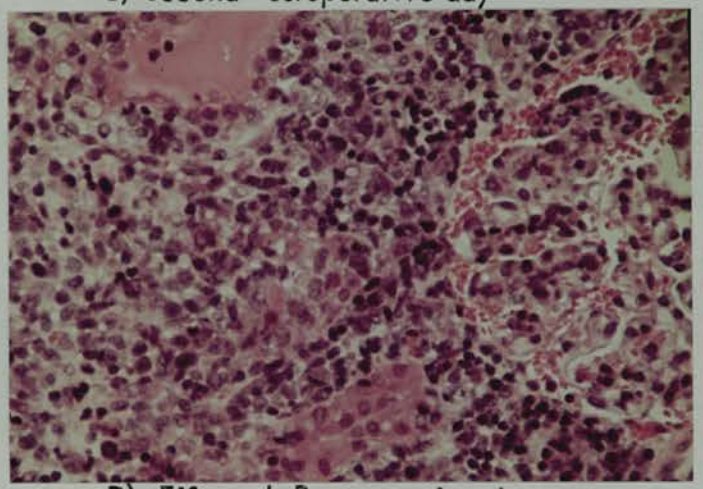
A) At operation



B) Second Postoperative day



C) Seventh Postoperative day



D) Fifteenth Postoperative day

Plate 10 REJECTION OF ALLOGRAFT IN SHEEP - HISTOLOGY

Magnification x 300

pyroninophilic cytoplasm (plate 11b). Sections of the postmortem kidney on day 15 show severe tubular damage with necrosis (plate 10d) and an intense lymphoid cell infiltrate, and in some cases the small vessels showed fibrinoid change (plate 11a) and thrombotic occlusion. There was no occlusion of the renal artery or its main branches but in several cases the renal vein was thrombosed.

At the time of postmortem the severity of rejection varied from sheep to sheep. It was possible to assess the degree of rejection on a semiquantitative basis as mild, moderate and severe. This assessment was carried out without knowledge of whether the sheep were receiving immunosuppressive therapy. In mild rejection there was little change in the renal structure, with a scanty infiltration of lymphoid cells in the interstitial tissues. In moderate rejection there was a heavier lymphocyte infiltration, with moderate tubular damage and focal tubular necrosis. In severe rejection there was extensive tubular necrosis and tubular degeneration, with an intense lymphoid infiltration and in most cases fibrinoid damage of the arterioles, with occlusion of the small renal vessels and the renal vein.

A total of 23 allograft experiments were carried out on sheep not receiving immunosuppression; 12 kidneys showed changes of rejection and 11 were ischaemic. Most of the ischaemic kidneys occurred in the early part of the study, prior to routine anticoagulation with heparin.

There were 18 allografts in sheep also receiving immunosuppressive drugs (Table XVIII). Only 5 of these were ischaemic. There were 3 sheep receiving a low dose of azathioprine (100 mgs. per day) and prednisolone and 3 sheep receiving prednisolone alone, and

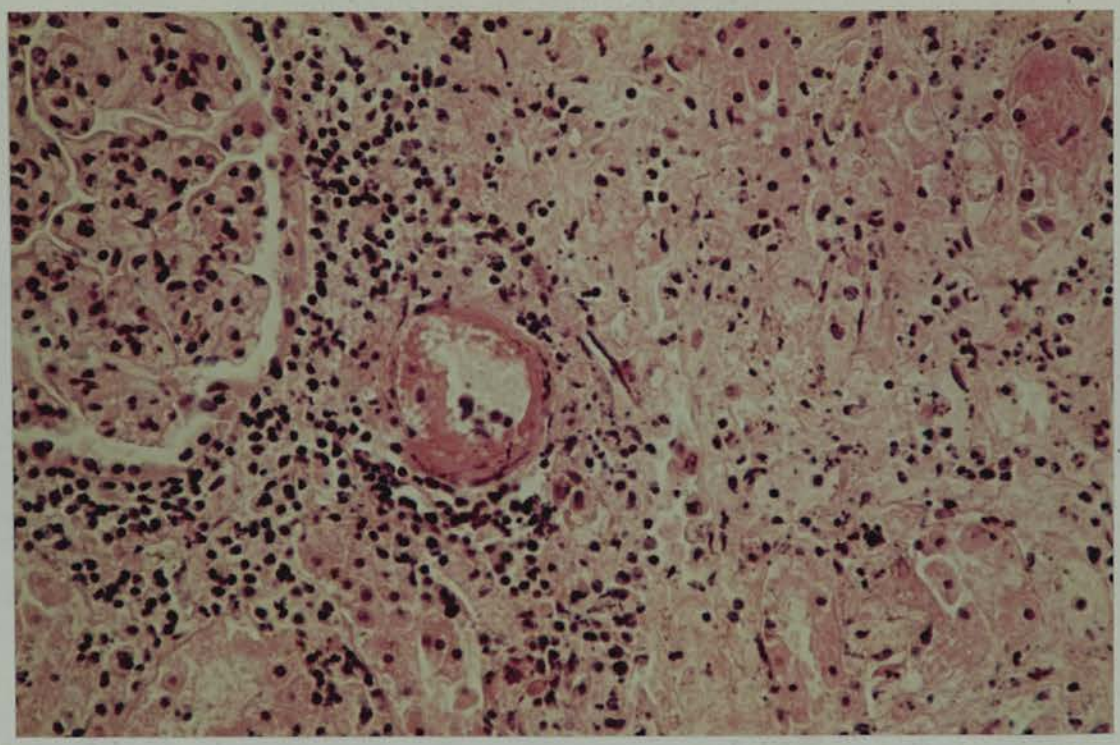


Plate 11a) SEVERE REJECTION IN SHEEP RENAL ALLOGRAFT SHOWING FIBRINOID CHANGE IN ARTERIOLES

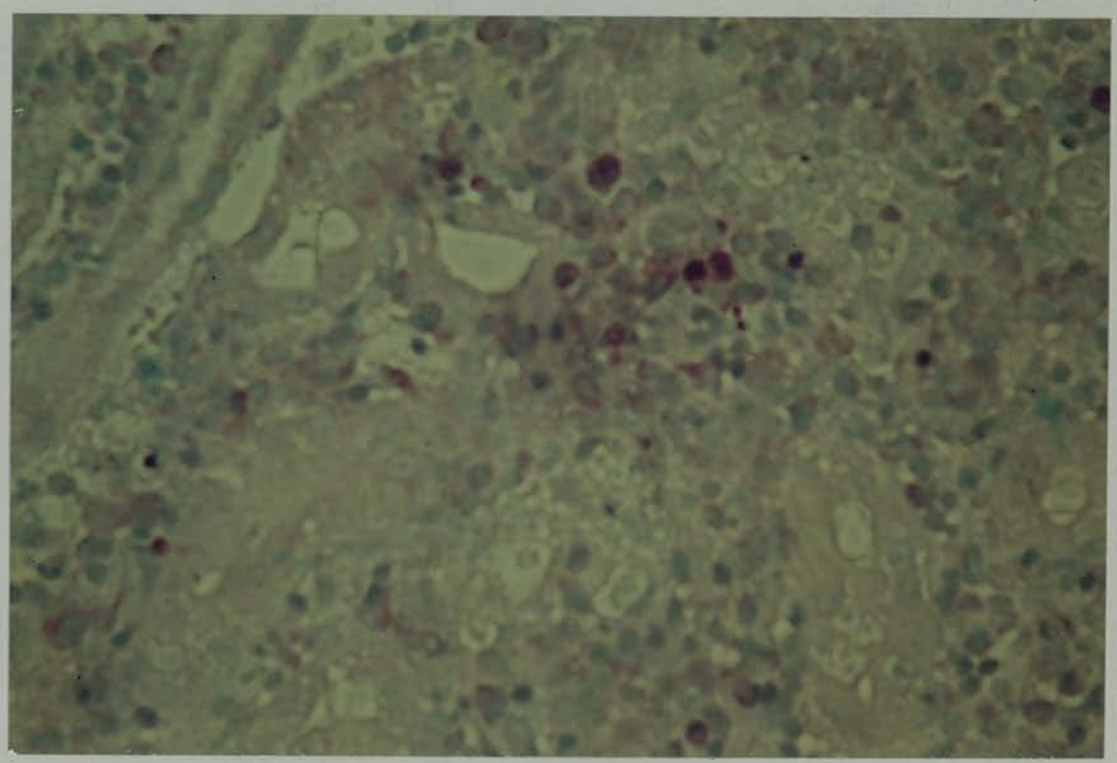


Plate 11b) SEVERE REJECTION, STAINED WITH METHYL-PYRONIN GREEN SHOWING LYMPHOBLAST CELLS

Magnification x 400

Group	Dose of Azathioprine mgs/day	Heparin 20,000 units/day	Degree of Rejection: Severity on 7th day	Degree of Rejection: Thrombosis of vessels on 14th day
Allograft		-	++	
"		-	++	
"		-	+	+
"		+	+++	+
"		+	++	with abscesses
"		+	++	+
"		+	++	+
"		+	++	+
"		+	+++	
"		+	+++	
"		+	+++	
Allograft + Immuno-Suppression	100	+	++	with abscesses
"	100	+	+++	
"	200	+	+++	+
"	400	+	++	
"	400	+	+	
"	400		++	
"	400	+	++	
"	400	+	+	+
"	400	+	++	
"	400	+	+	+
Allograft and Prednisolone only		+	+	
"		+	+++	
"		+	++	+

Histology of rejected kidneys. The severity of rejection on the 7th post transplant day: + mild; ++ moderate; +++ severe (see text).

Table XVIII

there was no reduction in the severity of the rejection. There was a reduction in the severity of rejection on histological assessment in many of the kidneys transplanted into sheep receiving 400 mgs. of azathioprine each day and prednisolone (plate 12).

Autografts

There were 7 autografted kidneys. Four were ischaemic and apart from early mild tubular damage the other three showed no histological abnormality.

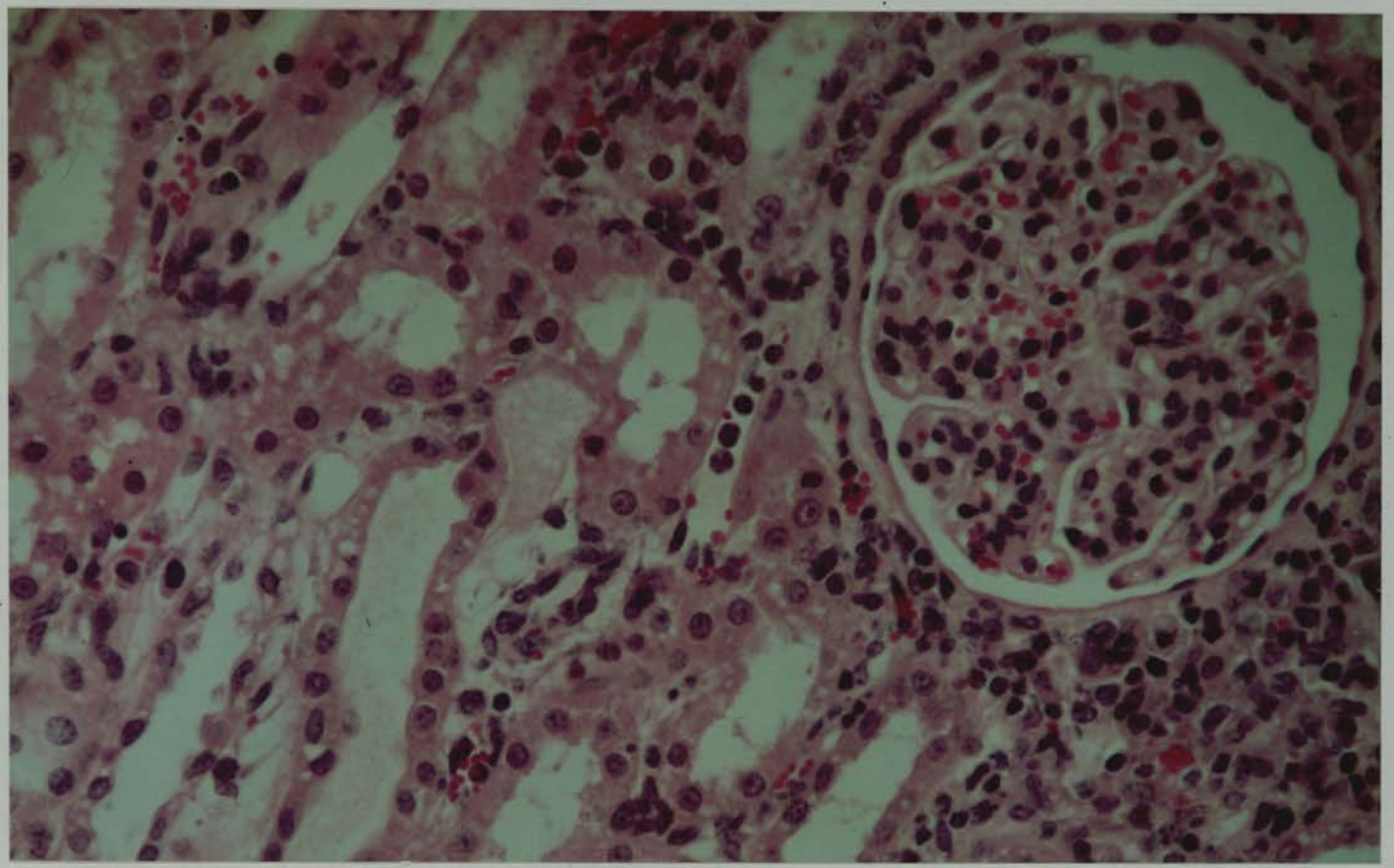


Plate 12 HISTOLOGY - REJECTION ABROGATED
in a sheep on Immunosupression (Azathioprine and Prednisolone) Day 7
Magnification x600

	AUTOGRAFTS		ALLOGRAFTS		ALLOGRAFTS PLUS IMMUNO-SUPPRESSION	
	0 - 7 days	7 - 14 days	0 - 7 days	7 - 14 days	0 - 7 days	7 - 14 days
REJECTED or NON ISCHAEMIC KIDNEYS		117.4	92.2	64.8	86.3	80.7
			110.3	80.9		
			138.2	84.4		
				mean = 76.7 ± 6 (n ± S.E.)		
ISCHAEMIC KIDNEYS		54.7	90.0	143.3	64.4	145.6
		106.0		146.9		107.5
				177.2		
				n. = 155.8 ± 11		

The total lymphocyte flow and ischaemia of kidneys. The figures represent the 7 day total lymphocyte flow of each sheep. Student T test for unpaired data gave $p < 0.01 > 0.001$ for the difference between the total lymphocyte flow in allografted kidneys with either ischaemia or rejection.

Table XIX

Total lymphocyte flow in relation to the histological findings

The total lymphocyte flow in allografted sheep varied according to the histology of the kidney. The lymphocyte flow during the second week after transplantation in those sheep whose kidneys had rejected was reduced (Table XIX).

Renal lymphocyte flow in relation to the severity of rejection

The use of immunosuppression reduced the severity of rejection on histological examination. The severity of rejection was for the main part determined by lymphocyte infiltration. A less marked lymphatic infiltration into the renal parenchyma is reflected by a reduction in the renal lymphocyte flow (Table XX).

Group	Renal lymphocyte flow x 10^9 for 5 days	Histology of Allograft 7th day
Allograft	4.8	moderate lymphocyte infiltration
"	12.3	moderate infiltration + haemorrhagic necrosis
"	19.8	severe rejection
Allograft + azathioprine and prednisolone	1.1	mild infiltration, focal groups of cells
"	1.6	moderate infiltration
"	6.6	moderate infiltration
Allograft + prednisolone	8.2	mild infiltration, vessels full of debris, ischaemic rejection
"	9.3	marked infiltration
"	1.4	moderate infiltration but early ischaemic changes

Renal lymphocyte flow and the histology of the kidney on the seventh day.

Table XX

F. DISCUSSION

1. Renal Transplantation in sheep

The reason for the choice of sheep for these experiments was their docility; they can be kept in restraining pens with ease for two weeks. They also have large enough, and easily identifiable, renal lymphatics and thoracic ducts for cannulation. Male sheep were chosen in order to prevent any previous sensitization from pregnancy, which might alter the course of rejection. Sheep have considerable drawbacks from the point of renal transplantation. Studies on renal transplantation in sheep (Mitchell, 1959) showed that of nine renal autografts, three were technical failures leading to ischaemia of the kidney from renal artery and vein thrombobis; three were successful and passed small amounts of blood stained urine during the first two weeks, and after two weeks, when the opposite normal kidney was removed, sustained normal life and renal function in these sheep for many months. There were also three sheep in which the autograft failed to function adequately to sustain life after removal of the controlateral single remaining kidney. In this work twelve renal allografts were also performed, of which three were again technical failures due to vascular thrombosis; the remaining grafts functioned initially; small quantities of blood stained urine were passed for two to nine days. Rejection was considered to have occurred when the animals were anuric, and the kidneys were then removed for histological examination, three to nine days after transplantation. The severity of rejection was marked and even by the fourth post-operative day there was heavy interstitial renal cell infiltration. Complete liquefaction of the kidney occurred if the removal of the graft was delayed more than 24 hours after the

onset of anuria.

It has been shown that periods of renal ischaemia longer than one hour cause a fatal uraemia in sheep (Mitchell and Woodruff, 1957). Mitchell (1959) felt that the problems arising in the autografted kidneys were due to the period of occlusion (up to 1 hr. 20 mins.) of circulation to the kidneys during the operation. Two methods of renal cooling were therefore tried, firstly intra-arterial perfusion with cold dextran and secondly surface cooling by immersion in cold saline. Three kidneys cooled by the first method all showed renal infarction. Surface cooling also described by Mitchell and Woodruff (1957) appeared much more satisfactory.

In the results of the present experiments renal ischaemia was also a considerable problem (see Appendix 3). The use of lignocaine added to the dextran for intra-arterial perfusion reduced considerable spasm of the arteries after circulation had been re-established. The kidneys were usually reanastomosed within one hour of removal and during that period were kept at 0^{o} C. in ice. The main factor which improved the blood supply to the kidneys and prevented thrombosis was full anticoagulation with heparin. Heparin has been used for the treatment of rejection episodes in man (Kincaid-Smith, 1967). There was no evidence to suggest that rejection was altered by the use of heparin in these experiments, since a marked degree of rejection had occurred by the second day in the sheep on anticoagulation but not receiving immunosuppressive therapy.

The histology of allograft rejection

Organ and tissue grafts transplanted to normal recipients behave like autografts for the first few days. First set renal allografts produce urine, and if the recipient has no kidneys of its own, the kidneys will pass considerable quantities of urine (Mitchell, 1969). Renal allografts transplanted into an animal previously sensitised to donor tissues are rejected in a rapid and dramatic manner (Pederson and Morris, 1970). In this hyperacute rejection the allograft becomes damaged within minutes of re-establishing the blood flow. The animals used by us were young male sheep, and there is no evidence to suggest that any of the recipients could be in any way presensitised to the donor rams. There was no effort made to identify or take into account the blood groups of the sheep although the results of the Coombs test showed no evidence of incompatibility between the donor sheep red cells and recipient sheep serum.

The macroscopic features of allograft rejection include oedema, changes due to cellular infiltration and circulatory disturbances (Herbertson, 1973). If rejection is rapid, necrosis of the parenchyma is a major feature. If rejection is more gradual, atrophy and eventual fibrosis occurs. The main microscopic changes during allograft rejection are mononuclear cell infiltration, oedema, vascular lesions and destruction of the parenchyma. As we have shown, these changes can vary considerably in different donor/recipient experiments. Herbertson (1973) described four types of mononuclear cell within a rejecting allograft - lymphocytes, blast cells, plasma cells and macrophages - most of these cells are derived from the recipient. He suggested that the blast cells and

plasma cells found in allografts were formed within the graft from infiltrating blood-bourne lymphocytes. The vascular lesions in an allograft are due to fibrinoid necrosis and thrombosis within the vessels. The parenchymal destruction may be due either to ischaemic injury or to direct destruction by immune cells.

Immunosuppressive treatment alters the changes of rejection by reduction in infiltration of mononuclear cells. The degree of abrogation of rejection varies considerably in each donor/recipient situation. Even in the presence of immunosuppressive therapy in humans, mononuclear cell infiltration has been observed on the third day in first set allografts. In the present experiments on sheep the routine use of steroids and azathioprine (3 mgs./Kg./day) did not alter the severity of the rejection process. Only by using high doses of immunosuppression (methylprednisolone 1 G., prednisolone 200 mgs./day and azathioprine 400 mgs./day) did the virulence of the rejection process appear to be reduced. Even this latter dose did not completely abrogate rejection in all cases.

2. Total body and renal lymphocyte flow

The experiments reported here have confirmed the findings of Pederson and Morris (1970) that there is an increase in the number of white cells, mainly lymphocytes, leaving a renal allograft. The peak flow from the kidney is around the third day, at which time histology shows lymphocyte infiltration of the interstitial tissues. The lymphatic drainage of white cells from the kidney then decreases to a very small number on the seventh day when severe rejection with ischaemic changes is present.

This massive rise in the number of lymphocytes flowing through a rejecting graft is not reflected in an increase in the total body flow of lymphocytes in the thoracic duct. This is surprising in that Porter, Joseph, Rendall, Stolinski, Hoehn and Calne (1964) showed that after canine renal allografts there was a generalised whole body lymphoid response, especially in the spleen. There may be two reasons for this. Firstly the daily renal lymphocyte flow is at maximum 8×10^9 cells/mm^3 whereas the daily total flow in the thoracic duct is up to ten times greater than this; thus the local increase in the area of rejection does not significantly after the massive total body lymphocyte circulating pool. Secondly at the time of transplantation into the neck only arterial and venous vascular reconnections are made. The lymph flowing from the lymphatics collects around the operative site. Most of this lymph no doubt is reabsorbed through the tissue spaces into the nearest draining node - the prescapular (Pederson, Adams and Morris, 1975) - but a proportion of the lymph collects around the graft and therefore is lost to the total lymphcyte flow. It has also been shown here that sheep with kidneys which were rejecting had a smaller total lymphocyte flow than sheep with kidneys

which were ischmaeic. The possible explanation for this is that the rejecting kidney is attracting and trapping many of the lymphocytes from the peripheral blood.

There is seen an increase in the number of lymphocytes flowing through the kidney in rejection; this increase is specific to the graft area alone.

The effect of immunosuppressive agents on renal and total lymphocyte flow

In the renal allograft, immunosuppressive drugs in the higher dose (see Methods) reduced the number of lymphoid cells collecting in the interstitial tissues as seen on histology. This reduction of lymphoid cells in the graft was also mirrored in a marked reduction of lymphoid cells in the renal lymph draining from the kidney. When steroids only were used the number of lymphocytes in the renal lymph was less than in the kidneys where no immunosuppression was used. The reduction was not as great as when a combination of steroids and azathioprine was given. This would appear to indicate that both steroids and azathioprine are effective in reducing the mononuclear cell infiltration which occurs at rejection.

Immunosuppressive drugs however did not alter the total body lymphocyte flow, when given either alone or in conjunction with a renal allograft. The measurement of the peripheral blood count did however show that a combination of immunosuppressive drugs and thoracic duct lymphocyte depletion reduced the peripheral white cell count more than in normal sheep. The fall in the peripheral blood count was four days after the marked fall in the thoracic duct lymphocyte count, as the bone marrow was probably maintaining the peripheral blood lymphocyte count during these four days.

It is considered that the main effect of the immunosuppressive drugs is to reduce the number of lymphocytes in the allograft and flowing from the graft. These drugs have little effect on the numbers of lymphocytes flowing through the whole body.

3. Transformed cells in the renal and thoracic duct lymph

The production of transformed cells is part of the central response of the body against antigen. The lymphocyte population is incited to proliferate and divide by the presence of an allograft: these dividing cells are given various names (see above - Background).

The renal lymph

There is a massive drainage of lymphoid cells from the kidney at the time of rejection. Some of these cells are large basophilic cells which we have called transformed cells. The percentage of transformed cells rose from 3.3% on the first post transplant day to a maximum of 16.5% on the sixth day. The maximum rise in the percentage of transformed cells occurred after the maximum lymphocyte flow (day 2) and does not constitute an actual increase in the number on the sixth day. The maximum flow of transformed cells occurred on the fourth day when a total of 4×10^8 transformed cells were drained. This drainage of transformed cells from a rejecting kidney confirms the findings of Pederson and Morris (1970) in sheep, and Hamburger, Dimitrui, Bankir, Debray-Sachs and Auvert (1971) in patients, with renal transplants. The percentage of transformed cells in allo-transplanted sheep in these present experiments is lower than the 60% recorded by Pederson and Morris (1970) and more in keeping with the 15% reported by Hamburger et. al., (1971) although the patients were also receiving immunosuppression.

The thoracic duct lymph

There was an increase in the percentage of transformed cells in the thoracic duct lymph of sheep receiving an allograft, from the

first to the seventh day of thoracie duct drainage. In none of the sheep did the actual total number (rather than the percentage) of transformed cells increase by the seventh day.

The effect of immunosuppressive drugs on transformed cells in renal and thoracic duct lymph

It has been suggested that one oftthe actions of azathioprine may be the inhibition of DNA and RNA synthesis and therefore the productinn of transformed cells (Hamburger, Crosnier, Dormont and Bach, 1972). We might expect a decrease in the percentage of transformed cells in sheep receiving immunosuppressive drugs.

Using azathioprine in conjunction with steroids, it has been shown that in sheep receiving immunosuppressive drugs alone there was an unexpected and considerable increase in the percentage of transformed cells after seven days thoracic duct drainage as compared with normal sheep. In those sheep receiving both an allograft and immunosuppressive drugs there appears to be a reduction in the percentage of trabsformed cells in the thoracic duct lymph during the first week, compared with sheep with an unsuppressed rejecting allograft, but not during the second week after transplantation. In these experiments we were unable to obtain results for the number of transformed cells in the renal lymph of immunosuppressed sheep.

The mode of action of combined immunosuppressive therapy does not appear to be upon DNA synthesis and cell division at the time of allograft rejection.

G. CONCLUSION

There is a large number of white cells (mainly lymphocytes) in a renal allograft and flowing through an allograft at the time of rejection. This is specific to the kidney and not reflected in an increase in total body lymphocyte flow. The daily renal lymphocyte flow however is considerably less than the total body flow and thus a local increase in the area of rejection does not alter the massive body circulating lymphocyte pool.

The large number of lymphocytes draining from the kidney correlate with the degree of rejection. Immunosuppressive drugs have been shown to decrease the severity of rejection and also the renal lymphocyte flow. The combination of immunosuppressive drugs is more effective than prednisolone alone.

The percentage of transformed cells in total lymphocyte flow is increased at the time of rejection of a renal allograft. This is to be expected as there is a known increase in DNA and RNA synthesis in animals and patients at the time of rejection as measured by ^{3}H thymidine uptake by the peripheral blood lymphocytes. The percentage of transformed cells after seven days thoracic duct drainage did not increase in sheep in which the thoracic duct alone was cannulated, yet unexpectedly there was an increase in sheep receiving immunosuppressive drugs. This is not fully explained but may be due to the increase in sepsis in the immunosuppressed sheep.

It would appear however that the mode of action of combined immunosuppression is not upon DNA synthesis and cell division at the time of rejection. Immunosuppressive drugs may possibly work mainly by diminishing lymphocyte flow through a transplanted organ.

APPENDIX H Difficulties in methods performed

Thoracic duct cannulation

There was a total of 74 sheep which had thoracic duct cannulations. As shown in the results 47 of these were successful, and there were also 27 other sheep in which the thoracic duct cannulation failed for various reasons, ranging from death to blockage of cannula, most of which occurred during the earlier part of the study.

Causes of failure of thoracic duct cannulation and drainage

Operative deaths	3
Early deaths within four days	8
Inability to cannulate	5
Cannula pulled out	3
Cannula blocked	7
Lymph leak	1

The causes of the operative and early deaths were not known, possibly some were due to infection or occult haemorrhage. The adequate fixation of the cannula (see Materials and Methods) prevented the continuation of the complication of the cannula being pulled out by the sheep. Blocking of the cannula was prevented by increasing the amount of intravenous fluid infused each day to ensure an adequate flow of lymph.

There were also a few post-operative complications which occurred in the 47 successful cannulations.

Post-operative complications

Sepsis (bacteraemia with pseudomonas aeroginosa)	2
Haemorrhage	1
Pneumonia	2
Severe dehydration	2
Bilateral obstruction of the external jugular vein	2

There were no wound infections.

Renal lymphatic cannulation

The lymphatics draining a renal transplant have been difficult to cannulate, owing to their small size and the tendency of the cannula to block. There have been 18 unsuccessful attempts due to inability to cannulate the lymphatic, blockage or displacement of the cannulae. Three attempts to collect lymph for five days from renal autografts failed due to the markedly reduced lymph flow, maximum 20 mls. per day.

Renal transplants

Forty-eight renal transplants have been performed. The kidneys became revascularised with arterial blood after the clamps were released from the carotid vessels. Initially there were occasions where the carotid or renal artery went into spasm, not at the anastomosis site but on either side of the anastomosis. The vessels were reopened and there was no evidence of intimal damage which could give rise to such narrowing. This complication disappeared after lignocaine was added to the perfusion fluid.

Among the first 18 transplants in all groups of sheep, there was a high number of ischaemic kidneys even though the blood supply was

satisfactory at the time of closure of the wound. Fourteen kidneys became necrotic very rapidly (within 7 days). The blood supply was satisfactory on the seventh day in 4 kidneys. Further sheep were therefore anticoagulated with 20,000 units of heparin daily, intravenously, and this complication was thereafter markedly reduced. A further 30 kidneys were transplanted into anticoagulated sheep, on the seventh day only 5 of these were considered on histology to be ischaemic, owing to arterial occlusion.

Transformed cell counts

The number of specimens of thoracic duct lymph from sheep receiving immunosuppression and an allograft in which the percentage of transformed cells was measured, was considerably lower than the number of successful cannulations in this group. The large number of bacteria in the lymph in a number of sheep from this group made identification of the transformed cells difficult. The renal lymph of sheep on immunosuppression also contained bacteria and no satisfactory transformed cell counts were obtained in these sheep after the second post-operative day.

CONCLUSION

The action of lymphocytes on both tumour and allografted tissue has been studied previously by many workers both by in vivo and in vitro tests. The ability of lymphocytes immunised against a tumour to specifically kill the tumour cells is well proven in animal experiments. With lymphocytes sensitised against transplantation antigens cytotoxicity has been claimed; however much of the previous work has been done using tumour grafts; these tumour cells are more easily killed than are cells derived from organ grafts.

Here using microcytotoxicity tests, in certain circumstances, sensitised lymphocytes reduced the number of remaining target cells in comparison with non sensitised lymphocytes, and we thus presume to have achieved in vitro killing by lymphocytes. This circumstance was found with hyperimmunization, where excellent cytotoxicity was achieved with spleen cells against the tissue cells of the donor; this fact comfirms that the technical basis of the experiments reported here is sound. Cytotoxicity has also been obtained in half the experiments where tumour allografts were performed, and the sensitised lymphocytes of the recipient tested against both donor tumour and tissue cells.

The limiting factors in obtaining cytotoxicity appeared to be (i) the use of skin grafting as a method of primary sensitization and (ii) the use of lymph node lymphocytes, rather than spleen cells, as effector cells. The fractionation of lymph node lymphocytes to obtain enriched population gave no greater cytotoxicity.

The cell counting technique (microcytotoxicity testing) proved to be more effective than the chromium release assay method for the determination of cytotoxicity, although other workers have found

this latter method to be more superior. Possibly the technique, culture conditions and the ratio of effector lymphocytes to targets cells may have been incorrect here.

In vitro cytotoxicity tests designed to measure the capacity of lymphocytes, sensitised in vitro, to kill either syngeneic tumour, allogeneic tumour or allogeneic tissue would appear to be limited. These tests do not reproduce the massive lymphocyte effect against allografts which is seen in vivo and they have at present no value in the measurement of the lymphocyte response against an allograft (for instance in the clinical situation for the prediction of rejection episodes).

The graft-versus-host popliteal node weight assay has theoretical advantages for the demonstration of the immune response, over in vitro lymphocyte cytotoxicity tests, in that the activity of the lymphocyte against antigenic tissue is observed in an in vivo situation, and thus incorporates features missing in vitro - such as tissue fixity, lymphokine activity, and recruitment of non specific lymphocytes and other cells. In allogeneic and xenogeneic experiments when donor lymphocytes are injected into non identical hosts the increase in node weight is mainly a host-versus-graft response. This in many experiments has been abolished by host irradiation. Even in the true graft-versus-host reaction between parent and F_1 hybrid rats there has been a suggested host component in the increase in node weight. This was not seen in the present experiments.

If the graft-versus-host popliteal node weight assay is to be used as a measurement of the allograft response then the observed action of the lymphocyte in vivo must be mirrored in this assay.

There should be a decrease in the lymphocyte activity on immunosuppressive drugs and an increase in lymphocyte activity during rejection of an allograft. Prior sensitization of lymphocytes by allografting did not however give an enhanced g-v-h response on injection to allogeneic rats. Immunosuppression of the lymphocytes by taking them from animals on immunosuppressive drugs appeared to slightly diminish the g-v-h response. The criteria mentioned above have therefore not been met by this assay. This assay does not measure the cytotoxic activity of the lymphocyte, but the ability of the lymphocyte to divide in the presence of a foreign stimulus and these actions are not the same.

The hypothesis that the g-v-h assay might be used as a test of immune competence of the donor cell in the clinical situation has not been confirmed.

The activity of the lymphocyte in the transplantation reaction lies in both its abilities to kill antigeneic cells and to proliferate in the face of an antigen. The effectiveness of each individual lymphocyte may be multiplied by an enormous increase in the concentration of lymphocytes at the graft site following transplantation. Indeed here a massive increase in the number of white cells (mainly lymphocytes) flowing through the graft has been shown to occur rapidly after grafting, when only early changes of rejection have appeared on histological examination. This increase in the lymphocyte flow appeared to occur only through the graft itself, as there is no alteration in the flow of lymphocytes through the thoracic duct.

Immunosuppressive drugs used in transplantation reduces the number of lymphocytes flowing from the graft, and reduces the severity of rejection. This action is specific to the renal lymphocyte flow, and is not reflected in a decrease in the number of lymphocytes flowing through the whole body. As might be expected the combination of azathioprine and prednisolone is more effective, than prednisolone alone, both in abrogating rejection, and in reducing the renal lymphocyte flow.

The percentage of transformed cells increased in the thoracic duct lymph over a 7 day period of drainage in sheep, both in immunosuppressed and in animals after an allograft, as compared with normal sheep. Other workers have found a rise in the percentage of transformed cells in the lymph during several days drainage of the thoracic duct and where no other procedure has been performed; this has not been confirmed in this work. The number of transformed cells also increased in the renal lymph from an allograft. Some infection was the rule in the immunosuppressed sheep, despite prophylactic antibiotics, and this may be the explanation for the increase in transformed cells in the thoracic duct lymph in these animals.

The activity of the lymphocyte is therefore shown to be concentrated in the area of the kidney after a renal allograft. This increase in activity is due to an increase in the number of lymphocytes in the graft and passing from the graft. This increase in lymphocytes may be initiated primarily in the kidney by the production of a lymphokine, or may be primarily a response from the whole body as the increase in the number of lymphocytes flowing

from the graft precedes the appearance of marked rejection changes in the renal histology.

One of the major actions of immunosuppressive drugs may be their ability to reduce the number of lymphocytes flowing through the transplanted organ.

GLOSSARY

Allograft - graft exchanged between two genetically dissimilar individuals of the same species (allogeneic - genetically dissimilar within the same species).

Autologous - derived from self.

Clone - a family of cells derived from a single ancestor. All the cells of a clone are genetically identical.

E rosettes - erythrocyte rosettes around T lymphocytes.

EA " - erythrocyte and antibody rosettes to detect B lymphocytes.

EAC " - erythrocyte, antibody and complement rosettes to detect macrophages and certain B lymphocytes.

F_1 hybrid - animal produced from parents of animals genetically dissimilar, first generation.

Lympholysis - destruction of a lymphocyte by disruption of the cell membrane.

Mitogens - substances which induce mitosis, i.e. the division of cells, e.g. phytohaemaglutinin (P.H.A.)

Passage - the successive transfer of tumour from one animal to another, in series.

Syngeneic - genetically identical animals, usually applied to grafts made within an inbred strain.

Xenogeneic - derived from different species.

EQUIPMENT LIST

1. Tissue Culture Techniques and Microcytotoxicity Tests

Anaesthetic Agents

Ether Anaesthetic (Macfarlane Smith Ltd., Edinburgh, Scotland)
Nembutal Veterinary 60 mgs/ml. (Abbott Laboratories, Queensborough, England)

Antibiotics

Gentamycin - Cidomysin Injectable 40,000/ml. (Rousell Laboratories Ltd., London, England)
Penicillin - Solupen, Benzyl Penicillin B.P. (Dista Products, Liverpool, England)
Streptomycin - Streptaquaine, Streptomycin Sulphate (Dista Products)
Terramycin (Pfizer Ltd., Sandwich, England)

Counters, Centrifuges and Microscopes

Centrifuge M.S.E. minor (M.S.E., Crawley, Sussex, England)
β counter - Backmann L.S. - 100 Liquid scintillation system (Beckmann, Croydon, England)
γ counter - Wallac 80,000 sample counter (L.K.B./Wallac, Sweden)
Cell counting microscope - Olympus model SZ11 (Gallenkamp, Birmingham, England)
Operating microscope - Nikon no. SMZ-6 (The Projectina Co. Ltd., Skelmorlie, Ayrshire, Scotland)
Constant infusion pump - Palmer (Analytical supplies, Derby, England)

Drugs (other)

Dextran 40 in saline - Lomodex (Fisons Ltd., Loughborough, England)
Heparin (with and without chlorocresol) (Weddel Pharmaceuticals Ltd., London, England)
Papaverine 40 mgs/ml. (Macarthys, Romford, Essex, England)

Enzymes and Additives

Bicarbonate 4.4% (Wellcome Research Laboratories, Beckenham, England)
Dnase (Sigma, Kingston upon Thames, England)
Foetal Calf Serum inactivated by heat (FCS) (Flow Laboratories Ltd., Irvine, Scotland)
5-fluorodeoxyuridine (Sigma)
Glutamine (Flow)
Hepes Buffer I.M. solution (Wellcome)
Trypsin (Difco Laboratories, West Molesly, Surrey, England)

Immunosuppressive Agents

Cortisone Acetate Injection B.P.; Cortistab (Boots Co. Ltd., Nottingham, England)
Cyclophosphamide - Endoxana (W. B. Pharmaceuticals Ltd., London, England)

Plastics etc.

Small tubes: clinical test tubes with rim 5.0 x 9.5 mm. (G.W.S.)
Microtest plates Type II (Dynatech Laboratories, Billinghurst, England)
Millex filters - 0.22 µm (Millipore (U.K.) Ltd., London, England)
Sterilin universal containers (Sterilin Ltd., Teddington, Middlesex, England)
Tissue Culture Bottles (Nunc U.K. Ltd., Stafford, England)

Radioactive Materials

Chromium ^{51})
5 I ^{125} Iodo 2 deoxyuridine) (The Radiochemical Centre, Amersham, England)
Thymidine 6 H_3)

Reagents

Azide, NaN_3 (B.D.H. Chemicals Ltd., Atherstone, Warwickshire, England)
Crystal violet - Revector microscopical stain (Hopkin and Williams, Chadwell Heath, Essex, England)
Ficoll 500 g. (Pharmácia, Upsalla, Sweden)
Glycerol - Analar (B.D.H.)
Methanol - Analar (B.D.H.)
Triosil 400 mgs/ml. (Nyegaard & Co., Oslo, Norway)
Trypan blue (B.D.H.)

Sutures etc.

Leucopak Leucocyte Filters (Fenwal Laboratories, Illinois, U.S.A.)
Disposable needles - Gillette scimiter (Gillette, Isleworth, Middlesex, England)
Scoville Lewis microvascular clips (Down Bros., Mitcham, Surrey, England)
Sutures - ethilon, silk (Ethicon, Edinburgh, Scotland)
Syringes (Sherwood Medical Industries, St. Louis, U.S.A.)
Tubing - silastic 0.5 mm. external diameter (Dow Corning Corp., Midland, Michigan, U.S.A.)

Tissue Culture Media

Eagles M.E.M. x 10 strength (Wellcome)
Hanks (Oxoid Ltd., London, England)
Phosphate Buffered Saline (Flow)
R.P.M.I. (Flow)
Waymouths without L-glutamine (Gibco Biocult Laboratories Ltd., Paisley, Scotland)
Glutamine (1 ml.) 200 mM. added to 100 mls. of Eagles and Waymouths media
Hepes buffer added to Eagles at a final concentration of 20 mM.

2. Lymphatic Cannulation and Renal Transplantation in Sheep

Anaesthetic Agents

Fluothane - Halothane B.P. (Imperial Chemical Industries I.C.I. Ltd., Macclesfield, England)
Nembutal Veterinary (Abbott Laboratories, Queensborough, England)

Antibiotics

Penicillin - Depocillin, Procaine benzyl penicillin (Mycofarm, Delft, Holland)
Streptomycin - Stryzolin, Streptomycin Sulphate (May and Baker, Dagenham, England)
Terramycin (Pzifer Ltd.)

Catheters etc.

1. Cannulae
   for thoracic duct cannulation:- Trocaflex 0.8 x 1.2 mm. diameter, 30 cms. length (Vygon U.K. Ltd., Uxbridge England)
   for renal lymphatic cannulation:- external diameter 0.75 mm. (Portex, Hythe, England)
   for intravenous and ureteric cannulation:- external diameter 1.65 mm. size 10 (Portex)
2. Collecting bag - Aldron urine drainage bag (Ashford, Kent, England)
3. Connection tubing - 60 cms. manometer line (Portex)
4. Netelast (Roussel, London, England)
5. Spiral cable wrapping bore (R.S. Components, London, England)

Other drugs

Heparin - Mucous B.P. (Weddel Pharmaceuticals Ltd.)
Pentazocine - Fortral (Winthrop, Surbiton-on-Thames, England)
Potassium Chloride 1 G. in 5 mls. (Macarthys Ltd.)

Immunosuppressive Agents

Azathioprine - Imuran (Wellcome)
Methylprednisolone _ Solumedrone (Upjohn Ltd., Crawley, England)
Prednisolone - oral - (Roussel, Wembley Park, Middlesex, England)

Intravenous Infusion Fluids

Aminosol Vitrium 10% (KabiVitrum Ltd., Ealing, England)
Dextran 70 Injection B.P. in 0.9% Sodium Chloride (Fisons Ltd.)
Mannitol 10% - 500 mls. Baxter (Travenol Laboratories, Thetford, Norfolk, England)

Intravenous Infusion Fluids (contd.)

Perfudex 5% - Dextran 40 for organ preservation (Pharmacia (G.B.) Ltd., London, England)
Sodium Chloride - 500 mls. Baxter (Travenol Laboratories)

Miscellaneous

Acridine orange (B.D.H.)
Chlorhexidine 0.5% in 70 spirit (Nottingham General Hospital Pharmacy)
Fluorochrome conjugated animal antiglobulin (Rash/FITC) (Sera Service, Maidenhead, England)
Hibispray: clear plastic dressing (Avlex, Wigan, England)
Nobecutane (B.D.H.)
P.P.O. 100 g. (N.E.N. Chemicals, Frankfurt, West Germany)
Savlon (I.C.I.)
Toluene (Koch Light Laboratories, Coinbrook, Bucks, England)
Trucut biopsy needle (Travenol)

Food for rats

41B DIET Rat and mouse cake

As supplied by Pilsbury's Ltd. (Edgbaston, Birmingham)

Barley)	
)	47%
Wheat)	
Oats	40%
Dried skim milk powder	3%
Dried yeast	1%
Mineral supplement	1%
Vitamin supplement	1%
English white fish meal	8%
Molasses to bind	

Food for sheep

Pregnant Ewe nut (made at Department of Agriculture, Sutton Bonington)

10 cwt.	56lbs.	Barley
7 cwt.		Oats
2 cwt.		Grass meal
	56lbs.	Neutranol
	56lbs.	Vitamins
20 cwt.	56lbs.	

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