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STUDIES IN XENOGENEIC GRAFT-VERSUS-HOST REACTIONS

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Human and murine immunocompetent cells when injected under the kidney capsule of Cyclophosphamide pretreated rats initiate a localized graft-versus-host reaction (GVHR). The cardinal feature of this reaction is the formation of tumorous mass of proliferating donor mononuclear cells that invade and destroy the host renal cortex. This xenogeneic graft-versus-host reaction (XGVHR) is specifically inhibited by antilymphocyte sera (ALS) raised against the donor lymphoid cells. The degree of inhibition of ALS is proportional to the number of donor lymphocytes injected as well as the amount of antilymphocyte serum given to each recipient. In addition, it was found that the capacity of antilymphocyte sera to inhibit this XGVHR correlated with their ability to inhibit the rejection of skin allografts. In other words, the capacity of ALS to inhibit this XGVHR does correlate with their immunosuppressive potency. Xenogeneic graft-versus-host reactions produced by human lymphocytes in rats are used as a basis for assaying the immunosuppressive potency of anti-human lymphocyte sera prior to their clinical use.

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by

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ORIGINAL CONTRIBUTIONS.

1. A graft versus-host reaction given by human immunocompetent cells in rats has been developed. i. e. graft-versus-host reaction in a xenogeneic combination.
2. This xenogeneic graft-versus-host reaction is used to assay the immunosuppressive potency of anti-human lymphocyte sera.
3. Immunocompetent cells from mice, dogs, rabbits and humans have been shown to be capable of invading and destroying the kidneys of Cyclophosphamide pretreated rats.
4. Antilymphocyte serum was most effective in inhibiting the graft-versus-host reaction when given to the host one day after the transfer of immunocompetent cells.
5. No correlation has been shown to exist between the capacity of anti-mouse lymphocyte serum to inhibit reactions produced by mouse cells and its titers of lymphocyte agglutinins and cytotoxins.

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Doctor J. Gordon closely supervised this project. All experiments were carried out in his laboratory and were discussed with him in detail prior to their performance. His contributions superpass that of any other contributor and were essential for the progress of this work.

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CHAPTER I

GRAFT VERSUS HOST REACTIONS

Murphy[1] grafted chick embryos on their seventh day of incubation with adult chicken spleen cells, and examined them on the eighteenth day. He observed round cell infiltrates in several tissues as well as marked splenomegaly.

Danchakoff[2] described in detail the histological changes in these spleens, but it seems that it did not occur to these investigators that round cell infiltrates were manifestations of the reaction of the graft against the host.

The concept of the graft reacting against its host was independently formulated by Dempster[3] and Simonsen[4] in 1953. These two pioneers while working on canine renal homotransplantation observed independently, a perivascular and periglomerular accumulation of mononuclear cells, comprising of lymphocytes and pyroninophilic cells in the interstitium of the grafted kidneys.

These cellular foci were observed as early as three days after transplantation, i. e. a few days prior to the usual infiltration by host mononuclear cells. When Simonsen removed the renal allograft after three-four days in the host and replanted it in the original donor, a very spectacular proliferation of the mononuclear cells occurred in the renal interstitium.

Simonsen at that time thought that this proliferation of the mononuclear cells in the original donor is consistant with the idea of continued proliferation of the donor kidney endogenous cells.

Since the pyroninophilic cells [23,24] were known to be antibody forming cells, Simonsen thought that the endogenous mononuclear cells of the kidney were reacting against the host.

Dempster [3] observed that irradiating the kidney prior to its grafting reduced the pyroninophilic reaction in the interstitium of the grafted kidney.

These observations by Simonsen and Dempster on the graft-versus-host hypothesis in renal homotransplantation were not confirmed by other investigators [5,6].

The graft-versus-host hypothesis gained a new momentum in 1957 when Simonsen [7] observed in chicken and in mice that adult spleen cells when grafted to embryos or neonates produce a disease which could be explained on the basis of grafts reacting against their hosts which were thought to be too immature to reject the grafted lymphoid cells, i. e. to defend themselves against the graft.

In the same year Billingham and Brent [8-10] found that the injection of adult lymphoid cells into new-born mice produced dwarfism, diarrhoea and death. They coined the term "Runt Disease" for this syndrome and concluded that a reaction of the graft against the host

is the most likely explanation for it. They also observed that the severity of the disease varies depending on the strain combination.

These observations created great interest amongst many investigators so that by 1962 voluminous literature accumulated on the phenomena of graft-versus-host reaction in fowl, rodents, animals and man. Several forms and combinations were unraveled. Simonsen[11] wrote a monumental review of the subject of graft-versus-host reaction which is the basic reference for workers who use graft-versus-host reactions as a tool for their research in the field of transplantation biology and immunology.

In this thesis no attempt is made to review the various aspects of graft-versus-host reactions, since this has already been excellently done by Simonsen.

Only the salient features which have a bearing on the presentation of this thesis will be reviewed in brief. For this reason only graft-versus-host reactions in rodents are summarized and attention is then focused on the localized form of graft-versus-host reaction, widely known as the Elkins form[12] of graft-versus-host reaction.

The pathological changes of graft-versus-host reaction in rodents: -

The outward manifestations of "Runting" so accurately described by Billingham are associated with changes in the internal organs that are more striking in the lymphatic and hemopoetic systems.

These pathological changes that occur in organs of "Runted" birds, rodents and other animals are used as criteria of assay for the graft-versus-host reaction, i. e. failure to gain weight, mortality, splenomegaly, hepatomegaly, dermatitis, depending on the model of graft-versus-host reaction used.

Simonsen[7,13] found that splenomegaly occurred in mice as well as in chickens. Splenomegaly reaches a peak in about eight to ten days after grafting and gradually subsides after that time. There may be hyperplasia of the germinal centres or disruption of the architecture. The increase in spleen weight is very frequently used by various investigators[14,15] to assay the severity of graft-versus-host reaction. Billingham found that the lymph nodes undergo changes similar to those of the spleen. He also observed that the lymph nodes may undergo extreme atrophy late in the disease.

Van Bekkum[16] observed that the thymus also undergoes reduction in weight concommitant with splenic enlargement.

Siskind and Thomas[17] directed attention to the frequent occurrance of widespread necrotic lesions in the livers of mice dying of "Runt Disease".

Howard[18], Gorer and Boyse[19] noted the accumulation of proliferating cells, mononuclear and pyroninophilic, in the periportal areas of the liver. These cells appeared to be the same type that they described in the enlarged spleens. They also found extensive cellular infiltration in the omentum and pancreas.

Simonsen[20,21] and Howard[18] found that hepatomegaly roughly follows splenomegaly but is less pronounced.

Billingham[14] noted that exfoliation of the skin is the initial manifestation of "Runt Disease". It begins around the urogenital and anal orifices and soon extends to the entire body surface. This is accompanied by the thickening and loss of elasticity, ruffled hair which grows slowly when clipped.

Billingham also described the profound blood and bone marrow changes, such as anemia, anisocytosis, poikilo cytosis and basophilia, leucocytosis and relative lymphopenia.

In mice Oliver[22] and his associates found that the most consistant hematological abnoramlity was leucopenia and lymphopenia.

The experimental evidence that "Runt Disease" is immunological in nature and is caused by the reaction of innoculated mature immunologically competent cells against the foreign transplantation antigens in their new host is briefly reviewed.

Simonsen[7,13] injected new-born inbred mice with adult spleen cells from the same inbred strain and found no splenomegaly or other signs of "Runting". But when he injected adult spleen cells from allogenic strain, splenomegaly occurred. He concluded that the maturity of the donor was not sufficient to cause splenomegaly in immature recepient mice. A genetical, hence also antigenic difference is needed

as well.

This antigenic difference was studied by Billingham and Brent[10]. They found that adult spleen cells from parental strain A mice produced "Runt Disease" in almost all (AuxA)F_1 hybrid mice that were injected as neonates with the parental cells.

But when they injected A strain new-born mice with spleen cells of adult (AuxA)F_1 hybrid mice no "Runting" occurred.

These observations were confirmed in rodents and other animals by several investigators and also applied to fowl[25].

Billingham and Brent[10] thus demonstrated that these reactions resulted from an immunological attack by the lymphoid graft against histocompatibility antigens of the cells of the host. When grafting lymphoid cells from a parent to F_1 hybrid, the parental graft reacted against those histocompatibility antigens of the host that were inherited from the other parent. No "Runting" occurred on grafting lymphoid cells from adult F_1 hybrid lymphoid cells into parental strain neonates.

Simonsen[11, 21] had predicted and confirmed that "One of the most obvious immunological predictions to be made from graft-versus-host hypothesis is that donor cells will cause a more violent reaction than normal cells of their own kind if they have been previously sensitized to the foreign antigens of the prospective host".

Simonsen and Jensen[21] studied in the same litters of infant

(C_3HxAKR) F_1 hybrid mice the effect on spleen weights produced by injection of $1x10^6$ adult spleen cells from normal AKR donors and from other AKR donors which had been previously immunized with (C_3HxAKR) F_1 tissue. The five test litters gave no splenomegaly with normal AKR cells but a doubling of spleen weights with pre-immunized AKR cells.

Billingham and Brent[10] injected new-borns of A strain mice with four to ten million spleen cells from adult normal CBA donors and adult CBA who were previously immunized with A cells. They found that the mortality rate from "Runting" increased from 51% to 95% when equal doses of cells from immunized donors were used. Thus pre-sensitized lymphoid cells produce more violent reactions than normal cells. Conversely cells made tolerant to a host antigen loose their capacity to produce a graft-versus-host reaction when injected in that host. Michie, Woodruff and Zeiss[26] found that spleen lymphoid cells from adult strain A mice, which had been rendered tolerant to CBA antigens by neonatal injection, were incapable of producing a graft-versus-host reaction when injected into new-born CBA mice.

Inactivating the donor immunocompetent cells by irradiation, cytotoxic drugs or specific antisera inhibits the development of graft-versus-host reactions. This point is discussed in detail in this thesis.

In mice Simonsen[11] found that lethal total body irradiation of mice immediately before their spleens were used for injection into infant F_1 hybrid mice, entirely abolished their capacity to produce graft-versus-host reactions. Damaging the grafted cells by irradiation inhibited the initiation of graft-versus-host reactions. Howard, Michie and Simonsen also found that irradiation of (AxC47bl) F_1 hosts prior to grafting of parental cells clearly inhibited the production of splenomegaly that is usually produced by grafting adult A strain spleen cells.

This observation confirmed the impression of Davies and Doak[28] that a substantial part of the spleen enlargement is due to host cell multiplication.

IMMUNOLOGICAL PREVENTION OF RUNT DISEASE.

1. Siskind and his associates[17, 17a, 29] injected eight to twelve million isologous adult spleen cells half an hour after the intraperitoneal injection of an equal number of allogeneic spleen cells. They found that the mortality from "Runting" dropped from 100% to 38% in the group treated with isologous spleen cells. Isologous adult liver cells provided no protection against "Runting". They also found that if the isologous adult spleen cells were injected one day after the adult allogeneic cells no protection occurred and 95% of the neonate mice died of "Runting". These observations implied that the isologous adult spleen cells protected

the new-born mice from the "Runting" effect of the allogeneic cells, presumably by destroying the allogeneic graft. This was confirmed by Russell [30, 31, 32] who in addition found that better protection from "Runting" could be achieved with isologous spleen cells from mice which had been immunized in advance with the skin grafts from the allogeneic donor strain.

2. Russell [31] was the first to observe that the administration of A-methopterin to neonatally grafted C47 bl mice over the first five days of life can reduce the mortality from "Runting" to about 50% as opposed to 100% of grafted controls.

Santos and Owen [33, 34] found that other cytotoxic drugs such as methotrexate and cyclophosphamide do inhibit the development of graft-versus-host reactions in rats. More important is their observation that hybrid adult mice injected with parental, allogeneic or xenogeneic immunocompetent cells following a preliminary injection of cyclophosphamide will develop a graft-versus-host reaction. The use of cyclophosphamide to make possible the development of graft-versus-host reactions in a xenogeneic combination in rats is the basis of experiments reported in this thesis.

Siskind and his colleagues [29] found in the C57 black/6 DBA/2 combination that passive transfer of serum from adult DBA/2 mice, which had been immunized against the donor strain tissue, could largely

abrogate the attack by the grafted cells. This anti-serum was given as a daily intraperitoneal injection in increasing dosages from days two to nine after grafting. Detailed review of the effect of antilymphocyte sera on graft-versus-host reaction is given in Chapters IIII and V.

CHAPTER II

THE ROLE OF LYMPHOCYTES IN THE REJECTION OF ALLOGRAFTS AND IN GRAFT-VERSUS-HOST REACTIONS.

Several investigators[35, 36, 38] had observed the presence of heavy infiltrates of lymphocytes on histological examination of rejecting grafts.

Da Fano[36] is credited to be the first to suggest that the lymphocyte may be important in rejection of foreign tissues.

Ehrlich[40] observed that a mouse tumor when transplanted into the rat would grow for about eight days then die.

Bashford[41] and his associates found that once a rat destroyed a mouse tumor that had survived for eight days, a second mouse tumor was destroyed in three or four days. They postulated that the rat had developed an immunity to mouse tumors as a result of the first transplant and became able to destroy the second transplant more rapidly.

Schone[42] coined the term transplantation immunitat, to point out that the failure of allografts is due to some form of immune response.

Murphy[37, 38, 39] also observed the presence of heavy infiltrates of lymphocytes in the rejecting grafts. He found that rat sarcoma can grow in chick embryos and that this growth could be inhibited by the grafting of adult chicken spleen or bone marrow cells into the same embryo. He also found that other tissues of adult fowl did not impede

the tumor growth. He observed that the histological changes in the tumors grafted into adults are similar to those in the embryos grafted with adult fowl lymphoid cells. He noticed that in one or two days prior to hatching, established tumor grafts began to degenerate and new tumor grafts did not take.

He concluded that the defence mechanism responsible for the destruction of the transplant was contained in the small lymphocytes. He tried to modify the rejection process by interference with the function of lymphocytes by radiation.

Murphy irradiated adult animals and found a much greater incidence of takes of mouse sarcoma in rats and mouse mammary adenocarcinoma in mice. While doing his experiments he observed splenomegaly and round cell infiltration of various organs in a chick embryo that was grafted on the seventh day of incubation with adult chicken spleen. He probably was unaware that this manifestation of what is now known as a graft-versus-host reaction.

Emile Holman [43], a clinical surgeon, is credited for suggesting the immunological basis for skin allograft rejection in burned patients. He skin grafted a twenty-eight month old child with extensive burns of the face and body. The skin grafts were taken from three different donors and were applied to the child at various intervals. He observed their rejection and deduced that "the agency which caused the first

homografts to disappear had no effect on the viability of the grafts from the third donor, and that the destroying agency is specific for each set of grafts. It seemed plausible to suppose, therefore that each group of grafts develops its own antibody which is responsible for the subsequent disappearance of the new epidermis".

Between 1925 and 1945 it gradually became evident[43a] that transplanted tissue must contain antigens which arouse an immune response in the new host that throws off the graft as rejection.

In 1942 and 1943 Gibson and Medawar[44] studied the fate of skin grafts on burned patients. They confirmed the observations of Holman and in addition they described the immunologic phenomenon of "memory" in homografting, i. e. the "second set" of grafts. They confirmed the Bashford[41] observation on tumor grafts.

In 1944 Medawar[45,46] studied this phenomena, using skin grafts in rabbits and he concluded that"The mechanism by which foreign skin is eliminated belongs to the general category of actively acquired immune reactions".

Converse and Rapaport[47] found that the first signs of rejection of the homograft appear in the deeper layer of the graft, in the form of perivascular lymphocyte infiltrates adjacent to the sweat glands. This lymphocytic infiltrate progressively intensifies and on about the ninth day or later the surrounded vessels thrombose and rejection of skin

grafts becomes rapid.

The observations of Medawar on skin graft rejection as well as the observations of Converse and Rapaport have been confirmed by several workers [48, 49, 3, 50] and have been shown to occur also in vascularized organ allografts. In fact it was during the study of the role of lymphocytes in canine renal allograft rejection that the concept of graft-versus-host reaction evolved. [3, 4]

This work of Medawar on skin allograft rejection was culminated by the discovery in 1953 by Billingham, Brent and Medawar [51], that the homograft rejection in experimental animals could be prevented by injection of recipients during their foetal or neonatal life with cells from prospective donors. The recipients, actively acquire tolerance to the donor tissues. Thus Billingham, Brent and Medawar has shown that the homograft barrier could be abolished by immunologic manipulation of the host during its early life.

Billingham, Brent and Medawar [52] further demonstrated that long surviving skin allografts on mice made tolerant by the neonatal injection of cells can be made to break down by injecting the tolerant recipients with normal lymph node cells from syngeneic mice. This observation established the key point that the deficiency of the tolerant animals lies in their lymphoid tissues.

They also demonstrated that a lymph node and spleen cell

suspension from syngeneic mice can prevent wasting disease and restore lymphocyte depletion and immune mechanisms when injected into neonatally thymectomized mice.

Miller [67,68] found that early removal of the thymus in mice delays or prevents allograft rejection. This role of the thymus in transplantation immunology was confirmed by Good [69,70,71] and his associates, in rabbits and mice. Waksman [72,73,74] and his associates showed that neonatal thymectomy in rats also impairs other cell mediated reactions such as delayed hypersensitivity.

Since neonatal thymectomy cause lymphopenia and atrophy of the lymphoid tissues, it is believed that the thymus is the regulator of the immunologically competent cells. This term "immunocompetent cell" is used according to the definition given by Medawar and Billingham [57, 12] and means those cells that are capable of initiating a graft-versus-host reaction and that can restore immunological competence to animals deprived of it whether through induced immunological tolerance, irradiation, cytotoxic drugs or neonatal thymectomy.

Miller [76,79] found that a suspension containing five million lymph nodes or spleen cells was effective in giving protection when given into recipients of the same strain within a week of neonatal thymectomy. Wasting disease that usually follows neonatal thymectomy was prevented in the majority of injected neonatally thymectomized C47bl mice. The

survivors were capable of rejecting allogeneic skin grafts, and of developing delayed hypersensitivity reactions. The same situation was also found in the rat [79a]. The immunological function of the thymus has been recently reviewed by Miller and Osoba [79]. There is now abundant evidence [69, 79, 80] that cellular immunity is mediated by lymphoid cells rendered immunocompetent under the influence of the thymus. A striking feature of all cellular immune reactions is the high rate of proliferation [11, 19, 56] of lymphoid cells participating in the process.

Following thymectomy, Miller [76] found a low index of mitotic activity in the lymphoid tissues in mice.

Good [81] and his associates found that neonatal thymectomy impairs the ability of rat lymphocytes to proliferate in vitro in response to phytohemagglutinin, pokeweed mitogen and allogenic lymphocytes treated with mitomycin C. They concluded that decreased lymphoid cell proliferation contributes to impaired cellular immunity found in the post thymectomy state.

Gowans and his associates reported in a series of papers [53-58] that chronic drainage of thoracic duct in rats produce a specific depletion of small lymphocytes. This lymphocyte depletion was associated with inhibition of skin homograft rejection. Permanent survival of some skin grafts occurred when the genetic disparity between donor

and recipient was small but skin grafts exchanged between distantly related strains survived only a few days longer than the controls.

This immunologic deficit following thoracic duct depletion could be reversed by reconstitution with syngeneic lymphocytes.[58]

Gowans, Gesner and McGregor[59] found that the injection of thoracic duct lymphocytes can break down a long standing skin allograft in tolerant rats.

Gesner and Gowans[60] demonstrated that rat thoracic duct lymphocytes can produce a graft-versus-host reaction in F1 hybrid rats, in lethally irradiated mice and can destroy a graft of insologous mouse bone marrow used to protect mice from lethal irradiation.

The use by Gowans and his associates[55, 58, 60] of inocula in which the proportion of large lymphocytes in the thoracic duct lymph had been greatly reduced provided strong evidence that the small lymphocyte was the effective cell type.

Wilson[61] sensitized donor rats with homografts of skin and nine days later added their thoracic duct cells to a culture of the donor kidney cells. Most of the kidney cells were destroyed after incubation for forty-eight hours, but the majority of the lymphocytes were still alive. The cytocidal effect did not require the presence of complement or immune serum and was associated with a clustering of lymphocytes around the target cells.[62] Wilson confirmed the observation of Govaerts

that thoracic duct lymphocytes from sensitized donors can kill target cells in vitro.

Gowans and his associates [55, 56, 57, 58] found that parental thoracic duct lymphocytes when injected intravenously into adult normal F_1 hybrid rats produce a lethal graft-versus-host reaction. They [60] demonstrated that this fatal reaction is initiated by the small lymphocytes in the parental lymphoid inoculum. They noted that during the first twenty-four hours in the host some of these small lymphocytes develop into large pyroninophilic cells which proliferate rapidly.

The work of Gowans and his colleagues have confirmed the initial impression of Billingham [14] that "Runt Disease" in rats is probably induced by homologous lymphocytes.

Gesner and Gowans [60] also found that an intravenous injection of rat thoracic duct cells hastened the death of lethally irradiated mice and produced changes in their splenic pulp identical to those found in the F_1 rats. They demonstrated by chromosome analysis that the great majority of the acitvely proliferating cells to be of donor origin.

Gorer and Boyse [63] transplanted spleen cells from parental into F_1 hybrid mice and described the pathological changes in the hosts which were identical to those in "Runt Disease". These observations have been confirmed in mice as well as in rats by other investigators [64, 65, 66].

The evidence presented so far incriminates the small lymphocytes as the initiators of homograft rejection and graft-versus-host reaction.

Gowans[58] has recently reviewed this subject and concluded that the role of lymphocytes in homograft rejection is identical to their role in graft-versus-host reaction.

CHAPTER III

THE ELKINS MODEL OF LOCALIZED GRAFT-VERSUS-HOST REACTION.

Elkins[82, 83] found that parental Lewis or Brown Norway strain rat immunocompetent cells when injected under the kidney capsule of (LBN) F_1 hybrid rats produce a localized graft-versus-host reaction.

The cardinal feature of this reaction is the formation of a tumorous mass of actively proliferating mononuclear cells resulting in invasion and destruction of the underlying renal parenchyma.

Elkins[83] observed that the histopathology of this localized graft-versus-host reaction mimics in many aspects the histopathology of the renal allograft rejection.

Since it is generally[58] believed that the histocompatibility factors recognized in the graft-versus-host reaction are also those involved in the rejection of allografts by the hosts, Elkins studied the activity of lymphoid cells from rats which differ only in their minor histocompatbility loci.

He[82] found that spleen and lymph node cells from parental Lewis or Fischer rats were incapable of inducing a graft-versus-host reaction when injected under the kidney capsule of (Lewis X Fischer) F_1 hybrid rats. The Lewis and Fischer strains differ only in their minor histocompatibility antigens. Elkins concluded that a population of up to fifty million Normal spleen cells was activated to produce a

graft-versus-host reaction ONLY if confronted with host tissues expressing a foreign antigen of the AG-B series. He found no evidence that antigens of the other thirteen loci by which the Lewis and Fischer rats may differ were sufficiently immunogenic to elicit this response under these conditions. Elkins[83] also found as expected, no graft-versus-host reaction on parent to parent, F_1 hybrid to isogenic F_1 hybrid and F_1 hybrid to parent transfer of lymphoid cells.

Parental rat thymocytes were also incapable of producing the reaction[83,86].

Elkins[83] demonstrated that lymphocytes from parental donors made tolerant by neonatal injection of F_1 hybrid cells do not give a graft-versus-host reaction when injected under the renal capsule of F_1 hybrid rats.

He concluded that the mere act of injecting parental strain lymphocytes under the kidney capsule of F_1 hosts does not result in any infiltrative reaction unless the cells are specifically competent to react against the isoantigens of the host.

Elkins[82,83] observed that the graft-versus-host reaction started about the third day, became well advanced around the seventh day, but most of the reactions showed signs of diminished activity on the fourteenth day of the great majority of them completely subsided by the forty - second day of cell injection. Using chromosome analysis of the inter-

acting cells, he found that in the seventh day reaction, about 90-96% of the dividing cells in the renal reaction were donor cells. In the host spleen and lymph nodes, only 4-8% of the proliferating cells were donor cells. When he injected the actively proliferating lymphoblasts from Leukemic Lewis rats, under the kidney capsule of F_1 hybrid rats, he found rapid proliferation of the malignant cells with invasion of renal cortex and medulla but no destruction of renal cortex as is usually seen in the localized graft-versus-host reaction.

Monaco, Russell[135] and their associates produced the Elkins type of graft-versus-host reaction in mice by injecting parental mice lymphoid cells into F_1 hybrid mice.

They also found that parental lymphoid cells from anti-mouse lymphocyte serum or (AMLS) treated donors were incapable of producing the reaction when injected under the kidney capsule of their F_1 hybrid mice.

Elkins[84] also found that total body irradiation of the F_1 host in doses of eight hundred to one thousand, two hundred r* prior to the parental cell transfer diminished or abolished the development of graft-versus-host reaction. He also stated on the basis of preliminary experiments that he could restore the reaction in the irradiated F_1 hybrid hosts by injection of host lymphoid cells together with the parental immunocompetent cells. Lumbar radiation of the host had only a minor effect on the graft-versus-host reaction. Thus far the Elkins form of localized

* r = rads

reaction has satisfied the criteria laid down by Simonsen about graft-versus-host reactions, i. e.

The direction of the immunologic process.

a. The initiation of the reaction is uni-directional, the injected parental cells initiate the reaction by interacting with the host mononuclear cells.

b. The host is genetically tolerent of the parental injected lymphoid cells and do not mount a host-versus-graft response against them.

Simonsen has also pointed out that successful transfer of a graft-versus-host reaction into a second F_1 hybrid which is isogenic with the primary host constitutes solid evidence for the continuing reactivity of the original donor component against host antigens.

To satisfy this criterion, Elkins excised the seventh day tumorous reactions from the F_1 hosts, or minces it and injected it into normal isogenic F_1 hybrid hosts, irradiated isogenic F_1 hybrid hosts and into other hosts.

He found that the reaction is successfully transferred only in normal isogenic F_1 hybrid hosts.

Because the florid seventh day reaction did not propagate on transfer to irradiated isogenic F_1 hybrid hosts, Elkins concluded that the development of localized graft-versus-host reaction depends on continuing interaction of immunologically activated donor cells with radiosensitive

population of host cells within the lesion.

Elkins and Guttmann[85] then transplanted kidneys of Lewis rats into (LxBN) F_1 hybrid recipients. The host as expected does not reject the parental kidney. Then they injected lymphoid cells from parental Lewis donors under the capsule of the Lewis kidney that were transplanted in the F_1 hybrid hosts. The injected lymphoid cells invaded and destroyed the transplanted kidney parenchyma producing a typical graft-versus-host reaction.

They concluded that the reaction results from the interaction between donor immunocompetent cells and host mononuclear cells that entered the transplanted kidney.

CHAPTER IV

ANTILYMPHOCYTE SERUM - A BRIEF HISTORICAL REVIEW

Antilymphocytic sera were first prepared by Elie Metchnikoff in 1899[86a]. He injected suspensions of guinea pig lymph node and spleen cells into rabbits and observed that sera from these rabbits agglutinated and lysed guinea pig lymphoid cells. He also found that these sera were active only against the cells of the species used for immunization.

Besredka[87] while working in Metchnikoff's laboratory demonstrated that these antisera produced leucopenia and toxicity when injected into normal animals. Flexner[88] studied the pathologic effects on animals of injecting these antisera. He noticed that occurance of both hyperplasia and hypoplasia of the lymphoid tissues together with varying degrees of cell death particularly in the germinal centres of lymph nodes.

These observations were confirmed by Bunting[89] and Christian[90]. Pappenheiner[91] injected rat thymocytes in rabbits and found by the trypan blue exclusion test that the immune sera were toxic to rat lymphocytes and thymocytes.

Chew, Stephens and Lawrence[92] immunized rabbits with lymph nodes from guinea pigs and found that the antisera produced were consistently capable of producing marked lymphopenia when injected into

guinea pigs.

These findings were later confirmed by Cruickshank[93] in rats injected with rabbit anti-rat lymphocyte serum. The production of lymphopenia and the in vitro cytotoxic properties of antilymphocyte sera have since been confirmed by several investigators[97, 108, 105] [111, 124, 126]

With the growing realization of the role of lymphocytes in homograft rejection during the past decade, widespread interest in antilymphocyte sera developed.

In 1955 Humphrey[94] was the first to use antilymphocyte serum as an immunosuppressive agent.[95] Three years later, Intebetzen, working in Humphrey's laboratory found that rabbit anti-guinea pig lymphocyte serum inhibited the tubercular reaction in guinea pigs. Waksman[96] and his associates confirmed the observations of Interbetzen and in addition found that rabbit anti-guinea pig lymphocyte sera inhibited each type of delayed hypersensitivity reaction such as the tuberculin reaction, allergic encephalomyelitis and skin allograft rejection. Woodruff[98] and Anderson found that rabbit anti-rat lymphocyte sera, prepared against rat thoracic duct lymphocytes greatly prolonged skin allograft survival in rats.[100] Gray, Monaco, and Russell demonstrated that rabbit anti-mouse lymphocyte sera greatly prolonged the first set skin allograft survival in serum treated mice. Levey and

Medawar[102,103] confirmed the effect of antilymphocyte serum on first set skin homograft survival and in addition they observed that antilymphocyte sera considerably prolonged the survival of second set skin homografts.

In 1966 several groups of investigators headed by Woodruff[99], Russell[105], Starzl[106] and Bliss[107] reported that canine renal allograft survival may be greatly prolonged by the administration of heterologous anti-dog lymohocyte sera.

Balner and Dersjant[108] found that antilymphocyte serum prolongs the life of skin homografts in primates.

Starzl[109,117,118] treated human recipients of renal and hepatic allografts with horse anti-human lymphocyte serum and globulin.

Ono[110] treated cardiac allotransplanted rats with heterologous antilymphocyte serum. He noted that the heart rate remained normal for thirty days compared to four days in the control group. He also noted that a single injection of antilymphocyte serum reversed the progressive deterioration of the heart as monitored on electrocardiographic studies.

Ono, Bell, Kashiwagi and Starzl[111], prepared rabbit antilymphocyte sera against the splenic, thymic and lymph node lymphocytes of inbred Fischer rats. They found that different antisera had the same ability to induce lymphopenia or to protect auxillary cardiac homografts

from rejection after transplantation from Wistar-Furth donors to Fischer recipients.

Barnard[112] used anti-human lymphocyte serum sucessfully to reverse an acute rejection episode in his second cardiac transplantation patient.

Shumway[113] used antilymphocyte globulin together with Azathioprine and Prednisone in the last eleven of his thirteen cardiac allotransplanted patients.

Cooley[114, 115] added antilymphocytic globulin to the routine immunosuppressive regimen given to all of his nineteen cardiac transplanted patients.

The above authors'[113, 115] felt that antilymphocytic globulin has a definite place in cardiac transplantation.

Kahn[116] and his associates used antilymphocyte globulin only for the rejection episodes of human heart transplantation.

Starzl[119] and his co-workers treated dogs with antilymphocyte globulin only after the onset of rejection of renal and hepatic allografts. He found that anti-dog lymphocyte globulin slowed the tempo and vigor of rejection even when treatment was started after this process was well advanced. They found that death from homograft failure was significantly delayed and in several experiments the rejection was at least partially reversed.

Pichlmayr[120] and his associates confirmed that the survival time of dogs following renal and liver homografts was significantly prolonged by using horse anti-dog lymphocyte serum.

Shanfield[130] and his associates noted that a short course of antilymphocyte treatment may lead to permanent survival of canine renal allografts.

Braf and Hume[121] found that significant functional survival of second set canine renal homografts was achieved by treatment with antilymphocyte sera. They stated that this could be of immense clinical importance in the management of multiple organ transplants.

Starzl[122] and his co-workers after using horse anti-human globulin as a adjuvant immunosuppressive agent for two years in patients with renal and hepatic homotransplantation concluded that the evidence is strong that there was a resulting improvement in patient care.

Medawar[124,125] has recently summed up the consensus of opinion gained from the extensive experimental investigation of antilymphocyte sera by several workers.

Medawar stated that antilymphocyte serum is the only true immunosuppressive agent that is distinguished from all other agents by three properties: Firstly, it has a much greater effectiveness on cell mediated reactions than on humoral antibody mediated reactions. Secondly, its power wholly to erase the record of prior sensitization i. e. abolish the

second set reaction completely and thirdly, its comparative indifference to whether the donor and host are distinguished by strong or weak histocompatibility differences.

Medawar stated that if the clinical use of antilymphocyte serum is to prosper, the following information must be sought without delay.

a. How to measure the potency of antilymphocyte sera by an in vitro test.

b. How best to raise antilymphocyte sera on a large scale for clinical use.

c. How antilymphocyte serum works.

Very recently improvements in the preparation of antilymphocyte sera has been made by Najarian[126] and his co-workers and by Starzl[122] and his associates.[127,128,129,141,142] In addition several workers developed tests that are claimed to assay the potency of antilymphocyte sera. This thesis deals with work initiated to develop a test that can assay and predict the immunosuppressive potency of anti-human lymphocyte sera.

CHAPTER V

THE USE OF GRAFT-VERSUS-HOST REACTIONS TO ASSAY THE IMMUNOSUPPRESSIVE POTENCY OF ANTILYMPHOCYTE SERA

Studies on graft-versus-host reactions[58] have provided compelling evidence that the small lymphocytes initiate the graft-versus-host reaction. They also provided strong evidence that the small lymphocytes may be involved in the inductive phase of the rejection of allografts.

Antilymphocyte sera has been known since the pioneering work of Metchnikoff in 1899 to be specifically toxic to lymphocytes, both in vitro and in vivo.[86-98]

Siskind[17a] and his associates has shown that mouse anti-mouse lymphocyte serum could protect neonatal mice from "Runt" disease.[17,29] Siskind also has shown that the serum was effective even when administration was delayed for two days, well after the lymphoid cells has settled in the tissues of the grafted neonatal mice.

Boak, Fox and Wilson[131,132] found that lymph node and spleen lymphocytes from parental C57bl mice treated with rabbit anti-mouse lymphocyte serum (RAMLS) were incapable of inducing a graft-versus-host reaction when injected into F_1 hybrid recipients. They used the splenic weight assay and the phagocytic index to measure the severity of graft-versus-host reaction. In another work the same authors prepared lymphocytes from spleens lymph nodes and thoracic ducts of normal

and RAMLS treated C57bl mice. They injected the lymphocytes into normal adult F_1 hybrids and into three to five day old recipients. They confirmed that lymphoid cells from parental donor mice treated with one, four or six daily injections of rabbit anti-mouse lymphocyte serum failed to induce a graft-versus-host reaction in F_1 hybrid mice. It was suggested that the lymphocytes became unable to react against histocompatibility antigens of the F_1 hybrid recipients.

Sauvette, Gitkens and Colofiore[133] used rabbit anti-mouse lymphocyte serum to modify "Runting" disease. In their model they injected six million viable adult C57bl spleen cells intraperitoneally within the first twenty-four hours of birth of Balb/c mice. In this model 80-85% of the injected mice developed fatal "Runting" within eleven - twenty days. Treatment of the hosts with rabbit antilymphocyte serum abolished or reduced the mortality from "Runting". They observed that RAMLS was most effective when treatment was begun on day one and continued for forty days. In addition donor cells could be demonstrated in about two-thirds of the survivors in the treated animals after cessation of ALS therapy. This finding suggested to them that ALS may be sufficiently specific to modify the graft-versus-host reaction without suppression of all immunological or hemopoietic activity of the grafted lymphoid cells.

This work has been confirmed by Brent, Courtenay and Gowland[134].

Monaco, Russell [135,136] and their associates in several excellent studies found that ten million adult (A/Jax) lymphocytes incubated for five minutes with rabbit anti-mouse lymphocyte serum failed to induce "Runting" in C3H/He neonates. The weight gain assay was used to measure the severity of this graft-versus-host reaction. The same authors also found that twenty-five million A/Jax lymph node cells after similar incubation with RAMLS failed to induce splenomegaly when injected into C57BL/6 x A/Jax)F_1 hybrid recipients. The spleen to kidney ratios were essentially similar to those after injections of isogeneic cells. A/Jax lymphoid cells incubated in normal rabbit serum produced profound splenomegaly when injected in the F_1 hybrid controls. They also grafted intact parental lymph nodes under the capsules of F_1 hybrid recipients mice. The degree of the local graft-versus-host reaction was graded on the basis of the histological gradings of Elkins in the rat.

They found that parental lymph nodes or dissociated lymphocytes from mice treated with rabbit anti-mouse lymphocyte serum lost the capacity to invade and destroy the underlying renal cortex of the F_1 hybrid recipients.

In the previous studies, no mention was made regarding a correlation between the capacity of antilymphocyte sera to prevent the graft-versus-host reaction and their ability to inhibit the rejection of homografts.

Gordon [137,138,143] and his associates developed a graft-versus-host reaction across species. They injected murine and human immuno-

competent cells under the kidney capsule of rats whose immunological capacity has been modified by prior treatment with Cyclophosphamide. A localized graft-versus-host reaction similar to that of Elkins[83] developed in the injected rats. These localized graft-versus-host reactions could be specifically inhibited by antisera raised against the donor lymphoid cells. They[141] used the reaction to assay the immunosuppressive potency of sera. Using mouse cells in rats, MacLean and his associates demonstrated that the capacity of anti-mouse lymphocyte sera to inhibit graft-versus-host reactions initiated by mouse cells correlated with their capacity to prolong skin allograft survival in mice. They[139, 140, 141, 142] found that the graft-versus-host reaction is also produced by human immunocompetent cells in Cyclophosphamide treated rats. This reaction was also specifically inhibited by anti-human lymphocyte serum. They demonstrated that this inhibition of the reaction by anti-human lymphocyte sera (AHLS) could be used to predict and assay the immunosuppressive potency of these sera.

A detailed description of the findings of MacLean and his co-workers is presented in this thesis.

CHAPTER VI

MATERIALS AND METHODS

1. Skin Grafting.

The method of Jeejeebhoy [159] of skin grafting in mice is used. Male CBA mice were killed by cervical fracture, and the hair clipped very closely off the ventral surface. The skin of the ventral abdominal and thoracic surface is removed and scraped scrupulously of any subcutaneous tissues and fat by using a sharp scalpel. The donor skin is divided into squares, each about one centimetre in diameter. The skin is kept in cold saline until it is placed on beds prepared on male $C_{57}BL$ mice.

These recipients were anaesthetized with ether and an area of skin removed by fine scissors from the right side of the thorax. Attention being paid to avoid nicking the subcutaneous vessels with consequent formation of hematoma in the graft bed and death of the graft.

Each graft, after being placed in its bed was sprayed with a plastic spray dressing (Aeroplast) and covered with a small square of gauze. A Band-Aid (Johnson and Johnson) was applied circumferentially around the recipient's thorax to keep the graft in place. Grafts were inspected on the ninth day and at daily intervals thereafter. Signs of rejection were considered to be moistness, edema, loss of epithelium, scabing, and in prolonged survival, scaling or contraction of the graft

to less than a quarter of its original size.

2. Thymectomy.

Thymectomy in rats and mice is modified from Jeejeebhoy's method[75]. Under ether anaesthesia a midline skin incision is made with fine scissors, extending from the lower half of the neck to the upper half of the thorax. The sternum is split in the midline by sturdy scissors and each side retracted laterally. Using a fine mosquito hemostat the strap muscles are separated in the midline until the trachea is exposed and dissection is continued towards the thymus. The thymus is grasped in the midline and pulled very carefully upwards. Using fine forceps, both lobes of the thymus are separated from the surrounding areolar tissues and treachea. The thymus must never be crushed and dissection must be very gentle done to avoid injury of the major vessels behind the thymus. As soon as the thymus is removed the rodent is held in the left hand, its chest is compressed to allow air of the pneumothorax to come out and the wound is closed by three or four stainless steel clips from an autoclip.

During the procedure, invariably the pleural cavity is accidently opened, the rodent immediately goes into respiratory distress. Little experience is required to perfect the technique and to do thymectomy with minimal mortality.

The thymus is then examined to confirm gross complete removal.

3. Technique.

The technique of intraperitoneal injection of cyclophosphamide (CY) chloral hydrate (C. H.) and antilymphocyte serum (ALS).

The technique described in this thesis was found to be essential for the proper performance of the experiments. During the course of an experiment, each rat is given three intraperitoneal injections - CY, C. H. and ALS. In the great majority of experiments, the results are quantited by the ratio of the weight of the left kidney to that of the right kidney. Hydronephrosis nullifies the accurate quantitation of the results. Hydronephrosis was quite frequent in early experience of the author and was thought to be due to retroperitoneal extravasation injection of drugs. The technique described in detail in this thesis drastically reduced the incidence of retroperitoneal injection and consequent hydronephrosis.

Rats were held by the left gloved hand. A thick leather glove is used to protect the ope rator from rat bites and scratches. Rats are held in such a way that their back fits into the palm of the left hand, and the operator applies pressure on the sides of the abdominal walls. When the abdomen is slightly squeezed between the thenar eminence on one side and the tips of the fingers on the other side, the anterior abdominal wall bulges forwards, becomes tense and ready for injection. The puncture site is in the left lower quadrant of the abdomen adjacent to the lower most nipple. A twenty-three gauge, half inch long needle is used and its

tip is not allowed to go deeper than a few millimeters.

When chloral hydrate is injected consecutively in each group five or ten rats, they are all under surgical anaesthesia within a few minutes. Quite unusually when a rat escapes the effect of anaesthesia it is due to extravasation in the abdominal wall.

The main advantage of this technique is:

1. Drastically lowers the incidence of hydronephrosis in the recipient.
2. Fast, reliable onset of anaesthesia in the groups of rats injected which allows rapid completion of the experiment and doing large numbers of rats in one sitting.

4. Animals.

a. Highly inbred Lewis, Brown Norway (B. N.) and (B. N. x L) F_1 hybrid rats each weighing 180-200 grams were purchased from Microbiological Associates, Bethesda, Md.

b. Outbred hooded rats were obtained from the Quebec Breeding Farm, Montreal, Quebec. The great majority of rats were females weighing 180 to 200 grams. These homogeneous body weights greatly facilitated the administration of anaesthetic to them and produced less variations in kidney weights and graft-versus-host reactions. The rats are kept in air conditioned rooms, five rats per cage, are fed standard chow diet and given water ad lib. All the cages are carefully labelled by the author and the labelling is checked by his associate.

c. Mice of the highly inbred strain of C57 black or CBA each weighing from 22 to 26 grams were purchased from the McIntyre Animal Centre, McGill University, or from The Jackson Laboratories, Bar Harbor, Me.

d. Pregnant Swiss Albino mice and pregnant Wistar rats in their last days of pregnancy were obtained from a local supplier. Their new born litter were injected with lymphocytes within twenty hours of birth.

e. Rats were irradiated in individual Perspex boxes using a Cobalt 60 source. Total body irradiation doses ranging from 100 to 1200 r were delivered at a rate of 58 r/minute.

5. Preparation of Cell Suspensions.

a) From rodents.

Mice were killed by cervical fracture. Rats were killed by a hard blow on the head. Mice were dead within seconds of cervical fracture. Rats were dead within a few minutes of cephalic damage. Groups of two or three mice were killed in immediate succession. Immediately after death the ventral surface of the body is clipped of hair by an electric shaver. The clipped area is moistened with alcohol. The rodent is placed in the supine position on a cork dissecting board. This board is covered by a clean disposable underpadding cloth which is changed whenever it is soiled with blood, excreta or hair clippings. The extremities of the rodent

were stretched centrifugally and pinned down to the dissecting board. The following steps were done in a sterile manner. A longitudinal midline skin incision is made that extends from the pubic to the submental area. In rats the incision is extended along all the extremities. The edges of the skin wound are held firmly by a strong toothed forcep and peeled laterally. In rats the use of scalpel dissection facilitates the peeling of the skin flaps. Once the skin flaps are peeled off the ventral and lateral surfaces, the skin edges are pinned to the dissecting board by three or more pins on each flap. Using a fine non-toothed forcep and fine dissecting scissors the lymph nodes in the groin, submental jugular paratracheal axillary and retrocubital areas are removed. Then the abdomen is opened carefully to avoid nicking the bowels and contaminating the instruments and lymph nodes suspension. A V type incision is made. The muscles in the pubic area were grasped by forceps and pulled upwards towards the ceiling. They were cut gradually and piecemeal by fine scissors until the peritoneal cavity overlying the urinary bladder is opened. Air enters and separates the bowel from the abdominal muscles. The two limbs of the V incision are completed by extending the incision on either sides towards the subcostal margin. The triangular flap of muscle is reflected on the thorax. where it can be used for wiping off excess blood from scissors or forceps. The spleen is excised first, followed by the mesenteric chain of lymph nodes, retrogastric and iliac

lymph nodes. All the lymph nodes must be removed, without the surrounding fat or areolar tissues - otherwise clotting of cell suspension occurs. The bowels should never be grasped with forceps and care is taken to avoid nicking of the mesenteric vessels, otherwise blood floods the field and renders identification of the mesenteric nodes difficult. The lymph nodes and spleen are placed in a 50 mesh stainless steel sieve which is positioned in the centre of a sterile glass petri dish that contains about 30 ml of cold Hanks balanced salt solution or medium 199. The nodes and spleen are minced with fine scissors and lightly squeezed through the sieve by a pestal or a piston of plastic syringe. The sieve is washed with cold Hanks balanced salt solution and removed. The cell suspension is transferred by pasteur pipette to a sterile 50 ml caped tube. The cell fragments that passed through the sieve are broken by passing the suspension many times through pasteur pipettes. The glass tube is placed vertically for two - three minutes until the undissociated fragments of tissue are sedimented. The supernatant cell suspension is transferred to another 50 ml sterile caped tube and centrifuged at 1000 rpm for seven minutes in 15° c temp. The supernatant is decanted and the sedimented cells are resuspended in 50 ml of the same solution Hanks balanced salt solution or culture medium 199 (Difco) (HBSS or med 199). Drops of the well mixed cell suspensions are taken into a small test tube for counting and the cell suspension is

re-centrifuged again as in the previous manner.

Counting the harvested lymphocytes.

All the cell counts in all the experiments reported in this thesis were done by the author, O.O5 ml of the cell suspension sample is taken to a calibrated pipette and added to 4 ml of Turk solution. A pasteur pipette is used to mix the suspension. Aliquots are put in the chambers of a neubauer hemocytometer. The hemocytometer is kept in a horizontal position for about two minutes until all the cells settle down, then is placed on the stage of the microscope. Only small and large mononuclear cells are counted. Polymorph nuclear leukocytes are disregarded. The number of cells in four large squares are counted with the aid of a denominator. The total cell count equals the count in the four large squares 1O X 8O X 1OOO X 5O.* No antibiotics or anti-coagulants are added to the cell suspension.

About three hundred and fifty million cells are obtained from each mouse and about nine hundred million lymphocytes are obtained from each rat. In most of the experiments one thousand five hundred to two thousand million donor lymphocytes are prepared. Once the total lymphocyte count is known, the sedimented cells of the main cell suspension are re-suspended in medium so that O.1 ml of cell suspension contained thirty million lymphocytes. In some experiments O.1 ml cell suspension contained from one to one hundred and fifty million lymphocytes. This

* please see next page.

final cell suspension is kept cold by immersing the caped tube in iced water.

Not infrequency at the termination of injection, the remaining cell suspension is counted to detect significant loss of cells during the final centrifugation.

$\frac{10}{4}$ = 4 squares counted = 0.4 mm^3

80 = dilution factor 0.05 in 4.

1000 = 1 ml = 1000 cmm.

50 = volume of cell suspension.

b) Preparation of peripheral human lymphocytes.

A phlebotomy is done on human volunteers and about 400 ml of heparinized venous blood is collected from each person into a graduated sterile flask.* Heparin (liquamin) is used in a concentration of 20 i. u. per ml of blood. Thirty ml of the heparinized blood is put into a 50 ml capacity glass tube with a screw cap. The tubes are centrifuged at 20 to 60 g for ten to six minutes. The plasma is collected and the sedimented blood is re-suspended and re-centrifuged at 60 - 20 g for six to ten minutes. Again the plasma layer is collected.

The speed of centrifugation depends upon the sedimention rate of the erythrocytes. As a general rule, females have a higher sedimentation rate than males. Therefore the blood obtained from females is

* International units.

centrifuged at a lower speed and for a longer time for the first spin and a higher speed with a shorter time for the second spin. i. e. 20 g - ten minutes for the first spin and 60 g - six minutes for the second spin. The reverse of this procedure is generally used for blood obtained from males. Not infrequently the sedimentation rate is very low. In this situation we spin the blood at 100 g for eight minutes, collect the plasma and buffy coat and then respin at 50 g for eight minutes. The lymphocytes in the collected plasma are counted in a hemocytometer by mixing 0.05 ml of the plasma into 1 ml of Turk white blood cell counting solution. The number of cells counted per ml is calculated from the following formula.

$$C \times 20 \times \frac{10}{4} \times 1000 = \text{number of cells per ml.}$$

Where C = number of cells

20 = is the dilution factor (0.05 in 1 ml).

$\frac{10}{4}$ = 4 squares counted = 0.4 mm^3

1000 = number of mm^3 per ml.

Only lymphocytes are counted and the total cell count is known. About four hundred million lymphocytes are usually obtained from each donor. Lymphocytes from three donors are usually mixed together after being washed once in medium 199 or Hanks balanced salt solution. This is achieved by centrifugation at 110 g for ten minutes. The supernatant plasma

and platelets are decanted. The cell button is re-suspended in medium and re-centrifuged. The cell button is re-suspended in medium so that O.1 ml of cell suspension contains thirty million lymphocytes. The procedure is done at room temperature and takes about two hours to complete. Once the lymphocyte cell suspension is in medium 199 or Hanks balanced salt solution, it is cooled to about 15° C and kept so until it is completely injected under the kidney capsule of rats. Occasionally another cell count is made of the final cell suspension to be sure that no significant lymphocyte loss occurred.

Anaesthesia.

Chloral hydrate was used for rats. A freshly prepared solution is made for every experiment. Four grams of chloral hydrate crystals are dissolved in 100 ml of saline solution (4% solution). It is administered intraperitoneally to rats in a dose of 1 ml solution per 200 gram body weight. Since the great majority of the recipient rats each weighed between 180 - 200 grams, 1 ml of anaesthetic is administered without the need to weigh them again. A ten milliliter syringe is filled with solution and ten consecutive rats are injected. Within a few minutes they are all under surgical anaesthesia.

Cyclophosphamide (Procytox, F.W. Horner, Ltd., Montreal) was dissolved in distilled water immediately prior to its use. It was obtained in one gram or two hundred milligrams , depending on the number of rats

to be injected. Fifty milliliters of distilled water is added to the one gram bottle, or ten ml to the 200 mgm vial. The vial is shaken vigorously for ten minutes until all the Cyclophosphamide is dissolved completely and the solution appears crystal clear. Each milliliter of Cyclophosphamide solution contains 20 mgms of Cyclophosphamide. The solution is injected into rats within one hour of its preparation. Solutions staying for more than two hours are discarded. Cyclophosphamide solution is injected intraperitoneally into the rats, according to body weight. Each rat is accurately weighed, the solution is aspirated into tuberculin syringes by the assistant, and is injected through a 23 gauge needle. An average of fifty rats are injected in one sitting.

6. The technique of injecting lymphocyte cell suspension under the kidney capsule of rats.

Groups of five to ten rats are intraperitoneally injected, each with 1 ml of freshly prepared solution of chloral hydrate crystals in saline (4% solution). Within a few minutes all the rats are in a state of surgical anaesthesia. The left side of the body is clipped of hair with an electric animal shaver, and rubbed off with the hand to remove the hair clippings. The rats are laid on their right side, serially one behind the other, so that their ventral aspect faces the operator and their head lies towards the left side of the operator. The left side which

is clipped of hair is cleaned with alcohol.

A two cm transverse skin incision is made just below the left costal margin. The underlying muscle is held by a toothed forcep and is incised along the wound with fine scissors. Care being taken to avoid cutting the spleen and the small intestine. A non-toothed forcep is inserted inside the peritoneal cavity to hold the tissues at the lower pole of the left kidney. This lower pole is exteriorized so that it overlapes the inferior edge of the wound. The superior pole of the kidney is led to overlap the upper wound edge. Inserting a cotton swab between the kidney and the ventral angle of the wound prevents the kidney from relapsing back inside the abdominal cavity.

A tuberculin plastic syringe with black graduation markings is filled up to O. 6 with the well mixed cell suspension which is kept cold in caped glass tubes. A 30 gauge half inch long stainless steel hypodermic needle is fitted tight to the syringe in such a way that the short bevel of the needle is in line with the graduation markings of the tuberculin syringe. Prior to each injection of O. 1 ml the cell suspension must be well mixed within the syringe and then the air bubbles are removed. Lymphoid cells tend to sediment rapidly within the syringe. These steps were found effective in assuring equal doses of cell suspension to each recipient. For right handed persons, the tuberculin syringe is held so that the plunger lies in the centre of the right palm,

and the syringe shaft near the hub is surrounded by the first three fingers of the hand. The needle bevel and syringe graduation markings point towards the ceiling and are easily seen by the operator.

The rat with the exposed kidney is held in the left hand and is raised for a few centimeters above the dissecting board. Only the head, neck and chest are surrounded by the left hand and fingers, the right lumber region is supported by the left index and thumb. The hind limbs, by effect of gravity fall towards the dissecting board but are not allowed to touch it. The kidney is punctured from its inferior pole and the needle is advanced in the cortex towards the superior wall. The bevel of the needle can easily be seen under the capsule if there is a spot light behind the left shoulder of the operator.* Once the needle bevel is seen UKC, the exact volume of cell suspension can be easily administered. The needle plunger is pushed by the mid palm. A bleb of cell suspension is seen forming gradually and its edges spread centrifugally. It is preferably to allow the needle bevel to be seen in the area of maximum convexity of kidney so that the bleb of cell suspension and the resulting reaction form in the area of maximum convexity of the rat kidney.

Not infrequently the needle bevel punctures a vessel in the capsule. In this situation no bleb forms and the cell suspension will be seen streaming through the vessel. The needle bevel has to be retracted and re-positioned for injection. Sometimes the inferior edge of the bleb

* under kidney capsule.

spreads and reaches the puncture site near the inferior pole with consequent loss of lymphoid cells and disappearance of the bleb, and in the case of inadvertant puncture of the capsule by the needle tip, the right kidney is exposed and injected. In the vast majority of rats the left kidney is injected. It is much more easily accessible for injection than the right kidney.

Once the injection is completed, the needle is withdrawn, no reflux of cell suspension is seen because of the valvular manner of the bleb formation. The kidney is allowed to relocate inside the abdomen by removing the cotton swab and retracting the cephalic wound edge from underneath the superior pole of the kidney. The assistant closes the wound with continuous OOO silk sutures on a straight needle and the skin edges are approximated with 8 mm size stainless steel clips. The rats are placed on their right side in their labelled cages, with their neck extended to avoid respiratory obstruction. This method requires little experience to perfect it, and the standardization of the technique made it possible to inject forty rats in two hours, inject accurately at least 95% of the rats, and obtain reliable and consistant results.

7. Harvesting the results of experiments.

On day six, each group of rats were put under ether anaesthesia. Both kidneys and spleen from each rat were excised, cleaned of connective tissue and perirenal fat, blotted of excess blood and urine. The

specimens were placed in a petri dish which has five labelled compartments. They were immediately weighed to the nearest milligram by the assistant. Meanwhile the surgeon prepared the kidneys and spleens of the next group and put them inside a labelled and covered petri dish. Once the specimens were weighed, the left kidney and spleen were fixed in 10% of formalin for twenty-four hours in carefully labelled bottles. The next day each kidney is bisected, the reaction is grossly graded and prepared for histological studies. Histological sections 5 microns in thickness were stained with hematoxylin and eosin. In each experiment histological grading was done blindly according to the criteria of Elkins.

The severity of graft-versus-host reaction is objectively quantitated by the ratio of the weight of the injected kidney (ki) to that of the non-injected contralteral kidney (Kc).

The results for each experiment are tabulated in the following manner.

B. W.	Kc	Kc	Mean Ki/Kc	Spleen weight	Spl/ Kc	Histological grading O	I	II	III

In certain instances, particularly after injection of human lymphocytes under the kidney capsule of rats, there is marked capsular and pericapsular thickening. The capsule should be very carefully peeled

prior to weighing the kidney to avoid false positive weight differences. In a few experiments the regional lymph node of the injected kidney were excised, weighed and histologically examined.

The mean of the Ki/Kc ratio and the 95% confidence limits were calculated for each control and experimental group.

CHAPTER VII

STUDIES ON THE ELKIN'S MODEL OF GRAFT-VERSUS-HOST REACTION.

The development of the localized graft-versus-host reaction by Elkins has already been briefly discussed.

The experiments described in this section were intended to familiarize the author with the Elkins model prior to attempting to modify it in such a way that immunocompetent cells from species other than rat cells would be allowed to induce a similar reaction.

Plan of Experiment:

A. Lewis lymph node and spleen cells were injected under the kidney capsule of non-irradiated adult (LxBN)F_1 hybrid rats.

B. Parental lymphocytes were injected in (LxBN)F_1 hybrid rats that were given 800 to 1200 r total body radiation or given Cyclophosphamide prior to the grafting of cells.

C. Adult hybrid rats were given Cyclophosphamide one to three days prior to injection of 50 X 10^6 parental lymphoid cells.

D. Parental lymphocytes were injected into lethally irradiated F_1 hybrid rats. Syngeneic or allogeneic lymphocytes were also injected under the kidney capsule of these recipients along with the parental cells. In some experiments the syngeneic or allogeneic cells were injected intravenously or intraperitoneally in the hosts.

E. Mouse lymph node and spleen cells were injected under the kidney capsule of lethally irradiated rats, either alone or together with host lymphoid

cells.

Results. TABLE I.

A. Lewis lymph node and spleen cells when injected under the kidney capsule of unmodified (LxBN)F_1 recipients produce the classical reaction as described in detail by Elkins.[83] In twenty-one hybrid rats that were injected each with 50 X 10^6 parental cells the mean Ki/Kc** ratio was 1.7 + 27 (S.D.)* and the mean spleen to the Kc ratio was 1.24. In nineteen hosts the reaction was grossly visible and of grade III according to Elkins grading. Two of the recipients had non-significant reactions by gross and histological criteria.

B. Lethal irradiation of the hosts prior to the injection of parental cells did inhibit the reaction in all the fourteen (LxBN)F_1 hybrid rats thus treated. The mean Ki/Kc ratio dropped to 1.1+ 0.09 and the mean S/Kc ratio to 0.460. Grossly none of the hosts exhibited the typical tumorous mass in the kidney, but histological examination showed that six reactions were grade II, six were grade I. Even in grade II reactions, the invading tongues of mononuclear cells showed little activity, i.e. mitosis were rare and the damage to renal parenchyma was minimal.

*S.D. = standard deviation.
** Kc = injected kidney.
Kc = non-injected contralateral kidney.
S/Kc = spleen to Kc weight ratio.

Pre-treatment of the F_1 hybrid recipients with Cyclophosphamide also did inhibit the development of the graft-versus-host reaction. The inhibition produced by the higher dose of Cyclophosphamide was complete and was comparable to that of lethal irradiation.

Comment:

These results confirm the observations of Elkins regarding the host contribution to the localized graft-versus-host reaction. This host component is sensitive to irradiation as well as to cytotoxic drugs. In new-born mice injected with allogeneic adult spleen cells, Davies and Doak[28] found exceedingly few cells in mitosis, these were identified as donor cells in the enlarged host spleens. Miller[76, 78, 80] observed that the donor cells contribution could be increased to about half the spleen cell mitosis at the peak of splenomegaly, if the new-born recipient mice had been thymectomized prior to grafting. Howard, Michie and Simonsen[27] showed that irradiation of the adult (AxC57BL)F_1 hybrid hosts prior to grafting them with adult A strain mice inhibited the development of graft-versus-host reactions.

In the Elkins form of graft-versus-host reactions the majority of proliferating cells in the renal lesion were donor cells but in the enlarged spleen very few mitotic figures were of donor origin. Irradiation and cytotoxic drugs inhibited the development of both the renal as well as the splenic manifestations of the graft-versus-host reactions. Elkins[84]

stated that it is possible to achieve renal graft-versus-host reactions in heavily irradiated hosts by inclusion of hybrid host type lymphoid cells along with the parental components in the inoculum.

Our experience with reconstitution of the graft-versus-host reaction is very limited and is summarized in Table II and tends to suggest that the inclusion of non-irradiated host lymphocytes along with the parental cells would at best, only partially restore the reaction.

Mouse lymph node and spleen cells injected either alone or together with host lymphoid cells into lethally irradiated (LxBN)F_1 hybrid rats did not initiate a reaction. Table III.

The failure to reconstitute the graft-versus-host reaction in irradiated hosts lend support to Elkins suggestion that the host contribution in the graft-versus-host reaction is a continuing process. Elkins[84] reached this conclusion after unsuccessful attempts to transfer the renal reaction into irradiated syngeneic F_1 hybrid hosts. Radiation apparently irreversibly and completely interferes with the host component required for continuation of the reaction.

TABLE I: The effect of total body irradiation and Cyclophosphamide treatment of the recipients prior to transfer of parental lymphocytes.

Total No. of recipients.	Treatment of host.	Mean ** Ki/Kc±S. D.	Mean S/Kc	Histologic Grading. O	I	II	III
21	None	1. 7±O. 27	1. 24	O	2	1	18
14	800-1200 r	1. 1±O. 09	O. 460	2	6	6	O
8	100mgm/kgm Cycloph*	1. 470±O. 15	O. 754	O	O	O	8
6	200mgm/kgm Cycloph.	1. 090±O. 09	O. 480	O	2	4	O

* Cyclophosphamide.

** Ki/Kc ratio of weight of inoculated to that of contralateral kidney.

S. D. standard deviation.

TABLE II: Reconstitution of the graft-versus-host reaction in irradiated hosts.

No of Rats.	Reconstituting lymph node and spleen cells. Donor	Dose $\times 10^6$	Route	Mean ** Ki/Kc	Mean *** S/Kc	Histologic Grading O	I	II	III
2	$(LxBN)F_1$	100	1. p. daily	1. 095	0. 296	O	2	O	O
5	Lewis	100	I. V. at day O	1. 18	0. 565	O	3	2	O
2	Lewis, irradiated in vitro with 2000 r.	100	I. V. at day O	1. 13	0. 433	O	O	2	O
2	$(LxBN)F_1$	50	UKC*	1. 60	0. 633	O	O	O	2
2	$(LxBN)F_1$ irradiated in vitro with 2000 r.	50	UKC*	1. 2	0. 450	O	2	O	O

*UKC = under kidney capsule.
** Ki/Kc = ratio of weight of injected kidney to that of contralateral kidney.
***S/Kc = ratio of splenic weight to that of control kidney.

TABLE III: Mouse lymphocytes *** injected into lethally irradiated rats.

No of recipients.	Reconstituting cells*		Mean Ki/Kc	S/Kc	Histologic grading O	I	II	III
8*	None		1.03	0.780	4	4		
4	Lymph node and spleen cells.	2000 r in vitro	1.24	0.377		4		
5	"	**	1.14	0.466		5		
2	None		1.14	0.290		2		
5	Thymocytes	2000 r	1.13	0.309	1	2	2	0
5	Thymocytes	Non irrad.	1.03	0.280	0	3	2	0

* (LxBN)F_1 lymphoid cells.
** Received no irradiation.
*** 100 to 300 million lymphocytes.

CHAPTER VIII

CYCLOPHOSPHAMIDE IN GRAFT-VERSUS-HOST REACTIONS.

Cyclophosphamide is a powerful immunosuppressive drug with a high therapeutic index.[147] Stender[148,149] and his associates showed that antibody formation is suppressed by this agent even when it was given as late as four days after the antigenic stimulus.

Sutton[150] and his associates reported prolonged survival of skin allografts in mice treated with Cyclophosphamide.

Salvin and Smith[150] demonstrated that Cyclophosphamide can induce a very specific solid immunologic tolerance in guinea pigs. These observations were confirmed and extended by others[151-156] to rabbits, rats and man.

Santos and Owen's[33] has shown that hybrid adult mice injected with parental, allogeneic or xenogeneic immunocompetent cells following a preliminary injection of Cyclophosphamide will show a graft-versus-host reaction.

He also reported that Brown Borway (BN) rats, injected intravenously with Lewis immunocompetent cells four hours after a single intraperitoneal injection of 100 mgm/kgm body weight of Cyclophosphamide developed a graft-versus-host reaction manifested by dermatitis and death.

Santos work was extended by Gordon[137,139] and his co-workers.

They showed that Murine, canine and human lymphoid cells when injected under the kidney capsule of Cyclophosphamide pre-treated rats initiate a graft-versus-host reaction similar to that in the parent to F_1 hybrid combination. This xenogeneic graft-versus-host reaction reflects the immunological function of the donor cells and is specifically inhibited by antilymphocyte sera directed against the donor lymphoid cells.

A detailed study of this xenogeneic graft-versus-host reaction as well as its use to assay the immunosuppressive potency of antilymphocyte sera is given in this thesis. Page 61 - 112.

a. The Pathology of the Xenogeneic Graft-Versus-Host Reaction:

The cardinal feature of the renal reaction is the formation of a tumorous mass that causes enlargement and increase in weight of the kidney.

The kidney capsule thickened but could be easily peeled off the underlying tumorous reaction. On bisecting the kidney, the reaction appears as a diffuse, grey, homogeneous mass infiltrating the renal cortex. The degree of invasion and destruction of the renal cortex reflects the severity of the reaction. The renal medulla is usually spared and the cortico-medullary junction is usually identifiable. No areas of hemorrhage or necrosis are usually seen.

The regional lymph node which usually lies between the renal and the adrenal vein tributary is invariably enlarged and firm. Histologically, there is massive infiltration of the renal cortex with pleomorphic mononuclear cells which has large nuclei and prominant neocleoli. Frequent mitotic figures are seen, one or more, per high power field. There is various degrees of changes in the convoluted tubules. These changes vary from cloudy swelling, to vaculation, disintegration and various manifestations of cell death. Although the glomeruli usually retained their shape, the glomerular capillaries were devoid of blood. The tufts appeared collapsed, but the glomerulus filled the Bowman's capsule. The basement membrane is thickened.

The glomeruli beyond the margins of the reaction are congested.

At the advanced border of the reaction the mononuclear cells tended to collect around the vessels and the glomeruli . There is usually no infiltration beyond the cortico-medullary junction.

The histological grading of the localized xenogeneic graft-versus-host reaction. The criteria of Elkins[83] are as follows: -

1. No reaction: Grade O.

No cortical infiltration. The cells deposited under the kidney capsule lie in a thin layer underneath a thickened fibrous capsule. There is no evidence of activity in these cells which may be replaced by fibroblasts. There is a sharp line of demarcation between the cell layer and the renal cortex. Figure X.

2. Grade I.

The cell layer shows little tendency for invading the renal cortex. There is usually some degenerative changes in the outermost layer of the tubules. There may be one or two oblique tongues of mononuclear cells. These are usually produced by the needle track.

3. Grade II.

Appears grossly as grey tongues invading the renal cortex. Microscopically the penetrating tongues of nononuclear cells with mitosis invade and destroy the renal cortex, but the kidney tissue between the cortex looks intact. At least more than two tongues of infiltrate should

be present before considering a reaction as Grade II Reaction.

4. Grade III.

The classical reaction as described previously. The regional lymph node is enlarged. There is loss of lymph node architecture which is replaced by actively proliferating large mononuclear cells similar to those invading the renal cortex.

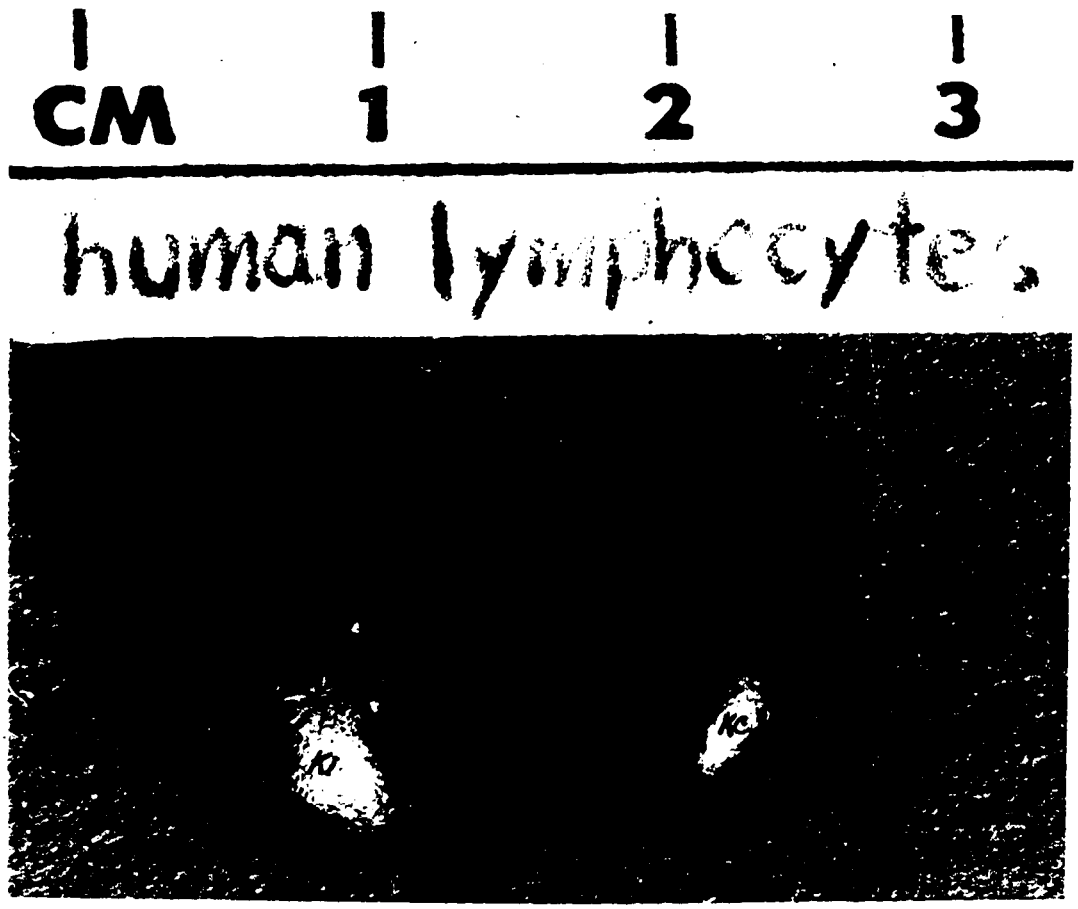
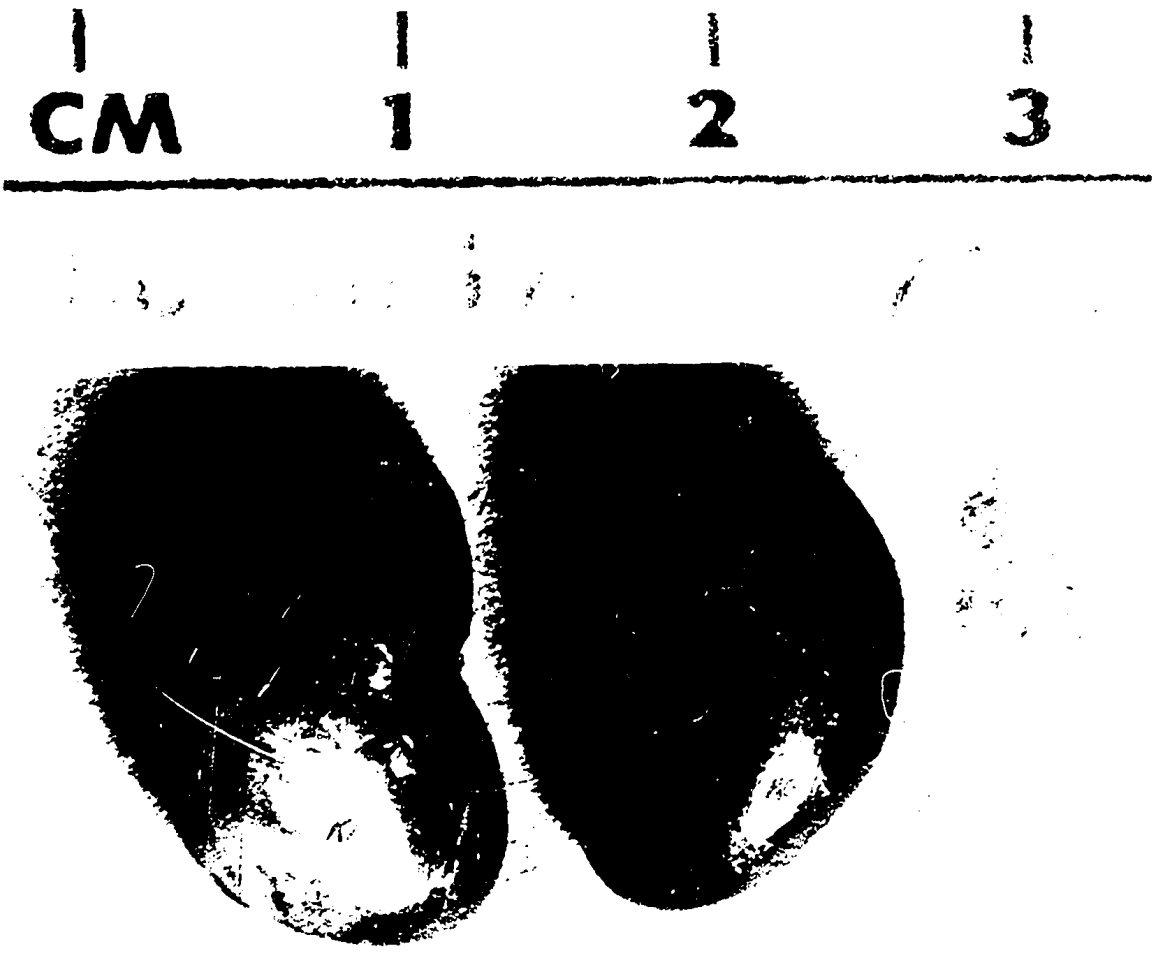


FIGURE: 1.

Gross appearance of a reaction produced by human lymphocytes in rat kidney (Ki). The grey homogeneous tumorous mass in the kidney causes enlargement and increase in its weight over the contralateral kidney (Kc).



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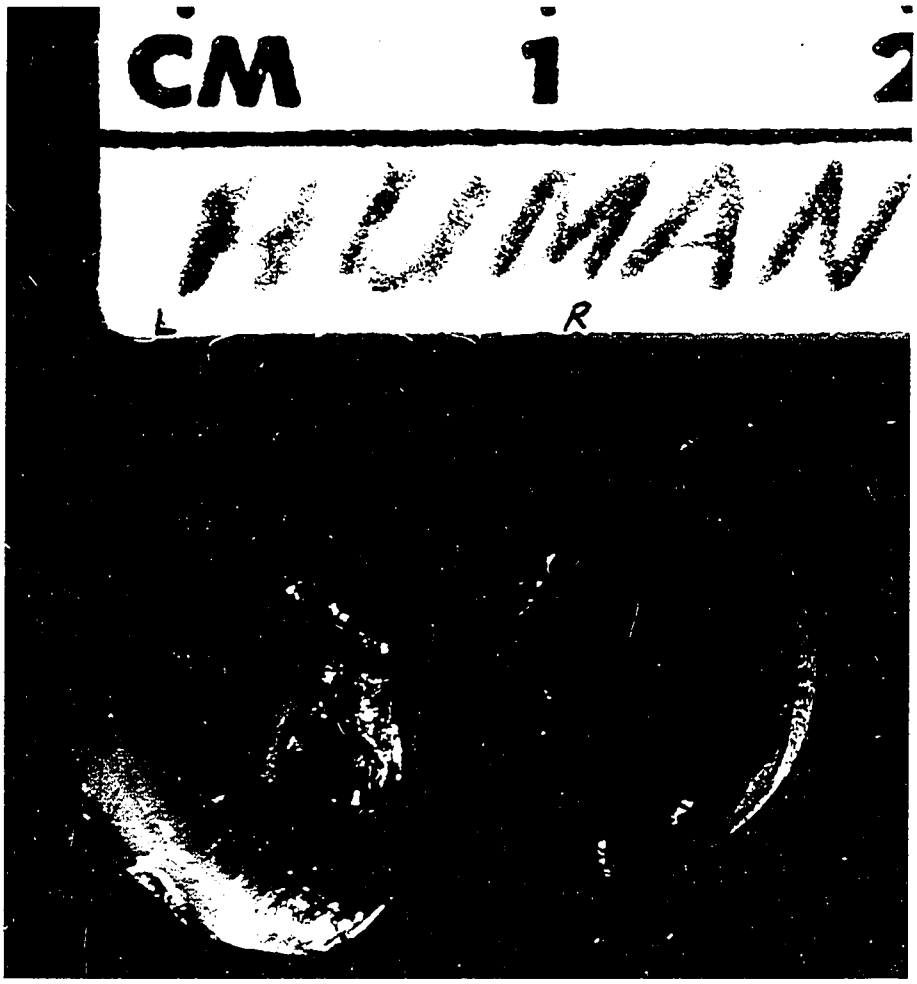
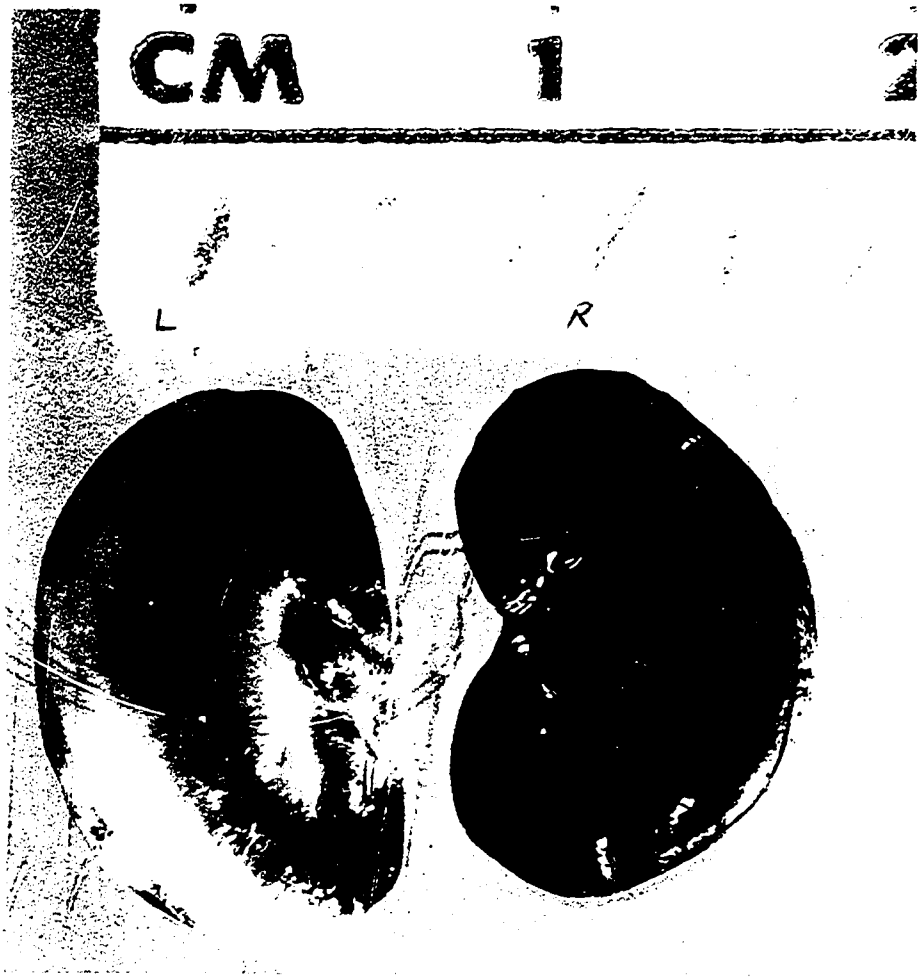


FIGURE: II.

Shows a graft-versus-host reaction produced by human peripheral lymphocytes in rat kidney (L). (R) is in the non-injected contralateral kidney.

The reaction consists of grey homogeneous tumorous mass that invaded the renal cortex. Reactions produced by human cells are similar to those produced by mouse or rat lymphocytes.



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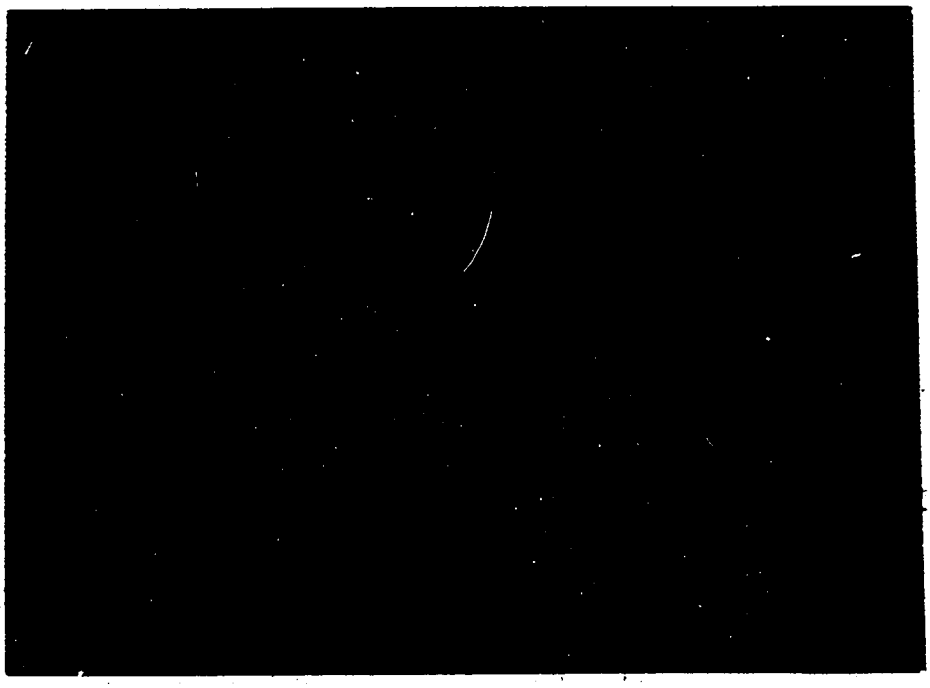


FIGURE: III.

Low power microscopic view of a reaction in rat kidney produced by human lymphocytes. The reaction consists of diffuse intense cellular infiltrate that destroyed and replaced the convoluted tubles of the kidney. The glomeruli look intact.

Hematoxylin and Eosin X25.

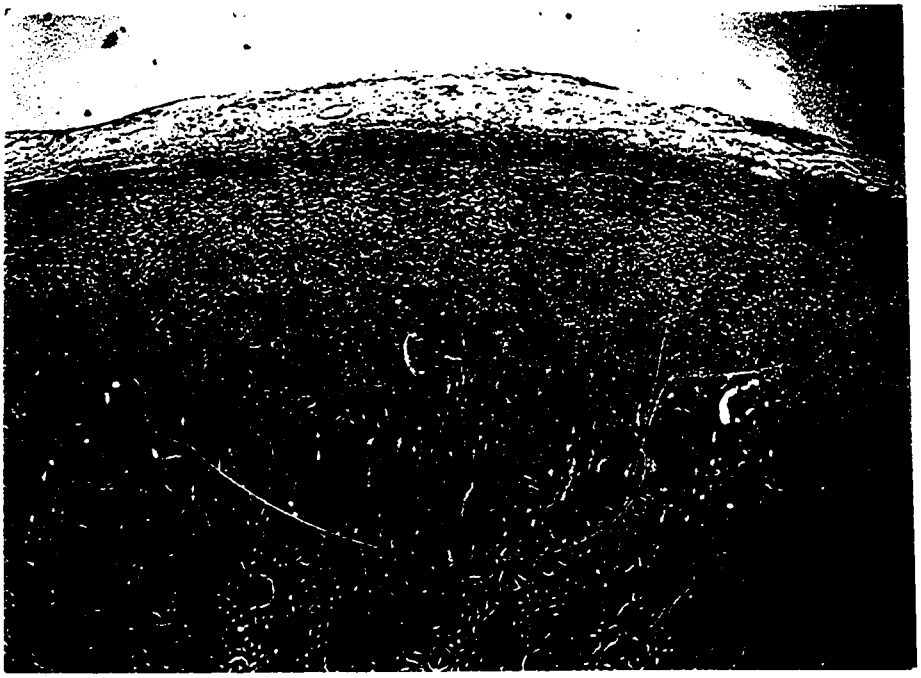


FIGURE 111.

Low power microscopic view of a reaction in rat kidney produced by human lymphocytes. The reaction consists of diffuse intense cellular infiltrate that destroyed and replaces the convoluted tubles of the kidney. The glomeruli took intact.

Hematoxylin and Eosin. X45.

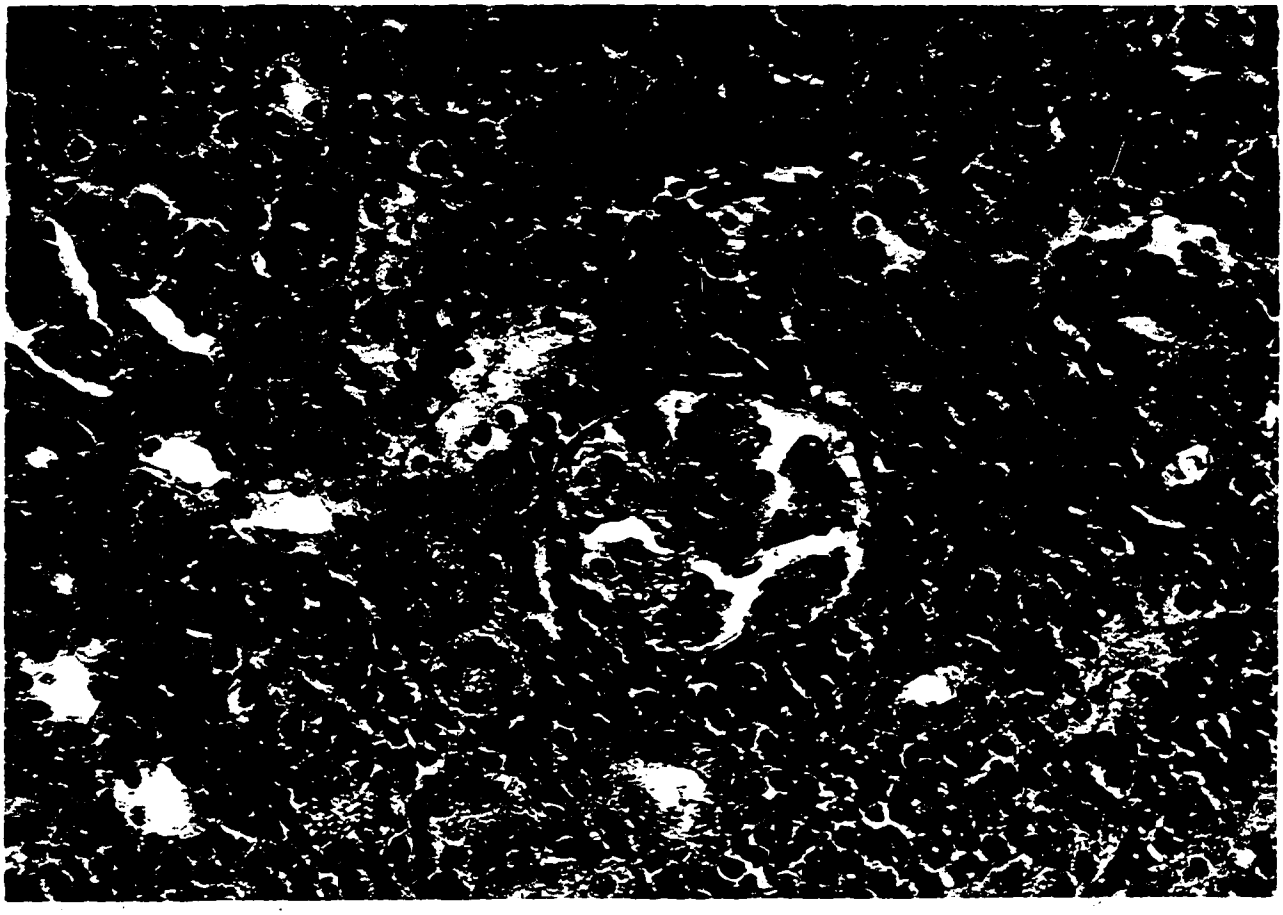
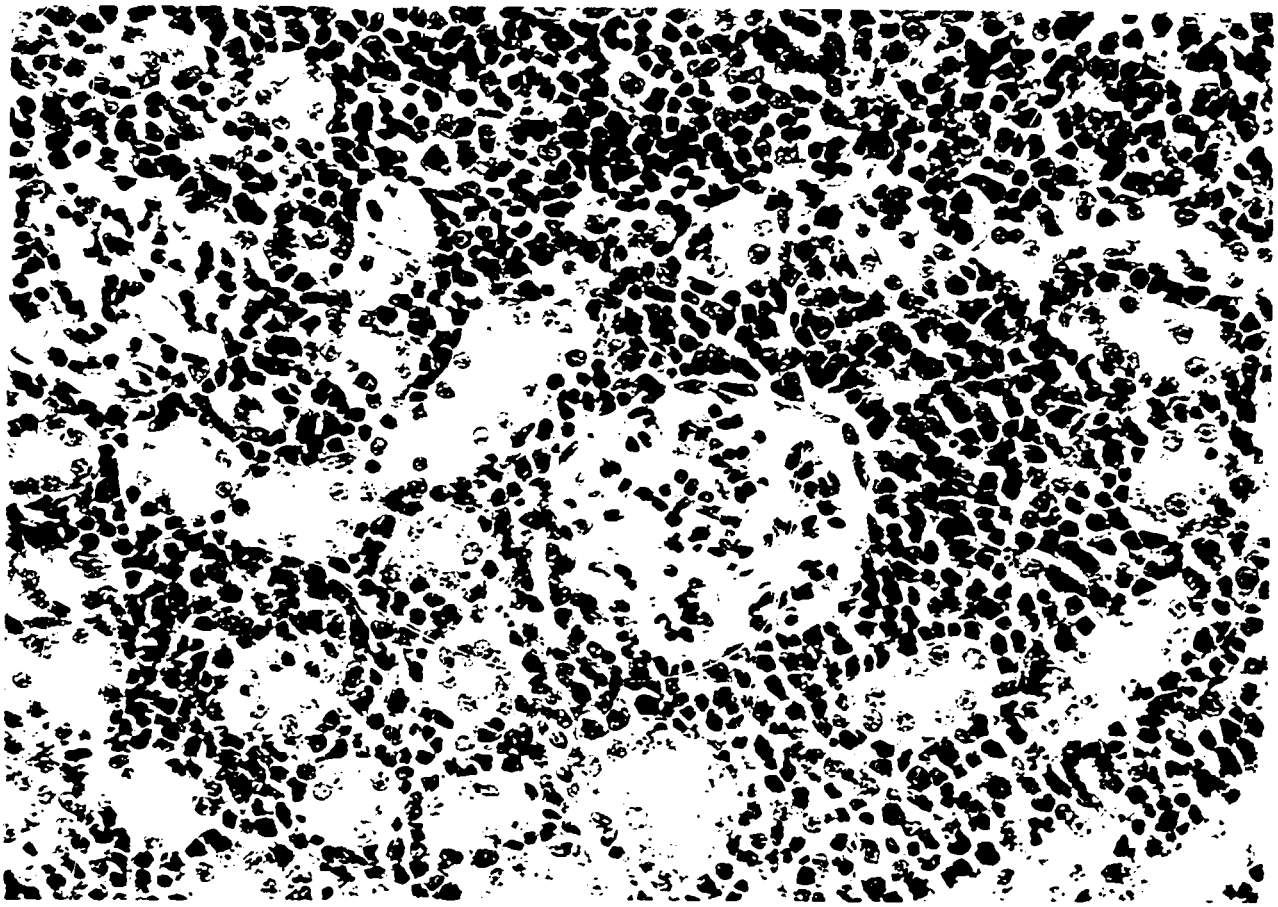


FIGURE: IV.

Shows typical Grade III reaction in a rat kidney injected with mouse lymphocytes. The renal tubules are destroyed and replaced by active mononuclear cell infiltrate. Convoluted tubules in various stages of degeneration are seen. The ischemic glomeruli fills Bowman's capsule.

Hematoxylin and Eosin X250.



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Hematoxylin and Eosin X250.

b. Immunologic evidence.

The following experiments were done to investigate the nature of the reaction produced by mouse cells in rats.

Experimental Plan.

Five groups, each consisted of ten rats were injected under the kidney capsule with 50 X 10^6 of spleen and lymph node cells from adult CBA mice.

Group I:

The rats were not treated with Cyclophosphamide.

Group II:

Cyclophosphamide in a dose of 100 mgm/kgm per body weight was given to each rat immediately after the injection of mouse cells.

Group III:

Donor mouse lymphocytes were irradiated with 4000 r in vitro prior to injecting into each Cyclophosphamide pretreated recipient.

Group IV:

Fifty million thymocytes from adult CBA mice were injected in each Cyclophosphamide pretreated rat.

Group V:

Fifty million mouse lymph node and spleen cells were injected in Cyclophosphamide pretreated rats.

All the recipients were sacrificed on the seventh day. The mean

Ki/Kc ratio, its 95% confidence limits and the histologic grading is shown in Table IV.

TABLE IV:

Experimental Group.		Number of Rats.	Mean Ki/Kc o	95% C. L. *	Mean S/Kc**	Histologic Grading O	I	II	III
No Cyclo	I	10	0.979	0.9544-1.0036	1.412	10	0	0	0
Cyclo Imm.	II	9	1.072	0.9888-1.1552	1.460		8	1	0
4000 r	III	10	1.086	1.0387-1.1323	1.233	9	1	0	0
Thymocytes	IV	5	0.989	0.923-1.055	1.576	5	1	0	0
Cyclo	V	10	1.30	1.182-1.450	-		1	1	8

* 95% C. L. = 95% confidence limits of the Mean.

** spleen to Kc ratio.

o Ki/Kc = ratio of weight of injected kidney.

Cyclo = Cyclophosphamide.

No xenogeneic graft-versus-host reaction developed when mouse cells were injected into rats whose immunological capacity to reject the injected cells has not been modified by prior treatment with Cyclophosphamide. The host is capable of rejecting the grafted cells. In recipients whose immunological reactivity has been modified by prior injection of a single dose of Cyclophosphamide, mouse cells induced the tumorous mass typical of graft-versus-host reaction in the kidneys of the recipients.

Mouse immunocompetent cells that were inactivated by irradiation in vitro or exposed to the effect of cytotoxic drugs in vivo lost the capacity to initiate the graft-versus-host reaction.

No reaction was initiated by mouse thymocytes into Cyclophosphamide treated rats.

Comment:

Graft-versus-host reactions have as their common basis, the direction of the immunological attack which precipitates the very complex immunopathological changes in the host. Simonsen[13,14] demonstrated that no such reaction can occur unless the grafted tissue contains immunologically competent cells, the host is incapable of completely destroying the graft which in turn reacts against the host antigens.

Billingham has shown that thymocytes have a very feeble ability to induce Runt disease. Elkins also showed that parental thymophocytes are

incapable of producing a virulent graft-versus-host reaction in F_1 hybrid rats.

The xenogeneic graft-versus-host reaction has fulfilled the criteria laid down by Simonsen i. e. it occurred when the injected lymphoid cells were viable, immunologically competent and the recipient is incapable of rejecting the injected cells.

Two other lines of evidence indicate that the reaction of mouse cells in rats is a graft-versus-host.

Firstly: The majority of the proliferating cells in the renal reaction were donor cells.

Secondly: The reaction was specifically inhibited by antilymphocyte sera raised against the donor cells. The reaction initiated by mouse lymphocytes could be inhibited by horse-anti-mouse lymphocyte serum, but not by horse anti-human lymphocyte serum. In addition reactions produced by human lymphocytes were inhibited by anti-human lymphocyte serum but not with anti-mouse lymphocyte serum. This capacity of horse-anti-mouse lymphocyte serum to inhibit reactions initiated by mouse cells could be removed by incubating the anti-serum with mouse but not with rat lymphocytes.

c. Chromosome analysis.

Method:

Three groups of Cyclophosphamide pretreated rats were injected with 50 X 10^6 mouse lymph node and spleen cells. The hosts were sacrificed on successive days starting on day four. Thirty minutes prior to their sacrifice, 0.75 mgm of Colchicine in 0.1 ml of normal saline was intraperitoneally injected into each host. The reaction tissue in each kidney was excised and minced with fine scissors. The resulting cell suspension was then processed according to the method of Nowell[74]. In Figures V and VI rat and mouse chromosomes are easily identifiable by the presence of x chromosomes in rat cells and its absence in mouse cells.

The results are shown in Table V.

Distribution of mouse and rat dividing cells in the cell suspensions prepared from the renal reactions.

Day of reaction.	Metaphase scored. Mouse	Rat	Total.	% of donor cells.
4	54	1	55	98
5	12	6	18	66
6	9	82	91	10

FIGURE V: Rat chromosomes.

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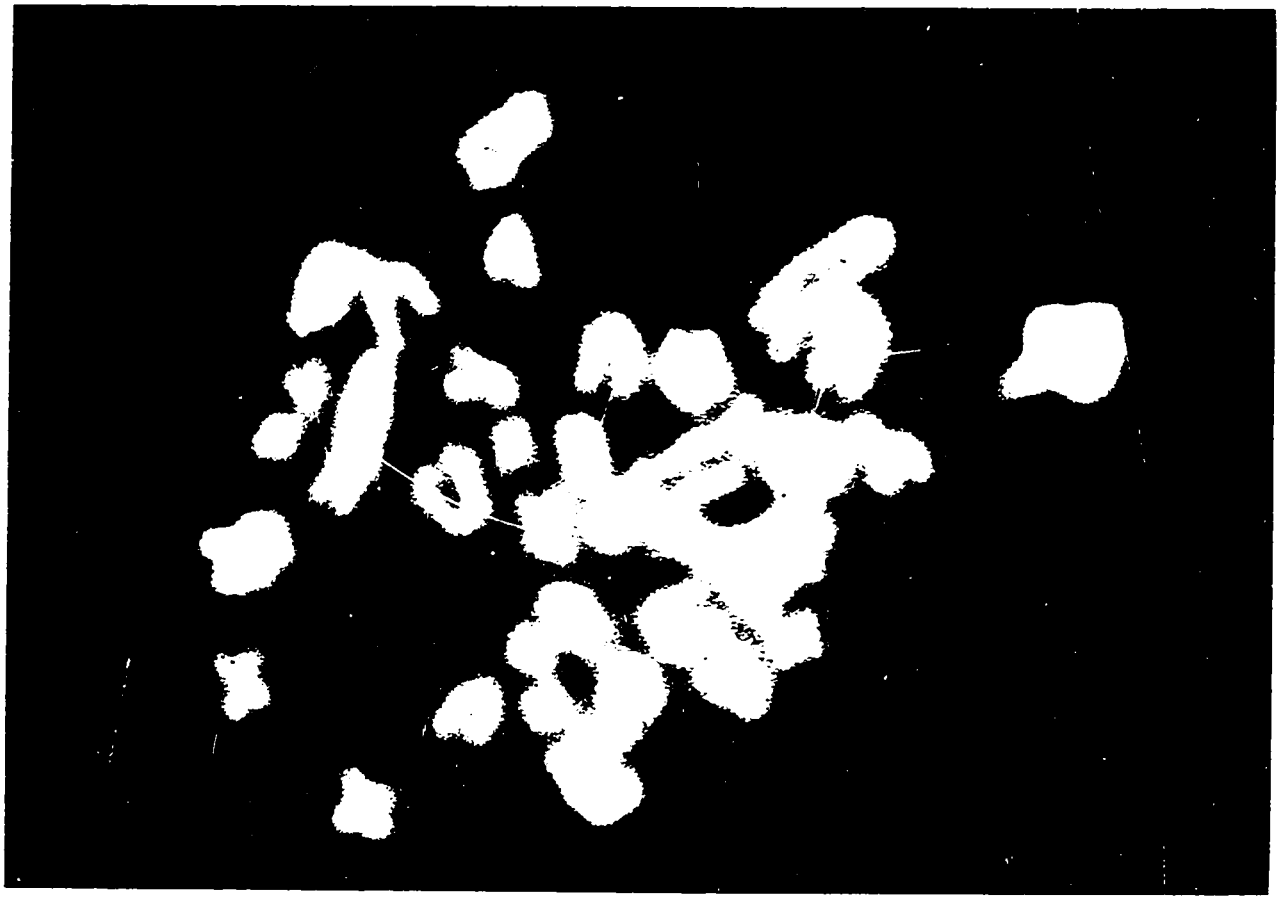

FIGURE VI: Mouse chromosomes.

FIGURE VI: Mouse chromosomes.

These results show that the majority of the proliferating cells in the early days of the reaction were donor cells, but on day six only 10% of the proliferating cells were of donor origin. Elkins[83] found that on the seventh day a reaction of 93% of the proliferating cells were of donor origin but by day fourteen the ratio dropped to 40%. These findings indicate that the reaction is initiated by the donor cells and support the observation by several investigators that "a striking feature of all cellular immune reactions is the high rate of proliferation of lymphoid cells participating in the process".

d. The donor cell dose response.

Elkins has studied the dose-response effect in two parental to F_1 hybrid combinations and found that renal graft-versus-host reactions can usually be observed with doses of five to ten million parental lymphoid cells when the hybrid host possesses a foreign Ag-B factor.

Fifty million lymphocytes gave a near maximum response but in parent to F_1 hybrid transfer where no Ag-B disparity existed fifty million parental cells did not induce graft-versus-host reactions.

In a xenogeneic combination it assumed that donor and recipient differ in all their major and minor histocompatibility antigens. A study of the cell dose response on the severity of the localized xenogeneic graft-versus-host reaction is shown.

Method:

Nine groups, each consisted of ten female rats were injected under the kidney capsule with lymph node and spleen cells from adult $C_{57}BL$ mice. The groups were given increasing doses of mouse lymphocytes from one to one hundred million cells. On the seventh day, the rats were sacrificed. Their kidneys and spleens were excised and weighed. The localized graft-versus-host reactions were grossly and histologically graded. The reactions were quantitated by the Ki/Kc ratio.

Results:

Are shown in Table VI. Illustrated in Figure VII.

TABLE VI: The donor mouse lymphocyte cell dose response. . $C_{57}BL$ mice.

No. of Rats.	Cell dose X 10^6	The Mean Ki/Kc ratio.*	95% confidence limits of the Mean.			S/Kc	Histological Grading O	I	II	III
9	1	1.022	0.9735	-	1.0705	1.452	5	4	0	0
9	5	1.077	1.0101	-	1.1439	1.552	6	3	0	0
6	10	1.294	1.1707	-	1.4173	1.294	0	0	1	5
9	20	1.3534	1.2033	-	1.5035	1.837	0	2	3	4
10	30	1.2970	1.1863	-	1.4077	1.636	0	1	1	8
10	40	1.288	1.1841	-	1.3919	1.700	0	2	2	6
10	50	1.300	1.2399	-	1.3461	1.835	0	0	1	8
10	100	1.380	1.2716	-	1.4884	1.890	0	1	1	8

* Ki/Kc ratio of weight of inoculated to that of control kidney.
* S/Kc ratio of weight of spleen to that of control kidney.

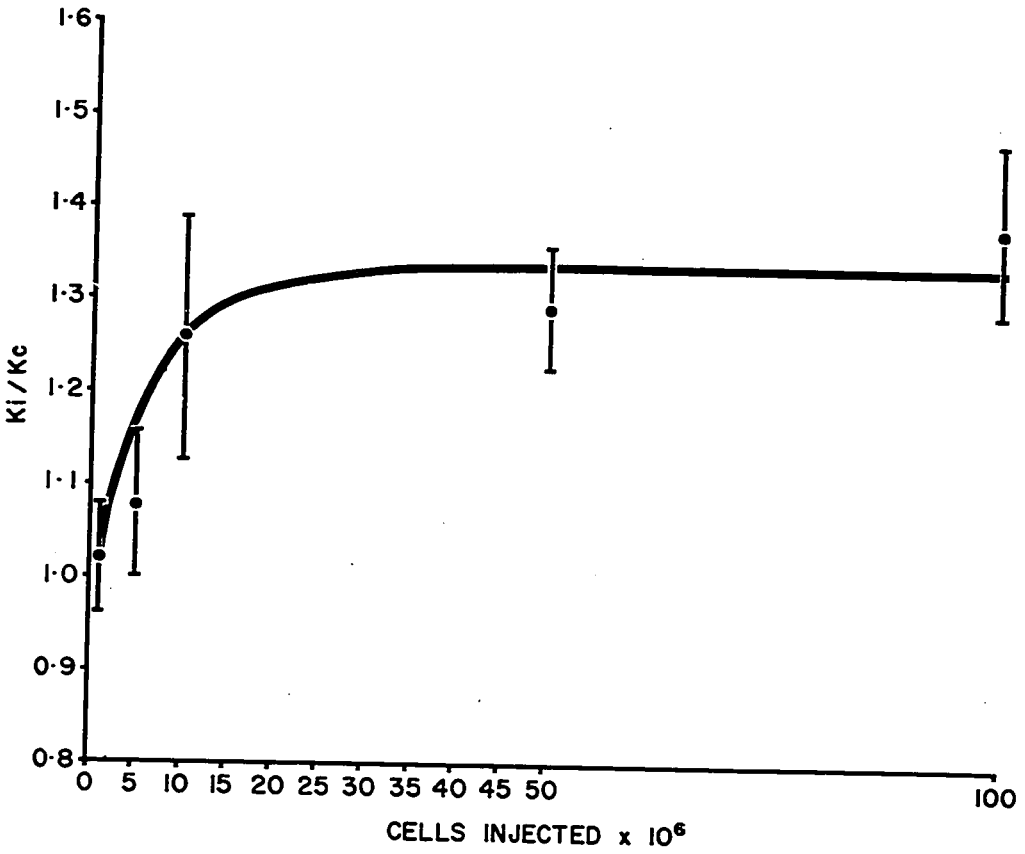

FIGURE VII: The effect of mouse lymphoid cell dose on the severity of the graft-versus-host reaction.

Comment:

The donor mouse cell dose response curve of this xenogeneic graft-versus-host reaction is similar to that demonstrated by Elkins[82] in the parent to F_1 hybrid graft-versus-host reaction. Cell doses lower than ten million lymphoid cells were incapable of producing a reaction. Mouse and human lymphocytes in doses of 20×10^6 cells or higher, consistently gave grade III reactions. Cell doses between ten and twenty million lymphoid cells frequently produced good reactions.

e). Chronology.

In the parent to F_1 hybrid transfer, Elkins [83] found that the injected cells started their activity and invasion of the host kidney on the third day. The graft-versus-host reaction was optimal at day seven, but by the fourteenth day Elkins noticed that the cellular infiltrate showed signs of diminishing activity. By the forty-second day, none of the injected F_1 hybrid kidneys showed the classical graft-versus-host reaction. On the contrary there was a saucer-like cortical atrophy at the site of cell inoculations.

In the xenogeneic graft-versus-host reaction one expects that the host recovers rapidly from the immunosuppressive effect of Cyclophosphamide with subsequent diminution of the activity of the donor cells.

The following experiments were done to study the chronology of the xenogeneic graft-versus-host reaction.

Method:

Ten groups of Cyclophosphamide pretreated rats were injected with lymph node and spleen cells from adult $C_{57}BL$ mice. Each group consisted of ten rats and five groups were completed in each of two experiments.

One group of rats was sacrificed daily starting on day three. In each of the two experiments a group of rats was sacrificed at day six and day seven. The mean Ki/Kc ratio and its 95% confidence limits, as

well as the spleen to the Kc ratio and histologic grading is shown in Table VII. and FIGURE VIII.

TABLE VII: The chronology of the xenogeneic graft-versus-host reaction.

No. of Rats.	Day of Reaction.	Mean Ki/Kc ratio.	95% C. L. of the Mean.	Mean S/Kc.	Histologic grading. O	I	II	III
A 9	3	1.0900	1.0325 - 1.1475	1.0114	O	1	O	8
B 9	4	1.1422	1.1032 - 1.1812	O.5119	O	O	2	7
C 9	5	1.2217	1.1719 - 1.2715	1.1790	O	O	1	8
D 9	6	1.2677	1.1894 - 1.3460	1.5604	O	1	4	5
E 9	6	1.3570	1.2549 - 1.4591	1.4838	O	O	1	8
F 8	7	1.2247	1.1590 - 1.2892	1.5675	O	O	2	6
G 10	7	1.2015	1.1112 - 1.2918	1.7210	O	O	2	8
H 10	8	1.1395	1.0867 - 1.1933	2.2423	1	1	2	6
I 8	9	1.0287	O.9782 - 1.0792	2.0488	O	2	3	3
J 8	10	1.0516	O.9560 - 1.0980	1.9552	1	4	3	2

*the normal Ki/Kc ratio for the rats used in these experiments O.9790

(O.9544-1.003)

The results are also illustrated in Figure IX and show that invasive reactions are discernible from the third day and progressively increase in vigor. They are optimal on the sixth day and subsequently diminish gradually in activity.

The majority of the dividing cells in the day four and five reaction were donor cells but in the day six reaction, the majority of the proliferating cells were host cells.

Comment:

This study of the xenogeneic graft-versus-host reaction suggests that in the early days (i. e. three to six) , the direction of the reaction is graft-versus-host but in the later part i. e. six to ten, the direction is host versus graft.

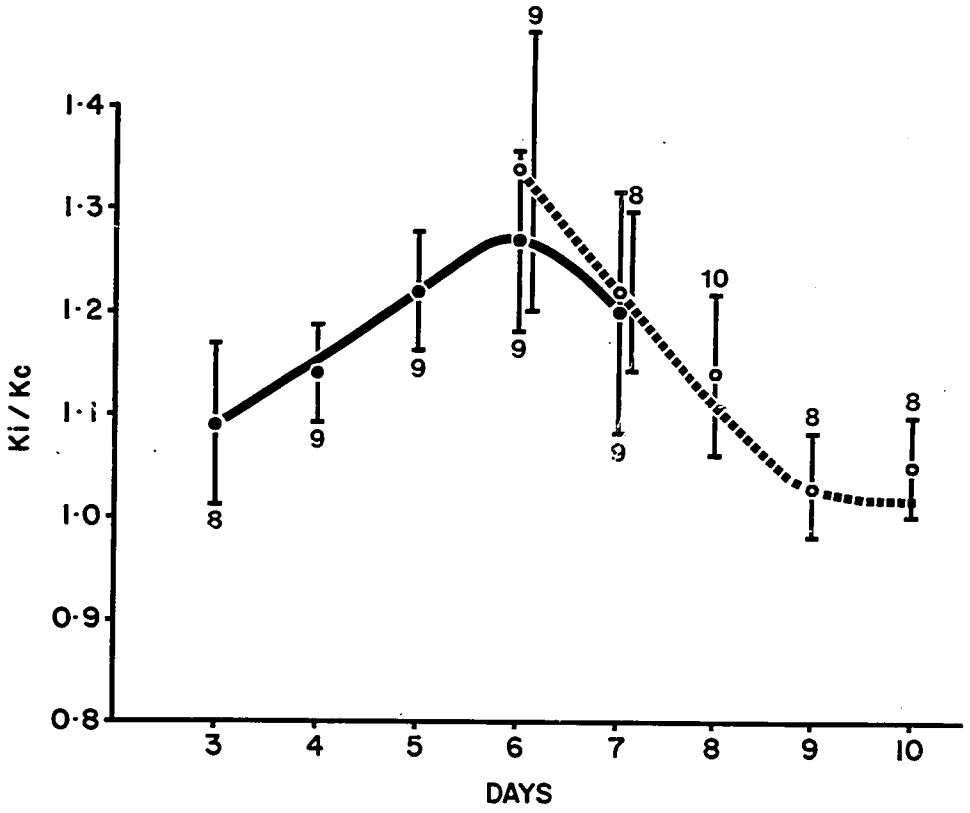

FIGURE VIII: The results of the two experiments are shown (dotted and solid lines): 50 X 10^6 mouse lymphocytes were injected into each rat on day O. Groups of rats, the number per group is indicated on the graph, were sacrificed starting on day three. The mean Ki/Kc ratio and 95% C. L. per group are shown in the graph.

f. Inhibition by antilymphocyte sera.

Introduction:

Monaco, Russell [135,136] and their associates were the first to show that anti-mouse lymphocyte sera inhibited the localized graft-versus-host reaction that occurs when parental mouse lymphoid cells are injected under the kidney capsule of F_1 hybrid mice.

They injected the rabbit anti-mouse lymphocyte serum into the parental donor mice prior to transfering their lymphocytes to the F_1 hybrid mice recipients.

Boak, Fox and Wilson [131,132] have also treated the parental mice with rabbit anti-mouse lymphocyte serum perior to transfering their lymphoid cells into F_1 hybrid recipients and demonstrated inhibition of graft-versus-host reaction.

Sauvette [133] and his associates treated the neonatal hosts with anti-mouse lymphocyte serum after their inoculation with adult allogeneic cells. In this situation, antilymphocyte serum presumably acts on both the donor and host components of the graft-versus-host reaction.

The experiments described in this section were planned to study the effect of antilymphocyte sera on the xenogeneic graft-versus-host reaction.

Preparation of Anti-mouse lymphocyte plasma:

A. Rabbit anti-mouse lymphocyte plasma.

200 X 10^6 lymph node and thymus cells from adult male CBA and C_{57} BL mice were injected intravenously once weekly into New Zealand rabbits. A total of three injections of mouse lymphocytes were given for each rabbit, followed in one week after the last injection by exsanguinating the heparinized rabbits via cannulating the carotid artery.

B. Rat anti-mouse lymphocyte plasma.

Lewis rats were bled after a three weekly intraperitoneal injection of 100 X 10^6 mouse and lymph node thymus cells.

C. Horse anti-mouse (anti C_{57}BL) lymphocyte sera.

This was prepared by Doctor G. Lamourex of the Institute of Microbiology and Hygiene of the University of Montreal.

All the sera and plasma were kept frozen until used.

In these preliminary experiments, the anti-mouse lymphocyte plasmas were injected intraperitoneally into rats that were subsequently used as recipients of mouse cells. Antisera was given in three daily injections, beginning one day prior to injection of mouse cells (day O). Fifty percent, 25% and 25% of the serum dose was given to the recipients at day - one, day O and day+ one respectively. Day O, is the day of injection of lymphocytes under the kidney capsule. Portions of the horse

anti-mouse lymphocyte sera were absorbed with mouse or rat lymphocytes. Five millilitres of serum were incubated for forty-five minutes at 37°C with 4 X 10^9 mouse or rat lymphocytes. The sera cleared by centrifugation were used according to the injection schedule described.

Plan of Experiment:

CBA and C_{57}BL mice were used as donors of lymphoid cells depending on whether the anti-mouse serum used was anti CBA or anti C_{57}BL strain mice.

The experimental group was injected with anti-mouse lymphocyte serum and the control group with equal amounts of normal rabbit or horse plasma.

The amount of antilymphocyte serum injected into each recipient were large and the dosage schedule was arbitrarily chosen to assure the presence of anti-serum immediately prior, during and following the injection of mouse cells. Sera absorbed with mouse and rat lymphoid cells were also tested in the same dosage. Groups of Cyclophosphamide pretreated rats were each injected with 50 X 10^6 mouse cells.

The hosts were sacrificed on the seventh day, and the kidneys were weighed, grossly and histologically examined. The results are shown in Tables VIII and IX.

TABLE VIII: The inhibition of xenogeneic graft-versus-host reaction by antilymphocyte plasma.

No of recipients.	Mouse cells injected $_{-6}$ No X 10	Plasma.	Mean Ki/Kc and 95 C. L.	S. Kc.	Histological grading. O	I	II	III
9	50	Normal horse plasma* (NHP*)	1.4653(1.3198-1.6108)	1.230	O	O	O	9
11	50	Horse anti-mouse lymphocyte plasma (HAMLP*)	1.0373(0.9950-1.0796)	1.046	9	I	I	O
8	50	Normal rabbit plasma (NRP*)	1.580(1.1722-2.0038)	1.3207	O	O	I	7
7	50	Rabbit anti-mouse lymphocyte plasma (RAMLP*)	1.0280(0.9092-1.0052)	1.077	7	O	I	O
11	30	Rat anti-mouse lymphocyte plasma**	1.1387(1.0913-1.1861	1.0126	O	2	3	6
5	30	Control	1.3259(1.1800-1.4718)	1.3259	O	O	O	5

* Dose of NHP and HAMLP, NRP and RAMLP were O.8, O.5, O.5 ml at day -one, O and + one respectively.

** Dose of rat anti-mouse lymphocyte plasma was O.5, O.5 ml at day O and day + one.

TABLE VIIII: Absorption of horse anti-mouse lymphocyte plasma (HAMLP) with cells.

No of recipients.	Absorption of HAMLP.	Mean Ki/Kc** (95% C. L)	S/Kc	Histologic grading O	I	II	III.
10	No HAMLP given	1. 2096 (1. 1577-1. 2615)	0. 8468	0	0	1	9
10	HAMLP* not absorbed.	1. 1104(1. 0482-1. 1726)	1. 0401	0	7	2	1
9	1 ml of HAMLP* with 800 X 10^6 mouse lymphocytes.	1. 194(1. 1366-1. 2516	0. 9838	0	1	1	7
4	No HAMLP given.	1. 2152 (1. 0813-1. 3491)	0. 6236	0	0	0	4
10	1 ml of HAMLP* with 100 X 10^6 mouse lymphocytes.	1. 0890(1. 0623-1. 1157)	0. 939	0	8	2	0
10	1 ml of HAMLP* in 800 X 10^6 rat lymphocytes.	1. 0907(1. 0457-1. 1357)	0. 879	0	7	3	0
6	No HAMLP given.	1. 3259(1. 1800-1. 4718)	1. 3259	0	0	0	6

* Dose of HAMLP

0. 25 ml day -one
0. 125 ml day 0
0. 125 ml day + one.

** Donor cell dose 30 X 10^6 mouse lymphocytes.

This showed that rabbit anti-mouse lymphocyte plasma and horse anti-mouse lymphocyte plasma when injected into the recipients inhibited the development of the xenogeneic graft-versus-host reaction in the rat kidney. The mean Ki/Kc ratio was O.96 (O.91-1.OO) and 1.O4(O.99-1.O8) respectively. Gross examination of the kidneys that were inoculated with mouse cells showed absence of the tumorous mass, characteristic of the localized graft-versus-host reaction. However the renal capsule overlying the deposited mouse lymphoid cells were thickened, opaque and whitish in colour, histologically. There was a thin rim of lymphocytes, histiocytes and fibroblasts overlying the renal parenchymal and is separated from it with a distinct demarcation line. These lymphocytes showed no mitotic activity nor any tendency to invade the underlying kidney as is shown in Figure X.

The control groups injected with normal rabbit serum and normal horse serum showed the classical tumorous mass of a graft-versus-host reaction. Figure IX.

The capacity of horse anti-mouse lymphocyte serum to abolish the xenogeneic graft-versus-host reaction was abolished by incubation with mouse lymphoid cells but not with rat cells. Table IX.

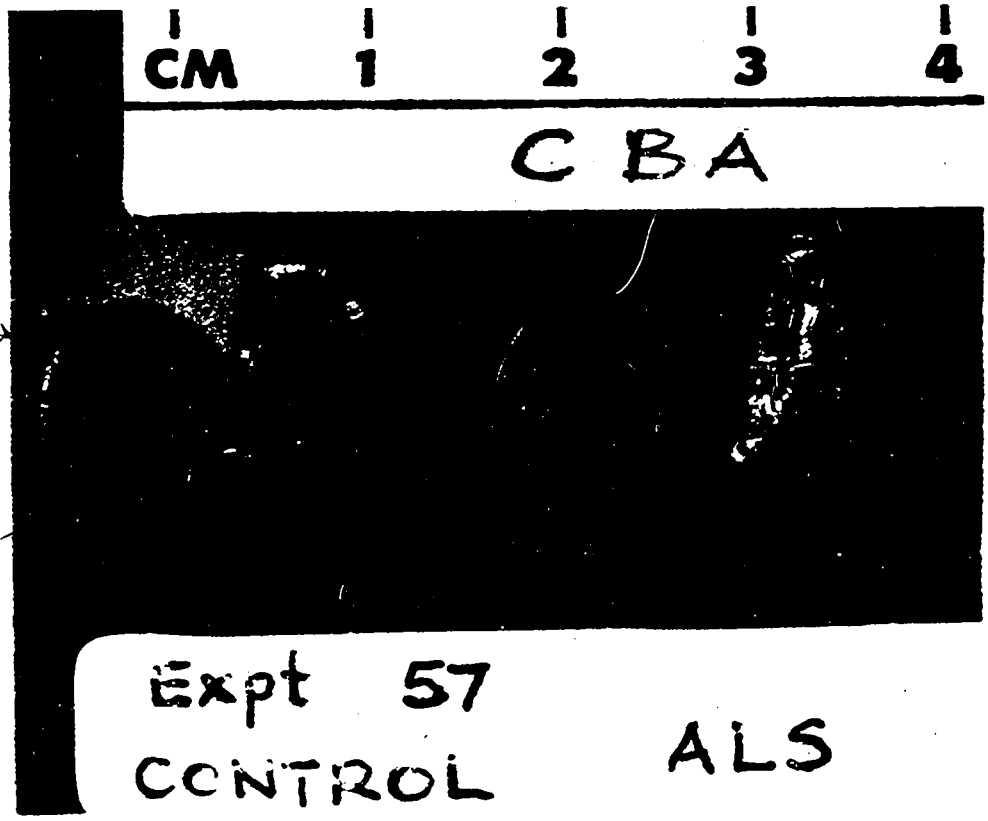

FIGURE IX: Bisected rat kidneys that were injected with CBA mouse lymph node and spleen cells. The kidney from the group treated with normal horse plasma(control) shows the tumorous mass pathognomic of graft-versus-host reaction. The reaction was inhibited by the administration of horse anti-mouse lymphocyte plasma.

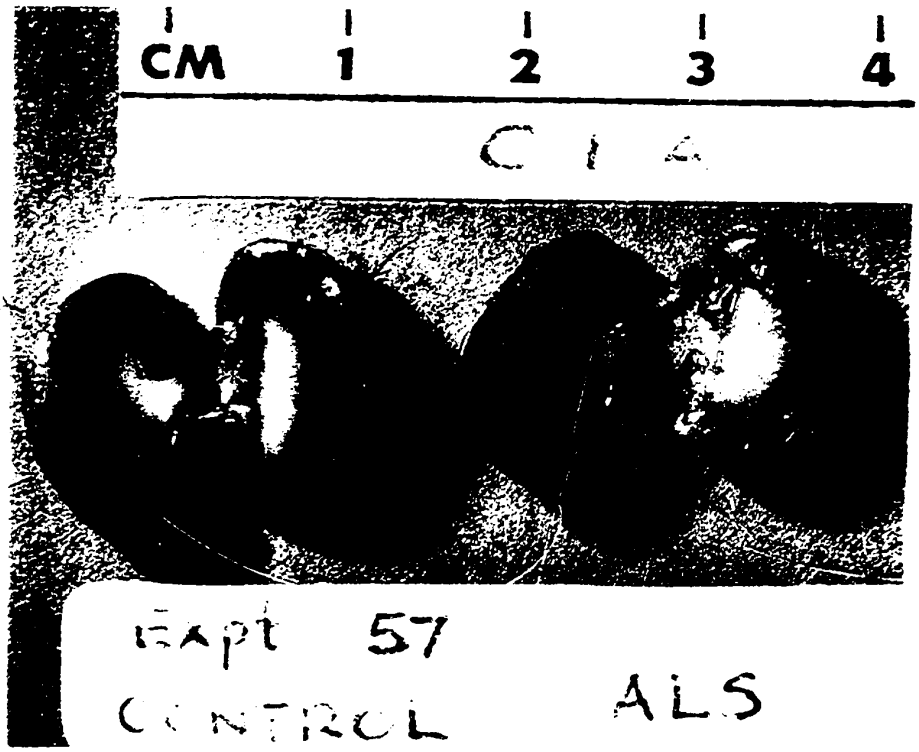

FIGURE IX: Bisected rat kidneys that were injected with CBA mouse lymph node and spleen cells. The kidney from the group treated with normal horse plasma(control) shows the tumorous mass pathognomic of graft-versus-host reaction. The reaction was inhibited by the administration of horse anti-mouse lymphocyte plasma.

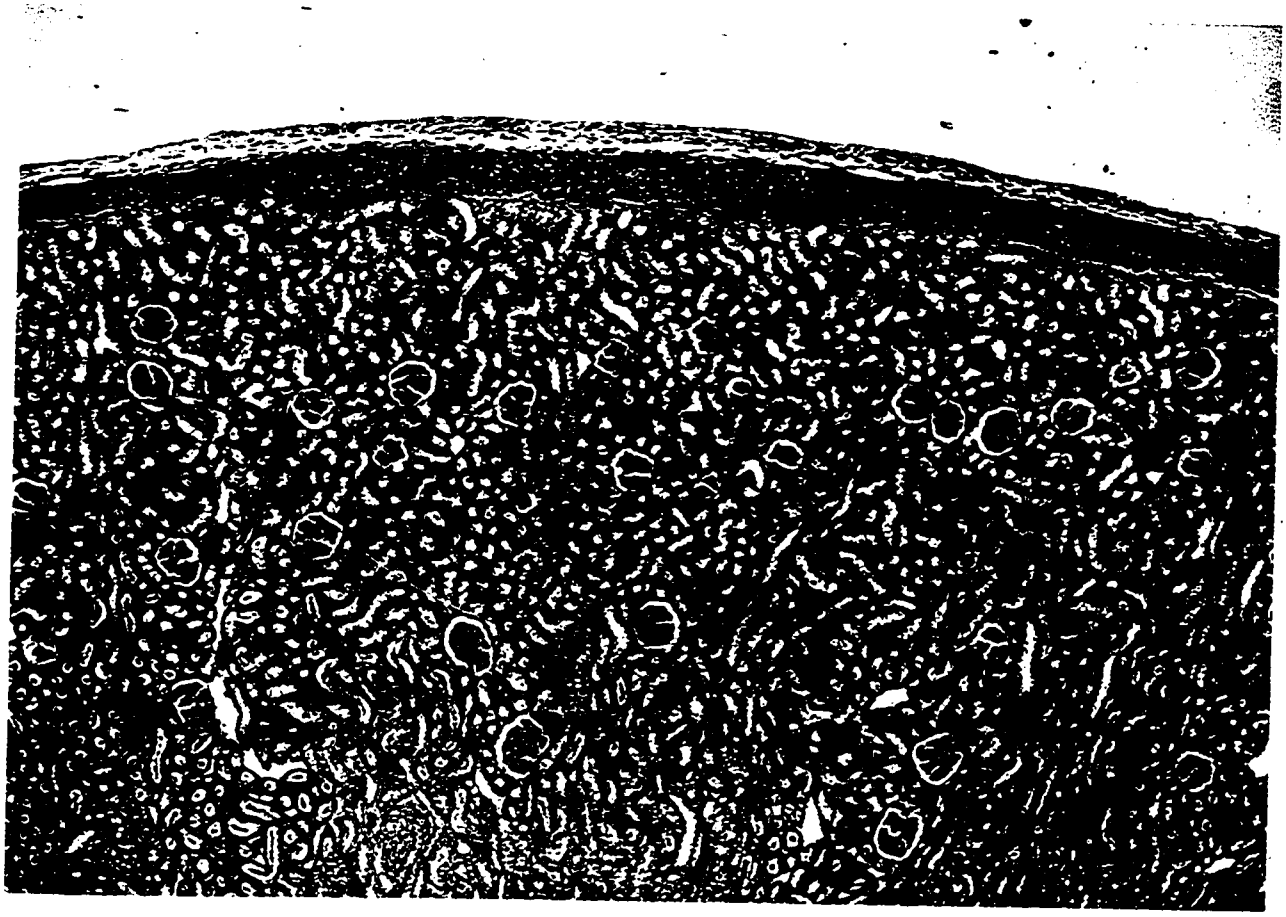

FIGURE X: Histological section of a kidney from a rat treated with anti-mouse lymphocyte serum. The mouse cells that were injected under the kidney capsule show no activity nor any tendency to invade the underlying renal cortex. Note the sharp line of demarcation between the rat kidney and the mouse cells. The renal capsule is also thickened. Hematoxylin and eosin X50.

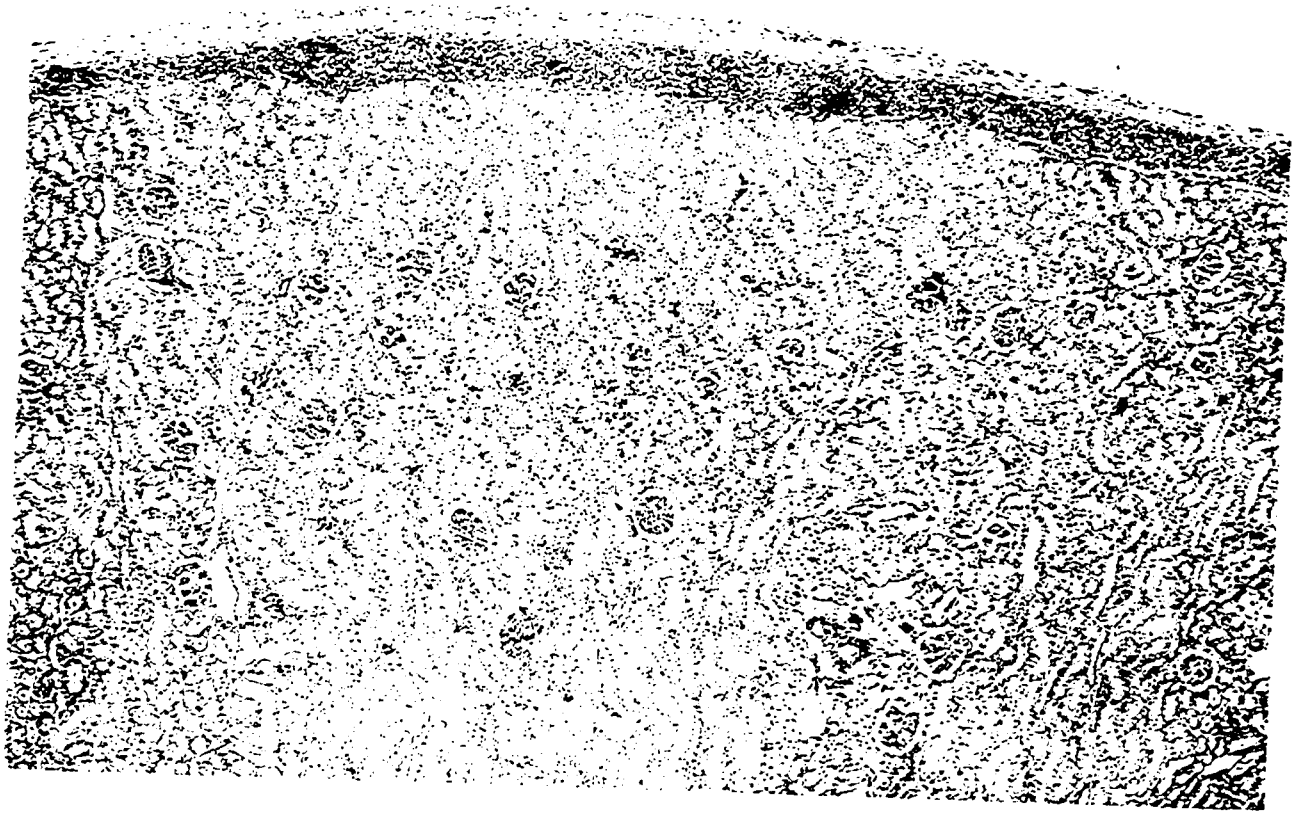

FIGURE X: Histological section of a kidney from a rat treated with anti-mouse lymphocyte serum. The mouse cells that were injected under the kidney capsule show no activity nor any tendency to invade the underlying renal cortex. Note the sharp line of demarcation between the rat kidney and the mouse cells. The renal capsule is also thickened. Hematoxylin and eosin X50.

CORRELATION BETWEEN CAPACITY OF HORSE ANTI-MOUSE LYMPHOCYTE SERA TO INHIBIT THE XENOGENEIC GRAFT - VERSUS-HOST REACTION AND THEIR ABILITY TO PROLONG SURVIVAL OF ALLOGRAFTS.

Introduction:

The evidence presented thus far strongly suggested that this xenogeneic graft-versus-host reaction was initiated by mouse lymphoid cells in rats. The reaction reflected the immunological activity of the grafted immunocompetent mouse cells and was specifically inhibited by anti-sera directed against the donor cells. In order to use this graft-versus-host reaction in a model for testing the immunosuppressive potency of anti-lymphocyte sera, one needs to show, Firstly: - that there is a quantitative correlation between the dose of immunocompetent cells producing a certain reaction and the amount of anti-lymphocyte serum required to inhibit it. Secondly: to demonstrate a correlation between the capacity of anti-lymphocyte serum to inhibit this xenogeneic graft-versus-host reaction and its ability to prolong survival of allografts.

Method:

A. Groups of rats were injected with graded doses of mouse lymphocytes and were treated with constant doses of horse anti-mouse lymphocyte serum.

CHAPTER IX

B. Groups of rats were injected with constant doses of mouse lymphocytes but were given varying doses of anti-lymphocyte serum.

The results are presented in Table XI and demonstrate a correlation between dose of mouse lymphocytes producing a graft-versus-host reaction and the amount of anti-serum required to inhibit them.

TABLE XI: The relationship between the cell dose producing a graft-versus-host reaction and the amount of anti-lymphocyte serum required to inhibit it.

No of Rats.	Cell dose $X10^{-6}$	HAMLS* dose in ml.	Mean Ki/Kc	95% C. L.	Histologic grading O	I	II	III
8	20	Nil	1.264	(1.2003-1.3277)	O	O	I	7
9	20	O.5	1.104	(1.0370-1.1710)	O	2	3	4
10	30	O.5	1.100	(1.0616-1.1384)	O	3	2	5
10	40	O.5	1.219	(1.138-1.300)	O	O	1	9
10	50	O.5	1.226	(1.1198-1.3322)	O	O	O	10
8	50	Nil	1.335	(1.2666-1.4034)	O	O	O	8
9	30	Nil	1.1888	(1.1566-1.2526)	O	O	1	8
9	30	O.25**	O.9941	(O.9698-1.0184)	O	9	O	O
6	30	O.1	1.0962	(1.0317-1.1607)	O	2	O	4
7	30	O.05	1.0668	(1.0320-1.1016)	O	5	O	2
9	30	O.03	1.1617	(1.0349-1.2885)		2	3	4

* Horse anti-mouse lymphocyte serum pool 921-22
** Horse anti-mouse lymphocyte serum pool B
The serum was given in three daily doses beginning at day -one.

CORRELATION BETWEEN PROLONGATION OF SKIN ALLOGRAFT SURVIVAL AND INHIBITION OF THE XENOGENEIC GRAFT-VERSUS-HOST REACTION BY ANTI-LYMPHOCYTE SERA.

Introduction:

Antilymphocyte serum (ALS), antilymphocyte plasma (ALP) and antilymphocytic globulin (ALG) contain a great variety of antibodies, only some of which possess immunosuppressive potency (ISP), i. e. capacity to suppress cell mediated immune reactions measured as prolongation of allograft survival. Jeejeebhoy[159,160] has shown in rodents that the immunosuppressive potency of a heterologous anti-lymphocytic plasma is related to factors other than its content of agglutinating and cytotoxic antibodies directed against lymphocytes. Jeejeebhoy's observations were confirmed by many investigators[161,162] and it is now generally held that the ability of ALS or ALP to agglutinate and lyse lymphocytes in vitro does not reliably measure or predict its immunosuppressive potency.

Method:

Eight horse anti-mouse lymphocyte serum (HAMLS) preparations, six individual sera and two serum pools were studied. All these horse sera were prepared and characterized by Doctor Gilles Lamoureux of The Institut de Microbiologie et Hygiene, University de Montreal. The leuko-agglutinin and cytotoxic titers of these sera were determined by

Doctor Lamoureux according to the method of Gray, Monaco, Wood and Russell [163]. The capacity of each serum to prolong skin allograft survival was measured in $C_{57}BL$ mice carrying CBA skin grafts. Each mouse was given 2 ml of HAMLS in four equal doses on day O, three, six and nine.

The eight HAMLS were tested, for their capacity to inhibit the reactions produced by mouse lymphocytes in rats. Two sera were tested in each of four experiments. In each experiment, three groups of Cyclophosphamide treated rats that were each injected 30×10^6 mouse cells. The HAMLS to be tested were injected into the rats in three divided doses O.25, O.125 and O.125 ml given at day -one, day O, and day + one.

Day O is the day of injection of mouse lymphocytes. Two groups were injected with HAMLS and a control not injected with ALS.

On day six the rats were sacrificed and the results are shown in Table XII.

TABLE XII: Capacity of eight anti-mouse serum preparations to inhibit the xenogeneic graft-versus-host reaction, prolong skin allograft survival and to agglutinate or lyse lymphocytes in vitro.

No. of Rats.	HAMLS No.	Titers. Agglutinin	Cytotoxin	Median skin* allograft survival in days.	Mean Ki/Kc 95% C. L.	Histologic grading O	I	II	III
7	control				1.2429(1.1680-1.3178)	O	O	O	7
14	Pool A	128	81	12	1.2381(1.1860-1.2902)	O	2	2	10
12	Pool B	1.024	243	22	1.0088(0.9828-1.0348)	O	11	O	1
10	control				1.3007(1.1742-1.3542)	O	1	O	9
12	229	256	81	13	1.2573(1.210-1.310)	O	O	O	12
12	232	512	81	24	0.9788(0.960-1.008)	O	11	1	O
8	control				1.1948(1.1148-1.2748)	O	1	O	7
11	230	256	243	11	1.1634(1.1011-12255)	O	2	O	9
11	233	4096	243	20	1.0334(1.0077-1.0591)	O	10	1	O
9	control				1.2360(1.1905-1.2815)	O	O	O	9
12	234	256	243	20	1.0408(0.9862-1.0954)	O	8	2	2
9	231	128	243	12	1.2180(1.1529-1.2831)	O	1	1	7

* The median skin allograft survival on control mice injected with normal horse serum was 9.5 ± 1.6 days.

Comment:

It is evident that the antibody content of the eight horse anti-mouse lymphocyte serum preparations measured as agglutinins and cytotoxins did not clearly reflect their immunosuppressive potency measured in terms of prolongation of skin allograft survival. The four antilymphocyte serum preparations Pool B, 232, 233, 234 which prolonged the survival of test skin allografts in mice to beyond twenty days did inhibit the capacity of mouse lymphocytes to invade and destroy the renal parenchyma of Cyclophosphamide treated rats. On the other hand, the other four antiserum preparations Pool A, 229, 230, 231, which marginally prolonged skin allograft survival did not inhibit the development of the reaction of mouse cells in rats. See Figure XI.

Subsequent experiments, see Tables XI and XIII revealed that as little as 0.05 ml of the immunosuppressive serum, Pool B, was sufficient to completely abolish the xenogeneic graft-versus-host reaction but 2 ml of Pool A were required to achieve similar inhibitions.

On the basis of these results it is concluded that the capacity of anti-mouse lymphocyte sera to inhibit the reaction of mouse cells in rats correlated with their immunosuppressive potency.

TABLE XIII: Comparison between Pool A and Pool B of HAMLS.

No of Rats.	HAMLS*	Mean Ki/Kc 95% C. L)	Histologic grading O	I	II	III.
6	Nil	1.2906 (1.1259-1.4556)	O	I	I	4
12	2 ml of Pool A.	1.0499(1.0050-1.0948)	O	9	2	1
11	1 ml of Pool A.	1.112 (1.0633-1.591)	O	4	1	6
9	O.25 of Pool B.	O.9941(O.9698-1.0184)	O	9	O	O

* Horse anti-mouse lymphocyte serum.

CHAPTER X

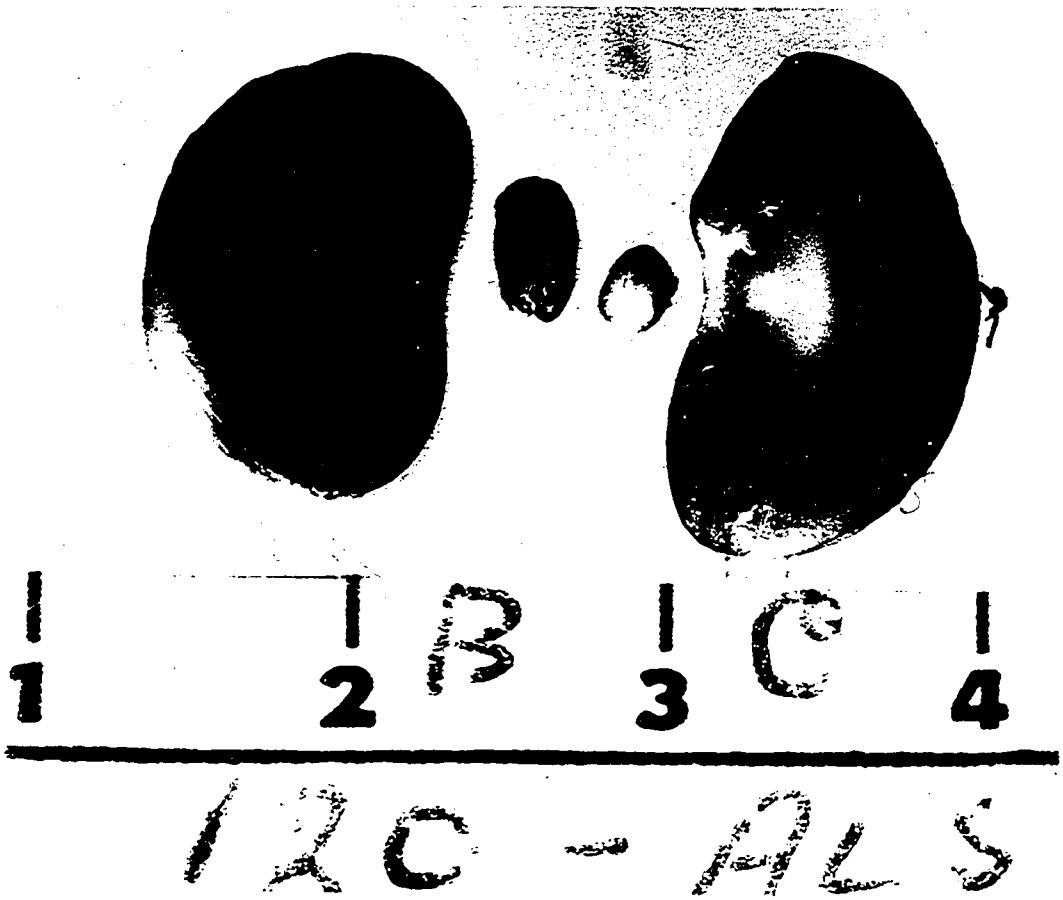

FIGURE XI:

Rat kidneys and their regional lymph nodes. Both were injected with mouse cells and anti-mouse lymphocyte sera. The kidney on the left hand side is from the group treated with Pool A, of HAMLS, and shows the presence of typical tumorous reaction.

The kidney on the right hand side was from the group treated with Pool B of HAMLS and shows inhibition of reaction - compare with Table XII.

GRAFT-VERSUS-HOST REACTIONS INITIATED BY HUMAN IMMUNOCOMPETENT CELLS IN RATS.

Introduction:

The reaction of mouse lymphocytes in Cyclophosphamide treated rats was readily applicable to human lymphocytes. Human spleen lymphocytes were obtained in emergency situations and the interval between the injection of Cyclophosphamide and the injection of lymphocytes in the rats was at least four hours which is the minimal time required for the disappearance of Cyclophosphamide from the host circulation [33,34].

Human peripheral lymphocytes were obtained from human volunteers and rats were injected with these lymphocytes twenty-four hours after injection of Cyclophosphamide.

Human lymphocytes when injected under the kidney capsule of Cyclophosphamide pretreated rats produced the classical tumorous reactions seen in Figures I, II which were grossly and histologically indistinguishable from those produced by mouse cells. The reactions were evaluated in a manner identical to that described for mouse cells. The results are presented in Tables XIIII - XXI.

The experience with human lymphocytes in rats were similar to that of mouse cells.

The experiment summarized in Table XIIII shows that as with mouse to rat system no reaction occurred when the injected human

lymphocytes were dead and the hosts were not depressed by prior injection of Cyclophosphamide. The reaction was initiated by human immunocompetent cells in Cyclophosphamide treated rats, the optimal dose of Cyclophosphamide seemed to be about 100 mgm/kgm and the reactions did not develop when Cyclophosphamide in doses of 200 mgm/kgm was given to the hosts.

The experiment in Table XV shows that no reaction developed when only ten million spleen cells were injected. The reactions produced by fifty million human spleen cells were inhibited by anti-human lymphocyte sera.

The two experiments shown in Table XVI demonstrates the correlation between the dose of human spleen cells producing a graft-versus-host reaction and the amount of anti-human lymphocyte serum required to inhibit them. The antilymphocyte serum preparation used was capable of inhibiting the reactions produced by human spleen cells only when given in a total dose of 1 ml or higher. A dose of 0.5 ml or lower of antiserum preparation did not inhibit the reaction.

In Table XVII, three experiments are presented, each experiment has its own control group of rats not treated with antiserum. In the first experiment three sera were tested and all inhibited the graft-versus-host reaction, but as is seen from the control group the reactions were weak and the amount of antiserum used was relatively large. The second

experiment shows good reactions which were not completely inhibited by Lot 8 of horse anti-human lymphocyte serum. The third experiment illustrates two points. Firstly: Lot 3 of horse anti-human lymphocyte serum has very little activity as compared to the serum labelled (R. V. H.). Secondly: The specificity of inhibition of the reaction by AHLS. The very potent Pool B of anti-mouse lymphocyte serum had no inhibitory effect on reactions produced by human cells.

Human peripheral lymphocytes consistently produced the xenogeneic graft-versus-host reaction in rats. The rats were injected with Cyclophosphamide twenty-four hours previously and the experiments were done under elective conditions.

The three experiments shown in Table XVIII demonstrated the cell dose response of human peripheral lymphocytes and the inhibition of the graft-versus-host reaction by anti-human lymphocyte sera.

Table XVIIII shows the results of preliminary testing of four anti-human lymphocyte sera. Lots 8 and 11 were used in a high dose since previous testing showed that they did not abolish the reaction when only 10 mgm of IgG was used. An impression is gained that these two lots of sera has a low immunosuppressive potency.

The serum labelled MRC - Pool 3 was capable of inhibiting the xenogeneic graft-versus-host reaction when given in 0.25 ml doses.

Comment:

One would not try to correlate the capacity of anti-human lymphocyte sera to inhibit the xenogeneic graft-versus-host reaction and their ability to prolong allograft survival in man. However in view of the fact that the reaction is applicable to both murine and human immunocompetent cells, that with mouse lymphocytes the inhibition of the reaction by anti-mouse lymphocyte sera correlated with their immunosuppressive potency, it would seem reasonable to assume that it would also be predictive of the immunosuppressive potency of anti-human lymphocyte sera.

TABLE XIIII: Reaction of human spleen cells in rats.

No of Rats.	CY* dose.	Day of sacrifice.	Mean Ki/Kc.	Histologic grading . O	I	II	III
10	Nil	-	0.9582	8	2	0	0
10	50	7	1.1778	0	1	1	8
10	75	7	1.2278	0	0	1	9
10	100	7	1.2176	0	0	1	9
9	125	10	1.1436	0	1	4	4
7	150	11	1.2175	0	0	3	4
10	200	7	0.9728	6	4	0	0
8	50	7	1.0235 **	8	0	0	0

* Cyclophosphamide dose in milligrams per kilogram body weight of rats.

** Human cadaver spleen cells.

TABLE XV: Reaction of human spleen cells in rats.

No of Rats.	Cell dose $X10^{-6}$	AHLS.	Mean Ki/Kc (95% C. L.)	Histologic grading. O	I	II	III
8	10	Nil	1.042(0.978-1.106)	2	5	1	0
9	25	Nil	1.130(1.069-1.1910)	0	2	3	4
7	50	Nil	1.210(1.0931-1.3269)	0	1	0	6
7	50	2 ml *	1.0461(1.0018-1.0804)	0	0	3	4
4	50	2 ml **	1.065(0.9316-1.2032)	0	0	0	4
5	50	2 ml ***	1.0940(1.0529-1.1351)	0	5	0	0

* Rabbit anti-human lymphocyte serum. A
** Rabbit anti-human lymphocyte serum. B
*** Human anti-human lymphocyte serum.

The antilymphocyte sera were given in two equal doses at day O and day +one/
Cyclophosphamide dose 50 mgm/kgm.

TABLE XVI: Correlation between the cell dose* and the amount of antilymphocyte serum required to inhibit them.

No of Rats.	AHLS*** dose.	Mean Ki/Kc (95% C. L.)	Histologic grading. O	I	II	III
8	Nil	1. 1875(1. 1264-1. 2486)	O	O	1	7
10	2 ml	1. 0477(1. 0152-1. 0802)	O	4	3	3
7	1 ml	0. 9998(0. 9755-1. 0241)	O	7	O	1
9	0. 5 ml	1. 1433 (1. 1000-1. 1867)	O	O	5	4
10	Nil	1. 1710(1. 1032-1. 2388)	O	1	4	5
9	1 ml	1. 0788(1. 0174-1. 1402)	O	3	3	3
9	0. 5 ml	1. 1043(1. 0551-1. 1535)	O	5	4	1
10	0. 25 ml	1. 0914(1. 0315-1. 1513)	O	4	2	5

* Cell dose 50X 10^6 human spleen cells.
Cyclophosphamide dose 50 mgm/kgm 4 hours prior to injection of cells.

*** Human anti-human lymphocyte serum given in two equal doses at day O and day + one.

TABLE XVII: Reaction of human spleen cells in rats.

No of Rats.	AHLS*	Dose.	Mean Ki/Kc (95% C.L.)	Histologic grading O	I	II	III
7	Nil		1.1379(1.0336-1.2322)				
9	Lot*6	10 mgm	1.0341(0.9695-1.0987)				
8	Lot 8	10 mgm	1.0244(0.9803-1.0685)				
9	HAHLS**	1 ml	1.0098(0.9588-1.0608)				
8	control		1.2247(1.1485-1.3004)				
7	Lot 8	10 mg	1.1569(1.1293-1.1875)				
13	control		1.1760(1.1296-1.2224)	0	1	1	11
10	Lot 3	0.5 ml	1.1718(1.1234-1.2188)	0	0	5	5
8	R.V.H.****	0.5 ml	1.0841(1.0392-1.1302)	0	7	0	1
9	HAMLS**** Pool B	0.25 ml	1.1876(1.1372-1.2380)	0	1	2	6

50 X 10^6 human spleen cells 4 hours after injection of Cyclophosphamide.

* horse anti-human lymphocyte sera.

** human anti-human lymphocyte sera.

*** horse anti-mouse lymphocyte serum Pool B.

R.V.H. horse anti-human lymphocyte serum used clinically at the Royal Victoria Hospital.

TABLE XVIII: Reaction of human peripheral lymphocytes in rats. (H. P. L.)

No of Rats.	H. P. L. X 10^6	ALS in ml.	Mean Ki/Kc (95% C. L.)	Histologic grading. O	I	II	III
5	40	Control	1. 2368(1. 1667-1. 3069)	O	1	1	3
5	40	O. 5*	1. 1408(1. 0855-1. 1991)	O	1	1	3
6	50	Control	1. 2795(1. 153-1. 4052)	O	O	3	3
10	50	1. 8 **	1. 0884(1. 0262-1. 1506)	O	5	3	2
7	20	- nil -	1. 150(1. 0837-1. 2283)	O	2	O	5
6	30	- nil -	1. 2201(1. 1649-1. 2753)	O	O	O	6
8	40	- nil -	1. 3197(1. 2048-1. 4346)	O	O	2	6

Cyclophosphamide 100 mgm 24 hours prior to cell injection.

* human anti-human lymphocyte serum given in one dose at day O.
** rabbit anti-human lymphocyte serum given in three doses.

TABLE XVIIII: Testing of anti-human lymphocyte sera.

No of Rats.	ALS	Dose.	Mean Ki/Kc (95% C. L.)	Histologic grading. O	I	II	III
9	Control		1. 2309(1. 1330-1. 3288)	O	1	2	6
8	Lot 11	.15 mgm	1. 0284(0. 9751-1. 0817)	O	5	2	1
9	Lot 8	.15 mgm	1. 0526 (1. 0011-1. 1041)	O	5	3	1
8	HAHLS	O. 5 ml	1. 095(O. 9463-1. 0727)	O	2	2	4
8	MRC Pool 3	O. 25 ml	1. 0781(1. 0304-1. 1258)	O	7	1	O
8	MRC Pool 3	O. 75 ml	1. 0603(O. 9614-1. 1592)	O	6	1	1

* 30 X 10^6 human peripheral cells given to each rat.
* all the sera were given in one dose one day after the cell transfer.
* Lot 8 and 11: horse anti-human lymphocyte globulin.
* MRC Pool 3: horse anti-human lymphocyte serum prepared by the Medical Research Council, of Canada.
* HAHLS: human anti-human lymphocyte serum.

SUMMARY:

Human and murine immunocompetent cells when injected under the kidney capsule of Cyclophosphamide pretreated rats initiate a localized graft-versus-host reaction (GVHR). The cardinal feature of this reaction is the formation of tumorous mass of proliferating donor mononuclear cells that invade and destroy the host renal cortex. This xenogeneic graft-versus-host reaction (XGVHR) is specifically inhibited by antilymphocyte sera (ALS) raised against the donor lymphoid cells. The degree of inhibition of ALS is proportional to the number of donor lymphocytes injected as well as the amount of antilymphocyte serum given to each recipient. In addition, it was found that the capacity of antilymphocyte sera to inhibit this XGVHR correlated with their ability to inhibit the rejection of skin allografts. In other words, the capacity of ALS to inhibit this XGVHR does correlate with their immunosuppressive potency. Xenogeneic graft-versus-host reactions produced by human lymphocytes in rats are used as a basis for assaying the immunosuppressive potency of anti-human lymphocyte sera prior to their clinical use.

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