THE CELLULAR REQUIREMENTS FOR GRAFT REJECTION

Cheryl Denise WATERS

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SUMMARY

The studies described in this thesis were designed to develop a model in which the capacity of various lymphoid cells subpopulations to cause graft rejection could be tested and correlated with their capacity to effect *in vitro* lysis of appropriate target cells. Cell populations specifically sensitized in MLC were injected locally into RT1 incompatible neonatal heart grafts implanted into the hind foot pad of rats syngeneic with the original responder cells. Local injection of MLC sensitized cells specifically accelerated graft rejection. The cells exerted both a systemic and a local effect. Cells injected in the grafted footpad procured more rapid rejection than similar populations injected systemically or into the footpad contralateral to the graft. The greater efficiency of local inoculation appeared to be mainly a consequence of immediate re-exposure of sensitized cell populations to the sensitizing alloantigen *in vivo*. Studies of the capacity of MLC sensitized cells to cause graft rejection in irradiated DA hosts suggested that rapid rejection in normal DA recipients involved collaboration between the injected MLC sensitized cells and two populations of radiosensitive host cells, *viz*. cells of the mononuclear phagocyte system and cytotoxic/suppressor T lymphocytes. This was not the case in WF hosts in which rapid graft rejection could be procured in irradiated hosts by the injection of MLC sensitized cells alone. Examination of the *in vitro* cytotoxic capacity of cell populations with the capacity to cause rapid graft rejection *in vivo* also highlighted differences between WF and DA strain rats. Following MLC sensitisation under identical conditions WF cells developed significant *in vitro* cytotoxicity whereas DA cells did not. WF lymphoid cell populations contained a significantly higher proportion of cells of the cytotoxic/suppressor T cell subset than did DA cell populations. However redressing this inbalance by adding excess cytotoxic/suppressor cells to DA cultures did not improve the cytotoxic capacity of DA MLC sensitized cells. Studies of *in vivo* and *in vitro* effector cell activity of DA T cell subsets

separated either before or after MLC stimulation failed to elucidate the nature of the apparent differences in effector function between WF and DA rat strains. Both the cytotoxic/suppressor and the helper T cell subsets separated from DA lymph node cells proliferated in MLC and were subsequently capable of procuring rapid graft rejection in normal DA hosts. Neither was as efficient as whole MLC cells in procuring rejection in irradiated, grafted hosts.

T cell subsets separated subsequent to stimulation in MLC also caused rapid rejection in irradiated DA hosts when supplemented with naive cytotoxic/suppressor cells and a source of mononuclear phagocytes. In the absence of added host strain cells neither subset procured rapid rejection. Both restored rejection with a slow tempo, which was significantly slower in the case of the cytotoxic/suppressor subset than with the helper T cell subset.

In irradiated WF strain rats, in which rapid graft rejection could be procured by whole MLC cells in the absence of additional host strain cells, the helper T cell subset separated after stimulation in MLC also caused rapid rejection in the absence of added cells. The cytotoxic/suppressor subset did not. These experiments provide evidence that the observed interstrain differences in the in vivo effector function were unlikely to simply reflect differences in cytotoxic/suppressor T cell numbers or functions. In addition, the finding that the helper T cell subset of MLC sensitized WF lymphocyte populations appears to be the effector of rapid graft rejection in vivo makes it difficult to conclude that the observed differences between the DA and WF strain rats in in vivo effector funcion have the same basis as the differences between them in in vitro effector function. These experiments provide data which supports the essential role of the T helper cell subset in the effector phase of graft rejection. They serve to underline the complex nature of the cellular interactions involved in all phases of the allograft response including the effector phase. They also suggest there may be significant interstrain differences which determine the nature of the final effector pathway.

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C.D. WATERS
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IGNORANCE

Strange to know nothing, never to be sure
Of what is true or right or real,
But forced to qualify or so I feel,
Or well, it does seem so:
Someone must know.

Strange to be ignorant of the way things work:
Their skill at finding what they need,
Their sense of shape, and punctual spread of seed,
And willingness to change;
Yes, it is strange.

Even to wear such knowledge - for our flesh
Surrounds us with its own decisions -
And yet spend all our life on imprecisions,
That when we start to die
Have no idea why.

by Phillip Larkin
in "Whitsun Weddings"
Penguin, London

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ABBREVIATIONS

ADCMC	antibody dependent cell mediated cytotoxicity
APC	antigen presenting cell
ATXBM	adult thymectomized, irradiated and bone marrow reconstituted
BM	bone marrow
BSA	bovine serum albumen
CML	cell mediated lysis
Con A	concanavalin A
CPM	counts per minute
Cr^{51}	^{51}chromium
CSA	cyclosporin A
DAB	Dulbecco's A and B salts
DC	dendritic cell
DTH	delayed type hypersensitivity
E:T	effector to target
FCS	foetal bovine serum
FITC	fluorescein isothiocyanate
FP	foot pad
GVH	graft versus host
H	histocompatibility
HEV	high endothelial venule
H^3TdR	tritiated thymidine
HVG	host versus graft
IA	intra arterial
Ig	immunoglobulin
IL1	Interleukin 1

IL2	Interleukin 2
IP	intra peritoneal
Ir	immune response
$I^{125}UdR$	^{125}Iodinated uridine
IV	intra venous
K	killer
LD	lymphocyte defined
LFP	left foot pad
LN	lymph node
LNC	lymph node cell
MHC	major histocompatibility complex
MLC	mixed lymphocyte culture
MoAb	monoclonal antibody
MR	maximum release
MST	median survival time
NK	natural killer
NNH	neonatal heart
PBS	phosphate buffered saline
PE	peritoneal exudate
PHA	phytohaemagglutin
PMN	polymorphonuclear
RFP	right foot pad
SD	serologically defined
SG	specific gravity
SR	spontaneous release
Tc	cytotoxic T cell
TC	tissue culture
TCM	tissue culture medium

Tdth	T cell involved in DTH reactions
TD	thoracic duct
TDL	thoracic duct lymph
Th	helper T cell
Ts	suppressor T cell
UV	ultra violet
VE	vascular endothelium
WBI	whole body irradiation

CHAPTER 1

LITERATURE REVIEW

1.1 HISTORY OF TRANSPLANTATION

Man has ever sought physical perfection, eternal youth and immortality and in this context the vision of transplantation for the replacement of damaged or worn out organs and tissues has existed for thousands of years. Autogenous pedicle grafts were developed in India several centuries B.C. to treat mutilations of the nose, ear and lips which were inflicted as punishment for crime and thus carried a social stigma greater than that merely resulting from disfigurement. In Western culture the concept of replacing lost or diseased parts exists in third century legends of ministering saints replacing amputated limbs. In Italy in the 15th and 16th centuries a method for skin grafting for the treatment of facial injuries was actually refined, formalised and published. However, following this, the practice of grafting underwent a further long period of neglect in the western world until the 18th century when the English became aware of the continuing practice of rhinoplasty in India. This coincided with a period in Britain during which the practice of surgery gained a new reputation as a discipline. This resulted mainly from the work of John Hunter (1756-1832) who elevated the status of surgery to that of an experimental science. The major thrust of the 19th century with regard to transplantation was towards the development of free grafts. The success of the evolutionary changes associated with the development of techniques, such as pinch grafts and tubular grafts, at this time was aided by the development of aseptic surgical procedures. Unfortunately, however, the early

triumphs with autografts encouraged surgeons into the use of allogeneic and even xenogeneic tissue. Unrealistic and often bizarre claims for the potential of this new form of surgery, associated with its often disastrous consequences, did much to retard the subsequent progress of clinical transplantation.

Man's enduring dream of rejuvenation and immortality impinged on the history of transplantation in real terms in the 19th century. During the early 20th century, a giant step was achieved when work with free grafting technique led to the observation that autografts were usually successful and allografts and xenografts were not. Cognisance of this biological principle began a search for the species and individual differences which led to graft failure. Transplantation entered a period of biological and surgical investigations designed to identify the factors which influence graft survival. These included attempts to identify the factors controlling the success or failure of both organ and tissue transplants and of blood transfusions.

As early as the 16th century analogies had been drawn between grafting in man and similar practices in agriculture where, though not always impossible, it had been found difficult to graft from one species to another. It had been concluded then that xenografts were not within the realm of the probable in man or animals. This conclusion forecast the later observed failure of xenografts in the 19th century. At the turn of this century transplantation was being used as a tool for the study of hereditary, phylogenetic relationships and hybridizability. In addition a number of workers were using transplantation as a method to explore the nature of cancer.

Studies of the biological basis for graft rejection depended on

adequate grafting technique. Organ grafting was initially hampered by the technical difficulties associated with vascular and arterial anastomoses and successful blood transfusion was bedevilled by the problem of coagulation. Thus significant progress in the study of tissue incompatibility had to wait on advances in related technical skills.

Early work in transplantation was made difficult not only by technical problems but also by the lack of genetically defined populations of experimental animals. Experimental work in the early 1900's depended on the use of outbred experimental animals, and was plagued by the inability to quantify the graft reaction. The inception of inbred colonies of experimental animals heralded the real beginning of the study of transplantation immunobiology and genetics.

Gorer (1938) recognized that genetically determined factors present on a graft but absent from the host were capable of eliciting a rejection response.

In 1945 Loeb reviewed earlier experimental and clinical work, and also concluded that the transplantability of tissues and organs was based on the requirement for biological identity. Little (1922, 1924) had earlier established that the susceptibility of hybrid and back cross generations of inbred mice to transplantable tumours was inherited according to Mendelian laws and was attributable to independent segregation of multiple genes. He concluded that there must be a large number of "susceptibility" factors, each determined by an independent gene. These theories led to the designation of "H" or 'histocompatibility' genes by Snell (1948).

Medawar (1958) drew a distinction between major and minor or strong and weak "histocompatibility genes" - the strong genes

governing the expression of antigens which caused allografts to break down very rapidly and the weaker governing antigens, the recognition of which led to slow, chronic graft rejection. He postulated that "when they act in unison, the many minor genes that govern compatibility are additive in their effect and therefore behave as a polygenic system". Similar differences between strong and weak histocompatibility genes were observed by Simonsen in the graft versus host reaction. Strong transplantation antigens were identified to be coded for in a discrete genetic region and constitute what is known as the major histocompatibility complex (MHC) and which can be identified in all species. In man it is called HLA, in rats RT1 and mice H-2. The power of the MHC antigens in determining the fate of transplanted tissue has been confirmed in numerous clinical and experimental studies, which show that the greater the degree of compatibility for MHC antigens the greater the chance of successful transplantation.

The involvement of antigens, other than those of the MHC, in graft rejection is clear from the fact that HLA identical siblings grafts, which would be expected to differ at a substantial number of minor H loci, are rejected unless the host is immunosuppressed and even if immunosuppressed often some recipients reject the grafts. Experimental work in mice has shown that multiple minor differences have a summative effect (Klein, 1975a) and some minor antigens are stronger than others (Bailey, 1971) thus confirming the prediction of Medawar (1958).

The recognition that antigenic differences between graft and host determined the susceptibility to rejection threw little light on the nature of the process involved in tissue destruction. A belief popular at the turn of the century, was that grafts did not take

because the host environment, the body fluids and the biochemistry, was poisonous to the graft and led to toxic injury. This idea was not incompatible with the observed failure of xenografts. Viewpoints from immunology and serology were then applied to the transplant problem, and differences in the chemical constitution of the host and transplant were seen to be due to differences in the structure of proteins or a single protein which would give rise to antibodies in a way comparable to haemolysin and agglutinin reactions. This led naturally enough to the idea that grafts could be adapted or their antigenicity modified so that they did not elicit a response in the prospective host. Loeb's review of tissue transplantation in 1945 presented evidence from a variety of workers that the graft itself could be altered so that the "organismal differentials" would not evoke a reaction from the host. Evidence, albeit somewhat vague and often unsubstantiated, was presented that the survival of a variety of homo and hetero transplants (amphibian and avian [but not mammalian] skin, and rat and murine tumours) could be prolonged if the tissues were first cultured in the "body fluids" of the recipient strain or species. The tissue culture technique was applied in the hope that following culture the graft would come to approach more nearly the constitution of the host. Results were variable and unreliable. However graft prolongation was apparently observed in some cases.

Other contemporary theories, reviewed by Loeb (1945) favoured the role of the host in transplantation rejection which had been shown to lead to active immunity at least in the case of tumour allografts and of xenografts. Workers demonstrated active immunization of the host as a result of the growth and regression of tumour allografts and the latent period before regression was viewed

as the time necessary for the production of an immune state. It was also shown that mouse organ xenografts led to more rapid rejection of mouse skin xenografts in a rat host. It was observed that the more closely related the donor and host, the more difficult it was to demonstrate an immune state. Attempts to demonstrate transplantation immunity to normal tissue allografts were inconsistent and often unsuccessful. However, histological examination of primary and secondary tumour grafts, had revealed the presence of cellular infiltrates consisting of lymphocytes, plasma cells and macrophages. These were seen as indicative of the immunological nature of the response and thought to be instrumental in its production.

Loeb (1945) observed no differences in the cellular infiltrates in primary and secondary grafts of normal (thyroid, cartilage and fat) tissues and therefore concluded that there was no obvious evidence for a role for immunity in rejection. While some workers were trying to prolong graft survival by treating the graft, those who believed in active host immunity as a mechanism of graft rejection sought ways of manipulating the host immune system as a means of procuring graft survival. Among the methods tried were desensitization by treating the host with serum plasma or tissue extracts from the donor, parabiosis between host and donor, reticulo-endothelial system blockade with trypan blue, splenectomy and X irradiation of the host. The latter was somewhat successful with tumour grafts but not with normal tissues. In general the results of all these studies were variable. Although there were occasional reports of prolonged graft survival, none of the methods described gave consistent results.

Thus, the situation at the time Loeb published his review was that the early work with tumour grafts had produced results which

were very suggestive of a host immune response against the grafted tissue, and many workers believed the host response towards all foreign grafts was an immunological one. The use of partially and/or fully outbred populations of animals made it very difficult, however, to conclusively demonstrate host immunity towards normal tissue.

A year before the publication of Loeb's review, Medawar (1944) had used a unique experimental design which overcame the problems inherent in the use of outbred animals, and had confirmed that graft rejection was the result of an immune response. He described the allograft response as a host reaction to donor strain antigens expressed on the grafted tissues. The response resulted in the rejection of grafted tissue. Sensitization of the host against graft borne antigens was demonstrated by the fact that the tempo of graft rejection was accelerated in animals that had previously rejected a graft from the same donor. Accelerated graft rejection was specific for tissues from the primary donor and was systemically propagated. These observations established that the host response to grafted normal tissue had all the characteristics of an immune response.

In the decades since Medawar established that the allograft response as an immune response, many of the features which differentiate it from the response to conventional antigens have been elucidated and the cellular response to allografts has been extensively studied. The wheel has turned the full circle since the mid 1940's. Once again workers in the field of transplantation are investigating both adaptation of the graft (Lafferty et al, 1983) and adaptation of the host as methods by which long term graft acceptance may be procured. In spite of very significant advances, many of the questions provoked by the turn of the century observations on the differences between autografts and allografts are still unanswered.

The precise mechanisms by which grafts are rejected are still not fully understood. Until these are elucidated it is unlikely that the clinical goal of achieving permanent graft survival without long term non-specific immunosuppression will be achieved.

1.2 THE ALLOGRAFT RESPONSE AS AN IMMUNE PROCESS

1.2.1 Antigen recognition and the tissue distribution of alloantigens

The observation by Medawar that a second skin graft from the same primary donor was rejected in accelerated fashion by the graft recipient, whereas one from a third party donor was rejected in normal first set time, not only allowed identification of the allograft response as an immunological one, it also provided a basis for the design of experimental protocols which would provide information on the nature of the antigen against which the response was directed.

Medawar (1946) demonstrated that second set skin graft rejection was brought about equally well by prior immunisation of the host with a skin graft or with blood leucocytes from the skin graft donor. He proposed that leucocytes and skin expressed common antigens. Billingham et al (1956 b) were able to demonstrate antigenic activity in totally disintegrated splenic cells and solutions made from them. Subsequently using an adoptive transfer assay in tolerant mice, Billingham et al (1963), confirmed the specificity of immunisation and showed that the degree and duration of immunity depended upon the mode of sensitisation.

The phenomenon of immunological tolerance was predicted in a theoretical model which was proposed by Burnet and Fenner in 1949 and

which approached the question of antigen recognition in a much broader sense. The model proposed that exposure to antigen during early life leads to a state of specific non reactivity in later life which accounts for tolerance to self determinants, and thus the discrimination between self and non self. Although Burnet et al (1950) failed to induce tolerance to a wide range of protein antigens injected in the neonatal period, their postulate excited wide interest; particularly on workers' seeking ways to overcome the barrier to allograft survival.

Within a few years Billingham et al (1953, 1954, 1956b) demonstrated that foetal mice of inbred strains injected in utero with a cell mixture of allogeneic spleen, kidney and testis subsequently showed a life long incapacity to reject skin grafts from donors of the same inbred strain as the donors of the cell mixtures. This provided powerful experimental support for the hypothesis put forward by Burnet and Fenner (1949). In addition the finding that skin graft rejection could be prevented by in utero exposure to donor antigens on tissues other than skin, provided further evidence for the widespread tissue distribution of alloantigens.

1.2.2 The cellular nature of allograft rejection

Once graft rejection was established as a manifestation of an immune response against antigens present on the surface of nearly all cells, attention was turned to the mediators of the response. Evidence for the complicity of cells from the regional lymph node in the rejection of allogeneic tumour cells was provided by Mitchison (1954) who found that regional lymph node cells from tumour bearing mice conferred a state of sensitivity upon naive adoptive hosts. This observation was confirmed and extended to the rejection of

normal tissue by Billingham et al (1954, 1956a) who showed that immunity to murine skin allografts could be transferred with lymph node cells or spleen cells from animals which had previously rejected a graft bearing the same alloantigens. They also demonstrated that the spleen is an important centre of response in animals sensitized by the intraperitoneal (IP) injection of dissociated cells. Neither the Mitchison nor the Billingham group were able to transfer sensitivity with serum, whole blood, peripheral blood leucocytes or non regional lymph nodes. The inability to transfer sensitivity with blood leucocytes was later seen to be due to the lack of sensitivity of the skin graft assay and a quantitative factor (low cell numbers) rather than to the absence within the blood of cells capable of transferring immunity (Billingham et al, 1963). It is likely that the failure to transfer immunity with non regional lymph nodes was similarly a consequence of the insensitivity of the assay and also of the timing of cell transfers. These were done soon after the primary host had rejected its graft and sufficient time might not have elapsed for immunity to be fully disseminated.

From the results of adoptive transfer experiments it was concluded that the "homograft" reaction was mediated not by humoral antibodies but by activated cells of lymphoid origin (Medawar, 1958; Brent et al, 1962).

A further very important contribution to the study of allo-immune responses arose from the observation of Simonsen (1957) that the injection of lymphoid cells into hosts which were incapable of mounting an immune response against the injected cells, led to a fatal wasting disease. Simonsen suggested that the observed response was the result of an attempt on the part of the injected cells to reject the unresponsive host. This reaction, the graft versus host

(GVH) reaction, became generally accepted as representing an immune response (Medawar, 1958) illicited by the introduction of antigen reactive cells into a host, the tissues of which carry different transplantation antigen and which is incapable of rejecting the grafted cells. It has become a valuable tool in the elucidation of the cellular interactions involved in responses to alloantigens.

Billingham and Brent (1959) showed that lymph node cells and peripheral blood leucocytes could cause GVH disease. Experiments investigating the capacity of thoracic duct lymphocytes to do so confirmed that the small lymphocyte was the cell which initiated the GVH response (Gowans, et al 1961; Gowans, 1962). Gowans (1962) established beyond doubt that lymphocytes could react to alloantigen and initiate an immune response by injecting labelled parental strain lymphoid cells into semi-allogeneic F_1 hybrid rats and following the fate of the injected cells within the recipient. He observed that they underwent blast transformation and division upon contact with host alloantigen. The similarity of these changes in cell morphology to those observed in the regional lymph node after skin allografting (Scothorne and McGregor, 1955) led to the suggestion that the cellular events which initiate the GVH response are the same as those that initiate graft rejection (Gowans, 1962; Gowans and McGregor, 1965).

The ability of syngeneic normal or immune small lymphocytes to break neonatally induced tolerance to transplantation antigens and to effect the rejection of long standing skin allografts on specifically tolerant hosts (Billingham et al, 1954; Billingham et al, 1956a; Gowans et al, 1962; Gowans et al, 1963) further implicated the small lymphocyte as the initiator of the immune response to transplantation antigens. Proper understanding of the nature of the contribution of

lymphocytes to the rejection process was however impeded by the popular concept at the time, that all lymphocytes were the precursors of antibody forming cells (Gowans et al, 1962). Gowans et al (1963) noted that the histological changes accompanying the rejection of tolerated grafts following the injection of immune lymphocytes occurred in all the host lymph nodes and in the spleen. This was in contrast to the strictly regional response to skin allografts on normal hosts described by Scothorne and McGregor (1955). The observed cytological changes in the lymph nodes and spleen of tolerant animals given syngeneic lymphocytes were the same as those seen in the GVH reaction when parental cells were injected into F_1 hybrid hosts. Gowans concluded that the mechanism which destroyed the graft in the former was generated during a cellular reaction against the chimeric F_1 progeny of the original tolerance conferring inoculation.

Thus, in the two decades following Medawar's identification of graft rejectionas a manifestation of an immune response, the GVH response was described and was also identified as a manifestation of an immune response against transplantation antigens. In addition it was firmly established that the small lymphocyte initiated the GVH response. This, and the experiments in tolerant hosts, strongly suggested, but did not prove conclusively, that the small lymphocyte was involved in initiating the graft rejection response.

Subsequent studies by Dorsch and Roser (1974a) showed that small lymphocytes can adoptively restore the capacity of irradiated rats to reject skin allografts. They also established that the adoptively transferred cells had to contain specific alloreactive cells by showing that small lymphocytes which had been clonally deleted failed to restore rejection (Dorsch and Roser, 1974b).

1.2.3 Basic concepts of the transplantation reaction

Pioneer work in the field of transplantation immunology (Sections 1.2.1. and 1.2.2) led to the formulation of a traditional concept of graft rejection that, with some modifications has remained a cornerstone of immunological studies. The concept that alloreactive cells recognize and respond to antigen either at the site of grafting or in the regional lymph node and that following this effector cells are produced which disseminate to procure antigen removal and systemic sensitisation sprang from the early studies on transplantation immunology. The postulate that all the somatic cells of an individual express the same transplantation antigens had its origin in the same studies.

The fact that specific alloreactive cells recognize and respond to antigen at any location and that sensitization is disseminated systemically suggests that cells involved in the immune response are migratory. The discovery of lymphocytic recirculation provided an explanation for the efficiency of the immune system as a surveillance mechanism against the entry of foreign antigen.

1.2.3.1 Lymphocyte recirculation

It is now well established that small lymphocytes recirculate between blood and lymph through the lymphoid tissues. The mechanism by which lymphocytes are maintained in a constant number in blood was not known until Gowans (1957) observed that the output of lymphocytes draining from the thoracic duct (TD) cannula of a rat begins to fall after 48 hours and that this drop in output can be prevented by reinfusion of cells from the thoracic duct. Radioactive labelling of thoracic duct lymphocytes (TDL) showed that approximately 80% of these cells recirculated between blood and lymph (Gowans, 1959).

Further experiments (Gowans, 1959; Gowans and Knight, 1964) established that although the majority of lymphocytes in thoracic duct lymph are small, long lived, recirculating cells there is also a minority population which is short lived, does not recirculate and includes large and medium lymphocytes.

The circulating pool of lymphocytes is heterogeneous with respect to both morphology and function. This heterogeneity may be ascribed not only to the primary differentiative processes, but also to recurrent exposure to various antigens which disturbs the stable physiological balance by altering the migration patterns of cells and inducing secondary differentiative changes in certain cell classes.

The immune response is initiated by the selection of precommitted lymphocytes by antigen (Jerne, 1971; Burnet, 1959). The recirculation of lymphoid cells which was so elegantly demonstrated by Gowans (1959) is an essential prerequisite to the selection, recruitment, activation and dissemination of these precommitted cells. Although many of the events which are essential to selection, recruitment, activation and dissemination have been elucidated there are still gaps in our understanding of the cellular interactions which lead to the ultimate elimination of antigen and the specific sensitization of the host.

1.2.3.2 Cellular events of the transplantation reaction

The cellular events which take place in a graft recipient which culminate in the rejection of foreign tissue may be divided into two phases. The first phase is represented by the initial inductive events which take place in the graft and/or the regional lymph node when lymphocytes come in contact with graft antigen and involves the reactions between antigen and lymphoid cells which subsequently takes

place. The second phase is represented by the effector arm of the response.

*** Contact by antigen reactive cells with graft antigen**

Scothorne and McGregor (1955) noted that the regional lymph node draining an allograft showed signs of marked hyperplasia with an associated weight gain. Greaves and Janossy (1972) found that the most prominent changes histologically were the appearance of pyroninophilic cells in T dependent areas, however activity was also observed in B dependent areas with increased numbers of plasma cells appearing (Micklem and Loutit, 1966).

The proliferative reaction which is initially observed in the draining lymph node is later also seen in the spleen (Scothorne and McGregor, 1955; Andre et al, 1962). This suggests that the allograft response is systemically propagated, however gives no information regarding the initial site of interaction between antigen and antigen sensitive lymphocytes. The fact that for normal rejection to occur intact lymphatic drainage from the graft site is essential was demonstrated by the finding that allogeneic skin grafted on a lymphatic pedicles (Tilney and Gowans, 1971) enjoyed greatly prolonged survival. The fact that most of these grafts did eventually reject implies that peripheral activation of lymphocytes in the blood circulating through the graft did eventually occur; however the very slow tempo of the response suggests that this mechanism is of little importance to the normal first set allograft response in the case of indirectly vascularised grafts.

The transport of either graft antigen or activated lymphocytes to the regional node via lymphatics is apparently critical to the normal inductive events in the immune response to indirectly vascularised grafts. It has been demonstrated that following the

implantation of a renal allograft there is a large scale redirection of lymphocytes from the bloodstream into the graft. These are predominately cells which carry no surface immunoglobulin and produce no antibodies. They leave the graft by way of the lymph (Pedersen and Morris, 1970; Pedersen et al, 1975; Miller and Adams, 1977) and at this stage have no demonstrable reactivity against the graft donor in vivo. However when they enter the regional lymph node they provoke a vigorous cellular responses there, and cells leaving the node in the efferent lymph do have specific reactivity against the graft donor when tested in vivo (Hay and Morris, 1975). The nodal response is thought to represent an amplification mechanism. It does not appear to be essential for the rejection of directly vascularised grafts. If lymph from the graft is diverted from the body rejection still occurs with approximately normal tempo. Thus with directly vascularised allografts, the recognition of graft antigen and the induction of the alloimmune response to it may occur within the graft itself and antigen and activated lymphocytes may leave the graft via both the blood and lymphatics to initiate the allograft response in the spleen and the regional lymph nodes.

It is clear that lymphocytes may make first contact with antigen either in the graft or in the draining lymph nodes. It is possible that the importance of these alternative sites of initiation of the response may vary according to the mode of vascularisation.

Lymphocytes may be nonspecifically or specifically accumulated within antigenically stimulated lymph nodes by the process of recruitment.

Non-specific recruitment

The injection of antigenic material into the lower leg of the

sheep leads to an acute but transient fall in the number of lymphocytes emerging in the efferent lymph from the popliteal node which drains the injected area. This effect continues for only about 4 hours after which time the cell numbers in the efferent lymph increase (Hall and Morris, 1963).

A second effect of antigen stimulation is to increase the entry of lymphocytes into the lymph node (Cahill et al, 1976). The increased traffic is primarily from the blood (Hay and Hobbs, 1977).

The initial fall in the efflux of lymphocytes, as a result of non-specific trapping, closely followed by increased traffic through the node, ensures that localised antigen is exposed not only to the sessile population of lymphocytes resident in the node, but also to large numbers of migrating lymphocytes. Among both populations there are presumably cells with specific receptors for the relevant antigen.

Specific recruitment

Antigen specific trapping of recirculating lymphocytes increases the number of antigen sensitive cells available for the induction of the immune response. One of the consequences of specific recruitment is the depletion of specific reactive cells from other lymphoid compartments. This has been shown to occur in a variety of circumstances and with a variety of antigens.

Experimental evidence for specific recruitment of antigen reactive cells and the consequent selective depletion of the recirculating pool of cells is provided by the finding that shortly after intravenous challenge of mice or rats with heterologous erythrocytes (Ford, 1968; Rowley et al, 1972; Sprent and Miller, 1973) or allogeneic cells (Sprent et al, 1971; Ford and Atkins, 1971)

lymphocytes specific for the antigens expressed on the injected cells are significantly decreased in or absent from thoracic duct lymph. The deficiency is usually apparent a few hours after the injection of antigen and lasts for approximately 2 days. Cells reactive to other antigens are not affected. During this period it would be expected that in the spleen, the main site of antigen localization after IV injection, there would be a net increase in the number of cells with specificity for the injected antigen.

When Sprent and Miller (1973) transferred TDL, spleen or mesenteric lymphnode cells from mice primed one day previously with heterologous red cells to irradiated mice challenge with both relevant and irrelevant heterologous red cells and measured the irradiated recipient's antibody responses 7 days later they could detect antibody to the irrelevant red cells but not to the red cells used to prime the original donors. The unresponsiveness observed following the transfer of spleen cells was however only temporary. If challenge with the priming antigen was delayed for 5 or more days full recovery in the reactivity of spleen cells occurred, however, the unresponsiveness to the priming antigen of recipients of TDL and mesenteric lymph nodes was not abrogated by delaying antigenic challenge of the transferred cells. This suggests that the unresponsiveness of TDL and lymph node cells taken 1-2 days after antigen injection reflects a deficiency of antigen reactive cells resulting from their sequestration in the spleen, the site of antigen localization following intravenous injection (Sprent and Miller, 1973). There is no clear explanation for the observed temporary non-reactivity of spleen cells in the above experiments.

Ford and Atkins (1971) and Dorsch and Roser (1974a) have shown, using different experimental approaches, that when normal lymphocyte

populations are injected intravenously into allogeneic or semi-allogeneic recipients, donor lymphocytes with specific reactivity for recipient alloantigens are sequestered in the spleens of the recipient animals. The donor cell population which recirculates in the recipient is specifically deficient in cells with reactivity for recipient alloantigens at a time when the cell population in the spleen shows increased reactivity.

Hopkins et al (1981) demonstrated that continued lymphatic drainage of a lymph node undergoing chronic antigenic stimulation with purified protein derivative of tuberculin (PPD) causes a gradual loss of PPD reactivity from the blood (as measured by an in vitro transformation assay) and eventually systemic unresponsiveness to PPD was shown by the absence of a delayed type hypersensitivity (DTH) skin response to PPD. No loss of reactivity to other non-cross reacting antigens was observed.

All these experiments show that antigen reactive cells are selected out from the recirculating pool as they pass through lymphoid tissue containing antigen and are activated to induce an immune response at this site. Subsequent migration by these activated cells disseminates the response systemically and allows effector cells to reach the antigen in other parts of the body.

*** The effector phase**

The effector phase of the immune response to grafted tissue may be defined as the stage during which alloactivated lymphoid cells are disseminated from the site of activation to the site of antigen deposition where they presumably make the intimate contact with graft cells which is apparently necessary for allograft destruction (Wilson, 1974; Cerottini and Brunner, 1974). The ability of

lyphocytes to migrate is obviously critical to this stage if the lymph node is indeed the primary site of alloactivation.

On the premise that cells with in vitro cytotoxic activity (Tc) represent the effector cells of the allograft response, many experiments have been done monitoring the appearance of Tc after grafting. Tc have been observed in the lymph node draining H-2 incompatible skin grafts in mice from 3-7 days after graft implantation. In the same animals Tc appeared in the spleen, blood and non regional lymphoid tissue a few days later than in the draining lymph node and had disappeared from these tissues by 21 days (Canty and Wunderlich, 1971). In the rat Tc activity does not appear in the draining lymphoid tissue for 4-5 days after skin or kidney allografting, is maximal at 7-8 days, which is the time of rejection, and is not detected 2-4 days after rejection (Biesecker et al, 1973; Peter and Feldman, 1972). These findings suggest that lymphocytes are activated and Tc are generated in the regional lymph node and migrate from there via the efferent lymphatics to the thoracic duct and there after are transported to the graft and other lymph nodes via the blood (Gowans, 1970). Transport of alloantigen in the blood and seeding into other lymphoid tissue or direct entry of activated lymphocyte into the blood can not, however, be excluded as a further mechanism (Pedersen and Morris, 1970) particularly in the case of directly vascularised grafts.

Returning to the experiments of Pedersen and Morris (1970; 1974a) discussed earlier, it is interesting to note that there appear to be significant functional differences between the cell population in the lymph draining a renal allograft and that in the efferent lymph from the regional lymph node. When each of these cell populations was tested for specific reactivity against donor

alloantigens *in vivo*, the cells in lymph from the allograft were found to have somewhat reduced specific responsiveness as early as 40-60 hours after grafting, when there was no evidence of blast transformation. By the fifth day, when large numbers of blast transformed cells were present, the population was quite anergic to graft antigens although able to respond to third party alloantigens. At the time when cells leaving the graft showed loss of specific reactivity, those emerging from the regional lymph node exhibited heightened reactivity. Cells from distant lymph nodes showed equal reactivity against donor and third party alloantigens for the life of the graft (Hay and Morris, 1975).

The *in vitro* responses of the two cell populations were also different. Cells from the regional lymph node taken 5 days after grafting displayed a specific increase in the magnitude of responsiveness to donor lymphocytes in one way MLC, however cells draining from the graft gave variable results. In some experiments there was a marked reduction in the response to donor strain cells while in others there was no depression of responsiveness. These results all indicate a qualitative difference between the cells in the peripheral lymph leaving the graft, and the cells in the central or efferent lymph leaving the node regional to the graft. These differences could be attributable to a selection process that operates to retain specifically reactive cells within the graft or they could indicate that specific cells that leave the graft and enter the regional lymph node via the afferent lymphatics undergo a functional or differentiative change within that node. The reactive cells that leave the regional lymph node may be cells newly formed in the node in response to interactions between alloantigen and/or cells from the graft and a sessile nodal population, or they may be cells

(or the progeny of cells) which leave the graft and undergo a differentiative change in the environment of the lymph node (Trevella and Morris, 1980). A further possibility is that cells leaving the graft after direct contact with graft antigen are temporarily refractory to specific antigen until they recuperate or are "modified" by a period of residence in the regional lymph node.

Further evidence suggesting a vital role for the regional lymph node in the allograft response is provided by the work of Dorsch et al 1983. Using a neonatal heart graft model, in which indirectly vascularised neonatal heart grafts are implanted subcutaneously in the footpad of adult rats, they found that removal of the regional lymph node on any of the days between 2 and 7 after grafting prolonged graft survival. This effect was maximal at day 4. Node removal at this time consistently resulted in very prolonged graft survival. In some cases grafts survived indefinitely. The induced "tolerance" was so effective that implantation of a second graft, of the same strain as the first, did not alter the prolonged survival of the first graft and the second graft was also retained for a prolonged period. The observation by the Morris group (discussed in Trevella and Morris, 1980) that alloreactive cells appear to require a second contact with the lymphoid system to develop full function, is obviously pertinent to the effect the removal of the regional lymph node has on graft survival. The removal of the regional lymph node at day 4 may remove antigen in its immunogenic form or may remove an important subset of cells that are required to synergise with other alloreactive cells for an allograft response to occur.

It is obvious from the foregoing discussion that the cellular events in the graft and its regional lymph node which culminate in the production of the effector cells necessary for graft rejection

are by no means simple. These experiments emphasize the importance to the outcome of grafting of lymphocyte traffic and sequential interactions between migratory and fixed elements of lymphoid tissues. Alterations in the distribution of specific subclasses of reactive cells, shifts in the patterns of lymphocyte migration into certain regions in response to antigen, the withdrawal of specific lymphocytes from the circulating pool by their retention in tissues following contact with antigen are all essential components of the immune response to grafted tissue. These depend upon physiological and anatomical relationships which cannot be reproduced in conventional in vitro assays of alloimmune function.

1.2.4 Evidence supporting T cells as the primary mediator of graft rejection

The capacity of cells of lymphoid origin to transfer graft rejection (Section 1.2.2) established the involvement of these cells in the rejection of allografts. Work done in the last 2 decades has provided ample evidence for the requirement for thymic function in the response to grafted tissue. The inability of nude mice (Wortis, 1971; Manning et al, 1973), neonatally thymectomised mice (Miller, 1961; 1962; Miller and Osoba, 1967) and thymectomized, sublethally irradiated and bone marrow reconstituted (ATXBM) mice and rats to reject allografts or to mount other T cell dependent alloimmune responses (Loveland et al, 1983; Lear et al, 1983), coupled with the ability of transplanted foetal or neonatal thymus (Miller, 1961; 1962; Wortis et al, 1971) to restore the deficit resulting from the athymic state, all indicate that the thymus plays a vital role in the response to transplantation antigens.

Direct evidence that T cells can mediate the rejection of

grafted tissue in the absence of B cells was obtained in experiments which examined the capacity of adoptively transferred lymphocyte populations to mediate graft rejection in heavily irradiated rodents.

Using an adoptive transfer model Sprent and Miller (1972a) attempted to show that T cells alone could mediate graft rejection using thymocytes activated against alloantigens by passage through lethally irradiate F_1 hybrids. The activated T cells when adoptively transferred to syngeneic, neonatally thymectomized mice accelerated the rejection of skin grafts of the sensitizing strain. These results, however, did not exclude the possibility that the transferred sensitized T cells functioned in the athymic recipients as helper T cells for the production of alloantibodies which effected rejection either directly or via a macrophage mediated effect (Cerottini and Brunner, 1974). Subsequent adoptive transfer experiments performed in recipients depleted of lymphocytes by whole body irradiation established that T cells appeared to be able to effect rejection in the absence of B cells. Hall, et al (1978a) transferred populations of TD or lymph nodecells to sub-lethally irradiated, cardiac allografted rats. They demonstrated that the capacity to restore allograft responsiveness resided with the Ig negative, recirculating, long lived lymphocyte population and that allograft rejection ocurred in the absence of either detectable serum antibody or a plasma cell infiltrate within the rejecting tissue. The tempo of rejection following the transfer of Ig negative cells was similar to that observed with whole cell populations containing the same number of Ig negative cells suggesting that only the Ig negative cells within whole lymphocyte populations contributed to graft rejection.

Many studies of rejecting tissues have identified that T cells

and macrophages rather than B cells are the predominant infiltrating cells confirming that effector cells are in the graft at the time of rejection (Strom et al 1975, Tilney et al, 1975).

Subsequent to these experiments it has been recognized that T cells may be divided on the basis of their responses to alloantigens, cell surface markers and *in vitro* function into at least two well characterized subsets.

1.3 THE MECHANISM OF T CELL ALLOACTIVATION

1.3.1 The role of graft antigens

MHC antigens are uniquely powerful primary immunogens and in this respect differ from conventional antigens and minor hiscompatibility antigens. The powerful proliferative and cytotoxic T cell responses provoked by foreign MHC antigens makes successful transplantation across MHC barriers a formidable challenge. Incompatibility between graft donor and recipient for antigens determined by the MHC is the single most important factor in rapid graft rejection, although non MHC antigens also induce graft rejection (Hildemann 1970; Bach and Van Rood, 1976).

1.3.1.1 The major histocompatibility complex of the rat

The structure and function of the major histocompatibility complex (MHC) in the rat, the RT1 complex, is less well understood than the MHC of man and mouse (HL-A and H-2 respectively) which have both been more extensively studied. Recent developments however have increased knowledge of the RT1 complex to a point which allows comparisons to be made between it and the HL-A and H-2 complexes. The rat MHC has recently been the subject of reviews by Gill and co-

workers (1982, 1983), the information from which served as the basis for the following discussion.

The RT1 complex codes for Class I and Class II membrane bound antigenic determinants. It can be divided into a number of different regions or loci. These, the A, B, D, E and C loci have been defined by the presence of glycoprotein products that are classified on the basis of homology in primary structure, tissue distribution and functional activity.

Genetic differences between donor and host in Class I antigens lead to acute graft rejection. Twelve unique rat Class I specificities have been identified to date (Cramer et al, 1978; Arenas et al, 1981; Gill et al, 1982, 1983). The RT1 A locus was considered to be the only locus coding for Class I antigens, however recent biochemical (Blankenhorn et al, 1978), serological (Misra et al, 1983) and functional (Livingstone et al, 1983) studies have produced evidence that more than one gene in the MHC may be responsible for encoding Class I antigens. The recent discovery of two new recombinant strains and the use of congenic rat strains has led to the identification of a second locus in the RT1 complex controlling the expression of Class I antigens, the E locus (Gill et al, 1982, 1983; Cramer et al, 1983). The RT1 A and RT1 E loci define the conventional boundaries of the MHC in the rat, separated by approximately 0.45 centimorgans (cM).

The Class I antigens coded for by the RT1 A and RT1 E loci are considered analogous to the mouse K and D region gene products. They are glycoproteins with a widespread tissue distribution, a molecular weight (M.W.) of 45,000 D and are associated on the cell membrane and in solution with a beta-2 microglobulin of a M.W. of 12,000 D. N terminal amino acid sequencing has shown remarkable homology in the

Class I antigens between the rat and the human, mouse and guinea pig (Blankenhorn et al, 1978; Gill et al, 1983). Like their mouse and human analogues, rat RT1 A antigens can also serve as restricting elements in T cell mediated immune responses to minor historcompatibility antigens (Marshak et al, 1977) and to viral antigens (Zinkernagel et al, 1977).

A third locus, the RT1 C (Kohoutova et al, 1980), appears also to code for a Class I like antigen which has been shown to serve as a target for skin graft rejection and has been mapped 2-3 cM from RT1 A. RT1 C disparity however does not lead to acute skin graft rejection (about 10 days) but to rejection with an intermediate tempo (17-32 days).

The product of the RT1 C locus is a glycoprotein of M.W. 40,000-43,000 D in association with a smaller 12,500 D M.W. component. Antigens encoded by RT1 C display limited tissue distribution (primarily lymphocytes) and do not restrict cell mediated lysis (CML) directed against minor histocompatibility antigens and in these respects they resemble the mouse Qa antigens.

The RT1 B and RT1 D loci are defined by Class II antigens which have a restricted tissue distribution and are expressed predominantly on lymphoreticular cells. Currently there appear to be 9 unique antigenic specificities (Gill, 1983). The rat Class II loci are comparable to the 1A and 1E loci of the mouse both in terms of their function and their biochemistry. Rat Ia (Class II) antigens closely resemble those of mice in N terminal amino sequences (Cecka et al, 1980) and cross react with mouse allo-antisera to 1A and 1E antigens (Blankenhorn et al, 1983). The above studies indicated that there are two rat Ia molecules encoded by the RT1 complex. This was confirmed by a recent analysis of a laboratory derived RT1

recombinant for phenotypic expression of I region related function (Lobel and Cramer, 1981) which indicated that the recombinant event had separated one locus responsible for mixed lymphocyte culture (MLC) reactivity (RT1 B) from a second locus (RT1 D) that regulated both the MLC and the immune response to synthetic simple polypeptides.

Rat Class II molecules are dimers composed of two subunits, α and β, with an apparent molecular weight of 31,000-35,000 D and 27,000-28,000 D respectively (Gill, 1983).

The studies using RT1 recombinant rats (Gill, 1982) which provided the information necessary to construct a detailed map of the rat MHC revealed that the gene order and size of the MHC in the rat is very similar to that in the mouse and different from that in man. In the mouse and rat, the MHC loci controlling Class II antigen expression are located between the Class I loci and the distances separating the Class I loci are very similar, approximately 0.5 cM. In man Class I loci are grouped together adjacent to the Class II loci. The polymorphism of the Class I antigens in the rat is extremely limited, the number of identified alleles of the two major Class I loci being 12 compared with more than 100 in the mouse (Klein, 1979). Even so Class I antigens are the most polymorphic molecules identified in all species. To date the mechanisms responsible for the restricted polymorphism in the rat MHC has not been established, but may be related to the relatively limited work done on these antigens or to the fact that the known inbred strains of rats come from a limited number of colonies.

1.3.2. Restriction of T cell subclasses

T cells may be divided into two basic subsets on the basis of

their response to Class I and Class II antigens and their cell surface markers.

1.3.2.1 T cell subsets

The phenotypic heterogeneity of rat T lymphocytes has been established by the use of a number of monoclonal antibodies (MoAb) derived from mouse myeloma cells fused with spleen cells from mice immunized with rat lymphocyte membrane antigens (Mason et al, 1983). These antibodies have defined two major non overlapping peripheral rat T lymphocyte subsets, W3/25$^+$ and MRC OX8$^+$, which have been shown to be functionally distinct. In addition two pan T cell MoAb for rat lymphocytes, W3/13 and MRC OX19 have also been produced. W3/13 MoAb binds to all T cells (Williams et al, 1977), some bone marrow cells (Brideau et al, 1980), neutrophils and plasma cells (Dallman et al, 1983). MRC OX19 apparently only binds thymocytes and peripheral T cells (Dallman et al, 1983). Thus MRC OX19 is a better antibody for the identification of T cells.

The W3/25 mouse MoAb binds to most rat thymocytes and a subset of rat peripheral T cells (Williams et al, 1977), while MRC OX8 mouse MoAb binds to most thymocytes and the majority of the peripheral T cells that do not label with W3/25 MoAb. A high proportion (80-90%) of thymocytes express both W3/25 and MRC OX8 antigen, however a small population (10-20%) of thymocytes resembles peripheral T cells in expressing either W3/25 or MRC OX8 exclusively (Brideau et al, 1980).

A further MoAb, MRC OX22, has recently been described that binds to all rat B cells and a subset of T cells (Spickett et al, 1983; Dallman et al, 1983). It appears to bind to all MRC OX8$^+$ T cells but only about 2/3 of the W3/25$^+$ subset (Spickett et al, 1983).

Functional studies of rat T cell subsets, positive or negative

for a particular monoclonal antibody marker, have indicated that MRC $OX8^+$ and $W3/25^+$ cells are functionally distinct in *in vivo* and *in vitro* assays. Further fractionation of $W3/25^+$ cells into MRC OX22 positive and negative populations has shown that the phenotypic division in this subset can also be correlated with functional differences.

Using the popliteal LN assay of Ford et al (1970) to determine GVH reactivity Brideau et al (1980) found that the MRC $OX8^-$ subpopulation in TDL from PVG rats produced LN enlargement in (DA x PVG) F_1 hybrids, whereas the $W3/25^-$ population did not. Similar results were obtained by White et al (1978) indicating that the phenotype of the cells involved in this GVH assay is $W3/25^+$ MRC $OX8^-$. Studies using $W3/25^+$ TDL fractionated into MRC $OX22^+$ and MRC $OX22^-$ subsets showed that only MRC $OX22^+$ subset caused popliteal LN enlargement in GVH assays (Spickett et al, 1983). Mason (1981) showed that lethal GVH disease in irradiated F_1 hybrids could be mediated by both the $W3/25^+$ and the MRC $OX8^+$ parental T cell subsets. Spickett et al (1983) transferred fractionated parental TDL into irradiated F_1 hybrids and found that only the recipients of MRC $OX22^+$ cells developed GVH disease. Further examination of the subset mediating GVH disease showed that $W3/25^+$ MRC $OX22^+$ cells had the ability to mediate the disease in the absence of MRC $OX22^-$ cells.

The $W3/25^+$ subset of peripheral T cells has been shown to be the cell which proliferates strongly in the rat MLC against either fully or semi allogeneic stimulator cells (Mason et al, 1981) and to be the major cell responsible for the produciton of inerleukin 2 (IL2) *in vito* (Cantrell et al, 1982). MRC $OX8^+$ cells have also been shown to proliferate in MLC, however the culture conditions are more stringent, MRC $OX8^+$ cells only proliferate in the presence of fully

allogeneic stimulator cells which contain W3/25$^+$ cells (Mason et al, 1981; Dallman et al, 1982). Examination of the MRC OX22 phenotype of MLC responder cells showed that MRC OX22$^+$ TDL proliferate as well as unfractionated cells against F_1 hybrid stimulator spleen cells. This suggests that the primary alloreactive cell in the rat MLC has the W3/25$^+$ MRC OX22$^+$ phenotype (Dallman et al, 1983).

In order to establish the phenotype of T cells with the ability to provide help to B cells in vivo hapten primed B cells were transferred to syngeneic irradiated recipients together with antigen and primed fractionated T cells. Anti-hapten plaque forming cell assays were performed 7 days later. On the basis of positive (White et al, 1978) or negative (Brideau et al, 1980) selection procedures, it was concluded that the ability to provide help to B cells resides in the W3/25$^+$ subset of T cells. Initial work characterizing the MRC OX22 phenotype of T helper cells for B cell responses suggests that cells with the W3/25$^+$ MRC OX22$^-$ phenotype play an essential role in the provision of B cell help, however weak responses were obtained with W3/25$^+$ MRC OX22$^-$ cells (Mason et al 1983).

Cytotoxic T cells and their precursors have been shown to have the MRC OX8$^+$ phenotype in in vitro studies. Spleen cells taken from rats 7 days after skin grafting were negatively selected for the W3/25$^-$ MRC OX8$^+$ subset. The resulting population was shown to contain all the cytotoxic activity of unfractionated spleen cells when it was tested in vitro in the CML (Duarte et al, 1982). Studies using TDL stimulated in vitro in MLC under conditions that provided maximum T cell help demonstrated that purified MRC OX8$^+$ cells, comprising only 5.5% of the TDL in this study, were as potent as unfractionated TDL and the MRC OX8$^-$ fraction was totally inactive (Dallman et al, 1982). The same workers also showed that if effector

cells were fractionated after MLC into MRC OX8 positive and negative populations only the MRC $OX8^+$ fraction contained the cytotoxicity. An integral part of this study was the demonstration that $W3/25^+$ cells in the responder or stimulator cell population provided help for the generation of Tc and the cells with the $W3/25^+$ phenotype were essential for the induction of MRC $OX8^+$ Tc (Dallman et al, 1982). Fractionation of $W3/25^+$ T cells into MRC OX22 positive or negative populations before addition to MRC $OX8^+$ cells in MLC against F_1 hybrid stimulator cells showed that only cultures supplemented with MRC $OX22^+$ cells developed cytotoxicity equal to that obtained using unfractionated TDL (Mason et al, 1983). These results indicate that the helper cells for the generation of Tc are of the $W3/25^+$ MRC $OX22^+$ phenotype. Other evidence (Mason et al, 1983; Spickett et al, 1983) suggests that the MRC $OX22^-$ subset of the $W3/25^+$ population provides T help for B cell responses.

Therefore in summary the Tc subset, identified as MRC $OX8^+$ (vide supra) in rats is comparable with the Lyt $1^-,23^+$ subset in mice, and contains Tc precursor cells, Tc effector cells and the suppressor cell for antibody responses (Cantor and Boyse, 1975; Brideau et al, 1980). The Th subset, identified as $W3/25^+$ in rats (vide supra) is comparable with the Lyt $1^+,23^-$ subset in mice, and includes the T cells responsible for help in antibody responses, help in the maturation of Tc cells, the mediation of DTH responses and proliferation in GVH and MLC assays (Cantor and Boyse, 1975; Brideau et al, 1980; Wright and Remshaw, 1983).

1.3.2.2 MHC restriction

The subpopulation of T lymphocytes which can be identified by different phenotype and function also have different reactivity

towards Class I and Class II MHC antigens.

Class II antigens, which stimulate allogeneic T cells to divide in MLC and GVH (Bach et al, 1972; Klein and Park, 1973) also regulate the response of Th cells to specific antigens. The I region of the mouse H-2 complex, which determines Class II antigens, was originally defined by its regulatory role in the immune response to synthetic polypeptides (McDevitt et al, 1972; Beraceraff and McDevitt, 1972). Later, Miller and Vadas (1977) showed that DTH responses mediated by Ly 1^+2^- cells were restricted; the response could only be transferred to hosts bearing the Class II MHC of the original sensitized cell.

In general differences in Class I antigens do not lead to a large T cell proliferative response in MLC (Bach et al, 1976). They are however the target antigens for Tc derived from primary MLC (Bevan, 1975a; Bach et al, 1976) and Class I disparity leads to rapid rejection of allografts (Bevan, 1975b). The lytic function of Tc directed against syngeneic cells was shown to be controlled by the H-2 gene complex. Tc from viral infected animals were shown to specifically lyse target cells only when the target shared the same H-2 as the Tc. The region of H-2 important in the restriction of target cell lysis mapped to the K and D (Class I) regions (Zinkernagel and Doherty, 1974; Doherty and Zinkernagel, 1975).

MHC restriction has now been demonstrated in a variety of systems so that activation and effector function of helper/inducer ($W3/25^+$ or Ly 1^+2^-) T cells is directed against Class II MHC antigens and cytotoxic (MRC $OX8^+$ or Ly 1^-2^+) T cells is directed against Class I.

Responses to minor antigen are of the MHC restricted type. Cytotoxic T cells (Tc) recognize minor antigens plus self-MHC Class I antigen (Bevan, 1975b; Pilarski and McKenzie, 1981), and the helper T

cells for Tc responses recognize minor antigen plus self MHC Class II antigen (Brenan and Muhlbacher, 1981; La Rosa and Talmage, 1983). The response to minor histocompatibility (H) antigens requires that the responding population be primed in vivo for in vitro challenge to generate cytotoxic T cells (Wilson, 1967; Bevan, 1975b). This differs from the requirements for priming against MHC antigens in which sensitization in vitro is usually sufficient to generate Tc. As far as MHC restricted responses are concerned knowledge of the T cell receptor is not yet sufficiently precise to allow us to know whether the complex of exogenous antigen and self MHC is recognised as two separate entities (dual recognition) or as a neoantigenic determinant created by proximity in the cell membrane of the two molecules (altered self). The circumstantial evidence currently favours the latter alternative (Bevan, 1981).

1.3.3 The role of antigen presenting cells

There is an increasing amount of evidence that dendritic cells (DC) play a special role in the induction of immune responses and the activation of T cells. DC have been demonstrated to be potent stimulators of the rat, human and mouse (Wong et al, 1982; Steinman et al, 1983b) primary MLC (discussed Section 1.4). There are practical difficulties associated with studies of the properties of DC. Much of the early work on immune interactions and antigen presentation was concerned with the role of the macrophage in the immune response and investigators were content to utilise heterogeneous adherent cell mixtures in experiments. Work by Tzehoval et al (1981) indicated that the macrophages used in such studies may be a heterogeneous mixture of cells and that phagocytosis and antigen presentation might represent distinct functions performed

by different subsets of macrophages; one Ia negative and phagocytic, the other Ia positive and immunogenic. With hindsight it could be concluded that these early workers may have identified DC in the latter subset.

It is thought today that macrophages and DC, although both bone-marrow derived non lymphocytic elements of the immune system (Steinman et al, 1983b), have little in common phenotypically or functionally. Morphologically DC are characterised by the presence of cytoplasmic processes which are obvious when the cells are attached to a surface. Because of these they have been referred to as "veiled cells". DC are obtained from the low density fraction of spleen, LN, TD, lymph, thymus and bone-marrow cell suspensions and in several species they apparently lose their capacity to adhere firmly to glass or plastic after several hours. They are non phagocytic, lack Fc receptors as detected by EA-rosetting, do not stain for non-specific esterase and do not express surface or intracellular Ig. An important feature of DC is that they are all strongly Ia positive (Mason et al, 1981; Bowers et al, 1983; Steinman et al, 1983b).

Bowers and co-workers (1983) demonstrated tha DC restore responsiveness in accessory cell depleted MLC cultures and are necessary for oxidative mitogenesis in periodate treated rat lymphoid cells. Macrophages on the other hand had no accessory activity. They were neither synergistic nor inhibitory for the accessory cell dependent mitogen response, and although Ia positive, were inert in MLC. Bowers reported that DC accessory activity was unaffected by 1,000 rads gamma irradiation, but was totally abolished by UV irradiation. This was interesting in view of earlier observations that stimulator cells in MLC must be metabolically active and that the ability to stimulate is destroyed by UV irradiation (Lafferty et

al, 1974; Kelso and Boyle, 1982a).

Several other groups have reported the accessory activity of DC in oxidative mitogenesis (Klinkert et al, 1980; Phillips et al, 1980). Austyn et al (1983) described a two stage mechanism of activation. The first stage occurred in the first 20 hours of culture, required live DC and involved the progressive release of IL-2. The second stage of proliferation was DC independent and took place in response to the IL-2 present in the culture medium. This work offers evidence that DC may function as accessory cells by stimulating responding T cells to produce IL-2.

The in vivo findings of Lechler and Batchelor (1982a and b, 1983) are consistent with the evidence for a central role for DC in alloimune response and strongly support the notion that DC are highly immunogenic. These authors found that long surviving enhanced kidney allografts, assumed to be devoid of donor type passenger cells, were not acutely rejected when retransplanted into secondary recipients syngeneic with the primary host (Batchelor et al, 1979) but were promptly rejected when DC obtained from the TD lymph of donor strain rats were injected intravenously into graft bearing hosts (Lechler and Batchelor, 1982a, 1982b, 1983).

With respect to their function in the immune response macrophages and DC have been broadly described as accessory cells. In the light of current evidence this term is not sufficiently informative. Concepts like "antigen presentation" and "antigen processing" are central to an understanding of alloactivation. Current evidence points to a dichotomy of accessory cell function, the macrophage processing either conventional or shed transplantation antigen and presenting it in an MHC restricted fashion to Th cells and dendritic cells presenting the foreign MHC alloantigen they bear

in an immunogenic form.

In man (Daar et al, 1983), rat (Hart and Fabre, 1981) and mouse (Nussenzweig et al, 1981) DC are widely distributed in interstitial connective tissue. Daar et al (1983) showed that in man a substantial subpopulation of DC express the OKT8 (suppressor/cytotoxic) T cell antigen. This is in agreement with findings in the mouse (Nussenzweig et al, 1981) in which a subpopulation of DC apparently express the Lyt-2 antigen which is expressed by the suppressor/cytotoxic subset of T cells. It is possible that DC are a heterogeneous population and like T cells may be divided into different functional subsets.

1.3.4 Role of soluble mediators

As has already been discussed the antigen specific activation of T cells is a complex process in which a variety of cell types and the soluble mediators IL1 and IL2 participate. A necessary prerequisite for production of soluble mediators is an antigen stimulus in a "recognizable" form. The antigen presenting macrophage, postulated in the model of interleukin mediated activation of T cells, is central to the process. What the relationship between the antigen presenting macrophage and the DC discussed in the previous section has not yet been elucidated and thus the role that DC may play in the production of IL1 and IL2 is not clear.

Antigen specific activation of T cells appears to be dependent on two distinct but interdependent macrophage signals (Schwartz et al, 1978). Macrophages, or their Ia^+ subpopulation, either present foreign MHC antigens to allogeneic T cells and also bind, process and present conventional antigens in immunogenic form to T cells in a

genetically restricted way. T cell binding acts as a stimulus for the production of IL1 by the macrophage (Oppenheim et al 1979). The release of IL1 stimulates the T cells in such a fashion that they becomes responsive to antigen or to secondary mediators, proliferate and secrete IL2 (Smith et al, 1980; Larsson et al, 1980). Therefore macrophages provide both a source of antigen in a "recognisable" form and a primary maturational signal (IL1). The primary maturational signal makes T cells sensitive to antigen and to a secondary T cell derived proliferative signal (IL2). The addition of IL1 or IL2 to spleen cells in the presence of alloantigen results in the enhanced generation of cytolytic T cells (TC) (Gillis and Smith, 1977; Wagner and Rollinghoff, 1978) and the effect of IL1 on the appearance of TC occurs in part via the induction of IL2.

Non activated T helper cells are unresponsive to IL1 and do not produce IL2, however they become sensitive to IL1 and synthesise IL2 following interaction with Ia antigen on the antigen presenting cell (APC) (Palacios, 1982). Therefore non activated T helper cells are unresponsive to IL1 (and IL2), however antigen specific and non specific Tc, suppressor (Ts) as well as activated Th lymphocytes can be maintained in a state of continuous proliferation in vitro when cultured in medium containing IL2 (Bach et al, 1981; Palacios, 1982; Farrar et al, 1978; Gillis and Smith, 1977; Glasebrook et al, 1981).

The importance of IL2 in allograft rejection is implied by the effect of cyclosporin A (CSA) on allograft survival. CSA has been shown by several groups to enhance graft survival and to be a potent immunosuppressive (Borel et al, 1976; Calne et al, 1978; Calne et al, 1981). It is currently thought that CSA operates by preventing the T cell activation that usually results in the production if IL2 (Bunjes et al, 1981; Wagner, 1983). In vitro work provides evidence that CSA

does not appear to affect the activation of precursor cytotoxic T cells per se but selectively impairs the release of IL2 from activated T helper cells. It may also have an effect on the release of IL1 from macrophages (Bunjes et al, 1981).

Further evidence supporting an in vivo role for the interleukins was obtained by Clason et al (1982) in adoptive transfer experiments in ATXBM rats. They found that the efficiency with which alloimmune spleen cells procured graft rejection was significantly increased by the addition of IL2 rich supernatants to the spleen cell inoculum; graft survival time was reduced by a factor of 2 in animals receiving both sensitized cells and IL2.

1.3.5 T cell alloactivation

The mechanism by which MHC gene products activate T cells is unclear. MHC antigens present on the surface of an antigen presenting cell (APC) may directly activate any T cell which binds them or the act of binding antigen may trigger the APC to send an activation signal to the bound T cell. The observation that not every cell expressing MHC molecules and antigen can act as a T cell inducer supports the latter suggestion (Batchelor et al, 1978; Lafferty et al, 1983).

While it is clear that MHC gene products constitute the major barrier to tissue transplantation, abundant evidence indicates that mere recognition of MHC antigens is not sufficient to induce alloactivation and the dual questions of what constitutes the stimulus to alloactivation and what is the target for effector cell function are still not fully answered.

The answers are being sought in two broad areas of research. The first encompasses studies of the relative roles of antigens

(either Class I or Class II) expressed on graft passenger cells (leucocytes, endothelial cells, dendritic cells etc.) and those expressed on parenchymal cells in the initiation and procurement of graft rejection. The second area is that of studies designed to identify the cell or cells responsible for actual tissue destruction.

In 1957, on the basis of rather tenuous evidence, Snell suggested that donor lymphocytes carried in grafts might play a role in invoking the immune response to the graft. More convincing evidence for a possible role for donor passenger leucocytes in immune induction derived from the demonstration that skin from tolerant mice has the capacity to sensitise naive syngeneic secondary hosts to the tolerizing antigen and that this capacity was related to the degree of leucocyte chimerism of the tolerant skin donor (Steinmuller 1967, 1969). Studies on the local GVH reaction in the rat kidney model (discussed in 1.4.3) were also interpreted as suggesting that passenger leucocytes in grafts may play an important role in alloimmune induction (Elkins and Guttmann, 1968; Elkins 1971).

Early attempts to enhance graft survival by inducing leucopenia in graft donors with whole body irradiation, cyclophosphamide or antilymphocyte serum and thereby reduce the passenger leucocytes in grafts of kidney, hearts and skin were only marginally successful (Guttmann et al, 1967; Freeman et al, 1971; Stuart et al, 1971; Kyger and Salyer, 1973).

The failure of these attempts to induce significant prolongation of graft survival by depleting the grafts of passenger leucocytes may have been a result of the types of tissue grafted. More recent work has indicated that the Langerhans cells present in skin can present foreign antigen to primed cells <u>in vitro</u> (Stingl et al, 1978) and are the major antigen presenting cell in contact hypersensitivity

(Streilen and Bergstresser, 1980). These may therefore represent the fixed immunogenic component of skin which is recognizable after graft revascularization takes place. Similarly organ-allografts which have been depleted of blood borne leucocytes, still contain non-parenchymal fixed tissue elements such as dendritic cells and vascular endothelium which are unaffected by the early methods employed to deplete immunogenic passenger cells and which probably play a critical role in immune induction.

The demonstration that tissues such as ovary, thyroid, parathyroid and pancreatic islets (Lafferty et al, 1975, 1976a and 1976b; Sollinger et al, 1977; Talmage et al, 1976; Talmage and Dart, 1978) may be permanently accepted by non-immunosuprressed allogeneic hosts, if they are kept in organ culture for a period prior to grafting, has finally consolidated the concept of the importance of passenger cells in the allograft response. Both mouse or rat thyroid glands which have been cultured <u>in vitro</u> prior to grafting into mice, enjoy long term survival (Lafferty et al, 1975, 1976 a and b; Sollinger et al, 1977; Talmage and Dart, 1978). This is in spite of the fact that the cultured tissue still express alloantigens because if graft recipients are immunized with a single injection of 10^3 peritoneal exudate cells from the thyroid donor, the cultured thyroid grafts are rejected (Lafferty et al, 1975, 1976b, Talmage et al, 1976).

Lafferty and his colleagues were among the first to question the assumption that the first step in alloimmunization against MHC antigens involves the simple recognition and activation of recipient T cells by graft antigens (Lafferty and Cunningham, 1975; Lafferty et al, 1978). They demonstrated that MHC incompatible cells did not always activate T cells <u>in vitro</u> and put forward a two signal

hypothesis for lymphocyte activation adopted from a model described by Bretcher and Cohn (1970). The hypothesis was that potentially responsive T cells were activated only if they received a second inductive stimulus at the time antigen was bound. This signal was later called lymphocyte co-stimulator.

As has already been mentioned in vitro MLC and CML studies with rat, human and mouse cells had lead to the definition of two separate lineages of T cells (Cantor and Boyse, 1975), effector cells and inducer or initiator cells. These findings also suggested that graft rejection was the result of a more complex series of cellular interactions than initially envisaged (Bach et al, 1976). A number of earlier studies had also shown that MHC incompatibility did not always activate T cells in vitro. It had been reported that cell proliferation in MLC appeared to be stimulated by a set of antigens designated at the time 'lymphocyte defined' (LD) antigens and that Tc were generated and directed against another set designated 'serologically defined' (SD) antigens (Bach et al, 1972; Alter et al, 1973; Brondz et al, 1975). Antigens previously included under the term SD are now called Class I antigens and those previously called LD are now referred to as Class II antigens.

Using HLA recombinant families, Eijsvoogel et al (1973a) were able to demonstrate that human cells which had proliferated in MLC in response to cells from a sibling with different Class II and identical Class I antigens were unable to cause lysis of PHA blasts from that sibling. Only when MLC stimulator cells differed from responder cells with respect to both Class I and Class II determinants was CML activity demonstrable and it was then directed against Class I and not Class II determinants. It was further shown by Eijsvoogel et al (1973b) that CML directed against Class I

antigens could be induced if two sets of stimulating cells were used, one differing from the responder with respect to Class II antigens only and the other differing with respect to Class I antigens only. Comparable results were obtained by Bach's group (reviewed Bach et al, 1976) who demonstrated in similar "three cell experiments" with mouse lymphocytes that responses between lymphocytes which differ for Class II antigens result in proliferation but for the generation of cytotoxic cells the presence of stimulator cells bearing Class I antigens is required.

As a result of experiments such as these Bach and co-workers (1976) suggested that T cell activation to alloantigen involves different T cell subsets which have different requirements for stimulation and play different roles in the response. Activation of T helper cells requires a single signal, the recognition of foreign Class II antigens. Activation of cytotoxic T cells on the other hand requires 2 signals, recognition of foreign Class I antigens and the presence of activated helper cells (Bach et al, 1976).

The Bach model defined the roles of different functional Tcell subsets but was incomplete with respect to Th cell induction. More recently, with the elucidation of the role in alloimmunity of soluble factors, particularly the interleukins, it has become apparent that Th cells, also require two signals for activation, namely foreign MHC antigen and Interleukin I (IL1). Cellular preparations such as UV irradiated or glutaraldehyde fixed cells, or allomembrane fragments, which have been shown to contain ample quantities of Class I and Class II antigens (Kelso and Boyle, 1982a; Batchelor et al, 1978) do not induce T cell proliferation <u>in vitro</u> unless either IL1 containing supernatants (Talmage et al, 1977; Lafferty et al, 1978), or small numbers of metabolically active allogeneic dendritic cells are added

to the cultures (Mason et al, 1981; Steinman et al, 1983a). IL1 will not however induce Th cells in the absence of foreign antigen.

The dendritic cell, which is found in nearly all tissues except the brain (Hart and Fabre, 1981) and which expresses both Class I and Class II antigens, probably represents the potent immunogenic passenger cell in a variety of tissues. Batchelor and colleagues (1978) demonstrated that two signals were also necessary for in vivo T cell activation in mice. In addition they found that sensitization with cell membrane fragments from strains disparate for either Class I or for both Class I and Class II antigens did not generate cytotoxic T cells. However, strong Tc responses were generated in animals sensitized with gamma irradiated cells disparate for both Class I and Class II antigens. The implication was that the second signal was provided by the irradiated, but intact cells. It has also been shown that B cells which bear both Class I and Class II antigens do not restore the immunogeneic status of passenger leucocyte depleted surviving kidney grafts (Lechler and Batchelor, 1982a), but immunogenicity is restored by the transfer of small numbers of viable dendritic cells.

These results indicate that the induction of Th cells is dependent on two signals, and in addition that strong immunogenicity cannot be explained merely on the basis of the presence of an incompatibility involving only Class II antigens as Bach's postulate would infer. On the other hand although the generation of Tc in MLC may require collaboration between Th cells responsive to Class II antigens and Tc precursors responsive to Class I antigens, grafts between strains disparate for only Class I or only Class II antigens or for minor antigens are rejected (McKenzie et al, 1980; Simpson, 1982). This implies either that in vitro methods for raising and/or

assaying Tc may not always reflect the conditions that operate in vivo, or that grafts may be rejected by a mechanism which does not involve Tc.

Batchelor and colleagues (Lechler and Batchelor, 1982a and 1982b; Batchelor, 1983) noticed that there was a strain dependent variability in the fate of retransplanted long surviving enhanced rat kidney allografts in which donor strain passenger cells were replaced by those of the primary enhanced host before they were retransplanted to secondary recipients syngeneic to the first. They found in some strain combinations, grafts survived indefinitely with minimal rejection responses but in other donor-recipient combinations destruction of the retransplanted graft occurred. Batchelor (Lechler and Batchelor 1982b; Batchelor, 1983) therefore proposed that there are two routes by which alloimmunization can occur. 'Route one' activates recipient T helper cells directly via the allodendritic cells present in the graft tissue. Most evidence is consistent with the hypothesis that allo-Class II-MHC antigens are equivalent to altered self Class II and in conjunction with IL1 constitute the molecular trigger that activates the Th cells of the recipient. Allodendritic cells act via 'route one' as a very powerful form of Th cell immunization (Lechler and Batcheler, 1982b). 'Route two' involves the less efficient breakdown, phagocytosis and presentation of alloantigen by the recipients own antigen presenting cells (APC) and is the same route of immunization employed for minor H antigens or conventional T dependent protein antigens. The density of altered self MHC molecules on APC could be assumed to be far less than the density of MHC "triggers" on allodendritic cells and therefore less efficient at T cell activation (Lechler and Batchelor, 1982a).

Lechler and Batchelor (1982b) further speculated that the reason

MHC incompatible grafts induce such strong rejection responses compared to non-MHC ones is that they immunize via both 'route one' and 'route two' and minor H incompatibilities only immunize via the relatively inefficient route two. In support of this, La Rosa and Talmage (1983), using a murine cultured thyroid graft model noted a strain dependent variation in the degree of leucocyte infiltration in the grafts. Cultured grafts between strains incompatible for only the MHC showed no infiltrate, or at best small foci of infiltration, 5 weeks after grafting, while grafts disparate for only minor H antigens showed marked infiltration in the same time period. This result is explicable in terms of the route of host immunization and the fact that the responses to minor H antigens are MHC restricted (vide supra). Cultured thyroid grafts are deficient in allodendritic cells and other Ia positive graft elements. Where only an MHC incompatibility exists cultured grafts would only express allo-class I antigens. Grafts disparate for only minor H antigens still bear Class I for which host and donor are matched; they may therefore sensitize the host in an MHC restricted manner by presentation of graft minor H antigens on host APC in association with self Class I and Class II, or more precisely via Lechler and Batchelor's (1982b) 'route two'.

Theoretically grafts depleted of allodendritic cells, and disparate only for Class I antigens should not lead to alloactivation via 'route two'. If host presenting cells pick up alloantigens shed from the graft and activate T cells against them in an MHC restricted manner, where only a Class I disparity exists the Tc produced would be of the wrong specificity to destroy the Class I alloantigen bearing graft parenchymal cells. Graft antigen would be treated like any conventional antigen, activating only those T cells which

recognize it in association with self MHC. The graft which does not express host MHC molecules should thus not be destroyed.

The fact that normal uncultured Class I disparate grafts are rejected in the absence of a Class II disparity is difficult to reconcile in the light of current theories of T cell alloactivation. If Th cells for allogeneic responses respond to allo-Class II antigens and IL1 from immunogenic passenger cells and induce Tc against allo-Class I, then how are grafts disparate for only Class I rejected?

In some congenic rat strains desparate for only Class I antigens grafts are rejected, however the rejection of Class I incompatible skin allografts was shown to be under stringent MHC-linked immune response (Ir)-gene control (Howard and Butcher, 1981; Butcher and Howard, 1982). It was found that the immune response to a given RT1A haplotype could be classified as high or low as skin graft rejection responses (rapid rejection vs permanent acceptance) were restricted by the MHC determinants of the responder (Howard and Butcher, 1981). Therefore the fate of Class I desparate grafts appears to rest on the MHC association that donor antigens form on host APC. This is somewhat difficult to reconcile with current theories regarding alloactivation in that Butcher and Howard (1982) were dealing with an MHC incompatibility which is considered to be immunogenic in its own right. What they (Butcher and Howard, 1982) postulate is that Class I antigens immunize independently of host MHC products at the effector cell level while apparently immunizing the same animal at the same time in association with host MHC products at the level of regulator cell induction. It would appear then that the difference between high and low responders is the degree of development of the regulatory cell pathway which would depend on the Th cell response

generated by reactions to self Class II antigens on donor APC or on host APC which have acquired donor Class I antigens. It is therefore possible that only antigen carried on viable cells of donor origin can activate an unrestricted allospecific T effector cell while regulatory T cells are activated by processed antigen on recipient APC (Butcher and Howard, 1982). The mechanism described here may also explain the strain dependent variability observed by Lechler and Batchelor (1982b) in the survival of retransplanted, long surviving, enhanced kidney allografts. It may be that some of the recipient strains were low responders with respect to Class I disparities however the possibility that all donor passenger cells were not replaced by host cells in the primary recipient cannot be excluded.

The anomalies observed in vivo have parallels in vitro which also suggest that the current concepts of alloactivation may be an oversimplification of the possible interactions which follow contact between immunologically competent cells and alloantigen. Using an in vitro system, Luger et al (1982) demonstrated that mitogen stimulation of either purified human T cell subset will produce substantial amounts of IL2. Addition of IL1 to macrophage depleted mitogen stimulated OKT8 or OKT4 subsets led to significantly increased production if IL2 by both subsets implying that both subsets are also sensitive to the inductive signal of IL1. Similar results were obtained by Andrus et al (1981) in a murine system. In the case of K and D region T cell activation, lymphokine production was dependent on the presence of Lyt 2^+ cells, however, lymphokine production by I region activated T cells was Lyt 2^- T cell dependent.

Antigen driven helper cell independent cytotoxic T lymphocyte clones have also been shown to secrete IL2 in the absence of T helper cells (Widmer and Bach, 1981, 1983; Widmer et al, 1983).

Helper T cells primed against Class I alloantigens that are not restricted to self Class II antigens have been described (Swain, 1981) as have allohelpers for antibody producing B cells which can be generated across K or D region differences alone and have the Lyt 2^+ phenotype (Panfilli and Dutton, 1978; Swain and Panfilli, 1979). In addition the generation of IL2 by stimulation with K and D alloantigens was not blocked by anti Ia antibodies which were effective at blocking factors generated in response to I region MHC antigens implying that Class II antigens were not recognised during the response of T helpers to Class I antigens (Swain, 1981). There is also some earlier work which produced evidence that cytotoxic T cells can be generated and directed against Class II alloantigenic differences alone (Wagner et al, 1975; Andrus et al, 1981) and Tc stimulated by Class II are Lyt 1^+2^- for I-A disparities but Lyt 1^+2^+ for I-E disparities (Vidovic et al, 1981) while Tc restricted by Class I are Lyt 1^-2^+ (Cantor and Boyse, 1975).

Not only does the response to antigens of the MHC and that to minor antigens appear to differ in the requirement for antigen presentation and induction, but the lymphocyte subsets involved in regulation of the two responses appear to differ. There is evidence which suggest that the cell which inhibits the cytotoxic response to minor HC antigens (Lyt 1^+2^-) is quite different to the cell that inhibits the response to major HC antigens (Lyt 1^-2^+) (Pilarski and Mckenzie, 1981).

More recent work has demonstrated that suppressor cells activated by heat treated allogeneic stimulator cells and capable of suppressing <u>in vitro</u> MLC responses are of the Lyt 1^+ phenotype (Holan and Mitchison, 1984). Suppression is independent of the presence of Lyt 2^+ cells either in the suppressor cell population or in the cell

population that stimulates suppression. Lyt 1^+ suppressor cells were also found to operate in the absence of Lyt 2^+ cells in the MLC responder or stimulator cell populations against which the suppression was directed (Holan and Mitchison, 1984), thereby ruling out the possibility that the Lyt 1^+ suppressor cell was in fact a T suppressor/inducer cell.

These findings, taken together, indicate that Class I and Class II antigens may serve reciprocal functions in the manner in which they activate T cells. The distinction between the separate lineages of T cells as distinct functional subsets while applying in most cases, does not appear to be absolute. Current dogma does not satisfactorily answer the problems posed by all the experimental evidence, which can only be explained by postulating alternate pathways for T cell alloactivation. Preliminary evidence suggests that the notion of alternate pathways for activation of T cells, and reciprocal functions of Class I and Class II MHC antigens in T cell activation may provide the answer to many of the puzzles presented by the experimental models which do not satisfy the precepts of current dogma.

1.4 IN VITRO AND IN VIVO ASSAYS FOR ALLOREACTIVITY

In the years since Medawar (1944) first identified the allograft response as an immune response, a huge body of data which is relevant to the mechanisms of alloactivation and tissue destruction has accumulated.

Realization of the complexity of the cellular interactions involved in the allograft response in vivo has led to much effort being invested in the development of in vitro models of the response

and of both *in vitro* and *in vivo* assays which are capable of separating various stages in its development. These have provided much useful information.

1.4.1 The MLC and CML assays

These *in vitro* assays were developed in the hope of solving the mystery surrounding the effector phase of the allograft response. They are based on two important early findings, namely that allogeneic lymphoid cells in mixed culture undergo blast transformation and synthesise DNA and proliferate (Schrek and Donelli, 1961; Baine et al, 1963) and that immune lymphoid cells are cytotoxic to allogeneic target cells *in vitro* (Govaerts, 1960; Rosenau, 1963).

Early studies of mixed lymphocyte culture (MLC) (Wilson, 1967; Wilson et al, 1967; Bach and Amos, 1967) and of cell mediated lympholysis (CML) (Brunner et al, 1970) established that both responses obey the rule of immunology with regard to selectivity and specificity (Hayry et al, 1972). Those observations have led to the development of the MLC and CML as standard assays for alloreactivity and as tools for unravelling the complex series of interactions which follow contact between immunocompetent cells and foreign histocompatibility antigen. The MLC has been studied extensively as a model of the proliferative or afferent phase of the allograft response (Hayry and Defendi, 1970; Wilson, 1974; Bach et al, 1976) and the CML assay has similarly been used as a model for the effector or efferent phase of the response (Hayry et al, 1972; Andersson and Hayry, 1975).

The belief that the proliferative response in MLC was immunological in nature derived from the observation that the

reactive cells in leucocyte cultures were lymphocytes, known to be important participants in immune phenomena, and that the magnitude of the proliferative reaction depended on the degree of immunogenetic disparity between the leucocyte co-donors (Bain et al, 1964).

One way MLCs are routinely performed using as the stimulating population either genetically tolerant F_1 lymphocytes with parental responder cells or fully allogeneic stimulator cells which are treated with either Mitomycin C or ionizing radiation prior to culture to prevent proliferation. The extent of the proliferation in the responder cell population is assayed by measuring the amount of radioactive DNA precursor incorporated in the cultures.

The parameters of the MLC in the rat were established by exhaustive studies done by Wilson (1967), Wilson and Nowell (1971), Wilson and Fox (1971) and later by Antczak et al (1979) and Antczak and Howard (1979).

The MLC assay has long been accepted as a quantitative measure of the response of lymphocyte populations to transplantation antigens. It has however, been known for some years that the capacity to stimulate in MLC is mainly the property of lymphoid cells. Most studies have shown that alloantigen bearing non lymphoid cells such as erythrocytes, fibroblasts, cultured epithelial tumour cells and polymorphs do not stimulate in MLC (Hardy and Ling, 1969; Greineder and Rosenthal, 1975; Talmage et al, 1977).

A further requirement for primary lymphocyte activation in vitro is that lymphoid stimulator cells be metabolically active. A study by Kelso and Boyle (1982a and 1982b) demonstrated that UV irradiated cells, glutaraldehyde treated cells or splenic membrane fragments, although carrying an amount of Class I and Class II MHC antigens similar to that on X irradiated or untreated stimulator cells, do not

stimulate in MLC or induce a primary cytolytic response.

It has also become clear that not all lymphoid cell types have similar stimulatory capacity; Milford et al (1979) demonstrated that Ia positive rat thymus cells appeared to be good stimulators in MLC whereas Ia negative thymus cells were good responders.

Recent work by Wong et al (1982) has clarified this issue by delineating two populations of cells in the rat thymus. One, a high density fraction (greater than 90%), is not stimulatory in MLC but cells in the low density fraction (4-10%) are potent MLC stimulators. Cells in the low density fraction were found to be Ia positive, Fc receptor negative and Thy 1.1 negative with the morphological features of dendritic cells.

The importance of dendritic cells as MLC stimulators has also been recognized in more detailed studies of this cell performed in the mouse. Steinman et al (1983b) showed that dendritic cells (DC) present in spleen cell populations act as potent stimulators in MLC. Their selective removal dramatically reduces the stimulatory capacity of both unfractionated spleen cell populations and spleen adherent cell populations and stimulatory capacity is restored by the addition of small numbers of DC to the cultures.

These results all suggest that the proliferative response observed in cells in MLC is not a direct consequence of simple recognition of alloantigens in any form but is dependent on the presence of metabolically active mononuclear cells of a distinct cell lineage.

Early work on MLC responsiveness by Bach et al (reviewed in Bach et al, 1976) also suggested that the proliferative response in MLCis directed against a particular class of determinants on the stimulator cell population. In addition these studies suggested that

the responding cells might be a subset of the T lymphocyte population. Other workers identified the subset of cells that proliferate most vigorously in MLC as the Th cell subset (Cantor and Boyse, 1975; Mason et al, 1981).

The MLC measures responder cell proliferation in response to foreign alloantigens on appropriate stimulator cells. This is not necessarily an accurate reflection of the allografts reactivity of a potential host against donor strain transplantation antigens. Attempts to correlate MLC reactivity with the allograft response in vivo in various strain combinations of host and donor have shown that the degree of proliferation in vitro is not always a good indicator of the rapidity with which grafts are rejected in vivo (Guttmann, 1977). The MLC response correlates more precisely with the proliferative response observed in the local GVH assay and like it, has come to be regarded as presenting the inductive phase of the immune response (Wilson, 1974; Bach et al, 1976).

During MLC, lymphocytes with specific cytotoxicity towards cells bearing the alloantigens expressed on the stimulator cells are generated (Hayry and Defendi, 1970; Solliday and Bach, 1970; Lindquist and Guttmann,1971). On the basis of their expression of the Ø antigen the cytotoxic lymphocytes generated in MLC were identified as thymus derive T cells (Cerottini et al, 1970; Wagner et al, 1972). Their in vitro responsiveness was shown to be similar to that of the cytotoxic T cells which are generated in vivo during allograft rejection (Brunner et al, 1970; Brunner and Cerottini, 1971). This is measured in the CML assay. In vivo or in vitro sensitised cytotoxic lymphocytes (Tc) are incubated with target cells that have been labelled with ^{51}Chromium, a cytoplasmic radioactive label that binds to proteins of the cytosol and is released when

cells are lysed. The ability to cause target cell lysis is the basis of the functional definition of the T cytotoxic (Tc) cell. The CML assay is governed by strict requirements regarding the nature of the target cells (Brunner et al, 1970; Lightbody and Bach, 1972; Howard, 1973; Bevan, 1975). In general only blast transformed or tumour transformed cells appear to serve as target cells. Small lymphocytes are poor targets for Tc but mitogen transformed lymphocytes are susceptible to cell mediated lysis and are widely used as target cells in CML assays. The most commonly used mitogens are phytohaemagglutinin (PHA) or concanavalin A (Con A).

Effector cells and target cells are incubated together in ratios ranging from 100:1 to 1:1 for a period of 4 to 6 hours. The effector to target cell ratio, as well as the incubation period varies from worker to worker and is dependent mainly on the ease with which the chosen target cell is lyse. Extensive study of the kinetics of target cell lysis has shown that lysis may occur at any time from several minutes to hours after contact between effector and target cells (Sanderson and Taylor, 1975; Zagury et al, 1979; Berke, 1980).

Studies on the target antigens for Tc have shown that Class I antigens are needed for the induction of these cells and are the target for their effector function. The CML assay provides proof that T cells, or more particularly the Tc subset, are able to destroy cells bearing foreign alloantigens to which they have been sensitized. The demonstration that Tc cells can kill target cells in vitro was instrumental in implicating these cells as the effector cells in graft rejection.

There are, however, some objections, based on theoretical and technical grounds, to the conclusion that the CML is the in vitro correlate of the efferent of effector phase of the immune response.

Many of these will be discussed in a later section (1.5.3). At this stage however it is appropriate to mention objections which are based on the technical requirements for demonstrating CML.

It is hard to envisage that the effector to target cell ratio, which commonly has to be employed to achieve significant target cell lysis, could reflect the in vivo situation unless effector cells display extreme discrimination in their choice of graft borne target cells and inflict their lethal hit at optimal sites. No evidence is currentlyavailable on the site of Tc action in vivo. Experiments designed to determine the degree to which sensitized T cells localize to a site of antigen have indicated an apparent lack of discrimination in localisation following IV injection (Section 1.5.3).

More important is the matter of the nature of the target cells required to demonstrate in vitro lysis. Graft parenchymal cells in no way resemble the ideal in vitro target cell (Parthenais et al, 1979). This might suggest that the Tc either achieves graft cell destruction by a different mechanism in vivo than which operates in vitro, or that the Tc is not the primary effector of destruction of solid tissue grafts.

Not withstanding the doubts which exist as to the legitimacy of accepting the MLC and CML assay as an absolute correlate of in vivo effector function, there is no doubt that these assays have provided convenient models in which to analyse the requirements for T cell activation and the differentiative events which may follow activation.

1.4.2 Assays demonstrating antibody mediated effector mechanisms

In vitro assays have provided evidence for a number of antibody

dependent mechanisms by which cellular damage may be procured. These have potential relevance to in vivo graft rejection.

*** Complement mediated lysis**

Specific alloantibody bound to alloantigen on target cells may effect target cell lysis via complement activation and direct cytolysis (Carpenter et al, 1976). The possible role of this effector mechanism in graft rejection is discussed in a later section (1.5.2).

*** Antibody dependent cell mediated cytotoxicity (ADCMC)**

In the presence of IgG alloantibody naive lymphoid cells have been shown to lyse target cells bearing alloantigens against which the alloantibody is directed. Very low concentrations of antisera are required for this form of cytotoxicity (Cerottini and Brunner, 1974) and it is apparently operative in the absence of complement. Thus, theoretically tissue damage might be effected by this mechanism in the presence of a lower concentration of antibody than is required for complement mediated lysis (MacLennan, 1972; Perlmann et al, 1972). The effector cell involved, designated a K cell, as originally described by Moller (1965) is an Ig negative, thymus independent, Fc receptor bearing cell found in all lymphoid compartments (MacLennan, 1972). K cells have been harvested from rejecting grafts in rats (Tilney et al, 1975) and man (Strom et al, 1975). Their presence has been interpreted as indirect evidence suggesting that ADCMC may contribute to the effector arm of the alloimmune response.

The results of recent work using spleen cells and alloantibody obtained from rats which had recently rejected a cardiac allograft

suggest that different structural components of a graft may differ with regard to their susceptibility to either Tc or ADCMC mediated damage (Parthenais et al, 1979). Endothelial cells from cultured neonatal hearts were shown to be susceptible to Tc mediated damage whereas myocardial cells were damaged by ADCMC mechanisms (Parthenais et al, 1979).

1.4.3 The graft versus host reaction as an assay for alloreactivity

The work of Billingham and Brent (1959) and Simonsen (1957; 1962) established that the graft versus host (GVH) reaction results from an immunological response by the cells of a lymphoid graft against the histocompatibility antigens of the host. In the years since this work, results have emerged which cannot be reconciled with the view that the GVH reaction simply represents an attack on a functionally defenseless host resulting from the recognition of host MHC antigens. There is certainly good evidence that the GVH reaction is initiated by the response of T lymphocytes to the foreign histocompatibility antigens of the recipient (Elkins, 1971) however, as with the MLC, it is apparent that there are specific requirements relating to both the nature of the alloantigens and the form in which they are presented. Elkins and Guttmann (1968) studied the local GVH reaction resulting from the injection of parental strain lymphoid cells injected under the kidney capsule of semi-allogeneic F_1 hybrid hosts. They found that irradiation of the hybrid host, prior to the subcapsular injection of parental cells, inhibited the subsequent development of the local renal GVH response, but local irradiation of the kidney did not (Elkins, 1971). They also showed that the injection of syngeneic parental cells beneath the capsule of a parental renal graft in a semi allogeneic F_1 hybrid led to a local

GVH reaction (Elkins and Guttmann, 1968). These results were interpreted as suggesting that lymphoid cells of host origin are a necessary stimulus to the induction of GVH type responses and that the recognition of foreign antigen on parenchymal cells only, will not, as had earlier been supposed (Simonsen, 1957; Billingham et al, 1958; Medawar, 1958), produce a GVH reaction.

One of the conditions for the manifestation of GVH disease is that the host is unable to reject the grafted cells. This condition is satisfied when allogeneic lymphocytes are injected into animals rendered unresponsive by irradiation or drug induced leucopenia or into animals whose immune system is immature. In these cases any observed host cell division can be interpreted as residual (or developing) immunocompetence that is a host versus graft (HVG) reaction, which may be eventually aborted by the GVH response. This explanation cannot account for host cell proliferation in "genetically tolerant" semi-allogeneic F_1 hybrids injected with parental strain lymphoid cells. Elkins (1970) showed that in such hosts there is an initial phase of donor cell proliferation followed by a phase in which the majority of proliferating cells appear to be of host origin (Elkins, 1970). This was confirmed by Rolstad (1976) who demonstrated that the increase in lymphoid tissue weight accompanying GVH disease in F_1 hybrids is caused by proliferation and trapping of both F_1 cells and parental cells.

Adult F_1 hybrids are comparatively resistant to the induction of GVH disease and large numbers of parental strain lymphocytes are needed to induce the disease in normal adult hybrids (Gowans, 1962). Irradiated adult F_1 hybrids are, however, susceptible to disease induction with small numbers of parental cells (Gowans, 1962). These two observations taken with the fact that F_1 cell proliferation is a

feature of the GVH response in F_1 hybrids (Elkins, 1970) suggest that a response by F_1 hybrid cells against parental cell receptor for the unshared haplotype may occur and might account for the resistance of normal adult F_1 animals to GVH disease.

Support for this hypothesis came from the demonstration that F_1 hybrids which have been preimmunised with very small numbers of parental T cells, show a reduced local popliteal LN GVH response when challenged with large numbers of parental cells (Woodland and Wilson, 1977). The observed resistance to the local GVH reaction is specific for cells of the immunizing parental strain and is dependent on the presence, within the immunizing cell population, of T cells with reactivity towards the unshared parental alloantigens (Woodland and Wilson, 1977).

Further work (Bellgrau and Wilson, 1978) demonstrated that immunization also protects against systemic GVH disease and that T cells from immunised F_1 hybrids can transfer protection to irradiated syngeneic recipients. These workers went on to show that resistance cannot be induced in irradiated F_1 hosts, that once induced in normal hosts resistance is radioresistant and that it is the result of a response directed against the receptors for the unshared parental MHC antigens on the donor T cells. Immunologically specific suppression of GVH reactions has also been achieved by immunization of F_1 hybrid hosts to produce antibody reactive with receptors on donor lymphoid cells for unshared host antigens (Binz et al, 1973; McKearn, 1974). Anti receptor (anti-idiotypic) antibody has been detected in resistant F_1 hybrid rats but not those rendered susceptible to GVH disease by irradiation (McKearn et al, 1974).

Bellgrau and Wilson (1978) make the point that most studies which have directly or indirectly, provided evidence for the presence

of anti idiotypic immune responses have involved extensive and repeated immunizations, often in the presence of adjuvants. In their experiments, where as few as 10^6 parental T cells administered to F_1 hybrids 1-3 days prior to irradiation rendered them resistant to the effect of a supra lethal dose of parental lymphocytes (Bellgrau and Wilson, 1978), there is some doubt about attributing the protective effect to the action of anti idiotypic antibody. It has however, been reported that anti-parental lymphocyte reactivity in F_1 hybrid rats is characterised by the rapidity with which it may be induced (McCullagh, 1977). Earlier work done by Sprent and Miller (1972a) may have also some relevance to the question of interaction between parental and F_1 cells. They showed that the parental T cells recovered fom the TD of irradiated F_1 mice injected 4-6 days previously with parental TDL or thymocytes did not induce GVH splenomegaly or wasting GVH disease in neonatal F_1 mice although they were strongly cytotoxic in vitro and could mediate skin graft rejection in neonatally thymectomized mice syngeneic with the parental strain. Rolink et al (1982) have reported that the population of cells present in the spleen on the fifth day after the injection of parental cells into irradiated F_1 hybrid recipients, has quite different functional activity in vivo to the population present a day later. Whereas both cell populations are very efficient at lysing F_1 target cells in vitro, day 5 spleen cells are able to induce lethal GVH disease in non irradiated secondary F_1 mice and day 6 spleen cells cause chronic GVH disease. Rolink et al (1982) proposed that between 5 and 6 days after injection of parental cells a physiological reassortment of cells in the F_1 spleen occurs such that cells taken at 5 days are enriched for alloreactive T suppressor (Ts) cells and those taken at 6 days are enriched for alloreactive Th

cells.

There is other evidence that there may be different forms of the GVH response and these may be induced by different T cell populations. It has been shown that the T cell subpopulation that mediates the popliteal LN GVH reaction in the rat is the $W3/25^+$ $MRCOX8^-$ (Th) cell subset (White et al, 1978; Brideau et al, 1980), whereas lethal GVH disease is mediated by pure populations of either $W3/25^+$ (Th) or MRC $OX8^+$ (Tc/Ts) T cell subsets (Mason, 1981). Lethal-acute GVH disease is characterized by thymic involution, hypoplasia of lymphoid tissue and hypogammaglobulinaemia; chronic GVH disease on the other hand, is characterised by lymphoid tissue hyperplasia, hypergammaglobulinaemis and the development of autoantibodies and the symptoms of connective tissue disease (Rolink et al, 1982). Rolink et al (1982) suggest that induction of lethal GVH disease depends upon a suppressor mechanism which occurs in the presence of specific CTL and operates to suppress cellular proliferation in the F_1 host and thus cause depletion of host lymphoid tissue. Work by Gleichmann et al (1983) provides support for this postulate. They showed that chronic GVH disease was mediated by T cells of the Lyt 1^+1^- phenotype selectively activated by Class II alloantigenic differences whereas with lethal GVH disease they found that neither unprimed Lyt 1^+2^- nor Lyt 1^-2^+ donor T cells alone were capable of inducing the disease. Unseparated donor T cells were required to mediate lethal GVH disease in non-irradiated F_1 recipients disparate for both Class I and Class II alloantigens. They postulate that in lethal GVH disease, Class I alloreactive Ts cells which, in the precursor state at least, express the Lyt 1^+2^+ phenotype, are activated by Class II reactive Lyt 1^+2^- donor Th cells to cause pancytopoenia (Gleichmann et al, 1983).

The fact that Sprent and Miller (1972a) were unable to induce lethal GVH disease or splenomegaly with TD lymphocytes from irradiated F_1 hosts injected with parental cells whereas the Rolink group (1982) were able to do so with host spleen cells may indicate that the cells responsible for the effect in the latter experiments did not recirculate.

1.5 EFFECTOR MECHANISMS IN GRAFT REJECTION

1.5.1 Concepts

Early experimental evidence that the passive transfer of immune lymphoid cells transferred allograft immunity (Mitchison, 1954; Billingham et al, 1954; Sprent and Miller, 1972a) and serum did not do so (Billingham et al, 1954, 1963) led to the concept that cell mediated mechanisms are more important in allograft rejection than humoral ones. There is ample evidence however that both T and B cell populations respond specifically to foreign historcompatibility antigen and infiltrate allografts. In addition both alloantibody and a variety of cell types have been shown to have the capacity to lyse alloantigen bearing target cells in vitro.

A prominent histological feature of an allograft undergoing rejection is its infiltration by lymphocytes of varying sizes (Medawar, 1944; 1945). Both T (Balch et al, 1973) and B (Strom et al, 1975) lymphocytes infiltrate rejecting allografts. In addition, macrophages and monocytes are present in the cellular infiltrates, and in the necrotic areas of the grafted tissue granulocytes are seen (Simonsen et al, 1953). Analysis of the cells recovered from rejecting cardiac allografts in the rat demonstrate that although T cells can be identified, 35%-47% of the mononuclear cells present

bear surface Ig, and a further 15%-25% are macrophages (Tilney et al, 1975). The variety of cell populations identified in grafts which have undergone rejection is equalled only by the variety of cell types with the capacity to cause, either directly or indirectly, in vitro lysis of target cells. Alloantibody can do so in the presence of complement or via antibody dependent cell mediated cytotoxicity. T cells (Hayry et al, 1972; Cerottini and Brunner, 1974), non T lymphocytes (Trinchieri et al, 1973), and macrophages (Evans and Alexander, 1970) can all function as cytotoxic cells to relevant allogeneic target cells in vitro. Studies of the kinetics of cellular differentiation in the allograft response have not helped in the identification of the effector mechanisms involved.

There isexcellent correlation between the time course of Tc formation and graft rejection (Canty and Wunderlich, 1971), however, the course of development of alloantibody forming cells and circulating alloantibodies also correlates with graft rejection (Carpenter et al, 1976).

There is currently no good reason to exclude the possibility that T cells, alloantibody and macrophages may all have a role in effecting graft rejection under different circumstances. The experimental evidence relating to the possible mechanisms by which the destruction of grafted tissue may be implemented will now be dealt with in detail.

1.5.2 The role of antibody as the effector of graft rejection

At one time humoral immunity was thought to play little if any role in rejection, a belief largely based on skin grafting experiments and the failure of early attempts to induce rejection by the passive transfer of serum (Billingham et al, 1954, 1963; Stetson,

1963). However in these early experiments demonstrating that second set rejection could be transferred adoptively with lymphoid cells and not sera, the cell populations transferred contained both T and B cells, and thus the possibility that B cells with the capacity to produce local alloantibody after infiltrating the graft may have contributed to graft destruction was not excluded (Stetson, 1963).

It is clear that humoral immunity is an important factor in the elimination of dispersed cellular grafts, such as lymphoid and bone marrow cells, which are freely accessible to circulating antibody (Moller and Moller, 1962; Stetson, 1963; Waksman, 1974). The relevance of humoral factors to the destruction of organised tissue allografts is in doubt. There are a number of mechanisms identified in vitro by which alloantibody may procure tissue damage (section 1.4.2), and as large numbers of B cells are present in rejecting allografts (Tilney et al, 1975) antibody produced, and acting within the graft itself, cannot be excluded as an effector mechanism in graft rejection.

There is little doubt that alloantibody can mediate hyperacute rejection. This has been demonstrated in man (Kissemeyer-Neilsen et al, 1966), rats (Lindquist et al, 1971; Guttmann, 1974), sheep (Pedersen and Morris, 1974b) and rabbits (Hobbs and Cliff, 1973). It may also be the effector of the damage which leads to the arterial lesions of chronic rejection in organ transplants in man (Winn, 1970; Rossen et al, 1971). Hyperacute rejection is characterised by an extremely rapid onset, usually within minutes or hours following vascular anastomosis (Forbes and Guttmann, 1984). Microscopic examinations of grafts reveal a vasculitis, the severity and extent of which correlates with prognosis (Tilney et al, 1984). It is believed that the lesions are mediated by the deposition of IgG and

complement which are frequently found in association with the vascular lesions (Lindquist et al, 1968; McKenzie and Wittingham, 1968). There is also an accumulation of polymorphonuclear (PMN) cells within the graft vasculature during the early rejection period, and later fibrin deposition in vessels and thrombosis. Lymphocytic infiltrates are not characteristic (Kissmeyer-Neilsen et al, 1966; Williams et al, 1968; Milgrom, 1977) of hyperacute rejection. The sensitivity of grafts to antibody mediated attack appears to be dependent on the nature of the tissue grafted and the time, following transplantation, at which antiserum is given (Gerlag et al, 1975; Jooste and Winn, 1975; Burdick et al, 1979). Hyperacute rejection is complement dependent. Procedures which deplete graft hosts of complement abrogate the response, and it is not seen following the transfer of non complement fixing $F(ab')_2$ antibody fragments (Winn et al, 1973). PMN cells, which feature prominently in the histological appearance of the intravascular inflammatory reaction, appear to represent an obligatory component of antibody mediated graft destruction as hosts depleted of PMN also do not develop hyperacute rejection (Winn et al, 1973).

As previously discussed (section 1.2.3.2) critical differences exist between the manner in which directly and indirectly vascularized grafts sensitize the host. There may also be differences in effector mechanisms between the two modes of grafting. Neither vascular nor lymphatic connections are made at the time of implantation of a skin graft, whereas directly vascularized grafts are immediately exposed to host lymphoid cells in the circulation. Skin allografts elicit a prompt and vigorous response in regional lymph nodes (LNs) with lesser changes occurring in the spleen; vascularized organ grafts elicit vigorous responses in both spleen

and LN. Furthermore the survival of indirectly vascularized grafts may be prolonged by regional lymphadenectomy but not splenectomy (Dorsch et al, 1983) while the converse has been reported for organ grafts (Souther et al, 1974). That humoral immunity may be more important in the rejection of organ grafts than skin grafts is suggested by the finding that anti-B cell serum failed to have any effect on skin graft survival, but prolonged heart graft survival, whereas T cell specific antisera administered to graft recipients prolonged skin but not heart graft survival (Birinyi et al, 1981).

The apparent differences in the effect of antibody with respect to skin and organ grafts, taken with the well known differential survival of skin and organ grafts (White and Hilderman, 1969; Warren et al, 1973) may reflect basic differences in effector mechanisms dependent on the type of tissue grafted. These differences may depend on non specific differences in the mode of transplantation (secondary vascularization and prolonged ischaemia in the case of skin, compared with prompt primary anastomosis in the case of organ grafts), quantitative or qualitative differences in the antigens expressed on the tissues or different modes of sensitization.

The inability of early workers to demonstrate accelerated rejection following the transfer of immune serum may be attributable toa deficiency of endogenous complement in mice and rats (French, 1972). Hyperacute skin graft rejection in mice has been shown to occur only when an exogenous source of complement is transferred with the specific serum (McKenzie and Henning, 1978; Burdick et al, 1979). More interestingly the time at which antibody is administered to skin graft recipients appears to be crucial to the outcome.

Rat skin xenografts on mice shown marked variability in their susceptibility to antibody. Antibody administered up to the fifth or

sixth day after grafting does not cause graft rejection in T cell depleted mice. Resistance to the effect of antibody apparently then decays and peak susceptibility to antibody mediated damage occurs at 14-16 days after grafting. Subsequently susceptibility waned (Jooste et al, 1981a). The latter was indirectly shown to be related to the replacement of donor endothelium by host cells (Jooste et al, 1981b). Primarily vascularized grafts on the other hand remain highly sensitive to antibody for prolonged periods (Hart et al, 1980) and the vascular endothelium of organ grafts has been shown to remain of donor origin and express donor alloantigens for long periods (Hart et al, 1980; Jooste et al, 1981b).

ADCMC has also been postulated as a possible antibody mediated effector mechanism in graft rejection in vivo (Perlman et al, 1972; Strom et al, 1977). It has been reported that 16-18% of cells infiltrating rat heart allografts are Fc-receptor positive cells, distinct from macrophages, which have the capacity to lyse target cells coated with anti-donor IgG (Strom et al, 1977). The observed high frequency of Fc-receptor bearing cells in the infiltrates of rejecting human kidney allografts (Strom et al, 1975; Von Willebrand and Hayry, 1978) together with reports of IgG deposits (Carpenter et al, 1976) suggests that antigen-antibody complexes may contribute to the entrapment of Fc-receptor positive cells at the rejection site.

Whether or not alloantibody normally plays a role in graft destruction, there is no doubt that under some circumstances it may protect grafts from rejection. It was observed many years ago during attempts to transfer rejections of allogeneic tumours in mice with anti sera that rather than accelerating rejection the sera enhanced tumour growth (Kaliss et al, 1953). More recently it has been shown that specific alloantisera can also significantly prolong the

survival of directly vascularized organ grafts (Stuart et al, 1968; French and Batchelor, 1969; Fabre and Morris, 1974; Tilney and Bell, 1974; Gerlag et al, 1975). This effect, termed enhancement, like the destructive effect of antibody, is more easy to induce in relation to directly vascularised grafts.

White and Hildeman (1969) observed that skin grafts were resistant to the induction of enhancement, whereas kidney grafts in the same strain combination were easily enhanced. Similarly, Warren et al (1973), using indirectly vascularized neonatal heart grafts and directly vascularized adult heart grafts observed that the indirectly vascularized grafts were rejected acutely whereas the surgically anastomosed hearts in the same strain combination were readily enhanced.

In summary it is clear that the specific antibody produced in response to an allograft may influence the survival of the graft. Its capacity to enhance the survival of primarily vascularised organ grafts is well established as is its role in hyperacute rejection. Its relative contribution to normal primary first set rejection is not clear.

1.5.3 Cell mediated effector mechanisms

There is no doubt that both T cells and macrophage-type cells are essential for the initiation of the allograft response. Their respective roles in the effector arm is less clear. That T cells infiltrate grafts, can adoptively transfer immunity, break neonatally induced tolerance, produce GVH reactions and cause target cell lysis in vitro, confirms that they play a vital role in the rejection process. Whether they are the actual effectors of tissue destruction and if so, which of the subsets is the effector cell is less clear.

Billingham et al (1954, 1963) having demonstrated that graft rejection was cell mediated, postulated that it might represent a DTH type reaction. This thesis was supported by the demonstration that the intradermal injection of cells from a skin graft donor in a guinea pig which had rejected a skin graft from that donor caused a tuberculin like reaction at the injection site. There was, however, a swing away from the belief that allograft immunity was an expression of a DTH type reaction following the discovery that cytotoxic T lymphocytes have the capacity to specifically lyse target cells in vitro. It became generally accepted that the CML assay was the in vitro correlate of graft rejection and Tc were the primary mediators of tissue destruction with the Th cell or T_{dth} cell being necessary for the optimal induction of Tc in vitro, but playing no direct role in the effector phase of graft rejection. The involvement of cells such as macrophages, mast cells, granulocytes and natural killer cells in graft rejection was considered to be at a secondary level. Recently several independent groups have challenged this view (Loveland et al, 1981; Dallman et al, 1982), and suggested that Th/T_{dth} cells, not Tc, are responsible for graft destruction. This represents a return to the traditional concept of cell mediated rejection which links the process to the DTH reaction.

Experiments designed to identify the cell population responsible for graft destruction, have examined the nature and functional characteristics of cells in the lymphoid tissue draining allografts and of the cells infiltrating grafted tissue.

In vitro characterisation of the cells present in the lymphoid tissues of graft hosts have shown that T lymphocytes (Hayry et al, 1972; Cerottini and Brunner, 1974) and non T lymphocytes (Trinchieri et al, 1973; Roberts and Hayry, 1977) and macrophages (Evans and

Alexander, 1970) present in this tissue can all manifest cytotoxicity towards relevant allogeneic target cells in vitro.

The characteristics of Tc recovered from the lymphoid tissue of graft recipients have been examined in considerable detail (Cerottini and Brunner, 1974). Tc are generated in response to intraperitoneal tumour cell allografts and are detectable in the spleen 3-4 days after challenge. A peak response occurs at day 10-11 after grafting after which time it gradually subsides. Activity is however still detectable for several months. There is a 48 hour delay between the appearance of Tc in lymphoid organs and their subsequent release into the blood (Brunner et al, 1970; Canty et al, 1971). The Tc response to skin grafts is of much briefer duration. Tc are detectable in the LN draining the graft several days before graft rejection, subsequently they can be found in the spleen, blood and contralateral LN. Peak Tc activity coincides with graft rejection and is greater in the draining LN than the spleen. No Tc activity is detectable several weeks after rejection (Canty and Wunderlich, 1971; Peter and Feldman, 1972).

The identification of cells with lytic activity and the demonstration of their enrichment in local lymphoid tissue at the time of graft rejection was instrumental in implicating Tc as the primary effector cell in graft rejection. This assumes however, that cells removed from lymphoid tissues are representative of the entire lymphoid population. It says little about what is actually happening at the graft site. Several workers (Snow and Hilgard, 1981; Ascher et al, 1983) have shown that the appearance of Tc within local LN and spleen does not correlate with their peak appearance within sponge matrix allografts. Other studies have shown that high concentrations of sensitized cells appear in the peripheral blood following graft

rejection but not during the rejection process (Sprent and Miller, 1976; Burton and Russell, 1981).

A more direct approach to identifying the mechanism of graft rejection has been adopted by workers who have examined the type and function of cells infiltrating allografts during rejection. Tilney et al (1975) characterised infiltrating host cells isolated from the parenchyma of rat cardiac allografts. The cells were obtained by mechanical disintegration of the tissue followed by enzymatic treatment and then purification of the infiltrating cells. T cells isolated in this manner from grafts displayed specific cytotoxicity in vitro for cells bearing graft alloantigens. The accumulation of specifically cytotoxic cells commenced three days after implantation and peaked just prior to graft rejection. Subsequent to graft rejection the proportion of cytotoxic cells among the infiltrating population fell. Roberts (1977), using sponge matrix allografts, confirmed the presence of specific Tc among the graft infiltrate. Cytotoxic macrophages have also been identified within cell population extracted from sponge matrix grafts (Roberts and Hayry, 1977), but not in populations extracted from rejecting rat-heart allografts (Tilney et al, 1975).

A somewhat more sophisticated protocol was used by Strom et al (1977). They grafted two mutually allogeneic hearts of different RT1 types into a naive rat to resolve the question of whether the accumulation of sensitized T cells within allografts is a consequence of specific antigen recognition or not. The harvested host T cells infiltrating both grafts had the capacity to lyse cells bearing the alloantigens expressed by either graft. Entirely compatible results were obtained by Ascher et al (1979; 1980) who examined the specificity of the Tc cells which accumulated in two unrelated sponge

allografts in the same murine host. They showed that each allograft contained T cells cytotoxic for both the relevant and the irrelevant graft, with only marginal preference being demonstrated towards antigens of the relevant graft. These studies support the notion that the actual migration of Tc into rejecting allografts may be non specific and the small degree of specificity evident in some studies is due to preferential retention of the relevant Tc within the graft.

Hancock et al (1983) reported a large number of infiltrating macrophages and a significant number of T cells in the interstitium of rejecting human renal allografts. In cases showing a moderate rejectionresponse theproportion of T cells and macrophages were approximately equal and the majority of the T cells were of the $OKT8^+$ suppressor/cytotoxic phenotype. In contrast, kidneys showing a severe rejection response with marked destruction, were characterised by a preponderance of infiltrating macrophages and polymorphs. In these studies the function of the $OKT8^+$ cells in the infiltrates was not examined.

In view of the evidence that the proportion of antigen specific T cells infiltrating grafts is low in comparison with the total number of infiltrating cells (Strom et al, 1977; Ascher et al, 1983), morphological studies demonstrating high proportions of T cells infiltrating grafts must be interpreted with caution unless accompanied by functional studies which clearly implicate these cells in the rejection process. Indeed the results of a large number of studies designed to demonstrate antigen specific homing of specifically sensitised lymphocyte populations suggest that caution should be exercised in attributing particular roles to cell types on the basis of their localisation in tissues. Early studies suggested that activated lymphocytes transferred to syngeneic hosts did not

accumulate specifically in skin grafts bearing the antigen to which the lymphocytes were sensitized (Najarian and Feldman, 1962; Billingham et al, 1963). More recently, in similar systems, small selective accumulation in the specific graft has been demonstrated by some workers (Emeson and Thursch, 1971; Tilney and Ford, 1974a). Sprent and Miller (1976) found that homing of specifically sensitized T cells to the relevant graft was never more than twofold greater than to third party grafts which is in agreement with the findings of Tilney and Ford (1974a). On the other hand, Bhan et al (1975) separated and labelled alloimmune T and B cells in vitro and found that sensitized T cells migrated into both specific and third party allografts and B cells did not enter either.

The small amount of specific 'homing' of sensitized cells into grafts observed by some workers has been interpreted as suggestive evidence that cytotoxic T cells are the effectors of allograft rejection in vivo (Tilney and Ford, 1974a). The objection to this is the finding that the migration of sensitised cells is to a large extent random and there is always a significant infiltration of sensitized cells into irrelevant third party grafts (Strom et al, 1977). The presence of non specific T cells at a site of irrelevant alloantigen is explicable in terms of the role of lymphocytes in immune surveillance. In order to fulfil this, T cells must be able to migrate into all tissues to contact antigen at sites distant form the site of first entry. For sensitised T cells to migrate specifically to the site of first contact with alloantigen would require that they possess a highly sophisticated homing mechanism. Thishas not been demonstrated. It is likely that effector T cells directed at one set of alloantigens enter grafts as a consequence of normal recirculation and migration patterns and the increased traffic

in the area.

A number of studies have shown that there is a substantial outflow of white cells from a recently transplanted renal allograft (Pedersen and Morris, 1970; Nemlander et al, 1982; 1983). Nemlander et al (1983) have used a model that involves pulsing either the whole animal or a renal allograft *in situ* with tritiated thymidine to investigate the traffic of allograft responding leucocytes between host and graft. An important aspect of this model is that the responding cellsare not handled *in vitro*. It was found that the first host derived labelled cells appeared in the graft, and the first graft derived labelled cells appeared in the host, on the day after transplantation. Following this there was an exponential increase in the rate of labelled cell traffic in both directions. All mononuclear cell types participated in the traffic. The entrapment of labelled cells which was observed in allografts was not observed in autografts. The peak of traffic took place on day four (2-3 days prior to graft rejection) and then suddenly declined, however proliferation of labelled cells in their original compartments continued.

Other workers have similarly shown, in immunocompetent hosts, that effector cells enter directly vascularized grafts as early as four days after grafting and proliferate there (Tilney et al, 1976; von Willebrand et al, 1979a). Hayry et al (1979) reported a marked proliferation of lymphoid cells, and to a lesser extent monocytes, in rejecting kidney parenchyma in rats during the first week after transplantation. Taken together these results suggest that once specific effector cells enter the graft, there is a second phase of proliferation and clonal expansion (Ascher et al, 1982; Nemlander et al, 1982). Therefore proliferation *in situ* may help to amplify the

host versus graft reaction. The in situ expansion of the response is associated with the release of various lymphokines and inflammatory mediators which increase vascular permeability and attract leucocytes including lymphocytes from the circulation into the graft (Hopt et al, 1980, 1983).

There is thus abundant evidence that an allogeneic graft does accumulate sensitized lymphocytes directed against the alloantigens of the graft, but that this population is invariably accompanied by a great number of cells which lack immunological specificity. Most studies of recruitment of cells to graft sites favour the hypothesis that recruitment is largely non specific and the presence of specifically sensitized cells is the result of specific retention. It is likely that cells are sensitized either in lymphoid tissue or the graft, and expand and mature into effector cells primarily in the latter site. There is no doubt that T cells accumulate in rejecting allograftsand that a proportion of these are Tc with the relevant specificity. However a distinction has to be made between the capacity of T cells to directly effect graft rejection and their capacity to initiate a process which is ultimately mediated by other effector mechanisms.

In vitro models have elucidated the complex interactions between different functional and phenotypic subsets of T cells, macrophages, dendritic cells and soluble mediators which may follow contact between alloantigen and immunocompetent cell populations. Histological and ultrastructural studies have shown that all the above cell types may be represented in the graft at various times during the rejection response. Neither group of studies has clearly defined which of the possible cellular interactions normally effect graft rejection.

A further model which has been extensively employed, in attempts to resolve the question of which cell mediates graft rejection, is the adoptive transfer of cell populations with recognised in vitro function to grafted immunologically inert recipients. Sublethally irradiated animals or adult thymectomized, irradiated and bone marrow reconstituted (ATXBM) animals have been used as adoptive hosts in such studies.

Evidence for the role of Th cells in the rejection of indirectly vascularized grafts was provided by the work of Dallman and Mason (1982) using ATXBM rats. They showed that the rejection of skin grafts could be procured by TDL or the Ig^- MRC $OX8^-$ subpopulation separated from it. The Ig^- $W/25^-$ subset of cells was ineffective at procuring graft rejection. Skin grafts on animals restored with this subpopulation lasted the life time of the recipient. Subsequently in similar experiments testing T cell subsets from naive rats in adoptive transfer, it was shown that the Th subset restored the rejection of cardiac allografts to irradiated recipients. The Tc subset failed to restore graft rejection (Hall et al, 1983, 1984).

As far as sensitised cell populations are concerned, evidence from adoptive transfer assays suggests that both the Th and the Tc subpopulation can transfer rejection. Results obtained by Loveland et al (1981) indicated that the Lyt 1^- subpopulation of immune T cells restored skin graft rejection to ATXBM mice. They did not however exclude the possibility that Tc cells (Lyt 2^+3^+) might also be capable of mediating rejection. The demonstration that Lyt-1-specific antibody plus complement, used to deplete immune spleen and LN cells of Th cells, depleted effector cells is not surprising as, in the strain used (CBA mice expressing the Lyt 1.1 allele), the anti-lyt-1 sera probably killed all T cells (Le Francois and Bevan,

1984) and certainly the majority of Tc cells which, in this strain, are Lyt $1^+2^+3^+$ (Baldwin, 1981; Loveland et al, 1982).

Gurley and Lowry (1983) demonstrated that the adoptive transfer of Ig^- MRC $OX8^-$ immune spleen cells to sublethally irradiated rats led to the rejection of renal allografts in a time comparable with that procured by whole spleen cells. The same group (Lowry et al, 1983a, 1983b) using a similar model showed that both the Th and the Tc subset of immune splenocytes could effect rejection of cardiac allografts. Cells of the Tc subset appeared less potent than those of the Th subset, on a per cell basis.

The foregoing experiments might be considered as providing good evidence that first set rejection is procured by the Th lymphocyte subsets, were it not for the fact that neither the ATXBM or the heavily irradiated animals use as adoptive hosts are totally devoid of cells with the potential to contribute to rejection.

ATXBM animals contain B cells and macrophages. In addition, examination of TD lymph of these animals has shown that 3% of the cells label with MRC OX8 MoAb. The cells which bind MRC OX8 are large blastic cells. Further examination showed that TD lymphocytes of ATXBM rats can be stimulated in MLC to generate alloreactive cytotoxic cells; these are however, anomolously non specific in their action and have been tentatively identified as natural killer cells which are known to have the MRC $OX8^+$ phenotype (Dallman et al, 1982).

Histological examination of grafts in ATXBM rats given Ig^- MRC $OX8^-$ TDL has revealed that within the grafts there are $W3/25^+$ and MRC $OX8^+$ cells and numerous $W3/25^+$ Ia^+ cells. The latter were thought to be macrophages (Dallman and Mason, 1982).

Further work (Dallman and Mason, 1983) in which acutely reirradiated ATXBM rats were reconstituted with Ig^- MRC $OX8^-$

lymphocytes, indicated that tissue damage leading to graft rejection take place in the absence of macrophages as the rejecting grafts contained only W3/25$^+$ Ia$^-$ cells. These could have been Th cells or non activated macrophages however, the authors maintained that if the latter was the case, it was unlikely that these macrophages mediated rejection because macrophages are induced by a local immune response tobecome Ia$^+$ (Steinman et al, 1980). They proposed that Th cells could mediate graft destruction in the absence of macrophages. However as the ATXBM recipients were re-irradiated several weeks after skin grafting and just prior to cell transfer, the possibility exists that activated radioresistant cells (Tc's for example, the presence of which were not excluded in this study) were present in the graft host as a result of contact with graft alloantigen prior to the second dose of irradiation and these cells may have collaborated with the injected Th cells in the rejection of the grafted skin. Therefore the evidence provided by the model that Th cells can mediate graft rejection in the absence of macrophages or other collaborating cell types is tenuous.

Results obtained using heavily irradiated, reconstituted hosts are equally equivocal. In acutely irradiated rats restored with only W3/25$^+$ Th cells, the cellular components of the cardiac graft infiltrate are similar to those in rejecting grafts from non-irradiated hosts (Hall et al, 1983, 1984). Over one third of the cells infiltrating rejected grafts in irradiated hosts given inocula of cells of which 99% were W3/25$^+$ and less than 1% MRC OX8$^+$ cells were MRC OX8$^+$ cells. Examination of the lymphoid tissue in these animals showed there had been significant regeneration of T cells by the time of graft rejection. As the heart grafts were from heavily irradiated donors, the authors (Hall et al, 1983, 1984; Hall and

Dorsch, 1984) concluded that the MRC $OX8^+$ Tc cells in the hearts were most likely of host origin and probably arose from the division of radioresistant T cells in peripheral lymphoid tissue. It is equally possible that they were NK cells as they were not assayed for either specific or non-specific cytotoxicity.

Further evidence that cells present in the rejecting grafts of adoptively restored recipients may be of host origin is provided by experiments in which Lowry et al (1983a) grafted WF hearts into acutely irradiated Lewis recipients restored with Lew x BN cells sensitized in vivo to WF antigens. They found that 95% of the infiltrating cells were of host origin and less than 5% were Lew x BN cells. Christmas and MacPherson (1982) found that in acutely irradiated rats restored with TDL, there was a marked regeneration of monocyte/macrophage cells and these cells had infiltrated the rejected grafts.

Experiments reported by Le Francois and Bevan (1984) suggest that in ATXBM mice specific cytotoxic T cells of host origin may be generated following grafting. These workers transferred alloantigen primed Thy 1.1 T cells to Thy 1 congenic Thy 1.2 ATXBM skin grafted murine hosts. Using this model the authors could distinguish activity due to the adoptively transferred Thy 1.1 and host derived Thy 1.2 T cells in the immune response to minor H antigens. Evidence that either Lyt 2^- or Lyt 1^- immune spleen cells could mediate skin graft rejection in animals restored with T cell depleted bone marrow (BM) either one week or 6 weeks prior to skin grafting and reconstitution with T cell subsets is in accord with other reports that when immune populations are adoptively assayed both the Tc and the Th subset may mediate rejection (Lowry et al, 1983a). More interesting was evidence for the presence of Tc of host origin in

ATXBM mice restored with T cell depleted BM. Spleens from mice receiving Lyt 2^- immune spleen cells 6 weeks after BM grafting were shown to generate specific cytotoxic T cells of host origin when spleen cells were taken approximately 3 weeks after skin grafting and restimulated in MLC. This was most obvious in those animals receiving Lyt 2^- cells. The predominantly host derived cytotoxicity implies that Lyt 1^+ cells or accessory cells in the Lyt 2^- adoptive cells triggered naive resident host Tc (Le Francois and Bevan, 1984).

If animals were grafted and reconstituted 1 week after BM grafting, the host Tc response was absent and only donor derived Tc specific for graft minor H antigens were detected, whether the restorative inoculum contained Lyt 1^- or Lyt 2^- cells. The adoptively transferred cell populations were greatly enriched for one or other of the T cell subsets, however they both contained between 2 and 5% contaminating T cells of the other T cell subset. The Tc cells of donor origin generated in recipients of Lyt 2^- T cells may reflect expansion of the contaminating Lyt 2^+ cells present in the original inoculum (Le Francois and Bevan, 1984), or the activated Lyt 1^+ donor T cells may themselves have been cytotoxic. Neither possibility was explored as the effector cell phenotype was not examined beyond establishing the Thy 1 phenotype.

Le Francois and Bevan (1984) showed conclusively that ATXBM hosts contain resident cells with the capacity to generate specific Tc against minor antigens. This is in agreement with Duprez et al (1982) who demonstrated that spleen cells from ATXBM mice contain Thy 1^+ pre cytolytic T cells, originating from the BM grafts, that are able to respond to antigen <u>in vitro</u>, in the presence of exogenous IL2, and develop full Tc effector function. Both these results suggest strongly that ATXBM hosts lack sufficient 'help' to generate

Tc function as the addition of IL2 or Th cells, with the capacity to produce IL2, is sufficient to generate antigen specific Tc reactivity in ATXBM hosts.

It is apparent from the foregoing that in using the conventional models of ATXBM or acutely irradiated animals restored with pure subpopulations of lymphocytes, neither the presence or absence of graft rejection nor the morphological or phenotypic identification of infiltrating cells allows direct conclusions to be drawn regarding effector mechanisms in allograft rejection.

Less direct evidence relevant to the relative role of Tc and Th/Tdth cells in graft rejection is provided by the work of Loveland et al (1981). They reported good correlation between DTH responses and graft rejection in ATXBM CBA mice grafted with C57BL/6 skin and adoptively restored with various cell populations. There also appears to be correlation between the appearance of DTH reactions and the rejection of H-Y incompatible skin grafts although no such correlation is found between the appearance of Tc and rejection. Some mouse strains reject H-Y incompatible skin grafts but do not make detectable Tc responses, whereas others incapable of rejecting H-Y skin grafts, make <u>in vitro</u> Tc responses (Simpson, 1982). Similarly, in the experiments of Le Francois and Bevan (1984) already cited, no correlation was found between the degree of lysis in CML and the rapidity of skin graft rejection. Earlier work by Lubaroff (1973) which showed that irradiation abolished the capacity of rats to mount a DTH response to tuberculin following the transfer of sensitized cells but did not abolish the capacity to reject skin allografts is evidence that a DTH mechanism is unlikely to be the sole mechanism of graft destruction. In addition, rejection takes place in the virtual absence of macrophages (Waksman, 1974; Dallman

and Mason, 1983). The mere presence of macrophages in grafts is not proof that they are directly involved in graft destruction. Lymphokines released by immune T cells alter vascular permeability and attract leucocytes (Hopt et al, 1980, 1983). These include large numbers of macrophages. The lymphokines which are produced after the activation of T helper cells by antigen bearing Ia^+ macrophages induce Ia antigen expression on previously immature Ia negative macrophages (Scher et al, 1982), an example of the symbiotic relationship between Th cells and macrophages (Unanue et al, 1984). The presence of large numbers of macrophages in allografts may merely represent the operation of an amplification mechanism whereby continued alloactivation of specific T cells is ensured.

There is no doubt that the presence of macrophages does not necessarily indicate rejection and their absence does not preclude rejection. Large numbers of Ia^+ macrophages are found in both rejectingallografts and healthy syngeneic skin grafts on ATXBM rats (Dallman et al, 1982). If ATXBM skin graft recipients are irradiated after skin grafting and immediately prior to the injection of T cells of the helper phenotype, the grafts reject in spite of the fact that the infiltrates contain very few Ia^+ cells (Dallman and Mason, 1983).

Convincing evidence against tissue destruction in the allograft response being the result of a conventional DTH type response mediated by macrophages which have been rendered non-specifically cytotoxic by lymphokines released following contact between specifically sensitized T cells and alloantigen is provided by the experiments reported by Minz and Silvers (1970). These workers showed that when skin is grafted from allophenic mice to parental strain recipients, melanoblasts and hair follicle cells expressing the same H-2 antigens as the host survive whereas contiguous cells

expressing H-2 antigens foreign to the host are destroyed. It is hard to imagine that specificity of this nature could be achieved in DTH mediated destruction.

It is possible that Th cells may operate to cause graft destruction not through a DTH mechanism but directly. It has been reported that cells of the Th phenotype may directly kill cells bearing class II antigens (Meuer et al, 1982; Spits et al, 1983). Although class II antigens have a restricted distribution in normal tissue, activated T cells can induce class II antigens on rodent vascular endothelial cells (Pober et al, 1983) rat epidermal cells and gut epithelium (Barclay and Mason, 1982). This increase in class II antigens during the immune response would provide a target for tissue destruction mediated by Th cells as well as providing amplification for the inductive phase of the response.

There is no doubt that T cells are central to the process which culminates in the destruction of grafted tissues. Whether or not the ultimate mediator of destruction is sometimes, always or never a T cell is still not clear. The cytotoxic T lymphocyte was first identified in about 1970 on the basis of its capacity to cause specific lysis of the target cells in vitro. At this time it was assumed to be the mediator of graft rejection in vivo. Since then in vitro studies have revealed a number of other possible effector mechanisms. Lack of an appropriate model in which cells with demonstrable in vitro effector function may be directly tested for in vivo effector function (i.e. capacity to cause tissue destruction) has prevented any firm conclusion as to whether any of these mechanisms including Tc mediated lysis, are capable of causing the destruction of solid tissue grafts in vivo. The studies described in this thesis were designed to develop a model in which the capacity

of various cell populations to cause graft rejection *in vivo* could be tested and then to use the model to examine the relationship between *in vitro* cytotoxicity and graft rejection *in vivo*.

CHAPTER 2

MATERIALS AND METHODS

2.1 ANIMALS

Inbred rats of the DA ($RT1^a$), PVG ($RT1^c$), WF ($RT1^u$), BN ($RT1^n$), LEW ($RT1^1$) strains were used. They were obtained from the Inbred Rodent Facility, Blackburn Building, University of Sydney.

2.2 SOLUTIONS

2.2.1 PBS

Dulbecco's phosphate buffered saline (Oxoid Ltd., London, England).

2.2.2 DAB

PBS with added A and B salts (Oxoid).

2.2.3 DAB/FCS

DAB containing 2.5% (v/v) foetal bovine serum (FCS) (Flow Lab., Sydney, Australia).

2.2.4 DAB/FCS/P+S

DAB/FCS supplemented with 100 units Benzylpenicillin sodium (Glaxo P/L, Boronia, Vic., Australia)/ml and 100ug Streptomycin Sulphate BP (Glaxo)/ml.

2.2.5 DAB/FCS/Az

DAB/FCS containing 0.1% (w/v) sodiom azide.

2.2.6 Tissue culture medium (TCM)

RPMI 1640 (Gibco, Grand Is, NY, USA) buffered with 2g sodium bicarbonate/litre. Supplements added just prior to use were 2mM L-glutamine (Sigma Chemical Co., St Louis, MO., USA) diluted from 200mM frozen (-20°C) stocks and $5x10^{-5}$M 2-Mercaptoethanol (Sigma) diluted from $2.5x10^{-2}$M stock solution kept at 4°C.

* **TCM-1** - TCM was supplemented with either 5% normal DA rat serum (drawn from large pools stored at -20°C in 5ml aliquots) or 5% FCS (stored at -20°C in 5ml aliquots).

* **TCM-2** - TCM was supplemented with 0.6g Hepes (N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid; Sigma)/litre and 10% FCS.

2.2.7 Belmont Buffer

0.4g Hepes (Sigma) and 0.5g sodium bicarbonate were dissolved in 250ml of triple distilled water.

2.2.8 Ficoll diatrizoate

8 parts, 21% (w/v) Ficoll (Ficoll 400; Pharmacia Fine Chemicals, Uppsala, Sweden) in triple distilled water was mixed with 5 parts 33% (w/v) sodium diatrizoate (Sigma) in Belmont Buffer and the specific gravity was adjusted to 1.096 with triple distilled water.

2.3 LYMPH NODE AND SPLEEN CELL PREPARATION

All single cell suspensions were made in DAB/FCS. Excised peripheral lymph nodes (LNs) (cervical, deep cervical and mesenteric), spleen or thymus were minced with scissors and the cells freed from the connective tissue by careful crushing in DAB/FCS. Clumps and debris were removed by filtering through 4 layers of

sterile gauze. Cells were then washed twice and gently resuspended in an appropriate volume of DAB/FCS for counting.

Cells recently obtained from lymphoid tissue were counted on a Coulter DN electronic particle counter (Coulter Australia) with a 100u aperture. Cell viability was determined by eosin dye exclusion in the counting chamber of a haemocytometer.

2.4 PERITONEAL EXUDATE (PE) CELL PREPARATIONS

PE cells were obtained 4 days after the intra peritoneal injection of 5ml of 10% v/v Proteose Peptone (Difco Lab., Detroit Michigan, USA) in PBS.

The peritoneal cavity was washed by injecting 30 ml of cold PBS containing 2 units sodium heparin (Weddel Pharmaceuticals Ltd., London, UK)/ml through the abdominal wall after the overlying skin had been removed. The abdomen was then vigorously massaged. Cells were harvested through an 18 gauge needle inserted through the lateral wall of the abdomen by creating a tent in the muscle wall and carefully withdrawing the injected fluid. The washing procedure was repeated once. Cells were transferred to glass centrifuge tubes over ice and washed once at 4°C. Cells were resuspended and pooled in PBS/5% FCS at 4°C. In this way 50-80x10^6 cells/rat could be obtained.

2.4.1 Enumeration of phagocytosing cells in PE cell populations

The number of phagocytosing cells was determined using a method adapted from that used by Roser (1968). 1x10^6 PE cells were resuspended in 80ul of DAB/FCS to which 20ul of a seed colloid gold (10mg/ml; pH 7.7) suspension was added. The cells were rapidly mixed

and transferred to the chamber of a haemocytometer which was then placed in a petri dish on moistened filter paper. Cells were incubated for 90 minutes at 37°C. Cells which had phagocytosed colloidal gold were identified by the presence of dark pink or black cytoplasmic granules.

2.5 IRRADIATON

Animals or cells to be irradiated were placed on a platform which rotated at 16rpm around the vertical axis in a horizontal beam from a ^{60}Co source at approximately 25cm from the source. The beam was filtered with shaped lead discs to an isodose uniformity of greater than 96% and was directed into the lead cave where irradiation occurred under conditions of maximum backscatter. The time of exposure was calculated daily on a programme which corrected for isotope decay.

Rats were restrained in a perspex box, 15 x 15 x 5cm, holding 3 animals. Animals routinely received 900 rad ^{60}Co irradiation. Cells were irradiated at $10-20x10^6$ cells/ml in 15ml medium in 30ml plastic tubes.

2.6 IN VIVO PROCEDURES

2.6.1 Anaesthesia

All surgical procedures were performed under ether anaesthesia. Animals were kept anaesthetised by ether drips onto cotton wool plugs in polyethylene nose cones. Animals were killed by ether overdose.

2.6.2 Neonatal heart grafts

The hearts from rats less than 24 hours old were used. Neonates were anaesthetised and a V-shaped incision was made below the sternum and up through the ribs with pointed fine scissors. The sternum was pulled back to reveal the thoracic cavity. The pulsating heart was removed by grasping its vascular roots with blunt forceps and gently pulling. It was then placed in DAB/FCS in which it continued beating and thus became perfused with medium. Any adherent lung tissue was removed with fine scissors and the neonatal heart (NNH) transferred to fresh DAB/FCS.

The graft site was prepared by swabbing the footpad with 70% ethanol. A transverse incision was made with a scalpel through the skin at the base of the foot, and a pocket was made by blunt dissection of the subcutaneous tissues, towards the toes. The NNH was blotted with gauze to remove excess fluid, and then aspirated into a wide necked pipette, so shaped that the tip could enter the incision. The heart was expelled into the pocket and the pipette withdrawn.

After excess air and fluid were carefully expelled, the incision was sealed with "Supa-Glue" (Selleys Chemical Co., Bankstown, Australia) and liberally sprayed with Neotracin (Ethnor Pty. Ltd., Sydney, Australia) antibiotic spray.

Survival of NNH grafts was monitored daily or second daily by electrocardiography (ECG). The grafted foot was liberally swabbed with alcohol, and then two 30 gauge needles, which were attached to limb leads, were inserted intradermally on either side of the grafted heart. A third limb lead acted as the earth and was connected to the animal subcutaneously in the flank. The foot was sprayed with Neotracin antibiotic spray after ECG. The endpoint of rejection was

recorded as the first day that ECG activity ceased.

2.6.3 Local injection of cells into NNH graft sites

Immediately after grafting, and as soon as the glue sealing the graft incision had dried, cells were injected in 0.1ml into the subcutaneous pocket containing the graft.

2.6.4 Intravenous injections

Cells were injected intravenously in 1ml through a 23 gauge needle into the lateral tail vein.

2.6.5 Intra-arterial injections

Animals were anaesthetised. A 3cm midline incision through the skin to the underlying muscle was made below the sternum. The underlying muscle was then opened with fine scissors. The incision was held open with retractors. To expose the underlying descending aorta the intestines were held to the left hand side with gauze moistened with DAB. Connective tissue overlying the length of exposed aorta was stripped using dry cotton buds. The aorta was clamped proximal to the heart with a very fine vascular clamp. Cells were injected in 1ml through a 27 gauge needle that was bent perpendicular to the syringe so that it was parallel to the artery during injection. Just prior to injection, traction was applied to the artery by firmly holding the clamp up and towards the head, and the injection was performed over 1 minute in the direction of blood flow. After injection a cotton bud was placed firmly over the site, the needle carefully withdrawn and the clamp removed. The cotton bud was kept over the injection site for 8-10 minutes with a gradual reduction in pressure. At the end of the procedure the muscle and

the skin were closed separately using 3-0 silk.

2.7 STATISTICAL ANALYSIS OF RESULTS

The advice of Dr. N. C. Weber (Lecturer, Department of Mathematical Statistics, The University of Sydney) was sought. As the tempo of graft rejection does not follow a normal distribution, graft survival times for 2 different experimental groups were compared according to the Wilcoxin/Mann-Whitney rank sum test for non-parametric data. The calculated approximate normal deviate was referred to the tables of the normal distribution (N (0,1)) to give the significance probability p. The null hypothesis was rejected if $p<0.05$.

Comparisons involving more than 2 groups were performed by the Kruscal-Wallace test which is the non-parametric correlate of the analysis of variance. The calculated test statistic was referred to the tables of the cumulative distribution of Chi-square. The null hypothesis was rejected if $p<0.05$. If the null hypothesis was rejected the groups were compared in a pairwise fashion by the Wilcoxin/Mann-Whitney test to establish which group differed.

Results are expressed as the survival time (days) of NNH grafts in individual rats with the median survival time (MST).

2.8 CELL CULTURE

2.8.1 Preparation of cells for culture

Cells for culture prepared from tissues as described in Section 2.3 were washed twice in DAB/FCS/P+S and once in TCM. They were then counted and a measured volume was then removed and added to an

appropriate volume of TCM to make up the highest concentration required for assay or dilution series. All procedures were carried out at room temperature.

Cells for culture were added either to round bottom microtitre trays in a total volume of 0.2ml, or to tissue culture flasks. Cells were cultured in 5% Co_2 in air atmosphere at 37°C in a nonhumidified incubator. Prior to incubation, flasks were gassed with 5% CO_2 in air to ensure adequate buffering of TCM. Flasks were incubated upright.

2.8.2 Phytohaemagglutinin stimulation of lymphocytes

2.8.2.1 Preparation of stock phytohaemagglutinin solution

Phytohaemagglutinin (PHA) -P form (#3110 - Difco Lab.) was dissolved in 5ml of sterile water to give the stock solution of PHA. This was then diluted 1:10 with DAB and stored in 1ml frozen aliquots at -20°C.

2.8.2.2 PHA stimulation in vitro

Lymph node cells (LNC) were used for all PHA stimulations. LNC were prepared as described in Section 2.8.1, and resuspended in TCM-1 for culture. PHA was added to the resuspended cells just prior to transfer to culture vessels. Cells were cultured in 3-6ml at $2.5x10^6$ cells/ml in tissue culture flasks for 3 days with 1.25ul stock PHA/ml of TCM-1.

2.8.3 Concanavalin A (Con A) stimulation of lymphocytes

2.8.3.1 Lymph node cells were cultured in 5ml at $2x10^6$ cells/ml in tissue culture flasks (Falcon 3013; Beckton Dickenson and Co., Oxnard, Calif., USA) for 2 days with 11.5ug Concanavalin A (Con A)

(type IV, C2010, Sigma)/ml of TCM-1.

2.8.3.2 Thymocytes were cultured in 10ml at $4x10^6$ cells/ml in tissue culture flasks for 2 days with 13ug Con A/ml of TCM-2.

2.8.4 Mixed lymphocyte culture (MLC)

* **Method 1:** Responder cells were prepared as outlined in Section 2.8.1 and diluted to the required concentration in TCM-1.

A mixture of spleen cells and LNCs were used as the stimulator population. They were prepared as outlined in 2.8.1. The cells were resuspended in DAB/FCS/P+S at $10-20x10^6$ cells/ml and inactivated by irradiation with 950 rads. After irradiation the cells were washed twice with DAB/FCS/P+S and resuspended in TCM-1.

* **Method 2:** Responder cells were prepared as outlined in Section 2.8.1 and diluted to the required concentration in TCM-2.

A mixture of spleen cells and LNCs were used as the stimulator population. Lymph nodes and spleen were minced and then crushed through sterile stainless steel mesh into 30ml DAB/FCS/P+S. Debris was allowed to settle for 5 minutes before 6ml was transferred to each of 5 sterile, 10ml, plastic centrifuge tubes. The cell suspension was then underlayed with 2ml Ficoll diatrizoate. The tubes were spun at 1700g for 15 minutes. The resulting interfaces were collected, combined and then washed twice in DAB/FCS/P+S. The cells were resuspended at $10-20x10^6$ cells/ml and irradiated with 3000 rads. After irradiation cells were washed twice with DAB/FCS/P+S and resuspended in TCM-2.

2.8.4.1 Mixed lymphocyte culture in microtitre trays

When cells were cultured in Linbro microtitre trays (Flow Lab.),

the stimulator cells were dispensed to the wells in triplicate in 0.1ml volumes using a micropipette with sterile tips, followed by 0.1ml of responder cells, beginning with the lowest dose and using a single sterile tip for each dilution series.

2.8.4.2 Mixed lymphocyte culture in tissue culture flasks

The sensitisation of rat lymphocytes *in vitro* for adoptive transfer to grafted rats was performed in bulk mixed lymphocyte culture (MLC) in tissue culture flasks. Equal volumes of responder and stimulator cells, at 5-10x10^6 cells/ml, in TCM-1 or TCM-2 were mixed in a sterile container and aliquoted in 10 or 15ml to tissue culture flasks. Flasks were incubated upright for 4 or 5 days after which time the cells were harvested. Using this method the cell yeilds were 50-75% of starting cell numbers.

2.8.4.3 Marbrook mixed lymphocyte culture

Cells were cultured in Marbrook culture vessels using a modified method of Wilson et al (1976b). Responder cells at 10x10^6 cells/ml were added to the inner chamber of the vessel in approximately 1ml of TCM-1. Stimulator cells at 6x10^6 cells/ml were added in an equal volume. The inner chamber was then gassed with 5% CO_2 in air and plugged with sterile cotton wool. The outer chamber contained approximately 70ml of RPMI 1640/P+S medium. Cultures were maintained for 6 days.

2.8.4.4 Assessment of cell numbers after culture

Cultured cells were harvested, spun once at 300g and resuspended in an appropriate volue of DAB/FCS for counting. Cells were counted using a haemocytometer and cell viability was estimated by eosin dye

exclusion.

2.8.4.5 Assessment of mitotic activity in cultures

0.1uCi of methyl-^{3}H-thymidine ($H^{3}TdR$) with a specific activity of 25 Ci/mmol (TRK-120; Amersham Int. Plc., Amersham, UK) was added in 25ul of PBS to each well 16-20 hours prior to harvest, or 0.5uCi, 4 hours prior to harvest.

Cells were harvested onto Titer Tek 78-105-05 glass filter strips (Flow Labs), using a Scatron A.S. multiple automated sample harvester (Flow Lab.).

Sample discs, after drying at room temperature, were placed in counting vials with 5ml of scintillation fluid (5mg-diphenyloxazole [PPO] /litre of toluene) and counted in an LKB Wallac 1215 Rackbeta II liquid scintillation counter (LKB Wallac, Finland). Results are expressed as the mean CPM incorporated $H^{3}TdR \pm 1$ SD (standard deviation) of 3 replicate wells. To assess the proliferative activity of cultures raised in tissue culture flasks, cells were resuspended by gentle swirling and transferred in 0.2ml aliquots, in triplicate, to the wells of a microtitre tray for $H^{3}TdR$ pulsing and then treated as above.

2.8.4.6 Mitomycin C treatment of stimulator cells

Cells were resuspended at $3x10^{7}$ cells/ml in DAB/FCS/P+S containing 50 ug/ml Mitomycin C (Sigma) and incubated at 37°C for 30 minutes. The cells were then washed three times, counted and resuspended in an appropriate volume of medium.

2.9 CELL MEDIATED LYSIS ASSAY

2.9.1 Labelling of target cells

Target cells were harvested and counted on a haemocytometer. The appropriate number of viable target cells was then transferred to a disposable 30ml tube and centrifuged once. Cells were labelled at $1x10^6$ cells/0.9ml TCM-1 or TCM-2, with 0.1ml of PBS containing 100uCi of Cr^{51} ($Na_2 CrO_4$, CJS4, Amersham Int.) with a specific activity of 350-600 Ci/mg. The tube was then gassed with 5% Co_2 in air and the cells incubated for 1-3 hours at 37°C. Cells were swirled every 15 minutes. After incubation the cells were washed three times in 30ml of TCM and made up to the required concentration. To avoid high spontaneous release values, target cells were not labelled until immediately before use.

2.9.2 Assay of Cr^{51} release

100ul of effector cells and 100ul of target cells in TCM-1 or TCM-2 were incubated for 4 - 6 hours in a round bottom microtitre tray, in triplicate, at varying effector to target cell ratios. After culture 100ul of supernatant was removed and the samples counted in an LKB Wallac Compu-Gamma counter.

2.9.3 Calculations of specific Cr^{51} release

The release of Cr^{51} from target cells, as a result of lysis by sensitized effector cells was calculated according to the formula.

% Cr^{51} release	=	$\frac{ER - SR}{MR - SR}$
ER (experimental release)	=	mean Cr^{51} released from triplicate aliquots of target cells incubated with effector cells
SR (spontaneous release)	=	mean Cr^{51} release from triplicate aliquots of target cells incubated alone
MR (maximum release)	=	mean Cr^{51} release from triplicate aliquots of target cells after 3 times freeze/thaw lysis
% specific Cr^{51} release	=	% Cr^{51} release by specifically sensitized cells incubated with relevant target cells - % Cr^{51} release by speficially sensitised cells incubated with third party target cells

2.10 SEPARATIONS OF SUBPOPULATIONS OF T CELLS

2.10.1 Preparations of plates

Lymphocyte subsets were separated by a modification of the panning technique of Mage et al (1981). Sterile plastic petri dishes (Lab. Tek, Miles Lab., Raperville, Ill., USA) were coated with antibody by adding 10ml of 0.05M Tris buffer, pH 9.5, containing 1mg of rabbit anti-rat immunoglobulin (Ig) or rabbit anti-mouse Ig (Dako, Denmark) to the dishes and allowing them to stand at room temperature for 40 minutes. The plates were washed 3 times with DAB and 3mls of 10% bovine serum albumin (CSL Melb., Aust.) in DAB was added and they

were left a further 30 minutes at room temperature. The latter procedure blocked any unoccupied protein binding sites on the plate. Plates were then washed 3 times in DAB and stored at 4°C covered with DAB until used, for up to 7 days.

2.10.2 Preincubation cells with monoclonal antibodies

All procedures during cell preparations and separations were performed at 4°C. Except during the period that cells were on plates, procedures were performed using glass or pyrex vessels.

LNCs in aliquots of $100x10^6$ cells were mixed with 0.5ml of a saturating concentration of the relevant monoclonal antibody (MoAb) and incubated for one hour at 4°C, with occasional shaking, before being washed once in a large volume of DAB/FCS.

MoAb (Sera Lab., Sussex, UK) for cell coating were diluted in DAB/FCS to a final concentration of 1:50 for MRC OX8 MoAb or 1:10 for W3/25 MoAb to give saturating concentrations.

2.10.3 Incubation of cells on plates

$100x10^6$ cells in 4ml DAB/FCS were added to the plates coated wtih anti-rat Ig. The plates were stood on a flat surface for 1 hour at 4°C, during which time they were rocked every 10 minutes to redistribute the cells. To remove nonadherent cell populations from the plates, the supernatant was poured off and the plates were washed 4 times with 4ml of DAB/FCS. The nonadherent cells recovered from each plate were combined, washed once, resuspended in 4 ml of DAB/FCS and then poured onto a second plate coated with anti-mouse Ig or, in the case of Ig^- separation, a second anti-rat Ig plate. The procedure was then repeated. To deplete Ig bearing cells the MoAb coating step was omitted prior to incubation on the first plate.

2.10.4 Identification of lymphocyte subpopulations

The purity of the cell subpopulations recovered at the end of the double plating procedures was asessed using cell membrane immunofluorescent staining.

Aliquots of $5x10^6$ cells were stained for T cell differentiation antigens by the addition of 50ul of a 1:50 dilution in DAB/FCS/Az of W3/13, W3/25 or MRC OX8 MoAb 30 minutes at 4°C. The cells were washed twice before 50ul of fluorescein conjugated goat anti-mouse Ig (Cappel Lab., Cochranville, P.A., USA) was added for 30 minutes at 4°C. The goat anti-mouse Ig had been diluted 1:5 with normal pooled rat serum, then a further 1:10 with DAB/FCS/Az. The normal rat serum blocked any cross reactivity against rat Ig.

Aliquots were stained for B cells by the addition of 50ul of a 1:80 dilution of fluorescein conjugated sheep anti-rat Ig (Wellcome Research Labs., Beckenham, England) for 30 minutes at 4°C.

In all cases, after staining with fluorescein conjugates, preparations were washed in DAB/FCS/Az and spun on a Ficoll diatrizoate cushion for 15 minutes at 1700g to remove dead cells and unbound aggregates of antibody. Cells were then washed twice in DAB/FCS/Az before smears were prepared.

Prior to incubation with cells all antibody reagents were spun for 5 minutes at 8740g in a Microfuge B (Beckman, Palo Alto, CA., USA) to remove large aggregates.

2.11 FLUORESCENT MICROSCOPY

2.11.1 Cell Smears

$5x10^6$ cells were resuspended in 10ul of calf bovine serum (Flow Lab.) containing 0.1% w/v sodium azide, smeared and air dried. After

fixation for 10 minutes in AR ethanol, slides were mounted in 80% glycerol v/v in PBS (pH 9.4).

2.11.2 Microscopy

Fluorescent cells were counted using a Zeiss Photomicroscope II with incident light fluorescence and combined transmitted light. At least four hundred cells were counted in each preparation.

2.12 LOCALIZATION STUDIES

2.12.1 Cell labelling with I^{125} UdR

Cells for labelling were suspended at 2.5×10^6/ml in 10ml of TCM-1. 0.2uCi I^{125}UdR (5-[I^{125}] Iodo-2'-deoxyuridine; IM.355, Amersham) with a specific activity of 5Ci/mg was added per ml of cells. Cells were incubated at 37°C in a 5% Co_2 in air atmosphere for 4 hours, then washed 5 times in DAB/FCS.

2.12.2 Estimation of radioactively labelled cells in tissues

At various times after the injection of labelled cells, animals were anaesthetized and bled by cardiac puncture to obtain a 1ml blood sample for radioactive counting. Animals were then killed by exsanguination and organs removed. Small and large bowel and stomach were counted after the contents were removed by flushing twice with PBS. Sliced organs were placed in 10ml sealed tubes and the presence of radioactively labelled cells was determined by counting in an LKB Wallac Compu-Gamma.

CHAPTER 3

EXPERIMENTAL FINDINGS

INTRODUCTION

A decade after Medawar (1944) demonstrated that graft rejection was an immunological response, Billingham et al (1954) confirmed that the response was a cell mediated one. Subsequently the similarities between the histology of allograft rejection and that of delayed type hypersensitivity reactions led Brent et al (1962) to suggest that the mechanisms of tissue damage were similar and that macrophages might play an important role in the destruction of grafted tissue. This idea became unfashionable with the discovery that lymphocytes raised in mixed lymphocyte culture could specifically lyse target cells bearing the relevant alloantigen. Hayry and Defendi (1970) suggested that the MLC-CML was an <u>in vitro</u> analogue of the <u>in vivo</u> allograft response and that Tc were the mediators of tissue destruction in the latter response. Since that time there have been enormous advances in our understanding of the complex series of interactions which are triggered by the contact between cells of the immune system and foreign alloantigen which follows the implantation of an allogeneic graft. In spite of this there is still no definitive data regarding the identity of the cell or cells which mediate tissue destruction or the mechanisms by which they do so.

Studies designed to identify the effector mechanisms in graft rejection have centred around adoptive transfer experiments and morphological and functional studies of the cells infiltrating rejecting allografts. Adoptive transfer experiments have produced evidence suggesting that Th cells are necessary for rejection and cytotoxic T cells play no essential part (Dallman etal, 1982).

Histological studies have revealed the presence of Th cells, Tc cells, B cells, macrophages and large granular lymphocytes (Nemlander et al, 1983) in grafts during the rejection process. The function of infiltrating cells has been investigated by isolating these cells from grafts. Each of the cell types identified has been shown by in vitro assays to have the potential to affect tissue destruction alone or in collaboration with other cell types.

There is no doubt that Tc generated in MLC or obtained from rejecting grafts or their draining lymphoid tissue, kill lymphoid blast cells bearing the relevant alloantigens. Their failure to lyse renal (von Willebrand et al, 1979) or cardiac (Parthenais et al, 1979) parenchymal cells has cast some doubt on their role in the rejection of solid tissue grafts. A further objection to accepting Tc, as identified in CML assays, as the possible mediators of in vivo graft rejection is the fact that usually, to obtain significant cytotoxicity in conventional CML assays, effector cells must exceed target cells by as much as 100 to 1. If these ratios reflect cellular requirements in vivo, Tc mediated destruction would appear to be a relatively inefficient mechanism to procure graft rejection.

It is possible that both the large effector to target cell ratio and the requirement for specific target cells which are necessary to demonstrate Tc function in CML assays, are a function of the assay system rather than a reflection of the real characteristics of Tc. To test this hypothesis and to obtain further information on the cellular requirements for graft rejection, in vivo experiments were designed to develop a model to study the capacity of Tc raised in in vitro MLC to cause the in vivo destruction of solid tissue grafts.

The neonatal heart (NNH) graft model has several advantages as a graft rejection model to study the effector arm of the allograft

TABLE 1

REJECTION TIMES OF RT1 INCOMPATIBLE NNH GRAFTS IN ADULT RATS

Recipient	Donor	Graft survival time	
		Day of rejection	MST[a]
DA	PVG	12(3)[b], 13(3), 14(7), 15(4), 16(4), 17(3), 18	14
	WF	11, 13, 14(2), 17	14
WF	PVG	12, 14, 15(2), 16	15
	DA	11, 12, 13(3)	13
PVG	WF	10, 11(2), 13(2)	11
	DA	11(2), 12(2), 14	12

[a] Median survival time.

[b] In this and subsequent tables the numbers in brackets beside the day of rejection refers to the number of recipients in which the grafts rejected on that day.

response. Graft survival can be objectively monitored by electrocardiography and electrical activity is detectable on the first day after implantation. The grafts are implanted subcutaneously and therefore do not require protective dressings. Thus, unlike skin grafts, NNH grafts can be easily monitored daily from the day after grafting. Grafts implanted in a subcutaneous pocket in the footpad can be directly inoculated with cells at the time of grafting. Furthermore the footpad has a well defined lymphatic drainage, to the popliteal, and thence to the lumbar node, and an easily accessible regional lymph node. This facilitates simultaneous monitoring of cellular events in the graft and its draining node (Dorsch et al, 1983).

3.1 REJECTION OF RT1 INCOMPATIBLE NNH GRAFTS IN ADULT RATS

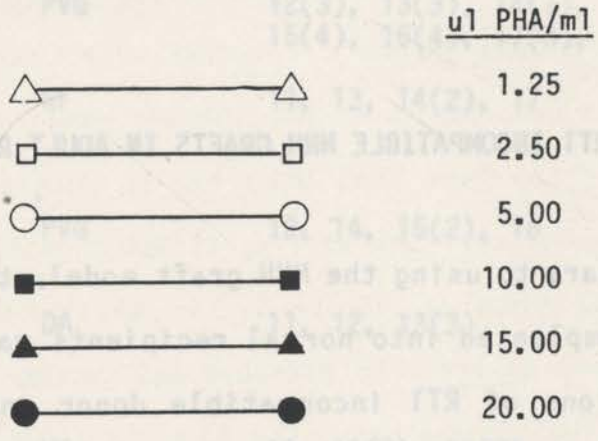
As a preliminary to using the NNH graft model, the rejection times of grafts implanted into normal recipients was studied in several combinations of RT1 incompatible donor and recipient. Greater availability of DA adults and PVG neonates than those of other strains dictated that the investigation be carried out predominantly in this strain combination. In this combination normal rejection occurs over a broad time range. Therefore a large experimental group was grafted to establish an accurate baseline for future experiments. The individual survival times and the median survival time (MST) of NNHs grafted into the footpads of adult recipients are shown in Table 1.

RT1 incompatible NNH grafts are rejected with a slower tempo than RT1 incompatible skin grafts. There is also a greater range of survival times in a given strain combination and a greater variation

FIGURE 1

IN VITRO PHYTOHAEMAGGLUTININ STIMULATION OF PVG LYMPH NODE CELLS

PVG LNCS were cultured at various cell concentrations in TCM-1 in microtitre trays in the presence of graded doses of PHA. Cells were harvested after 3 days in culture following overnight exposure to H^3TdR. Each point represents the mean of triplicate wells ± 1 SD.



The lowest concentration of PHA elicited the peak response. The maximum incorporation of H^3TdR was seen in cultures containing $4x10^6$ cells/ml to which PHA was added to a concentration of 1.25 ul/ml. The greatest incorporation of H^3TdR relative to the number of cells set up in the original culture was seen in cultures containing $2x10^6$ cells/ml to which PHA was added to a concentration of 1.25 or 2.5 ul/ml. PVG LNC cultured alone incorporated negligible counts of H^3TdR (less than 700 CPM).

CELLS (x10⁻⁶) / ml
8
4
2
1
0
20
40
60
80
100
CPM INCORPORATED (x10⁻³)

between strain combinations in the MST than has been reported for skin grafts (Dorsch et al, 1983). Rejection time in DA hosts grafted with PVG hearts ranged from 12-18 days with an MST of 14d. In no experiment reported in this thesis, or done by other workers in the laboratory, has rejection of PVG NNHs grafted into normal untreated DA recipients occurred in less than 12 days.

As a preliminary to testing the ability of putative Tc raised in MLC to effect the survival time of NNH grafts, experiments were done to determine the conditions under which a consistent and significant proliferative response could be obtained in MLCs of DA responder lymphocytes and PVG stimulator cells.

3.2 IN VITRO PROLIFERATIVE RESPONSES OF LYMPHOCYTES

The parameters of the response of PVG lymphocytes to the mitogen PHA were first examined so that ablation of the proliferative response to mitogens could be used to establish an irradiation dose suitable for mitotic inactivation of the stimulator cells to be used in MLC and also as a prelude to using blast cells as targets in CML assays planned for the future.

3.2.1 Response to PHA

Triplicate cultures of PVG lymph node cells (LNCs) at cell concentrations of 1, 2, 4 and 8×10^6/ml were treated with a range of doses of PHA from 1.25ul/ml to 20ul/ml. Two days later H^3TdR was added to cultures. Cultures were harvested 16 hours later and the amount of incorporated radioactivity in each culture was measured. The results appear in Fig. 1. The greatest incorporation of H^3TdR relative to the number of cells initially put up in culture was

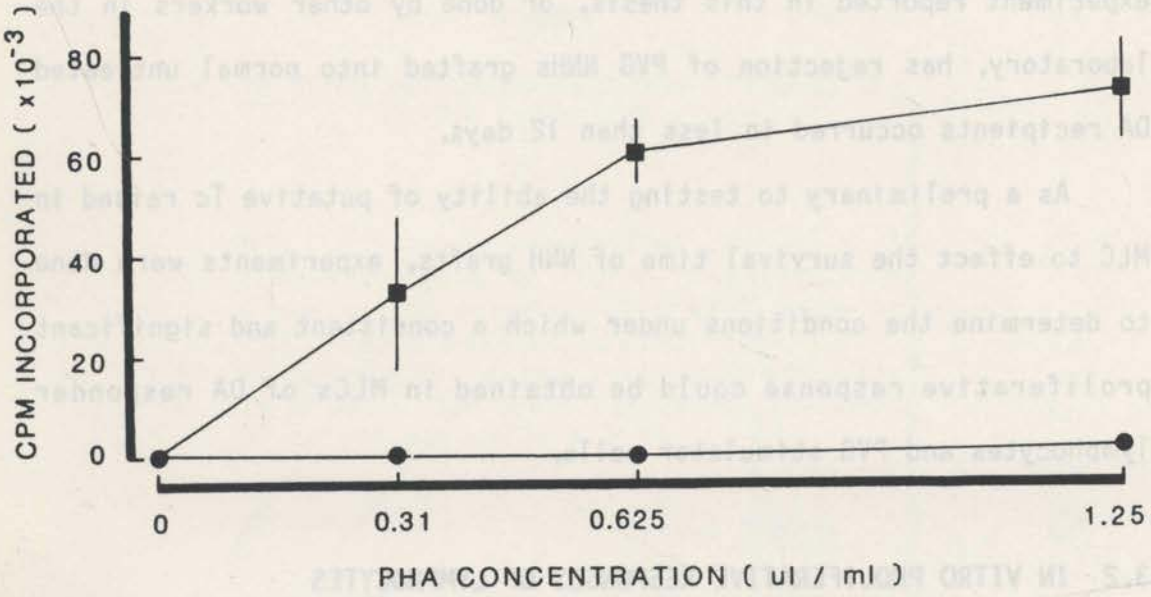


FIGURE 2

IN VITRO PHYTOHAEMAGGLUTININ STIMULATION OF PVG LNCs.

EFFECT OF IRRADIATION

Normal PVG LNC (■——■) and PVG LNC which had been irradiated with 950 rads (●——●) were stimulated at 2×10^6 cells/ml with various concentrations of PHA. Cells were harvested after three days in culture following overnight exposure to H^3TdR. Each point represents the mean of triplicate wells ± 1 SD. The proliferation response of normal LNC to 0.625 ul/ml did not differ significantly from that to 1.25 ul/ml. Irradiated cells showed extremely low levels of proliferation (less than 300 CPM maximum) at all doses of PHA.

observed in cultures containing cells at $2x10^6$/ml treated with 1.25ul PHA/ml.

The effect of PHA on PVG lymphocytes previously irradiated with 950 rad Co^{60} irradiation was next examined. Triplicate cultures of normal or irradiated PVG LNC at $2x10^6$ cells/ml were treated with 0.31, 0.625 or 1.25ul PHA/ml. Cells were again harvested after 3 days in culture following overnight exposure to H^3TdR. All cultures of irradiated cells showed extremely low levels of proliferation (less than 300 CPM). The normal LNC response to a dose of 0.625ul PHA/ml was not significantly different to the response to 1.25ul PHA/ml (Fig. 2).

It was concluded that optimal proliferation of PVG lymphocytes occurs when cells cultured at $2x10^6$/ml are exposed to 0.625 - 1.25ul PHA/ml of culture medium. As cells irradiated with 950 rad failed to show significant proliferation under these conditions, it was concluded that irradiation of PVG lymphocytes with 950 rad ablated the proliferative capacity of the majority of cells. This dose of irradiation was therefore routinely used to mitotically inactivate the stimulator cells in MLC.

3.2.2 Mixed lymphocyte cultures

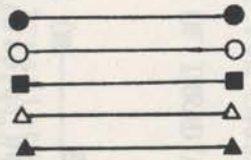
Experiments were next done to establish the conditions under which DA lymphocytes gave a maximum proliferative response when set up in MLC in microtitre trays with PVG stimulator cells using MLC method 1 (Chapter 2). To determine the optimum cell concentrations, DA LNCs at various concentrations were cultured with irradiated PVG cells at a concentration of either 1 or $2x10^6$/ml. Control cultures of DA LNCs at $2x10^6$/ml or DA LNCs at $2x10^6$/ml with $2x10^6$/ml irradiated syngeneic cells were also done. Cultures were terminated

FIGURE 3

PRIMARY MLC RESPONSES OF DA LNC
CULTURED WITH IRRADIATED PVG STIMULATOR CELLS

A $1x10^6$ PVG stimulator cells

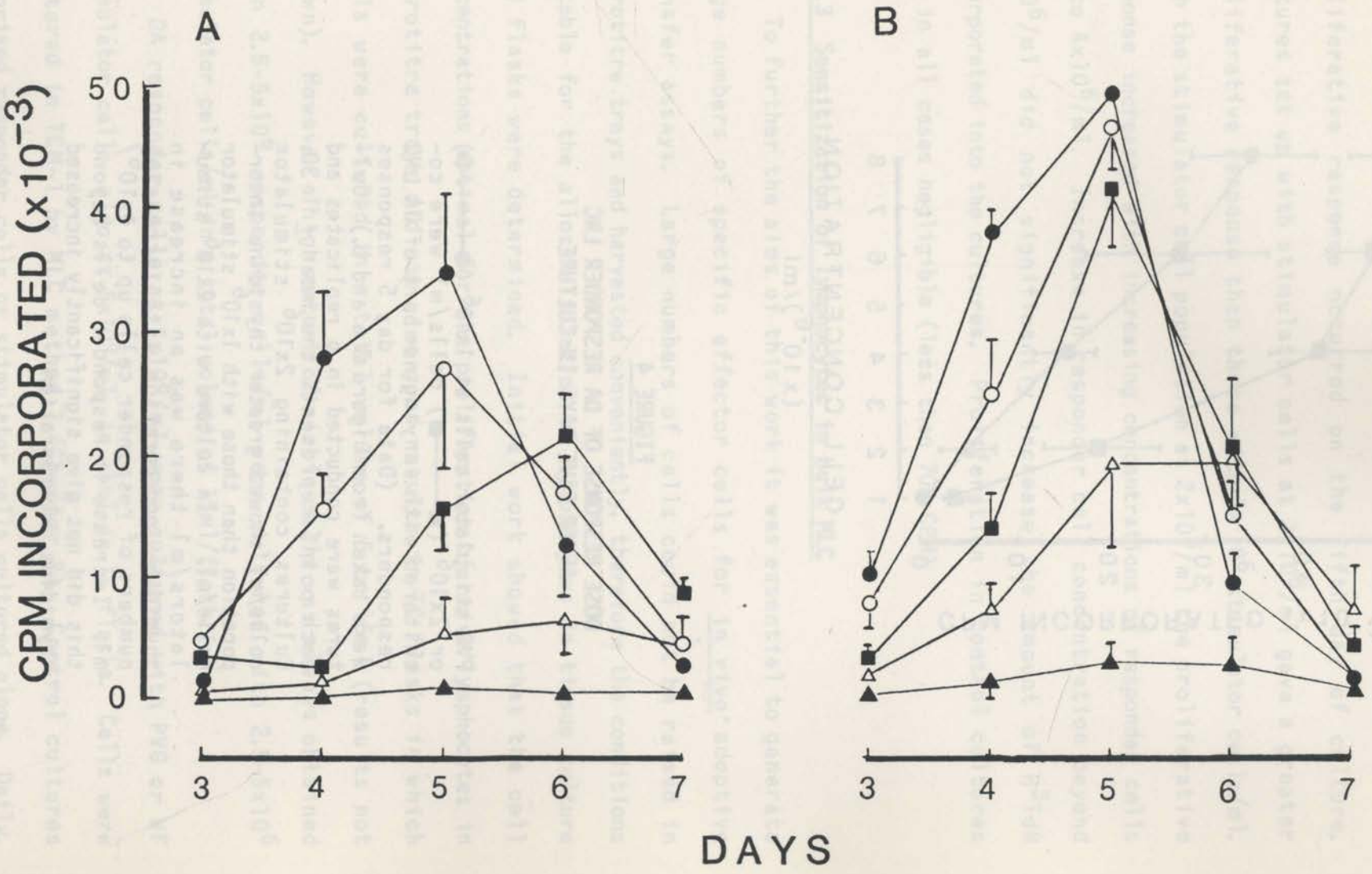
B $2x10^6$ PVG stimulator cells



DA responder cells/ml $(x\ 10^{-6})$

8
6
4
2
1

DA LNC were cultured at various concentrations with 1 or $2x10^6$ PVG stimulator cells/ml in microtitre trays by Method 1. MLC cultures were conducted in 6 replicates and harvested daily from days 3-7 after overnight exposure to H^3TdR. Each point represents the mean of 6 replicate wells $\pm$ 1 SD. The maximum proliferative response relative to the number of responding cells originally cultured was seen on the fifth day in cultures set up with $4x10^6$ responder cells with $2x10^6$ stimulator cells. Thymidine incorporated in control cultures of DA responder cells cultured alone or with irradiated syngeneic LNC is not shown. It did not exceed 660 CPM.


A
B
CPM INCORPORATED (x10⁻³)
50
40
30
20
10
0
3
4
5
6
7
DAYS

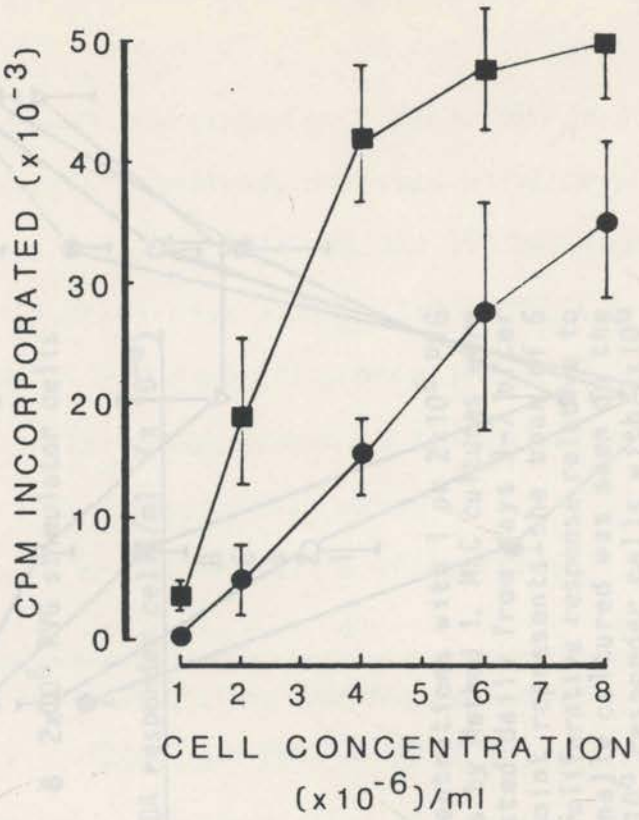


FIGURE 4

DOSE RESPONSE OF DA RESPONDER LNC AFTER FIVE DAYS IN CULTURE

PVG stimulator cells at $1x10^6$ (●——●) or $2x10^6$ (■——■) cells/ml were co-cultured with varying numbers of DA LNC responders. (Data for day 5 responses were taken from Figure 3 a and b.) Cultures were conducted in 6 replicates and each point represents the mean ± 1 SD. Cultures containing $2x10^6$ stimulator cells/ml showed greater thymidine incorporation than those with $1x10^6$ stimulator cells/ml. In culture with $2x10^6$ stimulators/ml there was an increase in thymidine incorporation with increasing numbers of responder cells up to $4x10^6$/ml. Increase in responder cells beyond this did not give significantly increased thymidine incorporation.

daily from day 3 to day 7 after overnight exposure to H^3TdR. The results are shown on Fig. 3 (a and b) and Fig. 4. The maximum proliferative response occurred on the fifth day of culture. Cultures set up with stimulator cells at $2x10^6$/ml gave a greater proliferative response than those with $1x\ 10^6$ stimulator cells/ml. With the stimulator cell population at $2x10^6$/ml the proliferative response increased with increasing concentrations of responder cells up to $4x10^6$/ml. Increase in responder cell concentration beyond $4x10^6$/ml did not significantly increase the amount of H^3TdR incorporated into the cultures. Proliferation in control cultures was in all cases negligible (less than 700 CPM).

3.2.3 Sensitization of lymphocytes in bulk MLC

To further the aims of this work it was essential to generate large numbers of specific effector cells for *in vivo* adoptive transfer assays. Large numbers of cells could not be raised in microtitre trays and harvested conveniently, therefore the conditions suitable for the allosensitization of lymphocytes in tissue culture (TC) flasks were determined. Initial work showed that the cell concentrations optimal for the proliferation of naive lymphocytes in microtitre trays did not give good results in TC flasks in which cells were cultured in a total volume of 5 to 15ml (results not shown). However a high return of viable cells was always obtained when $2.5\text{-}5x10^6$ responder cells/ml were cultured with $2.5\text{-}5x10^6$ stimulator cells/ml in a total volume of 15ml/flask.

DA responder cells at $5x10^6$/ml were cultured with PVG or WF stimulator cells, also at $5x10^6$/ml, in 15ml in TC flasks. Cells were cultured in TCM-1 by MLC method 1 (Chapter 2). Control cultures comprised responder cells or stimulator cells cultured alone. Daily,

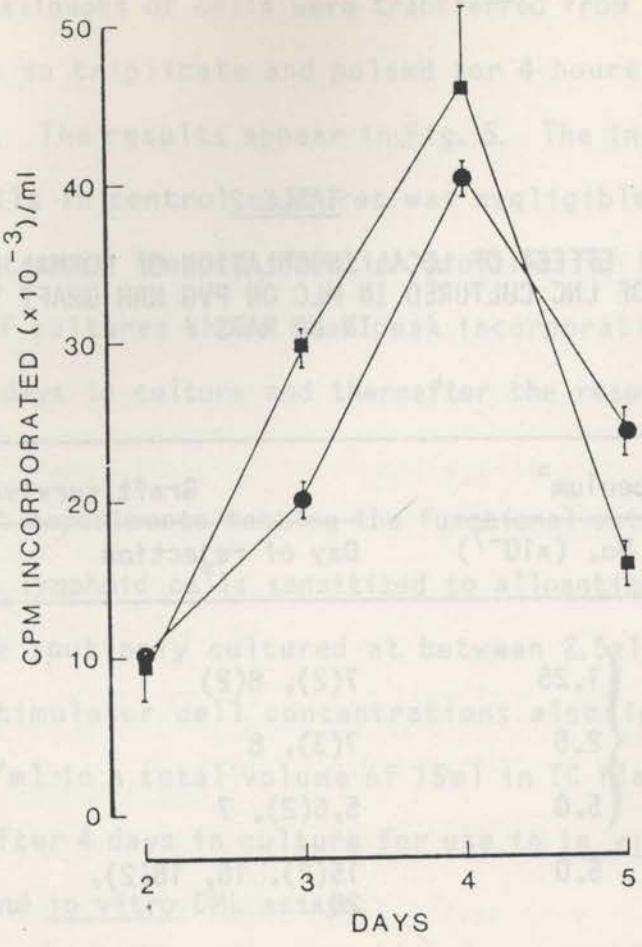


FIGURE 5

SENSITIZATION OF LYMPHOCYTES IN BULK MLC

DA LNC were cultured at $5x10^6$/ml with WF (●——●) or PVG (■——■) irradiated stimulator cells at $5x10^6$/ml in TC flasks containing 15ml. MLC wre performed by Method 1. Cells were aliquoted from TC flasks to microtitre trays and exposed to H^3TdR for four hours prior to harvest. Each point represents the mean of triplicate wells, derived from one TC flask, ± 1 SD. Note that both cultures show maximum proliferation after four days in culture. Thereafter the response falls away.

TABLE 2

EFFECT OF LOCAL INOCULATION OF NORMAL LNC AND OF LNC CULTURED IN MLC ON PVG NNH GRAFT SURVIVAL IN DA RATS

Cell inoculum[a]		Graft survival time		
Source	No. ($\times 10^{-7}$)	Day of rejection	MST	P[b]
A				
DA anti PVG MLC	1.25	7(2), 8(2)	7.5	NSD
	2.5	7(3), 8	7	
	5.0	5,6(2), 7	6	
DA LNC	5.0	15(2), 16, 18(2), 20	17	<.005[c]
-	-	13, 14(3), 17	14	<.025[c]
B				
DA anti PVG MLC	0.5	8, 9(3)	9	NSD
	1.0	7, 9(3)	9	
	2.0	7, 8(3)	8	<.005
	5.0	5, 6(3)	6	

[a] In this and all subsequent tables, cells locally injected at the graft site were given in 0.1 ml DAB/FCS.

[b] P = probability.

[c] Comparedto rejection time in animals injected with DA anti PVG MLC cells.

from day 2 to 5, aliquots of cells were transferred from TC flasks to microtitre trays in triplicate and pulsed for 4 hours with H^3TdR prior to harvest. The results appear in Fig. 5. The incorporation of H^3TdR into cells in control cultures was negligible (less than 1000 cpm) in all cases. The proliferation profiles for both DA anti PVG and DA anti WF cultures shows that peak incorporation of H^3TdR occurred after 4 days in culture and thereafter the response rapidly fell off.

In subsequent experiments testing the functional activity of MLC sensitized cells, lymphoid cells sensitized to alloantigens *in vitro* in bulk MLC were routinely cultured at between $2.5x10^6$ to $5x10^6$ cells/ml with stimulator cell concentrations also in the range $2.5x10^6$ to $5x10^6$/ml in a total volume of 15ml in TC flasks. Cells were harvested after 4 days in culture for use in *in vivo* adoptive transfer assays and *in vitro* CML assays.

3.3 EFFECT ON GRAFT SURVIVAL OF LOCALLY INOCULATED MLC CELLS

3.3.1 The effect of cell dose on graft survival

DA LNC were set up in bulk culture with irradiated PVG stimulator cells. The cells (DA anti PVG MLC cells) were harvested after 4 days in culture, washed and resuspended at the appropriate concentration for injection. Three groups of 4 DA recipients grafted in the footpad with PVG NNHs were inoculated in the graft site with 1.25, 2.5 and $5.0x10^7$ cells respectively. There were two control groups each of 4 similarly grafted animals. One of these was inoculated in the graft site with $5x10^7$ normal DA LNC and the other was not given any cells. The results appear in Table 2A.

All injected grafts showed decreased survival time (MST 7.5d)

TABLE 3

THE EFFECT OF LOCALLY INOCULATED MLC CELLS ON NNH GRAFT SURVIVAL

Recipient	Donor	MLC cell inoculum[a]	Graft survival time		
			Day of rejection	MST	P
WF	PVG	–	12, 14, 15(2), 16	15	
		WF anti DA	12, 13, 14(2)	13.5	NSD
		WF anti PVG	6(3), 7	6	<.005
PVG	DA	–	9, 10, 11(4)	10.5	
		PVG anti WF	10(2), 12(2)	11	NSD
		PVG anti DA	5(3), 6	5	<.005
DA	WF	–	11, 13, 14(2), 17	14	
		DA anti PVG	12, 13(3)	13	NSD
		DA anti WF	5(2), 6(2)	5.5	<.005
DA	PVG	–	13(2), 14, 15, 17	14	
		DA anti WF	12, 13(2), 14	13	NSD
		DA anti PVG	6, 7(2), 8	7	<.005

[a] 5×10^7 cells in 0.1 ml.

compared to the control uninjected grafts (MST 14d, $p<0.005$) or grafts injected with normal DA LNC (MST 17d, $p<0.005$). The degree of acceleration of rejection appeared dose dependent although this was only a trend, there being no significant difference in rejection times obtained with the cell doses tested. When the experiment was repeated using DA anti PVG MLC cells in doses of 0.5, 1.0, 2.0 and 5.0×10^7 cells similar results were obtained (Table 2B). As few as 5×10^6 cells caused some acceleration of graft rejection (MST 9d) and 5×10^7 cells caused marked acceleration (MST 6d) compared to all lower cell doses ($p<0.005$).

3.3.2 Specificity of rejection procured by MLC cells in different RT1 strain combinations

To establish whether the acceleration of graft rejection caused by inoculation of putative effector cells raised in MLC is specific, a series of experiments was done in which groups of rats in various combinations of recipient and donor were grafted with NNH grafts. Four recipients in each group were then inoculated in the graft site with cells which were in all cases syngeneic with the graft recipient but which had been sensitized in bulk MLC for 4 days either against the alloantigens of the graft donor or against third party alloantigens. The cell number injected was 5×10^7 cell per graft. The results appear in Table 3.

In all strain combinations tested, injection of cells raised in MLC against stimulator cells bearing the same alloantigens as the graft donor caused significant acceleration of graft rejection. Grafts injected with cells raised in MLC against stimulator cells bearing irrelevant alloantigens were rejected with the same tempo as uninjected control grafts.

TABLE 4

THE EFFECT OF DA ANTI PVG MLC CELLS INJECTED INTO ONE FOOTPAD ON THE SURVIVAL OF GRAFTS IN THE CONTRALATERAL FOOTPAD IN DA RECIPIENTS OF PVG NNH

Neonatal graft	MLC cell inoculum[a]	Graft survival time		
RFP / LFP	RFP / LFP	Day of rejection	RFP / LFP	P
– / PVG	– / DA anti PVG	–/5, –/6(2), –/7		<.01
– / PVG	DA anti PVG / –	–/8(4)		
PVG / PVG	– / –	12/12(2), 14/14, 17/17		
PVG / PVG	DA anti PVG / –	5/7(2), 5/9(2), 6/7, 6/8(2) 7/8		<.005
PVG / PVG	DA anti PVG / DA anti BN	5/7, 7/11(3)		<.01

[a] 5×10^7 cells in 0.1 ml.

It was concluded that lymphocytes sensitized in MLC against the alloantigen of NNH grafts acquire the capacity to cause specific acceleration of graft rejection following local inoculation into the site of graft implantation.

3.3.3 Site of action of MLC cells

To determine whether accelerated graft rejection procured by locally injected MLC cells is a purely local phenomena or follows systemic dissemination of the injected cells or their progeny, two groups of DA rats were grafted in the hind footpad with PVG NNHs. One group was injected in the grafted footpad with $5x10^7$ DA anti PVG MLC cells. The other group was similarly injected in the footpad contralateral to the grafted one. Graft rejection times appear in Table 4.

Both groups showed accelerated graft rejection. The group injected in the grafted footpad rejected their grafts with a more rapid tempo than the group injected in the footpad contralateral to the graft (Table 4; $p<0.01$). The difference in graft rejection time between the two groups was small. It was possible that earlier rejection in the group in which the grafted foot was inoculated reflected the fact that in these animals the MLC cells were re-exposed to specific antigen at the time of injection. Alternatively it may have been a consequence of non-specific injury accompanying the injection of cells into the graft site. To explore these possibilities 12 DA recipients were grafted in each footpad (FP) with PVG NNHs and injected in the right footpad (RFP) with $5x10^7$ DA anti PVG MLC cells. In eight animals the left footpad (LFP) was not injected; in the remaining 4 the LFP was injected with $5x10^7$ DA anti BN MLC cells. A further group of 4 DA recipients were bilaterally

TABLE 5

THE EFFECT OF SYSTEMICALLY INOCULATED MLC SENSITIZED CELLS ON SURVIVAL OF PVG NNHs IN DA RECIPIENTS

MLC cell inoculum		Graft survival time		
Route[a]	Source	Day of rejection	MST	P
IV	DA anti PVG	9, 10, 11(2)	10.5[b]	<.025
	DA anti WF	26, 28, 34, 35	31	
IA	DA anti PVG	9, 10(2), 11	10[b]	<.025
	DA anti BN	30, 34, 36, 37	35	

[a] In this and subsequent experiments employing systemically injected cells, $5x10^7$ cells were injected intravenously (IV) in the lateral tail vein or intra-arterially (IA) in the abdominal aorta in a volume of 1 ml.

[b] The rejection time procured by IV injected DA anti PVG MLC cells was comparable to that procured by IA injected cells ($P>0.5$).

grafted with PVG NNHs and were left uninjected.

In bilaterally grafted rats given no cells the grafts were simultaneously rejected. In all 12 rats given cells rejection of both grafts was accelerated, however in all cases the graft actually injected with DA anti PVG MLC cells was rejected before the uninjected graft. In the case of animals injected in the RFP with specific (DA anti PVG) and the LFP with non-specific (DA anti BN) cells, the grafts in the RFP rejected with a significantly ($p<0.01$) more rapid tempo than those in the LFP (Table 4). These results suggest that locally inoculated cells exert both a local and a systemic effect. The latter is presumably due to entry of the subcutaneously inoculated cells either into the systemic circulation via vessels disrupted during the process of graft implantation or passage of cells into the lymphatics draining the area.

The effect of systemically injected MLC cells on survival of NNH grafts was next examined.

Sixteen DA rats were grafted with PVG NNHs. One group of 4 was injected IV with $5x10^7$ DA anti PVG MLC cells and a control group of 4 was similarly injected with DA anti WF MLC cells. The other two groups of 4 recipients were injected intra-arterially (IA) into the abdominal aorta with DA anti PVG MLC cells and DA anti BN MLC cells respectively. The results appear in Table 5.

Systemic injection of syngeneic cells specifically sensitized against graft alloantigens accelerated the rejection of NNH grafts. Animals injected either IV or IA with DA anti PVG MLC cells rejected their PVG grafts with an MST of 10.5d and 10d respectively. This is more rapid than rejection in recipients given no cells (MST 14d; Table 1). Control groups injected with cells sensitized in MLC against third party antigen inexplicably showed prolonged survival of

grafts (Table 5) beyond that seen in normal uninjected DA recipients of PVG grafts (Table 1).

The acceleration of rejection observed after the systemic injection of MLC sensitized cells was significantly ($p<0.005$) less than that observed after the local inoculation of similar cell populations (Table 3). In addition graft rejection in recipients injected IV (MST 10.5d) or IA (MST 10d) was slower than the rejection of grafts in animals which are injected with similar populations of cells subcutaneously in the footpad contralateral to the grafts (MST 8d; Table 4). In the latter case there was no significant difference in the survival of the grafts in the uninjected footpad between groups in which there was a graft present in the injected footpad and groups in which there was no graft present in the injected footpad (Table 4). This suggests that immediate re-exposure to relevant alloantigen in the injected footpad is not a factor in the apparently increased systemic efficiency of locally inoculated cells over cells injected IV or IA. It is possible however that cells injected into the footpad contralateral to a graft encounter antigen in the lumbar nodes. Experiments tracing lymphatic drainage from the footpad, following the injection of colloidal carbon showed that after carbon is injected into the RFP a small amount reaches the left lumbar node (unpublished observation). It is possible therefore that MLC sensitized cells injected into the LFP of an animal grafted in the RFP encounter graft antigen in the lumbar node soon after injection and that it is early re-exposure to alloantigen which accounts for the fact that cells injected into the footpad contralateral to the graft cause more rapid graft rejection than similar cell populations injected into the systemic circulation.

TABLE 6

THE EFFECT OF MLC SENSITIZED CELLS INJECTED SYSTEMICALLY FOUR DAYS AFTER GRAFTING DA RECIPIENTS WITH PVG NNHs

MLC cell inoculum		Graft survival time		
Route	Source	Day of rejection	MST	P
IV	DA anti PVG	10, 12(4)	12	NSD
	DA anti WF	12(3), 13(2)	12	
IA	DA anti PVG	12, 13(2), 14(3), 16(2)	14	NSD
	DA anti BN	10, 12, 14, 15, 17, 18(2), 19	15	

Comparison of the time of rejection in all four groups by the Kruscal Wallace test for non-parametric data shows that there is no significant difference between them in graft survival times ($0.1<p<0.25$).

3.3.4 The effect on graft survival of the systemic injection of MLC cells after vascularisation of the NNH graft

Immediately following grafting no vascular or lymphatic connections exist between the graft and host. Thus, only those systemically injected MLC cells which were extravasated from the damaged vessels at the graft site or those which encountered shed antigen on their way through the draining LN would have immediate contact with antigen following injection. To examine whether immediate entry to the graft via the vasculature enhances the effect of cells injected by the systemic route, experiments were done in which effector cells were injected either IV or IA 4 days after graft implantation at which time it has been shown that connection between graft vasculature and host circulation are established (Dorsch et al, 1983).

Sixteen DA rats were grafted with PVG NNHs. Four days later all grafted rats were injected, by the systemic route, with MLC sensitized cells. One group of 4 was injected IV with $5x10^7$ DA anti PVG MLC cells and a control group of 4 rats was similarly injected with DA anti WF MLC cells. The other 2 groups of 4 recipients were injected IA with $5x10^7$ DA anti PVG and $5x10^7$ DA anti BN MLC cells respectively. The results appear in Table 6.

The systemic injection of specifically sensitized MLC cells by either the IA or IV route did not significantly accelerate graft rejection compared to the injection of cells sensitized to third party alloantigens (Table 6). When the graft rejection times for all 4 groups of animals are compared it is apparent that there is no significant difference between the groups irrespective of the injection route (IV or IA) or the specificity of the cells ($0.1<p<0.25$).

TABLE 7

EFFECT ON SURVIVAL TIME OF PVG NNH GRAFTS IN DA RECIPIENTS OF ADDING CELLS BEARING GRAFT ALLOANTIGEN TO IV INJECTED SPECIFICALLY SENSITIZED CELLS

Cellular inoculum		Graft survival time		
DA anti PVG MLC ($\times 10^{-7}$)	PVG LN and spleen cells ($\times 10^{-7}$)	Day of rejection	MST	P
A				
5	-	9,10(2),11	10	<.01
5	1.5	6, 7(3)	7	<.01
-	1.5	14, 15(2), 17	15	
B				
5	-	9(4)	9	<.01
5	0.7	5, 6, 7(2)	6.5	

Furthermore the majority of grafts rejected within the range observed in normal, untreated DA recipients of PVG NNH graft (Table 1). This may reflect the fact that with the delay of 4 days between grafting and cellular injection the system is not sensitive enough to detect any minor degree of acceleration of rejection brought about by the injected cells. It was apparent that some other means would have to be devised to examine the requirement of MLC sensitized cells for immediate re-exposure to specific alloantigen to maintain functional activity. For this reason experiments were done in which MLC sensitized cells injected systemically on the day of grafting, were inoculated together with a source of graft alloantigen in the form of lymphoid cells.

3.3.5 The requirements of MLC sensitized cells for immediate re-exposure to specific alloantigen

Groups of DA rats were grafted with PVG NNHs. On the day of grafting all animals were injected intravenously with cells as follows. Four recipients received $5x10^7$ DA anti PVG MLC cells mixed with $1.5x10^7$ PVG LN and spleen cells and four received $1.5x10^7$ PVG LN and spleen cells only. A further four recipients received $5x10^7$ DA anti PVG MLC cells only.

Recipients given DA anti PVG MLC cells rejected their grafts with MST of 10d (Table 7A). The addition of $1.5x10^7$ PVG lymphocytes to the inoculum of DA anti PVG MLC cells significantly accelerated graft rejection (MST 7d). Animals given $1.5x10^7$ PVG LN and spleen cells alone showed no acceleration of graft rejection (MST 15d). The experiment was repeated using lesser numbers ($0.7x10^7$) of PVG LN and spleen cells. The results were similar. Animals injected with PVG LN and spleen cells at the same time as DA anti PVG MLC cells

TABLE 8

EFFECT ON SURVIVAL TIME OF PVG NNH GRAFTS IN DA RECIPIENTS OF ADDING CELLS BEARING GRAFT ALLOANTIGEN TO LOCALLY INJECTED SPECIFICALLY SENSITIZED CELLS

Cellular inoculum [a]		Graft survival time		
MLC cells	PVG LN and spleen cells ($\times 10^{-7}$)	Day of rejection	MST	P
DA anti PVG	-	6, 7, 8(2)	7.5	
DA anti PVG	0.7	6(2), 7(2)	6.5	NSD
DA anti PVG	5	6, 7(2), 8	7	
				<.05
DA anti WF	-	12, 13(2), 14	13	
DA anti WF	5	12(4)	12	NSD
-	5	10, 12(3)	12	

[a] In all cases 5×10^7 cells were injected.

rejected their grafts more rapidly than those given MLC cells alone (Table 7B). Graft rejection, in both experimental groups given a source of graft alloantigen with the inoculum of sensitized MLC cells, was accelerated to the tempo seen in groups in which specific MLC sensitized cells are inoculated directly into the graft (Table 2).

Groups of DA recipients grafted with PVG NNHs were injected in the grafted footpad with $5x10^7$ DA anti PVG MLC cells or $5x10^7$ DA anti PVG MLC cells mixed with either $0.7x10^7$ or $5x10^7$ PVG LN and spleen cells. Control groups received $5x10^7$ PVG LN and spleen cells, $5x10^7$ DA anti WF MLC cells or $5x10^7$ DA anti WF MLC cells mixed with $5x10^7$ PVG LN and spleen cells.

The results appear in Table 8. DA anti PVG MLC cells accelerated the rejection of PVG NNHs (MST 7.5d). The addition of PVG alloantigen to the inoculum did not cause a significant further acceleration of rejection. As previously, acceleration of rejection procured by cells sensitized in MLC was specific. Graft rejection time in groups injected with PVG lymphoid cells and/or DA anti WF MLC cells was within the range of normal rejection times for PVG grafts in DA recipients.

The foregoing experiments showed that MLC sensitized lymphocytes had the capacity to cause accelerated rejection of NNH grafts bearing the sensitizing alloantigen. The finding that cells inoculated subcutaneously either directly into the graft, or into an area which drained into the same central nodes as the graft, caused more rapid rejection than intravenously injected cells suggested that early contact between MLC activated cells and relevant alloantigen in lymphoid tissue might be a prerequisite for demonstrable *in vivo* functional activity on the part of *in vitro* sensitized cells. The

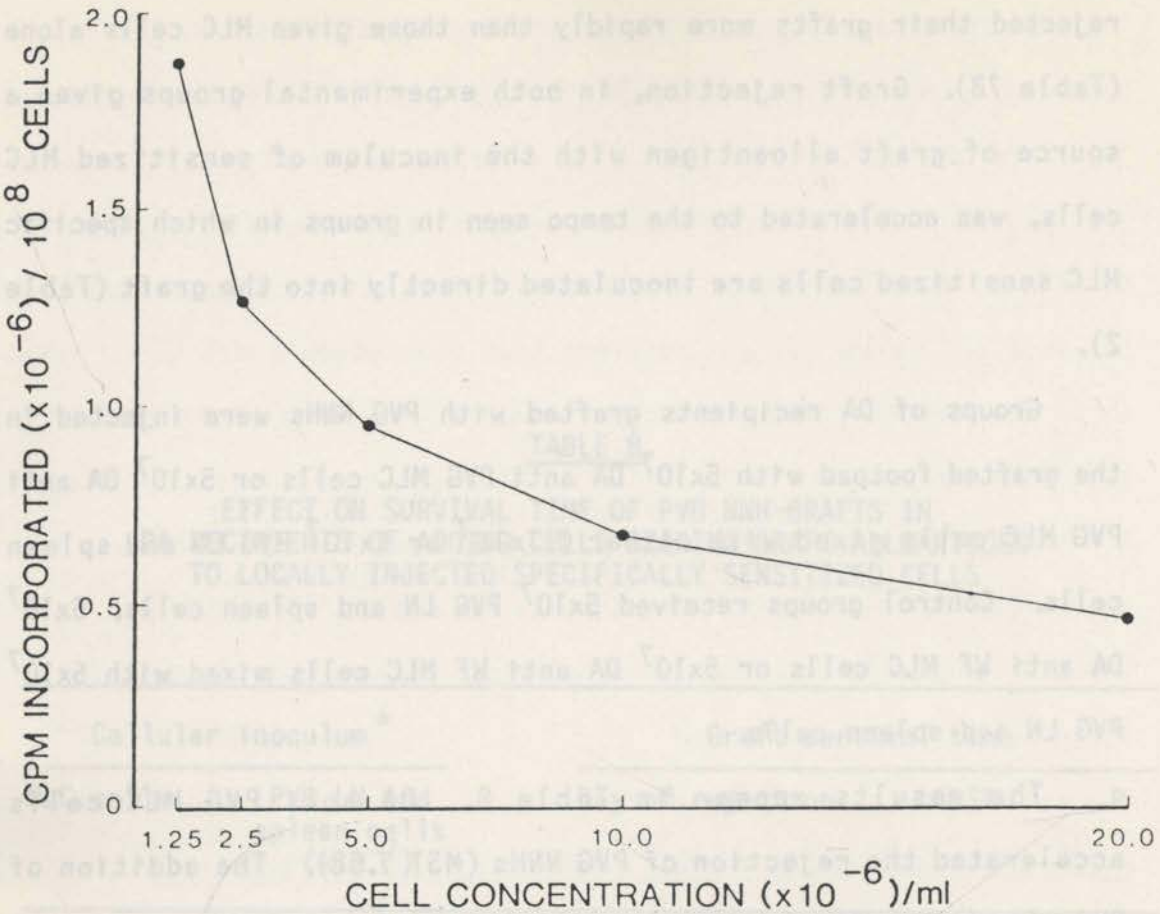


FIGURE 6

LABELLING OF MLC CELLS WITH I^{125} UdR

Cells from bulk MLC were harvested at four days and labelled in 5ml volumes containing 2 uCi I^{125}UdR/ml. Cells to be labelled were diluted at various concentrations from $2x10^7$ to $1.25x10^6$/ml. Cells were incubated for four hours at 37°C in TCM-1. Note the lower the labelling concentration of cells the higher the amount of label incorporated per cell.

finding that the addition of donor lymphoid cells to IV injected MLC activated cells accelerated graft rejection to a degree similar to that seen following the local inoculation of MLC activated cells supported this thesis. This data, taken together with the fact that even when MLC sensitized cells are inoculated directly into the graft site graft rejection does not occur for at least 5 days, might indicate that rejection is dependent on further division and/or differentiation of the MLC cells following in vivo contact with specific alloantigen and is not a consequence of the direct local action of the inoculated population. To throw further light on this question, the migration and localisation patterns of locally and systemically injected MLC sensitized cells were studied.

3.4 MIGRATION AND LOCALIZATION PATTERNS OF MLC CELLS

3.4.1 Parameters for labelling MLC cells with I^{125} UdR

I^{125}UdR as a label for dividing cells has the advantage that the I^{125} molecule is not reutilized but is excreted or concentrated in the thyroid after cell breakdown (reviewed van Rooijen 1977). In addition labelled cells present in various tissues can be quantitated by counting the radioactivity in whole tissue samples.

Studies were done to determine the optimum conditions for labelling MLC cells with I^{125}UdR.

DA anti PVG MLC cells were harvested after 4d in culture, resuspended at various cell concentrations from $2x10^7$ to $1.25x10^6$ cells/ml in 5ml of medium to which I^{125}UdR had been added at a final concentration of 2 uCi/ml. The cells were incubated for 4 hr at 37°C, following which they were washed 5 times in 30ml medium. After the final wash the cell pellet was counted to determine the amount of

TABLE 9

LOCALISATION OF I^{125} UdR LABELLED DA ANTI PVG MLC CELLS FOLLOWING LOCAL INJECTION INTO THE FOOTPAD OF DA HOSTS GRAFTED WITH PVG OR WF NNHs

Time (hr)	% Injected radio-activity recovered	Graft	% Total recovered radioactivity in various tissue			
			RFP	RPLN[a]	LFP	LPLN[b]
3.5	64	PVG	97.85	1.51	0.07	0.03
	80	WF	99.49	0.03	0.04	0.03
18	26	PVG	97.15	0.29	0.38	0.07
	28	WF	96.21	0.48	0.47	0.07
24	10	PVG	85.21	6.04	1.38	0.20
	18	WF	96.32	0.82	0.60	0.11

[a] Right popliteal LN.

[b] Left popliteal LN.

radioactive label which had been incorporated.

The results are shown in Fig. 6 and indicated an inverse relationship between cell concentration and the amount of label incorporated. As the planned experiments involved large numbers of cells it was not practical to label cells at a concentration of less than 2.5×10^6/ml and this was the concentration chosen for labelling in the studies reported below.

3.4.2 Localization of I^{125} UdR labelled MLC cells after local injection into NNH grafts

DA cells were sensitized to PVG alloantigen in MLC for 4d and then labelled with I^{125}UdR. Aliquots of 5×10^7 cells were injected into the grafted footpads of groups of DA recipients which had been grafted with either PVG or WF NNHs. The recipients were killed at intervals and both the grafted and the contralateral footpad, the nodes draining each, the spleen, liver, lung, cervical lymph nodes and mesenteric lymph nodes were removed. The amount of radioactivity present in each tissue was measured using a gamma counter.

The results appear in Table 9. The proportion of the injected radioactivity which was recovered fell progressively from 3.5hr to 24hr. In all animals the bulk of the recoverable radioactivity was found in the injected footpad and the proportion of the injected radioactivity was similar in PVG and WF graft sites. In both groups it declined slightly with time. At 3.5hr and 24hr the recipients of PVG grafts had a greater percentage of radioactivity localized in the PLN draining the graft than did recipients of WF grafts. At 18hr the percentage localization of radioactivity in the PLN draining the WF graft was slightly greater than that in the PLN draining the PVG graft.

TABLE 10

LOCALISATION OF I^{125} UdR LABELLED DA ANTI PVG MLC CELLS FOLLOWING LOCAL INJECTION INTO THE FOOTPAD OF DA HOSTS GRAFTED WITH EITHER PVG OR WF NNHs

Time (hr)	% Injected radio-activity recovered	Graft	% Total recovered radioactivity in various tissue			
			RFP	RPLN	LFP	LPLN
18	78	PVG	84.69	13.34	0.18	0.10
	76	PVG	86.56	11.45	0.18	0.11
	28	WF	87.12	8.97	0.38	0.26
	85	WF	89.45	8.34	0.22	0.09
24	39	PVG	72.44	21.59	0.65	0.19
	27	PVG	82.31	11.78	0.49	0.30
	70	WF	79.37	17.24	0.33	0.11
	50	WF	83.50	11.96	0.44	0.16
42	15	PVG	71.66	14.49	0.87	0.53
	9	PVG	70.26	14.30	1.16	0.90
	13	WF	57.47	24.35	1.17	0.65
	15	WF	71.36	17.73	0.89	0.56

The experiment was repeated with duplicate animals in each group. The results were similar (Table 10). At each time period the bulk of recoverable radioactivity was present at the injection site. The proportion of recovered radioactivity remaining localized in the specific (PVG) graft sites was not greater than that retained in the WF graft sites and indeed the tendency was the reverse. At both 18 and 24hr a greater proportion of total recovered radioactivity was detected in the PLN draining PVG than WF grafts but the difference was not great and by 42hr the reverse applied, the percentage recovery in PLN draining WF grafts was greater than that in PLN draining PVG grafts. The amount of radioactivity in the footpad and PLN contralateral to the injected grafted foot was similar in recipients of PVG and WF grafts. It increased in both over the period of observation but was never greater than 1.2% in the footpad and 0.7% in the node.

It was concluded that following local injection into a grafted footpad a high proportion of MLC stimulated cells remain localised to the injection site irrespective of whether the graft present bears sensitizing or third party antigens. Cells also travel to the LN draining the graft site and in the first 24hr after injection there may be a slight preferential accumulation in the node draining a graft bearing specific alloantigen compared with the node draining a graft bearing irrelevant alloantigen. The proportion of label found in sites distant from the injection site including the footpad and node contralateral to the injection site increased over the period of observation. The maximum observed in the contralateral footpad was approximately 1% at 42hr at which stage there was a total of up to 18% in the other organs examined.

TABLE 11

LOCALISATION OF I^{125} UdR LABELLED DA ANTI PVG MLC CELLS FOLLOWING ARTERIAL INJECTION INTO DA HOSTS BILATERALLY GRAFTED FIVE DAYS PREVIOUSLY WITH A PVG AND WF NNH

Time (hr)	% Injected radio-activity recovered	Graft	% Total recovered radioactivity in specific sites	
			FP	PLN
18	11.2	PVG	5.62	0.52
		WF	5.30	0.31
	11.0	PVG	4.61	0.57
		WF	3.11	0.19
	10.6	PVG	3.52	0.47
		WF	3.75	0.50
	9.9	PVG	4.22	0.54
		WF	4.53	0.52
24	3.7	PVG	12.63	0.74
		WF	10.61	0.66
	7.3	PVG	8.57	1.41
		WF	6.38	0.34
	4.0	PVG	5.91	0.81
		WF	5.53	0.85
	2.6	PVG	6.71	0.72
		WF	7.53	0.79

TABLE 12

LOCALISATION OF I^{125} UdR LABELLED DA ANTI PVG MLC CELLS FOLLOWING ARTERIAL INJECTION INTO DA HOSTS BILATERALLY GRAFTED FIVE DAYS PREVIOUSLY WITH A PVG AND WF NNH

Time (hr)	% Injected radio-activity recovered	Graft	% Total recovered radioactivity in specific sites	
			FP	PLN
18	17.6	PVG	2.97	0.24
		WF	2.35	0.32
	27.2	PVG	1.75	0.22
		WF	1.60	0.27
24	13.1	PVG	3.15	0.51
		WF	2.57	0.25
	18.0	PVG	3.03	0.26
		WF	2.78	0.36
42	1.8	PVG	7.39	1.12
		WF	6.54	1.03
	2.9	PVG	6.60	0.87
		WF	7.93	1.07

3.4.3 Localization of I^{125} UdR labelled MLC cells after systemic injection

The localisation pattern of systemically injected MLC sensitized cells was compared with that of locally injected cells.

Intra-arterial injection

DA recipients were grafted with PVG NNHs in the RFP and WF NNHs in the LFP. Five days were allowed to elapse to allow the graft to establish vascular connections with the host circulation and then $5x10^7$ I^{125}UdR labelled DA anti PVG MLC cells were injected into the abdominal aorta of each recipient. One group of 4 recipients was killed at 18hr and one at 24hr after injection. The tissues which were examined for their content of radioactivity were the grafted footpads and their draining PLNs, spleen, liver, lungs, cervical LNs, and small and large intestine. The results appear in Table 11. Although most of the recovered radioactivity was in tissues other than the grafted footpads there was a significant accumulation in the grafted footpads and the nodes draining them. This increased between 18 and 24hr relative to the total recovered radioactivity which fell during this time. Two of the four animals killed at each time had a slight increase in the proportion of radioactivity in the footpad and draining node on the side containing the graft bearing the alloantigen to which the injected cells were sensitized in MLC. One killed at 24hr had a slightly greater proportion of radioactivity recorded in the foot containing the PVG graft than in that containing the WF graft but the reverse in the respective PLNs.

The experiment was repeated with similar results (Table 12). In this case two recipients were examined at each of 18, 24 and 42hr. The same pattern emerged. There was a progressive accumulation of

TABLE 13

LOCALISATION OF I^{125} UdR LABELLED DA ANTI PVG MLC CELLS FOLLOWING IV INJECTION INTO DA HOSTS BILATERALLY GRAFTED FIVE DAYS PREVIOUSLY WITH PVG AND WF NNH

Time (hr)	% Injected radio-activity recovered	Graft	% Total recovered radioactivity in specific sites	
			FP	PLN
18	20.3	PVG	0.79	0.08
		WF	0.98	0.11
	21.3	PVG	1.00	0.11
		WF	1.26	0.11
24	9.0	PVG	2.30	0.25
		WF	2.17	0.22
	10.6	PVG	2.15	0.17
		WF	1.99	0.19
42	2.3	PVG	5.64	0.67
		WF	5.10	0.63
	2.3	PVG	4.36	0.56
		WF	4.25	0.52
48	1.0	PVG	8.36	1.02
		WF	8.96	1.10
	1.4	PVG	6.52	1.08
		WF	6.70	1.23

label in the grafted footpads and their draining nodes whereas the proportion of label recovered from other tissues decreased. There was a slightly greater proportion of radioactivity in the footpads grafted with PVG NNHs in each of the recipients killed at 18 and 24hr and one of the two recipients killed at 42hr. There was no consistent tendency in the relative amounts of label accumulating in the PLNs draining PVG and WF grafts.

Intravenous injection

To determine whether a similar localisation pattern follows the IV injection of MLC sensitized cells into bilaterally grafted hosts the experiment was repeated with $5x10^7$ DA anti PVG MLC cells being injected IV into DA hosts grafted 5 days previously with PVG NNHs in the RFP and WF NNHs in the LFP. These results appear in Table 13. Accumulation of label in the graft beds appeared to be slower than following IA injection otherwise the results were similar.

The results of localisation experiments shed little light on the question of whether locally injected cells procured graft rejection by a direct local effect or not. With each mode of injection there was a preferential accumulation of MLC sensitised cells at the graft site and its draining lymph node. The evidence that this accumulation was antigen specific was not convincing and with all three modes of injection including local, a proportion of the radioactive label could be detected at sites distant from the graft bed, presumably indicating systemic dispersal of the injected MLC sensitized cells.

To obtain further information on the mechanism by which MLC sensitized cells specifically accelerated the rejection of NNH grafts, experiments were done to determine whether they could cause accelerated rejection in hosts depleted of radiosensitve lymphoreticular cells.

TABLE 14

THE EFFECT OF LOCALLY INOCULATED MLC SENSITIZED CELLS ON SURVIVAL OF PVG NNHs IN IRRADIATED DA RECIPIENTS

Cellular inoculum[a]	Graft survival time		
	Day of rejection	MST	P
-	47, 52, 60, 68	56[b]	<.01
DA anti PVG MLC	10, 11(2), 12	11	<.01
DA anti BN MLC	56, 63(2), 67	63[b]	<.025
DA LNC	25, 28, 35(2), 41, 45, 53, 57, 59	41	

[a] $5x10^7$ cells.

[b] There was no significant difference between the survival time of grafts injected with cells sensitized in MLC against third party antigen (DA anti BN MLC) and that of uninjected grafts.

3.5 EFFECT OF MLC SENSITIZED LYMPHOCYTES ON GRAFT SURVIVAL IN IRRADIATED RECIPIENTS

Sixteen DA rats were irradiated with 900 rad whole body irradiation (WBI). This dose is routinely used in the laboratory for adoptive transfer experiments and is the maximum dose compatible with survival of recipients (see Chapter 2). Irradiated recipients were grafted with PVG NNHs. Groups of four recipients were then injected locally into the grafted footpad with $5x10^7$ normal DA LNC, $5x10^7$ DA anti PVG MLC cells or $5x10^7$ DA anti BN MLC cells respectively. Four control irradiated grafted recipients were given no cells.

The latter group showed prolonged survival of the NNH grafts (MST 56d, Table 14). Injection of cells sensitized in MLC against PVG alloantigen caused accelerated graft rejection, (MST 11d). In contrast the groups injected with DA anti BN MLC cells or normal DA LNC showed prolonged survival of PVG grafts. Animals injected with DA anti BN MLC cells showed graft survival comparable with that of uninjected controls. Those given normal DA LNC rejected their grafts with a slower tempo than recipients of DA anti PVG MLC cells ($p<0.01$) but more rapidly than controls given DA anti BN MLC cells ($p<0.025$).

The effect of systemically injected MLC sensitized cells on graft rejection in irradiated hosts was next studied.

DA rats were irradiated with 950 rad, grafted with PVG NNHs and injected IV or IA with $5x10^7$ DA cells sensitized in MLC against PVG alloantigens. Control groups were injected with cells sensitized against third party alloantigens or $5x10^7$ normal DA LNCs. The intra-arterial or intra-venous injection of MLC cells sensitized against PVG alloantigen caused acceleration of PVG graft rejection. Cells sensitized against third party antigen did not cause significant

TABLE 15

THE EFFECT OF SYSTEMICALLY INOCULATED MLC SENSITIZED CELLS ON SURVIVAL OF PVG NNHs IN IRRADIATED DA RECIPIENTS

Cellular inoculum[a]		Graft survival time		
Route	Source	Day of rejection	MST	P
IV	DA LNC	14, 17, 19, 20	18	
	DA anti PVG MLC	9(2), 10, 11	9.5	<.05
	DA anti BN MLC	30, 33(2), 34	33	<.01
IA	DA LNC	15, 19, 22, 23	20.5	
	DA anti PVG MLC	11, 12, 13(2)	12.5	<.05
	DA anti WF MLC	50, 53, 55, 62	54	<.025

[a] $5x10^7$ cells.

acceleration of graft rejection. (Table 15).

MLC sensitized cells accelerate graft rejection in irradiated hosts whether injected locally or systemically. This effect is specific. Animals injected with normal syngeneic LNC or MLC cells sensitized against irrelevant antigen did not reject their grafts with markedly accelerated tempo, although groups injected with normal LNC rejected grafts before those given MLC cells sensitized against irrelevant antigen. It was of interest that the rapid graft rejection (MST 5.5d; Table 3) procured by local inoculation of MLC sensitized cells in normal hosts could not be reproduced in irradiated hosts. The effect of systemically injected MLC cells was on the other hand similar in normal and irradiated hosts (MST 10.5 and 9.5d respectively; see Tables 3 and 13).

This suggests that the rapid rejection which follows local inoculation of MLC sensitized cells into the grafted footpad of normal hosts involves the participation of a radiosensitive host cell.

This hypothesis was tested by examining the effect on graft survival time of the addition of various normal cell populations to MLC sensitized cells inoculated locally in the graft site in irradiated hosts.

3.5.1 Effect on graft survival of the addition of peritoneal exudate (PE) cells to MLC cells

The macrophage is known to be implicated in allo-immune responses. The degree to which macrophages are radiosensitive in the resting state is not known however the number of monocytes in peripheral blood following WBI has been shown to be reduced (MacPherson and Christmas, 1984).

TABLE 16

THE EFFECT ON REJECTION OF PVG NNHs IN DA HOSTS OF THE ADDITION OF PE CELLS TO LOCALLY INOCULATED MLC SENSITIZED CELLS

Host Irradiation	Cellular inoculum: DA anti PVG MLC ($x10^{-7}$)	Cellular inoculum: DA PE cells[a] ($x10^{-7}$)	Graft survival time: Day of rejection	Graft survival time: MST	Graft survival time: P
+	5	-	12(2), 13, 14	12.5	
+	5	4	13, 14(2), 15	14	NSD
+	-	4	55(2), 58, 59	56.5	<.005
-	5	-	5(2), 6, 7	5.5[b]	
-	5	4	4(4)	4	<.01
-	-	4	6, 8(2), 10	8[b]	

[a] 70-80% phagocytic cells.

[b] NSD.

DA recipients were irradiated with 950 rad WBI and grafted with PVG NNHs. A control unirradiated group was also grafted. Four recipients in each group were injected in the grafted footpad with $5x10^7$ DA anti PVG MLC cells, four with $5x10^7$ DA anti PVG MLC cells and $4x10^7$ DA PE cells and four with $4x10^7$ DA PE cells. The latter were obtained after proteose peptone stimulation (see Chapter 2) and contained 80% adherent, phagocytic cells. The results appear in Table 16.

Grafts in irradiated rats locally inoculated with specific MLC sensitized cells showed a MST of 12.5d compared to a MST of 5.5d in normal hosts injected with the same cell population. The addition of stimulated PE cells to cellular inocula surprisingly further accelerated rejection times in normal hosts (MST 4d) but failed to do so in irradiated hosts (MST 14d).

A further surprising finding was that stimulated PE cells alone caused accelerated graft rejection in normal hosts (MST 8d) which was not significantly different to that procured by MLC sensitized cells (MST 5.5d). Irradiated hosts given PE cells alone (Table 16) however showed graft survival comparable with that in irradiated hosts not given cells, (cf Table 14; NSD) indicating that stimulated macrophages in the absence of radiosensitive lymphocyte populations have no effect on graft rejection.

3.5.2 Effect on graft survival of the addition of MRC $OX8^+$ cells to MLC cells

The helper lymphocyte subpopulation ($W3/25^+$) is the one which proliferates to the greatest extent in MLC (Mason et al, 1981; Dallman et al, 1982). It was possible that MLC sensitized cells lack sufficient cytotoxic/suppressor (MRC $OX8^+$) cells to effect rapid

TABLE 17

THE EFFECT ON REJECTION OF PVG NNHs IN IRRADIATED DA HOSTS OF THE ADDITION OF MRC OX8$^+$ CELLS TO LOCALLY INOCULATED MLC SENSITIZED CELLS

Cellular inoculum		Graft survival time		
DA anti PVG MLC ($\times 10^{-7}$)	DA[a] MRC OX8$^+$ ($\times 10^{-7}$)	Day of rejection	MST	P
5	–	8, 9, 10(2)	9.5	
5	1.8	9(4)	9	NSD
–	1.8	15, 25, 50(2)	37.5	<.005

[a] Contamination – 3% 1g$^+$ cells, 2% W3/25$^+$ cells.

graft rejection in vivo and that normal hosts provide this cell population whereas irradiated hosts do not. The effect of adding MRC $OX8^+$ cells separated from normal DA LNC populations to MLC sensitized cells was examined.

DA rats were irradiated and grafted with PVG NNHs. Groups of four animals were injected in the grafted footpads with $5x10^7$ DA anti PVG MLC cells, $1.8x10^7$ MRC $OX8^+$ cells separated from normal DA LNC (as described in Chapter 2) or a mixture of $5x10^7$ DA anti PVG MLC cells and $1.8x10^7$ MRC $OX8^+$ cells. The results appear in Table 17. DA MRC $OX8^+$ cells when added to DA anti PVG MLC cells did not markedly increase the capacity of the population to cause rejection (MST 9d compared to 9.5d with MLC cells alone). This was significantly more rapid than rejection in animals injected with naive MRC $OX8^+$ cells alone. The latter group rejected their grafts with a tempo (MST 37.5d) which was not significantly different to that in irradiated hosts given no cells (MST 56d; Table 14).

As neither PE cells nor MRC $OX8^+$ cells alone appeared to provide the cell population necessary to procure rapid graft rejection in irradiated hosts injected locally with MLC sensitized cells, the effect of adding both cell populations to MLC sensitized cells was examined.

3.5.3 Effect on graft survival of the addition of both MRC $OX8^+$ cells and PE cells to MLC cells

Groups of irradiated DA rats grafted with PVG NNHs were injected in the graft bed with either $5x10^7$ DA anti PVG MLC cells, $5x10^7$ DA anti PVG MLC cells mixed with $4x10^7$ DA PE cells and $2x10^7$ DA MRC $OX8^+$ LNC or a miscture of both $4x10^7$ DA PE cells and $2x10^7$ DA MRC $OX8^+$ LNC. The results appear in Table 18. The group injected with a

TABLE 18

EFFECT ON SURVIVAL OF PVG NNH GRAFTS IN IRRADIATED DA RATS OF THE ADDITION OF PE CELLS AND NAIVE MRC OX8$^+$ CELLS TO LOCALLY INOCULATED MLC SENSITIZED CELLS

Cellular inoculum			Graft survival time		
DA anti PVG MLC ($\times 10^{-7}$)	DA MRC[a] OX8$^+$ ($\times 10^{-7}$)	DA PE[b] cells ($\times 10^{-7}$)	Day of rejection	MST	P
5	–	–	10, 12(2), 13	12	<.01
5	2	4	5, 6(3)	6	<.005
–	2	4	30(2), 31, 33	30.5	

[a] Contamination – 3% 1g$^+$ cells and 0–1% W3/25$^+$ cells.

[b] 80% phagocytic cells.

TABLE 19

EFFECT ON SURVIVAL OF PVG NNH GRAFTS IN IRRADIATED DA RATS OF THE ADDITION OF PE CELLS AND NAIVE MRC OX8$^+$ OR W3/25$^+$ CELLS TO LOCALLY INOCULATED MLC SENSITIZED CELLS

Cellular inoculum				Graft survival time		
DA anti PVG MLC ($\times 10^{-7}$)	DA[a] MRC OX8$^+$ ($\times 10^{-7}$)	DA[b] W3/25$^+$ ($\times 10^{-7}$)	DA PE[c] cells ($\times 10^{-7}$)	Day of rejection	MST	P
5	–	6	5	11(2), 13, 14	12	<.01
5	1.8	–	5	6, 7(3)	7	<.01
5	–	–	–	10(3), 11	10	

[a] Contamination – 1% 1g$^+$ cells and 0–1% W3/25$^+$ cells.

[b] Contamination – 1.5% 1g$^+$ cells and 0–1% MRC OX8$^+$ cells.

[c] 80% phagocytic cells.

mixture of MLC sensitized cells, MRC OX8$^+$ cells and PE cells rejected their grafts with a MST of 6d which is comparable to that seen in normal hosts injected with MLC sensitized cells alone. The MST of grafts in the group injected with MLC cells alone was 12d; that of the group given PE cells and naive MRC OX8$^+$ cells was 30.5d.

This suggests that rapid graft rejection in normal hosts injected with MLC sensitized cells was a consequence of the interaction of these cells with host MRC OX8$^+$ (cytotoxic/suppressor) cells and host macrophages. To determine whether naive helper cells (W3/25$^+$) could substitute for the cytotoxic/suppressor population in this interaction, the capacity of an inoculum of MLC sensitized cells, PE cells and W3/25$^+$ cells to cause graft rejection in irradiated hosts was examined. (Table 19).

Groups of irradiated grafted animals were injected with $5x10^7$ DA anti PVG MLC cells either alone, mixed with $5x10^7$ DA PE cells and $1.8x10^7$ DA MRC OX8$^+$ cells or mixed with $5x10^7$ DA PE cells and $6x10^7$ DA W3/25$^+$ cells. Only the group given MLC sensitized cells with PE cells and MRC OX8$^+$ cells showed rapid graft rejection (MST 7d). The group given MLC cells alone and that given MLC cells with PE cells and naive W3/25$^+$ cells had MSTs of 10 and 12 days respectively.

3.6 THE CELL MEDIATED LYSIS (CML) ASSAY

Having established that putative DA cytotoxic cells raised in <u>in vitro</u> MLC have the capacity to accelerate the rejection of NNH grafts, but that in irradiated hosts rapid graft rejection only occurred if additional syngeneic naive MRC OX8$^+$ cells and PE cells were added to the MLC inoculum, experiments were designed to correlate the <u>in vivo</u> activity of MLC cells with the capacity of

these cells to procure target cell lysis in vitro. DA strain rats are known as low responders in a number of allo-immune assays and other workers have reported difficulty establishing CML assays with rat lymphocytes in general and DA strain cells in particular. Numerous attempts (summarized below) were made to develop a reliable and reproducible CML assay using DA anti PVG effector cells and PVG target cells. These were unsuccessful, although using the same methods cell mediated lysis with WF effector cells was reproducibly demonstrated.

Initial experiments tested the capacity of DA anti PVG MLC cells, harvested from bulk cultures on the fourth day of culture, to cause in vitro lysis. Although these cells were demonstrably capable of causing accelerated rejection of NNH grafts they failed to cause specific Cr^{51} release from labelled PHA or Con A blasts. Following this a large number of permutations and combinations, involving variations in both the conditions under which responder and stimulator cells were cultured and/or the conditions under which putative effector and target cells were incubated, were explored in attempts to obtain reproducible specific cytotoxicity. These are briefly summarised below.

3.6.1 Target cells for CML assay

*** PHA stimulation of rat lymphocytes**

The parameters of the in vitro responses of LNC to PHA had already been established in order to verify that an irradiation dose of 950 rad was sufficient to ablate the proliferative response of lymphoid cells in culture. These experiments also served as a preliminary to the CML assay as PHA blast cells were initially used as target cells for this assay. While many workers use PHA blasts as

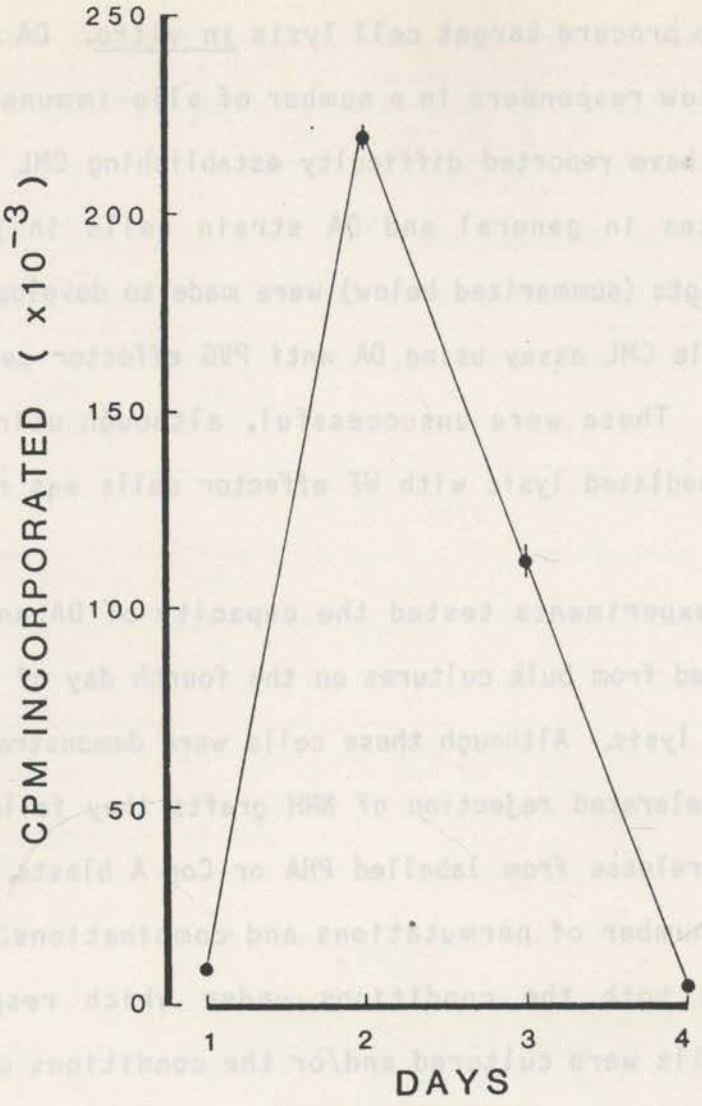


FIGURE 7

IN VITRO CONCANAVALIN A STIMULATION OF PVG LNC

PVG LNC were cultured in TCM-1 in micro-titre trays at a concentration of $2x10^6$ cells/ml. Concanavalin A was added to the cells at a concentration of 11 ug/ml. Cells were harvested daily for four days after a four hour pulse with H^3TdR. PVG LNC cultured alone showed negligible levels of incorporation of H^3TdR. CPM ranged between 120 CPM and 640 CPM with a mean of 280 CPM (results not shown for the sake of clarity). The addition of Con A to the cultures resulted in a vigorous proliferative response which peaked at day 2 of culture.

target cells in the CML assay it is also common to use Concanavalin A (Con A) stimulated blast cells. It was of interest to examine a variety of target cells in the establishment of a reliable CML assay, therefore experiments were done to determine the in vitro parameters of the response of LNC to Con A.

*** Con A stimulation of rat lymphocytes**

PVG LNC were cultured in U bottom microtitre trays at a concentration of $2x10^6$ cells/ml in 200ul of TCM-1 to which was added 11ug Con A/ml. Cultures were harvested daily for 4 days after a 4 hour pulse with H^3TdR. The results appear in Figure 7.

The results confirmed that culturing under these conditions for 2 days was sufficient to generate a vigorous proliferative response in rat LNC. A similar response was obtained when cells were cultured at $2x10^6$ cells/ml with 11ug Con A/ml in 5ml in TC flasks. After 2 days the cultures contained a high proportion of blast transformed cells (greater than 95%) with a very high yield of viable cells, returning 120% of the original cell number.

3.6.2 Variations in conditions of MLC to produce effector cells

*** Number of days in culture**

MLC cultures set up in TC flasks, in the same manner as described in Chapter 2 for the production of cells causing accelerated graft rejection, were harvested on days 3 - 10 after initiation and tested for their capacity to lyse Cr^{51} labelled PHA blasts. In all cases DA cells sensitized against PVG alloantigens were tested against both PVG and BN target cells. No specific Cr^{51} release was observed.

* **Cell concentrations and ratios of responder to stimulator cells**

It was considered that the MLC parameters which had been shown to provide the optimal conditions for proliferation might not necessarily be optimal for the development of demonstrable cytotoxic cells. DA responder cell concentrations from $10x10^6$/ml to $0.7x10^6$/ml in 1.5 times dilutions were set up with stimulator cells at 0.5, 1 or $2x10^6$/ml. None of the cultures yielded cell populations with demonstrable specific cytotoxicity.

* **Source of responder cells**

Spleen cells, TDL or purified recirculating T cells were used as responder cells in MLC. No cytotoxicity could be demonstrated in cultures in any case.

* **Culture vessels**

It has been reported that proliferation in primary MLC (Antczak et al, 1979; Knight, 1982) and cytotoxic T cell development (Wilson et al, 1976b; Marshak et al, 1977) may depend on the culture vessel used. Wilson et al (1976b) reported that MLC in Marbrook culture vessels produced effector cells with specific reactivity whereas culture using standard techniques did not.

MLCs were set up in microtitre trays at a responder cell concentration of $4x10^6$/ml. Cells harvested from days 2 - 10 after culture were assayed for their capacity to cause specific Cr^{51} release. Specific release of 3-11% was obtained with cells harvested on the fifth day of culture and tested at an effector: target (E:T) cell ratio of 100:1. The experiment was repeated using the same parameters. Specific cytotoxicity could not be reproducibly obtained. Attempts were made to raise Tc in Marbrook culture vessels following the method of Wilson et al (1976b) (See Chapter 2). No specific cytotoxicity was recorded.

* **Prior sensitization of responder cell populations**

In vitro

It has been reported that consistent, significant cytotoxicity can only be demonstrated with rat lymphocytes after secondary stimulation in vitro. DA responder cells were cultured with PVG stimulator cells. After 5 days the cells were harvested, washed and resuspended with fresh PVG stimulator cells. This was repeated after 2 further days in culture. Cells were harvested 2 days after tertiary stimulation and tested for CML. No Cr^{51} release was observed.

In vivo

(i) Cells, obtained from the popliteal LN of DA rats injected in the footpad 4 days previously with PVG lymphocytes, were set up in an MLC with PVG stimulator cells. The cultures were harvested 3d later. No specific cytotoxicity could be demonstrated in the population.

(ii) Cells obtained from the spleens of WF, BN, PVG and DA rats at days 4 to 16 following the IP injection of allogeneic lymphocytes were either tested immediately in a CML assay or restimulated in MLC with the relevant stimulator cell. MLC cultures were harvested 2 days later. Neither spleen cells nor cells restimulated in MLC produced reliable or reproducible specific cytotoxicity in vitro although occasional positive results were obtained.

* **Addition of conditioned supernatants**

Workers have reported the enhanced induction of Tc from precursor cells and the reexpression of cytotoxic activity from memory Tc (Le Francois et al, 1984) following culture in the presence of supernatants from cultures of alloantigen or mitogen stimulated lymphocytes. Bulk MLC and microcultures of MLC were conducted as described in Chapter 2 in the presence of 50%, 25%, 12.5% and 6.25%

(v/v) of supernatants derived from secondary MLC or Con A culture of rat lymphocytes. Although occasional cultures produced cells with specific lytic activity, this result was not reproducible.

3.6.3 Variations in conditions of CML assay

A number of variations in the methods used for CML assay were investigated in the search for a reproducible assay for Tc.

* **Incubation medium**

Other workers had observed greater specific Cr^{51} release when FCS was added to the medium in which effector and target cells were suspended. In different experiments cells were suspended in MLI medium (TCM 1, Chapter 2), RPM1 1640 + 10% FCS or RPM1 1640 + 20% FCS. In no case did the CML assay yield significant results.

* **Effector: Target cell ratios**

These were varied from 2/1 to 500/1 without any positive results.

* **Method of harvesting putative cytotoxic cells**

In some cases putative cytotoxic cells were harvested after culture and added in appropriate numbers to Cr^{51} labelled target cells. As the cells which react in MLC are blast cells and presumably fragile it was considered that the harvesting and washing procedures may have been too harsh and for this reason an alternative method was investigated. Cells cultured in microtitre trays were left in the trays in which they had been cultured or those cultured in TC flasks were gently aliquoted into microtitre trays several hours prior to assay. In each case 100ul of supernatant was removed from each well and the labelled target cells were added to the wells in 100ul of medium. No cytotoxicity was obtained.

* **Method of incubation**

In an attempt to maximise contact between effector and target cells after adding Cr^{51} labelled cells to the MLC cells, microtitre trays were centrifuged at 35g for 1 min to bring the cells into close contact. They were then incubated on a gently rocking platform, spun for 5 mins at 300g after incubation to mix released label uniformly throughout the supernatant and to pellet the cells. No Cr^{51} release above background was observed using this method.

* **Addition of macrophages to effector cell populations**

It has been reported that the addition of macrophages to the effector cells in CML assays enhances the cytotoxic effect of the effector cells. To examine this effect specifically stimulated PE cells were obtained by injecting $100x10^6$ DA lymphocytes, previously sensitized against PVG alloantigens, into the peritoneal cavity of DA rats. Peritoneal exudate cells harvested 4 days later contained 80% phagocytic cells. These were added in ratios of 1/2, 1/4, 1/8 and 1/16 to putative DA effector cells raised in MLC with PVG stimulator cells. PE cells were also tested alone for CML activity. In no case was significant cytotoxicity recorded.

* **Variation in target cells employed**

It was considered that failure to demonstrate cytotoxicity in cells raised in MLC cultures might have reflected the unsuitability of PHA blast cells as targets for CML. A number of other cell types were tested as target cells. These included PE cells obtained 4d after injecting the peritoneal cavity of PVG rats with 5ml 10% proteose peptone, cells from a PVG leukaemia (described in Dibley et al, 1975), dispersed cells from a mammary carcinoma and blast cells obtained after stimulation of PVG LNCs with Con A. None of these cell types appeared to have any advantage over PHA blasts as target

cells in CML assays.

The foregoing variations in the conditions and parameters of the MLC and the CML assay were exhaustively tested in various combinations. In most cases DA responder cells were used although other strain combinations of responder and stimulator cells were occasionally also tested. In the latter cases significant specific cytotoxicity was occasionally demonstrated but the results were inconsistent. In the case of DA responder/effector cells on no occasion was specific chromium release greater than 11% and was usually lower than 5%.

3.6.4 CML assay with Con A stimulated thymocyte targets

Subsequent to the aforementioned attempts to develop a reproducible and reliable CML assay, a method used by Lowry and workers (personal communication) was explored. As the target cells used in this method were Con A stimulated thymocytes the requirements for the Con A stimulation of rat thymocytes were first explored.

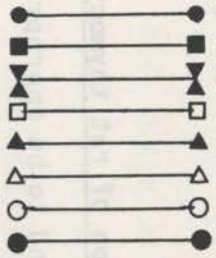
3.6.4.1 Con A stimulation of rat thymocytes

The conditions found to be appropriate for the stimulation of rat LNC with Con A proved to be unsuitable for the *in vitro* stimulation of rat thymocytes. The routine method that was being used by others (Lowry and workers, personal communication) also gave a very low yield of viable cells, none of which were blast transformed. It was apparent that the requirements for the culture of thymocytes were stringent and therefore a series of experiments were done to establish the Con A dose, time in culture and culture conditions which would give maximum blast transformation under the conditions prevailing in this laboratory.

FIGURE 8

IN VITRO CONCANAVALIN A STIMULATION OF WF THYMOCYTES

WF thymocytes at various concentrations were cultured in TCM-2 in the presence of graded doses of Con A. Cells were harvested daily for three days after a four hour pulse with H^3TdR.



ug Con A/ml

1.7
2.5
3.8
5.6
8.4
12.7
19.0
28.5

The peak proliferative response occurred after two days in culture. On a cell per cell basis the response in cultures containing $4x10^6$ thymocytes/ml treated with 12.7 ug Con A/ml was in the optimum range. WF thymocytes cultured alone incorporated negligible counts of H^3TdR (less than 1000 CPM). Each point represents the mean of triplicate wells $\pm$ 1 SD.

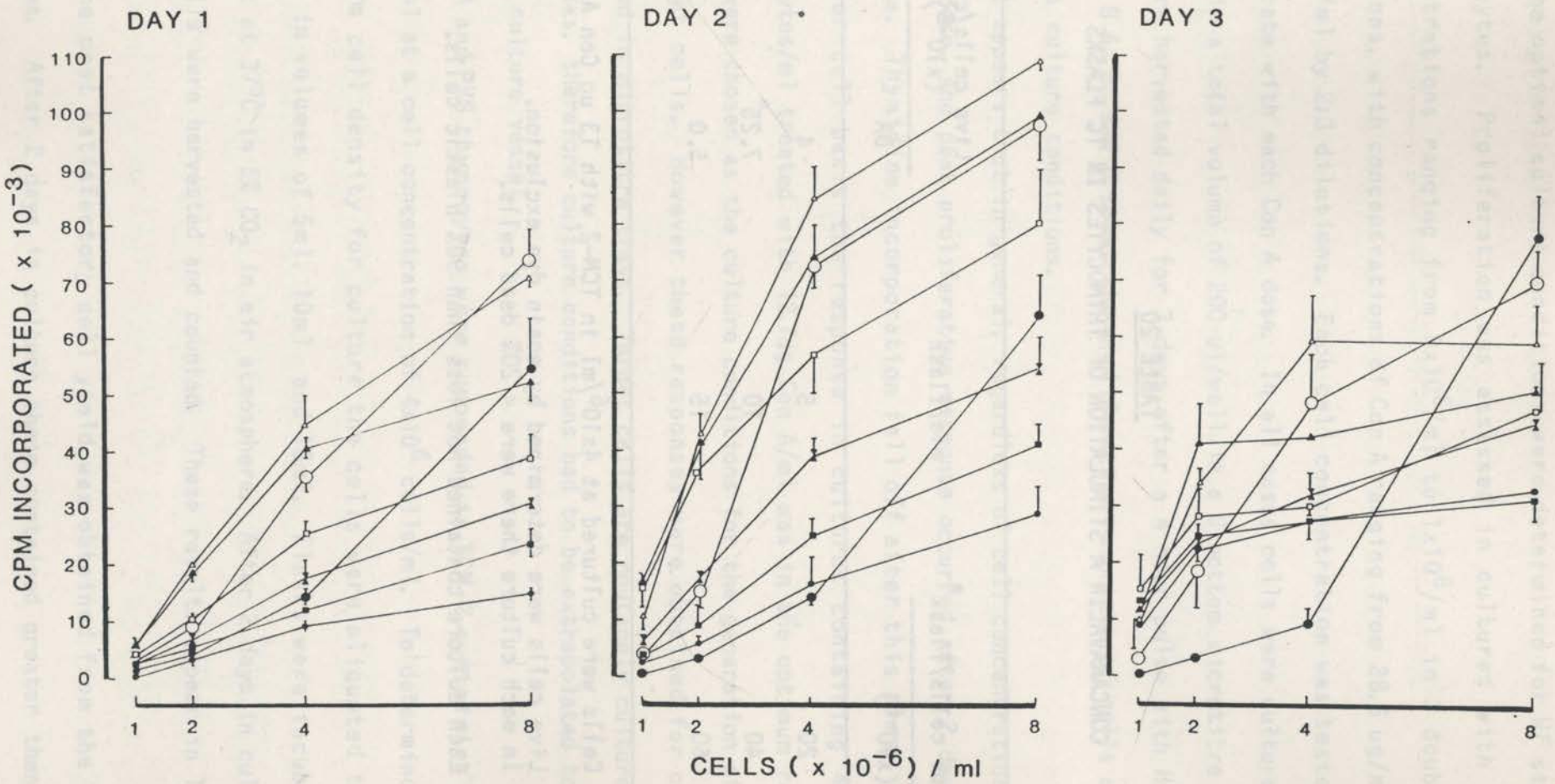

DAY 1
DAY 2
DAY 3
CPM INCORPORATED (x 10^{-3})
110
100
90
80
70
60
50
40
30
20
10
0
1
2
4
8
CELLS (x 10^{-6}) / ml

TABLE 20

CONCANAVALIN A STIMULATION OF THYMOCYTES IN TC FLASKS

No. of cells/flask[a] ($x10^{-6}$)	mls/flask	Live cells/culture[b] ($x10^{-6}$)	
		DA	PVG
20	5	4	4.7
40	10	7.25[c]	7.45[c]
60	15	5.0	8.5

[a] Cells were cultured at $4x10^6$/ml in TCM-2 with 13 ug Con A/ml.

[b] Live cells were determined by eosin dye exclusion. In each culture there were < 20% dead cells.

[c] Each culture contained not less than 90% blastic cells.

The optimal culture conditions were determined for WF strain thymocytes. Proliferation was assessed in cultures with cell concentrations ranging from $8x10^6$/ml to $1x10^6$/ml in 3 doubling dilutions, with concentrations of Con A ranging from 28.5 ug/ml to 1.7 ug/ml by 2:3 dilutions. Each cell concentration was tested in triplicate with each Con A dose. In all cases cells were cultured in TCM-2 in a total volume of 200 ul/well in a U bottom microtitre tray and were harvested daily for 3 days after a 4 hour pulse with H^3TdR. Figure 8 A, B, and C shows the proliferative responses of cells under various culture conditions.

It appears that in general, regardless of cell concentration and Con A dose, the peak proliferative response occurred after 2 days in culture. Thymidine incorporation fell off after this time. On a cell for cell basis the response in cultures containing $4x10^6$ thymocytes/ml treated with 12.7ug Con A/ml was in the optimum range. These were chosen as the culture conditions for the generation of Con A target cells. However these responses were obtained for cells cultured in microtitre trays. Target cells are routinely cultured in TC flasks, therefore culture conditions had to be extrapolated to the larger culture vessel.

DA and PVG thymocytes were suspended in TCM-2 containing 13ug Con A/ml at a cell concentration of $4x10^6$ cells/ml. To determine the optimum cell density for culture the cells were aliquoted to TC flasks in volumes of 5ml, 10ml and 15ml. Flasks were incubated upright at 37°C in 5% CO_2 in air atmosphere. After 2 days in culture the cells were harvested and counted. These results appear in Table 20.

The most satisfactory cell yield was obtained from the 10ml cultures. After 2 days in culture these contained greater than 80%

TABLE 21A

CAPACITY OF WF LNC TO RESPOND IN MLC AND CML ASSAYS

MLC cells[a]		CML assay[b]		
		% Cr^{51} Release		% Specific Cr^{51} Release
WF Responder cells/well ($x10^{-5}$)	PVG Stimulator cells/well ($x10^{-5}$)	DA	PVG	
1.25	1.25	–	17	17
	2.5	6	39	33
	5.0	2	48	46
	10.0	–	26	26
2.5	1.25	–	27	27
	2.5	7	52	45
	5.0	4	53	49
	10.0	–	24	24
5.0	1.25	–	24	24
	2.5	7	43	36
	5.0	3	55	52
	10.0	–	30	30
10.0	1.25	–	15	15
	2.5	6	35	29
	5.0	–	36	36
	10.0	–	21	21

[a] Cells were cultured in MLC for four days prior to assay in TCM-2 by Method 2 in microtitre trays. Total volume = 200 ul.

[b] MLC cells were not harvested. Prior to assay 100 ul of supernatant was removed from each well and 10^4 target cells were added in 100 ul. Plates were spun at 35g for two minutes before and after four hours incubation. Target cells were Con A stimulated thymocytes. Spontaneous release of Cr^{51} was 23% for DA target cells and 17% for PVG target cells of maximum releasable Cr^{51}.

viable cells of which not less than 90% were blasts.

3.6.4.2 The MLC and CML assay

The method used by Lowry et al (personal communication) for inducing effector cells in the MLC was also different from the methods previously explored. Stimulator cells were not squashed from diced lymphoid tissue but were expressed from the diced tissue through a wire mesh and the remaining connective tissue was well sieved. After collection of the mixed LNC and spleen cells, the cells were layered over Ficoll diatrizoate (S.G. 1.097) and spun for 15 min at 1700g. The collected interfaces were then washed once prior to irradiation with 3000 rad. The method is more fully outlined in Chapter 2 as MLC method 2. The TC medium used for both MLC and CML assay was TCM-2.

Effector and target cells were incubated together in microtitre trays. Prior to assay the microtitre trays were spun at 35g for 2 min to pellet the effector and target cells and after a 4 hr reaction period the plates were spun for 5 min at 300g to pellet cells.

The method was initially used in a further attempt to raise DA lymphocytes with demonstrable, reproducible *in vitro* cytotoxic activity. Once again this was unsuccessful. The experiments were repeated using WF responder cells.

3.6.5 WF MLC responder cells as effector cells

A variety of responder and stimulator cell ratios were employed to sensitize effector cells in MLC for the CML assay. 100ul of WF responder cells containing $10x10^5$, $5x10^5$, $2.5x10^5$ or $1.25x10^5$ cells were cultured in triplicate with 100ul of DA or PVG stimulator cells at one of the above concentrations. After 4 days in culture 100ul of

TABLE 21B

CAPACITY OF WF LNC TO RESPOND IN MLC AND CML ASSAYS

MLC cells[a]		CML assay[b]		
		% Cr^{51} Release		% Specific Cr^{51} Release
WF Responder cells/well ($x10^{-5}$)	DA Stimulator cells/well ($x10^{-5}$)	DA	PVG	
1.25	1.25	17	1	16
	2.5	44	8	36
	5.0	53	8	45
	10.0	38	3	35
2.5	1.25	20	1	19
	2.5	53	10	43
	5.0	60	8	52
	10	31	3	28
5.0	1.25	16	3	13
	2.5	52	9	43
	5.0	55	9	46
	10.0	30	4	26
10.0	1.25	-	-	-
	2.5	19	3	16
	5.0	39	5	34
	10.0	26	2	24

[a] Cells were cultured in MLC for four days prior to assay in TCM-2 by Method 2 in microtitre trays. Total volume - 200 ul.

[b] MLC cells were not harvested. Prior to assay 100 ul of supernatant was removed from each well and 10^4 target cells were added in 100 ul. Plates were spun at 35g for two minutes before and after four hours incubation. Target cells were Con A stimulated thymocytes. Spontaneous release of Cr^{51} was 23% for DA target cells and 17% for PVG target cells of maximum releasable Cr^{51}.

culture medium supernatant was removed from each well and 100ul of target cells containing 10^4 cells were added. Sensitized cells were tested on both PVG and DA target cells. Because cells were cultured in this way and not harvested before the CML assay it was not known what ratio of MLC effector cells to target cells was used in the CML assay. These results appear in Table 21, A and B.

These results indicate that WF LNCs sensitized to PVG or DA alloantigens in MLC develop the capacity to specifically lyse thymocyte target cells bearing the stimulator strain alloantigen. Most of the responder to stimulator cell ratios assayed led to the development of Tc with demonstrable specific lytic ability, however the optimal cell concentrations appeared to be 2.5 or $5x10^5$ responder cells per well cultured with 2.5 or $5x10^5$ stimulator cells per well.

3.6.6 The effect on CML of MLC stimulator cell treatment

A major difference in the methods of cell preparation for MLC between the two methods of MLC sensitization used, was that stimulator cells were expressed from lymphoid tissue by squashing in the initial method. The process of sieving used in the later method may have released cells from the LN and spleen connective tissue which would be absent from normal squash preparations of LN and spleen. The intrafollicular dendritic cell (DC) resident in the connective tissue of LN and spleen, has been shown to be the major immunogenic cell in the MLC (Nussenzweig and Steinman, 1980; Mason et al, 1981; Rollinghoff et al, 1982). Sieve preparation of stimulator cells may therefore have enhanced the development of Tc in MLC due to the increased numbers of DC present in the stimulator cell population as a result of the procedure. To determine what effect the preparation of stimulator cells would have on the generation of Tc in

TABLE 22

THE EFFECT ON TARGET CELL LYSIS BY MLC SENSITIZED EFFECTOR CELLS OF MLC STIMULATOR CELL TREATMENT

MLC cells		CML assay[b]		
Specificity	Stimulator cell Treatment[a]	% Cr^{51} Release PVG	BN	% Specific Cr^{51} Release
WF anti PVG	1	30	0	30
	2	45	2	43
WF anti BN	1	6	28	22
	2	4	15	11

[a] MLC were performed according to method 2 except with respect to stimulator cells treatment. Stimulator cells were prepared by either squashing diced lymphoid tissue (1) or sieving finely diced lymphoid tissue through wire mesh with the rubber tipped barrel of a sterile syringe (2).

[b] E/T ratio = 100/1; % Cr^{51} release determined from the mean of triplicate samples. Con A stimulated thymocyte target cells. Spontaneous release of Cr^{51} for PVG and BN targets was 19% and 23% of maximum release respectively.

bulk MLC, parallel cultures were conducted where in one set of cultures PVG and BN stimulator cells were squashed and in the other set of cultures they were sieved through wire mesh; in all other respects the culture conditions for MLC method 2 were followed. In both cases PVG and BN stimulator cells were cultured at 2.5×10^6/ml with WF responder cells, also at 2.5×10^6/ml.

After 4 days in culture WF anti PVG and WF anti BN cells were harvested and suspended at 10×10^6/ml and used as effector cells in the CML assay. 100ul of MLC effector cells were incubated for 6 hours with 100ul of Cr^{51} labelled PVG or BN Con A stimulated thymocytes at 10×10^4/ml. The assay was performed in triplicate. The results appear in Table 22.

Both WF anti PVG and WF anti BN MLC effector cells specifically lysed the target cells to which they were sensitized. In the case of WF cells sensitized to PVG a higher percentage of specific Cr^{51} release was observed using WF effector cells which had been sensitized to alloantigens in cultures containing sieved stimulator cells. The converse was true for WF anti BN MLC cells. However, both MLC stimulator cell treatments resulted in effector cells with specific lytic activity, something which had not been observed in earlier attempts to establish the CML assay. This result indicated that the success with the current method for MLC and CML assay did not reside exclusively in the preparation of stimulator cells for MLC but probably with a variety of other imponderables associated with the assay which had concommitantly been altered; i.e. the change in MLC culture medium or the use of thymocyte instead of LN derived blast cells as target cells in the CML assay. These were not further investigated.

TABLE 23

CAPACITY OF WF MRC OX8$^+$ LNC TO RESPOND IN MLC AND CML ASSAYS

MLC cells		CML assay[b]	
		% Cr^{51} Release	
Responder[a] cells/well ($x10^{-5}$)	Stimulator cells/well ($x10^{-5}$)	WF anti PVG	WF anti DA
1.25	1.25	–	4
	2.5	–	–
	5.0	2.4	–
	10.0	–	–
2.5	1.25	3	15
	2.5	3	9
	5.0	–	2
	10.0	–	–
5.0	1.25	32	11
	2.5	28	7
	5.0	18	6
	10.0	10	–
10.0	1.25	45	12
	2.5	51	14
	5.0	35	8
	10.0	28	6

[a] Population contained < 0.5% W3/25$^+$ cells and < 4.5% Ig$^+$ cells.

[b] Cultures were set up with either DA or PVG stimulator cells and MLC effector cells were tested on both PVG and DA target cells. Specific Cr^{51} release was calculated as described in Chapter 2. Spontaneous release of Cr^{51} for DA for PVG target cells was 27% and 15% of maximum release respectively.

3.6.7 MRC $OX8^+$ WF responder cells as effector cells

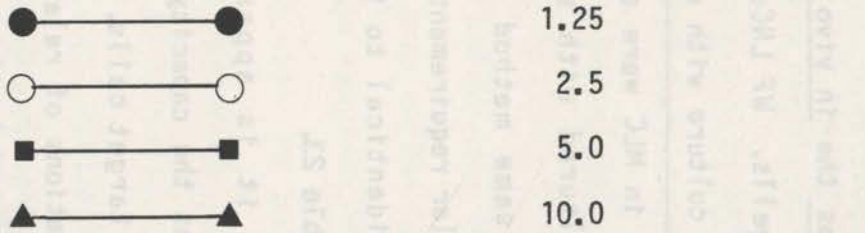
From the previous experiment, the method used for both the induction of effector cells in MLC and the assay for effector cells in CML appeared to be reproducible when WF lymphocytes were employed as responder/effector cells, therefore a method was established that could be used to test the responses of various lymphocyte subpopulations.

Of obvious interest was the *in vivo* and *in vitro* reactivity of the MRC $OX8^+$ subset of T cells. WF LNCs were depleted of Ig^+ and $W3/25^+$ cells and set up in culture with either PVG or DA stimulator cells. The responder cells in MLC were again titrated to a variety of concentrations and cultured with several concentrations of stimulator cells by the same method as described in 3.6.5 to determine whether the cellular requirements for the activation of MRC $OX8^+$ effector cells were identical to those of whole LNCs. The results are presented in Table 23.

From these results it is apparent that the MRC $OX8^+$ subpopulation of WF LNCs has the capacity, after allo-sensitization *in vitro*, to lyse specific target cells. The *in vitro* requirements for the activation of populations of relatively pure MRC $OX8^+$ cells appear to be somewhat different to those of whole LNC populations. In this case the culture of the higher concentrations of responder cells ($5x10^5$ and $10x10^5$ cells/well) with the lower concentrations of stimulator cells ($1.25x10^5$ and $2.5x10^5$ cells/well) appears to be optimal for the induction of Tc. Figure 9 (A and B) graphs the proliferative response of parallel MLC cultures of WF MRC $OX8^+$ cells that were harvested on days 3 to 5 after overnight exposure to H^3TdR. Those cultures which contained cells which maximally lysed specific target cells showed an early peak proliferative response at day 3,

FIGURE 9A

RESPONSES OF MRC OX8$^+$ WF LNC TO ALLOGENEIC STIMULATION IN MLC



MRC OX8$^+$ WF LNC at 2.5, 5 or 10x10^6/ml were cultured with various concentrations of PVG stimulator cells by MLC Method 2 in microtitre trays. Cultures were harvested after 4hr exposure to H^3TdR. Any population of cells which caused more than 20% specific target cell lysis showed an early peak proliferation on day 3. This usually occurred in cultures containing 5 or 10x10^6 WF cells/ml and where the responder cell to stimulator cell ratio was greater than 1:1. Control cultures of responder or stimulator cells cultured alone showed very low levels of incorporation of H^3TdR (less than 500 CPM and 1000 CPM respectively). They are not graphed for clarity. Each point represents the mean triplicate wells $\pm$ 1 SD.

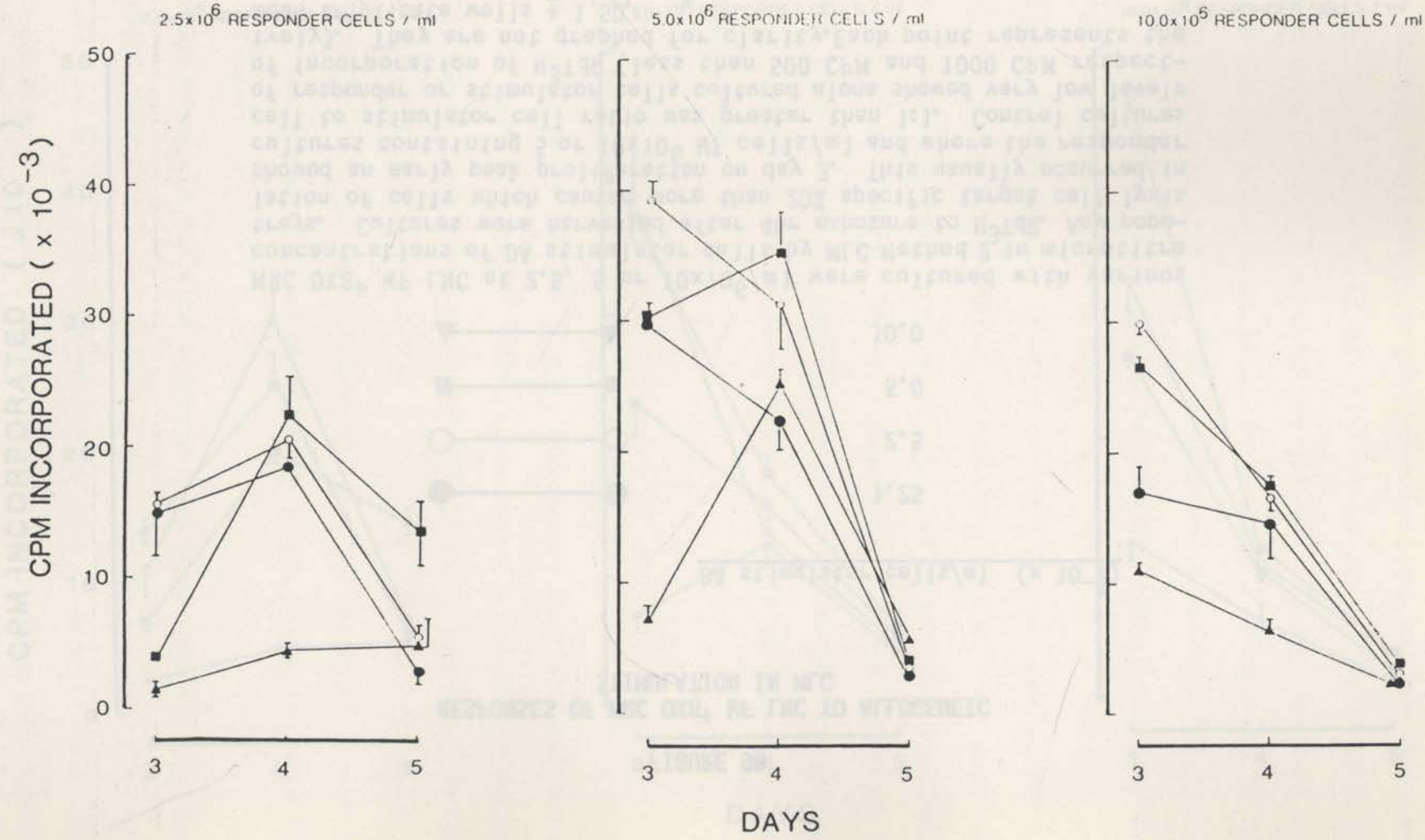
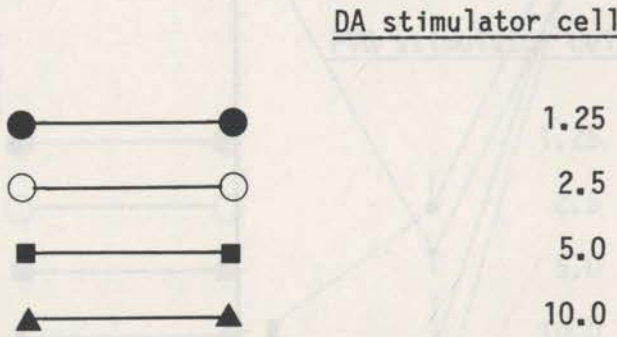
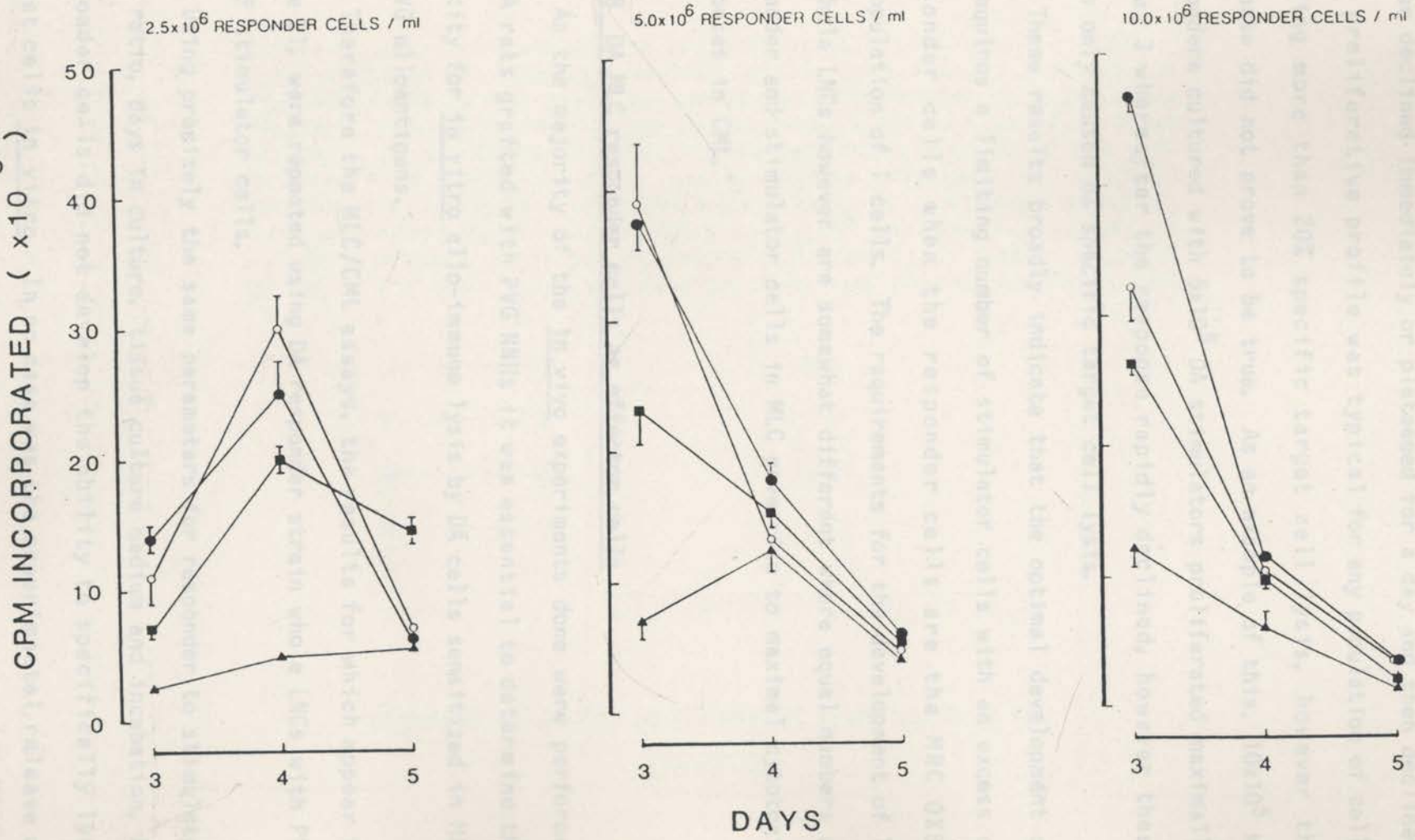

2.5x10^6 RESPONDER CELLS / ml
5.0x10^6 RESPONDER CELLS / ml
10.0x10^5 RESPONDER CELLS / ml
CPM INCORPORATED (x 10^-3)
50
40
30
20
10
0
3
4
5
DAYS

FIGURE 9B

RESPONSES OF MRC OX8$^+$ WF LNC TO ALLOGENEIC STIMULATION IN MLC



MRC OX8$^+$ WF LNC at 2.5, 5 or 10x10^6/ml were cultured with various concentrations of DA stimulator cells by MLC Method 2 in microtitre trays. Cultures were harvested after 4hr exposure to H^3TdR. Any population of cells which caused more than 20% specific target cell lysis showed an early peak proliferation on day 3. This usually occurred in cultures containing 5 or 10x10^6 WF cells/ml and where the responder cell to stimulator cell ratio was greater than 1:1. Control cultures of responder or stimulator cells cultured alone showed very low levels of incorporation of H^3TdR (less than 500 CPM and 1000 CPM respectively). They are not graphed for clarity. Each point represents the mean triplicate wells $\pm$ 1 SD.


2.5x10^6 RESPONDER CELLS / ml
5.0x10^6 RESPONDER CELLS / ml
10.0x10^6 RESPONDER CELLS / ml
CPM INCORPORATED (x10^-3)
50
40
30
20
10
0
3
4
5
DAYS

one day before CML assay. Thereafter the proliferative response either declined immediately or plateaued for a day and then declined. This proliferative profile was typical for any population of cells showing more than 20% specific target cell lysis, however the converse did not prove to be true. As an example of this, $10x10^5$ WF responders cultured with $5x10^5$ DA stimulators proliferated maximally on day 3 whereafter the response rapidly declined, however these cells only caused 8% specific target cell lysis.

These results broadly indicate that the optimal development of Tc requires a limiting number of stimulator cells with an excess of responder cells when the responder cells are the MRC $OX8^+$ subpopulation of T cells. The requirements for the development of Tc in whole LNCs however are somewhat different where equal numbers of responder and stimulator cells in MLC gave rise to maximal cytotoxic responses in CML.

3.6.8 DA MLC responder cells as effector cells

As the majority of the *in vivo* experiments done were performed in DA rats grafted with PVG NNHs it was essential to determine the capacity for *in vitro* allo-immune lysis by DA cells sensitized in MLC to PVG alloantigens.

Therefore the MLC/CML assays, the results for which appear in Table 21, were repeated using DA responder strain whole LNCs with PVG or WF stimulator cells.

Using precisely the same parameters for responder to stimulator cell ratio, days in culture, tissue culture medium and incubation, DA responder cells did not develop the ability to specifically lyse target cells *in vitro*. In no case was the experimental release of Cr^{51} from target cells incubated with sensitized cells greater than

FIGURE 10A

RESPONSES OF DA LNC TO ALLOGENEIC STIMULATION IN MLC

DA LNC at 2.5, 5 or $10x10^6$/ml were cultured with various concentrations of PVG stimulator cells by MLC Method 2 in microtitre trays. Cultures were harvested after 4hr exposure to H^3TdR. Maximum proliferative responses at day 4 were obtained with $2.5x10^6$ DA responder cells/ml cultured with $2.5x10^6$ or $5x10^6$ PVG stimulator cells/ml. All cultures of $10x10^6$ DA responder cells/ml showed peak proliferation at day 3 or earlier. Control cultures of DA LNC cultured alone or stimulator cells cultured alone showed very low levels of incorporation of H^3TdR (less than 2000 CPM and 1000 CPM respectively). They are not graphed for clarity. Each point represents the mean of triplicate wells $\pm$ 1 SD.

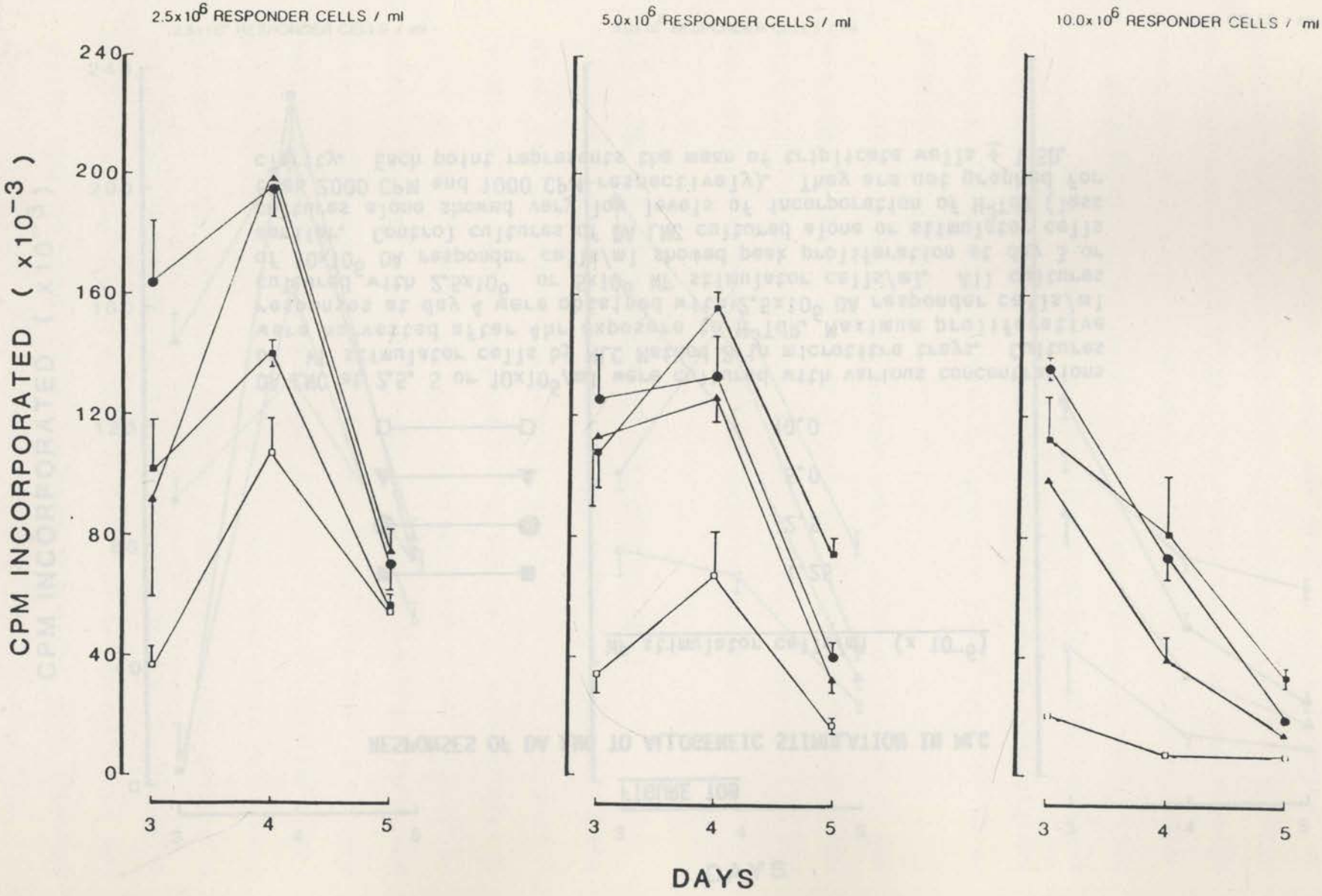
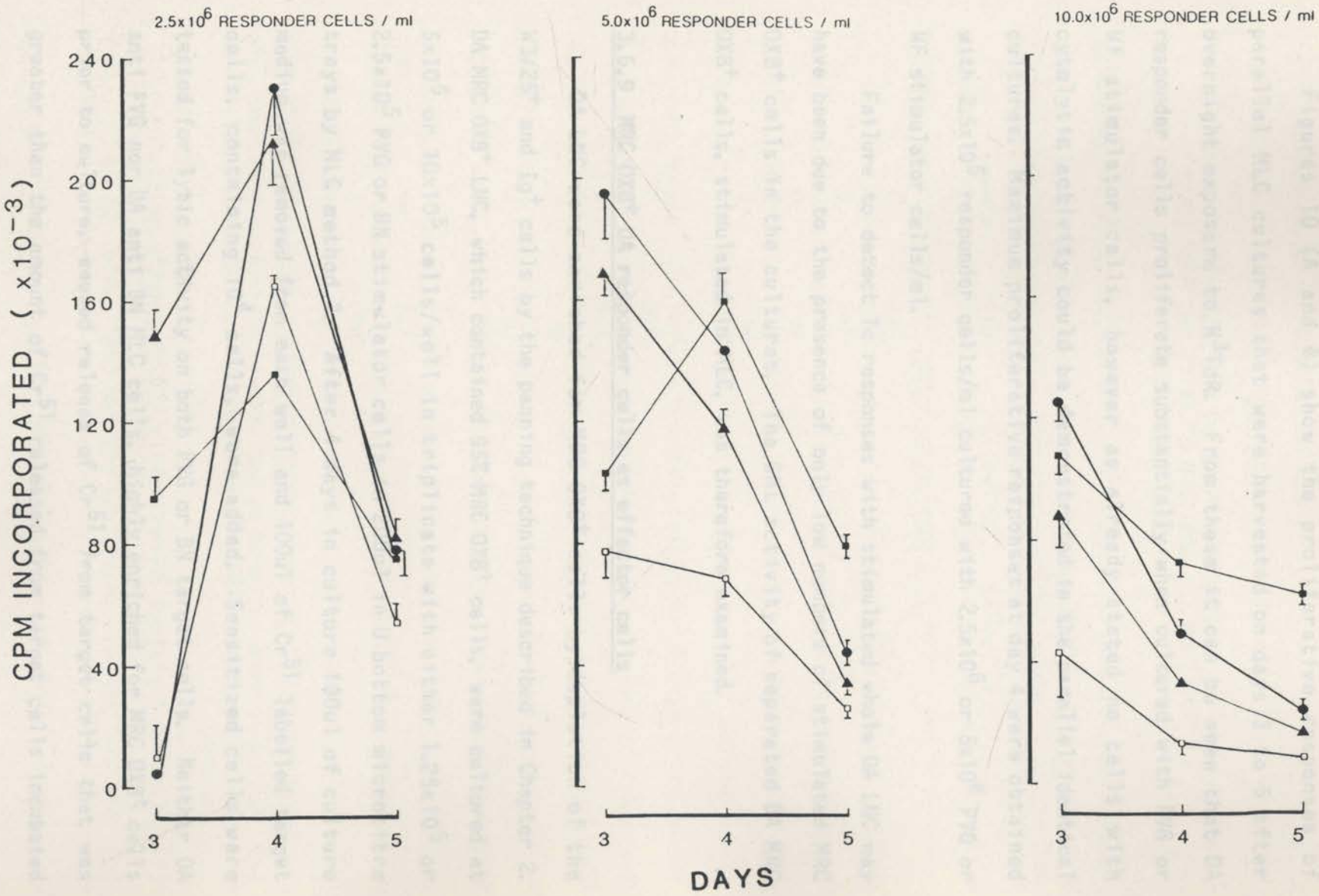

2.5x10^6 RESPONDER CELLS / ml
5.0x10^6 RESPONDER CELLS / ml
10.0x10^6 RESPONDER CELLS / ml
CPM INCORPORATED (x10^-3)
240
200
160
120
80
40
0
3
4
5
DAYS

FIGURE 10B

RESPONSES OF DA LNC TO ALLOGENEIC STIMULATION IN MLC

DA LNC at 2.5, 5 or $10x10^6$/ml were cultured with various concentrations of WF stimulator cells by MLC Method 2 in microtitre trays. Cultures were harvested after 4hr exposure to H^3TdR. Maximum proliferative responses at day 4 were obtained with $2.5x10^6$ DA responder cells/ml cultured with $2.5x10^6$ or $5x10^6$ WF stimulator cells/ml. All cultures of $10x10^6$ DA responder cells/ml showed peak proliferation at day 3 or earlier. Control cultures of DA LNC cultured alone or stimulator cells cultures alone showed very low levels of incorporation of H^3TdR (less than 2000 CPM and 1000 CPM respectively). They are not graphed for clarity. Each point represents the mean of triplicate wells $\pm$ 1 SD.


2.5x10^6 RESPONDER CELLS / ml
5.0x10^6 RESPONDER CELLS / ml
10.0x10^6 RESPONDER CELLS / ml
CPM INCORPORATED (x10^-3)
240
200
160
120
80
40
0
3
4
5
DAYS

the SR of Cr^{51} from target cells cultured alone.

Figures 10 (A and B) show the proliferative responses of parallel MLC cultures that were harvested on days 3 to 5 after overnight exposure to H^3TdR. From these it can be seen that DA responder cells proliferate substantially when cultured with PVG or WF stimulator cells, however as already stated no cells with cytolytic activity could be demonstrated in the parallel identical cultures. Maximum proliferative responses at day 4 were obtained with $2.5x10^6$ responder cells/ml cultured with $2.5x10^6$ or $5x10^6$ PVG or WF stimulator cells/ml.

Failure to detect Tc responses with stimulated whole DA LNC may have been due to the presence of only low numbers of stimulated MRC $OX8^+$ cells in the cultures. The CML activity of separated DA MRC $OX8^+$ cells, stimulated in MLC, was therefore examined.

3.6.9 MRC $OX8^+$ DA responder cells as effector cells

DA LNC were enriched for MRC $OX8^+$ cells by depletion of the $W3/25^+$ and Ig^+ cells by the panning technique described in Chapter 2. DA MRC $OX8^+$ LNC, which contained 95% MRC $OX8^+$ cells, were cultured at $5x10^5$ or $10x10^5$ cells/well in triplicate with either $1.25x10^5$ or $2.5x10^5$ PVG or BN stimulator cells in 200ul in U bottom microtitre trays by MLC method 2. After 4 days in culture 100ul of culture medium was removed from each well and 100ul of Cr^{51} labelled target cells, containing 10^4 cells, were added. Sensitized cells were tested for lytic activity on both PVG or BN target cells. Neither DA anti PVG nor DA anti BN MLC cells, highly enriched for MRC $OX8^+$ cells prior to culture, caused release of Cr^{51} from target cells that was greater than the amount of Cr^{51} released from target cells incubated alone.

TABLE 24

CML ASSAY WITH DA MLC CELLS;
EFFECT OF THE ADDITION OF NAIVE MRC OX8$^+$ CELLS
AND/OR PE CELLS

MLC cells[a]	Added DA cells		CML assay		
	PE[b] (10^5/well)	MRC OX8$^+$[c] (10^5/well)	Target cells (10^4/well)	% Cr^{51} release	Specific Cr^{51} release
DA anti PVG	–	–	PVG	0	
	+	–	PVG	0	
	–	+	PVG	0	
	+	+	PVG	0	
	–	–	WF	0	
	+	–	WF	0	
	–	+	WF	0	
	+	+	WF	0	
DA anti WF	–	–	PVG	0	
	+	–	PVG	0	
	–	+	PVG	0	
	+	+	PVG	0	
	–	–	WF	0	
	+	–	WF	0	
	–	+	WF	0	
	+	+	WF	0	
WF anti PVG	–	–	PVG	50	48
	–	–	DA	2	

[a] MLC cells were cultured as per text in microtitre trays and were not harvested. Therefore effector cell concentration was unknown.

[b] PE cells contained approximately 75% phagocytic cells.

[c] MRC OX8$^+$ LN cells contained 96% MRC OX8$^+$ cells, 3% W3/25$^+$ cells and 1.5% Ig$^+$ cells.

3.6.10 Effect on CML of adding naive DA MRC OX8$^+$ cells and PE cells to DA MLC effector cells

The inability of maximally stimulated whole DA lymphocytes or MLC activated DA MRC OX8$^+$ LNC to exhibit cytolytic activity prompted further investigation of the *in vitro* cellular requirements for target cell lysis. *In vivo* studies had shown that rapid graft rejection in irradiated hosts was procured by DA MLC cells mixed with DA PE cells (rich in macrophages) and naive DA MRC OX8$^+$ LNCs but not by MLC cells alone. Therefore these same cell populations were added to MLC cells immediately prior to the CML assay.

DA LN responder cells were cultured at $5x10^5$ cells/well with PVG or WF stimulator cells at $2.5x10^5$ cells/well in a total volume of 200ul. MLC method 2 was followed. At the same time WF responder LNCs were cultured with DA or PVG stimulator cells at the above concentrations. Cultures were maintained for 4 days prior to CML assay. On the day of assay 150ul of medium was removed from each well. To each well was then added an aliquot containing 10^4 Cr^{51} labelled target cells. In addition some wells received an aliquot of 10^5 DA PE cells, some 10^5 DA MRC OX8$^+$ LNCs and some received cells of both types. The volume in each well was made to 200ul with TCM-2. Spontaneous release control wells contained medium alone or the same cell mixtures as experimental wells, without the MLC cells. All effector cells were tested against target cells of both the sensitizing and third party strain. SR values were never more than 15% of maximum releasable Cr^{51}.

The results (Table 24) showed that DA MLC cells were again ineffective in bringing about specific target cell lysis either alone or mixed with PE cells and/or MRC OX8$^+$ cells. WF cells sensitized to PVG in MLC however effected 48% specific Cr^{51} release from target

cells. This result indicated that the target cells used for this particular assay were prone to lysis and that under conditions where WF responder cells generate CML effector cells in vitro, DA responder cells do not. The results further indicate that under conditions where optimal in vivo cytotoxicity occurs using DA MLC cells, no in vitro cytotoxicity is demonstrable.

3.7 EFFECT OF ALTERATION OF MLC CULTURE METHOD ON THE IN VIVO REACTIVITY OF MLC CELLS

Subsequent to the alteration in the method of MLC which led to the development of effector cells with the ability to lyse target cells in vitro, it was of interest to determine if MLC effector cells generated under the new conditions of culture were as effective in mediating in vivo NNH graft rejection as MLC cells derived from cultures performed by the earlier method.

DA LNC were sensitized to PVG alloantigens in parallel cultures performed according to MLC methods 1 or 2 (Chapter 2). In both cultures DA responder cells and PVG stimulator cells were each cultured at 2.5×10^6 cells/ml. Cells were harvested after 4 days. Groups of DA rats were grafted with PVG NNHs and injected locally with 5×10^7 DA anti PVG cells generated by Method 1 or Method 2 respectively. A further 2 groups of DA recipients of PVG NNH grafts were injected IV with 5×10^7 DA anti PVG cells cultured by Method 1 or Method 2 respectively. Aliquots of cells were retained for testing in CML on PVG and BN Cr^{51} labelled target cells at E:T ratios of 100/1, 50/1 and 25/1.

Animals locally injected with DA anti PVG MLC cells rejected their grafts very rapidly and with a comparable tempo ($0.2<p<0.4$)

TABLE 25

EFFECT ON GRAFT SURVIVAL OF PVG NNH GRAFTS IN DA RATS OF THE INJECTION OF DA ANTI PVG MLC CELLS CULTURED BY DIFFERENT METHODS

MLC cell inoculum[a]		Graft survival time		
Route	MLC method[b]	Day of rejection	MST	P
Local	1	7(2), 8(2)	7.5	NSD
Local	2	6, 7(2), 8	7	
IV	1	9, 10, 11(2)	10.5	NSD
IV	2	9(3), 10	9	

[a] Cells generated by both Method 1 and Method 2 were tested in CML and did not cause specific target cell lysis above SR levels.

[b] See Materials and Methods.

irrespective of the method of culture of the MLC cells (Table 25). The MST of grafts procured by MLC cells cultured according to Method 1 and Method 2 was 7.5d and 7d respectively.

Graft recipients injected IV with MLC cells also rejected their grafts with similar tempo ($0.1<p<0.2$) whether the MLC cells were cultured by Method 1 or Method 2 (MST 10.5 and 9d respectively) however at a slower tempo than DA recipients of PVG NNH grafts locally injected with DA anti PVG MLC cells ($p<0.05$). When these cells were tested on PVG or BN target cells however, neither cell population demonstrated the capacity to specifically lyse the relevant target cells and no Cr^{51} release above SR values was observed.

Subsequent to these experiments all MLC were done using Method 2.

3.8 IN VIVO REACTIVITY OF WF MLC CELLS

The strain combination in which the cellular requirements for graft rejection had been examined in normal and irradiated hosts was DA strain hosts with PVG NNH grafts. In this strain combination it had proved extremely difficult to raise conventional Tc with the capacity to lyse target cells *in vitro*. On the other hand WF strain LNC cultured in MLCs reproducibly gave rise to Tc with the capacity to kill specific target cells *in vitro*. It had also been demonstrated (Table 3) that WF lymphoid cells stimulated in MLC against PVG alloantigens specifically accelerated the rejection of PVG NNH grafts following their local injection into WF recipients. Experiments were done to determine if the cellular requirements for rejection in irradiated WF hosts of PVG NNH grafts were comparable to the requirements for rejection of PVG NNH grafts in irradiated DA

TABLE 26

EFFECT OF LOCALLY INOCULATED MLC SENSITIZED CELLS
ON SURVIVAL OF PVG NNH GRAFTS IN IRRADIATED WF RECIPIENTS

Cellular inoculum		Graft survival time		
Source	No. ($\times 10^{-7}$)	Day of rejection	MST	P
WF anti PVG MLC	4.5	5(2), 6, 7	5.5	<.01
WF anti DA MLC	4.5	13(2), 14, 15	13.5	
WF anti PVG MLC	4.0	5(7), 6(5)	5	<.005
WF anti DA MLC	4.0	9, 11, 14, 15	12.5	

recipients.

WF recipients were irradiated with 950 rad WBI and grafted with PVG NNHs. Groups of 4 rats were then locally injected with either $4.5x10^7$ WF anti PVG MLC cells or $4.5x10^7$ WF anti DA MLC cells. Injection of cells sensitized to PVG alloantigens led to very rapid graft rejection (MST 5.5d; Table 26). Those animals injected with cells sensitized to DA alloantigens rejected their grafts more slowly ($p<0.01$) with a MST of 13.5d.

This result was unexpected when compared to the rejection time of PVG NNH grafts in irradiated DA recipients locally injected with DA anti PVG MLC cells (MST 11d; Table 14). The experiment was repeated on a further occasion by injecting $4x10^7$ WF anti PVG or $4x10^7$ WF anti DA MLC cells locally into groups of irradiated WF recipients of PVG NNHs. Graft rejection was again very rapid in the group receiving WF anti PVG cells (MST 5d; Table 26) and was significantly more rapid than graft rejection in hosts receiving WF anti DA MLC cells (MST 12.5d).

The rapidity of graft rejection in irradiated grafted WF hosts injected with MLC sensitized cells was surprising in the light of the finding that irradiated DA rats injected with MLC sensitized cells rejected their grafts with a tempo which was accelerated in comparison to uninjected controls but significantly slower than rejection in non-irradiated rats injected with similar cells.

Rejection time was directly compared in irradiated and non-irradiated WF hosts injected with aliquots of the same MLC sensitized cell population. $5x10^7$ WF anti PVG MLC cells were locally injected at the time of implantation of a PVG NNH into 4 normal WF and 4 irradiated WF hosts respectively. A control group of WF recipients of PVG grafts was left uninjected. A further 2 groups of irradiated

TABLE 27

EFFECT OF LOCALLY INOCULATED MLC SENSITIZED CELLS ON SURVIVAL OF PVG NNH GRAFTS IN WF RECIPIENTS

Host Irradiation	Cellular inoculum		Graft survival time		
	Source	No. ($\times 10^{-7}$)	Day of rejection	MST	P
-	-	-	13, 14, 15(2)	14.5	
-	WF anti PVG MLC	5.0	5(2), 6(2)	5.5	<.005
+	WF anti PVG MLC	5.0	5, 6(2), 7	6	NSD
+	WF LNC	5.0	22, 24, 25, 27, 2	25	<.005
+	-	-	42, 45, 49, 53	47	

WF were grafted with PVG NNHs. One group was injected with $5x10^7$ unstimulated WF LNC and the remaining control group was left uninjected. Graft survival times are presented in Table 27.

Irradiated animals given no cells showed very prolonged graft survival time (MST 47d). The injection of $5x10^7$ WF LNC led to more rapid graft rejection ($p<0.05$) but the tempo was much slower than that in normal control rats. The local injection of specifically sensitized WF anti PVG MLC cells into irradiated recipients led to very rapid graft rejection (MST 6d). This is comparable with the MST (5.5d) of grafts in unirradiated WF hosts with MLC cells sensitized against graft alloantigen (Table 27).

These results confirm the observation that MLC cells procure equally rapid graft rejection in irradiated and non irradiated WF hosts. This is in contrast to the situation in DA hosts in which rapid graft rejection in irradiated hosts was only observed in animals injected with naive MRC $OX8^+$ cells and PE cells in addition to specifically sensitized MLC cells.

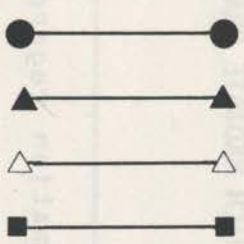
3.9 SUBPOPULATION OF MLC CELLS WHICH MEDIATE GRAFT REJECTION IN DA RATS

To obtain further information regarding the cellular requirements for rapid graft rejection in DA strain rats in vivo, experiments were done to determine which subpopulation of MLC sensitized cells collaborates with PE cells and naive MRC $OX8^+$ cells to cause rapid graft rejection in irradiated DA recipients.

T cell subpopulations were either separated from DA LNC and then set up in MLC with irradiated stimulator cells or LNC were depleted of Ig^+ cells and set up in culture and the subpopulations separated

FIGURE 11

THE RESPONSES OF DA LNCS TO ALLOGENEIC STIMULATION IN BULK MLC



DA LN responder cells

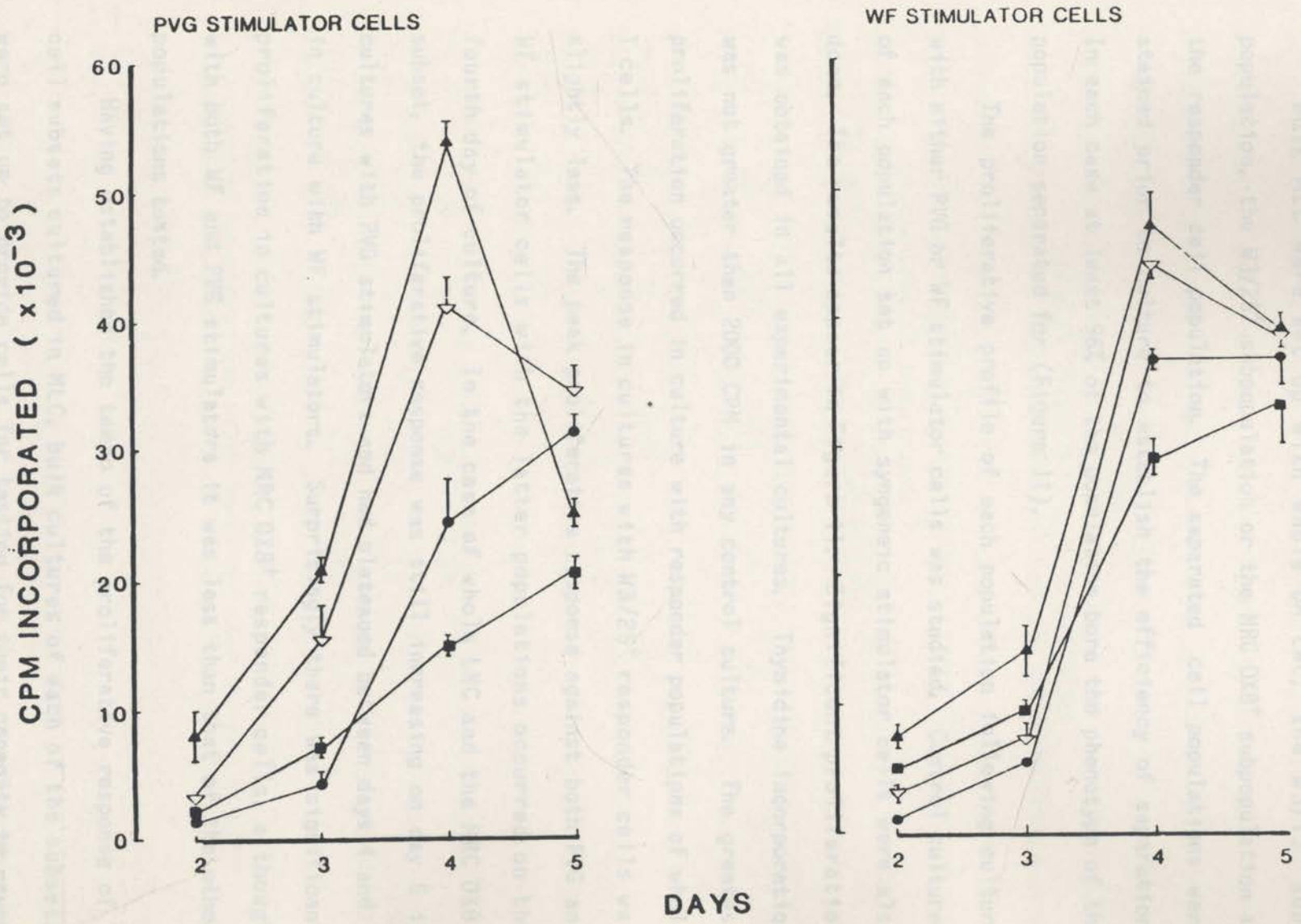
whole cells

W3/13$^+$ cells (97% W3/13$^+$)

W3/25$^+$ cells (98% W3/25$^+$)

MRC OX8$^+$ cells (96% MRC OX8$^+$)

DA responder and PVG or WF stimulator cells were cultured by MLC Method 2 at $5x10^6$/ml in TC flasks containing 15 ml. Cells were aliquoted from TC flasks to microtitre trays and pulsed for four hours with H^3TdR prior to harvest. The greatest proliferation occurred in cultures with responder populations of whole T cells. The peak proliferative response of T cells and W3/25$^+$ cells against both PVG and WF stimulator cells occurred on the fourth day of culture. The proliferative response of whole LNC and MRC OX8$^+$ cells was still increasing on day 5 in cultures with PVG stimulators and had plateaued between days 4 and 5 in cultures with WF stimulators. Control values of responders and stimulators cultured alone are not graphed and were never more than 2,000 CPM. Each point represents the mean of triplicate wells (derived from 1 TC flask) $\pm$ 1 SD.


PVG STIMULATOR CELLS
WF STIMULATOR CELLS
CPM INCORPORATED (x10⁻³)
60
50
40
30
20
10
0
2
3
4
5
DAYS

after 4 days in culture.

3.9.1 Separation of T cell subsets prior to MLC culture

Bulk MLC were set up with whole DA LNC, the W3/13$^+$ subpopulation, the W3/25$^+$ subpopulation or the MRC OX8$^+$ subpopulation as the responder cell population. The separated cell populations were stained prior to culture to establish the efficiency of separation. In each case at least 96% of the population bore the phenotype of the population separated for (Figure 11).

The proliferative profile of each population following culture with either PVG or WF stimulator cells was studied. Control cultures of each population set up with syngeneic stimulator cells were also done. The results appear in Figure 11. Significant proliferation was obtained in all experimental cultures. Thymidine incorporation was not greater than 2000 CPM in any control culture. The greatest proliferation occurred in culture with responder populations of whole T cells. The response in cultures with W3/25$^+$ responder cells was slightly less. The peak proliferative response against both PVG and WF stimulator cells with the latter populations occurred on the fourth day of culture. In the case of whole LNC and the MRC OX8$^+$ subset, the proliferative response was still increasing on day 5 in cultures with PVG stimulators and had plateaued between days 4 and 5 in culture with WF stimulators. Surprisingly there was significant proliferation in cultures with MRC OX8$^+$ responder cells, although with both WF and PVG stimulators it was less than that of the other populations tested.

Having established the tempo of the proliferative response of T cell subsets cultured in MLC, bulk cultures of each of the subsets were set up to provide cells for testing for their capacity to cause accelerated rejection of NNH grafts. Experiments were done

TABLE 28

PHENOTYPE OF CELLS HARVESTED FROM MLC USING WHOLE LNC OR T CELL SUBSETS AS RESPONDER CELLS

Responder cell population[b]	% Constituent Subpopulations					
	Before MLC			After MLC		
	Ig$^+$	MRC OX8$^+$	W3/25$^+$	Ig$^+$	MRC OX$^+$	W3/25$^+$
Whole LNC[a]	36±3	15±1	47±5	32±6	15±2	52±4
	1.0	0	98.5	0	0	99.0
W3/25$^+$ Subpopulation	2.0	0.5	97.0	0	1.0	98.0
	2.0	0	99.0	0	1.5	98.0
	1.5	94.5	4.0	0	98.0	2.0
MRC OX8$^+$ Subpopulation	1.0	97.0	3.0	0	98.0	3.0
	4.5	95.0	3.0	0	98.0	1.0

[a] Results are the mean and SD of seven separate experiments.

[b] In each case responder cells were bulk cultured in MLC at $5X10^6$ cells/ml by MLC method 2 (Chpt 2). 4 days later the total cell yield, as a percentage of responder cells put into culture, was 10-15% for the MRC OX8$^+$ subset, 40-45% for the W3/25$^+$ subset and 60-70% for whole LNCs.

concurrently to examine the changes in the proportions of various lymphocyte subsets which might occur during MLC. Aliquots of cells taken from whole DA responder cell populations or separated subpopulations were stained with anti Ig and the mouse monoclonal antibodies W3/25 and MRC OX8 prior to being put in culture to establish the purity of the populations. The cells were then cultured with irradiated PVG stimulator cells. The cultures were terminated on the 4th day and aliquots from each set of cultures were stained to identify the proportions of each subset present following stimulation in MLC (Table 28). The cells were then tested for their ability to cause rejection of PVG NNH grafts in DA recipients (Table 29).

The response of lymphocytes within a one way MLC is regarded as being primarily a T cell response involving mainly $W3/25^+$ cells but also MRC $OX8^+$ cells (Mason et al, 1981). The cell populations in 4d MLCs of whole DA LNC showed a surprisingly high proportion of B cells ($32\pm6\%$). Other workers have also reported this and suggested that the survival of B cells in MLC is due to the high concentration of growth factors resulting from the interaction between responder and stimulator cells (Garovoy et al, 1979; Hayry et al, 1984). The proportion of $W3/25^+$ to MRC $OX8^+$ cells present after 4d of MLC was similar to that of the starting population (Table 28). Examination of the phenotype of cells harvested from 4 day cultures of DA lymphocyte subpopulations with irradiated PVG stimulator cells confirmed that the majority of cells bore the phenotype of the subpopulation initially put up in culture. In no case had the small proportion of contaminating cells in the starting population overgrown the selected subpopulation during MLC (Table 28).

The capacity of the cells from the cultures to accelerate the

TABLE 29

EFFECT ON SURVIVAL OF PVG NNH GRAFTS IN DA RATS OF LOCAL INOCULATION OF T CELL SUBSETS SENSITIZED TO PVG ALLOANTIGEN IN MLC

DA anti PVG MLC cell inoculum			Graft survival time		
Responder cells	% Purity after MLC	No. injected ($\times 10^{-7}$)	Day of rejection	MST	P [a]
Whole LNC	–	5	6(3), 7(4), 8	7	
W3/25$^+$	99	3.2	7, 8(7)	8	<.05
MRC OX8$^+$	98	0.4	5, 6	5.5	NSD
W3/25$^+$	98	2	7(4)	7	NSD
MRC OX8$^+$	98	2	6(4)	6	NSD

[a] Compared with rejection time procured by whole LNC stimulated in MLC.

rejection of NNH grafts bearing the sensitizing alloantigen was examined. W3/25$^+$ and MRC OX8$^+$ subpopulations were injected in approximately the numbers equivalent to the numbers present in $5x10^7$ whole LNC populations. The effect of MRC OX8$^+$ and W3/25$^+$ populations given at a dose of $2x10^7$ was also compared.

The results appear in Table 29 and are somewhat difficult to interpret. Each subpopulation accelerated rejection to an extent similar to that resulting from the injection of cells from MLC of whole LNC cells containing the equivalent number of that subpopulation. Rejection was not further accelerated when the number of MRC OX8$^+$ cells was increased from $0.4x10^7$ to $2x10^7$. There was a slight but significant ($p<0.05$) difference between the effect of whole LNC (MST 7d) and the equivalent number of W3/25$^+$ cells (MST 8d).

The effect of MLC sensitized T cell subsets on graft rejection in irradiated recipients was next examined. W3/25$^+$ or MRC OX8$^+$ DA LNC were cultured for 4d with PVG stimulator cells and then tested, either on their own or with the addition of DA strain naive MRC OX8$^+$ cells and PE cells, for their capacity to cause rejection of PVG NNH grafts in irradiated DA recipients. Naive MRC OX8$^+$ cells and PE cells had previously been shown to be essential for rapid graft rejection in irradiated hosts injected with cells from MLC of whole cell populations (Table 18). Naive MRC OX8$^+$ cells and PE cells were added to locally injected T cell subset inocula in numbers of $2x10^7$ and $4x10^7$ respectively as these were the cell numbers shown to be effective in collaborating with the whole cell populations sensitized in MLC in causing rapid graft rejection in irradiated hosts. The results appear in Table 30.

MRC OX8$^+$ LNCs stimulated against PVG alloantigens in MLC and

TABLE 30

EFFECT ON SURVIVAL OF PVG NNH GRAFTS IN IRRADIATED DA HOSTS OF LOCAL INOCULATION OF T CELL SUBSETS SENSITIZED TO PVG IN MLC

Cellular Inoculum				Graft survival time		
DA anti PVG MLC[a]			Added DA cells	Day of rejection	MST	P
Phenotype of responder cells	Purity after MLC %	No. injected ($x10^{-7}$)	Naive[b] MRC $OX8^+$ ($2x10^7$) PE ($4x10^7$)[c]			
MRC $OX8^+$	98.0	1.8	-	22, 23, 25(2)	24	NSD
	98.0	1.8	+	15, 23(2), 25	23	
$W3/25^+$	98.0	4.5	-	10, 12, 13, 15	12.5	<.05
	98.0	4.5	+	8, 9, 10(2)	9.5	

[a] DA LNC were separated into T cell subsets prior to culture for four days in bulk MLC.

[b] Contamination - 1% Ig^+ cells and 0-2% $W3/25^+$ cells.

[c] 80-90% phagocytic cells.

injected locally caused rejection of PVG hearts in irradiated DA recipients in 22-25d (MST 24d). Although the number of MRC $OX8^+$ MLC stimulated cells exceeded the number of MRC $OX8^+$ cells present in populations of whole LNC stimulated in MLC and tested in doses of $5x10^7$ in irradiated grafted rats, the cells caused rejection with a tempo that was considerably slower ($p<0.05$) than rejection in irradiated hosts injected with whole LNC stimulated in MLC (Table 14). The addition of naive MRC $OX8^+$ cells and PE cells to MRC $OX8^+$ DA anti PVG MLC cells did not significantly alter their effect. Irradiated hosts injected with these cell populations rejected their grafts with an MST of 23d. MLC stimulated $W3/25^+$ LNC caused rejection with a more rapid tempo (10-15d) which was not significantly different from that seen in irradiated hosts restored with whole MLC populations (10-12d; Table 14). The addition of naive MRC $OX8^+$ cells and PE cells to inocula of $W3/25^+$ MLC stimulated cells resulted in slight further acceleration of rejection (8-10d, $p<0.05$). The acceleration was not as great as that observed when naive MRC $OX8^+$ cells and PE cells were added to populations of whole LNC stimulated in MLC (MST 5-6d; Table 18) and injected locally into grafted irradiated rats. It should be noted that $W3/25^+$ cells stimulated in MLC led to graft rejection with a more rapid tempo than MRC $OX8^+$ cells similarly stimulated irrespective of whether the MLC populations were given alone or with naive MRC $OX8^+$ populations.

The finding that neither MRC $OX8^+$ or $W3/25^+$ cells stimulated in MLC could substitute for stimulated whole LNC in causing rapid graft rejection in irradiated recipients suggested that rapid graft rejection required both stimulated populations. However this could equally well reflect the necessity for both T cell subsets to be present during MLC for the normal induction of either subpopulation

TABLE 31

PHENOTYPE OF CELLS HARVESTED FROM MLC PERFORMED WITH IG^+ DEPLETED DA LN RESPONDER CELLS AND THEIR PURITY FOLLOWING THE DEPLETION OF EITHER THE $W3/25^+$ OR MRC $OX8^+$ SUBSETS

% Population before separation			% Population after separation		
MRC $OX8^+$	$W3/25^+$	$1g^+$	MRC $OX8^+$	$W3/25^+$	$1g^+$
28	72	2	91	6	0
24	77	0	98	1	0
27	74	1	4	97	0
27	75	0	1	99	0

TABLE 32

THE EFFECT ON SURVIVAL OF PVG NNH GRAFTS IN IRRADIATED DA RATS OF LOCAL INOCULATION OF T CELL SUBSETS SEPARATED FROM DA ANTI PVG MLC CELLS

MLC cellular inoculum			Graft survival time		
Phenotype	Purity %	No. injected ($x10^{-7}$)	Day of Rejection	MST	P
MRC $OX8^+$	91	0.7	11, 12, 13(2)	12.5	<.025
$W3/25^+$	99	2.2	8, 10(3)	10	

to full effector function to occur. In view of this the *in vivo* action of the two subpopulations separated from MLC containing both subsets was examined.

3.9.2 Separation of T cell subsets subsequent to MLC

To reduce the number of cell separation steps required after MLC, Ig^+ cells were removed from populations of DA LNC prior to culture with irradiated PVG stimulator cells. The cells were harvested on the fourth day of culture and depleted for either the MRC $OX8^+$ T cell subset or the $W3/25^+$ T cell subset.

The composition of MLC cells prior to and subsequent to T cell subset depletion is shown on Table 31. Enriched populations of MRC $OX8^+$ cells or $W3/25^+$ cells separated from 4d MLCs were tested for their ability to cause rapid rejection when injected locally into NNH grafts in irradiated recipients (Table 32).

The effect of 0.7×10^7 MRC $OX8^+$ cells was compared with that of 2.2×10^7 $W3/25^+$ cells as this approximately represents the number of each present in 5×10^7 cells from 4d MLCs of whole DA LNCs sensitized to PVG alloantigens (see Table 27).

Rejection occurred with a slightly faster tempo in animals injected with $W3/25^+$ cells than those injected with MRC $OX8^+$ cells ($p<0.025$). In neither case was graft rejection very rapid.

The effect of adding naive MRC $OX8^+$ and PE cells to inocula of $W3/25^+$ and MRC $OX8^+$ cells separated from 4d MLC was next examined. The naive MRC $OX8^+$ and PE cells were added in the numbers which had been shown to accelerate rejection when given with whole MLC cell populations, *viz* 2×10^7 and 4×10^7 respectively. Separated T cells were given in numbers equivalent to those present in 5×10^7 DA anti PVG MLC cells. In the first experiment (Table 33A) subsets were

TABLE 33

EFFECT ON SURVIVAL OF PVG NNH GRAFTS IN IRRADIATED DA RATS OF THE ADDITION OF PE CELLS AND NAIVE MRC OX8$^+$ CELLS TO LOCALLY INOCULATED T CELL SUBSETS SEPARATED FROM DA ANTI PVG MLC CELLS

Cellular Inoculum					Graft survival time		
Separated DA anti PVG MLC cells			Added DA cells		Day of rejection	MST	P [c]
Phenotype	Purity %	No. ($\times10^{-7}$)	PE [a] ($\times10^{-7}$)	Naive [b] MRC OX8$^+$ ($\times10^{-7}$)			
A							
MRC OX8$^+$	95	0.75	4	2	5, 7(2), 8	7	NSD
W3/25$^+$	99	2.6	4	2	5, 6, 7(2)	6.5	
B							
MRC OX8$^+$	98	1.2	4	2	4, 5, 7, 8	6	NSD
W3/25$^+$	97	3.8	4	2	4, 6, 7, 8	6.5	

[a] 70–80% phagocytic cells.

[b] Contamination - 1.5% Ig$^+$ cells and $\leq$1% W3/25$^+$ cells.

[c] Comparison of the results from all 4 groups by the Kruscall Wallace nonparametric test shows there is no significant difference in graft survival time between them ($0.9<P<0.95$).

given in numbers equivalent to those present in MLC sensitized whole DA LNC (see Table 28) whereas in the second experiment (Table 33B) subsets were given in numbers equivalent to those present in MLC sensitized DA LNC depleted of Ig^+ cells (see Table 31).

MRC $OX8^+$ and $W3/25^+$ cells separated from 4d MLC both caused rapid rejection of NNH grafts in irradiated recipients when inoculated with host strain PE cells and naive MRC $OX8^+$ cells and both cell types procured graft rejection with a comparable tempo. The tempo of rejection in all 4 groups was similar irrespective of MLC cell type or dose ($0.9<p<0.95$).

An aliquot of MRC $OX8^+$ cells separated from 4 day DA anti PVG MLC cells set up with DA T cells (depleted of Ig^+ cells) was retained for testing in a CML assay. Effector cells at $20x10^6$, $10x10^6$ and $5x10^6$/ml were cocultured with PVG and WF target cells at $10x10^4$/ml, to give E/T ratios of 200/1, 100/1 and 50/1, for 6 hrs as described in Chapter 2. No specific target cell lysis was observed.

These experiments further demonstrate the stringent cellular requirements for graft rejection in irradiated DA recipients which, unlike irradiated WF recipients, require the presence of naive MRC $OX8^+$ and PE cells to bring about graft rejection at a tempo equivalent to that seen in normal DA graft recipients inoculated with specific MLC cells.

3.10 STRAIN DIFFERENCES IN CONSTITUENT T CELL SUBSETS

One obvious difference between the DA and WF strains is a constitutive difference in the numbers of MRC $OX8^+$ cells present in LNC populations. The WF strain LNC population contains $23\pm3\%$ MRC $OX8^+$ (see Table 34) cells while the DA strain LNCs contain only $15\pm1\%$

TABLE 34

PERCENTAGE OF VARIOUS SUBSETS OF CELLS IN NORMAL AND MLC SENSITIZED LNC POPULATIONS FROM WF AND DA RATS[a]

Subset	WF		DA	
	Normal LNC	anti PVG MLC cells	Normal LNC	anti PVG MLC cells
MRC OX8$^+$	23±3	29±3	15±1	25±2
W3/25$^+$	41±5	39±7	47±5	52±4
1g$^+$	34±3	31±4	36±3	32±6

[a] Represents the mean ± 1 SD of at least 7 experiments.

TABLE 35

EFFECT ON THE SURVIVAL OF PVG NNHS IN IRRADIATED DA RECIPIENTS OF SUPPLEMENTING MLC SENSITIZED CELLS WITH ADDITIONAL SPECIFICALLY SENSITIZED MRC OX8$^+$ CELLS

DA anti PVG MLC cell inoculum		Graft survival time		
Whole[a] ($\times 10^{-7}$)	MRC OX8$^+$[b] ($\times 10^{-7}$)	Day of rejection	MST	P
5	-	10(2), 12, 14	11	NSD
5	1.65	10, 12, 14(2)	13	

[a] 33.2% MRC OX8$^+$, 66.8% W3/25$^+$ and no detectable 1g$^+$ cells.

[b] 99% MRC OX8$^+$.

MRC $OX8^+$ cells (see Table 34). This difference is also reflected in the MLC subpopulations of either strain. Stimulation of whole LNCs in MLC against PVG alloantigens for 4 days results in cell populations containing 29±3% and 15±1% MRC $OX8^+$ cells for the WF and DA responder strains respectively (Table 34).

The inability of DA anti PVG MLC cells to procure rapid graft rejection *in vivo* in irradiated hosts and their failure to cause *in vitro* target cell lysis may be a reflection of the paucity of MRC $OX8^+$ cells in the MLC cell inoculum.

3.10.1 Effect on graft survival in DA rats of supplementing MLC cells with stimulated MRC $OX8^+$ cells

To test this hypothesis irradiated DA rats were grafted with PVG NNHs and locally injected with whole MLC cells and extra MRC $OX8^+$ cells separated from whole MLC cells. MLC cultures were derived from DA LNC depleted of Ig^+ cells prior to culture for 4 days with PVG stimulator cells.

8 irradiated DA rats were grafted with PVG NNHs. A group of 4 rats was injected in the grafted footpad with $5x10^7$ DA anti PVG MLC cells. The remaining 4 rats were similarly injected with $5x10^7$ DA anti PVG MLC cells mixed with $1.65x10^7$ MRC $OX8^+$ cells separated from an aliquot of the DA anti PVG MLC cells. This effectively more than doubled the number of MRC $OX8^+$ cells present in a normal inoculum of MLC cells. Graft rejection times appear in Table 35.

The addition of extra MRC $OX8^+$ cells separated from MLC cells to MLC inocula did not increase the capacity of the latter to accelerate the rejection of grafts in irradiated DA hosts. Both groups rejected their grafts with a similar tempo (MST 13d and 11d; Table 35).

TABLE 36

PHENOTYPE OF CELLS HARVESTED FROM MLC PERFORMED WITH IG$^+$ DEPLETED WF LN RESPONDER CELLS AND THEIR PURITY FOLLOWING DEPLETION OF EITHER THE W3/25$^+$ OR MRC OX8$^+$ SUBSETS

% Population before separation			% Population after separation		
MRC OX8$^+$	W3/25$^+$	1g$^+$	MRC OX8$^+$	W3/25$^+$	1g$^+$
44	59	0	0	99	0
42	60	2	98	1	1

TABLE 37

THE EFFECT ON SURVIVAL OF PVG NNH GRAFTS IN IRRADIATED WF RATS OF LOCAL INOCULATION OF T CELL SUBSETS SEPARATED FROM WF ANTI PVG MLC CELLS

MLC cellular inoculum			Graft survival time		
Phenotype	Purity %	No. injected ($\times 10^{-7}$)	Day of Rejection	MST	P
1g$^-$	>99	5	5(3), 7	5	
					NSD
W3/25$^+$	98	3	5(4)	5	
					<.005
MRC OX8$^+$	99	2	8(2), 9, 10	8.5	

3.11 SUBPOPULATION OF MLC CELLS WHICH MEDIATES GRAFT REJECTION IN IRRADIATED WF RATS

As large numbers of MRC OX8$^+$ cells separated from MLC did not accelerate graft rejection in irradiated DA rats it was of interest to determine which subpopulation of MLC cells was required to procure rapid graft rejection in irradiated WF recipients. The effect on graft rejection in irradiated WF hosts of the MRC OX8$^+$ and W3/25$^+$ T cell subsets separated from 4d MLC cells was examined. As with studies in DA hosts, MLCs were prepared from WF LNC depleted of Ig bearing cells prior to culture with PVG stimulator cells. The MLC cells were harvested on the fourth day of culture and depleted of either the MRC OX8$^+$ or W3/25$^+$ T cell subset.

The composition of the MLC cells prior to T cell subset depletion is shown in Table 36 which also shows the purity of the depleted populations. Enriched populations were tested for their ability to cause rapid graft rejection when injected at the site of implantation of a PVG NNH in irradiated WF recipients (Table 37).

The effect of $2x10^7$ MRC OX8$^+$ MLC cells and $3x10^7$ W3/25$^+$ MLC cells was compared both with each other and with the effect of $5x10^7$ whole MLC cells. MLC T cell subsets were given in approximately the numbers equivalent to those present in $5x10^7$ whole WF anti PVG MLC cells 4 days after the sensitization of Ig$^-$ WF LNC.

Rejection occurred with a faster tempo in animals injected with W3/25$^+$ WF anti PVG MLC cells than in those injected with MRC OX8$^+$ MLC cells. Animals injected with W3/25$^+$ cells rejected their grafts at the same tempo as animals receiving whole MLC cells (MST 5d and 5d respectively). Animals receiving MRC OX8$^+$ cells rejected their grafts at a significantly slower rate (MST 8.5; $p<0.005$).

CHAPTER 4

DISCUSSION

Fulmer et al (1963) first described the technique of neonatal heart transplantation. Since that time the grafting of free NNH tissue has been widely used as a model for the allograft response (Warren et al, 1973; Klein et al, 1976; Steinmuller and Lofgreen, 1977). The NNH graft model has advantages that graft models employing other secondary vascularized tissues do not; the main one being the precision with which the endpoint of survival may be determined. ECG activity may be monitored regularly and the cessation of the electrical activity of the graft defines the endpoint of survival. Skin grafts which are the traditional assay graft used in transplantation immunology are particularly difficult to assess for viability because of numerous objective and subjective interpretational errors. Dressings are needed to protect skin grafts from physical trauma. These may prejudice interpretation of results: if they are removed too early the graft may be damaged anyhow, if they are removed too late they may mask a rejected graft (Raju et al, 1974). In the rat the normal first set responses to MHC incompatible skin grafts may lead to graft rejection as early as 7 days. However unless protective dressings are left on for 7 days the skin grafts are difficult to read because the host animal scratches at the graft. For this reason accelerated rejection as a consequence of prior sensitization of the host, or the adoptive transfer of specifically allo-sensitized cells, is difficult to demonstrate using skin as an indicator graft. In addition, in many rat strain combinations of donor and host, sensitisation to MHC incompatibilities accelerates skin graft rejection by about 1 day only. For these reasons, in the

rat at least, assays which employ skin as an indicator graft lack sensitivity. With NNH grafts on the other hand grafts are implanted subcutaneously and do not require dressings, survival is monitored by ECG tracings which are more objective than the visual assessment usually used for skin grafts and there is a substantial difference between first and second set rejection times (Dorsch et al, 1983). Grafting NNH tissue subcutaneously to the hind footpad of adult recipients has the further advantage that the graft site has a well defined lymphatic drainage (Tilney, 1971) and the anatomical unit of graft site, draining lymphatic and popliteal LN lends itself to the study of local cellular function and mechanisms. The hind footpad of the rat drains by a single lymphatic to the popliteal LN, the efferent lymphatic draining to the iliac LN, thence to the renal LN and the thoracic duct (Tilney, 1971).

The parameters of the NNH graft model - the specificity, reliability and reproducibility of the graft system - have been thoroughly studied and the method is well established (Dorsch et al, 1983). All grafts implanted in the hind footpad of rats give positive ECG tracings within 2 days of grafting and syngeneic grafts continue to do so for the lifetime of the recipient (Dorsch et al, 1983). In the experiments reported in this thesis all the NNHs which were grafted healed in successfully. In all cases at least one positive ECG result was obtained prior to rejection. Thus it could be assumed with some degree of confidence that even when graft rejection was apparently very rapid it was a consequence of a host response and not of a technical failure.

In the rat the tempo of rejection of NNH grafts in naive recipients is slower than that of indirectly vascularised skin grafts (Dorsch and Roser, 1974a) or directly vascularised heart grafts (Hall

et al, 1978a) and the range of rejection times for individual animals in a given combination of graft donor and recipient is greater (Dorsch et al, 1983). The results of the experiments which were done establish the rejection times of NNH grafts in naive recipients in a variety of strain combinations of host and donor confirm the relatively slow tempo of rejection reported by others (Dorsch et al, 1983). This may reflect the sensitivity of the assay for the endpoint of survival: it is possible that only a few viable cardiac cells are required to give a positive ECG tracing. With skin grafts the nature of the graft tissue is such that once the corium of the graft breaks down the whole graft is doomed and small numbers of viable cells would obviously be difficult to detect. NNH graft tissue is exposed on all sides to the host vasculature and its size presumably allows for the diffusion of nutrients to groups of still viable cardiac cells the electrical activity of which would be detectable by ECG tracing until such time as total graft destruction occurs.

In addition to the foregoing reasons for apparent differences in survival times between skin grafts and NNH grafts, there may also be very real differences in survival which are a consequence of the relative lack of Class II antigen bearing cells in neonatal heart tissue. This possibility will be discussed later in the context of the immunological stimulus offered by NNH tissue to both host cells and adoptively transferred cells.

The aim of initial experiments was to demonstrate that putative effector cells raised in conventional MLCs had the capacity to cause graft rejection <u>in vivo</u>. The MLC has long been regarded as an <u>in vitro</u> correlate of the allograft reaction (Bach et al, 1969; Hayry and Defendi, 1970; Cerottini and Brunner, 1974) and more particularly

of the afferent or proliferative arm of the immune response to alloantigens (Howard and Wilson, 1974). Wilson (1967, 1974) recognized the difficulties experienced by many workers in the establishment of in vitro MLC assays using rat lymphoid cells. Prior to this time workers had not been able to routinely culture rat lymphocytes with any success. Wilson's exhaustive studies into the parameters of the rat MLC model established the guidelines for future workers (Wilson, 1967; Wilson and Nowell, 1971). Even so many later workers experienced difficulty in performing MLC with rat cells (Nemlander et al, 1979; Lindsay and Allardyce, 1979). Review of the extensive literature on MLC and CML in the rat makes it clear that for both these assays individual workers have had to establish methods and basic parameters which are apparently specific for the conditions which prevail within the investigating laboratory and apply to the sublines of rat strains available in that laboratory. Initial attempts at the in vitro sensitisation of rats for the work done in the studies reported in this thesis were performed following a variety of methods established in other laboratories for murine, human or rat lymphocytes. After a long and frustrating series of experiments success was finally achieved when the method used by Antczak et al (1979) was tried and modified for local conditions and rat strains. Using the same culture vessels, tissue culture medium and additives and the same method of cell preparation used by the Antczak group the parameters were established for time in culture and cell concentration in microtitre trays which routinely gave reproducible and optimal proliferative responses in MLC, with minimal background and a predictable yield of cells of high viability. The method was further modified for bulk MLC culture of rat lymphocytes in order to obtain the large numbers of cells which were necessary

for the planned in vivo adoptive transfer assays. The basic parameters for the in vitro stimulation of rat lymphoid cells with PHA also had to be established. Again the methods reported by Antczak et al (1979) gave the most reliable and consistent results under the prevailing conditions. Using the culture conditions which gave rise to maximal proliferation in the presence of the mitogen PHA, it was confirmed that 950 rad, which was the irradiation dose used by Antczak et al (1979), appeared to be sufficient to ablate proliferation.

Once the conditions for MLC which gave maximal proliferation were established, the basic parameters were rigorously adhered to in all experiments in which the alloreactive cells generated in MLC were tested in vivo for their capacity to alter the tempo of rejection of NNH grafts. This graft model was chosen not only for the reasons already discussed but also for the fact that it lent itself to an attempt to develop an adoptive allograft assay which avoided some of the difficulties inherent in the conventional adoptive assays in which the cells to be tested are injected IV. Because NNH grafts are implanted in a subcutaneous pocket, the cells to be tested for their in vivo effect can be injected into the graft pocket around the graft. The conventional adoptive transfer assays which employ the IV route of injection do not lend themselves to a study of local effector mechanisms in graft rejection. Precise knowledge of the normal migratory and recirculatory patterns of effector cells is lacking, however there is some doubt as to whether fully differentiated effector CTL are "normal" with regard to their ability to successfully navigate the adoptive hosts recirculation pathways (Rouse and Wagner, 1972, Kim et al, 1983). Although it is obvious from experiments designed to demonstrate specific localization of

sensitised cell populations (Rouse and Wagner, 1973; Sprent and Miller, 1976) that a proportion of IV injected putative effector cells find their way to the graft site, many may be retained in the first lymphoid or other tissue they encounter. One of the aims of these studies was to examine the relevance of the most commonly employed *in vitro* assay for Tc, the CML assay, to *in vivo* graft rejection. It was therefore desirable that the *in vivo* model used permitted the injection of the putative effector cell populations by a route which ensured the greatest possible opportunity for immediate contact with the grafted tissue. No claim is made that local injection into the graft site accurately mirrors the migration of effector cells into grafted tissue which normally occurs *in vivo*.

That the model is a useful one for assaying the allograft reactivity of cell populations was established. 50×10^6 MLC cells routinely procured very rapid graft rejection with a tempo that was reproducible between experiments. In all the MHC incompatible combinations of donor and host investigated, local inoculation with specific MLC cells resulted in very rapid rejection. The local injection of either normal lymphocytes or MLC cells sensitized against third party alloantigens did not significantly alter the tempo of rejection. Grafts injected with normal cells or cells sensitized against irrelevant alloantigen rejected with a tempo which was not significantly different from rejection in uninjected control animals. This model provides a very sensitive assay for alloreactive cells. Very low numbers of specific MLC cells (as few as 5×10^6) caused significant acceleration of the tempo of rejection when injected locally into NNH grafts.

The results of preliminary experiments examining the effect on NNH graft survival of locally inoculated MLC sensitized cell

populations provided more questions than answers as it turned out. Rejection was not immediate. Even with $5x10^7$ cells ECG activity was detectable for at least 5 days after injection. Thus it was not possible to assume that the injected cells simply acted locally in the same manner as Tc *in vitro*. Nor was it possible to conclude that the injected cells themselves were directly responsible for tissue damage. They may merely have induced effector cells. If the injected cells were indeed the mediators of rejection the lapse of time between injection and rejection was such that it could be postulated that they or their progeny exerted this effect only after migration and further differentiation in sites distant from the graft.

Experiments examining the effect of systemically injected MLC cells on graft survival indicated that while graft rejection was specifically accelerated following the IV or IA injection of effector cells, the tempo of rejection was not as rapid as that procured by the local injection route. This suggested a local factor in the mechanism of rejection procured by the MLC cells. Studies on the effect of MLC cells injected in one footpad on the survival of grafts in the contralateral footpad confirmed that at least some of the effect of the cells is a consequence of local action. Cells injected in an ungrafted footpad procured the rejection of a graft in the contralateral footpad in 8 days compared with 5-7 days for grafts in the injected foot. It was also shown that graft rejection proceeded more slowly in the contralateral footpad in bilaterally grafted recipients injected with specific MLC cells in only one footpad. The tempo of rejection of uninjected grafts was very similar to that procured by systemically injected MLC cells. This might suggest that subcutaneously injected MLC cells or their progeny are capable of

disseminating from the site of injection and procuring graft rejection systemically. The difference in the kinetics of the local and systemic rejection response elicited by locally injected MLC cells may be explicable merely as a result of the tempo of effector cell recirculation from the site of injection to a distant antigen depot. If this were the case then it would also imply that the cells that mediate graft rejection following local injection do so without need to recirculate and mature in the adoptive host's lymphoid system and that in fact they do act locally in the first instance. Cells injected at the site of grafting would have immediate access to the graft and if they directly mediate graft destruction, they are in the right position to do so from the moment of injection. As the grafted tissue takes about 4 days to vascularize, systemically injected cells could only gain access to the graft site by extravasation prior to this time. The delay in rejection seen in hosts systemically injected with putative effector cells may represent the time necessary for the local recruitment of sufficient cells to mediate graft rejection. If graft rejection was mediated by host cells which have undergone a qualitative change due to the presence of MLC alloactivated cells, then the concentration of MLC cells at the graft site would also lead to a high local concentration of soluble factors with the potential to incite a host response. The local accumulation of such mediators, both cellular and soluble, would be delayed if graft specific MLC cells were injected systemically.

Specific MLC cells, injected systemically 4 days after grafting, at which time vascular connections between graft and host can be shown to have been established (Dorsch et al, 1983) did not bring about rapid graft rejection. In fact, grafts in this case rejected with a similar tempo to grafts in naive uninjected hosts. These

experiments did not demonstrate an enhanced capacity of cells with direct vascular entry to the graft to bring about graft destruction. Taking into account the 4 day delay before MLC cells were injected, the cells still took at least as long and usually longer than cells injected locally at the time of grafting, to procure graft rejection.

This further supports the thesis that locally injected cells mediate their action, at least in part, locally.

The most likely explanation for the reduced efficiency of IV or IA injected putative effector cells is that systemically injected cells are not immediately re-exposed to graft alloantigen whereas locally injected cells are. The time difference may reflect a need for MLC sensitized cells to be re-exposed to graft antigen, proliferate and/or recruit other cells before graft rejection is accomplished. It was demonstrated that immediate re-exposure to a solid tissue graft following local injection did not accelerate the systemic effect of the MLC cells. When cells were locally injected in one footpad at the time of grafting both hind footpads, the rejection of the graft at the distant site was not as rapid as the rejection of the graft at the site of injection. Therefore, the systemic effects of locally inoculated cells appear to be independent of an immediate re-encounter between the MLC cells and the stimulating antigen. This was shown not to be the case for IV injected MLC cells. The addition of lymphocytes bearing the same alloantigens as the graft and the MLC stimulator cells to the injected MLC cell inoculum resulted in very rapid graft destruction. In fact the tempo of rejection in animals systemically injected with MLC cells plus graft antigen was not significantly different from that procured by locally injected MLC cells. The addition of dispersed cells bearing the same antigens as the graft to locally inoculated MLC cells did not accelerate the

rejection of injected grafts. This indicates that the antigen in the form of a solid tissue graft is sufficient to initiate or sustain whatever mechanisms are mediated by locally injected cells.

Experiments will be done in which MLC cells mixed with graft strain lymphoid cells are injected into one footpad to determine if free antigen accelerates the rejection of a graft in the contralateral footpad, i.e. increases the systemic effects of locally injected cells in the same manner as it increases the effect of IV injected cells. The results of the experiments which were done however, established that additional antigen, in the form of dispersed cells, had no effect on the survival of grafts injected with MLC cells. In addition it was shown that additional donor antigen alone had no apparent effect on the survival of NNH grafts in the footpad. This latter finding is of interest in view of the suggestion, vide infra, that the reason that NNH grafts are rejected more slowly than skin grafts is that they lack Class II antigens.

The mechanism by which the added antigen, in the form of single cells, acts to enhance the effect of systemically injected MLC cells was not investigated, however a number of mechanisms can be postulated.

The addition of single cells bearing graft antigen to the MLC cells immediately prior to IV injection may lead to qualitative or quantitative change in either the transferred cells or in host cells. The observation that injected allogeneic cells, syngeneic to the grafted NNH were not, on their own, able to accelerate graft rejection implies that any changes in host reactivity was insufficient to entirely account for the acceleration of rejection observed in hosts receiving both MLC and allogeneic cells. It does not, however, rule out the possibility that alterations in host

reactivity lead to synergism between the transferred MLC cells and alloactivated host lymphoid cells.

Antczak and Howard (1979) have shown that rat MLC cells continue to proliferate on re-exposure to stimulator cell alloantigen and may be maintained in exponential growth in vitro with continued stimulation. The effect of mixing MLC cells with single cells bearing stimulator strain alloantigens and then injecting the cells IV would, in effect, amount to creating an in vivo secondary MLC reaction, probably within the spleen of the adoptive host. This would serve to increase the number of alloreactive cells available to effect graft rejection or, if host derived cells are the final effector cell, it might increase the available "help" to host derived effector cells by expanding the MLC derived Th cell numbers.

The kinetics of proliferation in MLC demonstrate that after 4 or 5 days in culture there is a rapid decline in the number of cells which proliferate. It has been shown that the decline in cellular proliferation, following the peak of the response in the primary MLC, and the early curtailed proliferative response observed in secondary MLC, are a result of insufficient stimulator cell antigen in the cultures (Sugarbaker and Mathews, 1981). The longer MLC cells are left before restimulation, the more their in vitro responsiveness resembles that of memory T cells (Le Francois et al, 1984). With no apparent antigenic challenge IV injected MLC cells would probably seed to the spleen and LNs and behave more like memory cells, which have been shown to recirculate very slowly if at all (Hall et al, 1978c). Systemically injected MLC cells would be unlikely to be re-exposed to antigen until such time as the graft is vascularised, although a small number which reach the popliteal LN which drains the graft site would presumably encounter some shed antigen there. In

either case re-exposure to antigen would be dependent on the ability of MLC cells to successfully recirculate and migrate.

The reduced efficiency of systemically injected cells in this model may well be due to an inherent incapacity of stimulated cells to migrate appropriately after systemic injection. Experiments have shown that cytolytic cell lines which were capable of causing tumour inhibition in a Winn type assay (Giorgi and Warner, 1981; Cheever et al, 1981) were without effect on tumour growth if injected IV (Giorgi and Warner, 1981). It has also been shown that cells grown in IL2 or harvested from long term culture exhibit abnormal traffic patterns when injected systemically (Lotze et al, 1980; Dailey et al, 1982). The observed deficient homing patterns have been postulated to be due to a deficiency in the receptors which are necessary for normal migration (Dailey et al, 1982). The relationship between the behavior of long term cultured cell lines and MLC cells is not known but alterations to the cell surface are quite likely to result from in vitro culture irrespective of the time in culture, particularly so in MLC where the relevant cells undoubtedly undergo other differentiative changes. It is quite likely therefore that systemically injected MLC cells are incapable of normal physiological recirculation and that the acceleration of graft rejection which is observed following the systemic injection of MLC cells is mediated by progeny of the adoptively transferred cells. The effect of antigen administered with the injected cells may be to stimulate the production of these progeny. Thus the fact that locally injected MLC cells have immediate access to relevant alloantigen in the form of the graft may be the most important factor in determining the apparently more rapid action of cells injected in this manner.

The local inoculation of MLC cells at the graft site could be

followed by a variety of interactive events. It is possible that having been reexposed to graft antigen in the foot locally injected cells or their progeny travel to the popliteal LN, thence to the TD and return to the graft via the circulation to mediate rejection. If this is the case one would expect that they would procure rejection at the same tempo as IV injected cells and indeed it appears that if IV injected cells are provided an immediately accessible source of antigen they are as effective as locally inoculated cells in causing rejection. This is obviously not the entire story as grafts in the injected footpad reject much more rapidly than grafts in the uninjected footpad which suggests that at least some of the injected cells exert their effect locally.

If the MLC cells do mediate their effect locally, they could do so either alone or in concert with cells of host origin. The local injection of MLC cells could lead to a high local concentration of cells with the capacity to activate host cells. It would be expected that systemically injected cells or their progeny, being more widely dispersed, would be less effective than locally inoculated cells in inducing host dependent effector mechanisms.

Studies on the localization of radiolabelled DA anti PVG MLC cells were designed to answer the question of the site and mode of action of injected MLC sensitized cells by providing information on the migratory patterns and localization of locally and systemically injected cells. Cells were injected locally at the graft site on the day of implantation or systemically 5 days after rats were bilaterally grafted with a heart bearing the sensitizing antigen in one footpad and a third part graft in the other. Five days was allowed to elapse between grafting and system injection to ensure that the blood supply to the graft was well established by the time

the cells were given (Dorsch et al, 1983). The results of these studies are largely non-informative. A large variation both within experiments and between experiments in the proportion of the injected dose which was recovered from a standard selection of organs made interpretation of the localization experiments difficult. However a few general observations could be made.

MLC cells injected at the graft site tended to remain in the footpad for at least 42 hours (the longest period of observation) irrespective of whether the graft bore relevant or third party alloantigens. At 42 hours after the local injection of labelled MLC cells more than 80% of the recoverable counts were localized to the grafted footpad. However, by this time the recoverable counts represented only about 10-15% of the injected dose as opposed to 60-80% of the injected dose at 3.5 hr. Very little emigration of cells from the injected footpad to organs other than the popliteal LN could be demonstrated in the period of observation following injection, the proportion of total recovered radioactivity found in sites other than the injected leg in no case amounted to more than 20% even at 42 hrs. This implies that the decrease in counts in the footpad over the same period was probably largely the result of cell death or dissemination to organs which were not counted and not dissemination of cells to seed other lymphoid organs.

In contrast, the injection of MLC cells into the systemic circulation led to the dissemination of radioactivity throughout all organs of the animal with a very low proportion of the recoverable counts found in the graft bed (less than 6% at 18 hrs). There was a gradual increase in the amount of recoverable radioactivity in the grafts during the time of observation (up to 48 hrs), however, at no time was it as great as the proportion of counts recovered following

the local injection of radiolabelled cells and again specific localization was not apparent.

The pooled results for both grafted footpads at 42 hrs were 16% and 10-12% of recoverable counts for IA and IV injected cells respectively and 15-20% for IV injected cells at 48 hrs. This compares with 80% of recoverable counts being present in the grafted leg 42 hrs after the local injection of radiolabelled cells.

The results obtained following IV or IA injection of radiolabelled MLC cells were in most respects similar, however the accumulation of label in the graft beds following IV injection appeared to be slower than that following IA injection. The arterial route was chosen in the hope that cells injected into the descending aorta 5 days after grafting would encounter the capillary bed of the graft immediately after injection. Cells injected IV would first have to traverse the circulation of the lungs [where it is known many are held up (Rannie and Donald, 1977)] before they entered the arterial circulation and had a chance of passing through the graft. Earlier accumulation of radioactivity within the graft bed following arterial injection supports the idea that MLC cells injected by this route may contact graft antigen while still present in relatively high concentration in the circulation whereas those injected by the IV route may be thoroughly diluted in the arterial blood.

Although the studies were extended to locally injected cell populations the salient findings of these experiments were the same as that reported by many other workers studying specific cell localization. There was no marked consistent tendency for DA anti PVG MLC cells to accumulate preferentially in the PVG graft or its draining LN in individual rats grafted with both a PVG and a WF NNH and injected systemically with cells. Similarly when animals were

grafted with either a PVG or a WF heart and the graft site injected locally with DA anti PVG MLC cells there was no consistent difference between the groups in the extent of retention of injected cells at the graft site.

The level of counts retained in the FP following the injection of radiolabelled MLC cells at the graft site remained high for a long period, however the cells were retained equally in the footpads containing specific and those containing third party grafts.

The question of whether sensitized lymphocytes do specifically home to relevant allografts has been the subject of a number of investigations. Carefully controlled studies involving the differential labelling with dual isotopes of two cell populations with differing specificities have demonstrated a small amount of specific accumulation of sensitized cells to the relevant graft. The difference between the accumulation of specific cells and non-specific accumulation was in no case more than two fold (Tilney and Ford, 1974a; Chang and Sugarbaker, 1979). These results are in agreement with those obtained by other workers using different assay systems (Sprent and Miller, 1976; Strom et al, 1979). The small degree of specific localization demonstrated in any of these studies was always accompanied by a very large amount of cellular infiltration into the irrelevant third party grafts. The results presented in this thesis confirmed the latter finding but unfortunately failed to provide any consistent data indicating a degree of specificity in the localization of systemically injected cells or in the retention of locally injected cells. Taken together the results of experiments designed to follow the fate of locally injected MLC cells provided no information relevant to the site or mechanism of their action.

It seems likely that effector T cells enter any site of inflammation whether it be a graft site or not, probably as a consequence of the increased circulation and increased vascular permeability and perhaps also of the variety of non specific chemotactic factors in such areas. It is possible that specifically reactive cells may be retained in the graft preferentially, accounting for the specific localization which has been demonstrated by some workers.. It is also likely that specifically reactive cells divide in response to graft antigens and also produce further mediators. The proportion of cells which cannot be identified as being specific for graft antigens by the methods used would be increased by both these events.

Localisation studies shed little light on whether locally injected cells procured graft rejection by a direct local mechanism or not and provided no information on the mode of action of MLC cells, however they did demonstrate that locally injected MLC cells had a marked propensity to remain at the site of injection. This is an interesting corollary of other localization studies where endeavours to demonstrate the ability of systemically injected specifically sensitized cells to home to a relevant graft have met with little success and accumulation of cells in an irrelevant graft or accumulation of both specific and nonspecific cells within the one graft has been noted. A similar finding was made here when IV or IA injected cells accumulated equally in specific and third party grafts. It is interesting to note that if cells are injected at the site of implantation they tend to stay there and just as there appears to be little specific homing following systemic injection there also appears to be a low rate of emigration of non specific cells from a local site. This is in contrast to the specificity of

graft rejection procured by MLC cells. The retention of nonspecific MLC cells in the footpad following local injection is a further indication that the presence of a cell at a graft site does not necessarily implicate it in the rejection process.

The localization studies in this thesis were limited and a far more extensive investigation would be necessary before it could be unequivocally stated that MLC cells do or do not preferentially localize in the relevant graft following local inoculation.

The problem with localization experiments is that there is no cell label which is ideal, i.e. that is readily detectable, non-toxic and does not have the potential to alter cell migration or function. I^{125}UdR, an analogue of thymidine (reviewed van Rooijen, 1977) was used in these studies. It has advantages over many other radioactive labels. It is minimally re-utilized after cell death, it is preferable to H^3TdR because it is a gamma emitter and to Cr^{51} as it is only incorporated into DNA synthesising cells. Tissues in which labelled cells localise can therefore be counted whole without the necessity for autoradiography or the solubilization of the tissue for liquid scintillation. The greatest disadvantage of I^{125} UdR as a cell label is radiotoxicity, and to a lesser extent chemical toxicity. The former causes cell death following one or a very limited number of cell divisions (van Rooijen, 1977; Le Mevel et al, 1973). Sprent and Miller (1976) found labelling concentrations as low as 0.1uCi/ml toxic to cells in localisation studies and Sprent (1976) attributed the alteration of the migration characteristics which occurred with increasing doses of I^{125}UdR used to label murine activated T.TdL to radiation induced cell damage which appeared to cause poor splenic localization.

I^{125}UdR is therefore not considered an ideal cell label for long

term migration studies. The experiments presented in this thesis dealt mainly with the short term tracing of radiolabelled MLC cells, however the possibility that radiotoxicity affected cell viability and hence the results can not be excluded. In particular the incorporation of the label into the nucleus of dividing cells make it prone to affect adversely the processes of cell division. In secondary MLC peak proliferative responses may occur as early as 24hr following restimulation with the priming antigen (unpublished observation). It is likely that upon reexposure to antigen in vivo recently activated MLC cells rapidly divide and this may prejudice the results of localisation studies using I^{125}UdR as the label will be diluted out of relevant cells or may even cause death of this population.

Another potential problem with labelling sensitive cell populations, is that the actual manipulations in vitro necessary for labelling may adversely affect the cells so that they fail to fulfil their normal physiological role after injection. The ability of systemically injected cells to localize to a site of antigen deposition is dependent upon the degree to which that cell is able to navigate the adoptive hosts recirculation pathways and interact with the vascular endothelium (VE) of both the lymphoid tissue and the graft. Lymphocyte traffic is at least partially regulated by the interaction between the cell surface and the VE. High endothelial venules (HEV) have been shown to express a determinant for lymphocyte binding and, in the mouse, cell populations show organ specificity in their patterns of localization (Butcher et al, 1980). A striking parallel was observed between the preferential localization in vivo and adherence to HEV in vitro of lymphocytes from different lymphoid organs. This strongly suggests that selective interaction with HEV

is the primary determinant of the organ specificity of lymphocyte homing (Butcher et al, 1980). A variety of substances which alter the cell surface of lymphocytes, such as neuraminidase, trypsin and lipopolysaccharide, have been shown to change the normal circulation patterns of in vitro treated cells resulting in reduced entry to LN with a concomitant increase in the cells which enter the spleen, lungs and liver (Woodruff and Gesner, 1969; Freitas and de Sousa, 1976; Schlesinger, 1976).

Heat killed immunoblasts are mostly held up in the liver and spleen and are rapidly catabolized (Hall et al, 1972) and immune lymphocytes labelled with Cr^{51} and H^3TdR in vitro and retransfused into allograft bearing recipients are found to harbour in the lung and liver (Sprent and Miller, 1976). These results indicate an alteration in the migratory capacity of labelled cells which makes it difficult to interpret the significance of elevated cell numbers in a particular organ.

The ideal cell label would be permanent, not affect cell division and not be lost when division occurs. It would be non-toxic and would not alter the normal physiological behaviour of the cells, be easily identifiable and quantifiable in host tissues, would label all cells uniformly and not be reutilized by host cells. It does not exist. All labels used in localisation studies fall short of the ideal to a greater or lesser extent. As far as some which are used in lymphocyte studies are concerned, there is also doubt that all subpopulations of T cells are labelled uniformly. Only cells which are dividing at the time of labelling take up radioactive DNA precursors, for example. This would mean that to label effector cell populations adequately, all the cells would have to be in the S phase when exposed to the label. Recent work has indicated that Tc

precursors in vivo may not necessarily undergo cell division to reach maturity and effector cell status (Kimura and Wigzell, 1983). Furthermore in vitro analysis of alloreactive T cells shows that in the rat the $W3/25^+$ Th subset is the major cell population which proliferates in MLC, although MRC $OX8^+$ Tc do so to a lesser extent (Mason et al, 1981). Low indices of localization may therefore also be partially explicable in terms of the difficulties associated with labelling the relevant population while excluding all others.

The foregoing factor smake experiments to trace the fate of radiolabelled sensitized cells injected into graft recipients very difficult to design and interpret. Even so several reports do suggest that injected labelled cells will migrate and exhibit normal effector function.

Using murine cortisone resistant thymocytes which had been alloactivated in vitro in MLC, Rouse and Wagner (1972) observed that at least some Cr^{51} labelled cells entered the recirculating pool following IV injection into syngeneic recipients. In comparison to normal TDL and cortisone resistant thymocytes however a high proportion of the cells were found in the liver (about 50%) for prolonged periods (72 hrs) but moderate numbers did localise to the T dependent areas of the spleen and LNs and some cells recirculated from blood to TD lymph. This is in agreement to the findings of Sprent and Miller (1972b) who demonstrated that the highly cytotoxic population of activated T cells produced as a consequence of a GVH reaction continued to recirculate in a syngeneic recipient for at least 4 days.

A number of reports confirm that unlabelled MLC sensitized populations retain some functional activity following adoptive transfer. Rouse and Wagner (1973) showed that unlabelled MLC cells

injected IV into irradiated syngeneic mice and recovered from the LN and spleen 24 hr later were specifically cytotoxic to target cells in vitro thereby confirming the functional activity of the localized cells. The same authors also demonstrated that in vitro sensitized cells had the capacity to accelerate the rejection of murine skin allografts when transferred IV to T cell depleted syngeneic hosts further confirming the ability of these cells to function normally in the adoptive hosts (Rouse and Wagner, 1972). The experiments in T cell depleted mice suggest that the injected cells themselves procure graft rejection . However the participation of host monocytes and B cells was not excluded.

Experiments in irradiated grafted recipients locally injected with MLC cells strongly suggested that at least in the case of local inoculation in DA strain hosts, MLC sensitized cells require the presence of additional cell populations to effect rapid graft destruction. It is known that irradiation acutely depletes both small lymphocytes and all bone marrow derived cells. Small lymphocytes of both the T and B cell lineage are extremely radiosensitive and die in interphase (Anderson and Warner, 1976). Irradiation has thus been regarded as providing an immunologically inert milieu in which the infrastructure of the peripheral lymphoid tissue which is required to support lymphocyte migration is left intact (Roser and Dorsch, 1979). The immunosuppressive effect of a given dose of irradiation is rapid, reproducible and leaves no residue which will interfere with subsequently injected cells (Hall and Dorsch, 1984). To achieve reproducible, significant prolongation of survival of MHC-incompatible skin grafts in rats, near lethal doses (750-900 rad) of gamma irradiation are required (Dorsch and Roser, 1974a; Hall et al, 1978a). A variety of models of adoptive

allograft assays which depend upon the use of acutely irradiated rat hosts have been developed in this laboratory. The original model used skin as the indicator graft (Dorsch and Roser, 1974a) and has since been adapted for the use of directly vascularized cardiac grafts (Hall et al, 1978a) or indirectly vascularized neonatal heart grafts as indicator grafts (Dorsch et al, 1983).

Irradiation of DA rats with 900 rad whole body irradiation (WBI) prior to grafting a PVG NNH caused a marked delay in the tempo of rejection. Unmodified DA rats reject a PVG NNH in approximately 12-18d. Prior irradiation resulted in graft survival times of 47-68d.

MLC sensitized cells accelerated rejection in irradiated hosts whether injected locally or systemically. The response was specific for the sensitizing alloantigen. DA hosts grafted with PVG NNHs receiving normal DA LNC or MLC cells sensitized against third party alloantigens rejected their grafts with a much slower tempo. Normal LNC produced more rapid graft rejection than nonspecific MLC cells. The latter finding might be expected. Normal LNC populations presumably contain a normal clone of cells with specificity for graft alloantigen which expands following contact with graft antigen in the irradiated host and eventually produces sufficient cells with effector function to cause graft rejection. MLC cell populations which have been sensitized against third party alloantigen probably contain very few cells with receptor for graft alloantigen. The unexpected finding was that the tempo of graft rejection procured by locally injected MLC cells in irradiated hosts was much less rapid than that procured by similar cell populations in normal hosts. In contrast, the tempo of graft rejection procured in irradiated hosts by systemically injected MLC cells was not markedly different from that procured by systemically injected cells in normal hosts.

This result suggested that the rapid graft rejection which followed local inoculation of MLC cells in normal DA hosts was dependent upon collaboration between the injected cell population and radiosensitive host cells. The similarity in the rejection tempo procured by systemically injected cells in normal and irradiated hosts does not necessarily imply that systemically injected cells do not collaborate with host derived cells. The tempo of rejection following systemic injection of MLC cells in normal hosts is slower than that following local inoculation; 9-11d compared to 5-7d. It is possible that this time span might permit the regeneration of the relevant host cell population in irradiated hosts. In addition systemically injected cells would have more opportunity to contact a small number of remaining host radioresistant cells during their migration through lymphoid tissue than would locally inoculated cells.

There was no reason to presume that the radiosensitive cell, upon which rapid graft rejection following the local inoculation of MLC sensitized cells was apparently dependent, was necessarily a cell of lymphoid lineage. All bone marrow derived cells are sensitive to radiation effects (Anderson and Warner, 1976). Of these the nonlymphoid cell which has been most strongly implicated in graft rejection is the macrophage. Information regarding the radiosensitivity of cells of the mononuclear phagocyte lineage is imprecise, probably due to the considerable confusion which exists regarding the identification of the various differentiative stages of macrophages, particularly tissue macrophages. There is no doubt however that immature cells of the mononuclear phagocyte population are radiosensitive. Subsequent to sublethal doses of irradiation which cause a fall in blood lymphocytes to less than 1% of normal,

blood monocyte levels fall to below 5% of their preirradiation levels (MacPherson and Christmas, 1984). The ability of macrophages to migrate to an inflammatory site has also been shown to be impaired after WBI. The number of macrophages which adhere to glass coverslips implanted in a skin window is diminished to 10-30% of normal levels 4-10d following 750 rad WBI (MacPherson and Christmas, 1984) and murine peritoneal macrophages elicited by immune and non immune stimuli are reduced to less than 3% of normal levels 10 d after 900 rad WBI (Scher et al, 1982).

Macrophages have been reported by some workers to be the predominant infiltrating cell at the time of rejection of rat cardiac allografts and are apparently present in the graft in intimate relationship to myocardial cells (Christmas and MacPherson, 1982; Forbes and Guttmann, 1983). With the onset of rejection of renal allografts in the rat, blood borne monocytes which have entered the graft in the early cellular infiltrate of lymphocytes and monocytes (Hayry et al, 1979) have been reported to rapidly mature into tissue macrophages.

It is apparent that the macrophage is a major component of the graft infiltrating cell population in normal hosts, and is susceptible to the effects of irradiation. It was thus considered possible that the failure of irradiated recipients to display rapid graft rejection following the local inoculation of MLC sensitised populations was due to a deficit of host macrophages during the early days following irradiation. The finding that MLC cells do procure rejection in irradiated hosts by 10-12d would not deny this hypothesis as MacPherson and Christmas (1984) have reported that although prior irradiation of cardiac graft recipients reduces the proportion of macrophages infiltrating rejecting grafts, there are

identifiable macrophages present at 10-12d after irradiation and grafting. It was found that the addition of PE cells, rich in macrophages, to locally injected MLC cell inocula did not accelerate graft rejection in irradiated rats to the tempo seen in normal recipients injected with MLC sensitized cell populations. Two surprising findings arose from the control groups done in this experiment. The first was that the addition of PE cells to MLC sensitized cells enhanced their ability to cause rapid rejection when locally inoculated into normal recipients, although it had no enhancing effect in irradiated recipients. The second was that PE cells alone accelerated graft rejection when locally inoculated in normal recipients. Graft rejection was however significantly slower in groups injected with PE cells alone than it was in groups injected with PE cells and MLC sensitized cells.

The ability of PE cells to enhance both MLC cell mediated graft rejection and host cell mediated graft rejection in non irradiated hosts implied a central role for these cells in the alloactivation of host mediated graft rejection mechanisms as well as a synergistic role in the in vivo effector function of MLC cells. The PE cells which were responsible for accelerated rejection were obtained from the peritoneal cavity following stimulation with proteose peptone. Non-specific stimulation of macrophages has been reported to stimulate the production of IL1 (Oppenheim and Gery, 1982) and it is possible that this was responsible for the observed acceleration of rejection in normal hosts inoculated at the graft site with PE cells either with or without MLC sensitized cells.

Whether or not local production of IL1 or other lymphokines at the graft site was the mechanism by which PE cells accelerated rejection in normal hosts it was clear that the addition of PE cells

to MLC sensitized cells did not restore rapid rejection in irradiated recipients. Therefore the possibility that rapid graft rejection depended upon lymphocyte subpopulations which are deficient in irradiated hosts was examined by adding various T cell subsets to MLC sensitized cell populations.

The proliferative response which occurs in the rat MLC is primarily a response by the W3/25$^+$ subpopulation of T cells (Mason et al, 1981). The MRC OX8$^+$ subpopulation of T cells proliferates only minimally under normal circumstances (Dallman et al, 1982). It was therefore possible that to mediate rapid graft rejection MLC sensitized cells require the presence of host MRC OX8$^+$ cells which irradiated host rats cannot supply. The results of a series of experiments examining this possibility indicated that although the addition of naive MRC OX8$^+$ cells alone did not augment the capacity of MLC cells to cause rapid rejection in irradiated hosts, MLC sensitised cells supplemented with both syngeneic naive MRC OX8$^+$ cells and PE cells caused rapid graft rejection in irradiated hosts. The tempo of rejection procured by injection into the graft site of a mixture of MLC cells, naive MRC OX8$^+$ cells and PE cells was 5-6d which is comparable to that seen in normal hosts injected with MLC cells alone (5-7d). The naive W3/25$^+$ subset of LNC was ineffective when substituted for MRC OX8$^+$ cells in the interaction.

These experiments suggested that the rapid graft rejection in normal DA hosts injected with MLC cells was the result of interactions between the injected cells and host derived macrophages and T cells of the cytotoxic/suppressor subset.

It is possible to draw several conclusions from the results of studies of the effects on the survival of PVG grafts in DA hosts of MLC sensitized DA cell populations. Locally inoculated MLC

sensitized cells had the capacity to specifically accelerate graft rejection. Comparison of the effect of locally inoculated cells with those of cells inoculated subcutaneously in the footpad contralateral to the graft or systemically, suggested that there is a purely local component to the effect of locally inoculated cells. The finding that the addition of a source of graft antigen to inocula of systemically injected MLC cells increased the rapidity with which they procuredrejection, suggested that rapid reexposure to an adequate source of specific antigen is necessary to sustain the maximum effector function of MLC sensitised cells and is probably the most important factor in determining the rapidity with which cells injected directly into the graft bed procure tissue destruction. In addition the difference between the rejection time of the locally inoculated and contralateral grafts in bilaterally grafted animals implies that some of the large number of cells which are retained in the graft site, by what may be mainly non specific mechanisms, may exert a purely local effect. The observation that MLC sensitised cells do not effect rapid rejection in irradiated hosts unless supplemented with additional naive MRC $OX8^+$ cells and PE cells suggests either that the latter cell populations increase the efficiency of MLC sensitized cells or alternatively are essential for their effector function in vivo.

The latter is a very real possibility. Studies using directly vascularised grafts have shown that by 10–12 days after irradiation grafts in irradiated recipients restored with $W3/25^+$ cells contain large numbers of macrophages and MRC $OX8^+$ cells (Dallman et al, 1982; Hall et al, 1983a). This suggests that by this time there has been significant regeneration of macrophages and MRC $OX8^+$ cells which may be either NK cells (Dallman et al, 1982) or cytotoxic suppressor

cells (Hall et al, 1983). It is thus possible that MLC sensitised cells are incapable of procuring graft rejection in vivo in the absence of host macrophages and MRC $OX8^+$ cells and that the graft rejection observed 10-14d after MLC cells are locally inoculated in irradiated recipients is procured with the collaboration of regenerating host derived macrophages and MRC $OX8^+$ cells.

What role then do PE cells and naive MRC $OX8^+$ cells play in the rejection process mediated by locally injected MLC cells? Dealing first with the apparent requirement for macrophages, the presence of antigen presenting cells (macrophages and/or DC) in either the repsonding or stimulating cell populations is essential for the induction of both proliferation and cytotoxicity in vitro (Steinman et al, 1983a; Singer et al, 1984). The expression of Ia antigens on the surface of APCs which may in fact be increased by exposure to immune T cells (Unanue et al, 1984) has been well documented as playing an important role in both MHC restricted responses to protein antigens (Zinkernagel and Doherty, 1974; Klinkert et al, 1980, Bevan, 1981) and responses to MHC alloantigens (Eijsvoogel et al, 1973; Bach et al, 1976; Klinkert et al, 1980). The interactions between antigen reactive T cells and APC leads to the release of IL1, a primary maturation signal (Oppenheim and Gery, 1982) to which both Th and Tc cells may be responsive (Luger et al, 1982). In addition graft derived Ia^+ cell populations have been shown to be important in the induction of transplantation rejection responses and their removal has been shown to lead to marked prolongation in graft survival (reviewed Lafferty et al, 1983). The latter fact may be pertinent to findings with the NNH graft model. Preliminary work in this laboratory has revealed that NNH tissue contains very few cells expressing Class II antigen. Examination of adult cardiac tissue

reveals the presence of abundant numbers of cells expressing Class II antigens which are esterase positive and capable of phagocytosing fluorescein labelled dextran. Such cells are almost totally absent in neonatal cardiac tissue. This may be an explanation for the observed prolonged survival of NNH grafts compared to both skin and adult cardiac grafts which are both endowed with immunogenic Class II bearing cells which NNH tissue appears to lack.

The finding that NNH tissue is deficient in Class II bearing cells thus provides an alternative explanation for the mechanism by which local provision of a large number of stimulated macrophages in the form of PE cells influences the tempo of graft survival in both uninjected hosts and hosts simultaneously injected with MLC cells (the other explanation viz that stimulated PE cells provide a source of IL1 has already been discussed). Injected macrophages may rapidly process antigen and present it in an immunogenic form to host cells or to injected MLC cells. As far as the latter are concerned there is good evidence that the rapid fall in DNA synthesis which follows the day 4 peak proliferative response in MLC is a result of the loss of antigenic stimulation (Sugarbaker and Mathews, 1981). The decrease in proliferation can be prevented, and an exponential growth of alloactivated cells can be achieved, in MLC by the addition of alloantigen bearing stimulator cells every 48 hr or so after primary culture (Antczak and Howard, 1979). The fact that the efficiency in procuring graft rejection of systemically injected MLC cells was increased by the addition of graft strain lymphoid cells supports the notion that the reactivity of the MLC cells used in these studies was susceptible to the removal of the antigenic stimulus.

T cell proliferation, although initiated by antigen or mitogen appears to be mediated via IL2 (Smith et al, 1980; Larsson et al,

1980) and IL2 receptors must be induced for appropriate immune stimulation (Larsson, 1981; Robb et al, 1981). Thus the immune specificity of T cell clonal expansion is guaranteed by the restriction of IL2 receptor expression to the antigen reactive clone (Cantrell and Smith, 1983). It has been shown that the IL2 responsiveness of T cell clones decays with time but can be restored by reexposure to antigen (Engers et al, 1980; Smith and Ruscetti, 1981). Fluctuating IL2 responsiveness has been attributed to changes in cellular expression of IL2 receptors (Cantrell and Smith, 1983). It is possible that in order to proliferate and express their function in vivo DA MLC sensitised cells require reexposure to antigens to restore the expression of IL2 receptors and/or the production of IL2. NNH grafts may not present an appropriate antigenic stimulus for this in the absence of additional APCs with the capacity to deliver a stimulating signal in the form of IL1. The effect of the suboptimal immunogenicity of NNH grafts would be most pronounced in irradiated recipients in which neither graft nor host tissue contain adequate numbers of APCs until sufficient time has passed for host cells to regenerate.

With the present understanding of the role of lymphokines in the events which lead to T cell alloactivation and the apparent interdependence between antigen sensitive T cells and macrophages, the former producing monokines and immune interferon which stimulate macrophages and the latter producing IL1 which activates T cells (Unanue et al, 1984), it is not surprising that rapid graft rejection in irradiated hosts depends on the presence of both activated T cells and macrophages. Studies in ATXBM rats by Tilney's group illustrate the need for helper factors in the procurement of rapid graft rejection in cardiac allografted recipients. It was

found that the addition of IL2 conditioned supernatants to sensitized spleen cell inocula increased their effectiveness in inducing graft rejection (Clason et al, 1982; Lear et al, 1983). Initial work by these groups illustrated a requirement for either IL2 or adherent cells as well as sensitized cells to procure rapid rejection (Clason et al, 1982). Later work failed to confirm the requirement for adherent cell populations. IL2 plus T cells in numbers separated from spleen cell inocula which transferred rapid rejection in the presence of IL2, were not as effective as whole cell preparations (Lear et al, 1983), however adding back adherent cells had no effect. Only large numbers of T cells (10^8) or equal numbers of splenic cells, both with the addition of IL2, procured rapid rejection (Lear et al, 1983). Although ATXBM rodents may be deficient in macrophages (Lear et al, 1983) and it has been shown that macrophages harvested from ATXBM rats do not function well as APC in vitro compared to adherent cells from normal rats (Clason et al, 1982), it is possible that the injection of such large numbers of immune T cells into an animal bearing a specific allograft was sufficient to induce both proliferation of macrophages derived from the bone marrow inoculum and the release by them of IL1.

The observation that NNH tissue lacks Class II antigens suggests that the role of PE cells in the model may be one of antigen processing to present specific antigen in a form which ensures the continuation of alloreactivity on the part of MLC sensitised populations.

Why then is there not a requirement for the addition of PE cells toWF MLC cells to procure rapid rejection in irradiated WF hosts? The simplest explanation is that WF cell populations are more radio-resistant than DA cell populations. This may be so however there is

no evidence for it. A more convincing explanation for the inter-strain differences in the requirements for rapid graft rejection may lie in interstrain differences in immune responsiveness. Studies on the alloimmune responsiveness of various PVG ($RT1^c$) congenic rat strains have provided evidence that skin graft rejection, alloantibody production and the *in vivo* generation of Tc against alloantigens may be under MHC linked immune response gene control (Howard and Butcher, 1981; Butcher et al, 1982; Butcher and Howard, 1982). These authors showed that in the absence of a Class II antigenic disparity DA rats were low responders in alloantibody production to PVG alloantigens. Fabre and Morris (1972 and 1974) also reported that not only was the DA strain rat very easily enhanced compared to the Lew strain, it was also difficult to consistently raise cytotoxic alloantibodies in DA rats against either AS2 or Lew alloantigens without the use of adjuvants. This is in agreement with the observation that the subline of DA rats used in this laboratory are low cytotoxic antibody producers whereas (DAxWF) F_1 hybrid rats are commonly high responders producing high titre cytotoxic antibodies (unpublished observations). Taken together these results suggest that the DA strain rat may be a low responder in a number of alloimmune assays. This effect may be even more pronounced in the absence of appropriate stimulation by antigen in association with Class II bearing cells. One can draw the conclusion from the work presented here that WF MLC cells are less vulnerable to the effects of antigen withdrawal than DA MLC cells and it is thus possible that, even in the absence of APC and a source of IL1, NNH tissue presents an adequate stimulus for continued reactivity in WF MLC sensitized cells whereas DA MLC sensitized cells may revert to a quiescent state.

WF recipients differ in a further respect from DA hosts. Irradiated DA hosts require the addition of PE cells and MRC $OX8^+$ cells to MLC sensitized cells to restore rapid rejection. WF MLC sensitized cells alone caused rapid graft rejection in irradiated WF hosts. The inability of DA MLC sensitised cells to cause rapid graft rejection in irradiated hosts without the provision of additional MRC $OX8^+$ cells may be related to the apparent absence of *in vitro* cytotoxic activity in DA MLC sensitized cell populations. Initial studies of MLC were all done with DA responder cells and PVG stimulator cells as this was the strain combination of host and graft donor respectively which had been chosen for *in vivo* studies of graft rejection. DA lymphocytes consistently showed substantial proliferation in MLC and DA MLC sensitized cells specifically accelerated graft rejection *in vivo*. It was impossible to establish a reliable reproducible CML assay with DA strain putative effector cells. The cellular products of standard MLCs, as they were routinely performed by a method which gave predictable high yields of cells of high viability with established *in vivo* effector function, did not lyse target cells in a classical CML assay.

Various avenues were explored in attempts to establish CML assays. As it has been reported that the conditions which gave rise to optimal proliferation and cell survival do not necessarily provide optimal stimulation of Tc the conditions of culture for MLC sensitization of cells were altered. Wilson et al (1976b) had found that the culture conditions which were optimal for producing high levels of both H^3TdR incorporation and cell survival were also appropriate for the preparation of positively selected lymphocytic populations enriched for both MLC reactivity and potency in the popliteal LN GVH assay, but not for the generation of cells with

lytic activity in the CML assay. The latter MLCs were prepared in microcultures. When Marbrook culture vessels were used for MLC, effector cells with high specific lytic activity in the CML assay were produced although these culture conditions gave rise to very low levels of H^3TdR incorporation compared to standard culture methods. A number of different culture vessels including the Marbrook flask were used in the experiments reported here. Neither these measures nor the use of a great variety of combinations and permutations of tissue culture media, different cell concentrations, different periods in culture and different methods of treating stimulator cell populations reliably gave cells which were cytotoxic in vitro.

Another possible explanation for poor CML in DA MLC sensitized cells was explored. The curtailment of cellular proliferation seen in MLC following the peak of the response may be due to the removal of stimulator cell antigen as a result of specifically cytotoxic cells generated during the in vitro sensitization process. Sugarbaker and Mathews (1981) provided evidence that cytotoxic cells may feed back negatively on the immune response in vitro by effecting cellular antigen elimination early in the MLC response thereby reducing the proliferation and generation of further Tc during the culture period. This supported the work of Knoeber and Clark (1977) who demonstrated a requirement for stimulator cell antigen in primary MLC for at least 24 hrs, with a decreasing dependence up to 72 hrs, for the generation of Tc. Furthermore the maintenance of continued proliferation of alloactivated lymphocytes after sensitization in primary MLC has been shown to be dependent on the addition of stimulator cell antigen to the cultures every 48 hrs (Antczak and Howard, 1979). Examination of the kinetics and magnitude of

proliferation in MLC of DA lymphocytes showed that as responder cell numbers were increased, at some stimulator cell concentrations there was a shift in the time of peak proliferation from 4 days to 3 days whereafter the response abruptly declined or plateaued for 1 day then declined. If this early peak proliferative response seen in DA cultures was the result of the removal of stimulator cell antigen by cytotoxic cells, the presence of the latter was not demonstrable in the CML assay. Examination of the kinetics of the response by harvesting MLC cells on days 2 to 10 did not support the notion that Tc were generated early in the in vitro sensitization process and by the time of harvest had become quiescent. At no stage was positive specific cytotoxicity observed.

Variations in the treatment of stimulator cell populations were also explored. MLC stimulator cells were routinely mitotically inactivated with 950 rad Co^{60} irradiation for preparation of cells for adoptive transfer experiments. In a number of experiments designed to follow the methods employed by workers also using rat models, mitomycin C and a variety of irradiation doses between 950 and 3000 rad were used to inactivate MLC stimulator cells.

Failure to produce a reliable CML assay using any of these methods led to a search for a more appropriate target cell for CML assays. Kinetic studies of Cr^{51} release from various normal and tumour cells of the same H-2 genotype in the presence of the same population of immune lymphoid cells reveals considerable differences in target cell susceptibility to immune lysis (Brunner et al, 1970). Normal lymphocytes are poor target cells whereas a variety of single cell tumours are susceptible to lysis (Cerottini and Brunner, 1974). The original discovery by Nowell (1960) that PHA added to cultured leucocytes caused the appearance of large blastoid cells and mitosis

in cultures led to the use of PHA induced lymphoblasts instead of normal lymphocytes as target cells in the CML assay (Lightbody et al, 1971) and as a result of this the sensitivity of the assay was vastly increased (Cerottini and Brunner, 1974). It was subsequently found that Con A induced lymphoblasts provided very susceptible targets (Marshak et al, 1977).

A wide variety of target cells, including PHA and Con A induced lymphoblasts, single cell suspensions of mammary carcinomas and T cell leukaemias, were tested in the CML experiments described in this thesis. None of these produced satisfactory results. Although the initial aim of these futile and time consuming studies of the parameters required to establish a reliable CML assay was to correlate the assay with the in vivo activity of DA strain MLC responders, other rat strain (BN, WF and PVG) cells were tried as responder cells with similar negative or non-reproducible results.

When success was eventually achieved it was with MLC sensitized WF strain cells. The key to that success appeared to be the use of Con A induced thymocyte blasts as target cells in the CML assay and a variation in the method of preparing the MLC stimulator cells. The original method was to squash chopped LN and spleen pieces. Later stimulator cells were obtained by sieving the lymphoid tissue through wire gauze. It appears that the change in the source of target cells for CML assay was the most important factor. Examination of the effect of the method of preparing MLC stimulator cells on the generation of Tc showed that the sieving method did not consistently yield better CML results than the squashing method. There is ample evidence regarding the critical importance of the nature of the target cell in the CML assay (vide supra) and it was apparent that with the rat strains used and under the conditions prevailing in this

laboratory the thymocyte blast was the target cell of choice for the CML assay. The former statement has however to be qualified. With all the rat strains used except DA, Tc with demonstrable low levels of specific in vitro cytotoxicity for Con A induced thymocyte blasts could be raised in MLC. The maximum specific cytotoxicity obtained with DA effector cells however was 5-11% and frequently no specific lysis could be detected. This has not been the experience of other laboratories using DA strain rats. Methods which had proven successful with DA strain lymphocytes (Dr B. Roser; personal communication) were followed meticulously on a number of occasions without successful demonstration of specific lysis. It can only be concluded that with the particular subline of DA rats used, and under the conditions prevailing in the laboratory, the generation of Tc in this strain was not possible. One explanation is that DA rats are low responders for Tc. The fact that DA cells showed strong proliferative responses suggests that the strength of the proliferative response is not necessarily a reflection of the strengh of the effector response where MHC determined antigens are concerned.

In an attempt to confirm that the failure of DA lymphocytes to develop demonstrable cytotoxicity was under Ir gene control, experiments were done using (DAxWF) cells sensitized in MLC against PVG alloantigens (unpublished results). It might be expected that if failure to produce Tc was a consequence of DA rats being low responders, lymphocytes from F_1 hybrids between DA and WF, demonstrably a high responder, should develop cytotoxicity. This was not the case.

It has been established that the great majority of the cells which proliferate in MLC are of the T helper subpopulation. In the studies presented in this thesis it was found that DA lymphocytes

proliferated extensively in MLC but did not apparently produce effector cells with the capacity to cause measurable cytotoxicity in CML assays or rapid graft rejection in irradiated hosts unless the latter were provided with PE cells and additional naive DA MRC $OX8^+$ cells. In contrast WF lymphocytes sensitised in MLC under identical conditions produced cells with the capacity to cause both *in vitro* lysis and rapid rejection of NNH grafts in irradiated hosts. An apparently simple explanation for these findings was suggested when it was found that the proportion of MRC $OX8^+$ cells in both unstimulated and MLC stimulated DA lymphocyte populations was considerably less than that in similar WF lymphocyte populations. This suggested that the limiting factor in both CML assays and *in vivo* graft rejection might be the number of MRC $OX8^+$ cells present. This thesis was not supported by experimental results. The addition of extra MLC stimulated MRC $OX8^+$ cells to DA MLC sensitized cells did not increase the efficiency of DA MLC cells in graft rejection in irradiated recipients. Furthermore, DA MRC $OX8^+$ LNC, cultured at the same concentration as whole LNC, in MLC with PVG stimulator cells did not develop cytolytic activity whereas WF MRC $OX8^+$ cells cultured under the same conditions were demonstrably cytotoxic *in vitro*. Therefore, boosting the number of MRC $OX8^+$ cells present in DA MLC cells to the numbers present in WF MLC cells did not increase the efficiency of DA MLC sensitized cells either *in vitro* or *in vivo*. Nor did the addition of both DA PE cells and naive DA MRC $OX8^+$ cells to DA MLC effector cells in the CML assay increase specific cytotoxicity, although they did restore rapid graft rejection *in vivo* when added to MLC sensitized cells.

It is possible that the conditions used for culture, though suitable for the production of WF effector cells, were suboptimal for

the production of DA effector cells. The evidence previously cited regarding low responsiveness of the DA strain in a number of situations may suggest that the requirements for induction of effector cells in this strain are more stringent and that effector mechanisms are easily subverted.

The proliferative response of DA cells in MLC is substantial. MLC sensitized DA cells specifically accelerate rejection in normal hosts and transfer specific rejection to irradiated hosts, albeit with a slower tempo than in normal hosts given similar cell populations. It is likely that these functions reflect the activity of a specifically induced helper cell population. Kim et al (1983) showed that cloned T cells expanded 8-10 times in the presence of IL2 and injected IV were able to mediate specific skin graft rejection in normal and irradiated mice. If the cells were depleted of Lyt 2^+ cells prior to sensitisation, the cloned Lyt 1^+ cells had no demonstrable cytotoxicity but retained the capacity to restore skin graft rejection even when expanded more than 100 fold. Lyt 1^-2^+ cloned lymphocytes with high levels of specific cytotoxicity however were not capable of mediating rejection in this model (Kim et al 1983). This is in agreement with the findings of other groups that although cell populations used in adoptive transfer might contain cytotoxic cells the levels of <u>in vitro</u> cytotoxicity do not correlate with the capacity to mediate graft rejection (Lowry et al, 1983a; Le Francois and Bevan, 1984).

There is increasing evidence that naive cells depleted of Tc or their precursors adoptively transfer first set cardiac and skin graft rejection to T cell deprived rats (Hall et al, 1983; 1984) and mice (Loveland et al, 1981) whereas naive cells depleted of Th cells do not do so. Although one study found that both the Th and Tc subset

of sensitized spleen cells were equally effective in procuring skin graft rejection in ATXBM mice (Le Francois and Bevan, 1984) in another study it was reported that in cardiac grafted irradiated rats the W3/25$^+$ subset of sensitized spleen cells was more potent on a per cell basis than the MRC OX8$^+$ subset although the latter were demonstrably cytotoxic in vitro in the CML assay (Lowry et al, 1983a). What both studies found however was that there appeared to be a large host component in the response. Host derived cytotoxic cells were demonstrable in the spleens of restored ATXBM mice (Le Francois and Bevan, 1984) and the vast majority (>90%) of cardiac graft infiltrating cells in irradiated restored rats were found to be of host origin 7 days after cell transfer (Lowry et al, 1983a and b). As graft recipients received high doses of irradiation prior to grafting and cell transfer (Lowry et al, 1983a) it appears either that host cells have the capacity to regenerate rapidly or that there are sufficient peripheral radioresistant lymphocytes to proliferate and be recruited to the graft site following adoptive cell transfer.

None of the above models defined a role for Tc in graft rejection. Superficially it appears that the transfer of either naive or sensitized Th cells will cause graft rejection in T cell deficient rodents (Loveland et al, 1981; Kim et al, 1983; Hall et al, 1983; 1984). In the DA rat strain used in the studies presented here whilst MLC sensitized cells alone accelerated rejection in irradiated rats, both Tc precursors and macrophages appeared to be essential to procure rapid rejection. This suggests that the MRC OX8$^+$ cell observed in rejecting organ grafts (Dallman et al, 1982; Hall et al, 1983; 1984) might not be, as has frequently been suggested, merely part of the non specific infiltrate accompanying graft rejection. They may play a vital role in procuring rejection. To examine this

further the activity of purified populations of the Th and Tc subset, separated either before or after stimulation in MLC, was investigated.

It was found that LNC populations depleted of W3/25$^+$ cells proliferated substantially in MLC though not to the same extent as did the same number of the whole T cell population or the W3/25$^+$ population. Studies done by Mason et al (1981) of the phenotype of the T cells which proliferate in the rat MLC indicated that both the W3/25$^+$ and the MRC OX8$^+$ subsets do proliferate but the latter only in the presence of W3/25$^+$ cells. In later studies (Dallman et al, 1982) it was shown, using congenic animals and cultures in which responder cells of one parental strain were cultured with irradiated F_1 cells and irradiated cells of the other parental strain, that the helper activity of the W3/25$^+$ T cell population is radioresistant, is not MHC restricted and can be provided by a background MLC in the stimulator cell population. Thus in the fully allogeneic MLCs between MRC OX8$^+$ responder cells and irradiated stimulator cells, sufficient help might be provided by the response of the irradiated T helper cells in the stimulator cell population to alloantigens on responder cells.

Both T cell subsets following stimulation in MLC developed the capacity to procure very rapid graft rejection in normal hosts. In irradiated hosts, however, the W3/25$^+$ subset of cells caused graft rejection in 10-15d (similar to the tempo of rejection procured by stimulated whole LNC in irradiated hosts), whereas stimulated MRC OX8$^+$ cells did not cause rejection for 22-25d. Although the addition of syngeneic naive MRC OX8$^+$ and PE cells to MLC stimulated W3/25$^+$ cells marginally increased their efficiency in irradiated hosts, added cell populations did not similarly increase the efficiency of

MLC stimulated MRC $OX8^+$ populations. Thus neither T cell subset, separated and then sensitized to graft antigens in MLC, had the same capacity as whole T cells stimulated in MLC to collaborate with added PE cells and MRC $OX8^+$ cells and procure rapid graft rejection in irradiated DA rats. Although each subset proliferated in MLC in the absence of the other their in vivo functional activity was apparently impaired. This indicates either that the conditions for the generation of in vivo effector cells were not satisfied in cultures in which either the helper or cytotoxic subset of the responding strain was absent although the conditions for proliferation were met, or that both subsets are required to collaborate with host strain cells and bring about in vivo graft rejection.

Heidecke et al (1984) reported that individual T cell subsets separated from sensitized spleen cells had reduced capacity to cause rejection when compared with unseparated cells but showed normal capacity when recombined. This suggests that both subsets are required for maximum efficiency in vivo. This conclusion was not supported by the studies reported here. It was found that when T cell subsets were separated after stimulation in MLCs set up with whole T cells, each subset showed the same capacity to accelerate graft rejection as the equivalent number of whole MLC cells when each was given to irradiated hosts with PE and MRC $OX8^+$ cells. However, although both T cell subsets mediated equally rapid graft rejection when given with added host cells (MST 6-7d in both cases), the $W3/25^+$ subset when given alone procured more rapid graft rejection than the MRC $OX8^+$ subset alone (MST 10d and 12.5d respectively).

The latter studies were done with responder cells from DA strain rats. The MRC $OX8^+$ cells separated from MLC were tested for in vitro cytotoxicity and found to be non-reactive which supported the

previous conclusion that failure to demonstrate CML with MLC sensitised DA cells was not merely due to the relatively lesser number of cytotoxic/suppressor cells in this strain. WF cells sensitized in MLC to PVG alloantigens which were demonstrably cytotoxic *in vitro* to PVG target cells were also separated into their constituent W3/25$^+$ and MRC OX8$^+$ subpopulations and tested *in vivo*. As has been previously discussed MLC sensitised cells of the WF strain have the capacity to cause rapid graft rejection in irradiated recipients in the absence of host cells. When each of the T cell subsets was injected locally into PVG grafts in irradiated WF hosts it was found that the W3/25$^+$ cells procured rejection at the same rate as whole MLC cells containing the same number of W3/25$^+$ cells (5d) but MRC OX8$^+$ cells also given in the numbers present in the whole inocula which were tested resulted in the slower rejection (8.5d).

One of the explanations which has been proffered for findings in adoptive transfer models in which the rejecting hearts in irradiated animals restored with W3/25$^+$ cell populations are found to contain large numbers of MRC OX8$^+$ cells is that Th cells procure graft rejection *in vivo* as a consequence of their capacity to recruit unstimulated host cells to the graft site. This is supported by studies on the capacity of both MLC sensitized spleen cells and *in vivo* primed spleen cells to recruit cells into sponge matrix allografts. It was found that sensitized cells with no demonstrable cytolysis were slightly more effective than cytotoxic cells in mediating the recruitment of unstimulated lymphocytes to a site of interaction between antigen and specifically sensitized cells in vivo (Hanto et al, 1982; 1983). Depletion of Lyt 2$^+$ (Tc) cells from day 5 MLC cells abrogated cytotoxicity but not the capacity of the cells to

mediate recruitment whereas depletion of Lyt 1^+ (Th) cells slightly but significantly reduced recruitment mediated by the remaining cells (Hanto et al, 1982). This group had earlier shown that the interaction between sensitized cells and specific alloantigen resulted in an increase in vascular permeability and regional blood flow which suggests that the recruitment process is mediated by lymphokine release from sensitized cells (Hopt et al, 1981).

The notion that the greater efficiency of Th cells in procuring in vivo graft rejection is a consequence of their greater capacity to recruit unstimulated host cells to the site of interaction appears to be supported by the findings in irradiated hosts. In these, when host PE cells and MRC $OX8^+$ cells, which might abrogate the necessity for additional host cell recruitment, are given with the inoculum of MLC sensitised cells, whole MLC cells, the $W3/25^+$ or MRC $OX8^+$ cells separated from whole MLC cells led to rejection with the same rapid tempo. When MLC sensitized populations are given without additional host cells, inocula containing the $W3/25^+$ subset cause rejection more rapidly than those from which this subset has been removed. In irradiated WF animals the $W3/25^+$ subset also mediates rejection more rapidly than the MRC $OX8^+$ subset.

The experiments in DA strain animals suggest that both the Th and Tc subsets of MLC sensitised cells may be equally efficient at inducing the differentiation of naive Tc to effector Tc via IL2 release when the cells are injected with PE cells and both the Th and Tc subset have been shown to release IL2 (Luger et al, 1982; Andrus et al, 1984). As the provision of naive MRC $OX8^+$ cells appears to restore rapid graft rejection in irradiated DA hosts provided with MLC sensitised cells and PE cells it is tempting to conclude that the cells which actually effect rejection are derived from the MRC $OX8^+$

population, the PE cells simply providing a source of lymphokine and the MLC cells the helper factors required to promote the response.

In DA hosts one can postulate the need for naive strain MRC $OX8^+$ cells to generate killer cells which ultimately take out the graft. This postulate is possible on the basis that DA MLC cells had no demonstrable cytolytic activity and therefore it can be inferred that *in vitro* conditions for sensitization were not optimal for the generation of *in vitro* effector Tc in this strain whereas given the right circumstances *in vivo* they could be generated and may in fact be the *in vivo* effector cell. Experimental results certainly demonstrate an absolute requirement for naive MRC $OX8^+$ cells. This hypothesis unfortunately does not translate well to the WF strain where WF MLC cells were demonstrably cytotoxic *in vitro* and the $W3/25^+$ subset, not the MRC $OX8^+$ subset, of MLC cells was found to procure very rapid graft rejection *in vivo*. If one postulates that $W3/25^+$ MLC cells function to recruit regenerating and/or radioresistant host cells more efficiently than MRC $OX8^+$ cells do, then the question to be addressed is - which cell population is being recruited and what is its function? It would be simpler to conclude that $W3/25^+$ MLC cells themselves mediate graft rejection however experimental data from the DA strain does not support this.

It is clear that the data reported in this thesis does not allow the identification of the cell population responsible for effecting the destruction of allogeneic grafts. The finding that the $W3/25^+$ subpopulation of WF MLC cells mediated rapid rejection in irradiated hosts was made recently and thus the cellular requirements for rapid graft rejection in this strain still require further investigation. It will be interesting to see if host strain macrophages and/or naive MRC $OX8^+$ cells have an effect on the slower tempo of rejection

procured by the MRC $OX8^+$ subset of WF MLC cells.

In the graft model described here it is not possible to exclude a role for macrophages in the direct mediation of graft rejection however there is suggestive evidence that they do not themselves mediate graft rejection. If macrophages were the primary mediators of graft rejection in this model then the injection of both MLC cells and macrophages should have been sufficient to cause rapid graft rejection. Most evidence indicates that macrophages are activated by immune T cells or their products or by a local inflammatory reaction to both phagocytic and cytocidal function (Cerottini and Brunner, 1974; Biondi et al, 1984; Ununue et al, 1984). The presence of MLC cells and specific antigen should have provided sufficient stimulation for macrophages to mediate tissue damage via a DTH type mechanism. This was apparently not the case. The fact that whole MLC cells did not stimulate macrophages to mediate graft rejection also makes it highly unlikely that either MLC T cell subset would do so. T helper cell clones can very effectively induce DTH reactions in vivo (Bianchi et al, 1981) so it could be assumed that MLC activated cells have the capacity to stimulate macrophages to non-specific tissue damage. The evidence in the model presented here, however, is not suggestive of a direct role for macrophages, activated by immune MLC cells and graft antigen, in the mediation of graft rejection as there was a strict requirement for the concomitant provision of naive MRC $OX8^+$ cells.

The CML assay is at best a simple representation of in vivo graft rejection and single cell targets in no way resemble the obstacle that a solid tissue graft presents by virtue of its size and cellular composition. Consequently the in vivo requirements for graft rejection may be quite different from the cellular requirements

necessary to maintain the "cytotoxic state". Experiments demonstrating the enhanced activity of sensitized spleen cells and T cell subsets in the presence of IL2 rich supernatants in the mediation of graft rejection (Clason et al, 1982; Lear et al, 1983; Heidecke et al, 1984) suggest a requirement for further "boosting" of the response and this need for continued immune stimulation may well be the major difference between the requirements for in vivo and in vitro cellular cytotoxicity. The multiplicity of cell types which are present at a graft site may be a reflection of this.

Of particular interest therefore, is the role, if any, of soluble mediators in this model. It would be of great interest to establish if IL1 may substitute for macrophages or IL2 for immune T cells in the cell mixing experiments in irradiated DA rats. If the major role of PE cells is in the provision of a further soluble signal to boost the alloreactivity of quiescent DA MLC cells then IL1 containing supernatants should substitute for macrophages in the cellular inoculum of PE cells, MLC cells and naive MRC $OX8^+$ cells. Similarly if graft rejection is mediated directly by added naive MRC $OX8^+$ cells which have differentiated in the presence of immune MLC cells and specific antigen then it is possible that the soluble products from immune T cells may be sufficient to activate naive MRC $OX8^+$ cells to graft destruction. These experiments are technically difficult. Because of the short half-life of IL1 and IL2 and their rapid diffusion in vivo it may be impossible to mimic the physiological production of these products in an experimental model.

In the NNH graft model it is difficult to ascribe a direct effector role to sensitized $W3/25^+$ (Th) cells. Most authors who postulate the Th cell as the mediator of graft rejection envisage a DTH-type reaction in which non-specific tissue damage is mediated by

macrophages which have been activated by specifically sensitized cells. In this model even when there was a very high local concentration of macrophages and immune Th cells graft rejection did not occur. Other workers have ascribed a direct cytotoxic function to Th cells. In this case cytotoxicity has been directed against Class II alloantigens on target cells (Swain and Panfilli, 1979; Vidovic et al, 1981; Meuer et al, 1982, Spits et al, 1983) in contrast to Tc mediated cytotoxicity which is directed against Class I alloantigens (Cantor and Boyse, 1975). As has already been discussed, NNH tissue is difficient in Class II bearing cells; this would presumably make it a poor target for Th cell mediated cytotoxicity. However local immune reactions have been shown to induce Class II or Ia antigens on VE cells (Pober et al, 1983) and epidermal cells (Barclay and Mason, 1982). This could then increase the potential targets for Th cell mediated graft damage and is an attractive premise. Such a mechanism could well operate in WF strain rats in which rapid graft rejection in irradiated hosts can be procured by sensitized W3/25$^+$ cells but not by sensitized MRC OX8$^+$ cells. The requirements for naive MRC OX8$^+$ cells and PE cells in DA hosts is harder to reconcile with the concept that W3/25$^+$ cells are the mediators of rejection unless it is suggested that the additional cells are required to sustain the local immune response which induces Class II antigens on the graft and thereby provides targets for the sensitized T cells.

An understanding of the biological basis for the differences between DA and WF strain rats in the cellular requirements for rapid graft rejection may allow a more rational interpretation of the findings in the NNH graft model. Examination of the requirements for rejection in other strain combinations, and in particular in those RT1 dissimilar combinations which other workers (Howard and Butcher,

1981; Butcher and Howard, 1982) have used to study the genetic control of allo-immune responsiveness, may reveal whether the differences between DA and WF responses reflect genetically determined differences in immune responsiveness. The other possibility is that they reflect differences in radiosensitivity or regenerative capacity of the lymphoid and mononuclear phagocyte populations in different rat strains. The relative radiosensitivity and tempo of regeneration following WBI of the lymphocyte subsets and of mononuclear phagocytes will be compared in various rat strains. The changes in the pattern and tempo of regeneration which might be brought about by the implantation of an allogeneic graft and/or the local or systemic injection of various cell populations will be examined.

The results reported in this thesis confirm that the T helper cell population plays a central role in allograft rejection. They strongly suggest that the cytotoxic/suppressor T cell population and cells of the mononuclear phagocyte system, which have been observed by other workers to be present in rejecting grafts, are not mere bystanders but are also essential for graft rejection. It would appear that all three cell types may be required to sustain the immune response for the period of time necessary to effect tissue destruction. The question of which of them is the direct mediator of destruction remains unanswered.

CHAPTER 5

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