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LYMPHOID EFFECTOR CELLS IN INTESTINAL  
GRAFT-VERSUS-HOST REACTION

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Doctor in Philosophy to the Faculty of Science of  
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## SUMMARY.

The aim of this thesis was to examine some of the lymphoid effector cells which are involved in intestinal cell-mediated immune responses as exemplified by the intestinal phase of graft-versus-host reaction (GvHR). The cells which were studied were natural killer (NK) cells and intraepithelial lymphocytes (IEL), both of which are found<sup>in</sup><sub>^</sub> increased numbers during a GvHR.

The first experiments examined whether a GvHR in experimental mice could be used to assess the cell-mediated immune effector potentials of different types of donor lymphoid cell. Both parental MLN and spleen cells caused splenomegaly and enhancement of NK activity in unirradiated F<sub>1</sub> hybrid mice, but MLN showed a greater ability to induce the systemic changes of GvHR. This was not merely due to a larger proportion of T cells in MLN but was related to the greater ability of MLN cells to recirculate into host lymphoid tissues. The induction of splenomegaly and NK cell activation in GvHR were shown to require donor cell proliferation, as these alterations were prevented by treating donor cells with mitomycin C. These findings confirmed that the GvHR could be used to assess donor cell effector potential in vivo and highlighted the importance of donor cell proliferation and recirculation.

The role of donor NK cells in the development of systemic and intestinal GvHR was examined by using NK cell-deficient beige mice as a source of donor cells.

Beige spleen cells were unable to induce either acute or proliferative forms of systemic GvHR in unirradiated mice and could not produce a local GvHR as measured by popliteal lymph node hypertrophy. Furthermore, beige spleen cells induced a milder form of intestinal GvHR than that found after injection of normal, congenic C57BL/6 (B6) spleen cells. However, beige spleen cells were as efficient as B6 cells in inducing a lethal GvHR in irradiated hosts. Although these results suggested an important role for NK cells in the effector phase of GvHR, subsequent examination showed that beige mice have an additional defect in the generation of cytotoxic T lymphocytes (CTL) *in vitro* and *in vivo*. In contrast, beige mice had normal DTH responses *in vivo* and high proliferative responses *in vitro*. Therefore, the inability of beige spleen cells to mediate a GvHR in unirradiated mice may not only reflect their lack of NK cell function but may also be due to an associated defect in T cell function. These findings may also indicate that cytotoxic lymphocytes play an important part in the development of GvHR.

The role of IEL in the development of intestinal damage was also approached by studying their alloreactivity *in vivo*. The data presented here confirmed that IEL were capable of inducing a local GvHR as measured by popliteal lymph node hypertrophy or after intraperitoneal injection. Nevertheless, they failed to induce a systemic GvHR after intravenous injection into irradiated recipients. The fact that donor cells require to enter host lymphoid tissue in order to

mediate alloreactive immune responses led me to compare the migration pathways of intravenously injected IEL with to those of other conventional lymphoid populations. These studies showed that IEL failed to enter lymphoid tissues in vivo, and accumulated predominantly in the liver. This abnormal recirculation was not due to the procedure used to isolate IEL and could not be explained by poor viability or low proliferative capacity. Furthermore, IEL did not migrate in vivo like immature lymphocytes or activated peripheral lymph node cells. In contrast to these findings, IEL had a polarised morphology and an excellent locomotor capacity in vitro. These characteristics of activated cells led me to examine if IEL could migrate into sites of inflammation in the same way as other activated lymphocytes. These last series of experiments showed that IEL were able to localise in sites of inflamed skin but failed to accumulate in intestine which had been inflamed by a GvHR or a parasite infection.

Together, my results are consistent with the idea that the heterogenous population of IEL contains a large proportion of fully differentiated effector cells.

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A Alberto, por supuesto.

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# DECLARATION.

These studies represent original work carried out by the author, and have not been submitted in any form to any other University. Where used has been made of material provided by others, due acknowledgement has been made.

October, 1987.

Maria Eugenia Baca Trespalacios.

## PUBLICATIONS

Parts of this thesis have been used in the following publications.

Mowat, A. McI., MacKenzie, S., Baca, M. E., Felstein, M. V. and Parrott, D. M. V. (1986). Functional characteristics of intraepithelial lymphocytes from mouse small intestine. II. In vivo and in vitro responses of intraepithelial lymphocytes to mitogenic and allogeneic stimuli. *Immunology* 58: 627-634.

Baca, M. E., Mowat, A. McI., MacKenzie, S. and Parrott, D. M. V. (1987). Functional characteristics of intraepithelial lymphocytes from mouse small intestine. III. Inability of intraepithelial lymphocytes to induce a systemic graft-versus-host reaction is because of a failure to migrate in vivo. *Gut* 28: 1267-1274.

Mowat, A. McI., McInnes, I. B., Baca, M. E. and Parrott, D. M. V. (1987). Proliferative capacity and recirculation pathways of murine intraepithelial lymphocytes. *Proc. NFIC Research Conference*. In press.

# Abbreviations used in the text.

ALN	:	axillary lymph nodes
B cell	:	bone marrow derived lymphocyte
CCPR	:	crypt cell production rate
CMI	:	cell-mediated immunity
Con A	:	concanavalin A
cpm	:	counts per minute
<sup>51</sup> Cr	:	chromium-51
CTL	:	cytotoxic T lymphocyte
DLN	:	draining lymph nodes
DTH	:	delayed-type hypersensitivity
F1	:	first generation
FCS	:	foetal calf serum
FITC	:	fluorescein isothiocyanate
GALT	:	gut associated lymphoid tissue
GvHR	:	graft-versus-host reaction
H-2	:	murine major histocompatibility complex
H&E	:	haematoxylin and eosin
IFN	:	interferon.
<sup>3</sup> H-TdR	:	tritiated thymidine
i.d.	:	intradermal
Ig	:	immunoglobulin
IEL	:	intraepithelial lymphocytes
IL-2	:	interleukin 2
i.p.	:	intraperitoneal
LGL	:	large granular lymphocytes
LPL	:	lamina propria lymphocytes
<sup>125</sup> IUdR	:	<sup>125</sup> I-iododeoxyuridine
Lyt	:	T lymphocyte associated antigen
MHC	:	major histocompatibility complex
MLN	:	mesenteric lymph nodes
MLR	:	mixed lymphocyte reactions
NCS	:	newborn calf serum
NK	:	natural killer
p	:	probability
PLN	:	peripheral lymph nodes
T cell	:	thymus derived lymphocyte
Th/i	:	helper/inducer T lymphocyte
Ts/c	:	suppressor/cytotoxic T lymphocyte
TDL	:	thoracic duct lymph
Thy 1.2	:	murine T lymphocyte-specific alloantigen
i.v.	:	intravenous
MMC	:	mucosal mast cells.
APC	:	antigen presenting cell.

Abbreviations of measures:

weight	:	g = gram
		mg = milligram
		µg = microgram
volume	:	l = litre
		ml = millilitre

$\mu\text{l}$  = microlitre  
length :     $\text{mm}$  = millimetre  
              $\mu\text{m}$  = micrometre

CHAPTER 1  
INTRODUCTION

## CELL-MEDIATED IMMUNITY AND THE SMALL INTESTINE.

Cell-mediated immunity (CMI) is that form of the immune response which involves T lymphocytes, rather than immune reactions in which antibody is involved and is probably the principal host defence mechanism against many intracellular pathogens, including viruses, parasites and several bacteria (Roitt 1984). Although activation of antigen-specific T cells is the necessary initiator of a CMI reaction, activation and recruitment of many other, non-specific effector cells, such as macrophages, NK cells, mast cells, polymorphonuclear cells and eosinophils is also a characteristic feature of these responses (Roitt, 1984). Under certain conditions, the activation of these non-specific effector mechanisms can cause severe damage to host tissues (Roitt, 1984).

The small intestine is constantly exposed to an enormous number of potentially pathogenic intracellular organisms and has evolved a complex local immune system to cope with this antigenic load. Therefore, it might be anticipated that CMI responses are an important component of intestinal defences against invasion. This hypothesis has been confirmed in several parasitic diseases (Ferguson and Jarrett, 1975; MacDonald and Ferguson, 1978; Manson-Smith, Bruce and Parrott, 1979) and it has been shown that these local responses are associated with severe intestinal damage (MacDonald and Ferguson 1978). Furthermore, certain clinical

enteropathies such as coeliac disease and cow's milk protein intolerance, as well as the intestinal changes found in bone marrow transplantation patients with GvHR, are associated with intestinal as well as a local CMI response (Mowat, 1984). Therefore, it is important to understand the mechanisms by which initially useful intestinal immune responses can result in local pathology.

The pathological features of intestinal CMI responses have been studied in several animal models including graft-versus-host reaction, allograft rejection and induction of CMI to dietary protein antigens (Ferguson and Parrott, 1973; MacDonald and Ferguson, 1977; Mowat and Ferguson, 1981;1982). These studies have revealed a characteristic pattern of mucosal alterations which includes villus atrophy, crypt hyperplasia and increased lymphocytic infiltration of the epithelium (Mowat and Ferguson, 1981) and which may be accompanied by recruitment of local NK cells and MMC (Borland, Mowat and Parrott, 1983; Mowat and Ferguson, 1982). Although, studies of murine GvHR have suggested that a local DTH response causes the pathology, it is not known which particular effector mechanisms are responsible for the tissue damage. The purpose of this thesis is to examine the possible role of two effector mechanisms in intestinal CMI responses, intraepithelial lymphocytes (IEL) and NK cells.

## GRAFT-VERSUS-HOST REACTIONS.

Elucidation of the effector mechanisms involved in the pathology of CMI responses requires a reproducible experimental model in which defined alterations in immune function are associated with different forms of tissue damage. Graft-versus-host reactions comprise the pathological and immunological consequences of the recognition of host tissues by grafted immunocompetent T cells and have interested both clinicians and scientists, partly because GvHR is the principal complication of allogeneic bone marrow transplantation (Slavin and Santos, 1973) and partly because it provides a useful model to study mechanisms of immune regulation and immunopathology (Gleichmann et al., 1984; Bril and Benner, 1985). This is especially so for the intestine, which is a major target organ in GvHR.

The outcome of a GvHR involves a wide spectrum of pathological features and reflects complex interactions between both donor and host elements. The consequences depend on the immunological state of the host, as well as the nature of the donor T cells used to induce the reaction and on the degree of histoincompatibility between donor and recipient. Furthermore, a variety of specific anti-host immune mechanisms have been described in the effector phase of the GvHR including cytotoxic T lymphocyte (CTL) activity (Cerottini, Nordin and Brunner, 1971; Singh, Sabbadini and Sehon, 1972; Kubota, Ishikawa and Saito, 1983), autoantibodies (Gleichmann et al., 1984) and delayed type hypersensitivity (DTH) (Wolters and Benner, 1978),

while several non-specific mechanisms such as NK cells and macrophages (Zinkernagel, 1980) are also activated as a consequence of the initial T cell activation. The following sections will review briefly the immunobiology and pathological consequences of this complex reaction in experimental models.

#### Immunological mechanisms in GvHR.

##### Genetic basis of GvHR.

It has long been known that one of the most important requirements for the initiation of GvHR is a difference between the host and donor at the MHC (Billingham, 1955). More recently, the development of MHC congenic strains and mice carrying defined MHC mutations have allowed more detailed study of the MHC differences which induce a GvHR. Although both Class I (H-2 K/D) and Class II (Ia) MHC differences can lead to systemic GvHR in mice, Class II MHC differences tend to be more efficient in terms of mortality (Klein and Park, 1973; Elkins, 1976; Klein and Chiang, 1976; Klein, 1978; Jadus and Peck, 1983b), splenomegaly (Klein, 1977), macrophage activation (Zinkernagel, 1980), and anti-host DTH activity (Wolters and Benner, 1979). In addition, the intestinal phase of GvHR is entirely Class II restricted (Piguet, 1985; Mowat, Borland and Parrott, 1986). The ability of Class I MHC differences to induce a GvHR is more variable, and depends on the assay used, as well as the particular antigen involved. Allelic Class I differences in congenic mice elicit

minimal T cell proliferation in vivo (Elkins, 1976), and cause only very mild GvHR in sublethally irradiated recipients (Klein and Chiang, 1976). Nevertheless, severe mortality can occur in heavily irradiated recipients (Korngold and Sprent, 1985). The induction of anti-host DTH activity or splenomegaly across a Class I difference is normally of low or moderate intensity, depending on the allele concerned (Klein and Park, 1973; Mowat et al., 1986a) and there are usually no intestinal or other specific pathological consequences during a GvHR in Class I congenic mice (Gleichmann et al., 1984; Mowat et al., 1986a). In contrast, mutant Class I molecules elicit quite strong responses as measured by the induction of splenomegaly and lethal GvHR (Klein, 1978; Korngold and Sprent, 1985). Furthermore, several studies have shown that a combination of Class I and Class II differences is required for full-blown GvHR (Wolters and Benner, 1981; Gleichmann et al., 1984). Finally, it has also been shown that a strong GvHR can be induced across some minor histocompatibility antigens (Korngold and Sprent, 1978), with the Mls locus being a particular strong inducer of GvHR (Bril and Benner, 1985).

#### Role of T cell subsets in GvHR.

The relative roles of Tc/s and Th/i in GvHR is highly controversial and depends on many of the factors discussed above. Most studies have approached this issue by separating Th/i and Tc/s on the basis of their expression of specific surface markers. In the initial

studies of Cantor and Boyse (1975a, b) it was shown that  $\text{Lyt } 1^+2^-$  ( $\text{CD8}^-$ ) cells were the principal responders in both MLR and GvHR whereas  $\text{Lyt } 1^-2^+$  ( $\text{CD8}^+$ ) cells accounted for the resulting allospecific CTL responses. Subsequently, it was shown that both  $\text{Lyt } 1^+2^-$  T and  $\text{Lyt } 1^-2^+$  cells were required for full splenomegaly and mortality, although  $\text{Lyt } 2^-$  cells sometimes are effective alone (Mage and Hugh, 1973; Mage et al., 1981; Valleria, Soderling and Kersy, 1982; Glinchmann et al., 1984). In the rat, it has also been shown that  $\text{CD4}^+$  cells are the principal inducers of systemic GvHR in irradiated hosts, although  $\text{CD8}^+$  cells may also play an important secondary role (Mason, 1981; Barclay and Mason, 1982). In parallel with its Class II restriction, the intestinal phase of murine GvHR appears to depend on  $\text{Lyt } 2^-$  cells (Mowat et al., 1986a).

More recent studies in mice have used the expression of L3T4 as a non-overlapping marker of Class II-restricted murine T helper cells (Korngold and Sprent, 1985). This work has shown that depletion of  $\text{L3T4}^+$  cells from the donor inoculum consistently prolongs host survival during a GvHR across a full MHC incompatibility. In contrast, depletion of  $\text{Lyt2}^+$  cells had a more variable effect depending on the strain used (Korngold and Sprent, 1985). These results have been confirmed in our own laboratory. When highly purified  $\text{L3T4}^+$  and  $\text{Lyt } 2^+$  T cells subsets were compared for their capacity to induce a GvHR across isolated Class I or Class II MHC barriers in congenic or mutant mice, it was

shown that  $\text{Lyt } 2^+$  cells responded strongly and uniquely to Class I differences, while  $\text{L3T4}^+$  cells could not induce mortality or intestinal GvHR across Class I differences but showed strong responses to Class II alloantigens (Korngold and Sprent, 1985; Sprent *et al.*, 1986). Thus, it appears that both subpopulations of T cells are capable of inducing GvHR across the appropriate MHC antigens (Sprent *et al.*, 1986), but it is not clear whether the mechanisms utilised by each subpopulation are identical. Nevertheless, it should be noted that most studies of GvHR across full MHC differences emphasise the role of Class II restricted cells, which are believed to be Th/1.

#### Pathological features of GvHR.

The exact pathological consequences of a GvHR depend on the age and immunological status of the host, as well as on the MHC disparity between host and donor and the type of T cell used to elicit the reaction. However, the principal target organs in most cases are the bone marrow, the lymphoid system, the liver, the gut and the skin. In addition, there may be lesions in other tissues including the pancreas and the salivary glands (Elkins, 1971; Grebe and Streilein, 1976; Seemayer, Lapp and Bolande, 1977; Beschorner, Tutschka and Santos, 1982a; Lapp *et al.*, 1985).

Two distinct forms of GvHR have been described following allogeneic bone marrow transplantation (BMT) in man. In the acute disease there is necrosis of the liver, skin, gut and lymphoid tissue, while the chronic

form is characterised by proliferative changes in the same organs leading to sclerosis and fibrosis. Finally, a state of severe immunodeficiency may result (Seemayer *et al.*, 1977; Lapp *et al.*, 1985). which may be due to  $T_S$  or thymic damage (Seddik, Seemayer and Lapp, 1984; Lapp *et al.*, 1985). Although similar patterns of disease have been described in rats (Beschoner *et al.*, 1982ab), the pathogenic mechanisms have not been established in this system. There is no real equivalent of the human disorder in mice, but Gleichmann and co-workers have identified a model in which different pathological consequences occur and appear to be due to different effector mechanisms (Gleichman *et al.*, 1984). The most severe form of this GvHR is an acute disorder associated with weight loss, liver and gut necrosis, as well as lymphoid hypoplasia, bone marrow failure and frequently death. Both  $Lyt\ 2^+$  and  $Lyt\ 2^-$  cells recognising a combination of Class I and II MHC differences are required for this acute GvHR and it has been suggested that suppressor T cells, rather than CTL are the effector cells involved (Gleichmann *et al.*, 1984). The chronic form of this GvHR model is an entirely proliferative disease which results in persistent hyperplasia of B lymphocytes leading to hypergammaglobulinemia and the formation of several auto antibodies as well as immune complexes. As a result, affected mice develop glomerulonephritis, and immunoglobulin deposits along the basement membrane of the skin (Gleichmann *et al.*, 1984). Other signs characteristic of collagen vascular diseases also occur,

including arteritis, polyarthrititis, Sjogren and scleroderma-like lesions, and liver disease (van Rappard-van der Veen et al., 1983; Fals, Radaszewicz and Gleichmann, 1984). This chronic GvHR is induced by Lyt 2<sup>+</sup> cells recognising isolated Class II MHC differences and appears to be mediated by prolonged activation of allospecific donor T helper cells. These findings illustrate the wide range of pathological consequences which can occur in a GvHR and the potential usefulness of the GvHR as a flexible model to study the immunopathological functions of different CMI effector cells.

As the outcome of a GvHR depends on the model used, techniques to measure the intensity of GvHR must be chosen carefully and must be appropriate to the particular model. The assays used most often to assess an acute, destructive GvHR are weight loss and mortality assays, while more proliferative forms of GvHR are usually assessed by assays of lymphoid hyperplasia, such as splenomegaly or popliteal lymph node hypertrophy (Simonsen, 1957; Ford, Burr and Simonsen, 1970).

#### Effector mechanisms in GvHR.

Although, CTL have been generally assumed to play a major role in tissue lesions during a GvHR (Elkins, 1976, 1978), many workers have found no correlation between lethal GvHR and the development of CTL (Jadus and Peck, 1983b; Hamilton, 1984). However, it has been reported that the development of GvHR does correlate

with anti host DTH responses (Wolters and Bener, 1978); as has also been suggested for in allograft rejection (Loveland and McKenzie, 1982).

Immunosuppression is a feature of various models of GvHR (Howard and Woodruff, 1961; Blaese, 1964; Tutschka et al., 1980; Gleichmann et al., 1984;). As noted above, allospecific anti-host suppressor T cells have been shown in some models (Pals et al., 1984) but non-specific suppression is a more frequent finding and seems to be mediated by an increase in prostaglandins (Lapp et al., 1980), IFN (Zawatsky et al., 1979). The role of disordered immunoregulation in the tissue pathology is not clear, but the studies of Gleichmann illustrate the potential effects of Ts and Th in mediating specific immunopathology. In addition to these specific mechanisms, activation of non-specific mechanisms such as NK cells of GvHR (Roy et al., 1982; Borland et al., 1983; Kubota et al., 1983) and macrophages (Zinkernagel, 1980) correlate with the development of GvHR. Prevention of lethal GvHR by anti-AsGM1 treatment supports the evidence that NK cells may contribute to the pathological disorders of GvHR (Charley et al., 1983).

#### Intestinal phase of GvHR.

Intestinal damage is an almost invariable clinical and experimental feature of GvHR, but the degree and nature of the damage depends on the severity of the reaction. At one extreme, the intestinal alterations may consist only of minor changes in mucosal architecture

such as transient increases in crypt cell production rate (CCPR) and crypt length (Mowat and Ferguson, 1982). In a severe GvHR, the early stages are characterised by a similar proliferative phase (Mowat et al., submitted) but this is followed by the development of more destructive changes such as villous atrophy and mucosal degeneration and necrosis (Reilly and Kirsner, 1965; MacDonald and Ferguson, 1977). The resulting malabsorption is an important cause of death in animals with GvHR (Reilly and Kirsner, 1985) and the intestinal phase of GvHR contributes significantly to the morbidity and mortality of patients developing GvHR following bone marrow transplantation (Slavin and Santos 1973; Glucksberg et al., 1974). The changes in mucosal architecture are accompanied by infiltration of lymphocytes and other mononuclear cells (Slavin and Santos, 1973; Woodruff et al., 1976; Guy Grand et al., 1978) includes MMC and NK cells. An increased density of IEL is a particular feature (Mowat and Ferguson, 1982) and infiltrating IEL in GvHR have an increased mitotic activity (Ferguson et al., 1984) and increased NK activity (Borland et al., 1983).

The pathological changes of intestinal GvHR are similar to those occurring during clinical enteropathies (Ferguson, McClure and Townley, 1976; Phillips et al., 1979) the intestinal phase of GvHR has been suggested as a good model to study the mechanisms of intestinal damage due to local CMI. The mechanisms responsible for the intestinal damage in GvHR are still poorly

understood but of studies in unirradiated mice show that intestinal damage occurs in the absence of specific CTL (Borland et al., 1983). In addition, intestinal pathology evolves in parallel with the proliferative changes of systemic GvHR (Mowat and Ferguson 1982) and occurs as a bystander phenomenon in grafts of foetal intestine which are syngeneic to the donor cells used to induce the GvHR (Elson, Reilly and Rosenberg, 1977; Mowat and Ferguson, 1981). Finally, the intestinal damage is induced by Lyt 2<sup>+</sup> cells which are activated by Class II MHC alloantigens carried on bone marrow-derived cells (Mowat 1986; Mowat et al., 1986a). CTL are found in association with the more destructive intestinal GvHR which occurs in irradiated mice, this intestinal pathology is also I-A restricted (Piguet 1985) and occurs as a bystander phenomenon (Mowat et al., submitted for publication). Together, these findings suggest that DTH responses are responsible for the intestinal phase of GvHR and it has been proposed that T cell derived lymphokines may be directly responsible for the tissue damage (Mowat et al., 1986a). However, the recruitment of NK cells and MMC during the the mucosal phase of GvHR raises the possibility that non-specific effector cells may be the final mediators of the intestinal pathology. This thesis attempts to address the role of local effector cells in the pathogenesis of intestinal CMI responses, by investigating the immune effector potentials of IEL and NK cells.

## NATURAL KILLER CELLS.

Natural killer (NK) cells are effector cells which are capable of rapid, non-specific cytotoxicity against a range of target cells, without evidence of MHC restriction or immunological memory (Becker, Fenyó and Klein, 1976). Natural cytotoxicity was first discovered when it was found that lymphocytes from normal unimmunised individuals could lyse certain tumour cells *in vitro* (Herberman *et al.*, 1973; Oldham *et al.*, 1973; Rosenberg *et al.*, 1974). Since then, natural killer cells have been recognised as a distinct subpopulation of lymphocytes in virtually all species examined (Herberman *et al.*, 1973; Oldham *et al.*, 1973; Rosenberg, 1974). Furthermore, although initial studies concentrated on the possibility that this new lymphocyte population represented a natural immunosurveillance mechanism against tumours *in vivo*, more recent work has indicated that NK cells have a wide range of regulatory and effector functions in many different types of immune response (Reynolds and Ortaldo, 1987).

### Morphological and phenotypic characteristics of NK cells.

NK activity is associated with a distinct subpopulation of lymphocytes which can be identified morphologically as large granular lymphocytes (LGL) (Timonen *et al.*, 1981; Reynolds *et al.*, 1981; Kumugai *et al.*, 1981). LGL have been found in all vertebrates tested (Herberman and Holden, 1978). and are larger than

conventional small lymphocytes with a diameter of 16-20um (Ortaldo and Herberman, 1984), a slightly indented nucleus and several azurophilic cytoplasmic granules. These granules appear to play an important role in the target lysis and they have been shown to contain a variety of cytotoxic factors such as cytolytins, perforins, proteases and esterases (Koren *et al.*, 1987; Herberman, Reynolds and Ortaldo, 1986).

NK cells are non-adherent and non-phagocytic cells which express a complex range of surface markers, some of which are shared with other cell types and others which are partly or uniquely restricted to NK cells. The situation is complicated by the fact that natural cytotoxicity can be mediated by a heterogeneous group of cells, including activated T cells and macrophages (Grimm *et al.*, 1982; Roitt, Brostoff and Male, 1985). However, studies using highly defined populations show that the majority of human NK cells express the pan T cell markers CD2, but not CD3 (Ortaldo and Herberman, 1984). In rodents, a small proportion of NK cells carry the Thy 1 marker (Ortaldo and Herberman, 1984). In rats, NK cells are CD8<sup>+</sup>, but most NK cells in man and mice do not express Tc/s markers (Reynolds and Ortaldo, 1987). NK cells are uniformly negative for CD4 marker (Ortaldo and Herberman, 1984) and surface immunoglobulin (sIg) but express high levels of the IL-2 receptor (Trinchieri *et al.*, 1984). These findings are complicated by the fact that NK cells also express a variety of myeloid cell markers such as OKM1, Mac1, Fc

receptors and receptors for the C3b fragment of complement (Ortaldo and Herberman 1984). However, they do not express MHC class II antigens. There are now several markers which appear to be NK-cell specific, including CD16 (Leu 11) and NKH-1 (Leu 19) in man and asialo GM1 (AsGM1) and NK-1 in rodents (Reynolds and Ortaldo, 1987).

#### Origin and lineage of NK cells.

Despite the presence of several T cell markers, NK activity is elevated in thymectomised or congenitally athymic animals and their exact lineage is still a matter of controversy (Ortaldo and Herberman, 1984). Recent reports have shown that true NK cells do not express mature T cell receptors (Lanier, Cwinla and Phillips, 1986; Herberman *et al.*, 1986), but like early thymocytes, may express truncated forms of T-cell receptor beta chain mRNA, but do not express alpha chain mRNA (Ritz *et al.*, 1985). These features indicate that NK cells are not related to mature T cells but do not exclude the possibility that NK cells represent an early stage in the T cell lineage. As noted above, NK cells are not dependent on the presence of a thymus, and it has been suggested that NK cells are derived directly from a stem cell in the bone marrow (Haller *et al.*, 1977). This is supported by the fact that NK cell activity is exquisitely sensitive to agents which destroy the bone marrow selectively, like  $^{89}\text{Sr}$  (Haller and Wigzell, 1977).

### Ontogeny of NK cells.

Age has been shown to have an important and consistent effect on the expression of NK cell activity in rodents. NK activity is absent at birth, with detectable levels appearing at 3 weeks of age, before reaching a peak between 5-8 weeks of age and slowly declining thereafter (Kiessling, Klein and Wigzell, 1975a). In germ-free animals NK activity levels are lower than in control animals (Kim *et al.*, 1982; Roder, Karre and Kiessling, 1981). However, it should be noted that NK activity is present in humans from birth (Savary and Lotzova 1986).

### Recognition and lysis of target cells by NK cells.

As noted earlier, NK cells do not exhibit immunological specificity and can react against a wide variety of syngeneic, allogeneic and xenogeneic cells. Furthermore, unlike cytotoxic T lymphocytes, NK cells do not demonstrate MHC restriction (Becker *et al.*, 1976) and, in fact, have strong reactivity against MHC deficient targets. In addition, unlike CTL, the activity of NK cells does not appear to be dependent on antigenic sensitization and no specific memory response has been demonstrated (Ortaldo and Herberman, 1984). Susceptibility to cytotoxic activity is not restricted to malignant cells, as foetal cells, virus infected cells and subpopulations of normal lymphoid or haematopoietic stem cells such as thymus and bone marrow cells, may also be susceptible (Nunn, Herberman and

Holden, 1977; Welsh, Zinkernagel and Hallenberck, 1979; Hansson et al., 1979).

For these reasons, differentiation antigens may be an important group of NK cell-specific target structure. Although this is supported by studies using maturational agents in target cells (Kabelitz and Kunkel, 1983) and with a wide variety of target cells (Herberman, 1982) the nature of the target structure which is recognized by NK cells is not known. The transferrin receptor has been suggested as one such structure (Vodinelich et al., 1983, but recent results do not support this idea (Dokhelar et al., 1984). NK cell activity can be inhibited by lectin-binding sugars and the possibility remains that differential glycosylation of surface proteins on dividing or differentiating cells is the target molecule (Werkmeister, Pross and Roder, 1983). Despite the wide spectrum of tumour target cells that can be lysed, it should be noted that NK cells do show some selectivity in their pattern of killing (Herberman and Ortaldo, 1981). Many tumour cell lines are relatively insensitive to lysis by NK cells and there is some evidence of clonal heterogeneity amongst different subsets of NK cells (Herberman et al., 1986).

The structure which NK cells use to recognise target cells is unknown, but is not the Fc receptor (Perussia et al., 1983). The evidence of limited clonality in some NK populations and the presence of some T cell markers has lead workers to investigate the expression of genes coding for the T cell receptor

(Herberman et al., 1986). Although initial studies suggested that  $CD3^+$  lymphocyte clones with non-specific lytic activity did rearrange T cell receptor genes (Ritz et al., 1985), these cells were probably T cells. More recent work using highly purified rat LGL and freshly isolated  $CD3^-$  LGL in mice, rats and humans have shown no evidence of T cell receptor rearrangements (Reynolds et al., 1985; Herberman et al., 1986), supporting the view that NK cells are not related to mature T cells. The nature of the recognition structure on NK cells remains unclear.

Lysis of sensitive targets is divided into a number of distinct steps and is similar to the mechanism used by CTL (Herberman et al., 1986). The initial step is the binding of the effector to the target cell. After formation of this conjugate, the next step involves triggering of the intracellular process required for lysis. This seems to involve orientation of cytoplasmic granules towards the area of membrane contact with the target cell, followed by release of granule material into the target cell (Herberman et al., 1986). The granules contain a potent calcium-dependent cytolytic material and it has been suggested that these include perforins which cause membrane lesions in the target cells by polymerisation and formation of pores in the lipid layer (Herberman et al., 1986). The ultrastructural appearance of the ring structures which are found in the target cell membrane is thus analogous to the insertion of the terminal components of the

complement cascade (C9). However, NK cells contain many other potentially cytotoxic molecules such as esterases, beta-glucuronidase and an NK-cell specific cytotoxic factor (NKCF) which shares the target cell specificity of the cells themselves. The exact role of all these mediators in target cell lysis remains obscure (Koren et al., 1987).

#### Regulation of NK cell activity.

Several studies have indicated that there may be a genetic influence on the levels of NK cell activity. In mice, high NK activity appears to be inherited as a dominant trait (Petranyi, Kiessling and Klein, 1975). Mouse NK cell activity may be regulated partly by a gene within the H-2 complex, as well as by other non-H-2 genes (Roder et al., 1981). A similar MHC linkage of NK activity has also been identified in man (Santoli et al., 1976). The nature of most of the non MHC-linked genes is not known, but one gene of particular interest is found on chromosome 13 in mice (Lane and Murphy, 1972). First identified as a spontaneous mutation in C57Bl/6 mice, this "beige" mutation leads to abnormalities in skin pigmentation (Windhorst and Padgett, 1973) and in NK cell activity (Roder and Duwe, 1979) and will be discussed later in more detail. Other mutations affecting NK activity and pigmentation have been independently reported (Shultz and Sidman, 1987).

Endogenous NK activity can be regulated by a variety of agents particularly the interferons and their

inducers. Although initial studies suggested that all types of IFN could augment NK cell activity, more recent work with highly purified or recombinant products, has shown that alpha and beta IFN are the most active in this respect (see below). Although IFN gamma was originally reported to activate mouse NK cells in vivo and in vitro (Welsh and Doe, 1980), more recent evidence suggests that this lymphokine has little, if any, enhancing effect on cytotoxic activity by NK cells (Sayers, Manson and Ortaldo, 1986). IFN alpha and beta appear to act by increasing the proportion of LGL that bind to target cells, as well as by activating the lytic mechanism (Timonen et al., 1981,1982) and increasing the numbers of effector cells (Ortaldo and Herberman, 1984).

NK cells express the interleukin 2 (IL-2) receptor (Trinchieri et al., 1984) and they proliferate in response to IL-2 in the absence of other stimuli (Timonen et al., 1982; Trinchieri et al., 1984). In addition to its effect on NK cell growth, IL-2 activates the lytic activity and produces marked changes in morphology and lytic specificity (Henney et al., 1981). Although this was originally thought to reflect induction of IFN gamma (Henney et al., 1981), more recent results indicate that IL-2 acts directly on NK cells (Burns et al., 1985). One potential source of difficulty in interpreting some of these results is that T cells grown in IL-2 exhibit enhanced non-specific cytotoxic activity (Burns et al., 1985), but recent

studies using highly purified and well defined NK cell preparations have confirmed the role of IL-2 on NK cells (Herberman et al., 1987).

A variety of agents inhibit the lytic activity of NK cells. This inhibition of cytolysis may occur either at the level of recognition and binding to target cells (as demonstrated by conjugate formation) or at postbinding or lytic stage. Agents known to inhibit NK activity at the binding phase are EDTA and proteases (Kiessling and Wigzell, 1979) while ATP, cAMP, PGE<sub>2</sub> or monensin (an inhibitor of microfilament and microtubule movement) are potent inhibitors at the postbinding stage, possibly by preventing the production and/or release of NK cytotoxic factors (Herberman et al., 1986).

#### Functional relevance of NK cells.

##### Resistance to tumours.

The ability of NK cells to lyse tumour cells in vitro led many workers to examine the role of NK cells in resistance to tumours in vivo. Early studies showed a strong correlation between NK activity in vitro and resistance to <sup>n</sup>transplantable haemopoietic tumours in vivo in a variety of mouse strains (Kiessling et al., 1975a; Sendo et al., 1975). In addition, the fact that nude mice have enhanced NK activity and a normal or increased resistance to tumour growth, supports the view that NK cells may be an important T-independent surveillance mechanism against tumours. Beige mice with defective NK

activity also show poor rejection of transplanted and metastatic tumours of haemopoietic origin (Karre et al., 1980; Talmadge et al., 1980), while human with the analogous Chediak-Higashi syndrome have impaired NK function and exhibit a high incidence (85%) of spontaneous lymphoproliferative disorders (Roder et al., 1980). However, beige mice do not have a higher than normal incidence of spontaneous tumours and NK cells are rarely able to lyse freshly isolated solid tumours in vitro. Thus, it remains to be proven that NK cells normally play a role in protecting the animal from spontaneous solid tumours, but they may be important in resistance to tumours of haemopoietic origin.

#### Protection against infectious organisms.

There is increasing evidence that NK cells play a role in resistance to some microbial infections, particularly those of viral origin (Lotzova and Herberman, 1986). Several investigations have shown that NK cells can lyse virally infected cells and resistance to infection by some viruses is correlated with NK activity (Herberman and Ortaldo, 1981). NK cells may also be involved in resistance against some other types of infection, such as malaria, Babesia microti (Ruebush and Burgess, 1981; Eugui and Allison 1982) and the fungus Cryptococcus neoformans (Herberman and Ortaldo, 1981).

#### Immunoregulatory functions of NK cells.

NK cells show an unusual pattern of distribution in the animal, with the highest levels found in the spleen

and blood (Roder *et al.*, 1981). Furthermore, as noted above, NK cells can lyse thymocytes and bone marrow cells. Therefore, considerable attention has focussed recently on a possible role of NK cells in regulating the activity of the immune and haemopoietic systems (Nunn *et al.*, 1977; Riccardi *et al.*, 1981). This is supported by the fact that the colony forming potential of bone marrow stem cells can be decreased by preincubation with NK cells (Hannsson *et al.*, 1982).

A specialised form of this activity may underlie the ability of certain, heavily irradiated  $F_1$  hybrid mouse strains would reject bone marrow cells from one or other parent strain. This phenomenon appears to be due to H-2 linked, recessive hybrid histocompatibility (Hh) genes which are expressed only in the homozygous parent and not in the heterozygous  $F_1$  (Cudkowicz and Stimpfing, 1964). That NK cells mediate  $F_1$  resistance is suggested by the facts that the ability of  $F_1$  mice to resist parental bone marrow cells matures during the 4<sup>th</sup> week of life, is radioresistant and thymus independent, but requires a functioning bone marrow. Furthermore, agents which deplete NK cells, such as  $^{89}\text{Sr}$ , steroids and anti-AsGM1 or anti-NK1 also prevent  $F_1$  resistance, while both can be activated by IFN (Kiessling *et al.*, 1977; Welsh, 1984).

Some mouse strains can also reject fully allogeneic bone marrow, but although it was originally suggested that NK cells also mediated this rejection, recent data indicate that allogeneic resistance is due to classical, MHC-restricted T cells (Dennert, Anderson and Warner,

1985). Finally, there is some evidence that certain F<sub>1</sub> mice can also resist a GvHR induced by parental lymphoid cells and therefore, host NK cells may help to protect an animal from the consequences of a GvHR (Kiessling et al., 1977; Dennert et al., 1985)

A further potential immunoregulatory role for NK cells may be the ability to modulate ongoing immune responses. NK cells in lymph nodes are found predominantly within germinal centres (Itoh, Suzuki and Umez, 1982; Ritchie, James and Micklem, 1983) and several reports have shown that cells with the phenotype of NK cells can suppress antibody production in vitro (Nabel, Allard and Cantor, 1982; Tilden, Abo and Balch, 1983). NK cells may also inhibit mixed lymphocyte reactions (MLR) and mitogen responses in vitro (Tilden et al., 1983) and other evidence suggest that NK cells may act in this situations by lysing <sup>ANTIGEN PRESENTING CELL</sup> (APC) (Gilbertson, Shah and Rowley, 1986). Although it is possible that these regulatory functions reflect the lytic functions of NK cells, recent work indicates that secretion of various cytokines may also be involved (Ortaldo and Herberman, 1984). Thus, highly purified LGL or NK cells have been shown to produce lymphotoxin (TNF beta) (Herberman et al., 1983); alpha, beta and gamma IFN (Kasahara et al., 1983; Herberman 1982; Djieu Timonen and Herberman, 1982); IL-2 (Kasahara et al., 1983); IL-1 (Scala et al., 1984) colony-stimulating factor (CSF) (Kasahara et al., 1983) and B-cell growth factor (BCGF) (Herberman et al., 1982). Whether

production of these factors by NK cells plays a major role during immune responses in vivo remains to be elucidated.

The beige mouse as a model to study the role of NK cells in vivo.

The findings discussed above indicate that NK cells are a distinct subpopulation of lymphocytes with many potential effector and regulatory functions. Of particular note there is evidence that NK cells are activated during CMI reactions and may be capable not only of lysing appropriate target cells, but may also produce a wide range of important soluble mediators. Therefore, it would be important to know the exact role of NK cells in the immune responses in vivo. One approach to this is to use animals which have very low levels of endogenous NK activity. Although this can be achieved by treating mice with antibodies to NK cells, many of these are not specific and a more flexible system is offered by using mice with a genetic lack of NK cells.

The beige mutation in mice is located on chromosome 13 (Lane and Murphy, 1972) and has been considered as a homologue of the Chediak-Higashi syndrome in man (Windhorst and Padgett, 1973). A similar mutation has also been reported in Aleutian mink, Hereford cows (Padgett et al., 1964), Persian cats (Kramer, Davies and Prieur, 1977) and killer whales (Taylor and Farrel, 1973). In each case, the syndrome is characterised by reduced pigmentation and increased size of lysosomal

granules in cells of myeloid origin (Oliver and Essner, 1975). Characteristically, the cells affected include neutrophils, macrophages and mast cells and they have a large, single lysosomal granule instead of the usual cluster (Shultz and Sidman 1987). It is believed that the defect lies in failure of lysosomal fusion and as a result, neutrophil chemotaxis, bactericidal activity (Gallin et al., 1974) and macrophage tumoricidal activity (Makóney, Morse and Morahan, 1980) have been reported to be defective in beige mice.

Further studies of mice carrying this mutant gene showed a profound and apparently selective decrease in natural killer cell activity (Roder et al., 1979; Roder, 1979). The defect in NK cell activity is predetermined at the level of stem cells in the bone marrow (Roder, 1979) and is not due to altered organ distribution of NK cells, abnormal target selectivity or to a lack of endogenous IFN. NK activity can be enhanced but not fully restored by IFN (Roder et al., 1980). The defect in cytotoxicity seems to be in the lytic mechanism, as the frequency of target-binding cells is normal (Roder, 1979). Other forms of cell-mediated cytotoxicity have been reported to be normal in beige mice (Roder et al., 1979), including alloimmune or lectin-generated CTL activity in vivo and in vitro, antibody dependent cell-mediated cytotoxicity (ADCC) mediated by promonocytes and macrophages, as well as cytolysis of tumour cells by activated macrophages (Roder et al., 1979). In contrast, NK cell mediated

ADCC activity against antibody coated P815 cells is defective in beige mice (Roder, 1979).

The defect in NK cell activity in beige mice is associated with decreased resistance to transplantable syngeneic leukemia and melanoma cells (Karre et al., 1980; Talmadge et al., 1980), as well as increased susceptibility to bacterial, parasitic and viral infections (Elin et al., 1974; Kirkpatrick and Farrell 1982; Shellam et al., 1981)

The availability of mice with an apparently NK cell-specific, single gene defect makes them a ideal model to study the role of NK cells in vivo. Furthermore, normal congenic mice of the C57BL/6 strain are available as appropriate controls for such studies.

## INTRAEPITHELIAL LYMPHOCYTES.

The presence of such a large number of lymphoid cells in close contact with the antigenic load of the gastrointestinal lumen, suggests that IEL may be an important first line of defence against local pathogens. The potential role of IEL in local immune responses is emphasised by the fact that an increased density of IEL is a characteristic of several experimental forms of enteropathy which are due to local T CMI, including GvHR, allograft rejection and experimental DTH responses to dietary proteins (Ferguson and Parrott, 1973; MacDonald and Ferguson, 1976; Mowat and Ferguson, 1981a,b, 1982). Increased IEL counts are also found in several naturally occurring enteropathies in man, which are associated with local CMI, including coeliac disease, cow's milk protein enteropathy and in active lesions of Crohn's disease (Ferguson, 1974; Ferguson *et al.*, 1976; Phillips *et al.*, 1979; Mowat, 1984), as well as in parasitic infections such as giardiasis in mice and man (MacDonald and Ferguson, 1978) and in murine trichinosis (Manson-Smith *et al.*, 1979). IEL appear activated or in mitosis in several of these cases (Marsh, 1980; Ferguson *et al.*, 1984). However, the exact role of IEL in local immune responses is not clear.

## Morphological characteristics.

IEL are heterogenous in size, but examination of

both tissue sections and isolated populations shows that the majority are medium sized lymphocytes (Collan, 1972; Rudzik and Bienenstock, 1974; Marsh, 1975; Ropke and Everett, 1976; Mayrhofer and Whately, 1983).

Electron microscopy has shown that IEL have a dense nucleus and granular cytoplasm, with some rough endoplasmic reticulum, ribosomes, lysosomes, mitochondriae, and a well developed Golgi apparatus (Ferguson, 1977). One interesting feature is that, in mice (Guy Grand, Griscelli and Vassalli, 1978; Davies and Parrott, 1981; Tagliabue *et al.*, 1982), rats (Collan, 1972; Mayrhofer, 1980; Lyscom and Brueton, 1982), rabbits (Rudzik and Bienenstock 1974) and man (Cerf-Bensussan, Schneeberg and Bhan, 1983) many IEL contain a few cytoplasmic granules. In mice, up to 60% of IEL may contain 2-5 membrane-bound granules, which are 0.4-0.9  $\mu$ m in diameter (Guy Grand *et al.*, 1978). The nature of these granules is unclear and it is not known whether granulated IEL represent a distinct subpopulation of IEL. Although initial studies suggested a relationship between IEL and MMC (Guy Grand *et al.*, 1978), recent biochemical studies indicated that these represent distinct cells types (see below).

#### Ontogeny and origin of IEL.

The origin and fate of IEL are still a matter of controversy and it has not even been proven whether this population is of lymphoid or non-lymphoid origin (Ferguson and Parrott, 1972; Guy Grand, Griscelli and

Vassalli, 1974, 1978; Mayrhofer, 1980). Although, IEL appear as early as day 28 of gestation in the rabbit (Orlic *et al.*, 1981) and by 11-12 weeks in the human foetus (Spencer *et al.*, 1986; Orlic and Lev, 1977), they are virtually absent until birth in rodents, with only a small increase during the first 2-3 weeks of life. However, after weaning, there is a sudden rise in the number of IEL, with adult levels of around 10-15/100 epithelial cells being attained by 6-7 weeks of age in mice (Ferguson and Parrott, 1972). This apparent influence of luminal antigen on the number of IEL is supported further by the very low number of IEL found in the intestine of germ-free animals or in antigen-free grafts of foetal intestine placed under the kidney capsule (Ferguson and Parrott, 1972; Ropke and Everett, 1976). In contrast, granulated IEL increase rapidly in number soon after birth (Lyscom and Brueton, 1983).

The origin of IEL remains controversial. Although only about 3% of IEL are labelled after a single pulse of  $^3\text{HTdR}$  *in vivo* (Darlington and Rogers, 1966), 80-90% of IEL are labelled within 48 hours of a repeated series of  $^3\text{HTdR}$  injections. The labelling index then remains at this level for at least 10 days (Darlington and Rogers, 1966). The number of IEL also regenerates fairly rapidly after bone marrow reconstitution of irradiated mice (Guy Grand *et al.*, 1978) and these features indicate that the majority of IEL are derived from a rapidly dividing precursor population. However, immediate source of IEL precursors is unknown. Guy

Grand and her colleagues have shown that after labelling of mouse PP *in situ* by topical application of  $^3\text{HTdR}$ , leads to the appearance of labelled IEL in the jejunal mucosa within one day (Guy Grand *et al.*, 1978). Similar findings have been reported after labelling of sheep ileal PP with FITC *in vivo* (Reynolds, 1984), and therefore, it is possible that IEL are derived directly from PP. This idea is also consistent with the fact that thoracic duct (TD) T lymphoblasts have been shown to migrate to the jejunal epithelium (Sprent, 1976).

A further area of confusion is the role of the thymus in the ontogeny of IEL. As discussed below, the majority of IEL carry one or more T cell markers (Parrott *et al.*, 1983) and there is a dramatic reduction in the numbers of IEL in congenitally athymic animals (Mayrhofer, 1980a) or after adult and neonatal thymectomy (Ferguson and Parrott 1972). However, studies by Mayrhofer in adult thymectomised, (lethally irradiated) bone marrow reconstituted, lethally irradiated rats have suggested that a large proportion of IEL are derived directly from the bone marrow and are thymus independent in origin (Mayrhofer, 1980a). Interestingly, granulated IEL appear to be most efficiently generated from the bone marrow and therefore it is possible that IEL are heterogenous in terms of origin as well as in appearance.

The fate of IEL is equally unclear. Early workers suggested that lymphocytes entered the gut epithelium merely to be excreted (Bunting and Huston, 1921). In

addition, it has been suggested that IEL are shed from the villus along with effete enterocytes (Heatley and Bienenstock, 1982). Although there is no direct evidence against this hypothesis, it should be noted that the majority of IEL are found on the basement membrane rather than at the luminal surface of the epithelium (Toner and Ferguson 1971). Furthermore, there is electron microscopy evidence of basement membrane disruption in both directions, suggesting that IEL may leave the epithelium by re-entering the lamina propria (Toner

1971). This concept is also supported by the and Ferguson fact that the proportion of labelled IEL remains constant for a considerable period after a single *in vivo* pulse of  $^3\text{HTdR}$ , suggesting that IEL are not shed with the enterocytes (Darlington and Rogers, 1966). The only study of the migration pathways of isolated IEL *in vivo*, suggested that IEL localised most efficiently in PP (Guy Grand *et al.*, 1978). However, this redistribution required 3-4 days after iv. injection and used poorly defined populations of IEL. Therefore, it would be important to investigate the circulation pathways of highly purified IEL.

#### Phenotype of IEL

The phenotype of IEL has been studied both in histological sections and in isolated populations, with essentially identical results within a species. Initial work in humans confirmed findings from athymic animals which virtually all IEL expressed the pan T cell marker CD3, while sIg<sup>+</sup> B lymphocytes were absent from this

compartment (Selby, 1981). The exact proportion of IEL which react with pan T cell markers appears to differ between species and depends on the marker and technique used. Thus, only 35-40% of mouse IEL express the Thy 1 marker (Tagliabue *et al.*, 1982; Parrott *et al.*, 1983; Ernst, Befus and Bienenstock, 1985a) and a similar proportion express W3/13 in the rat (Lyscom and Brueton, 1982). In the human epithelium, at least 80% of IEL have been shown to express the pan T cell marker, CD3 (Cerf-Bensussan, Guy Grand and Griscelli, 1985; Selby *et al.*, 1983) but other human pan T cell markers such as CD5 are not expressed in such a high proportion (Selby, 1983). Nevertheless, as the CD3 molecule is part of the T cell receptor complex, these findings indicate that most IEL should express the T cell receptor.

A consistent finding in all species is the predominance of CD8<sup>+</sup> IEL with very few CD4<sup>+</sup> IEL being present (Lyscom and Brueton, 1982; Parrott *et al.*, 1983; Selby *et al.*, 1983). A feature which is unique to IEL is that a large proportion of IEL in mice and rats express the CD8 antigens (Lyt2/OX8) but not pan T cell markers (Lyscom and Brueton, 1982; Parrott *et al.*, 1983; Dillon and MacDonald, 1984; Petit *et al.*, 1985). At present, the nature and function of this unusual cell type remains unknown. The finding that these cells are present in equivalent proportions in the epithelium of athymic mice (Parrott *et al.*, 1983) raised the possibility that pan T<sup>-</sup>, CD8<sup>+</sup> IEL are not thymus-derived. However, the absolute number of all IEL is

much reduced in nude mice (Mayrhofer, 1980b) and so it seems likely that this population are at least partially thymus dependent. Studies on the phenotype of granulated IEL showed by separation of  $\text{Lyt } 2^+$  ( $\text{CD8}^+$ ) and  $\text{Lyt } 2^-$  IEL by FACS, that 85% of IEL were  $\text{Lyt } 2^+$  ( $\text{CD8}^+$ ) and 80% of these were granulated (Petit et al., 1985).

Studies of other phenotypic markers have not added to the knowledge of the origin of IEL. As expected, IEL express class I MHC antigens (Selby, Janossy and Jewell, 1981) but do not express class II MHC antigens or other activation markers, even in inflammatory sites (Cerf-Bensussan et al., 1984; Selby et al., 1981; Dobbins, 1986). In addition, IEL do not express IL-2 receptors or the receptor for C3d component of complement (Selby et al., 1983, 1984). IEL rarely express antigens characteristic of natural killer cells such as HNK-1, Leu 7 and Leu 11, in man (Cerf-Bensussan et al., 1984; Selby et al., 1983) AsGm1 and NK1 in mice (Tagliabue et al., 1982).

Developmental studies in rats have shown that the predominance of  $\text{Tc/s}$  is already discernible at birth, while lymphocytes carrying the pan T cell or  $\text{Th/i}$  marker appear only after weaning (Lyscom and Breuton, 1983). An important point is that lymphocytes in the lamina propria have a markedly different phenotype, with equal numbers of T and B cells and a ratio of  $\text{T}_\text{h}/\text{T}_\text{c/s}$  which is similar to that in blood. Also not as many  $\text{Thy } 1^- \text{Lyt } 2^+$  cells and less granulated cells (Lyscom and Breuton, 1982; Parrott et al., 1983; Ernst et al., 1985a; ). These findings indicate that IEL appear to comprise a

distinct population of lymphocytes, whose functions are difficult to predict from phenotypic analysis or from studies of other peripheral lymphocytes.

#### Functional Characteristics of IEL.

The facts that IEL are found in close proximity to the antigenic load of the gut and some T cell characteristics, supports the view that IEL may be involved as effector cells in local CMI responses. Nevertheless, there is no direct evidence to support this idea. Several workers have attempted to study the functions of isolated IEL, but the results have frequently been conflicting. As might be anticipated from their phenotype, a considerable amount of cytotoxic T lymphocyte activity can be detected in IEL after ip or oral administration of allogeneic tumour cells (Davies and Parrott, 1980, 1981; Klein and Kagnoff, 1984). In addition, IEL have been shown to transfer DTH responses *in vivo* (Shields and Parrott, 1985) and to produce lymphokines *in vitro* (Dillon and MacDonald, 1986b). Although it has been reported that IEL proliferate in response to T cell mitogens or in mixed lymphocyte reactions, these responses are frequently small and require exogenous accessory cells or lymphocyte products (Arnaud-Battandier and Nelson, 1982; Greenwood, Austin and Dobbins, 1983; Dillon and MacDonald, 1984; Cerf-Bensussan *et al.*, 1984; Mowat *et al.*, 1986b). In addition, others have found that IEL exhibit no proliferative responses *in vitro* at all (Cerf-Bensussan

et al., 1984; Greenwood et al., 1983; Nauss 1984).

The studies described above have concentrated on T cell functions, but the presence of granules in a substantial proportion of IEL has led workers to investigate the relationship between IEL and other granulated effector cells such as NK cells and MMC. As noted earlier, initial studies suggested that virtually no IEL carry the NK cell specific marker (Greenwood et al., 1983; Selby, 1983; Cerf-Bensussan et al., 1983; Tagliabue et al., 1982), but more recently it has been suggested that 10-15% of murine IEL are AsGm1<sup>+</sup> (Carman et al., 1986; Flexman et al., 1983). There is similar confusion about the level of resting NK activity in IEL. Thus, early studies suggested that guinea pig (Arnaud-Battandier et al., 1978) and murine IEL (Tagliabue et al., 1981, 1982) had high levels of NK activity in conventional short term assays. In contrast, the majority of recent studies have shown that IEL from mice (Guy Grand and Vassalli, 1982; Mowat et al., 1983), and humans (MacDermott, Franklin and Jerkins, 1980; Chiba et al., 1981) have little or no NK activity under normal circumstances. However, reasonable levels of NK activity can be revealed by treating IEL *in vitro* with interferon (Flexman, Shellam and Mayrhofer, 1983) or by increasing the assay time to 18 hours (Mowat et al., 1983). Furthermore, IEL from mice with GvHR (Borland et al., 1983) or immunised with allogeneic tumour cells have enhanced NK cell activity (Mowat et al., 1983). Interestingly, in GvHR, the enhanced NK activity by IEL parallels the other features of intestinal pathology.

Therefore, it seems that although IEL may have an excellent potential for NK cell activity, their lytic mechanism requires to be activated to exhibit full activity. Finally, it has also been suggested that NK cells in the gut epithelium may be able to kill *Salmonella* organisms in association with secretory IgA antibodies (Nencioni *et al.*, 1983). These features indicate that intraepithelial NK cells may be important components of local defence mechanisms which are readily activated by a CMI reaction.

It is clear from all these studies that despite the circumstantial evidence that IEL may be involved directly in local, intestinal immune responses, their phenotype and role *in vivo* remains to be elucidated. In the experiments described in this thesis, I decided to investigate the functional relevance of IEL, by examining their ability to mediate a proliferative response *in vivo*.

## LYMPHOCYTE MIGRATION IN VIVO.

The complex cellular interactions which form the basis of the immune response takes place within the organized architecture of peripheral or secondary lymphoid tissues. This process is assisted by the continuous migration of many lymphoid cells through lymphoid and non-lymphoid organs via the lymph and blood (Gowans and Knight, 1964). As a result, the lymphocyte population is in a state of dynamic equilibrium, although the extent to which individual lymphocytes participate in the process of recirculation depends on the class of lymphocyte and its stage of differentiation (Ford, 1975; Parrott and Wilkinson, 1981).

The migration of lymphocytes through lymphoid organs is not merely a random phenomenon, but reflects specific interactions between lymphocytes and cells of the appropriate tissues. Furthermore, lymphocyte recirculation results in accumulation of individual lymphocyte subpopulations in highly defined and specific areas of each tissue.

### Mechanisms of lymphocyte recirculation.

All organised secondary lymphoid tissues have discrete T cell and B cell areas, with B cells occupying the follicles and T cells being found in the surrounding interfollicular areas (de Sousa, 1981). In most tissues, lymphocytes enter both from the bloodstream by migrating through postcapillary venules in the midcortex

of the lymph node (de Sousa, 1981) and from the afferent lymphatics draining local tissues. Thereafter, cells either remain in the lymphoid tissue or may leave via efferent lymphatics which drain into the thoracic duct (TD) and hence back into the bloodstream.

The processes which allow selective migration and localisation of lymphocytes in lymphoid tissues are not fully understood, but it is generally agreed that postcapillary venules with high endothelium are critically important. These high endothelial cell venules (HEV) (Gowans and Knight, 1964) are found throughout the cortex and inter-follicular regions of secondary lymphoid tissues, including lymph nodes and Peyer's patches (PP), but are not present in germinal centres or the medulla (Claesson, Jorgensen and Topke, 1971; Gowans and Knight, 1964). In addition, there are no HEV in the spleen, thymus or bone marrow and they are not normally found in non-lymphoid organs such as skin and gut mucosa (Woodruff, Clarke and Chin, 1987).

The first indications that specific cell surface molecules might be involved in lymphocyte recirculation were the findings that lymphocytes treated with glycosidase (Gesner and Ginsburg, 1964) trypsin or neuraminidase (Woodruff and Gesner, 1968) do not enter lymph nodes efficiently and are rapidly removed from the circulation in the liver. The high molecular weight polysaccharide fucoidin also inhibits lymphocyte recirculation *in vivo* (Spangrude, Baaten and Daynes, 1984). However, it should be noted that glycosidase or

neuraminidase do not alter the binding of lymphocytes to HEV in fixed sections in vitro. Pertussigen also inhibits lymphocyte recirculation in vivo without altering adhesion to HEV in vitro and therefore, it has been suggested that the extravasation of lymphocytes into lymphoid tissues involves two clearly distinguishable steps. First, a specific receptor-mediated binding event at the surface of the HEV, followed by an adenylate cyclase dependent activation of cell motility which is required for movement of the lymphocyte through the vessel wall (Spangrude et al., 1984).

More recent studies have attempted to define in greater detail the specific cell surface molecules on both lymphocyte and endothelial cell which mediate the binding to HEV. One lymphocyte-associated structure which has been identified, is recognised by the MEL-14 monoclonal antibody (Gallatin, Weissman and Butler, 1983). The presence of this marker is a characteristic of mature lymphocytes with the capacity to recirculate and the interaction between lymphocytes and HEV can be blocked by antibodies to MEL-14 (Jalkanen et al., 1986). Other studies have shown that HEV produce a specific sulphur-containing glycoprotein which may be involved in adhesion to lymphocytes (Andrews et al., 1982, 1983). The exact nature of these different recognition molecules and the relationship between them are unknown. There may also be further specialisation of HEV recognition structures within different tissues (Jalkanen et al., 1986). Thus, lymphocytes derived from

PP seem to adhere specifically to HEV from PP in vitro, while peripheral lymphocytes adhere only to peripheral lymph node HEV. HEV from MLN appear to be a mixture of both types. Recently, monoclonal antibodies which define discrete recognition molecules in HEV from peripheral lymph nodes or PP, in mice (Gallatin et al., 1983), humans (Woodruff et al., 1987) and rats (Rasmussen et al., 1985) have been produced.

Although specific recognition or adherence to HEV may be important in migration of lymphocytes into lymph nodes and PP, it cannot account for the selective distribution of T and B lymphocytes within lymphoid organs. This may involve interactions with discrete reticulum cells present in the T and B cell areas themselves (de Sousa 1981). In addition, HEV binding cannot explain recirculation of lymphoid cells through tissues which lack these specialised vessels, such as spleen, gut and skin. In the case of the spleen, migration of lymphocytes into T and B cell areas is consequent on cells passing out of the bloodstream in the marginal zone of the white pulp and so may reflect an alternative form of emigration from specialised vasculature. The recirculation pathways of lymphocytes through the intestinal mucosa presents a particularly difficult problem. Lymphoblasts derived from GALT accumulate specifically in the gut wall, whereas peripheral lymphoblasts do not (see below). However, this specific recirculation pathway cannot be explained by the presence of specific HEV-binding sites as

classical HEV are not found in the intestinal mucosa. Although recent work has identified mucosal blood vessels with some similarities to HEV, this remains to be confirmed (Jeurissen *et al.*, 1987).

In addition to the problems discussed above, assays of lymphocyte-HEV binding *in vitro* may not necessarily provide much information on normal recirculation pathways *in vivo*. For many years, the specific recirculation pathways of different lymphocyte populations have been studied by examining the distribution of radiolabelled cells after iv injection. This approach has provided evidence not only about the routes, but also about the kinetics and regulation of lymphocyte recirculation *in vivo*. As a route, intravenously injected, labelled lymphocytes from TDL, spleen or LN, are normally removed rapidly from the circulation in the lungs and remain there for a short period (<6 hours). Thereafter, there is a gradual accumulation of cells in the spleen and liver over the first 12 hours, while the appearance of cells in the lymph nodes and PP is relatively slow and does not peak until 12-24 hours after injection. Autoradiographic studies have shown that injected lymphoid cells accumulate both in the T and B cell areas after injection (Parrott and Wilkinson, 1981).

In contrast, intravenously injected bone marrow cells localise mainly in the red pulp and marginal zone of the spleen, in the medullary cords or primary nodules of lymph nodes, with only a small proportion appearing in T cell areas (de Sousa, 1981). Unfractionated

thymocytes also recirculate poorly in comparison with peripheral T lymphocytes (Goldschneider and McGregor, 1968b), with slower passage through lung capillaries and large numbers persist in the sinusoids of the liver (Goldschneider and McGregor, 1968b; Durkin, Carboni and Waksman, 1978). As might be anticipated the majority of thymus cells which do enter lymphoid tissues are found in T cell areas (de Sousa, 1981), but this is much less in numerical terms than with peripheral T cells (Goldschneider and McGregor, 1968a; Mitchell, 1972). These findings appear to correlate with the low levels of MEL-14 molecule in these cells (Jalkanen *et al.*, 1986).

The different proportions of T and B cells in different lymphoid cell populations are reflected in the efficiency migration of labelled cells to T and B cell areas after transfer. Furthermore, this may also explain selective organ distribution of different lymphocyte populations. Thus, B cells appear to localise selectively in PP and spleen while T cell accumulate in peripheral lymph nodes. These patterns correlate with the selective ability of T and B cells to bind HEV in the appropriate organs *in vitro* (Stevens, Wiessman and Butcher, 1982). An additional factor which may influence differential localisation of lymphoid cell populations may be the proportion of T cells subsets. It has been shown that  $CD4^{+}$  ( $Lyt\ 2^{-}$ ) T cells localise in PP more efficiently than  $CD8^{+}$  ( $Lyt\ 2^{+}$ ) whereas both subsets accumulate equally in PLN. In parallel,  $Lyt\ 2^{-}$  ( $CD4^{+}$ )

cells have been found to bind better to PP HEV than to those of PLN in vitro (Kraal, Weissman and Butcher, 1983). In contrast, recent work using human T cells has suggested that  $CD4^{+}$  (T4) cells may bind slightly better than  $CD8^{+}$  cells to HEV of PLN, and there is a preferential adherence of  $CD8^{+}$  to HEV from GALT, MLN and tonsils in vitro (Pals et al., 1986). The exact recirculation pathways of different T cell populations remains to be elucidated.

These experiments have defined the migration pathways of mature, small lymphocytes, which are normally referred to as the recirculating pool. However, the redistribution of activated lymphocytes are markedly different, these cells acquire the capacity to migrate into inflamed tissue while their ability to enter lymphoid tissue decreases (Parrott and Wilkinson, 1981). Furthermore, as discussed below, lymphoblasts from TD and GALT gain a selective ability to localise in the intestinal mucosa. The differences in recirculatory patterns between resting and activated lymphocytes are underlined by findings that T cells which have been cloned or stimulated in vitro by mitogens, antigen or lymphokines such as IL-2, are unable to enter lymph nodes or PP after transfer in vivo (Dailey, Gallatin and Weissman, 1985). As stimulated cells are also unable to bind HEV in vitro and lack the MEL-14 antigen, it has been suggested that HEV binding molecules are expressed at different stages of T cell differentiation and are lost during proliferation (Dailey et al., 1985). Interestingly a more recent report found that

administration of antigen plus IL-2 could overcome the inability of T cell clones to enter lymph nodes in vivo (Bookman, Groves and Matis, 1987).

#### Migration of lymphocytes to mucosal sites.

The mucosal surfaces are sites of continuous antigenic challenge and therefore it is not surprising that large numbers of lymphoid cells are present in several distinct compartments. These comprise the organised tissue such as PP, tonsil, appendix and MLN, while in the mucosa, lymphocytes are scattered throughout the lamina propria and epithelium.

The outstanding feature of mucosal tissues is the large predominance of IgA secreting cells and their precursors. In the <sup>LAMINA PROPRIA</sup>(LP), >80% plasma cells produce IgA and early studies showed that PP were a much richer source of IgA precursors than spleen, peripheral and mesenteric lymph nodes or blood (Craig and Cebra, 1971, 1975; Cebra et al., 1977). Later studies showed that the gut-seeking cells in PP were already activated and suggested that IgA precursor cells from PP reached the LP via thoracic duct and bloodstream after a period of residence in the MLN (Guy Grand et al., 1974; McWilliams, Phillips-Quagliata and Lamm, 1975). In parallel, PP appear to be the major source of mucosal T cell precursors as local irradiation of PP reduces the numbers of T cells in the gut mucosa and radiolabelling of PP in situ is followed by the appearance of labelled cells in the MLN and gut mucosa (Guy Grand et al.,

1978).

These studies of the recirculation of lymphocytes from PP are complemented by the findings that lymphoblasts from TDL or MLN localise selectively in the gut mucosa (Sprent, 1976; Rose, Parrott and Bruce, 1976a). In contrast, lymphoblast from peripheral sites do not enter the gut mucosa (Rose, Parrott and Bruce, 1976b; Hall, Hopkins and Orlands, 1977) unless the gut has been inflamed. Even under these circumstances, the peak accumulation of peripheral lymphoblasts is less than that of mesenteric lymphoblast (Cahill *et al.*, 1977). Whether, small lymphocytes from the GALT exhibit similar properties of selective localisation is not clear. In rodents, small lymphocytes from PP, TD and MLN do not localise in the mucosa but recirculate into PP, lymph nodes and spleen in the same manner as peripheral small lymphocytes (Gowans and Knight, 1964; Griscelli *et al.*, 1968; Hall, Parry and Smith, 1972; Parrott and Ferguson, 1974; Freitas, Rose and Parrott, 1977). However, in sheep, small T cells from intestinal lymph do appear to have a selective ability to return to the mucosa. (Scolley, Hall and Orlands, 1976; Hall *et al.*, 1977; Cahill *et al.*, 1977). The reasons for these discrepancies are not known, but may reflect the fact that the small lymphocytes which were used in sheep were derived from efferent lymph and had not yet entered an organised lymphoid tissue. The possibility remains that small T cells from rodent intestinal lymph may be similar.

The selective migration of T and B lymphoblasts may

also migrate to different compartments of the intestinal mucosa, as it has been shown that mesenteric T lymphoblasts localised predominantly in the intestinal epithelium and villus lamina propria, whereas B lymphoblasts localised mainly in the lamina propria around the crypts (McDermott *et al.*, 1986).

The mechanism responsible for the selective localisation of GALT-derived lymphocytes has engaged several groups of workers, but remains unresolved. One idea was that lymphoblasts bearing IgA were attracted towards secretory piece in the intestinal epithelium (McWilliams *et al.*, 1975). However, SC has no affinity for the monomeric IgA on the cell surface, while coating peripheral lymphoblasts with IgA does not permit them to enter the gut (Hopkins and Hall, 1975; Rose *et al.*, 1976b). In addition, treating mesenteric lymphoblasts with anti-IgA or anti-secretory piece does not inhibit their migration to the gut (McWilliams *et al.*, 1975). Moreover, a mechanism dependent upon secretory piece could not attract GALT B lymphoblasts producing IgG or T lymphoblasts which are known to accumulate selectively in the mucosa (Parrott and Wilkinson, 1981). The presence of specific antigen in the gut also does not explain the selective localisation, as thoracic duct or mesenteric lymphoblasts taken from cholera-toxin primed donors (Pierce and Gowans, 1975) migrate efficiently to the intestine of naive recipients. Furthermore, MLN lymphoblasts migrate to intestinal grafts which contain no luminal antigen (Moore and Hall, 1972; Parrott and

Ferguson, 1974). However, few plasma cells or IEL are found in the gut of germ-free animals (Halslead and Hall, 1972) or in the antigen free intestinal grafts (Ferguson and Parrott, 1972). In addition, specific antibody-forming cells accumulated more efficiently in areas of gut in which antigen is placed, than in non-stimulated regions (Pierce and Gowans, 1975). One possible explanation for these apparent discrepancies is that the initial localisation of lymphocytes in the mucosa is not dependent on antigen, but the presence of antigen enhances the subsequent retention and expansion of these cells. Similarly, it has been shown that peripheral and GALT lymphoblasts migrate out of blood vessels into the mucosa with equal efficiency, but only GALT cells are retained within the intestinal wall (Ottaway and Parrott, 1980). One possible local factor which influence localisation of lymphocytes in the mucosa are hormones such as vasoactive intestinal peptide (VIP). Recent work shows that T cells and mononuclear cells have receptors to VIP and that incubation with VIP *in vitro* not only alters their response to mitogens, but decreases their ability to localise in MLN and PP after transfer *in vivo* (Ottaway, 1984).

The fact that many lymphocytes derived from the GALT appear to localise selectively in other mucosal sites as well as the small intestine led to the concept of a "common mucosal immunological system" (McWilliams *et al.*, 1975; McDermott *et al.*, 1980; Befus and Bienenstock, 1982; ). This hypothesis suggested that

all mucosal tissues were rich in IgA cells because priming of lymphocytes within one mucosal site led to the redistribution of IgA precursors to distant sites, via a common recirculation mechanism. However, recent experiments have not confirmed this attractive idea and it is not clear whether T cells were governed by a similar system (McDermott *et al.*, 1986).

#### Migration of lymphocytes into other non-lymphoid tissues.

With the exception of the liver and mucosal surfaces, the traffic of lymphocytes through most non-lymphoid tissues is small, compared with that through lymphoid tissues (Smith, McIntosh and Morris, 1970; Cahill, Frost and Tranka, 1976; Hall, Scollay and Smith, 1976; Miller and Adams, 1977). However, two types of non-lymphoid tissue may be particularly relevant such as skin and areas of chronic inflammation.

Following skin grafting or antigenic challenge, rapid changes occur in the cellular components of the skin and its afferent lymph. After an increased influx of polymorphs within the first 24 hours, the number of macrophages and veiled cells increases after 24 hours (Hall *et al.*, 1977; Miller and Adams, 1977; Soeberg, Sumarska and Balfour, 1976). From 2 days onwards there is an increase in the number of lymphocytes and lymphoblast (Cahill *et al.*, 1976; Hall, 1967). After a secondary challenge with antigen, the influx of lymphocytes is much more rapid, with many lymphocytes appearing in the skin within 4 hours after the challenge

and peaking at 24 hours (de Sousa and Parrott, 1969). Thus, at least some lymphocytes can localise rapidly in inflamed skin and it has been shown that lymphoblasts are particularly active in this respect (McCluskey *et al.*, 1963; Asherson and Allwood, 1972; Rose *et al.*, 1976b). Furthermore, the accumulation of lymphoblasts in inflamed skin is not antigen-specific, as lymph nodes draining oxazolone sensitised skin accumulate equally in skin inflamed by the same contact sensitizer, by another antigen or even by a non-specific inflammatory agent such as cotton oil (Asherson, Allwood and Mayhew, 1972; Asherson *et al.*, 1973; Rose *et al.*, 1976b). These findings suggested that sites of cell mediated immune inflammatory responses are unusually receptive to the emigration of lymphoblasts and that this occurs in an efficient but non-specific fashion.

#### Regulation of recirculation.

##### Blood Flow.

Studies of blood flow and lymphoid cell after skin sensitisation with oxazolone have shown that the resultant increase in blood flow was directly related to the concomitant increased migration of lymphocytes to the skin (Ottaway and Parrott, 1979). Similar results have been reported with respect to the localisation of lymphoblasts within the small intestine, where there is a significant correlation between the distribution of lymphoblasts and the blood flow to different regions of the small intestine. In addition, the enhanced accumulation of lymphoblasts in the inflamed intestine

is associated with a parallel increase in blood flow (Ottaway and Parrott 1980).

#### Hormones.

Lactating animals and humans exposed to intestinal antigens can be shown to have specific IgA antibodies in milk in the absence of serum antibodies of any class (Montgomery, Rosner and Cohn, 1974; Goldlum, Ahlstedt and Carlsson, 1975), suggesting that sensitised lymphocytes from the gut migrated to mammary gland with subsequent local synthesis of IgA. Animal models have shown that this migration of specific IgA B cells to mammary glands was under hormonal control (Weisz-Carrington, Roux and McWilliams, 1978). Hormones such as oestrogen, progesterone and prolactin enhance the number of IgA plasma cells in the mammary glands of virgin female mice, and testosterone given to naturally lactating females depresses it (Weisz-Carrington *et al.*, 1978). Although these hormones have no effect on the localisation of lymphocytes in other tissues, it has been shown recently that the neuropeptide hormone VIP, can influence the redistribution of gut-derived lymphocytes *in vivo* (Ottaway, 1984).

#### LYMPHOCYTE LOCOMOTION IN VIRO.

The effective migration of lymphocytes to sites of immune responses *in vivo* not only requires that the cells have to be able to recognise and adhere to specific structures such as HEV or lymphoid accessory cells, but also assumes that lymphocytes possess an

efficient locomotor capacity to enable their movement through tissues. Since it is virtually impossible to examine the mechanisms of lymphocyte locomotion directly in vivo several in vitro systems have been devised to study this aspect of lymphocyte behaviour. The motility of neutrophils and macrophages has been investigated for many years (Wilkinson, 1981). Although early studies of lymphocyte locomotion in vitro were performed on lymphocytes moving out of explants of lymph nodes into plasma clots (Lewis and Webster, 1921), more detailed studies have been limited by various technical factors. Compared with macrophages, freshly isolated lymphocytes adhere poorly to serum coated glass or plastic and so it has been difficult to study lymphocyte locomotion using the conventional filter assays developed for phagocytes. This difficulty has been overcome by a system which takes advantage of the ability of lymphocytes to migrate through three-dimensional collagen matrices (Haston, Shields and Wilkinson, 1982). This method can employ lymphocytes which have not been precultured in vitro. Moreover, the transparency of the collagen matrix allows detailed study of the dynamic behaviour of individual cells as well as providing population data. Collagen is a more physiological substrate than glass, plastic or cellulose acetate which have been commonly used in studies of cell behaviour.

The morphological features of motile lymphocytes have been well characterised and can be summarised as follows: motile lymphocytes are characterised by the

presence of pseudopodia and under conditions in which locomotion is not possible, these appear randomly over the cell surface. However, as the cell begins to move, it puts out a broad pseudopodium in the direction in which motion will occur. Thereafter, a "constriction ring" appears at the base of the pseudopodium and remains fixed to the substratum until the entire lymphocyte has moved through it. Moving lymphocytes exhibit a pronounced "tail", with the nucleus being found immediately behind the leading edge, giving the front end of the cell an oval or rounded outline. However, considerable variation in this morphology can be observed when lymphocytes have to squeeze through narrow spaces, in which morphology may be delineated by physical mechanisms (Haston et al., 1982).

Lymphocyte locomotion through three-dimensional matrices is probably independent of adhesion (Haston et al., 1982). In contrast, the cells and the cells put out blebs into gaps in the gel, which they seem to use as fixed traction points for movement.

The mechanisms and regulation of lymphocyte locomotion have only recently been studied in detail. Several workers have shown that the locomotor capacity of lymphocytes increases with culture in vitro (Parrott and Wilkinson, 1981) while activation of lymphocytes with PHA (Rydgren et al., 1976; Scheiner, Braun and Unanue, 1976; Parrott and Wilkinson, 1981), in mixed lymphocyte reactions (McFarland and Heilman, 1965) or by anti-CD3 antibody leads to an increased proportion of cells with locomotor morphology. Anti-CD3 also leads to

increased lymphocyte locomotion in collagen gels (Wilkinson, 1987), while activated lymphocytes from a lymph node draining the site of oxazolone sensitization, show increased locomotor activity both in filters (Russell *et al.*, 1975) and in collagen gels (Shields, Haston and Wilkinson, 1984). Thus, stimulation *in vivo* as well as *in vitro* induces a lymphocyte population with enhanced locomotor capacity.

Few studies have defined the phenotype of locomotor lymphocytes. Human peripheral blood lymphocytes are capable of locomotion into filters after culture *in vitro* (O'Neill and Parrott, 1977). T lymphoblasts enriched from the draining lymph nodes of oxazolone sensitized mice (Russell *et al.*, 1975; Shields *et al.*, 1984) and B lymphoblasts were actively motile under the surface of other tissue cells (Russell *et al.*, 1975) and into micropore filters (Ponten 1975). Little is known about the locomotor capabilities of lymphocyte subsets but cytotoxic T lymphocytes have been found to be motile cells when observed in time-lapse films (Sanderson and Glauert 1977, 1979).

No specific chemotactic factors for lymphocytes have been defined. However, there are reports of a chemotactic effects of endotoxin-activated serum or plasma, casein and denatured protein on lymphocytes (Wilkinson *et al.*, 1976; O'Neill and Parrott, 1977). These studies require to be confirmed using more appropriated assays.

CHAPTER 2  
MATERIALS AND METHODS.

## Animals

Mice used in the experiments were of the following inbred strains: CBA/Ca (H-2<sup>k/k</sup>) C57B1/6 (H-2<sup>b/b</sup>) (B6), C57B1/6 beige bg/bg (H-2<sup>b/b</sup>), DBA/2 (H-2<sup>d/d</sup>), NIH (H-2<sup>q</sup>) (CBA X BALB/c)F1 (H-2<sup>kxd</sup>), (C57B1/6 X DBA/2)F1 (BDF1) (H-2<sup>bxd</sup>), (C57B1/6 X C3H )F1 (H-2<sup>bx k</sup>). Hybrids were bred from CBA and C57B1/6 mothers and DBA/2, BALB/c and C3H fathers. All mice were bred and maintained in the Animal Unit, Department of Bacteriology and Immunology, Western Infirmary, Glasgow, apart from C57B1/6 beige mice which were obtained originally from Dr. H.S. Micklem, Department of Zoology, Edinburgh. Adult mice were normally first used at 6-10 weeks of age.

Animals were maintained on a standard rodent diet (Labsure Maintenance) and had access to tap water ad libitum. In experiments using irradiated mice, 100mg/L of neomycin sulphate (Sigma) was incorporated in the drinking water.

## Anaesthesia and sacrifice of animals

Procedures such as footpad injections and intravenous injection into the tail vein were carried out under ether anaesthesia while mice were killed by cervical dislocation.

## Irradiation of mice

Adult mice, aged 12-16 weeks, were irradiated using a 300KeV X-ray source (Dynaray) at a distance of

100 cm and a dose rate of 125 Rad/min. In these experiments, (CBA X BALB/c)F1 mice received 900 Rad, while (C57Bl/6 X C3H)F1 mice received 450 rad, due to marked differences in radiosensitivity. After irradiation, all mice were maintained on drinking water containing 100 mg/L neomycin sulphate (Sigma).

#### Body and organ weights

Mice were weighed on an electronic pan balance (Oertling JC 12), which was accurate to 0.01g, while individual organs were dissected free of surrounding tissue and weighed on a pan balance (Starton Instruments), accurate to 0.2mg.

#### Preparation of cell suspensions

Spleen, thymus, mesenteric and peripheral lymph nodes were removed immediately after sacrifice. After washing in RPMI 1640 (Flow Labs.), they were dissected free of surrounding material and gently teased apart with forceps and scalpel. Spleens were additionally passed through a fine wire mesh filter using the plunger of a 5 ml syringe (BD Ltd). All cells were allowed to stand for a few minutes at room temperature to allow debris to settle and washed 3 times at 400g in RPMI 1640. In experiments using spleen cells for immunofluorescence or recirculation, these cells were first treated with 0.16 M  $\text{NH}_4\text{Cl}$  for 5 minutes at 4°C to remove red cells. After counting in a haemocytometer (Neubauer) the final cell pellet was made up to the

required concentration in RPMI for use. Cell viability was assessed by phase contrast microscopy (Nikon, Japan).

#### Preparation of intraepithelial lymphocytes.

IEL and LPL were isolated and purified as previously described (Davies and Parrott 1981). Small intestines were removed from animals and handled in batches of three. The guts were thoroughly washed through with 30 ml of cold calcium/magnesium free Hanks balanced salt solution (CMF) (Gibco) using a 50 ml syringe fitted with a short plastic cannula. This removed all food remains and much of the mucus. The small intestines were then placed on moistened paper towels, the mesentery stripped off and all Peyer's patches removed. The intestines were then opened longitudinally and cut into small pieces (0.5 - 1 cm), which were shaken repeatedly in 20 ml cold CMF until a clear supernatant was obtained.

The washed pieces of gut were washed once more in warm RPMI containing 2% newborn calf serum (NCS) (Gibco) and transferred to a 50 ml conical flask before incubation in 25ml RPMI/2% NCS for 30 min. at 37°C in a shaking water bath (Grant Instruments). The gut pieces were then transferred to a universal container, shaken vigorously for 3 min in warm medium and the supernatant removed. This shaking procedure was repeated twice and the supernatants obtained were washed and stored at 4°C. The gut pieces were reincubated for another 30 min and the shaking procedure was repeated. All supernatants

were pooled, centrifuged, resuspended in 10 ml RPMI/2% NCS and filtered through glass wool.

The resulting cell suspension which contained lymphocytes, epithelial cells, and debris was then resuspended in 4 ml of Percoll (Pharmacia) (specific gravity 1.055g), and then layered on to 3 ml of Percoll (specific gravity 1.085g) in a siliconised glass universal. 3 ml of RPMI was then layered on top, before centrifuging for 20 min at 600g at 4°C.

After removing epithelial cells from the interface between RPMI and 1.055g Percoll, IEL were obtained from the 1.055g/1.085g interface. After washing 3 times in a large volume of RPMI/2% NCS, the cells were counted and viability assessed. The yield was normally  $0.8 - 1.2 \times 10^7$  cells/intestine, with >90% lymphocytes and >95% viability as assessed by phase contrast microscopy.

#### Separation of lamina propria lymphocytes

Pieces of small intestine free of mesentery and Peyer's patches were prepared as described above, and were then incubated in 25 ml CMF containing 5mM EDTA (Sigma) at room temperature. A magnetic stirrer was used at 250 rpm to remove the epithelium. At intervals (usually every 15 min), the supernatant became cloudy due to released epithelial cells and this was removed and fresh EDTA/CMF added. This procedure was continued until no more cells appeared in the supernatant, indicating that all the epithelium had been removed (usually around 90 min). The pieces of gut were then

washed with CMF and incubated with stirring for a further 20 min in 25 ml RPMI/2% NCS to inactivate any remaining EDTA.

The fragments were then transferred to flasks containing 15 ml RPMI/2% NCS + 10 units/ml collagenase (Type C- trypsin free 2139, Sigma) and incubated at 37°C in an orbital incubator (Gallenkamp type IH 460) for 90 min at 180 cycles/min. The remaining pieces of intestine were then disrupted completely by repeated passage through a 5 ml syringe and the resultant suspension filtered through a glass wool column, before being washed twice with RPMI/2% NCS. Lymphocytes were then purified using a 1.055g and 1.085g Percoll gradient as described above.

#### Induction of systemic graft-versus-host reactions.

Adult CBA, C57B1/6 and C57B1/6 b6bg mice were used as sources of donor spleen cells, while recipients were either (CBA X BALB/c)F1, BDF1 or (C57B1/6 X C3H)F1 mice. In experiments using unirradiated recipients, adult mice received varying doses of parental spleen cells in 0.2 ml RPMI 1640 intraperitoneally (ip.) or intravenously (iv.) while neonatal hosts received  $10^7$  parental lymphocytes in 0.05 ml RPMI 1640 injected ip through the inguinal region, 5-7 days after birth. Previous experiments in this laboratory (M. V. Felstein, personal communication) had established that injection of syngeneic F1 cells and medium had identical effects on host mice and therefore, in all experiments, controls

received appropriate amounts of medium only.

To induce a GvHR in irradiated mice, host animals were given parental lymphocytes in 0.2 ml medium intravenously, within 24 hours of irradiation. Control mice in these experiments were either left unreconstituted or received appropriate numbers of syngeneic lymphoid cells.

#### Assessment of systemic graft-versus-host reaction

The spleen weight assay of Simonsen was used to assess the intensity of a systemic proliferative GvHR in unirradiated hosts (Simonsen, 1962). Mice were weighed, the spleen removed and weighed and the relative spleen weight expressed as mg /10g body weight.

The Spleen Index was given by the formula:

$$\frac{\text{Relative spleen wt. in individual mouse with GvHR}}{\text{Mean relative spleen wt. in control mice.}}$$

The ability of donor lymphocytes to induce an acute, lethal GvHR was assessed by weighing host mice at intervals after cell transfer and by observing the mortality rate up to 6 months later.

#### Assessment of a local graft-versus-host reaction in lymph nodes

A popliteal lymph node assay was used to assess the ability of locally transferred cells to induce a GvHR in the draining lymph node (Ford, 1970). The local GvHR was induced by injecting  $10^7$  parental lymphocytes in 50  $\mu$ l

medium into one footpad of adult F1 mice and was assessed 8 days later, by weighing the draining popliteal lymph node. As controls, separate mice were injected with syngeneic cells into one footpad and all mice received 50ul of medium in the opposite footpad.

The results were assessed by calculating the difference in weight between the popliteal lymph nodes draining footpads which had received cells and those which received medium alone.

### Histology

After removal, tissues were fixed immediately in 10% buffered formalin, embedded in paraffin wax, and 4um sections thick were cut. The tissues were then stained with haematoxylin and eosin (H&E). Histological processing was carried out by Mrs. Margaret Hardy and Mr. Hector Cairns and specimens were examined using a Leitz-Wetzlar microscope.

### Intraepithelial lymphocyte counts

Samples of jejunum (5mm<sup>2</sup>) were taken 10 cm from the pylorus and IEL were counted on H&E stained sections by the method of Ferguson and Murray (1971), under X40 magnification. Only well orientated sections with a single cell layer were counted and differential cell counts were performed by counting all epithelial and lymphoid cell nuclei lying above the basement membrane, ignoring goblet cells. A total of 600 epithelial cells were counted in each specimen and results were expressed

as IEL/100 epithelial cells.

#### Measurements of mucosal architecture by microdissection

The method of Clarke (1970) was employed to measure mucosal architecture. Mice were injected with 7.5 mg/kg colchicine (Sigma) ip. to cause metaphase arrest and were sacrificed at intervals of 20-120 min. thereafter. Pieces of jejunum (5mm X 5mm) were removed 10 cm from the pylorus, avoiding obvious lymphoid aggregates. The tissue was opened and placed villus surface upwards on pieces of card for fixation in 75% ethanol/25% acetic acid for 6-24 hours and then stored in 75% ethanol before use. Tissues were stained in bulk by the modified Feulgen reaction as follows: pieces of gut were washed in 50% ethanol for 10 min, followed by 10 min. in tap water before hydrolysing for 7 min. in 1 N HCl at 60° C. After a further 10 min. in tap water, the specimens were rinsed 3 times with fresh tap water and then stained with Schiff reagent (Difco Ltd.) for 30 min. at room temperature. The specimens could then be stored in tap water for a maximum of 48 hours before microdissection.

Using a dissecting microscope (X32 magnification: Carl Zeiss, E. Germany), the muscularis mucosa was first removed using fine forceps. Thin strips of mucosa, each one villus thick and containing a few villi and surrounding crypts, were then cut from the edge by dissection with a cataract knife (Weiss Ltd.). These fragments were placed on a microscope slide in 45% acetic acid, covered with a coverslip and examined by

microscopy (Leitz-Wetzlar, Germany) with a previously calibrated eyepiece micrometer. 10 villi and crypts were measured on each specimen and the results were expressed in  $\mu\text{m}$ . The pieces of gut were then gently squashed under the coverslip and the number of metaphases per crypt counted for 10 intact crypts.

To obtain the crypt cell production rate (CCPR), the number of metaphases per crypt was correlated with the corresponding time interval after colchicine injection, and the set of results for one group was subjected to linear regression analysis. Having established linearity, the CCPR was calculated from the slope of metaphase accumulation with time and presented as cell production/crypt/hour.

#### Assessment of systemic delayed-type-hypersensitivity

To examine the generation of allospecific DTH responses, mice were immunised with  $10^7$  allogeneic tumour cells or F1 spleen cells intradermally into one footpad. Systemic DTH was assessed 5 and 10 days later respectively by measuring the increment in footpad thickness 24 hours after an intradermal challenge with  $10^7$  cells in 50  $\mu\text{l}$  RPMI 1640 into the opposite footpad, using a pair of skin fold calipers (Carobronze Ltd.). The specific response was calculated by subtracting the increment in footpad thickness obtained by injecting unimmunised mice with  $10^7$  allogeneic cells. These schedules were known to be optimal from studies performed in this laboratory.

#### Identification of T lymphocytes by antibody dependent cytotoxicity.

A cell suspension containing  $10^7$  lymphoid cells/ml in RPMI was prepared and 100 $\mu$ l added to the wells of microtitre plates (Linbro). 100 $\mu$ l of a 1:1000 dilution of monoclonal anti Thy 1.2 (F7D5 Olac) was added and after incubation at 4 $^{\circ}$  C for 40 min, fresh guinea pig serum was added at a final dilution of 1:10 as a source of complement (C'), for 45 min at 37 $^{\circ}$  C. Control suspensions were incubated with C' alone and viabilities were assessed by phase contrast microscopy.

#### Identification of T lymphocyte classes by immunofluorescence.

Helper/inducer T cells ( $T_{H/I}$ ) were identified by direct immunofluorescence using phycoerythrin conjugated anti-L3T4 (GK1.5 Becton and Dickinson) antibody, while cytotoxic/suppressor T cells ( $T_{C/S}$ ) were identified by indirect immunofluorescence using a monoclonal rat anti mouse Lyt 2 antibody (kindly provided by Dr. T. MacDonald, St. Bartholomew's Hospital, London) and FITC-labelled sheep anti rat antibody (Sera Lab.), this FITC conjugate was absorbed by mixing it in a 1:1 v/v with mouse serum, to eliminate the cross reactivity with mouse Ig.

$2.5 \times 10^6$  cells were resuspended in either 100 $\mu$ l of 1:4 FITC-labelled anti L3T4 or 50 $\mu$ l of neat rat anti Lyt 2 using a drawn-out Pasteur pipette and incubated for 20 min on ice. After three washes in cold

phosphate buffered saline (PBS) pH 7.2, anti-Lyt2-labelled cells were then incubated with 100 $\mu$ l 1:32 FITC-labelled sheep anti rat for a further 20 min on ice. After washing 3 times in cold PBS, labelled cells were resuspended in 100 $\mu$ l of PBS, and a drop put on a microscope slide. Slides were allowed to dry at room temperature before fixing for 20 seconds in 95% ethanol at room temperature. A small drop of glycerol-phosphate buffer was then added, covered with a coverslip and sealed with nail polish.

The cells were examined for positive immunofluorescence using a Lietz-Wetzlar UV light microscope and the number of positive cells expressed as a percentage of the total cell number, as determined by simultaneous phase contrast examination. For Lyt 2<sup>+</sup> cells, background staining of the cells labelled only with FITC sheep anti rat Ig was subtracted from the values obtained.

#### Tumour cell lines

The YAC-1 cell line, a subline of a Moloney leukemia virus-induced tumour of A strain mice (H-2<sup>B</sup>) was grown and maintained in continuous culture in flasks (Costar 75 cm<sup>2</sup>) in sterile RPMI 1640 containing 10% heat-inactivated foetal calf serum (FCS) (Gibco), Hepes, 2mM L-glutamine (L-Glu), 100 u/ml penicillin and 0.1 mg/ml streptomycin (P/S) (Gibco). P 815, a methylcholanthrene induced mastocytoma of DBA/2 origin (H-2<sup>D</sup>), was passaged for one week in the peritoneum of DBA/2 mice and then maintained in vitro in sterile RPMI

with 5% NCS, 5% FCS, and L-Glu, P/S as above. Both cell lines were subcultured at least 3 times a week and if possible, 24 hours before use in cytotoxicity assays.

#### Cell mediated cytotoxicity assays.

YAC-1 and P 815 cell lines were labelled with sodium  $^{51}\text{Cr}$  Chromate (Amersham, England) at  $50\mu\text{Ci}/2.5 \times 10^6$  cells/ $0.5\text{ml}$  for 45 min at  $37^\circ\text{C}$ . The cells were then washed 6 times in RPMI/5% NCS, counted, and adjusted to  $2 \times 10^5$  cell/ $\text{ml}$  immediately before use. Quadruplicate aliquots of lymphoid effector cells in  $100\mu\text{l}$  RPMI/5% NCS were added to the wells of 96 well V bottomed microtitre plates (Titertek) and  $2 \times 10^4$  labelled target cells added in  $100\mu\text{l}$  aliquots to give final effector:target (E:T) ratios of 50:1, 25:1, and 12.5:1. NK assays were incubated for 4 hours at  $37^\circ$  in 5%  $\text{CO}_2$ /95% air in a humidified  $\text{CO}_2$  incubator (Flow Digital), while specific CTL assays were transferred to a  $45^\circ\text{C}$  water bath for 1 hour after 2 hours at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ , to maximise  $^{51}\text{Cr}$  release. After incubation  $100\mu\text{l}$  of the supernatant from each well was carefully removed and  $^{51}\text{Cr}$ -specific radioactivity counted for 3 min on a gamma counter (LKB 1282 Compugamma). In all assays, maximum release was obtained by incubating  $100\mu\text{l}$  target cells with  $100\mu\text{l}$  10% Triton-X 100 (Sigma) in distilled water. Spontaneous release from YAC-1 targets was determined using appropriate numbers of thymocytes as inert filler cells, while control spleen cells were used to obtain spontaneous release in specific CTL assays. In each case, the specific cytotoxicity was calculated as

follows:

$$\begin{aligned} & \% \text{ Specific cytotoxicity} = \\ & \frac{(\text{Release exp. effector cells} - \text{Spont. release})}{(\text{Maximum release} - \text{Spont. release})} \times 100\% \end{aligned}$$

Results shown are means of quadruplicate cultures and variation between wells was normally less than 10%. In some experiments the presentation of the results was simplified by expressing the NK activity in GVHR mice as a ratio of that found in control mice. This also allowed direct comparison with the Spleen Index in the some experiments.

#### Proliferative responses to Concanavalin A in vitro

$2 \times 10^5$  spleen cells in RPMI supplemented with mM sodium bicarbonate, 10% FCS, 2mM L-Glu, 100 u/ml penicillin, 100µg/ml streptomycin and  $5 \times 10^{-5}$  M 2-mercaptoethanol (2-ME) (Sigma) ("Complete RPMI") were cultured in a final volume of 100 µl in flat bottomed 96 well microtitre plates (Linbro) in the presence of 20µg/ml Concanavalin A (Con A, Sigma). Cultures were performed at 37°C in 5% CO<sub>2</sub> / air in a humidified incubator for 1 to 6 days. 1µCi <sup>3</sup>H methyl-thymidine (<sup>3</sup>H-TdR) (Amersham, England) was added to each well, 18 hours before harvesting cell bound DNA on to filters using a Skatron cell harvester. Filters were dried at 37°C, immersed in 1 ml scintillation fluid (LKB) and the

$^3\text{H}$ -TdR incorporated into DNA was measured on a scintillation counter (MINAXI). All cultures were performed in quadruplicate.

#### Mixed lymphocyte reaction

Stimulator cells were prepared by incubating  $10^7$  spleen cells/ml with  $40\mu\text{g/ml}$  mitomycin C (Sigma) for 30 min at  $37^\circ\text{C}$  in RPMI. After 3-4 washes,  $100\mu\text{l}$  aliquots containing  $2 \times 10^5$  stimulator cells were added to  $2 \times 10^5$  responder cells in  $100\mu\text{l}$ . The plates were cultured for 1 to 6 days and harvested as described above. Control cultures contained mitomycin-treated stimulator cells and syngeneic responder cells.

#### Preparation of collagen gels

Type II collagen was prepared from rat tails as described by Elsdale and Bard (1972). Rat tails were collected and placed in 70% ethanol for 5 min before removing the skin. Tendons were then stripped and dipped in RPMI containing  $50\mu\text{g/ml}$  penicillin and  $50\mu\text{g/ml}$  streptomycin for 5 min, before soaking overnight at  $4^\circ\text{C}$  in 3% v/v acetic acid (BDH Chemicals Ltd. England) in a sterile conical flask. The resulting mixture was stirred for 1 hour with a magnetic stirrer and centrifuged at  $800g$  for 30 min to remove undissolved material. The collagen was aggregated with 2 volumes of 20% w/v sodium chloride, then it was centrifuged at  $600g$  for 40 min and the collagen aggregates floated and were collected from the supernatant with a spatula. After washing with

distilled water, The collagen was redissolved in 3% v/v acetic acid and centrifuged twice at 800g for 20 min, to discharge the undissolved material collected in the bottom. The collagen solution was exhaustively dialysed against distilled water adjusting to pH 4 with HCl.

The concentration of collagen in the solution was determined by measuring the optical density value at 234 nm (using a Pye Unicam spectrophotometer) and comparing it to a standard curve of absorptions obtained using known concentrations of collagen. 10 ml aliquots containing 4.7 mg/ml of collagen were stored at  $-20^{\circ}\text{C}$  until use.

Collagen gels were prepared by the method of Shields et al. (1984). The osmolarity and pH of a solution containing 20 mg of collagen in 8.8 ml were adjusted to physiological levels by mixing with 1 ml of X10 RPMI and 200  $\mu\text{l}$  of 1M HEPES (pH 7.3). 2 ml of collagen solution were then added to 50 x 13 mm tissue culture dishes (Linbro), allowed to set for 20 min at room temperature and covered with medium for immediate use.

#### Determination of locomotor capacity in vitro.

$2 \times 10^6$  lymphocytes in 1 ml of complete RPMI were layered on to fresh collagen matrices and allowed to invade overnight at  $37^{\circ}\text{C}$ . The gels were then fixed with 2.5% glutaraldehyde in RPMI for 30 min, washed 3 times and covered with RPMI. Gels were examined at X40 by

phase contrast microscopy and the distribution of lymphocytes through the gel was determined by counting the number of cells in each of a series of focus planes, starting 20 $\mu$ m below the gel surface and moving down at 20 $\mu$ m intervals until there were no more cells to be counted. The leading front of migration was assessed by measuring the distance of the 2 leading cells in the same focus plane. In each case, ten randomly chosen fields were examined in two replicates matrices.

#### Determination of tissue distribution of lymphocytes in vivo.

Lymphocytes were resuspended at  $10^7$  cells/ml and were labelled with 40 $\mu$ Ci/ml of sodium  $^{51}$ Cr chromate (Amersham, England) by incubating at 37 $^{\circ}$  C for 45 min. After washing 4 times in RPMI, the radiolabelled cells were resuspended at  $1.5-2.5 \times 10^7$  cells/ml and 0.2 ml injected i.v. into recipients. At intervals after injection, mice were killed, their organs removed and weighed, and  $^{51}$ Cr-specific radioactivity measured in a gamma counter (LKB 1282 Compugamma), the radioactivity in blood was measured in a volume of 0.2 ml. The results obtained from tissues were expressed as the percentage of the total radioactivity recovered from all the organs removed calculated as follows:

$$\% \text{ Recovery} = \frac{\text{Mean organ radioactivity (cpm)}}{\text{Total radioactivity in all organs removed (cpm)}} \times 100\%$$

In each experiment, one 0.2 ml aliquot of the cell

inoculum was retained to obtain the level of injected radioactivity, to measure the % recovery of injected dose, calculated as follows:

%Recovery of            =  
injected dose

$$\frac{\text{Total radioact. in all organs removed (cpm)}}{\text{Radioactivity in injected dose}} \times 100\%$$

#### Infection of mice with *Trichinella spiralis*.

Infective larvae were recovered from the muscles of previously infected NIH mice by pepsin (Sigma) HCl digestion (0.5% in physiological saline) for 2 hours. The suspension was allowed to stand for a few minutes to allow the larvae to settle and the volume was reduced to 15 ml. After counting, 450 viable larvae were administered to 13-14 week old mice by oral intubation using a rigid steel tube. These experiments were performed in association with Dr. R. G. Bruce, Department of Zoology, University of Glasgow.

#### Lymphocyte migration in oxazolone treated mice

6 days before cell transfer, mice were sensitised with oxazolone and 1 day before transfer of <sup>51</sup>Cr-labelled syngeneic lymphocytes, recipient mice received 10mg of 2 ethoxy-4 methyl oxazolone (BDH) dissolved in olive oil (10% w/v) on both ears.

In experiments using sensitised PLN lymphocytes, donor mice were also sensitised on both shaved flanks with 10 mg of oxazolone dissolved in acetone (10% w/v),

3 or 4 days before removal of draining lymph node lymphocytes.

#### Statistics.

Results are expressed as means  $\pm$  1 standard deviation, unless otherwise stated. Student's t-test was used to compare differences between groups in most cases. In experiments where non-parametric distributions were observed such as mortality assays, results were compared by Wilcoxon's Rank Sum test.

Crypt cell production rates, calculated by linear regression were compared by covariance analysis to detect differences between the slopes. Calculations were performed on Casio PB-100 and 180P calculators.

CHAPTER 3  
INFLUENCE OF DONOR CELLS IN THE  
INDUCTION OF GRAFT-VERSUS-HOST REACTION

## Introduction.

The experiments in this thesis not only investigated the properties of non-specific effector cells in intestinal GvHR, but also examined the relative abilities of selected donor cell populations to induce a GvHR. The studies in this chapter were therefore designed to establish the type of systemic and intestinal effector responses which characterise a GvHR and to determine the effects of using different donor cell populations on a GvHR in the same host-donor combination. In this way, it was hoped to establish that GvHR could be used to measure the ability of different lymphoid cells to mediate CMI *in vivo*.

The features of GvHR induced by spleen cells in unirradiated hosts, include splenomegaly and enhancement of peripheral and intestinal NK activity (Borland *et al.*, 1983), as well as increases in CCPR, crypt length and in IEL count in the small intestine (Mowat and Ferguson, 1982). Therefore, I performed a series of GvHR experiments to establish the development of these features in my hands. In addition, I studied the effect of altering the donor cell population by inducing a GvHR with either spleen or MLN cells and also by using mitomycin C-treated donor cells.

## Induction of systemic GvHR by spleen or MLN cells.

A GvHR was induced with  $6 \times 10^7$  CBA spleen cells or MLN cells *ip.* and at intervals thereafter, animals were killed for assessment of splenomegaly and splenic NK activity against YAC-1 target cells in microcytotoxicity

assays.

Both groups of GvHR mice developed significant splenomegaly (Fig. 1-A), but at all times of GvHR, hosts receiving parental MLN cells showed higher levels of splenomegaly compared with that observed after injection of parental spleen cells. Furthermore, splenomegaly appeared to persist longer in recipients of MLN cells. Identical results were found when this experiment was repeated on two separate occasions (data not shown). Similar results were observed when NK activity was measured in these groups of mice (Fig. 1-B). Both groups of GvHR mice exhibited an enhancement of NK activity compared with control levels and these peaked on day 8 of GvHR. However, MLN donor cells induced a much greater activation of NK activity than spleen cells, (Relative Cytotoxicity on day 8, 3.0 vs 1.8 for spleen cells). In addition, recipients of MLN cells had prolonged enhancement of NK activity which persisted until day 15, whereas recipients of spleen cells had no NK cell activation after day 8. Once again, identical results were observed in repeated experiments (data not shown).

Intestinal phase of GvHR induced by parental spleen cells or MLN cells.

The intestinal changes in a GvHR were measured by performing IEL counts and by measurements of villus and crypt length and CCPR on days 6 and 9 of the GvHR (Table 1). In this experiment, mice given MLN cells had

significantly greater splenomegaly than those given spleen cells on both days 6 ( $2.24 \pm 0.20$  vs  $1.87 \pm 0.11$   $p < 0.025$ ) and day 9 ( $1.93 \pm 0.12$  vs  $2.56 \pm 0.31$   $p < 0.025$ ) confirming the previous experiments. As has been shown previously (Mowat and Ferguson, 1981;1982) villus atrophy is not a feature of this model of GvHR and this was confirmed in the present experiment, where both groups of GvHR mice had identical villus lengths to control animals at both times (Table 1). Both groups of GvHR mice had significant crypt lengthening on day 6 compared with controls, but there was no difference between the values in recipients of spleen and MLN cells ( $109.3 \pm 6.3$   $\mu\text{m}$  vs  $111.4 \pm 4.3$   $\mu\text{m}$ ) respectively vs  $95 \pm 5.3$   $\mu\text{m}$  for controls). At this time, a higher CCPR was also found in recipients of both MLN and spleen cells compared with controls ( $8.7 \pm 1.02$  for spleen cells  $11.1 \pm 1.06$  for MLN and  $6.9 \pm 0.95$  for control). Although this was somewhat more pronounced in mice given MLN cells, none of these changes reached statistical significance.

A similar pattern was observed on day 9 of the GvHR, when the two groups of GvHR mice had identical and significant increases in crypt depth compared with control animals ( $115.2 \pm 6.0$   $\mu\text{m}$  and  $117.1 \pm 9.0$   $\mu\text{m}$  for spleen and MLN GvHR respectively, vs  $98.8 \pm 4.4$   $\mu\text{m}$  for controls). Once again, although GvHR mice also appeared to have slightly higher CCPR than controls at this time, these changes did not attain statistical significance. There were no significant differences in IEL count between any GvHR mice and controls at either time during

a GvHR. This was surprising in view of previous studies where this has been reported as a feature of intestinal GvHR (Mowat and Ferguson 1982), unusually high counts in control mice were found.

These results show that although MLN cells appear to be more effective than spleen cells at inducing systemic changes of a GvHR in unirradiated mice, this phenomenon is not found as consistently when the intestinal phase of GvHR is examined. However, the experiments underline the potential differences between donor cell populations in the induction of GvHR.

#### The role of donor T lymphocyte numbers in determining the intensity of GvHR

The results in the previous section showed that compared with spleen cells, parental MLN were able to induce a more severe GvHR in  $F_1$  hosts when measured as splenomegaly and enhancement of NK activity. One reason for this could be that MLN contained a higher proportion of alloreactive T cells and this was supported by an experiment in which the proportion of T cells in the two populations was examined by complement-dependent cytotoxicity. These studies showed that MLN cells contained 50% Thy 1.2<sup>+</sup> cells whereas only 20% of spleen cells were T cells by this criterion.

I then assessed if this higher proportion of T cells could account for the greater ability of MLN cells to induce a systemic GvHR. Therefore, a GvHR was induced using preparations of MLN and spleen cells which

had been adjusted to give equal numbers of T cells in each donor inoculum. Accordingly, (CBA X BALB/c) $F_1$  mice received either  $6 \times 10^7$  CBA spleen cells or  $2.4 \times 10^7$  MLN cells and the development of splenomegaly in these groups is shown in Fig. 2. This reduced number of MLN cells still induced greater splenomegaly than that found in recipients of spleen cells, at all times of the GvHR. This was also more prolonged than in recipients of spleen cells. As these differences were similar to those found using unadjusted populations of MLN cells, the greater ability of MLN cells to induce a systemic GvHR cannot be attributed solely to the number of T cells present in the two populations.

Importance of donor cell proliferation in the induction of systemic and intestinal GvHR.

Several of the experiments described in later chapters used donor cells which appear to have abnormal abilities to proliferate. Therefore, I examined the importance of proliferative capacity in the ability of donor cells to induce GvHR. The proliferative ability of CBA spleen cells was inhibited by pretreatment with mitomycin C and these cells were compared with normal spleen cells for their ability to induce systemic and intestinal GvHR in (CBA X BALB/c) $F_1$  hosts.

As expected, significant splenomegaly occurred in mice receiving normal spleen cells with a peak on day 8 (SI  $2.5 \pm 0.13$ ) and remaining significant on day 10 (Fig. 3). In contrast mice receiving mitomycin C treated donor cells had no splenomegaly at any time during GvHR.

Intestinal pathology was assessed on day 8 of the GvHR (Table 2). At this time, mice given normal parental spleen cells had a significant increase in crypt depth compared with controls ( $116.6 \pm 4.1 \mu\text{m}$  vs  $103 \pm 4.7 \mu\text{m}$   $p < 0.005$ ) and also had an increased CCPR, although this was not statistically significant ( $18.3 \pm$  vs  $14.5 \pm$ ). Villus atrophy was not found in GvHR mice. In contrast, mice given mitomycin C treated cells had no alterations in either crypt length ( $105.1 \pm 57.3 \mu\text{m}$ ) or CCPR ( $15.22 \pm$ ) compared with controls. Thus, donor lymphocytes need to proliferate to be able to induce both systemic and intestinal GvHR.

#### Summary and Conclusions.

The experiments in this chapter showed that, compared with spleen cells from the same donors, parental MLN cells were more efficient at inducing a systemic GvHR in unirradiated adult mice and there were some indications that MLN cells also induced a more severe intestinal GvHR. These studies also confirmed the observations that systemic GvHR is accompanied by NK cell activation and reproduced some of the intestinal alterations which have been reported previously in this model of GvHR. Although MLN contained more T cells than spleen cells this did not account for the greater ability of MLN cells to induce a GvHR, as greater splenomegaly occurred even after adjusting for this excess of T cells. Finally, treatment of donor spleen cells with mitomycin C abolished their ability to induce

systemic and intestinal GvHR, indicating that donor lymphocytes proliferation is required for these phenomena.

These findings highlight some of the effector mechanisms induced by a GvHR and show that the systemic consequences in particular are highly dependent on the nature of the donor cell used to induce the GvHR. However, the gut changes were not as sensitive in this respect. These findings illustrate the usefulness of the GvHR as a means of assessing the cell mediated effector potential of different populations of donor lymphoid cells, a feature which will be explained in future studies.

DAY 6.

HOST	V.H.	C.D.	CCPR.	IEL counts
CONTROL	600.6 $\pm$ 50.5 $\mu$ m	95.3 $\pm$ 5.3 $\mu$ m	6.9 $\pm$ 0.95	19.0 $\pm$ 2.1
SPLEEN CELLS	565.2 $\pm$ 86.9 $\mu$ m	109.3 $\pm$ 6.3 $\mu$ m*	8.7 $\pm$ 1.02	22.7 $\pm$ 3
MLN CELLS	590.1 $\pm$ 20.3 $\mu$ m	111.4 $\pm$ 4.3 $\mu$ m***	11.1 $\pm$ 1.06	21.9 $\pm$ 2.8

DAY 9.

HOST	V.H.	C.D.	CCPR.	IEL counts
CONTROL	541.3 $\pm$ 43.8 $\mu$ m	98.8 $\pm$ 4.4 $\mu$ m	9.9 $\pm$ 1.00	22.3 $\pm$ 2.4
SPLEEN CELLS	574.5 $\pm$ 19.9 $\mu$ m	115.2 $\pm$ 6.0 $\mu$ m**	12.1 $\pm$ 2.09	23.4 $\pm$ 3
MLN CELLS	521.6 $\pm$ 35.7 $\mu$ m	117.1 $\pm$ 9.0 $\mu$ m*	12.2 $\pm$ 1.75	23.3 $\pm$ 4.0

Table 1. Comparison of intestinal GvHR in (CBA X BALB/c)F<sub>1</sub> mice induced by either 6X10<sup>7</sup> CBA spleen or MLN cells. <sup>1</sup> Results shown are means  $\pm$  1 s.d. for 4 mice per group. \* p<0.025, \*\* p<0.01 and \*\*\* p<0.005.

C. D. = Crypt depth  
V. H. = Villus height

HOST	<u>V.H.</u>	<u>C.D.</u>	<u>CCPR.</u>
CONTROL	547.2±28.5µm	103±4.7µm	14.5±1.00
NORMAL CELLS	566.5±17.9µm	116.6±4.1µm *	18.3±1.03
M.C. CELLS	585.2±45.2µm	105.1±57.3µm	15.22±1.23

Table 2. Comparison of intestinal GVHR in (CBA X BALB/c)F<sub>1</sub> mice induced by either 6X10<sup>7</sup> CBA normal or mitomycin C-treated spleen cells. Results shown are means± 1 s. d. for 5 mice/group. \* p<0.005.

C. D. = Crypt depth  
V. H. = Villus height

MC= Mitomycin-C

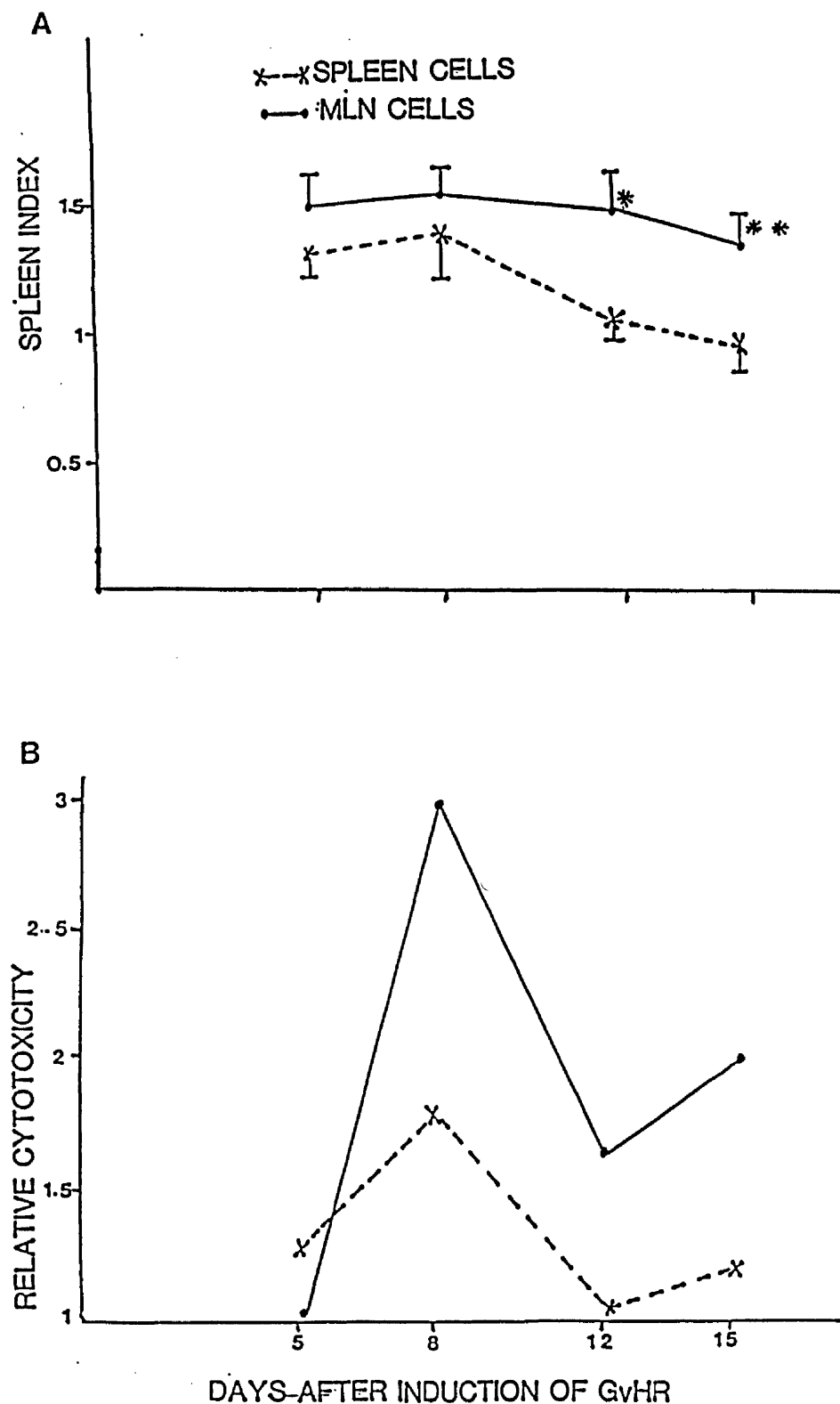


Fig. 1. Evolution of systemic GvHR in unirradiated (CBA X BALB/c) $F_1$  mice injected with CBA MLN or spleen cells. Development of: B) NK cell activity at 50:1 at E:T. Results in GvHR mice are expressed as a ratio to those in control mice using cells pooled from 3-4 mice/group. A) Splenomegaly during GvHR. Results are means  $\pm$  1 standard deviation for 3-4 mice/group. \*  $p < 0.025$  and \*\*  $p < 0.005$ .

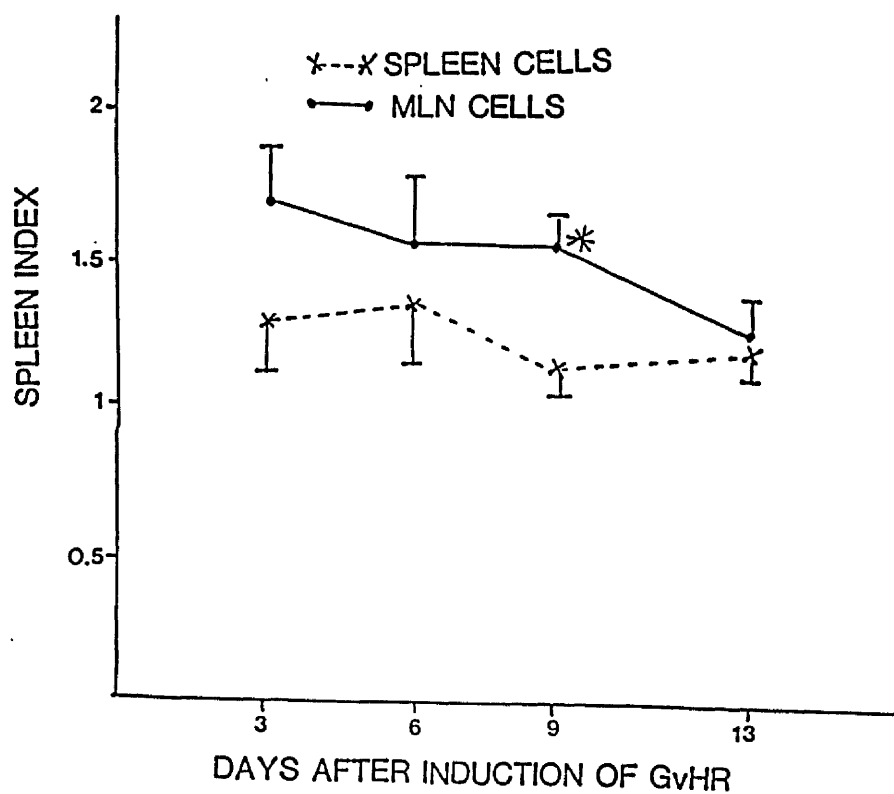


Fig. 2. Induction of GvHR in (CBA X BALB/c) $F_1$  mice by inoculation of CBA MLN cells and spleen cells containing the same number of T cells. The results shown are mean Spleen Indices  $\pm$  1 standard deviation from 3 mice/group after transfer of  $6 \times 10^6$  spleen cells and  $2.4 \times 10^6$  MLN cells. \*  $p < 0.005$ .

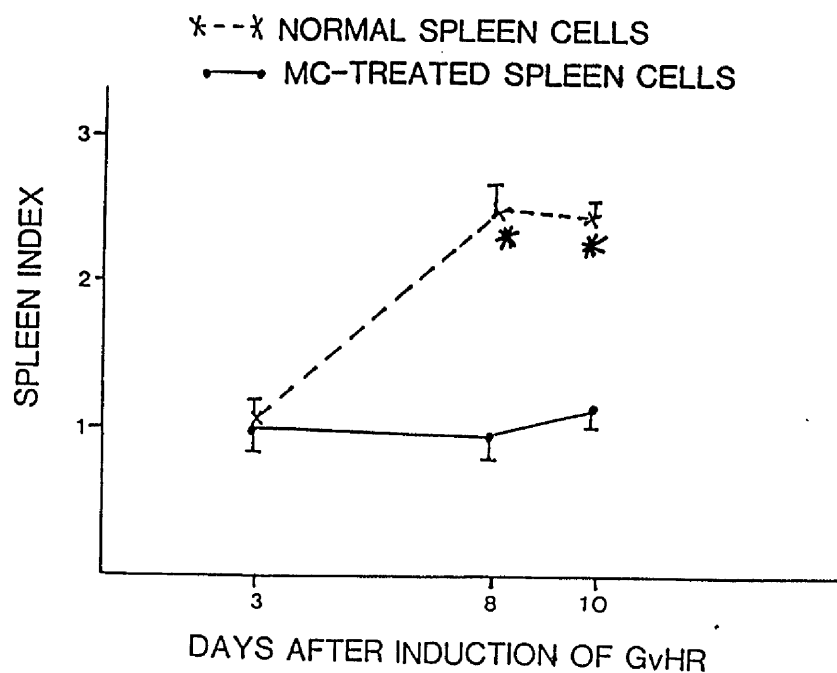


Fig. 3. Induction of GvHR in (CBA X BALB/c) $F_1$  mice by mitomycin C (MC)-treated and control CBA spleen cells. The results shown are mean Spleen Indices  $\pm$  1 standard deviation from 3 mice/group. \*  $p < 0.005$  vs MC-treated cells. Mice were infected with  $5 \times 10^7$  spleen cells.

## CHAPTER 4

### ROLE OF DONOR NATURAL KILLER CELLS IN INTESTINAL AND SYSTEMIC GRAFT-VERSUS-HOST REACTION

## Introduction.

As shown in the previous chapter and as reported (Roy *et al.*, 1982; Borland *et al.*, 1983; Kubota *et al.*, 1983), enhancement of NK activity is a characteristic feature of GvHR in unirradiated  $F_1$  mice. One interpretation of this enhanced NK activity is that NK cells are mediating a form of  $F_1$  resistance in GvHR, similar to that described in  $F_1$  hosts given parental haemopoietic cells (Cudkowicz and Stimpfling, 1964; Warner and Dennert, 1982; Harrison and Carlson, 1983; Lotzova, *et al.*, 1983). Alternatively, activated NK cells could play an important effector role in the pathogenesis of GvHR (Charley *et al.*, 1983, Borland *et al.*, 1983; Mowat and Felstein, 1987). That NK cells may be important in the effector phase of CMI responses is supported by the presence of NK cell infiltrates in rejecting allografts (Nemlander *et al.*, 1986) and by the fact that depletion of NK cells inhibits the development of autoimmune diabetes in rats (Like *et al.*, 1986). Both, host and donor NK cells are recruited in mice undergoing GvHR (Lapp *et al.*, 1985) and in this chapter, I studied the role of donor NK cells in the development of intestinal and systemic GvHR by assessing the ability of lymphoid cells from NK deficient beige mice to induce a GvHR.

## Experimental protocol.

Four separate models of GvHR were used to examine the potential of beige spleen cells to mediate a GvHR. First, an entirely proliferative, systemic GvHR was

induced in unirradiated (C3H X B6) $F_1$  mice; secondly, an acute and occasionally lethal GvHR, was induced in adult, unirradiated BDF $_1$  hosts; next, I examined the local GvHR which occurs in the popliteal lymph node after footpad injection of  $F_1$  hosts, and, finally, an acute, lethal GvHR was induced in irradiated hosts. In each experiment, the ability of beige spleen cells to induce a GvHR was compared with that of normal congenic B6 mice. The progress of systemic GvHR was assessed by mortality, weight loss and splenomegaly, while in some experiments these changes were accompanied by measurements of intestinal pathology.

Ability of beige mouse lymphocytes to induce a proliferative GvHR in unirradiated  $F_1$  adults.

In this first experiment, I examined the GvHR that occurs in adult, unirradiated (C3H X B6) $F_1$  hosts after injection of parental spleen cells. This produces an entirely proliferative disease with no weight loss and no destructive changes in the gut, but it is accompanied by splenomegaly and enhancement of splenic NK activity. Adult  $F_1$  mice were given  $6 \times 10^7$  B6 or beige spleen cells ip and were sacrificed from 2 to 16 days thereafter to assess NK and anti-host CTL activity and for measurements of splenomegaly.

Initial experiments confirmed that the beige mice used as donors had little or no NK activity compared with B6 mice (Fig. 4). Thus B6 spleen cells showed 25% lysis of YAC-1 target cells at 50:1 E:T, while beige

spleen cells showed only 2% lysis under the same conditions. Similar results were found at other E:T ratios (Fig. 4).

When B6 spleen cells were used to induce a GvHR in (C3H X B6) $F_1$  hosts, significant splenomegaly was found on day 6 (Fig. 5-A SI  $1.53 \pm 0.10$ ) which then declined to levels only slightly above normal on days 8 and 14. In contrast, beige spleen cells were completely unable to induce splenomegaly at any time after transfer to identical hosts. In parallel, (C3H X B6) $F_1$  mice given B6 spleen cells had enhanced splenic NK activity on day 6 of the GvHR compared with control mice (Fig. 5-B). This peaked on day 8 and fell thereafter. Beige spleen cells produced some enhancement of NK activity in host spleens, but this was only apparent on day 8 and was considerably less than found with B6 cells.

A similar experiment was conducted in BDF $_1$  hosts given  $6 \times 10^7$  parental spleen cells. As before, mice given B6 spleen cells developed significant splenomegaly in the first 2 weeks of GvHR (Fig. 6) SI  $1.45 \pm 0.25$  on day 12. In contrast, recipients of beige spleen cells had no splenomegaly compared with controls. This data is taken from controls in experiment shown in Fig. 10-4 three further experiments showed identical results (data not shown).

#### Ability of beige mouse lymphocytes to induce a local proliferative GvHR.

In the next experiment, I examined whether beige spleen cells were also defective in inducing a local

GvHR in the popliteal lymph node of  $F_1$  hosts. (C3H X B6) $F_1$  mice were injected into one footpad with  $2 \times 10^7$  B6 or beige spleen cells and the draining popliteal lymph node weighed 8 days later. B6 cells produced a significant increase in lymph node weight compared with control lymph nodes which had been injected with medium only (Table 3.  $10.02 \pm 3.2$  mg vs  $1.57 \pm 0.4$  mg  $p < 0.001$ ). A significant weight increase also occurred in lymph nodes of mice given beige spleen cells but this was significantly less than that found after injection of B6 cells compared to lymph nodes from beige cells recipients ( $3.50 \pm 0.4$   $p < 0.001$ ). Thus, beige spleen cells are also deficient in their ability to induce a local GvHR in unirradiated hosts.

Ability of beige mouse lymphocytes to induce an acute GvHR in unirradiated hosts.

In the next experiment, adult unirradiated BDF $_1$  mice were injected iv. with  $10^8$  B6 or beige spleen cells as a means of studying the ability of beige cells to induce the acute, often lethal GvHR which normally occurs in this model (Gleichmann et al., 1984). BDF $_1$  mice injected with normal B6 cells began to lose weight after day 10 and this continued until day 20 (Fig. 7). At this time, 2 mice died and most animals had a characteristic runted appearance and significant low weight compared to controls. These mice remained below normal weight and at the end of the experiment on day 50, had a mean weight of  $27.3 \pm 1.7$  g compared with

31.9±1.5g for controls ( $p<0.001$ ). In contrast, recipients of beige spleen cells showed only slight weight loss around day 14, which was not significant and their weights rapidly recovered to control levels thereafter. No deaths and no runting occurred in this group. At the end of the experiment, these hosts had similar weights to control animals (30.7±1.5g). Thus, beige spleen cells are markedly defective in their ability to induce either a proliferative or destructive systemic GvHR in unirradiated adult hosts.

#### Intestinal phase of GvHR induced by beige spleen cells in unirradiated hosts.

The intestinal changes in an acute GvHR induced by injecting BDF<sub>1</sub> either  $10^8$  B6 or beige spleen cells were assessed by measuring villus and crypt lengths as well as CCPR on days 14, 20 and 29 of GvHR.

The findings are shown in Table 4. On day 14, a significant increase in crypt depth was observed in recipients of both B6 and beige spleen cells (139.3±6.3µm and 126.7±6.8µm) respectively compared with (102.0±2.1 µm in controls  $p<0.001$ ). Significant villus atrophy was also found in both recipients of B6 and beige cells at this time (397.6±62.5 µm and 402.1±54.5 µm respectively compared with (493.4±38.5 µm for controls  $p<0.05$ ). Both these groups of GvHR mice had increases in CCPR, but this was only significant in mice receiving B6 spleen cells (24.5±3.5 vs 9.7±1.4 for controls  $p<0.02$ ) At this time, recipients of beige cells had a CCPR of 18.1±2.4.

On day 20, the increased in crypt depth ( $161.5 \pm 12.0 \mu\text{m}$   $p < 0.001$ ) and CCPR ( $29.6 \pm 3.5$   $p < 0.02$ ) as well as villus atrophy ( $399.0 \pm 45.2 \mu\text{m}$   $p < 0.001$ ) were still present in mice injected with B6 cells and if anything, had intensified. In contrast, crypt lengths ( $124.1 \pm 18.4 \mu\text{m}$ ) and CCPR ( $18.8 \pm 2.4$ ) in mice receiving beige cells were the same as day 14, while villus length had recovered slightly ( $486.7 \pm 26.6 \mu\text{m}$ ). Values in controls at this time were essentially identical to those on day 14.

On day 29, recipients of B6 cells still had significant intestinal damage with persistent increases in crypt length ( $124.1 \pm 6.2 \mu\text{m}$   $p < 0.001$ ) and CCPR ( $29.7 \pm 4.7$   $p < 0.025$ ) as well as <sup>VILLUS ATROPHY</sup> ( $456.5 \pm 6.2 \mu\text{m}$   $p < 0.001$ ). In contrast, recipients of beige cells now had crypt ( $101.3 \pm 3.4 \mu\text{m}$ ) and villus length ( $489.1 \pm 9.7 \mu\text{m}$ ) which were identical to controls values. However, these mice did have a significant increase in CCPR ( $27.9 \pm 1.1$  compared with  $9.9 \pm 2.1$  for controls  $p < 0.001$ )

These results show that although B6 and beige spleen cells were both able to induce the intestinal phase of GVHR in BDF<sub>1</sub> mice, the changes induced by beige cells were normally milder than those induced by B6 spleen cells and were also less sustained.

#### Ability of beige mouse lymphocytes to mediate a lethal GVHR in irradiated hosts.

The preceding studies showed that lymphocytes from beige mice were very poor at inducing many of the

characteristic features of GvHR in three different models in GvHR in unirradiated hosts. As a GvHR in intact hosts is markedly influenced by the involvement of host cells, it was important to study the behaviour of beige donor cells in an immunoincompetent host.

Adult BDF<sub>1</sub> mice were irradiated with 450 Rads and injected with  $2 \times 10^7$  B6 or beige spleen cells iv. As shown in Fig. 8, all recipients of B6 spleen cells died by day 22 (MST 22). Furthermore, mice given beige spleen cells had an identical mortality rate all recipients dying by day 21 (MST 17). All BDF<sub>1</sub> mice reconstituted with syngeneic spleen cells or left unreconstituted after irradiation survived the course of the experiment.

Thus, beige spleen cells are entirely normal in their ability to induce a lethal GvHR in irradiated mice.

#### Effect of Interleukin-2 on the ability of beige mouse lymphocytes to induce a GvHR in unirradiated mice.

The ability of beige spleen cells to cause a GvHR in irradiated mice indicated that these cells do have the potential to mediated alloreactivity in a GvHR. It was of interest therefore to investigate whether an immunomodulator would allow beige cells to produce GvHR in intact hosts.

Donor B6 and beige mice were injected with 1000 u of human recombinant IL-2 in 15% gelatine ip one day before their spleen cells were used to induce a GvHR in unirradiated BDF<sub>1</sub> hosts as described before.

The NK cell activity of the cell inocula was measured as a means of assessing the effect of the IL-2 treatment. As shown in Fig. 9, the NK cell activity of B6 mice given rIL-2 24 hours before was enhanced compared with B6 controls (15.9% vs 5.3% lysis at 50:1 E:T). In contrast, the NK activity of beige mice which received rIL-2 was the same as that in control beige mice (3.9% vs 3.3% cytotoxicity respectively). Nevertheless, these results indicate that the protocol of IL-2 treatment was capable of producing a biological effect in donor mice.

The ability of spleen cells from IL-2 treated B6 and beige mice to induce a GvHR is shown in Fig. 10-A. As shown previously, BDF<sub>1</sub> mice given B6 spleen cells had significant splenomegaly by day 12 ( $1.45 \pm 0.25$ ) and this was significantly greater in recipients of spleen cells from IL-2 treated B6 mice ( $2.08 \pm 0.3$   $p < 0.05$ ). In contrast, administration of IL-2 to donor beige mice had no effect on their inability to induce a GvHR in unirradiated hosts, with virtually no splenomegaly at any time in either group. The level of splenic NK activity was also enhanced on day 5 of a GvHR induced by B6 cells, compared with that in controls (Fig. 10-B). Spleen cells from IL-2 treated B6 mice not only produced enhancement of NK activity which was more marked than that induced by control B6 cells, but this also persisted throughout the GvHR. Recipients of control beige spleen cells had little or no change in splenic NK activity. However, IL-2 treated beige spleen cells

produced enhanced NK activity on day 5 of the GvHR with levels similar to those found in recipients of IL-2 treated B6 cells.

Thus, treatment of beige mice with rIL-2 had conflicting effects on the ability of beige donor cells to induce systemic GvHR in unirradiated mice with no effect in the induction of splenomegaly, but were able to induce enhancement of NK activity in host mice.

#### Summary and Conclusions.

In this chapter I have shown that, compared with congenic B6 cells, beige spleen cells are defective in their ability to induce different forms of GvHR in unirradiated  $F_1$  mice. This defect was manifested not only by the virtual absence of splenomegaly but also by the development of milder intestinal pathology. In addition, there was a lesser degree NK cell activation in host mice and beige spleen cells induced a defective local GvHR as measured by the popliteal lymph node assay. As well as this defective ability to cause a proliferative GvHR, beige spleen cells were unable to induce the acute, destructive disease which occurred in unirradiated BDF<sub>1</sub> mice given  $10^8$  B6 cells. Administration of rIL-2 *in vivo* enhanced the NK activity of B6 spleen cells and increased their ability to induce a proliferative GvHR, but rIL-2 had no effect on beige spleen to induce splenomegaly but it had an effect on the enhancement of host NK activity. In contrast to these findings in intact hosts, beige lymphocytes were as efficient as B6 lymphocytes in mediating a lethal

GvHR in irradiated hosts.

These results indicate that although NK cell deficient beige mice have the potential to induce a GvHR, this is markedly reduced compared with normal animals and is only revealed in immunoincompetent hosts. Together these findings could support an important role for donor NK cells in the induction of GvHR. However, before this hypothesis can be accepted, it is important first to exclude the possibility of other immune defects in beige mice.

DONOR CELLS	HOST	LYMPH NODE WT. (mg)
MEDIUM	BDF <sub>1</sub>	1.57±0.4
B6	BDF <sub>1</sub>	10.02±3.2 *
BEIGE	BDF <sub>1</sub>	3.50±0.4

Table 3. Induction of a local GvHR by B6 and beige spleen cells. BDF<sub>1</sub> mice were injected into the footpad with 10<sup>7</sup> B6 or beige cells and the draining popliteal lymph node weighed 8 days later. Result shown are means ± 1 standard deviation for 6 mice/group. \* p<0.001 vs beige cells.

# DAY 14

<u>HOST</u>	<u>V.H.</u>	<u>C.D.</u>	<u>CCPR</u>
CONTROL	493.0±38.5 $\mu m$	102.0±2.1 $\mu m$	9.7±1.4
B6 F <sub>1</sub>	397.6±62.5* $\mu m$	139.3±6.3***	24.5±3.5**
Bg F <sub>1</sub>	402.1±54.5* $\mu m$	126.7±6.8***	18.1±2.4

# DAY 20

<u>HOST</u>	<u>V.H.</u>	<u>C.D.</u>	<u>CCPR</u>
CONTROL	528.0±22.6 $\mu m$	100.9±1.5 $\mu m$	10.2±1.2
B6 F <sub>1</sub>	399.0±45.2*** $\mu m$	161.5±12.0*** $\mu m$	29.61±3.5**
Bg F <sub>1</sub>	486.7±26.6* $\mu m$	124.1±18.4* $\mu m$	18.8±2.4

# DAY 29

<u>HOST</u>	<u>V.H.</u>	<u>C.D.</u>	<u>CCPR</u>
CONTROL	503.2±7.8 $\mu m$	98.4±2.0 $\mu m$	9.88±2.1
B6 F <sub>1</sub>	456.5±6.2*** $\mu m$	124.1±6.2*** $\mu m$	29.7±4.7**
Bg F <sub>1</sub>	489.1±9.7 $\mu m$	101.3±3.4 $\mu m$	27.89±1.1***

Table 4. Mucosal architecture in jejunum of adult BDF<sub>1</sub> mice with GvHR induced with either B6 or beige spleen cells. Villus height (V.H.) and crypt depth (C.D.) are expressed in  $\mu m$  and results shown are means  $\pm$  1 standard deviation for 5 mice/group CCPR. \*  $p < 0.05$ , \*\*  $p < 0.025$  and \*\*\*  $p < 0.001$ .

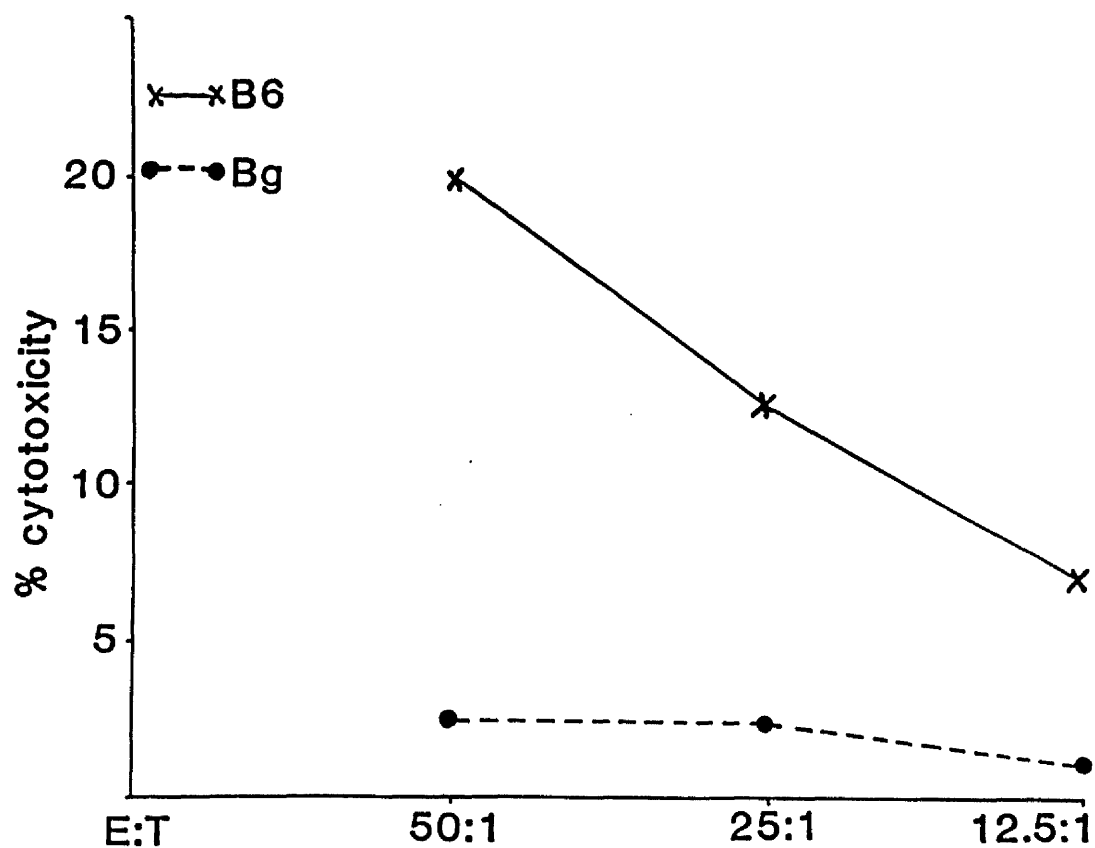


Fig. 4. Levels of NK activity in the spleen of normal B6 and beige mice. Results shown are % lysis obtained at different E:T ratios using cells pooled from 4-5 mice/group.

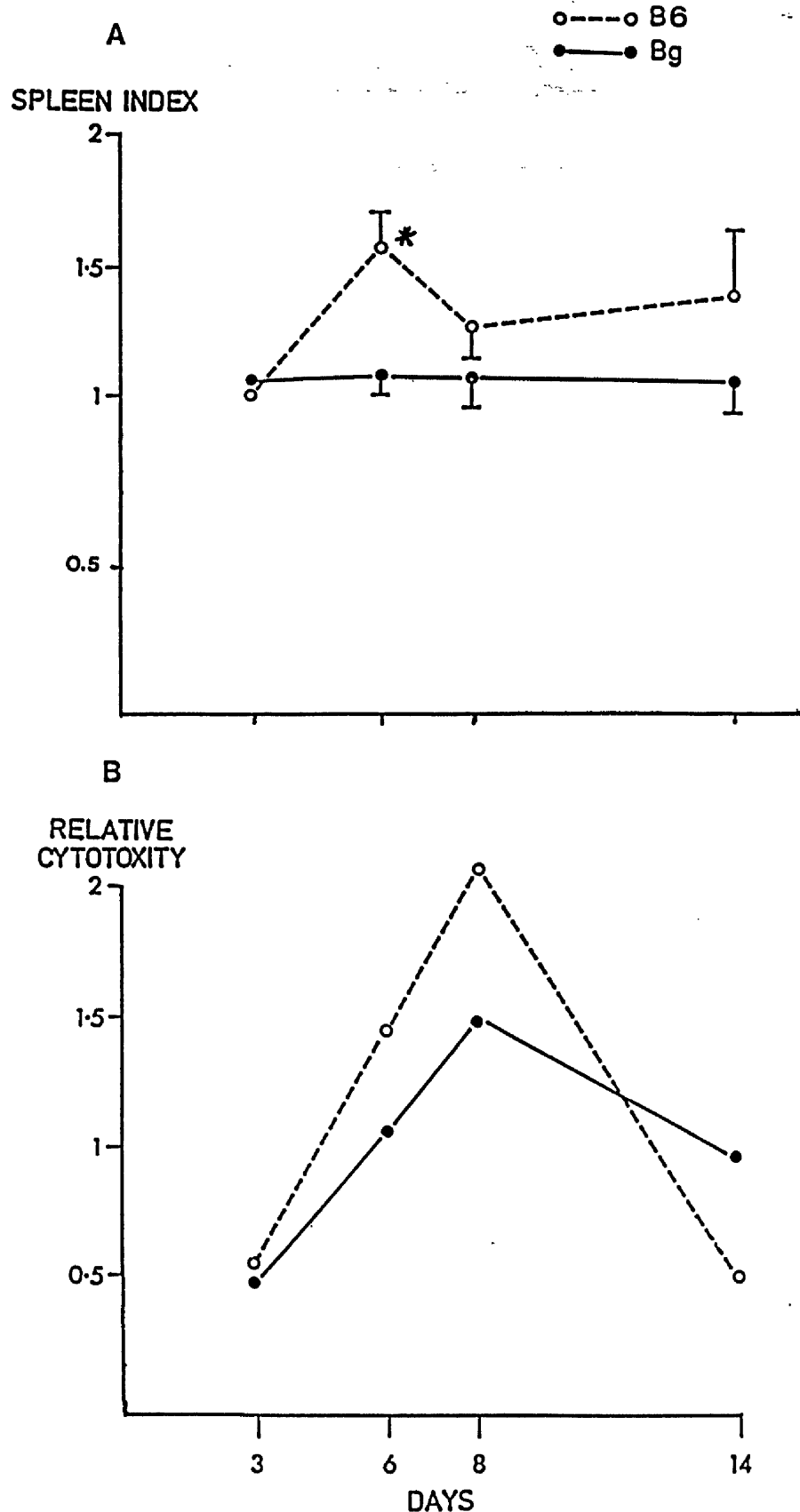


Fig. 5. Evolution of systemic GvHR in unirradiated (C3H X B6) $F_1$  mice, given either B6 or beige spleen cells. B ) Development of splenic NK cell activity during GvHR. Results shown are the NK activities at 50:1 E:T in GvHR mice expressed as a ratio to that in controls using cells from 3-4 mice/group. A ) Development of splenomegaly during GvHR. Results are mean Spleen Indices  $\pm$  1 standard deviation for 3-4 mice/group.

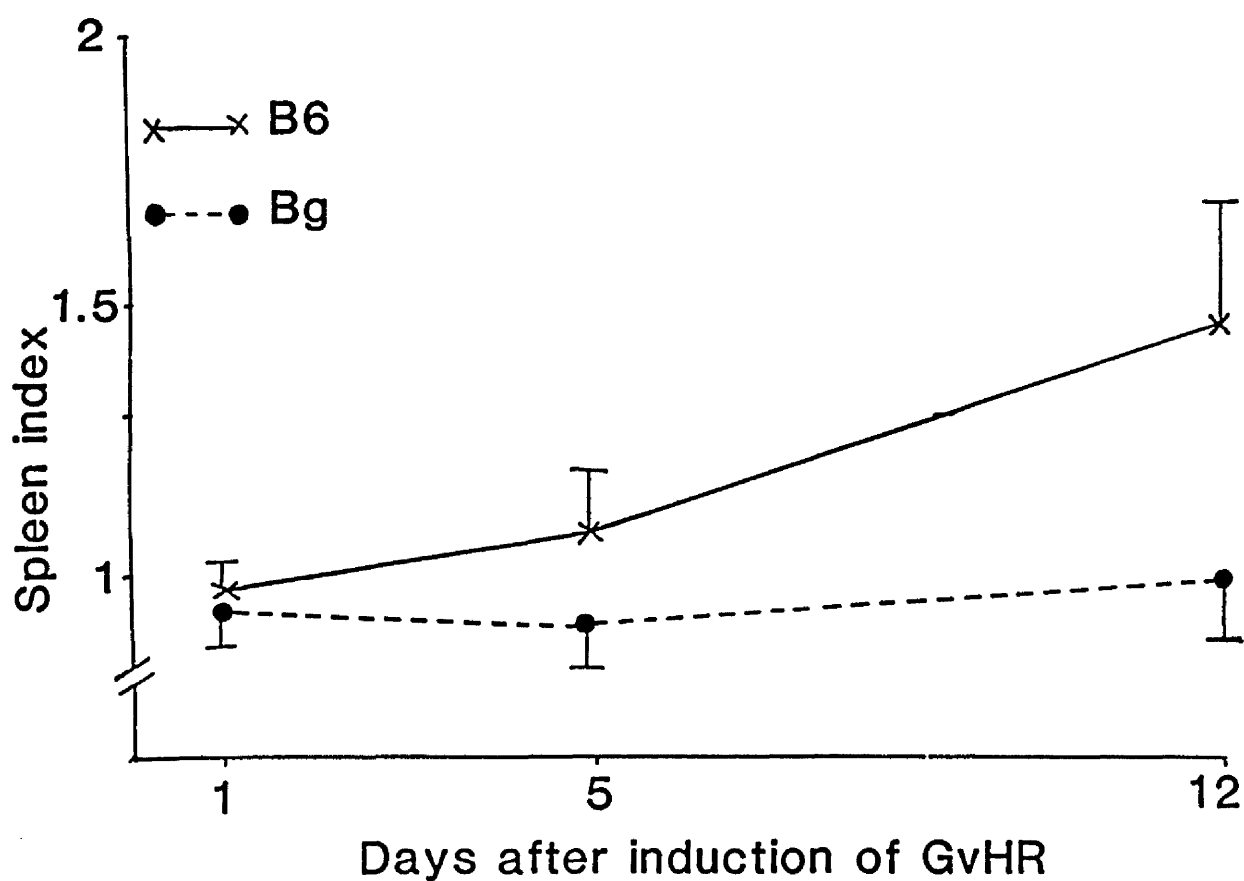


Fig. 6. Evolution of systemic GvHR in unirradiated BDF<sub>1</sub> mice given either B6 or beige spleen cells. The results shown are mean Spleen<sub>7</sub> Indices for 3 mice/group after ip.transfer of  $6 \times 10^7$  beige or B6 spleen cells.

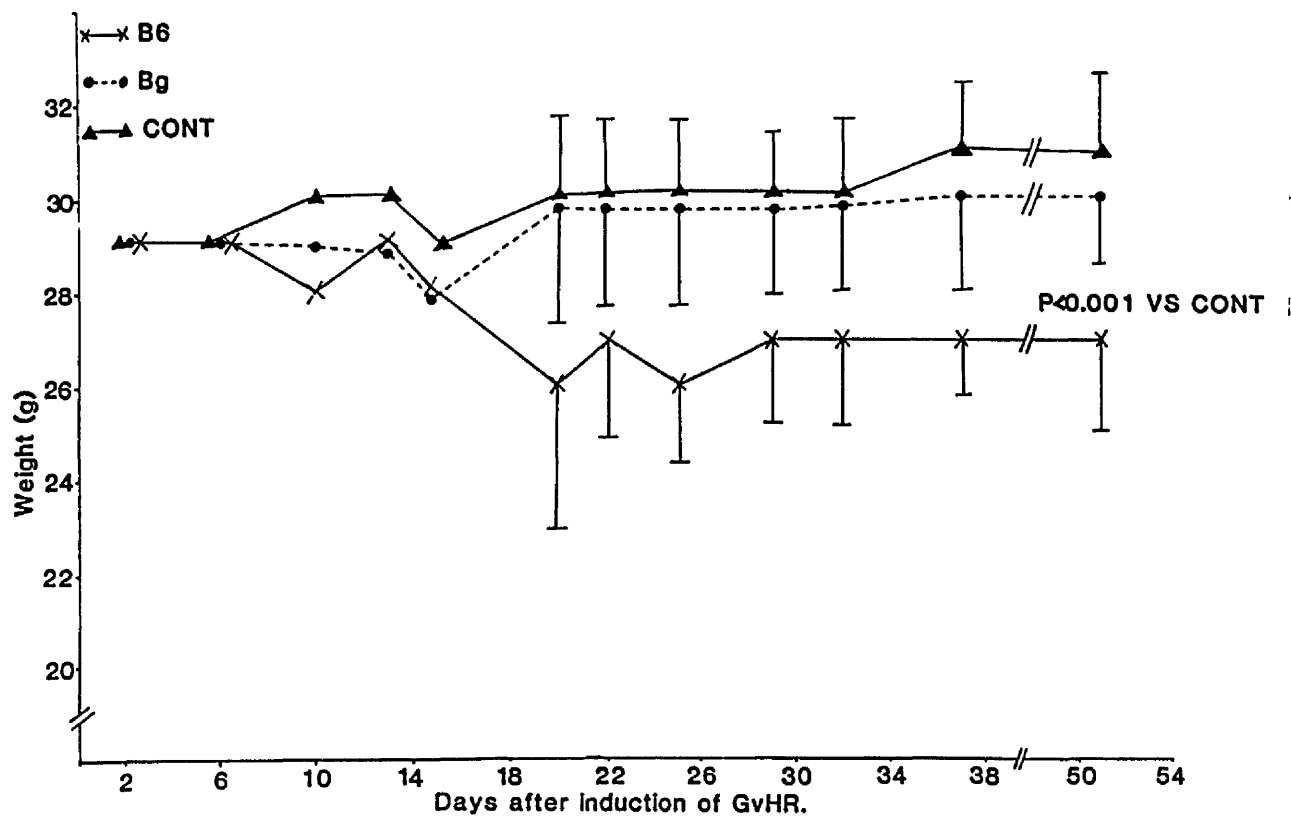


Fig. 7. Induction of acute GvHR in BDF<sub>1</sub> mice. BDF<sub>1</sub> hosts were inoculated with 10<sup>8</sup> B6 or beige spleen cells<sup>1</sup>iv and results shown are mean body weights  $\pm$  1 standard deviation for 11 mice/group. \*p<0.001 vs control.

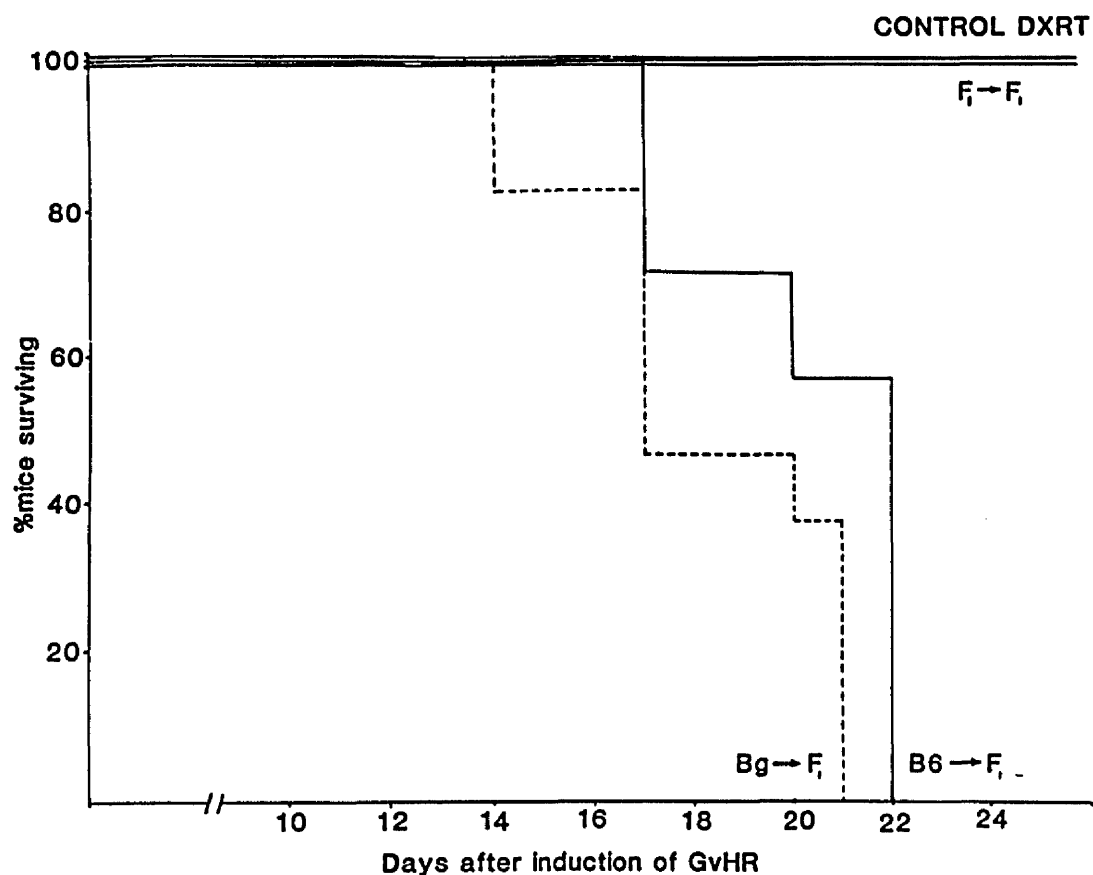


Fig. 8. Induction of a lethal GvHR in sublethally irradiated BDF<sub>1</sub> mice by 10<sup>7</sup> B6 or beige spleen cells. Control mice were either left unreconstituted after radiation (control DXRT) or received syngeneic cells. Results shown are % surviving animals at each time for 8-10 mice/group.

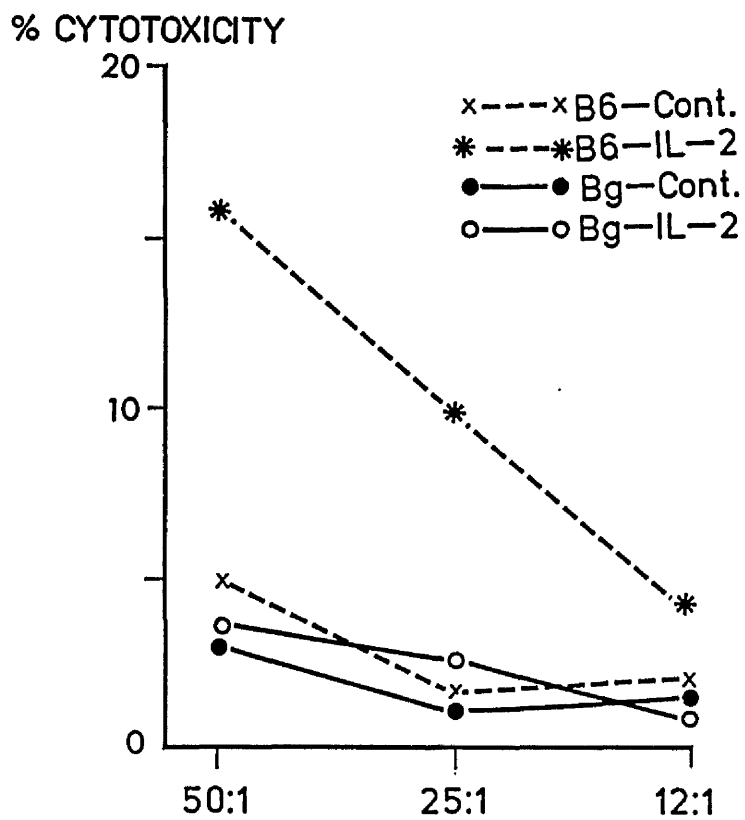


Fig. 9. Effect of IL-2 on the levels of splenic NK activity of B6 and beige mice. B6 and beige mice were inoculated with 1000 u of IL-2 ip and the levels of NK activity measured one day later. Results shown are % of lysis at 50:1 E:T ratios from cells pooled from 3 mice.

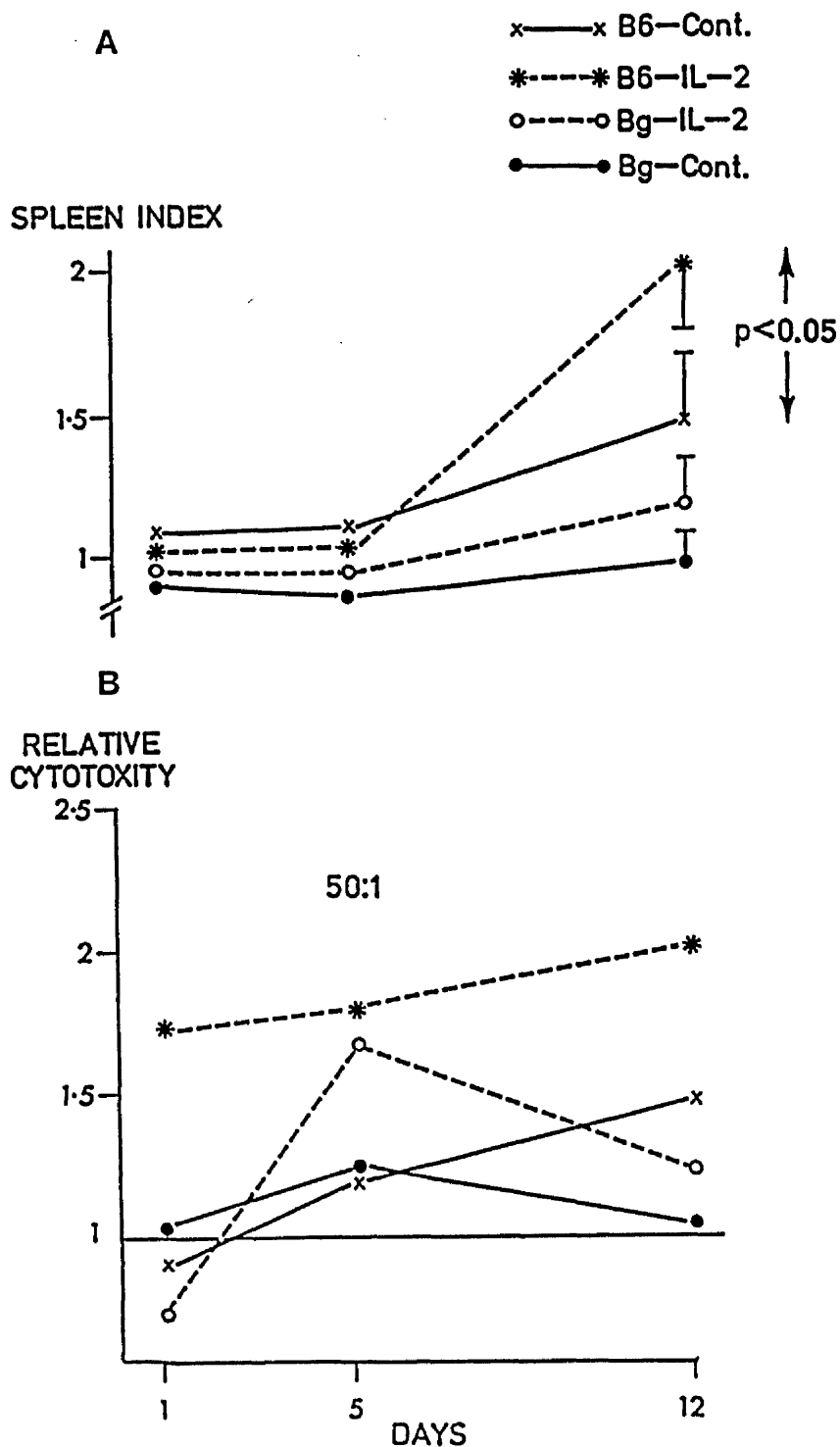


Fig. 10. Effect of treating B6 or beige donor mice with rIL-2 on the ability of spleen cells to induce GvHR in BDF<sub>1</sub> mice. Donor mice were inoculated with 1000 U of rIL-2 ip one day before removed of their spleen cells to induce GvHR. B ) Development of NK activity during GvHR. Results shown are the NK activities at 50:1 E:T in GvHR mice expressed as a ratio to that in controls, using cells from 3-4 mice/group. A ) Splenomegaly in GvHR. Results are mean Spleen Indices  $\pm 1$  standard deviation for 3-4 mice/group.

CHAPTER 5  
T CELL FUNCTION IN BEIGE MICE

## Introduction.

It is clear from the experiments in Chapter 4 that spleen cells from C57BL/6 mice carrying the beige defect have a defective ability to induce a GvHR in vivo. As beige mice are commonly used as a specific means of examining the role of NK cells activity, these results are circumstantial evidence that donor NK cells may be important for the induction of GvHR. However, while I was completing these experiments, I became aware of work (Saxena et al., 1982; Halle-Pannenko and Bruley-Rosset, 1985) which challenged the early reports that the beige defect was expressed exclusively in NK cells (Roder and Duwe, 1979).

In view of this possibility and because other T cell functions in beige mice have not been examined in detail, I considered it important to determine whether T cell defects could account for the inability of beige cells to induce a GvHR. Therefore, in this chapter, I have compared the T cell functions of beige lymphocytes with those of normal, congenic B6 mice.

T cell subsets in the spleen and lymph nodes of beige and normal B6 mice.

The number and type of T cells in a lymphocyte population can influence the outcome of GvHR (Bril and Benner, 1985) and in the first experiments, the proportion of helper/inducer and cytotoxic/suppressor T cells in the spleen and lymph nodes of beige and B6 mice were compared.

Spleen cells and MLN lymphocytes from B6 and beige mice were analysed for the proportion of  $T_{H/i}$  and  $T_{C/s}$  by immunofluorescence using anti L3T4 ( $T_{H/i}$ ) and rat anti Lyt2 ( $T_{C/s}$ ), as described in the Materials and Methods.

The percentage of T cells and the proportion of Lyt 2<sup>+</sup> and L3T4<sup>+</sup> cells in the spleen and MLN of B6 and beige mice are shown in Table 5. In the spleen and MLN of B6 mice there were 15.3±1.2% and 21.6±2% Lyt 2<sup>+</sup> cells respectively, while the percentage of L3T4<sup>+</sup> lymphocytes in these organs was 22.6±2% and 50±2.9% respectively. The proportion of Lyt2<sup>+</sup> cells in spleen and MLN of beige mice was 16.3±1.2% and 24.6±1.6%, while that of L3T4<sup>+</sup> lymphocytes was 22±0.8% and 47.6±2.8% respectively. Therefore, there are no differences in either the absolute numbers of T cells or the proportion of T cell subsets between beige and B6 mice.

#### Generation of specific and non-specific cell mediated cytotoxicity after alloimmunisation of B6 and beige mice.

The next series of experiments examined various aspects of cell-mediated immunity in beige mice *in vivo*.

Immunisation with allogeneic tumour cells is an excellent means of inducing both specific CTL activity and of enhancing NK activity *in vivo* (Herberman *et al.*, 1977; Cerottini and Brunner, 1974). Therefore, the ability of beige and normal B6 mice to generate specific CTL at intervals after immunisation with P815

(H-2<sup>d</sup>) cells was compared. In addition, I examined the degree of NK cell activation induced by this protocol. B6 and beige mice were immunised with  $2 \times 10^7$  P815 cells and 3-17 days later, spleen cells were assayed in vitro for CTL activity against P 815 targets and for NK activity against YAC-1 targets.

The results in Fig. 11 indicate that specific CTL activity against P815 increased rapidly in the spleen of immunised B6 mice from day 6, reaching a peak value of 79.1% cytotoxicity (50:1 E:T) on day 13. By day 17, the activity had declined considerably to levels of 26.5%. Immunised beige mice showed a similar pattern of CTL generation, but the levels observed were much lower, with a peak of 45% on day 10 cytotoxicity and declining thereafter to levels of 10.2% by day 17.

The results in Fig. 12 show splenic NK activity in the same mice, measured against YAC-1 target cells. As expected, splenic NK activity in control B6 mice remained fairly constant around 20% lysis at 50:1 E:T, throughout the period of the experiment, while unimmunised beige mice had little or no NK activity at any time. B6 mice immunised with P815 cells had a marked increase in NK activity after day 6, which paralleled the rise in CTL activity. This reached a peak of 57.9% on day 13 and declined to 30.5% by day 17. Immunised beige mice also had an increase in NK activity from day 6, with maximum levels of 35% on days 10-13, declining to 9.3% by day 17. As before, these values did not approach those in immunised B6 mice.

Thus, immunisation of B6 and beige mice with allogeneic P815 cells produced parallel generation of CTL activity and enhance<sup>7ment</sup> of NK cell activity. However, beige mice generated lower levels of CTL and had NK cell activation than B6 mice and these defects were quantitatively very similar.

Systemic delayed type hypersensitivity responses in alloimmunised beige and B6 mice.

In view of the defective CTL activity in beige mice, the ability of beige mice to generate specific DTH responses was examined after immunisation with fully or semiallogeneic cells. Beige and normal B6 mice were tested for allospecific DTH by footpad challenge with appropriate cells, either 5 days after intradermal immunisation with  $2 \times 10^7$  (C3H X B6) $F_1$  (H-2<sup>kxb</sup>) spleen cells or 10 days after intraperitoneal immunisation with  $10^7$  P815 (H-2<sup>d</sup>) tumour cells.

B6 mice immunised with P815 cells had a specific increase in footpad thickness of  $0.54 \pm 0.12$  mm 24 hours after challenge with P815 cells and a virtually identical response was obtained in immunised beige mice (Fig. 13.  $0.46 \pm 0.23$  mm). Similar results were obtained when DTH responses were examined in B6 and beige mice immunised with (C3H X B6) $F_1$  cells. Thus, after challenge, B6 mice had a specific footpad increment of  $0.28 \pm 0.3$  mm while beige mice had a specific increment of  $0.32 \pm 0.11$  mm. These results shown that beige mice generate normal allospecific DTH responses in vivo.

I next examined the capacity of beige mice to

mediate T cell functions in vitro.

#### Proliferative responses of beige lymphocytes to ConA.

In the first experiment B6, and beige spleen cells were cultured in the presence of 20ug/mg Con A and their proliferative responses measured on days 3-5 of culture. As shown in Fig. 14, the maximal responses for each type occurred on day 3, when beige lymphocytes had a stimulation index of  $88.1 \pm 3.4$  compared with  $40.6 \pm 5.1$  of B6 cells ( $p < 0.001$ ). Thereafter, the proliferative responses declined, beige cells always showed much higher responses than B6 cells ( $p < 0.005$ ).

#### Proliferation of beige lymphocytes in mixed lymphocyte reactions.

In the next experiments, beige and B6 spleen cells were compared for their ability to proliferate in an MLR in response to fully allogeneic DBA/2 ( $H-2^d$ ) stimulator cells. Cells were harvested after 3-5 days of culture and the results are shown in Fig. 15. The peak response by both cell types occurred on day 4, with Stimulation Indices of  $14.5 \pm 2.8$  and  $15.5 \pm 3.3$  for B6 and beige responder cells respectively. However, at other times, beige lymphocytes showed significantly better MLR responses than B6 lymphocytes, with a SI of  $14.2 \pm 2.5$  on day 3 (vs  $10.0 \pm 1.2$  for B6;  $p < 0.05$ ) and  $8.2 \pm 2.8$  on day 5 (vs  $4.0 \pm 0.9$  for B6;  $p < 0.05$ ). In three further experiments beige responder cells consistently exhibited higher proliferative responses at most times

of the MLR (data not shown).

These results indicate that beige spleen cells have a normal or increased capacity to proliferate in MLR and are consistent with the enhanced proliferative responses to Con A.

#### Generation of allospecific CTL by beige spleen cells in vitro.

As beige spleen cells proliferated well in an MLR, despite their defective ability to generate allospecific CTL *in vivo*, it was of interest to investigate whether specific CTL would develop during an MLR using beige lymphocytes as responders. Beige or B6 spleen cells were stimulated in an MLR with mitomycin-treated DBA/2 stimulator cells and, 3-6 days later, responding cells were harvested and tested for specific CTL activity against  $^{51}\text{Cr}$ -labelled P 815 targets.

In cultures using B6 responders, CTL activity was already detectable on day 3 (Fig. 16. 38% cytotoxicity at 25:1) and this peaked at 47% on day 4, before declining to 11.9% on day 6. In comparison, beige responders cells always had much less CTL activity than B6 responder cells, with 19% on day 3, 18.9% on day 4 and only 5.3% on day 6. Similar responses were found at 50:1 E:T ratios.

#### Redistribution of B6 and beige lymphocytes in vivo.

The results discussed in this chapter indicated that although beige mice exhibit defective generation of CTL, their lymphocytes are capable of many other cell

mediated effector functions. Therefore, it was not clear whether this restricted pattern of defects could account for the poor ability of beige mice to induce a GvHR. In Chapter 7 it will be shown that failure to recirculate through lymphoid tissues in vivo may explain the inability of IEL to cause GvHR. Therefore, the final experiments in this section examined the redistribution of beige and B6 cells after iv injection into syngeneic recipients.

Beige and B6 MLN lymphocytes were labelled with  $^{51}\text{Cr}$  and injected iv into syngeneic B6 mice. The distribution of radioactivity was assessed 18 hours after cell transfer as described in the Materials and Methods.

No significant differences were found in the organ distribution of these two cell types (Fig. 17), both B6 and beige cells accumulated efficiently in lymphoid organs such as in MLN, PLN and spleen.

#### Summary and Conclusions.

The experiments presented in this chapter show that beige mice not only had normal numbers and proportions of T cell subsets in their spleen and lymph nodes, but were also capable of normal or enhanced proliferative responses to mitogens or alloantigens in vitro. In addition, beige mice generated normal DTH responses to allogeneic cells in vivo and their lymphoid cells showed a normal redistribution pattern in vivo. In contrast to these findings, beige mice immunised with allogeneic

cells generate lower levels of specific CTL than normal B6 animals. Furthermore, although the very low levels of NK activity in beige mice could be enhanced by alloimmunisation, this did not approach the levels found in immunised B6 mice. Beige spleen cells also demonstrated a defective ability to generate specific CTL during an MLR in vitro.

It can be concluded that the immune defect in beige mice is not restricted to the NK cell lineage but also causes a quantitatively similar defect in specific CTL activity and there is a paradoxical enhancement of proliferative T cell function. These findings indicate that the defective ability of beige mice to induce a GVHR may not be due merely to the absence of NK cells from the donor inoculum.

STRAIN	TISSUE	% L <sub>yt</sub> 2 <sup>+</sup>	% L3T4 <sup>+</sup>	% TOTAL T CELLS
B6	MLN	21.6±2	50.0±2.9	71.6
	SPLEEN	15.3±1.2	22.6±2	37.9
BEIGE	MLN	24.6±1.6	47.6±2.8	72.2
	SPLEEN	16.3±1.2	22.0±0.8	38.3

Table. 5. Percentage of T cells and T cell subpopulations in the spleen and MLN of B6 and beige mice. Results are expressed as the percentage of total cells labelled by immunofluorescence and represent the mean of 3 experiments ± standard deviation, while the percentage of total T cells is the sum of the % of L3T4<sup>+</sup> and L<sub>yt</sub>2<sup>+</sup> cells.

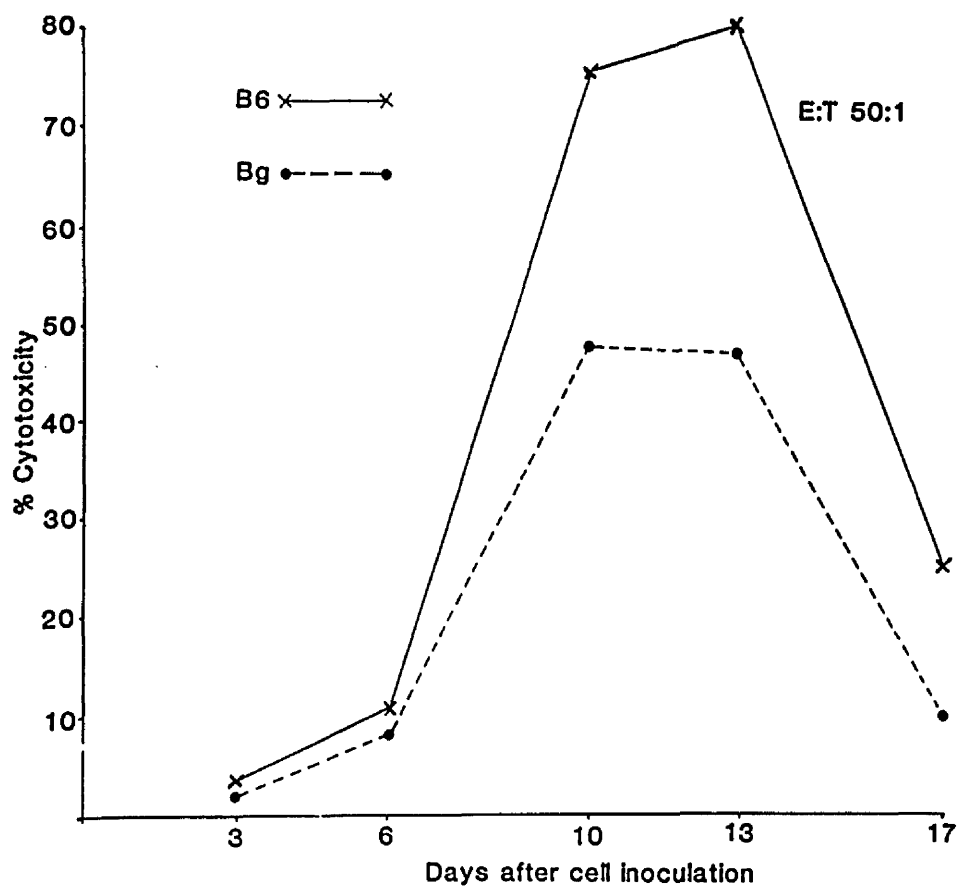


Fig. 11. Generation of allospecific CTL in B6 and beige mice after immunisation with  $2 \times 10^7$  P815 ip. Results shown are % lysis of P815 at 50:1 E:T ratio, using spleen cells pooled from 4 mice/group.

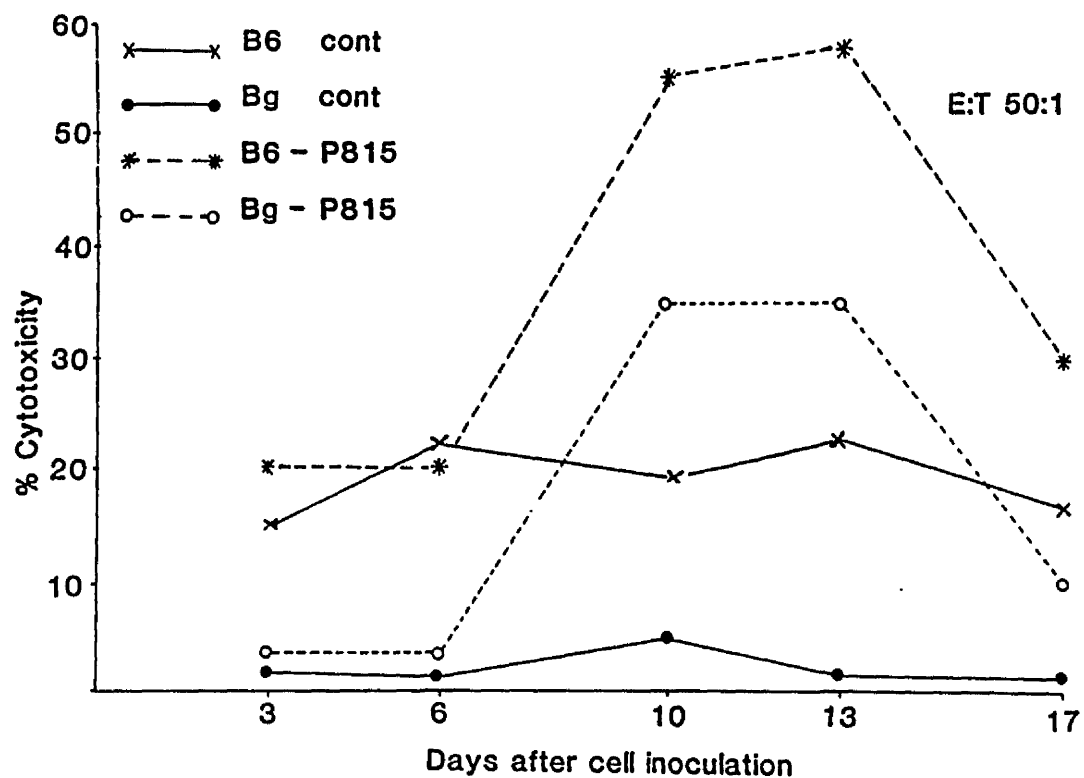


Fig. 12. Splenic NK cell activity in B6 and beige mice after immunisation with  $2 \times 10^7$  P815 cells ip. Results shown are % lysis obtained at 50:1 E:T ratios using spleen cells pooled from 4 mice/group.

Cytotoxicity measured against YAC-1 target cells.

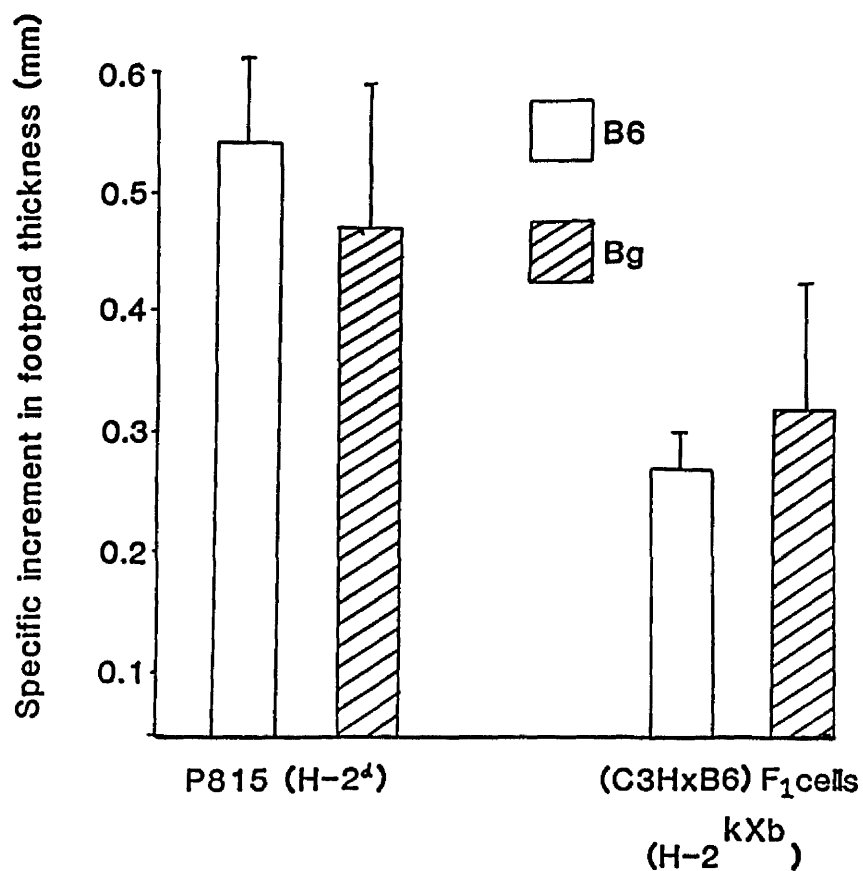


Fig. 13. Generation of delayed type hypersensitivity responses to alloantigens in B6 and beige mice. B6 or Bg mice were immunised with either P815 cells (H-2<sup>d</sup>) intraperitoneally or (C3H X B6)F<sub>1</sub> (H-2<sup>kXb</sup>) cells intradermally. Results shown are mean specific increment in footpad thickness  $\pm$  1 standard deviation 24 hours after challenge for 5-6 mice/group.

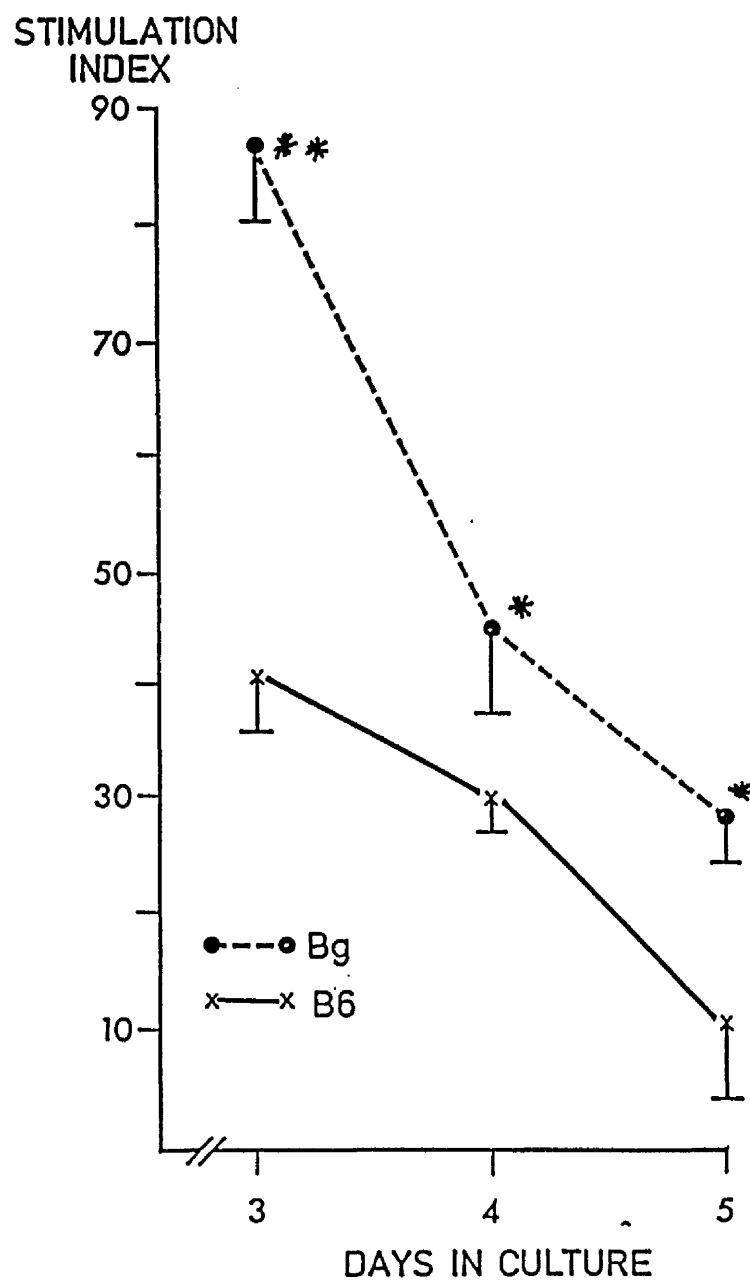


Fig. 14. Responses of B6 and beige spleen cells to Con A. Results shown are mean Stimulation Indices  $\pm$  1 standard deviation for quadruplicate cultures. Background responses in the absence of Con A were 100-150 cpm. \* $p < 0.005$  and \*\* $p < 0.001$ .

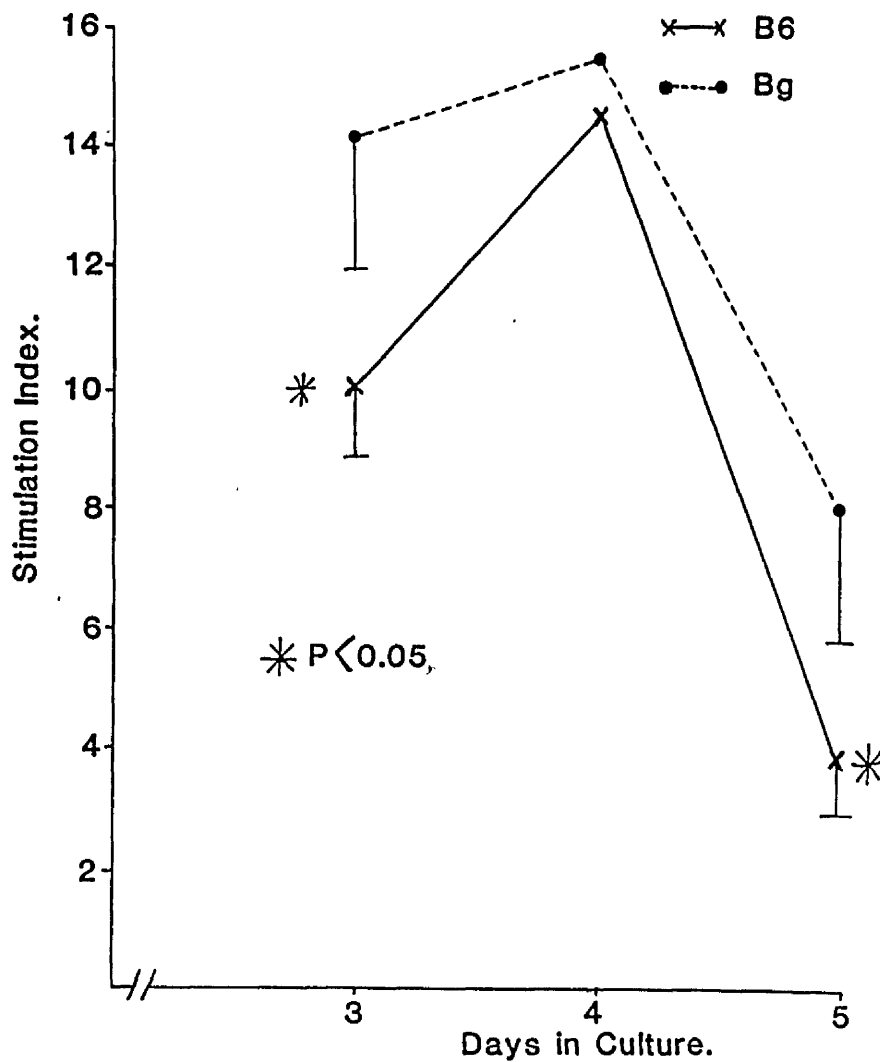


Fig. 15. Mixed lymphocyte responses of B6 and beige spleen cells to mitomycin C-treated DBA/2 (H-2<sup>d</sup>) spleen cells. Results shown are mean Stimulation Indices  $1 \pm$  Sd of quadruplicate cultures.

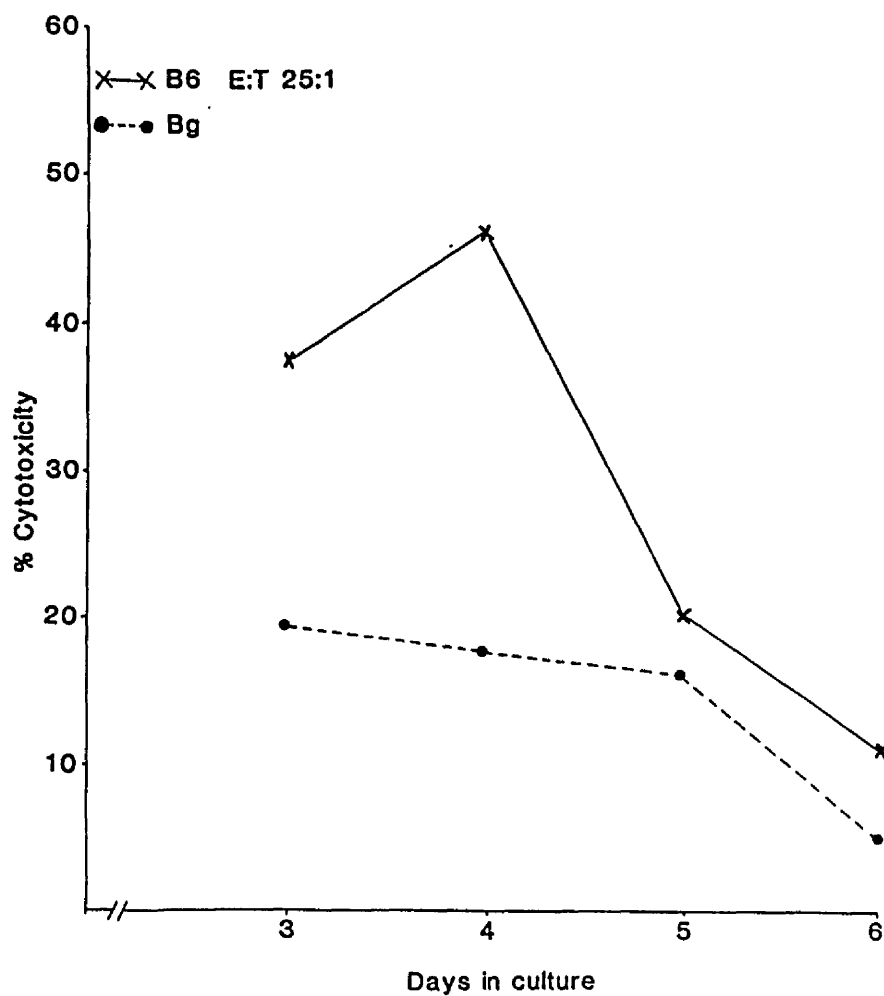


Fig. 16. Generation of allospecific CTL *in vitro* by B6 or beige responder spleen cells stimulated with mitomycin C-treated DBA/2 (H-2<sup>d</sup>) spleen cells. Results shown are % lysis of P815 cells obtained at 25:1 E:T ratio, using cells pooled from 4 cultures/group.

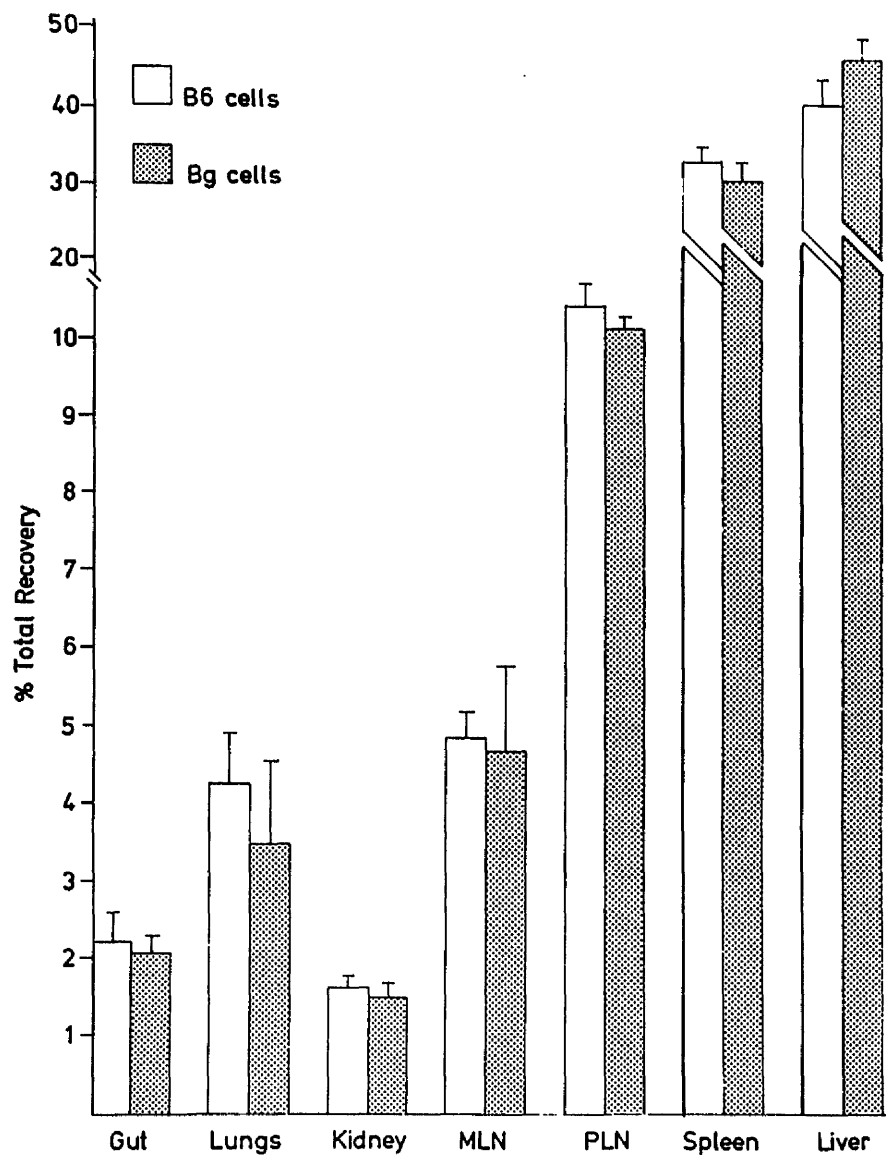


Fig. 17. *In vivo* migration of  $^{51}\text{Cr}$ -labelled B6 and beige MLN cells. Organ distribution of radioactivity 18 hours after iv transfer of  $5 \times 10^6$  cells. Each bar represents the mean percentage of recovered radioactivity  $\pm 1$  standard deviation for 5 mice/group.

CHAPTER 6  
RESPONSIVENESS OF INTRAEPITHELIAL  
LYMPHOCYTES TO ALLOANTIGENS IN VIVO.

## Introduction.

One of the major features of the intestinal phase of murine GvHR is increased infiltration of the epithelium by lymphocytes (Mowat and Ferguson, 1982) and this is paralleled by increased NK activity in IEL from mice with GvHR (Borland *et al.*, 1983). Furthermore, in GvHR and other conditions characterised by an increased proportion of IEL, some IEL have the morphological features of activated lymphocytes, and exhibit increased mitotic activity (Marsh, 1975). Together, these findings suggest that IEL may be involved as local effector cells in the intestinal pathology of local CMI responses, but this has not been proved directly.

As discussed earlier, the nature of IEL is highly controversial and studies of IEL *in vitro* have often produced conflicting information. Therefore, I decided to address the possible role of IEL in local immune responses by using an *in vivo* approach. In view of the association between intestinal pathology and the increased IEL count during a GvHR (Mowat and Ferguson, 1982), I thought it would be appropriate to investigate the ability of IEL to mediate alloreactivity *in vivo* using various models of GvHR. These models had to be suitable for the limited numbers of IEL that can be isolated and were therefore a local, proliferative popliteal lymph node GvHR, the induction of systemic GvHR in neonatal mice and the induction of acute lethal GvHR in irradiated hosts.

#### Induction of local proliferative GvHR by IEL.

The popliteal lymph node assay was chosen as the first means of assessing the ability of IEL to induce a proliferative GvHR in unirradiated hosts. (CBA X BALB/c) $F_1$  mice were injected with  $10^7$  CBA IEL or spleen cells into one footpad and the draining popliteal lymph nodes removed 8 days later. Control CBA mice received the same number of syngeneic IEL or spleen cells.

CBA IEL and spleen cells both produced significantly greater popliteal lymph node enlargement after injection into (CBA X BALB/c) $F_1$  mice than in syngeneic CBA hosts (Table 6 IEL:  $5.68 \pm 0.6$  mg vs  $3.9 \pm 0.91$  mg; spleen cells:  $3.84 \pm 0.52$  mg vs  $1.38 \pm 0.34$  mg). However, when the non-specific responses in syngeneic mice were taken into account, it could be seen that spleen cells produced more GvHR-specific popliteal lymph node hypertrophy than IEL (Table 6).

#### Ability of IEL to induce a systemic proliferative GvHR in unirradiated neonatal hosts.

The results of the popliteal lymph node assay suggested that IEL were capable of inducing a local proliferative GvHR, but this may not be as marked as found with spleen cells. It was important to confirm these findings using another assay of proliferative GvHR and so, I examined the ability of intraperitoneally injected CBA IEL to induce splenomegaly in neonatal  $F_1$  hosts.

5 day old (CBA X BALB/c) $F_1$  mice were injected with

$10^7$  IEL or spleen cells ip and, 8 days later, the mice were sacrificed and their spleens weighed (Table 7). Mice given IEL had significantly enlarged spleens compared with littermate controls given medium (Relative spleen weights  $44.2 \pm 6.0$  and  $30.9 \pm 5.9$  mg/10g body weight respectively  $p < 0.005$ ); and this was similar to the splenomegaly seen in  $F_1$  recipients of spleen cells (Relative spleen weight  $47.9 \pm 8.2$ ,  $p < 0.01$ ). Time did not permit these studies to be extended, but the results of this preliminary experiment confirm that IEL do possess the ability to induce a proliferative GvHR when transferred subcutaneously or intraperitoneally.

Ability of IEL to induce an acute lethal GvHR in irradiated hosts.

The induction of an acute, lethal GvHR in irradiated hosts may reflect immunological mechanisms which are different from those responsible for the mainly proliferative GvHR found in unirradiated animals. Therefore, it was of interest to determine whether IEL could produce a lethal GvHR as well as the proliferative disease discussed above.

Adult (CBA X BALB/c) $F_1$  mice were irradiated with 900 Rad and reconstituted with either  $10^7$  CBA spleen cells or IEL or with  $10^7$  syngeneic cells iv, or left unreconstituted to assess the effects of radiation alone. Mice receiving  $10^7$  CBA spleen cells lost weight rapidly and showed signs of runting, diarrhoea and skin disease within 4-5 days after cell transfer (Fig. 18). All these mice died within 8-9 days (MST 6 days),

whereas 93% of the  $F_1$  mice receiving syngeneic spleen cells survived indefinitely. In contrast,  $F_1$  recipients of CBA IEL showed no clinical evidence of GvHR and had identical survival curves to irradiated, unreconstituted mice. Interestingly, recipients of syngeneic IEL had slightly poorer survival than irradiated controls  $p < 0.02$ . Some  $F_1$  recipients of CBA IEL were still alive > 120 days after cell transfer with no signs of delayed GvHR. Thus, IEL were unable to cause either acute, lethal GvHR or haemopoietic reconstitution of irradiated animals.

Effects of allogeneic or syngeneic bone marrow on the inability of IEL to induce GvHR in irradiated mice.

The failure of IEL to induce an acute GvHR could reflect a similar requirement for accessory cells to that found for proliferative responses by IEL *in vitro* (Mowat *et al.*, 1986). Previous work in GvHR has shown that administration of either host or donor bone marrow could increase the ability of donor T cells to mediate a GvHR, either by providing an additional source of precursor T cells or due to accessory cell function, such as increased presentation of host alloantigens (Korngold and Sprent 1985). In the next series of experiments, I examined whether bone marrow of host or donor origin influenced the ability of CBA IEL to mediate a GvHR in lethally irradiated  $F_1$  hosts.

As before, all irradiated (CBA X BALB/c) $F_1$  recipients of  $10^7$  CBA spleen cells died within 4-8 days

(MST 5 days) and showed the characteristic features of systemic GvHR, with rapid weight loss, skin disease and diarrhoea (Fig. 19). In addition, mice given  $10^7$  CBA IEL had a survival pattern which was identical to irradiated unreconstituted mice, (MST 11 days  $p < 0.02$  vs spleen) and none of these mice showed evidence of clinical GvHR. Irradiated  $F_1$  mice given either  $10^7$  CBA or  $F_1$  bone marrow cells alone, survived indefinitely, confirming efficient haemopoietic reconstitution by the injected bone marrow cells, (Fig. 19) However, neither population of bone marrow cells allowed CBA IEL to induce a lethal GvHR in  $F_1$  mice and virtually all these mice survived for up to 200 days, without any clinical evidence of delayed GvHR.

#### Effect of IL-2 on the inability of IEL to induce an acute GvHR in irradiated hosts.

Proliferative responses by IEL *in vitro* are augmented by lymphocyte derived mediators (Dillon and MacDonald., 1984; Mowat *et al.*, 1986) and IL-2 is known to enhance a GvHR in experimental animals (Jadus and Peck, 1983b). As bone marrow had no effect on the inability of IEL to induce GvHR, I assessed whether concurrent administration of IL-2 would allow parental IEL to induce a GvHR in irradiated (CBA X BALB/c) $F_1$  mice.  $F_1$  mice were lethally irradiated, given  $10^7$  CBA IEL or spleen cells as before and were injected daily with 1000U of human rIL-2 *ip* thereafter.

As usual, all lethally irradiated  $F_1$  mice given  $10^7$  CBA spleen cells died within 6-7 days, with

characteristic features of GvHR (Fig. 20. MST 7 days). Daily administration of rIL-2 did not alter this highly aggressive form of GvHR (MST 7 days). In this experiment, insufficient IEL were available to transfer alone to  $F_1$  hosts but the experiments described above show clearly that this protocol produces a survival rate similar to that of unreconstituted hosts. The important finding from the present study was that  $F_1$  mice given CBA IEL plus daily injections of rIL-2 had a survival pattern which was identical to irradiated mice given rIL-2 alone (MST 13 and 15 days, respectively). In addition, mice given IEL + rIL-2 had no clinical evidence of GvHR and survived significantly longer than spleen cells recipients ( $p < 0.01$ ). That the schedule of rIL-2 dosage which was used had a biological effect in recipients was confirmed by the fact that the 2 groups of mice which received rIL-2 died more rapidly than unreconstituted, irradiated controls (Fig. 20).

Thus, administration of rIL-2 did not allow IEL to induce a lethal GvHR in irradiated hosts.

#### Summary and Conclusions.

The experiments described here were designed to examine the potential of IEL to mediate alloreactive cell mediated immune responses in vivo.

Although CBA IEL were capable of inducing a local GvHR measured by the hypertrophy of the popliteal lymph node and were able to induce splenomegaly in neonatal (CBA X BALB/c) $F_1$  mice, they were unable to induce a

systemic GvHR in irradiated F<sub>1</sub> mice. Administration of bone marrow cells of parental or host origin did not allow IEL to induce a GvHR and daily administration of rIL-2 also had no effect on the inability of IEL to mediate GvHR.

In conclusion, these results indicate that although IEL do possess a significant alloreactive potential in vivo, this can only be demonstrated using a relatively local GvHR in intact hosts. In contrast, intravenously injected IEL cannot produce a systemic GvHR in irradiated hosts and the next Chapter will attempt to define the reasons for this paradoxical reactivity.

DONOR CELLS	HOST	LYMPH NODE WT. (mg)	RATIO
MEDIUM	(CBA X BALB/c)F <sub>1</sub>	1.15±0.07	-----
IEL	(CBA X BALB/c)F <sub>1</sub>	5.68±0.60 *	1.46
	CBA	3.9±0.91	
SPLEEN	(CBA X BALB/c)F <sub>1</sub>	3.84±0.52 **	3.05
	CBA	1.38±0.34	

Table. 6. Induction of a local GvHR<sub>7</sub> in (CBA X BALB/c)F<sub>1</sub> mice after injection of with 10<sup>7</sup> CBA IEL or spleen cells. Results shown are mean popliteal lymph nodes weights ± 1 standard deviation for 6 mice/group 8 days after transfer. The ratios shown were calculated by dividing the mean lymph node weight in F<sub>1</sub> mice by that in syngeneic CBA mice. \*p<0.01 \*\*p<0.001 vs control lymph nodes.

<u>DONOR</u> <u>CELLS</u>	<u>RELATIVE SPLEEN</u> <u>WEIGHT (mg).</u>	<u>SPLEEN</u> <u>INDEX</u>
MEDIUM	30.9±5.9	-----
CBA IEL	44.2±6.0 **	1.43± 0.31
CBA SPLEEN.	47.9±8.2 *	1.55± 0.22

Table. 7. Induction of systemic GvHR<sub>7</sub> in 6 day old (CBA X BALB/c)F<sub>1</sub> mice by injection with 10<sup>7</sup> CBA IEL or spleen cells. <sup>1</sup>Results shown are means ± 1 standard deviation for 3 mice/group 8 days after induction of GvHR and the ratios shown calculated by dividing the mean relative spleen weight of control mice by that in IEL and spleen cell injected mice. \*p<0.01 \*\*p<0.005 vs control spleen

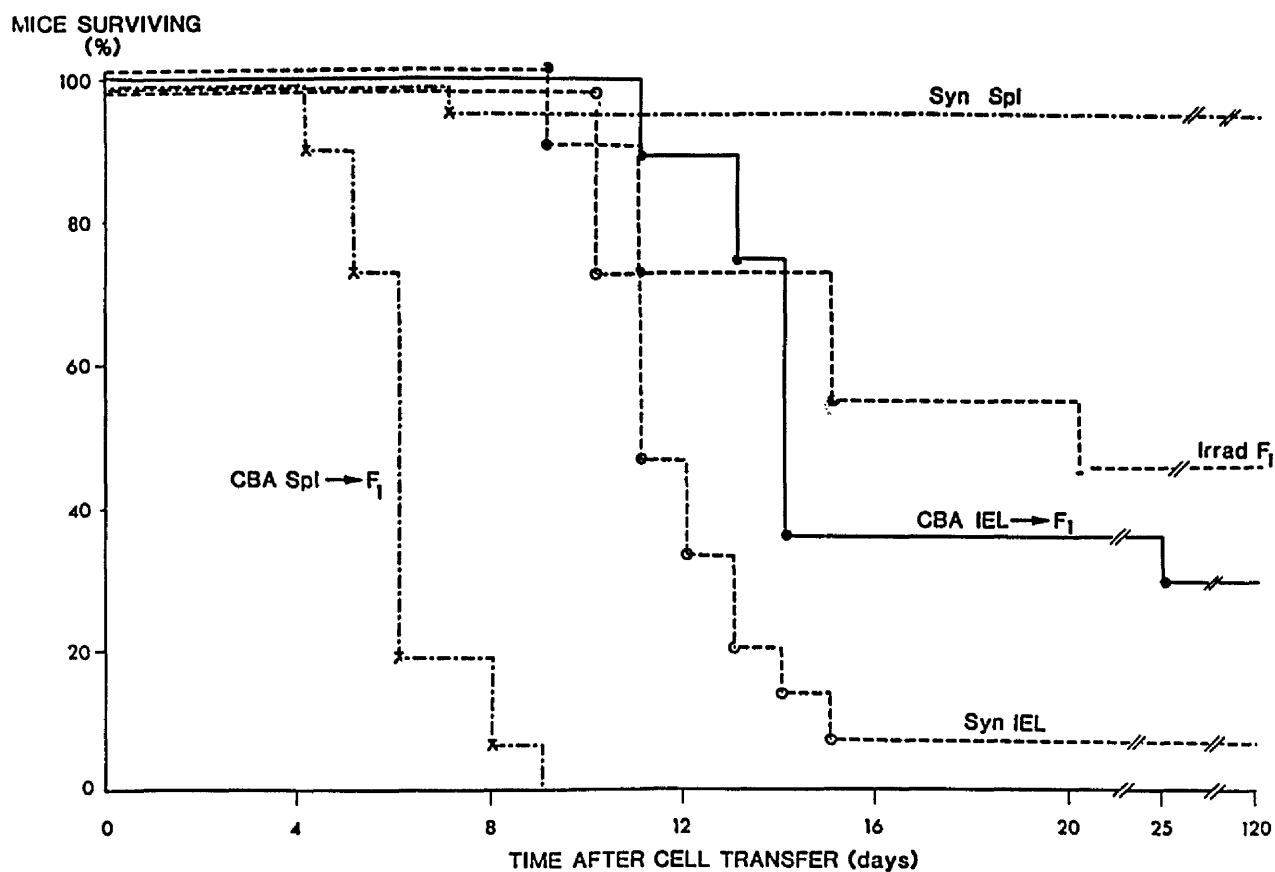


Fig. 18. Induction of lethal GVHR in lethally irradiated (CBA X BALB/c) $F_1$  mice with  $10^7$  CBA IEL or spleen cells. Control mice were either left unreconstituted after radiation or received syngeneic IEL or spleen cells. Results shown are % surviving animals at each time for 8-10 mice/group.

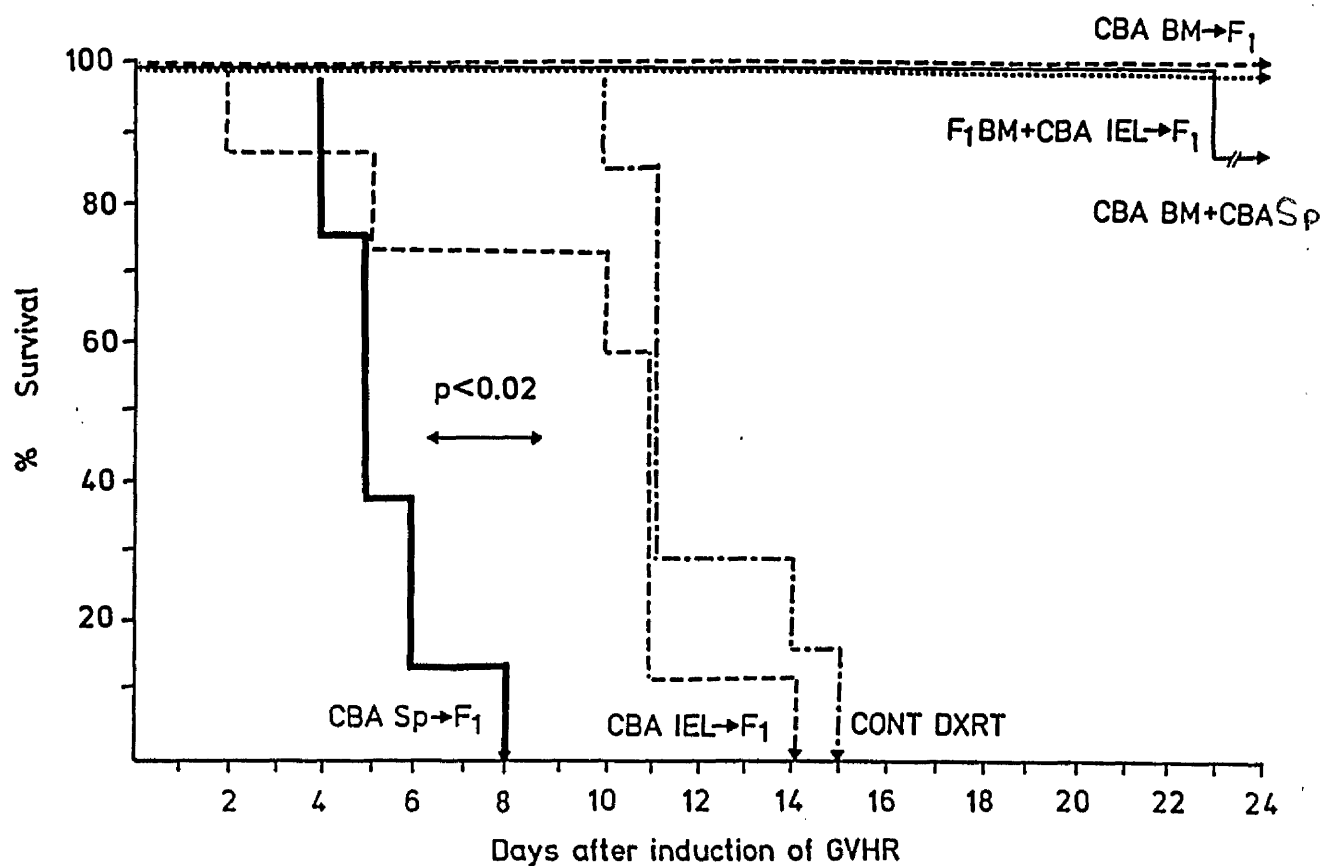


Fig. 19. Effect of bone marrow cells on the induction of GvHR by IEL. Lethally irradiated (CBA X BALB/c)F<sub>1</sub> mice were injected with 10<sup>7</sup> CBA spleen cells or IEL to induce GvHR and some recipients of IEL also received 10<sup>7</sup> F<sub>1</sub> or CBA BM iv. Control mice were either left unreconstituted after irradiation or received 10<sup>7</sup> BM cells alone. Results shown are % surviving animals at each time for 8-10 mice/group.

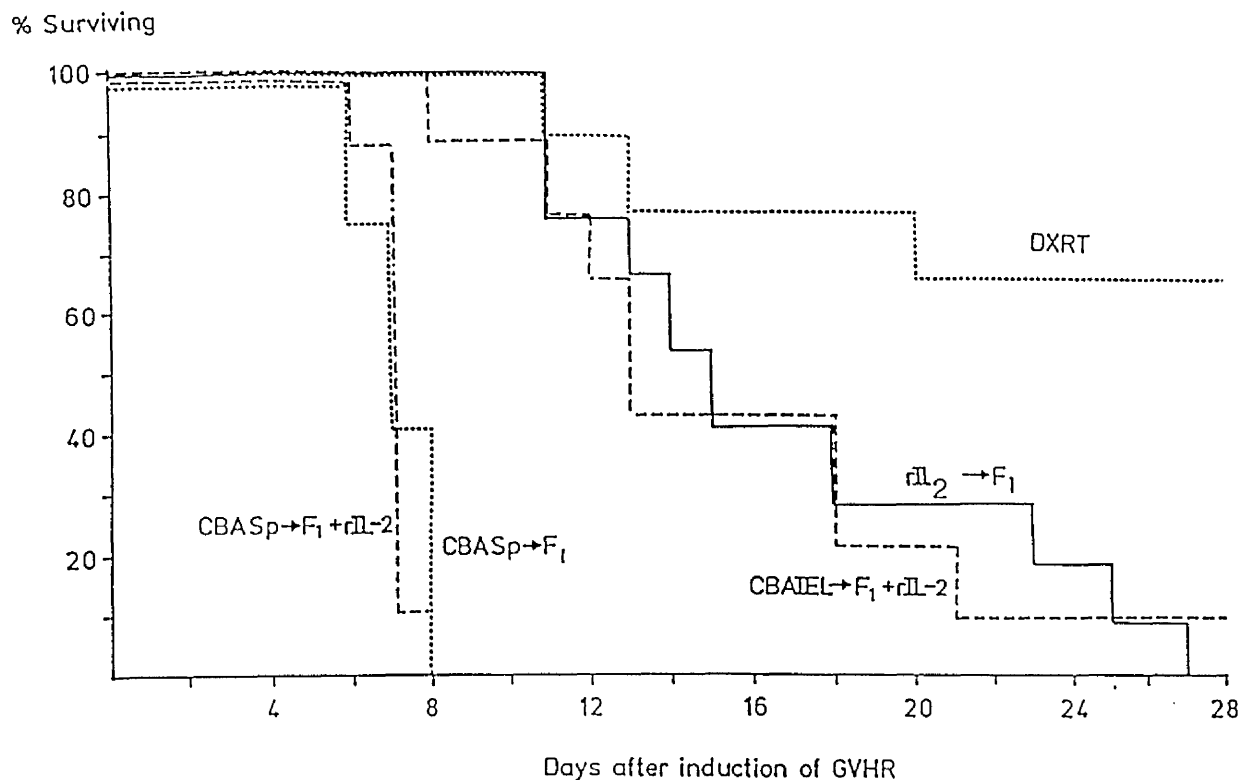


Fig. 20. Effect of human rIL-2 on the induction of GvHR by IEL. Lethally irradiated (CBA X BALB/c) $F_1$  mice were injected with either  $10^7$  CBA spleen cells alone or with spleen cells or IEL plus daily injections of 1000u rIL-2. Control mice were either unreconstituted or were given rIL-2 alone after irradiation. Results shown are % surviving animals at each time for 6-8 mice/group.

CHAPTER 7  
MIGRATION PATHWAYS OF INTRAEPITHELIAL  
LYMPHOCYTES IN VIVO

## Introduction

The results of the preceding chapter showed that IEL could not induce an acute systemic GvHR in irradiated F<sub>1</sub> hosts after intravenous injection, despite their ability to induce a local GvHR in unirradiated mice. As the induction of a systemic GvHR requires the donor lymphocytes to encounter host alloantigens within lymphoid tissues (Ford, 1975), one possible explanation for the failure of IEL to induce a systemic GvHR was because they were incapable of migrating through host lymphoid tissues.

The only report in the literature which had examined migration of IEL in vivo, suggested that mouse IEL were a gut-homing population, as they were found to enter PP 3-4 days after transfer in vivo, (Guy Grand et al., 1978). However, this study used poorly defined preparations of IEL and has not been confirmed.

Therefore, in this Chapter, I undertook a detailed study of the migration pattern of IEL after iv transfer into syngeneic recipients and compared the redistribution of IEL with that of other lymphoid cell populations.

## Distribution of IEL and spleen cells in the first 24 hours after intravenous transfer.

The choice of protocol for these experiments was determined by two factors. First, the studies of the ability of IEL to induce systemic GvHR, had used spleen cells as a positive control. Secondly, the requirement

for GvHR-inducing lymphocytes to enter host lymphoid tissues is known to occur within 18-24 hours after transfer (Ford, 1975). Therefore, in the first experiments, IEL and spleen cells were compared for their migration *in vivo*, 2-18 hours after iv injection into syngeneic recipients.

The organ distribution of radioactivity 2 hours after transfer of  $^{51}\text{Cr}$ -labelled CBA IEL or spleen cells is shown in Fig. 21. At this time, spleen cells were found predominantly in the liver, spleen and lungs ( $45.2 \pm 7.3\%$ ,  $35.34 \pm 4.3\%$ ,  $9.19 \pm 3.5\%$  recovered radioactivity, respectively) although, even at this early stage a small but consistent proportion was already present in the gut ( $2.1 \pm 0.54\%$ ), MLN ( $2.5 \pm 0.46\%$ ) and peripheral lymph nodes (PLN) ( $3.5 \pm 1.27\%$ ). There were virtually no cells in kidney. In comparison, IEL showed a very different pattern of redistribution. The vast majority of recovered radioactivity at this time was found in the liver and lungs, while very few IEL were found in the spleen ( $6.7 \pm 1.4\%$  vs  $35.3 \pm 4.3\%$  for spleen cells  $p < 0.001$ ). Furthermore, in comparison with spleen cells, there was virtually no accumulation of IEL in lymph nodes (PLN  $0.18 \pm 0.07\%$   $p < 0.001$ ; MLN  $0.15 \pm 0.02\%$   $p < 0.001$ ), but a larger proportion of IEL was present in the kidney ( $4.5 \pm 1.32\%$  vs  $0.99 \pm 0.11\%$   $p < 0.001$ ) and liver ( $76.0 \pm 24.6\%$  vs  $45.0 \pm 7.3\%$   $p < 0.025$ ). There was a similar accumulation of IEL and spleen cells in the gut ( $1.45 \pm 0.54$  and  $2.17 \pm 0.54\%$ ) respectively, lungs ( $8.88 \pm 4.1\%$  vs  $9.19 \pm 3.5\%$ ) and in blood ( $1.49 \pm 0.64\%$  and  $0.94 \pm 0.4\%$ ) were similar at this time.

The organ distribution of labelled IEL and spleen cells 6 hours after cell transfer is shown in Fig. 22. At this time, spleen cells were found predominantly in spleen and liver ( $40.6 \pm 3.3\%$  and  $47.1 \pm 3.6\%$  recovered radioactivity, respectively), while the proportion found in the gut ( $1.9 \pm 0.15\%$ ), MLN ( $2.2 \pm 0.29\%$ ), PLN ( $2.07 \pm 0.23\%$ ) and blood ( $1.4 \pm 0.27\%$ ) was similar to that found in these organs 2 hours after cell transfer. In parallel, there was a marked decrease in the radioactivity recovered from the lungs. Significantly more IEL than spleen cells were recovered from the lungs ( $11.2 \pm 2.0\%$   $p < 0.001$  vs spleen cells), kidney ( $5.4 \pm 0.82\%$   $p < 0.001$ ) and liver ( $66.4 \pm 7.87\%$   $p < 0.001$ ). In contrast, IEL had not accumulated in the spleen over the preceding 4 hours  $p < 0.001$  and there were still virtually no IEL in the MLN ( $0.9 \pm 0.08\%$   $p < 0.001$  vs spleen cells) or PLN ( $0.94 \pm 0.7\%$   $p < 0.001$ ). Thus, whereas spleen cells were accumulating in the spleen and lymphoid tissue of recipients by 6 hours IEL are either retained in the liver and lungs or had been eliminated completely.

The organ distribution 18 hours after cell transfer underlines the differences between spleen cells and IEL as shown in Fig. 23. At this time, although many spleen cells were still found in the liver ( $45.6 \pm 4.6\%$ ), a larger proportion were now present in the MLN ( $4.5 \pm 0.93\%$ ) and PLN ( $6.2 \pm 1.12\%$ ) and there was emigration of cells out of the blood stream ( $0.33 \pm 0.06\%$ ). Many spleen cells were still present in the spleen ( $38.05 \pm 2.1\%$ ). In contrast, many more IEL were

found in the liver ( $80.7 \pm 18.6\%$   $p < 0.001$ ) and kidney ( $5.17 \pm 0.18$  vs  $1.09 \pm 0.12$  for spleen  $p < 0.001$ ), while there was virtually no accumulation of IEL in lymph nodes (MLN  $0.24 \pm 0.04\%$ , PLN  $0.28 \pm 0.05$  and a considerable loss of radioactivity from the spleen  $9.3 \pm 0.87$   $p < 0.001$  vs spleen cells). In addition, significantly fewer IEL than spleen cells were found in gut ( $0.99 \pm 0.2\%$  vs  $2.17 \pm 0.54\%$  for spleen,  $p < 0.025$ ).

It is clear from these experiments that labelled IEL could not accumulate in recipients lymphoid tissues 18 hours after intravenous transfer.

#### Organ distribution of IEL and MLN cells 3-4 days after intravenous transfer.

The previous sections showed that IEL could not migrate into lymphoid organs 2, 6, or 18 hours after cell transfer. In view of the report that IEL could migrate into PP 3-4 days after transfer, I reexamined the migration pattern of IEL 72 and 96 hours after cell transfer. In this experiment, the behaviour of IEL was compared with that of MLN lymphocytes, as a source of known gut-homing cells.

The organ distribution of IEL and MLN cells 3 and 4 days after cell transfer is shown in Figs. 24 and 25. Although the distribution pattern of the cells is very similar at these times, the total proportion amount of the injected dose which was recovered decreased from 45.6% on day 3 to 33.7% on day 4, presumably reflecting elimination of the cells. At both times, MLN cells accumulated principally in the spleen ( $41.3 \pm 11.7\%$  and

47.5±5% on days 3 and 4 respectively) and liver (23.9±4.2% and 31.0±5.8%), but large numbers of cells were also found in MLN (8.7±2.8% and 11.5±1.4%) and PLN (16.2±5.7% and 18.1±2.8%). A small, but significant proportion of MLN cells was also present in the PP (3.5±0.5% and 3.1±0.63%) and gut (1.25±0.38% and 1.68±0.36%) at both times. In comparison, the majority of radioactivity from mice given labelled IEL was found in the liver on both days 3 and 4 (82.1±8.5% and 82.7±8%) and significantly fewer IEL accumulated in the spleen (10.2±1.54% and 10.7±2.1%). In addition, virtually no IEL were found in the MLN, PLN, PP and gut at either time, with a maximum recovery of 0.5% for each of these tissues. More radioactivity was present in the kidney of mice given IEL than MLN cells (3.2±0.49 and 3.39±0.55 vs 0.85±0.14 and 1.1±0.06%  $p<0.001$ ).

The findings in this section show that IEL not only fail to enter peripheral lymphoid tissues even up to 96 hours after cell transfer, but they also have no ability to enter PP or the intestine at any time.

The failure of IEL to migrate is not due to cell damage or to the isolation procedure.

The experiments described above indicate that, in comparison with other lymphoid cells, IEL do not accumulate into lymphoid tissues in vivo. As IEL are subjected to a long isolation procedure, it was important to determine whether their failure to migrate was an artefact of this process. Therefore, the

following series of experiments were designed to examine the redistribution pattern of normal lymphocytes subjected to the IEL isolation procedure, and of cells with low viability or in whom DNA synthesis had been inhibited with mitomycin C.

In the first experiment, CBA MLN lymphocytes were incubated and shaken for one hour at 37°C in the presence of a supernatant obtained previously during the isolation of IEL, before being labelled with <sup>51</sup>Cr and transferred into syngeneic recipients. The pattern of redistribution of these cells 18 hours after transfer is shown in Fig. 26, compared with that of normal MLN cells. It can be seen that both populations accumulated rapidly in lymphoid tissues such as MLN, PLN and spleen and there were no significant differences in the behaviour of MLN cells subjected to the IEL isolation technique.

The next experiment examined whether inhibition of DNA synthesis, without loss of viability, would alter the recirculation of MLN cells (Fig. 27). CBA MLN cells which had been treated for 30 minutes with mitomycin C showed a pattern of redistribution 18 hours after injection, which was identical to that of normal MLN cells.

Finally, although the populations of IEL which had been injected in previous studies were always of >90% viability, it was possible that the cells were unusually fragile. Therefore, I examined whether a rapid loss of viability after transfer could account for the failure of IEL to migrate in vivo. Thus, MLN cells were killed

by heating at  $65^{\circ}\text{C}$  for 1 hour before labelling, resulting in a final viability of 35% compared with 96% viability for normal MLN cells 18 hours after cell transfer of  $^{51}\text{Cr}$ -labelled heat-killed cells, (Fig. 28) significantly less radioactivity was found in the MLN ( $3.2 \pm 0.52\%$  vs  $8.6 \pm 2.12\%$  for normal MLN  $p < 0.025$ ), PLN ( $4.79 \pm 1.9\%$  vs  $25.3 \pm 4.5\%$   $p < 0.001$ ) and spleen ( $12.8 \pm 2.1\%$  vs  $40.0 \pm 4.16\%$   $p < 0.001$ ), compared with that found after injection of normal MLN cells. In contrast, significantly more radioactivity was recovered from the lungs ( $12.47 \pm 2.1\%$  vs  $2.78 \pm 0.91\%$   $p < 0.025$ ), kidney ( $5.6 \pm 0.5$  vs  $0.78 \pm 0.08\%$ ) and liver ( $44.0 \pm 2.6\%$  vs  $20.1 \pm 1.8\%$   $p < 0.001$ ) of mice injected with dead MLN cells. A notable feature of this experiment was that dead cells had very low uptake of  $^{51}\text{Cr}$  after radiolabelling ( $5067 \text{ cpm}/5 \times 10^6$  compared with  $63552 \text{ cpm}/5 \times 10^6$  for normal MLN cells) and this was also much less than normally found with IEL, which usually took up more label than normal MLN cells. It should also be noted that heat killed MLN cells still accumulated in lymph nodes and gut more efficiently than IEL, despite similar accumulation in the spleen, kidneys and lungs (Fig. 28).

These experiments indicate that the failure of IEL to migrate through lymphoid tissues is not due to the procedure used to isolate IEL, nor to the low viability nor to a lack of DNA synthesis in IEL. Therefore, it appears to be an inherent property of the cells and so it became important to investigate whether any other lymphoid cell population behaved like IEL in migration

experiments.

Distribution of intravenously injected thymocytes and activated peripheral lymph node cells.

Two possible explanations were considered for the failure of IEL to migrate *in vivo*. First, IEL may be an immature group of cells or secondly they may be partially activated population. Therefore, I compared the migration pathways of thymocytes and activated peripheral lymph node cells with that of IEL. The previous experiments indicated clearly that IEL never circulate through lymphoid tissues and in view of the technical difficulties associated with preparing sufficient large numbers of radiolabelled IEL, IEL were not included as a negative control in the present series of experiments. However, MLN cells were used as an actively circulating cell population. In addition, the studies of IEL had had to use  $^{51}\text{Cr}$  as a radiolabel, because IEL did not take up  $^{125}\text{IUdR}$  and so in the experiments using thymocytes  $^{51}\text{Cr}$  was used also as a label.

The distribution of labelled thymocytes at 18 hours is compared with that of MLN lymphocytes as shown in Fig. 29. Despite a significantly lower accumulation of thymocytes in the MLN ( $1.6 \pm 0.14\%$  vs  $8.6 \pm 2.12\%$  for MLN cells), PLN ( $4.15 \pm 0.45\%$  vs  $25.31 \pm 4.53\%$ ) and gut ( $1.4 \pm 0.14\%$  vs  $4.12 \pm 0.55\%$ ; all  $p < 0.001$ ), Thymocytes migrated equally well as MLN lymphocytes into the spleen ( $52.9 \pm 7.4\%$  vs  $40.0 \pm 4.16\%$ ). Slightly more labelled thymocytes were recovered from kidney ( $1.8 \pm 0.2\%$  vs

$0.78 \pm 0.08\%$   $p < 0.001$ ) and liver ( $36.3 \pm 5.3\%$  vs  $20.1 \pm 1.8\%$   $p < 0.025$ ). Therefore, although the redistribution pattern of thymocytes had some superficial similarities with that of IEL, they accumulated much more efficiently in lymphoid tissues such as lymph nodes and spleen (see above for IEL).

Activated PLN cells were obtained by sensitising CBA mice on both shaved flanks with 10 mg of oxazolone dissolved in acetone, 3 or 4 days before removal of draining lymph node lymphocytes. The distribution of MLN cells and activated PLN cells 18 hours after transfer is shown in Fig. 30. Although MLN cells accumulated more efficiently than PLN lymphoblasts in the gut ( $3.4 \pm 0.18\%$  and  $1.6 \pm 0.6\%$  respectively,  $p < 0.001$ ), no other significant differences were found in the organ-specific accumulation of these cells types, with both populations localising very efficiently in lymphoid organs such as PLN, MLN and spleen. Thus, the migration pattern of IEL could not be reproduced by  $^{51}\text{Cr}$ -labelled activated PLN cells.

#### Summary and Conclusions.

These experiments show that in comparison with peripheral and MLN small lymphocytes, IEL have a defective ability to migrate into lymphoid tissues after intravenous transfer into syngeneic hosts. The defect is apparent from 2 hours to 4 days after cell transfer and is associated with an unusually high accumulation of IEL in the lungs and liver. The procedure used to

isolate IEL did not account for their abnormal migration pattern, as incubation of MLN cells with supernatant obtained during the isolation of IEL had no effect on their migration in vivo. Furthermore, MLN cells treated with mitomycin C also accumulated normally. Although heat-treated MLN cells with low viability did exhibit an abnormal pattern of distribution, this had several differences from that exhibited by IEL. Therefore, the defective ability of IEL to migrate in vivo is an inherent property of these cells and is probably not an artefact of their isolation or due to poor viability or unusual fragility.

Experiments using thymocytes as a source of immature, "non-recirculating" lymphocytes (de Sousa 1981) showed that they also exhibited a low accumulation in lymph nodes, but, in contrast to IEL, thymocytes migrated readily to the spleen and more efficiently to lymph nodes. Furthermore, activated PLN lymphocytes had a pattern of localisation which was similar to that of MLN cells and was totally unlike that of IEL.

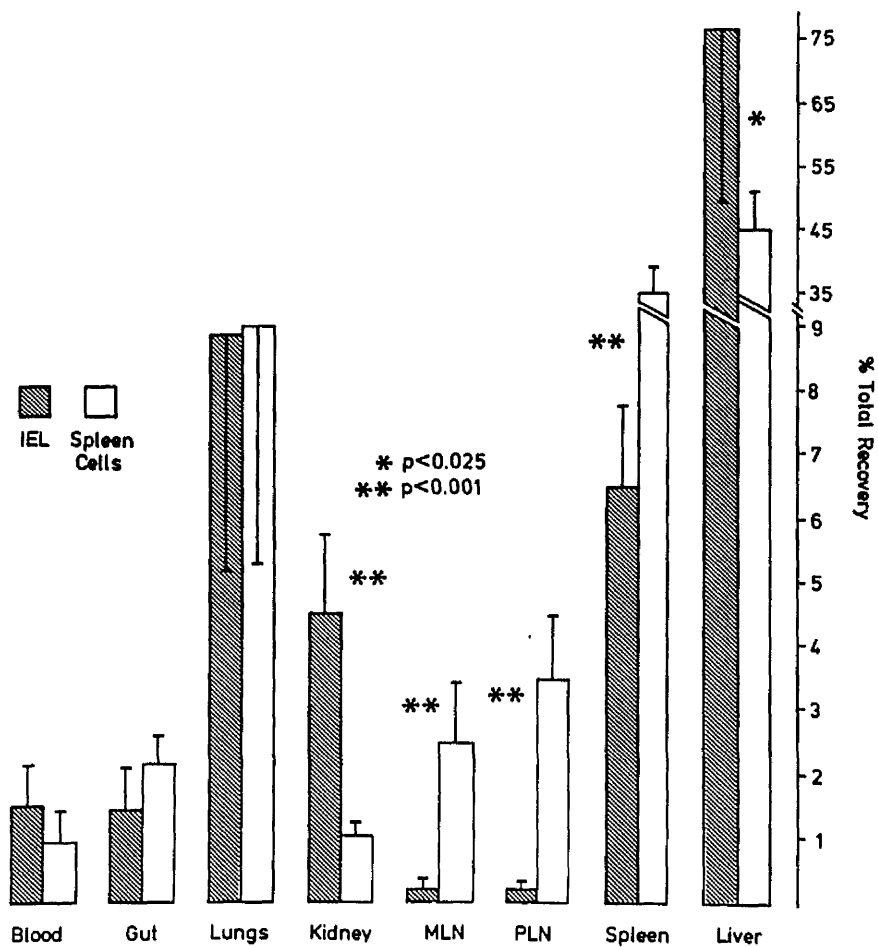


Fig. 21. Migration of  $^{51}\text{Cr}$ -labelled CBA IEL and spleen cells *in vivo*. Organ distribution of radioactivity, 2 hours after transfer of  $5 \times 10^6$  cells into syngeneic recipients. Each bar represents the mean percent recovered radioactivity  $\pm 1$  standard deviation for 5 mice/group.

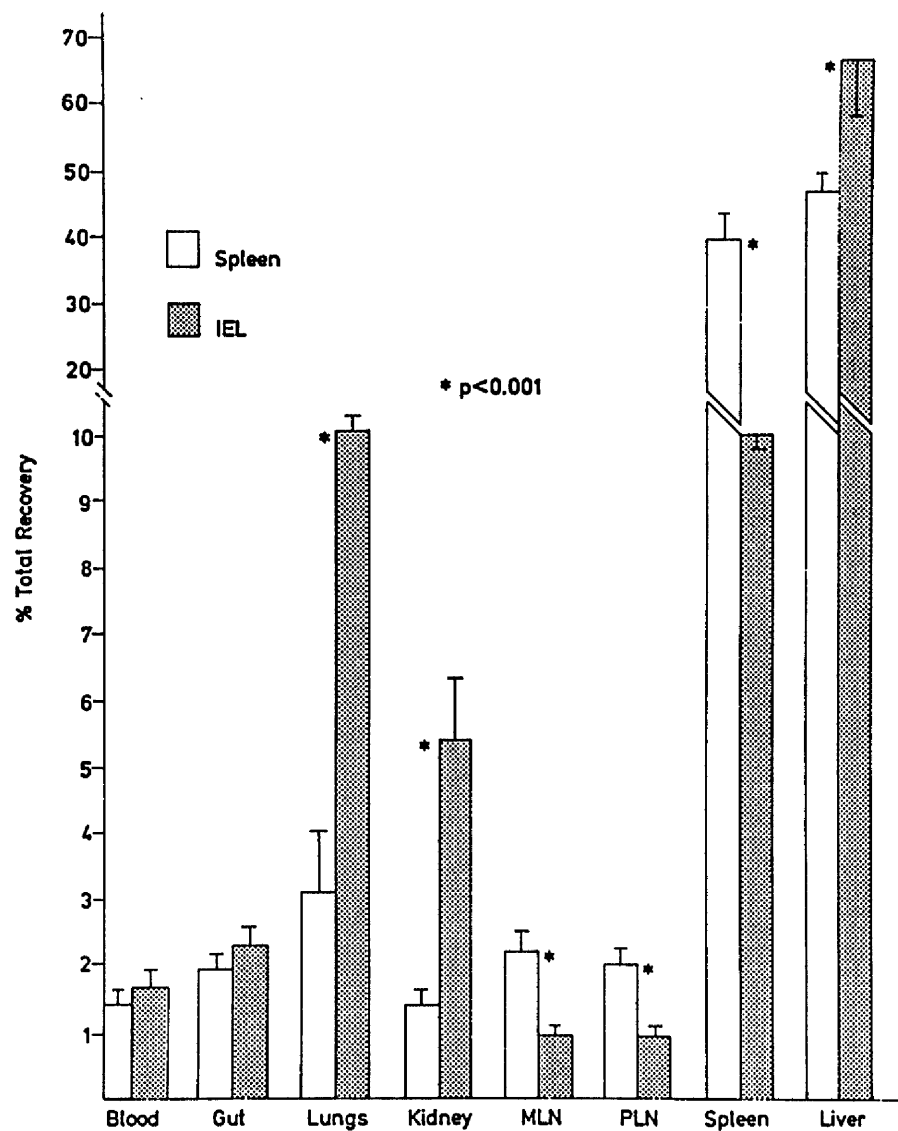


Fig. 22. Migration of  $^{51}\text{Cr}$ -labelled CBA IEL and spleen cells *in vivo*. Organ distribution of radioactivity, 6 hours after transfer of  $5 \times 10^6$  cells into syngeneic recipients. Each bar represents the mean percent recovered radioactivity  $\pm 1$  standard deviation for 5 mice/group.

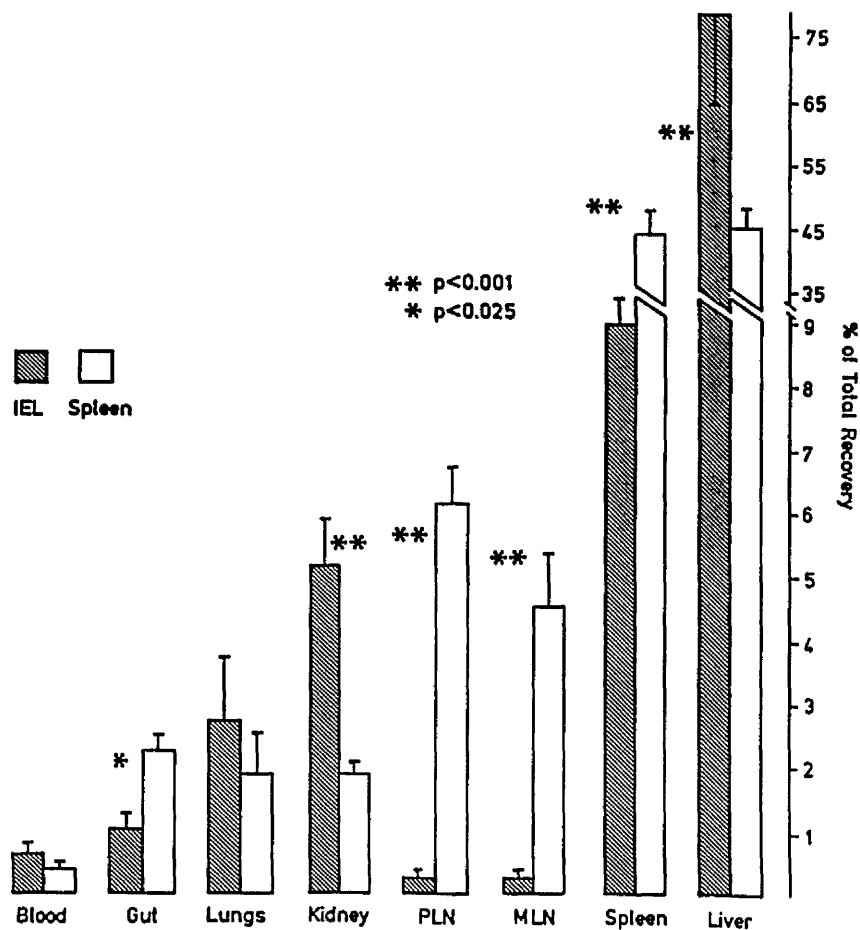


Fig. 23. Migration of  $^{51}\text{Cr}$ -labelled CBA IEL and spleen cells *in vivo*. Organ distribution of radioactivity, 18 hours after transfer of  $5 \times 10^6$  cells into syngeneic recipients. Each bar represents the mean percent recovered radioactivity  $\pm 1$  standard deviation for 5 mice/group.

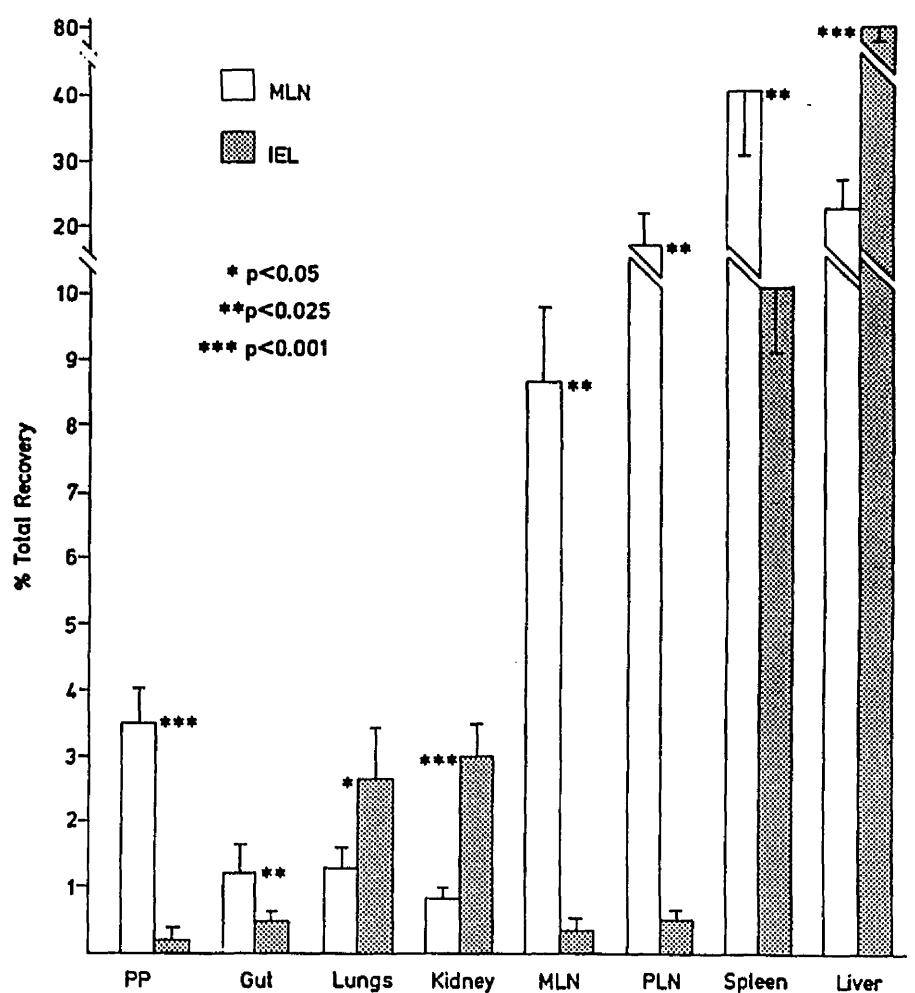


Fig. 24. Migration of  $^{51}\text{Cr}$ -labelled CBA IEL and MLN cells *in vivo*. Organ distribution of radioactivity, 72 hours after transfer of  $5 \times 10^6$  cells into syngeneic recipients. Each bar represents the mean percent recovered radioactivity  $\pm 1$  standard deviation for 5 mice/group.

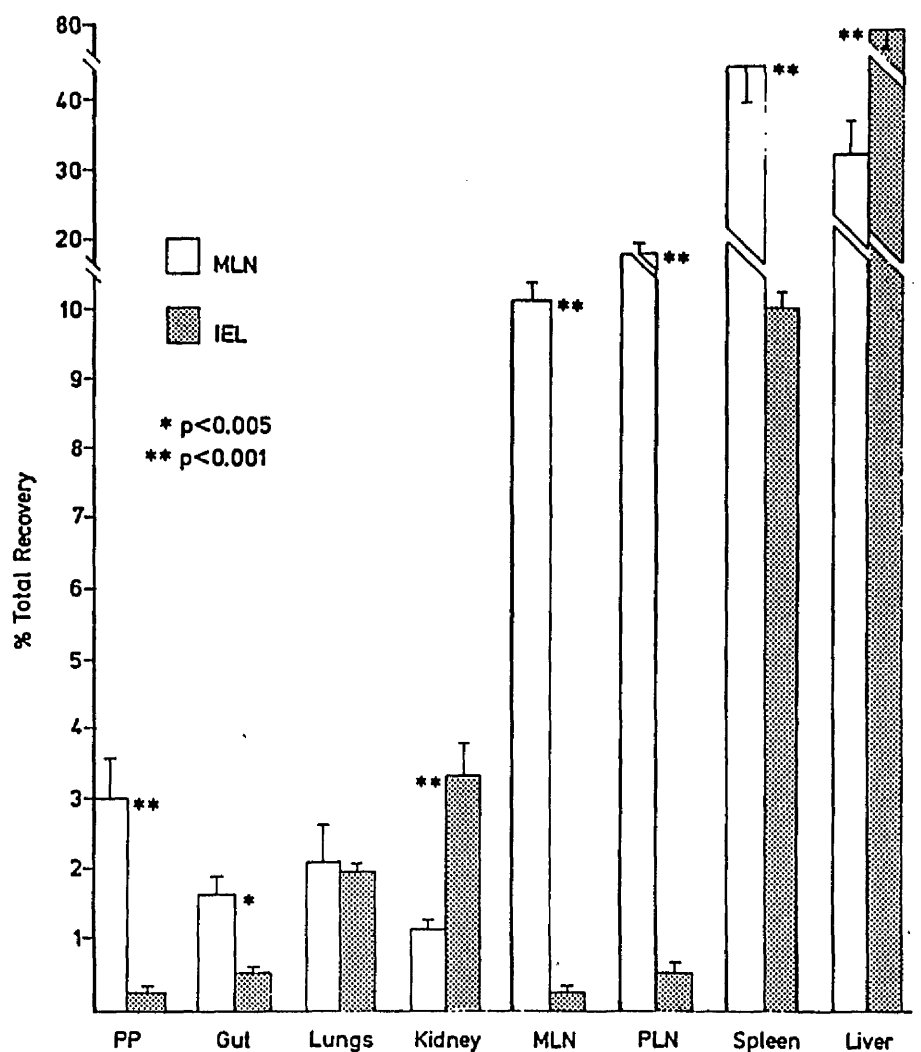


Fig. 25. Migration of  $^{51}\text{Cr}$ -labelled CBA IEL and MLN cells *in vivo*. Organ distribution of radioactivity, 96 hours after transfer of  $5 \times 10^6$  cells into syngeneic recipients. Each bar represents the mean percent recovered radioactivity  $\pm 1$  standard deviation for 5 mice/group.

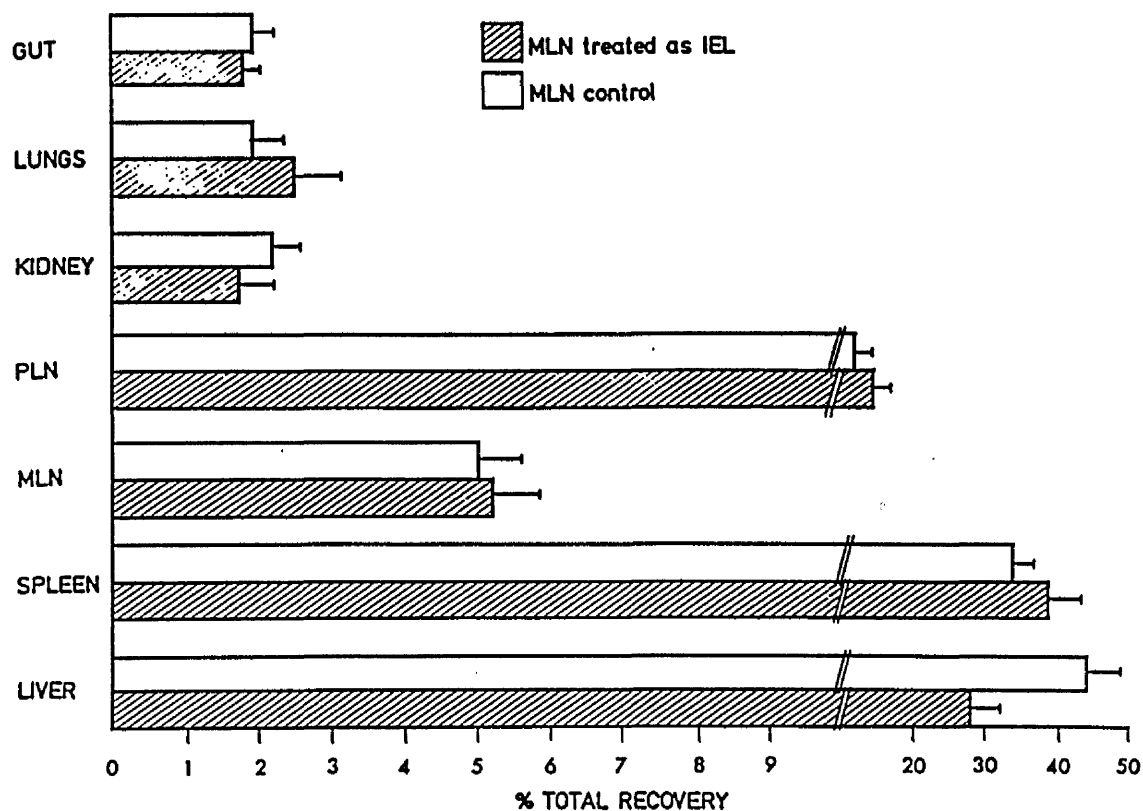


Fig. 26. Effect of the isolation procedure used to obtain IEL on the migration of MLN cells *in vivo*. CBA MLN were incubated with supernatant obtained during the IEL isolation procedure. Results shown are mean percent recovered radioactivity  $\pm$  1 standard deviation for each organ, 18 hours after transfer of  $5 \times 10^6$  syngeneic MLN cells 5 mice/group.

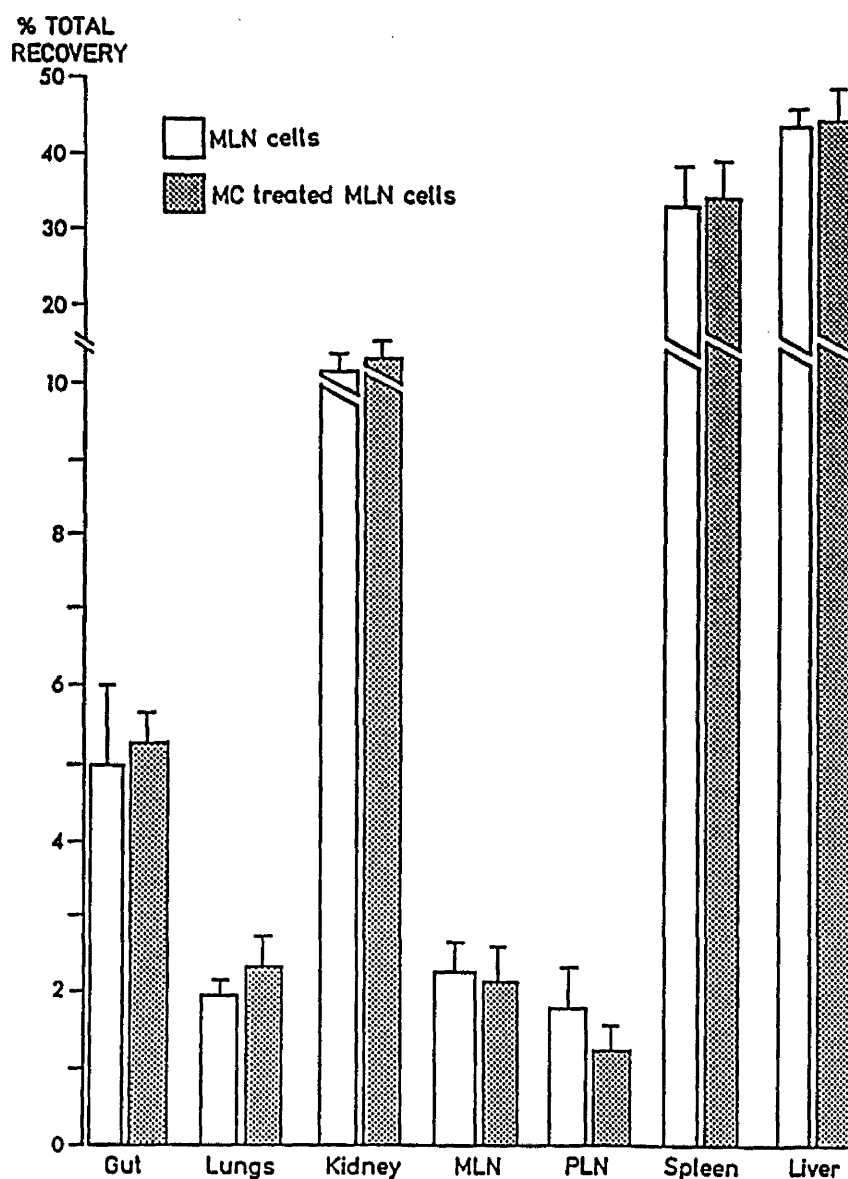


Fig. 27. Effect of mitomycin C treatment on the migration of MLN *in vivo*. Organ distribution of radioactivity, 18 hours after intraperitoneal transfer of  $5 \times 10^6$  CBA cells into syngeneic recipients. Each bar represents the mean percent recovered radioactivity  $\pm 1$  standard deviation for 5 mice/group.

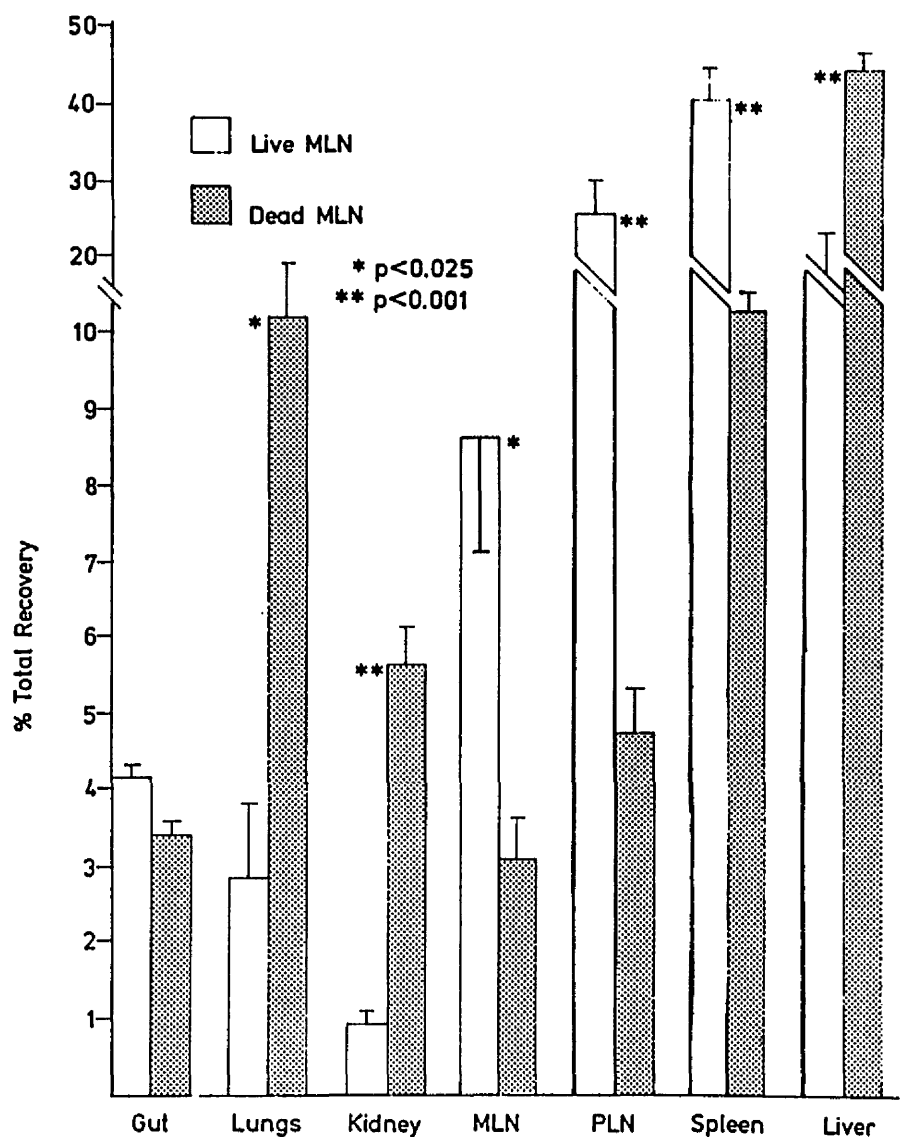


Fig. 28. Effect of heat-killing on the migration of  $^{51}\text{Cr}$ -labelled *in vivo*. Organ distribution of radioactivity, 18 hours after transfer of  $5 \times 10^5$  control of heat-killed MLN cells into CBA recipients. Each bar represents the mean percent recovered radioactivity  $\pm 1$  standard deviation for 5 mice/group.

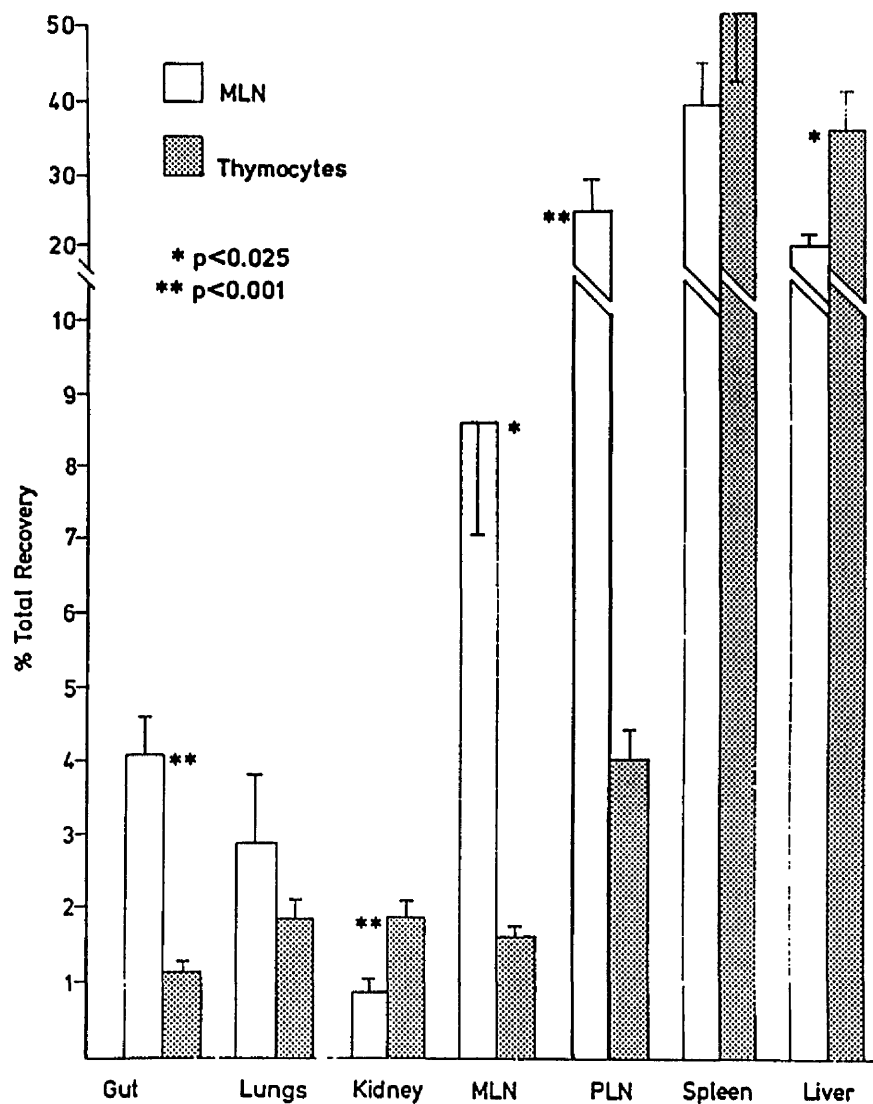


Fig. 29. Comparison of the migration pathways of thymocytes and MLN cells. (Organ distribution of radioactivity, 18 hours after transfer of  $5 \times 10^6$  syngeneic cells into CBA recipients. Each bar represents the mean percent recovered radioactivity  $\pm$  1 standard deviation for 5 mice/group.

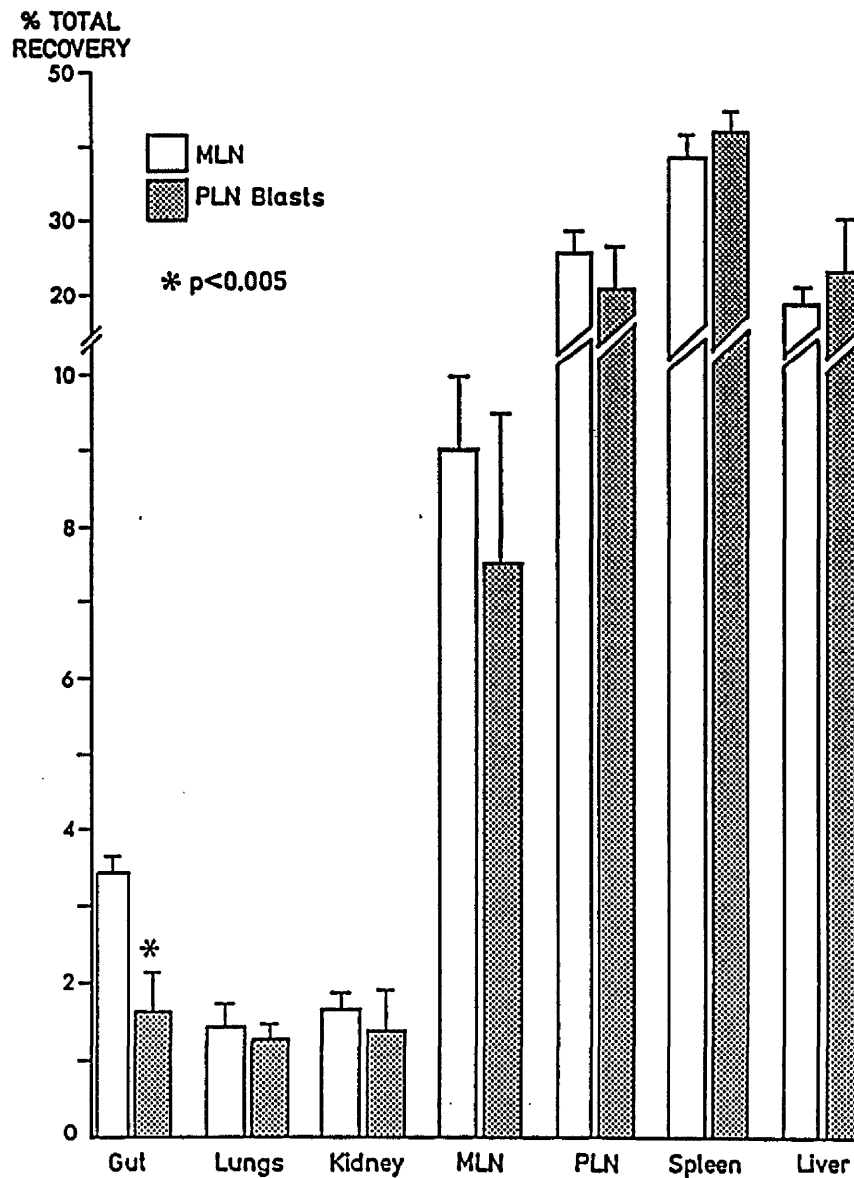


Fig. 30. Comparison of the migration pathways of activated PLN lymphocytes and MLN cells. Organ distribution of radioactivity, 18 hours after transfer of  $5 \times 10^6$  syngeneic MLN cells or PLN cells from oxazolone sensitised mice. Each bar represents the mean percent recovered radioactivity  $\pm$  1 standard deviation for 5 mice/group.

CHAPTER 8  
LOCOMOTOR CAPACITY OF INTRAEPITHELIAL  
LYMPHOCYTES IN VITRO.

## Introduction.

The results of the previous chapter demonstrated that IEL failed to migrate into lymphoid tissues in vivo. Nevertheless, their presence within the most superficial layer of the intestine suggests that IEL may be highly motile in situ. In addition, around the time these experiments were being performed, other workers in the laboratory noted that freshly isolated IEL showed many of the characteristics of motile cells in vitro, with marked polarisation and the formation of pseudopodia and constriction rings. Therefore, it was important to examine if the failure of IEL to migrate in vivo reflected the absence of all locomotor functions, or whether it was due to a specific defect in recirculation from blood to lymphoid tissues. The experiments described in this chapter compared the locomotion activity of IEL and other lymphoid cells in vitro, by assessing their ability to invade three-dimensional collagen gels. This method was developed recently in this department and is now considered the method of choice for determining lymphocyte locomotion in vitro (Shields et al., 1984).

## Determination of optimal conditions for migration through collagen gels.

It was first necessary to optimise the assay by determining the concentration of collagen which allowed the most efficient migration in vitro. Therefore, three-dimensional gels were prepared using different

concentrations of collagen and the efficiency of migration was assessed using a population of MLN cells. Gels prepared with concentrations of < 1.5 mg/ml collagen, were extremely fragile and disrupted completely during the washing stages. Efficient migration was observed using higher concentrations of collagen, but measurements of the leading front showed that the optimal concentration was 2 mg/ml (Fig. 31). Therefore, this concentration of collagen was used throughout subsequent experiments.

In all these studies, freshly prepared collagen gels were used for each experiment and the locomotor capacity of the lymphocytes was calculated 18 hours later, by measuring the proportion of cells at different levels in the gel, using the micrometer of an inverted microscope.

#### Morphological characteristics of IEL invading collagen gels.

Initial studies indicated that IEL would migrate into gels and exhibited the characteristic features of motile cells under these conditions. The morphology of locomoting IEL is shown in detail in Figs. 32 and 33 where it can be seen that a large proportion of migrating cells acquired a polarised morphology associated with the formation of transient pseudopodia and a constriction ring. As the cells moved through the gel, the pseudopodia entered small gaps in the gel matrix and were used by the cell as an anchor, this provides traction which allows the cell to move through

the gel (Haston et al., 1982). These features are identical to those observed in other populations of locomoting lymphocytes in collagen gels (Shields et al., 1984) and I went on to quantify the locomotor activity of IEL.

#### Locomotor activity of IEL, spleen cells and MLN cells in collagen gels.

The data presented in Fig. 34 show that IEL had an excellent locomotor capacity in vitro, migrating into collagen gels as readily as MLN cells and both of these populations migrated much more efficiently than spleen cells. After 18 hours incubation, IEL and MLN cells showed identical distribution through the gel and by this time, 30% of IEL had invaded more than 50  $\mu$ m into the gel, compared with 28% and 4% of MLN and spleen cells respectively. Furthermore, very few IEL remained near the upper surface of the collagen, while a large proportion of spleen cells showed little or no invasion whatsoever. These features are illustrated in Figs. 35, 36 and 37, which represent a series of photographs taken at 20  $\mu$ m intervals through the gel, starting 10  $\mu$ m from the gel surface. These photographs illustrated clearly the ability of IEL and MLN cells to migrate efficiently into the gels, while most spleen cells were found close to the gel surface, indicating their poor locomotor activity.

#### Locomotor activities of thymocytes and lamina propria

lymphocytes.

The next experiments examined the locomotor activity of thymocytes as another population with relatively poor recirculation in vivo, while LPL were used as a population of lymphocytes of more usual phenotype and function than IEL but nevertheless residing in the mucosa.

For practical reasons, it was imposible to use IEL as a direct comparison in these experiments and as MLN cells had identical locomotor activity in vitro, these were used as an indicator population in studies of thymocytes and LPL.

Fig. 38 shows the result from one experiment of this type and indicates that all three populations had similar abilities to migrate into the gel. After 18 hours, 46.6% of thymocytes, 42.9% of MLN and 43.9% of LPL of the cells had moved more than 60  $\mu$ m into the gel and all cell types showed a similar distribution through the gel.

#### Summary and Conclusions.

The results of this chapter show that IEL have an excellent capacity to migrate into collagen gels in vitro and exhibit the characteristic features of motile cells under these circumstances. Indeed, although most lymphoid cell populations show some ability to move into collagen gels, IEL are more active in this respect than spleen cells.

This is the first demonstration that mucosal lymphocytes are motile in vitro and indicates that the

failure of IEL to migrate into lymphoid tissues in vivo is not due to a generalised effect of poor viability or of limited metabolic activity. In contrast, these findings suggest that IEL are not part of the pool of recirculating lymphocytes and it will be important to determine if they exhibit any form of migration in vivo.

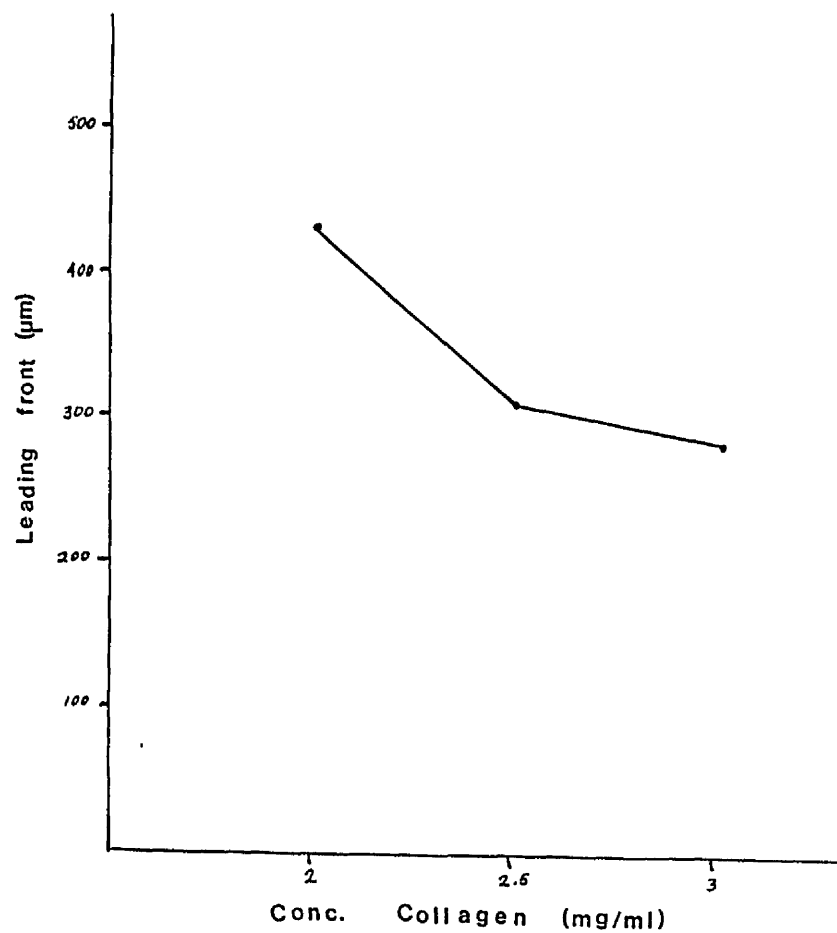
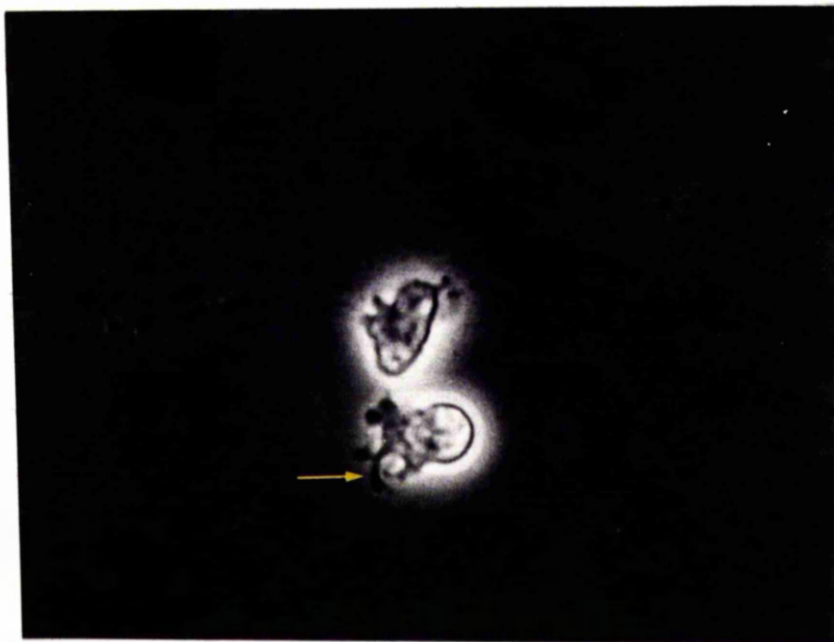


Fig. 31. Determination of optimal concentration of collagen for lymphocyte locomotion *in vitro*.  $2 \times 10^6$  MLN cells were allowed to migrate into gels of different concentrations of collagen. Results shown are the mean leading front ( $\mu\text{m}$ ) of ten readings in duplicate cultures.



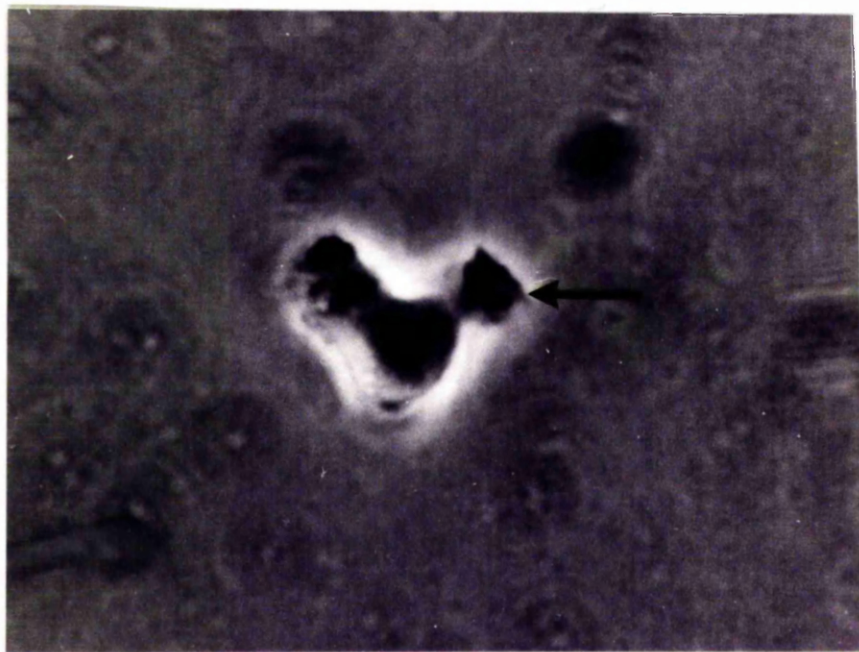
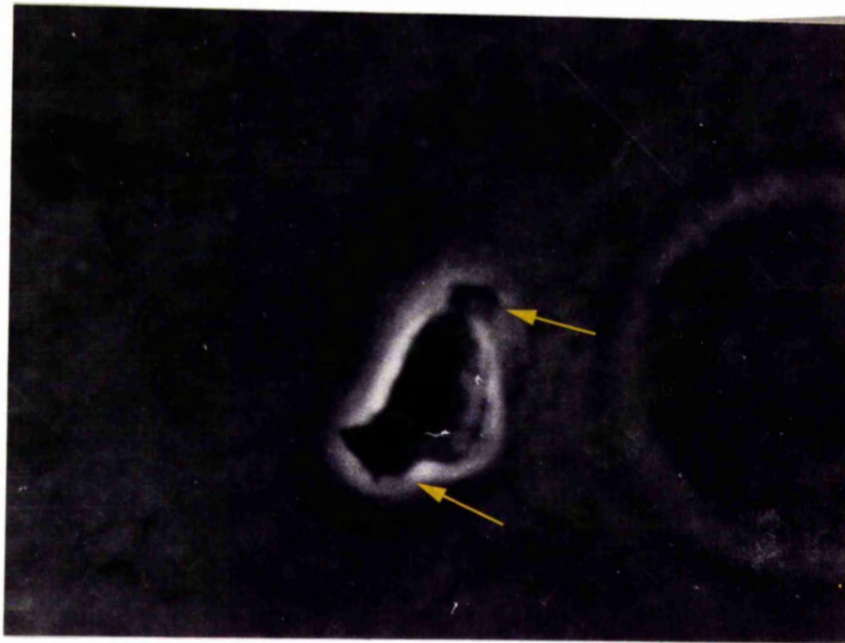


Fig. 32. Morphological characteristics of locomoting IEL. The formation of pseudopodia and constriction ring (arrow) during invasion of collagen matrices. Note the fibres of collagen.

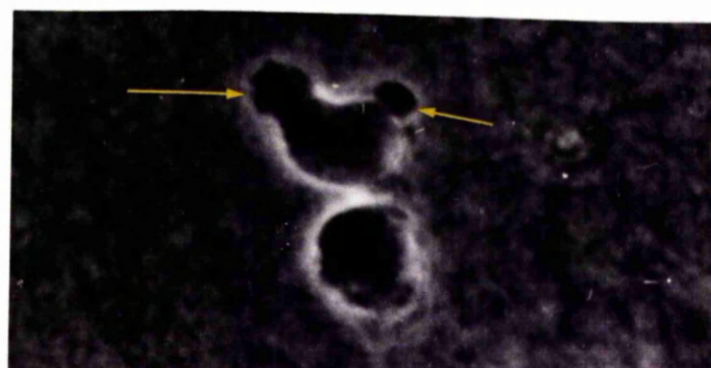
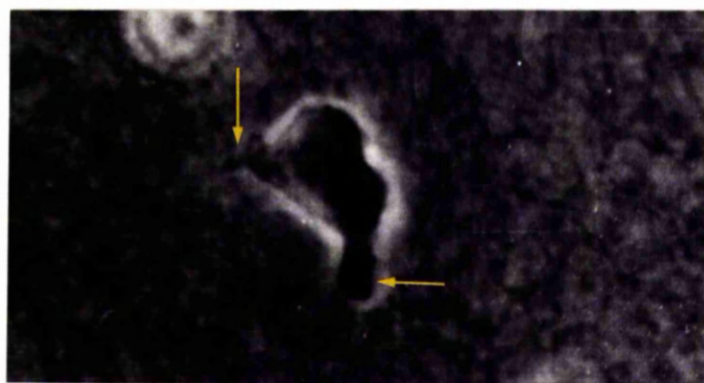


Fig. 33. Morphological characteristics of locomoting IEL. The formation of pseudopodia and constriction ring (arrow) during invasion of collagen matrices. Note the fibres of collagen.

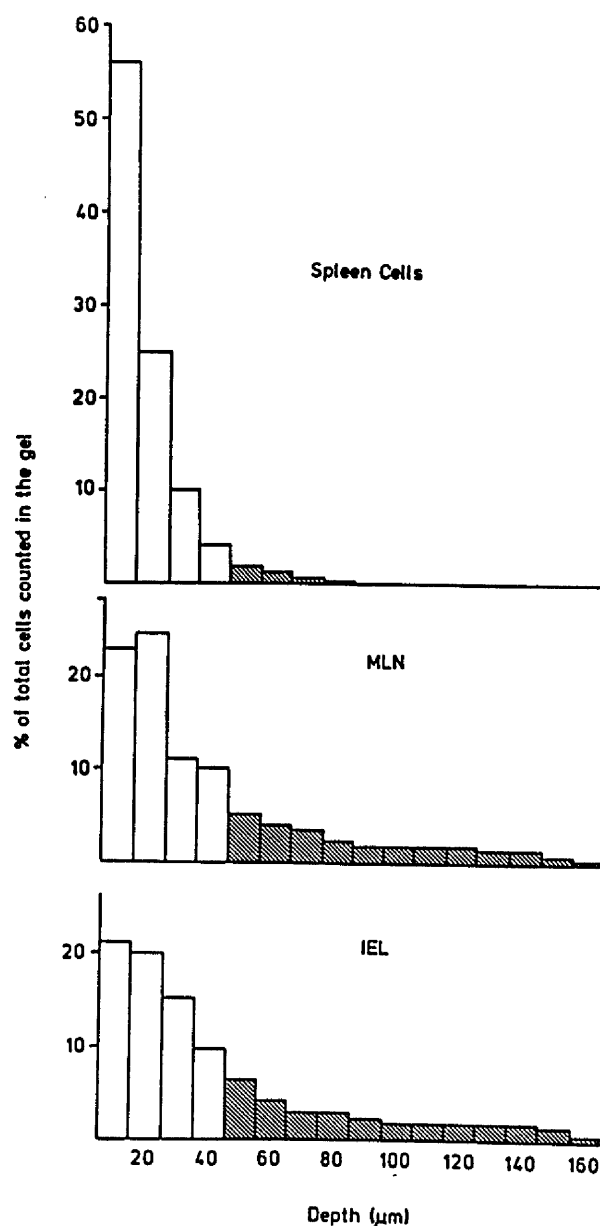
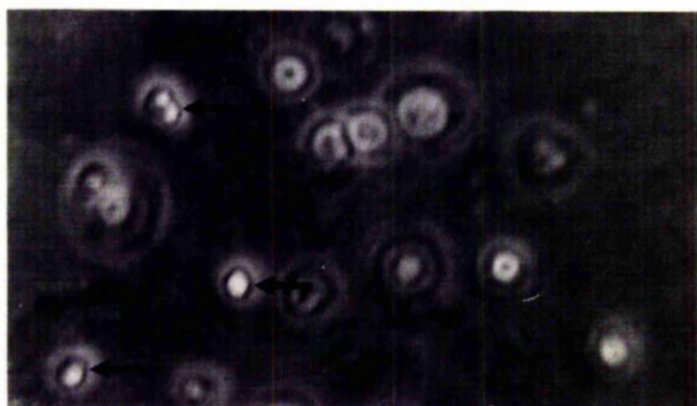
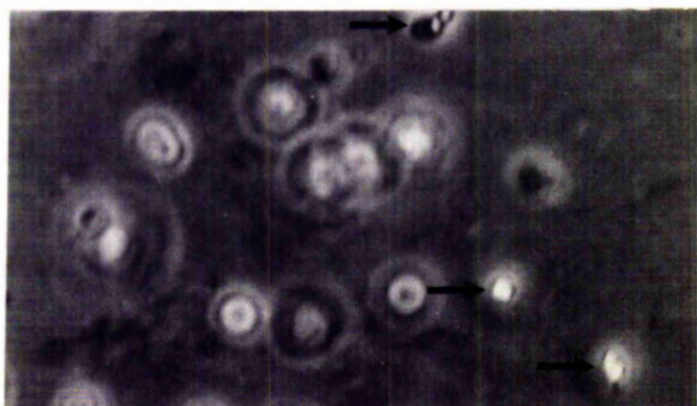
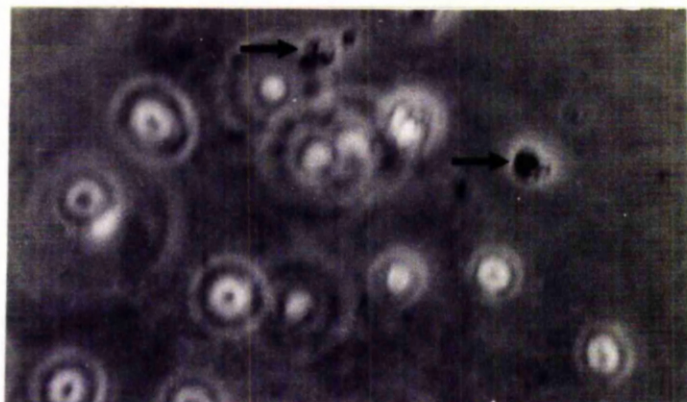
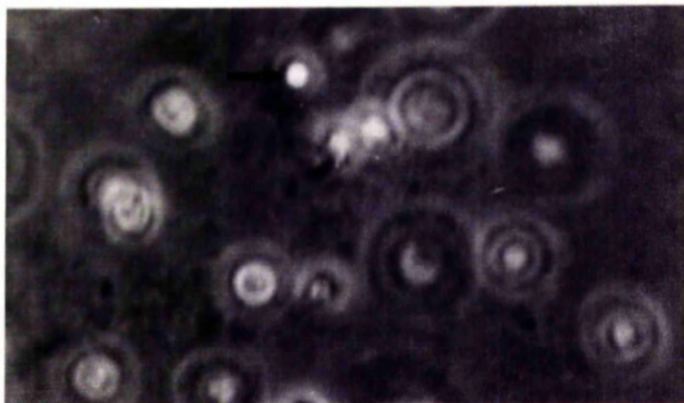
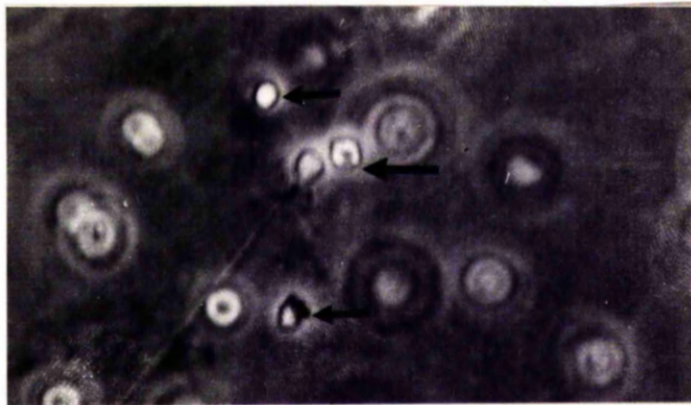


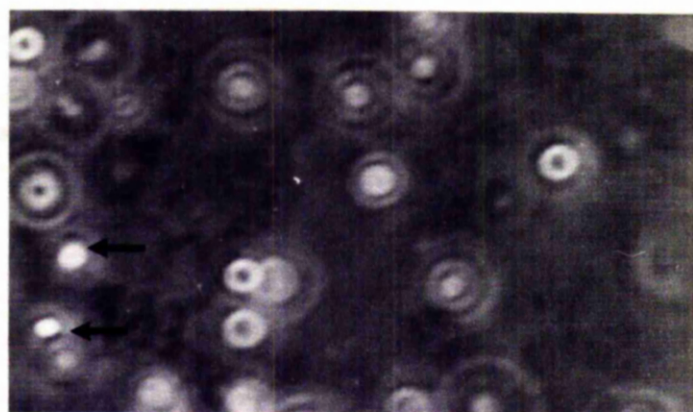
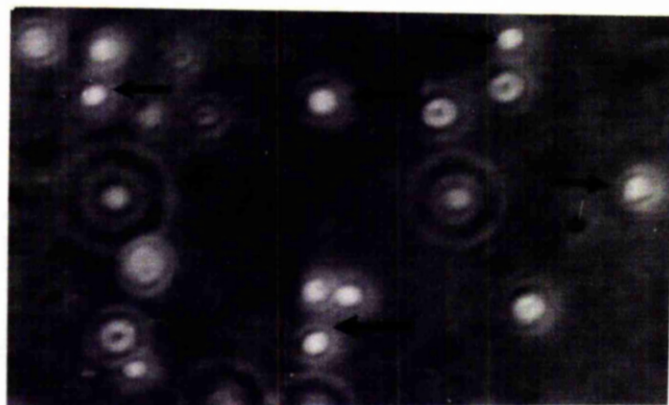
Fig. 34. Invasion of collagen gels *in vitro* by spleen cells, MLN cells and IEL.  $2 \times 10^6$  cells were allowed to migrate into gels for 18 hours and results shown are mean percentage of the total cells found in random fields at 10  $\mu$ m intervals throughout the gel. 50  $\mu$ m was chosen arbitrarily as the distance which discriminated between high (shaded area) and low locomotor capacity.





(120  $\mu$ m)

Fig. 35. Illustration of moving IEL within collagen matrices. Serie of photographs taken in the same field at intervals of 20  $\mu$ m starting from 10  $\mu$ m of the gel surface. Cells were layered on top of collagen matrices and were incubated overnight and then fixed with 2.5% glutaraldehyde. Note the appearance of motile cells in focus at different levels of the gel (arrow).



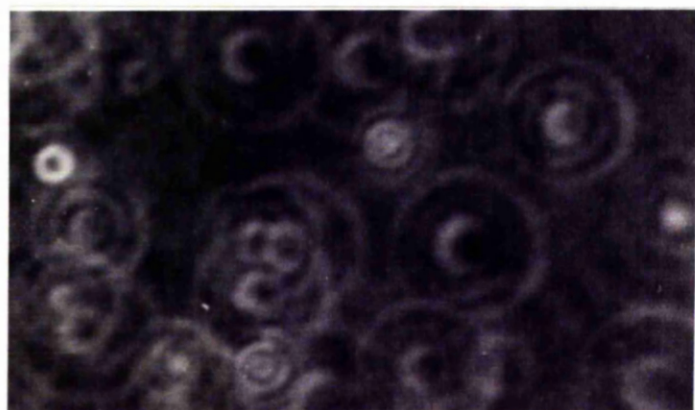
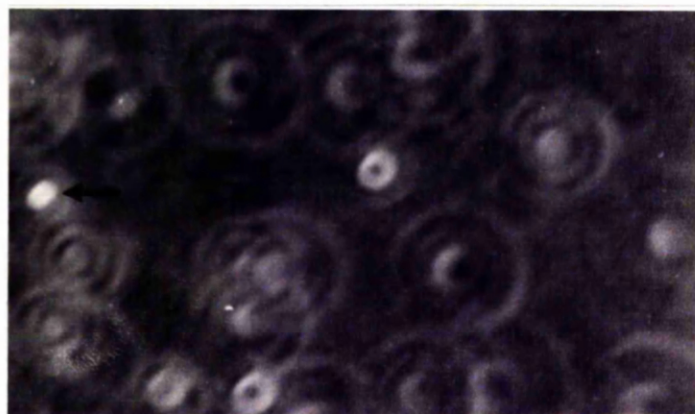
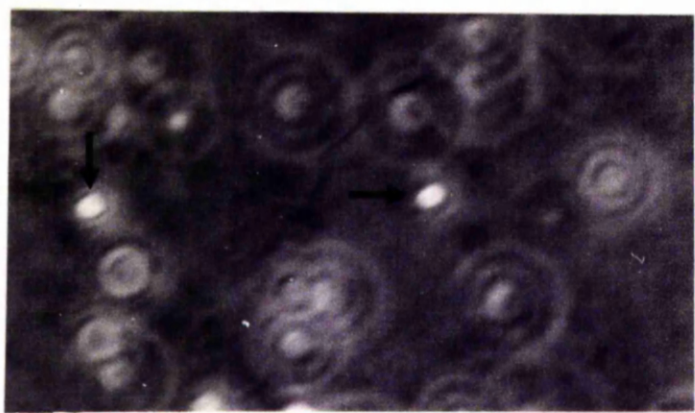


Fig. 36. Illustration of moving MLN cells within collagen matrices. Serie of photographs taken in the same field at intervals of 20  $\mu$ m starting from 10  $\mu$ m of the gel surface. Cells were layered on top of collagen matrices and were incubated overnight and then fixed with 2.5% glutaraldehyde. Note the appearance of motile cells in focus at different levels of the gel (arrow).

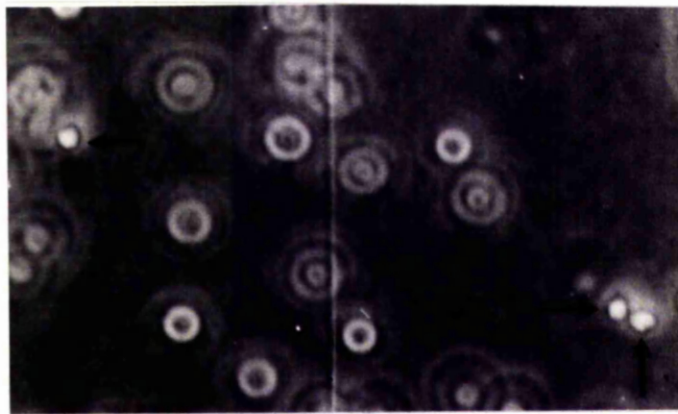
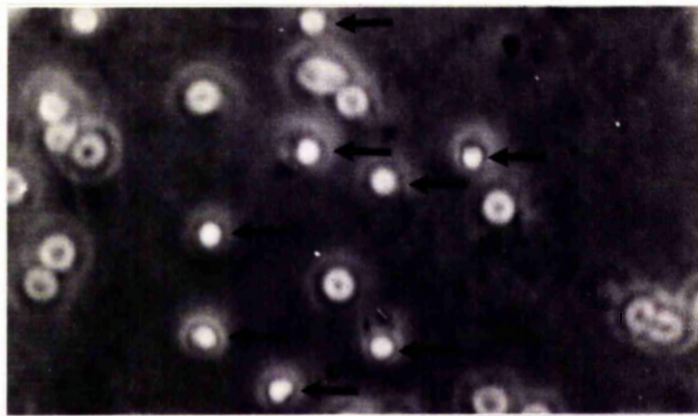


Fig. 37. Spleen cells within collagen matrices. Serie of photographs taken in the same field at intervals of 20  $\mu$ m starting 10  $\mu$ m from the gel surface. Cells were layered on top of collagen matrices and were incubated overnight and then fixed with 2.5% glutaraldehyde. Note the appearance of motile cells in focus at different levels of the gel (arrow) showing the poor locomotor capacity of spleen cells.

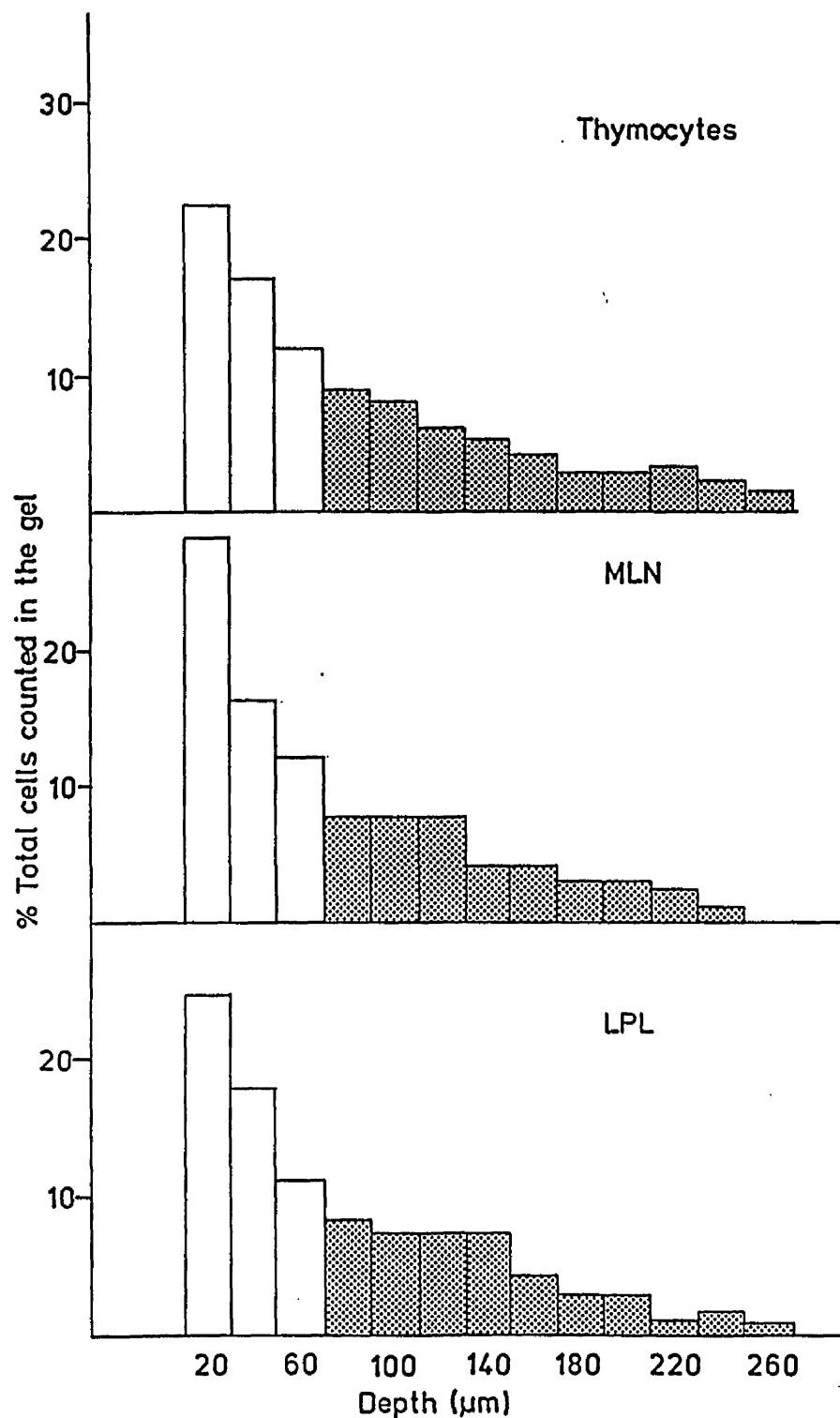


Fig. 38. Invasion of collagen gels *in vitro* by thymocytes, MLN cells and LPL.  $2 \times 10^6$  cells were allowed to migrate into gels for 18 hours and results shown are mean percentage of the total cells found in random fields at 20  $\mu$ m intervals throughout the gel. 60  $\mu$ m was chosen arbitrarily as the distance which discriminated between high (shaded area) and low locomotor capacity.

CHAPTER 2  
MIGRATION OF INTRAEPITHELIAL LYMPHOCYTES  
INTO SITES OF INFLAMMATION

## Introduction.

The results in Chapter 7 showed that IEL did not exhibit the migration pattern of small lymphocytes. However, a significant proportion of IEL in situ have the morphological appearance of activated cells (Marsh, 1975) and data in the preceding Chapter showed that IEL were extremely motile in vitro. Certain other inflammatory effector cells show a similar pattern of locomotor properties and these have been found to migrate into areas of inflammation rather than to lymphoid tissues (Parrott and Wilkinson, 1981; de Sousa, 1981). Therefore, I examined whether IEL would also show an ability to localise in inflamed tissues.

### Migration of IEL into inflamed skin.

In the first experiments, I examined whether IEL would accumulate in skin which had been sensitised and challenged with oxazolone to induce a local inflammatory response. Therefore, labelled-CBA IEL and MLN cells were transferred intravenously into mice which had been treated on both ears with oxazolone, 1 and 6 days previously, and into control mice. The accumulation of radioactivity was assessed 18 hours later in the skin and its draining lymph nodes, as well as other lymphoid tissues.

There were no significant differences between the accumulation of either labelled IEL or MLN in the liver, spleen, lungs, gut, kidney, PLN or MLN of control or oxazolone treated mice (data not shown). Neither IEL nor MLN cells accumulated well in the ears of control

mice (Table 8.  $0.2 \pm 0.005\%$  and  $0.25 \pm 0.051\%$  respectively), but significantly more IEL accumulated in the inflamed ears of oxazolone treated mice ( $0.74 \pm 0.11$   $p < 0.001$ ). MLN cells also showed slightly enhanced accumulation in the inflamed ears, but this was markedly less than that found with IEL ( $0.37 \pm 0.06\%$   $p < 0.025$  vs control ears).

As expected, MLN cells migrated efficiently into the auricular (draining) (DLN) ( $3.2 \pm 0.7\%$ ) and axillary (ALN) lymph nodes ( $9.14 \pm 0.25\%$ ) of control mice and this was greatly enhanced in lymph nodes draining oxazolone-treated ears ( $12.15 \pm 1\%$   $p < 0.001$  and  $11.53 \pm 1.3\%$   $p < 0.025$ ). In contrast, IEL showed little accumulation in control DLN or ALN and there was only a slightly enhanced accumulation of IEL in the ALN of oxazolone treated mice ( $0.33 \pm 0.02\%$  vs  $0.27 \pm 0.01\%$   $p < 0.005$ ).

#### Migration of IEL into inflamed intestine.

As IEL showed an enhanced ability to infiltrate inflamed skin, it was obviously of considerable interest to assess whether a similar phenomenon could be demonstrated under more physiological conditions. Therefore, the next experiments examined whether IEL would migrate efficiently into intestine which had been inflamed by conditions which appear to involve IEL.

#### a) Migration of IEL into *Trichinella spiralis* infected intestine.

*Trichinella spiralis* was chosen as a model of intestinal inflammation because its well-characterised immunopathology not only includes an increased density of IEL (Manson-Smith *et al.*, 1979) but is also

associated with markedly enhanced accumulation of MLN lymphoblasts (Rose et al., 1976ab).

NIH mice were infected orally with 450 infective stage larvae and, 4 days later,  $^{51}\text{Cr}$ -labelled IEL and MLN cells were transferred intravenously into both infected and control recipients. This time is known to be the peak of increased localisation of MLN lymphoblasts and, in the current experiments, the infected mice had an average worm count of 83.8/mouse, of which 68.9% were in the anterior part of the intestine and 31.1% in the posterior part of the intestine. Both IEL and MLN cells exhibited their usual migration pattern in control mice and there were no significant differences between the accumulation of either MLN or IEL in the organs of infected and uninfected recipients (Figs. 39 and 40). In particular, there was no increased localisation of IEL in the intestine of mice infected with *Trichinella spiralis*. But  $^{51}\text{Cr}$ -labelled MLN cells also did not show increased recruitment into the intestine of infected mice. In this respect  $^{51}\text{Cr}$ -labelled MLN differ from MLN lymphoblasts.

b) Ability of IEL to migrate into the intestine of mice undergoing a GvHR.

As labelled IEL failed to localise in the gut of *Trichinella spiralis* infected mice, an alternative means of inducing an inflammatory reaction in the gut was examined. The model chosen was the intestinal phase of GvHR which occurs in  $\text{BDF}_1$  mice given  $10^8$  B10 lymphocytes. This produces an acute GvHR with villus

atrophy, crypt hyperplasia and a marked intestinal inflammatory response, which includes a increased IEL count (Mowat and Ferguson, 1982). These changes become most apparent during the third week after induction of GvHR and (Mowat, personal communication) so I injected  $^{51}\text{Cr}$ -labelled BDF<sub>1</sub> IEL and MLN cells into mice on day 15 of the GvHR and assessed organ-specific radioactivity 18 hours later.

The results in Fig. 41, show that IEL followed their usual pattern of distribution in vivo irrespective whether recipients had GvHR or not. There were no significant differences between the radioactivity found in any organ taken from control or GvHR mice, and once again, there was no increased accumulation of IEL in the gut of mice with GvHR.

The GvHR also did not attract increased numbers of MLN cells into the intestine (Fig. 42) but there was significantly enhanced accumulation of MLN in the lungs of mice with GvHR compared with those of controls (Fig. 42,  $3.18 \pm 0.5\%$  vs  $1.4 \pm 0.42\%$   $p < 0.005$ ). In addition, significantly fewer MLN localised in the MLN of GvHR mice when compared with controls ( $2.6 \pm 0.11\%$  vs  $4.6 \pm 0.8$   $p < 0.01$ ). The significance of these findings is unclear.

Together, the results from these two models of intestinal inflammation indicated that IEL do not show a enhanced accumulation in inflamed gut, despite to their ability to migrate more efficiently into inflamed skin.

#### Summary and Conclusions.

The results of this chapter show that IEL exhibited

an enhanced ability to accumulate in the ears of mice which had been painted with oxazolone. In contrast, MLN cells did not localise as efficiently in inflamed skin, but had a marked tendency to migrate into lymph nodes draining inflamed ears. IEL showed only a little greater accumulation in draining lymph nodes. Paradoxically, when an inflammatory response was induced in the intestine by *Trichinella spiralis* or by GvHR, there was no enhanced localisation of either MLN cells or IEL in the gut, although some minor differences were observed in other tissues from mice with GvHR. Despite these discrepancies, these results provide some evidence that IEL can be recruited non-specifically into sites of inflammation and together with the findings of the previous Chapters, supports the view that IEL may be activated effector cells.

In conclusion, the findings in this Chapter suggest that IEL are a "non-recirculating" population of cells. However, their pattern of migration *in vivo* is not only completely different from that of small lymphocytes, but cannot be reproduced by immature cells or activated lymphocytes. Together, these results suggest that the failure of IEL to induce a systemic GvHR is due to their failure to migrate into the lymphoid organs of host mice.

% Total recovery of  $^{51}\text{Cr}$ -specific radioactivity.

INOCULUM HOST	IEL		MLN	
	Control	Oxazolone	Control	Oxazolone
Ears	0.2+0.005***	0.74+0.11	0.25+0.015*	0.37+0.06
FLN	0.27+0.01	0.22+0.01	4.8+1.8	4.10+0.77
ALN	0.27+0.01**	0.33+0.02	9.14+0.25*	11.53+1.3
DLN	0.29+0.03	0.31+0.01	3.2+0.7***	12.15+1.0
MLN	0.31+0.013	0.28+0.04	7.38+0.9	5.75+1.5

Table. 8 Migration of IEL and MLN cells into inflamed skin and its lymphoid tissues. Organ distribution of radioactivity 18 hours after transfer of  $5 \times 10^5$  syngeneic IEL or MLN cells into control mice and mice sensitised with oxazolone. Results shown are mean percent recovered radioactivity  $\pm$  1 standard deviation for 5 mice/group. \* $p < 0.025$ , \*\* $p < 0.005$  and \*\*\* $p < 0.001$  compared with controls.

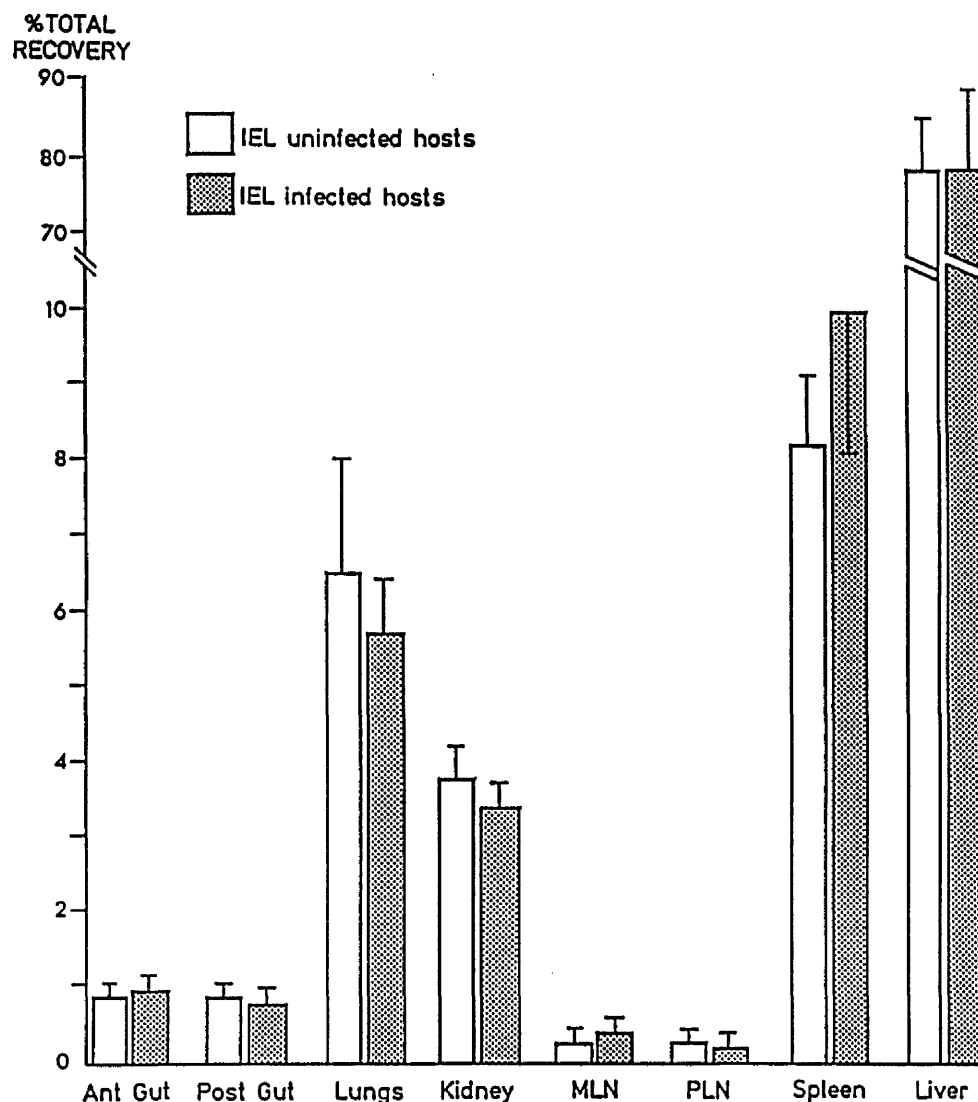


Fig. 39. Effect of intestinal infection with *T. spiralis* on the migration of IEL. Organ distribution of radioactivity, 18 hours after transfer of IEL into control NIH mice or mice 4 days after oral infection with *Trichinella spiralis*. Each bar represents the mean percent recovered radioactivity  $\pm$  1 standard deviation for 5 mice/group.

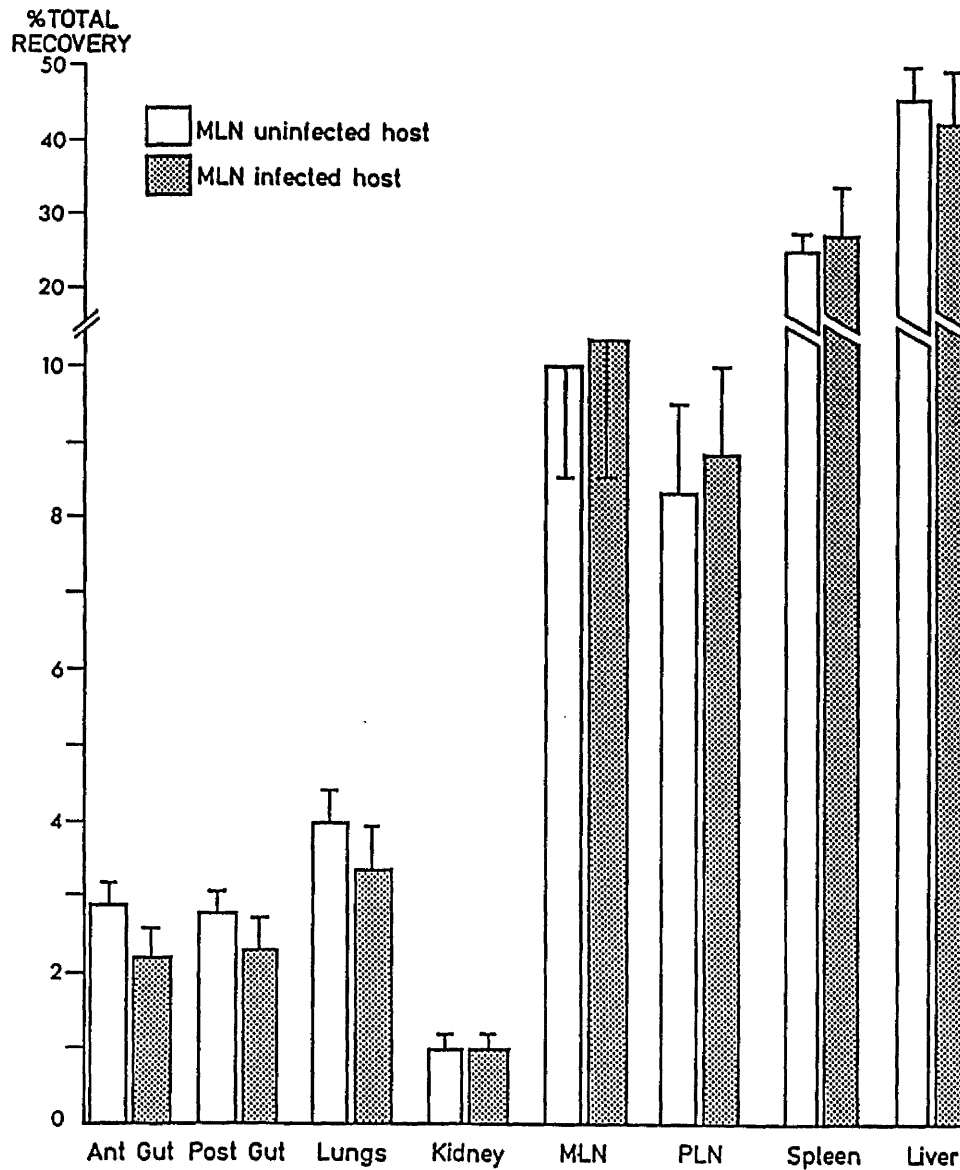


Fig. 40. Effect of intestinal infection with *T. spiralis* on the migration of MLN cells. Organ distribution of radioactivity, 18 hours after transfer of MLN cells into control NIH mice or mice 4 days after oral infection with *Trichinella spiralis*. Each bar represents the mean percent recovered radioactivity  $\pm$  1 standard deviation for 5 mice/group.

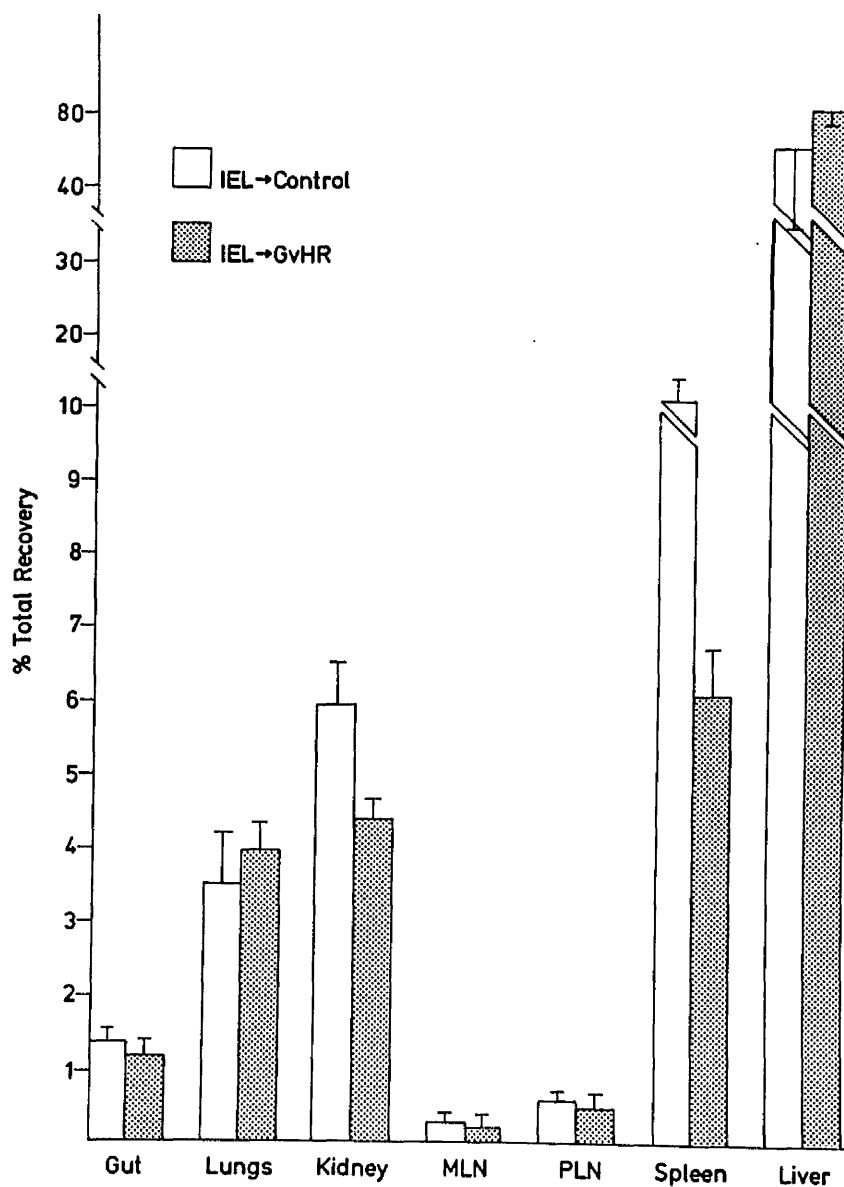


Fig. 41. Effect of graft-versus-host reaction on the migration of IEL *in vivo*. Organ distribution of radioactivity, 18 hours after transfer of IEL into control BDF1 mice or mice on day 15 of GvHR. Each bar represents the mean percent recovered radioactivity  $\pm$  1 standard deviation for 5 mice/group.

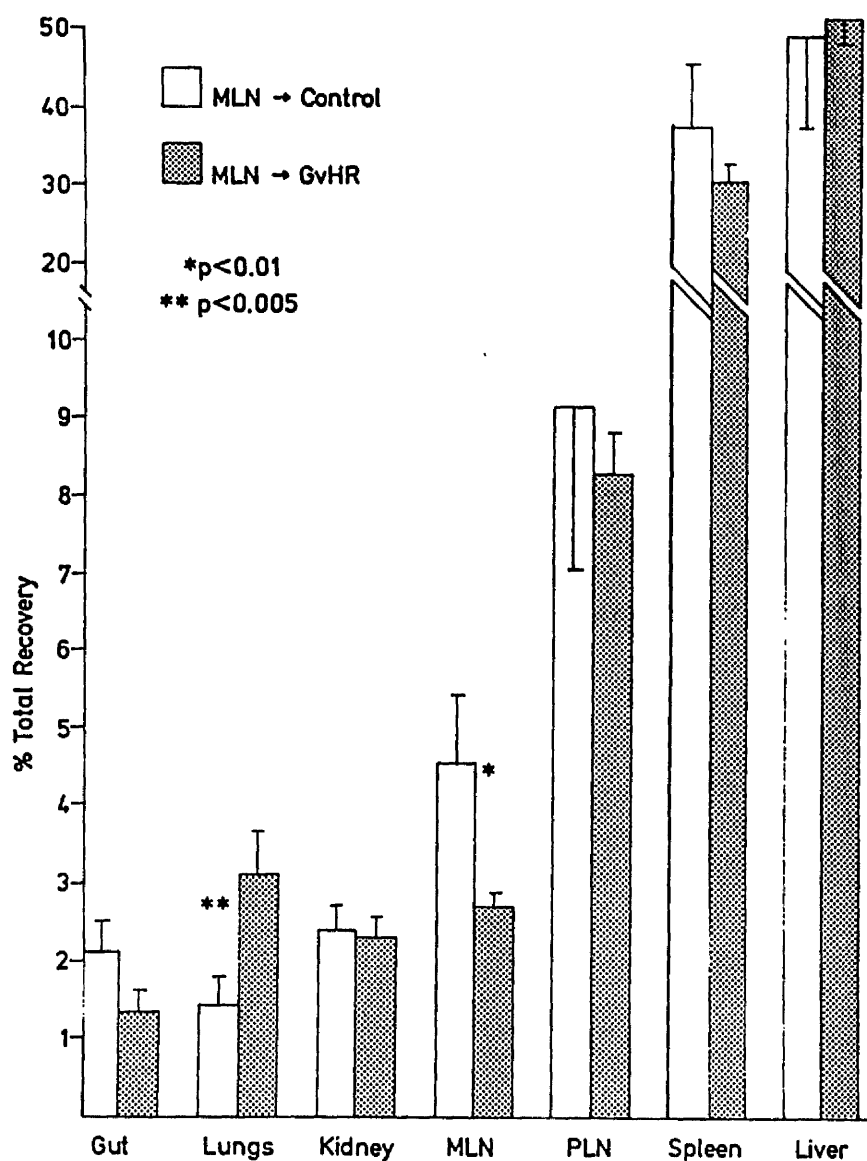


Fig. 42. Effect of graft-versus-host reaction on the migration of MLN cells *in vivo*. Organ distribution of radioactivity, 18 hours after transfer MLN cells into control BDF1 mice or mice on day 15 of GvHR. Each bar represents the mean percent recovered radioactivity  $\pm$  1 standard deviation for 5 mice/group.

CHAPTER 12  
GENERAL DISCUSSION.

## GENERAL DISCUSSION.

This thesis has dealt with the possible role of NK cells and IEL as effector cells in murine GvHR and the chapters on each of these topics will be discussed separately. However, many of the experiments examined the ability of donor IEL and NK cells to induce a GvHR as an indicator of their immunological potential *in vivo*. Therefore, I will first discuss the findings of Chapter 3 which investigated the capacity of a GvHR to reveal differences in the donor cell population.

It is well known that lymphocytes from different anatomical origins display different capacities to induce GvHR (Bril and Benner, 1985). Using the popliteal lymph node assay, it has been shown that blood lymphocytes are the most effective inducers of GvHR while, lymphocytes from lymph nodes and spleen were less effective and bone marrow and thymus cells were the least effective (Yoshida and Osmond, 1971). Similar data have been presented by other workers using different assays (Heim *et al.*, 1972; Kerckheart, Benner and Willers, 1973) and may reflect different maturational stages of the lymphoid populations. Although differences of this type have not been noted previously using lymphocytes from secondary lymphoid tissues, the first experiments in Chapter 3 showed that the splenomegaly and enhanced NK cell activity which characterise a GvHR in unirradiated F<sub>1</sub> hybrid mice were

induced more efficiently by parental MLN cells than by spleen cells. Although MLN cells contained a higher proportion of T cells than spleen cells, the greater ability to induce a systemic GvHR was still observed, even when donor inocula were adjusted to contain equal numbers of T cells. Furthermore, despite the fact that use of individual subsets of T cells can influence the outcome of a GvHR (Korngold and Sprent 1985), it seems unlikely that this could account for my results, as MLN and spleen T cells contained identical proportions of  $\text{Lyt } 2^+$  and  $\text{L3T4}^+$  cells. The reason for this phenomenon remains to be established, but the results in Chapter 7 indicated that injected MLN cells may migrate more efficiently than spleen cells into peripheral lymphoid tissues. That this may explain the greater ability of MLN to induce a GvHR is supported by further results in Chapter 7 which showed clearly that the development of GvHR is influenced by the ability of donor cells to migrate into peripheral lymphoid tissues (see below). The high ability of peripheral blood lymphocytes to induce a GvHR could also be because blood lymphocytes are enriched for recirculatory cells.

One unusual feature of the experiments using MLN and spleen cells was that MLN did not induce more severe intestinal changes of GvHR. However, it should be noted that in my studies, neither donor cell population caused the increases in IEL counts which have been shown previously to characterise the intestinal phase of GvHR in this model (Mowat and Ferguson, 1982). This

discrepancy might be explained by the fact that several mouse strains from the departmental Animal Unit were affected by intestinal parasites at the time these experiments were being performed. This is supported by the unusually high IEL counts in controls and therefore, the changes induced by GvHR may have been masked the presence of a preexisting intestinal inflammatory response. In view of the different ability of MLN and spleen cells to migrate into the small intestine (Chapter 8) it would be of interest to reexamine the intestinal phase of GvHR caused by these donor cell populations in hosts of defined cleanliness.

The next experiments in this Chapter showed that mitomycin-C-treated spleen cells were incapable of inducing a GvHR in unirradiated hosts indicating that GvHR-induced splenomegaly and NK cell activation requires the ability of donor cells to proliferate. These results were not due to defective migration of mitomycin-C-treated cells, as experiments in Chapter 7 demonstrated that these cells migrated as efficiently as normal cells *in vivo*. Furthermore, at the concentrations used, mitomycin C inhibits cell division, but leaves other immunological functions intact (Meuwissen and Good, 1967). Previous studies using T6-chromosome marked donor cells have shown that acute GvHR in mice is associated with proliferation of donor cells in host lymphoid tissues (Fox, 1962). My findings also confirm earlier reports that treatment of donor cells with mitomycin C prevents the induction of splenomegaly in GvHR (Meuwissen and Good, 1967), while

elimination of specifically activated, proliferating T cells by  $^3\text{H}$ -TdR suicide in vitro greatly diminished the ability of the residual cells to induce GvHR (Cheever et al., 1977). The exact effector mechanism which requires donor cell proliferation has not been identified, but it should be noted that the full development of specific antihist DTH during an acute GvHR is dependent on proliferation of the reactive T cells (Wolters and Benner, 1979). In contrast to these reports, other workers have found that mitomycin C-treated or irradiated donor cells could cause GvHR in the chorioallantoic membrane assay (Lafferty et al., 1972), and in explants of newborn  $F_1$  spleen (Scolley, Hofman and Globerson, 1974) and could cause splenomegaly in newborn  $F_1$  mice, provided they were mixed with newborn  $F_1$  spleen cells (Scolley et al., 1974). Hence, donor cell proliferation may not be required to produce mild changes of GvHR, but may be essential to cause maximal disease.

These findings highlight the potential importance of donor cell recirculation and proliferation in the induction of murine GvHR and confirm that GvHR-based assays can be used to detect functional abnormalities in lymphoid cell populations.

#### Role of donor NK cells in GvHR.

The results discussed in Chapters 3 and 4 confirmed previous studies which showed that enhancement of NK activity was a feature of a GvHR in unirradiated hosts

(Roy *et al.*, 1982; Borland *et al.*, 1983; ; Kubota *et al.*, 1983). Furthermore, it has been shown that the intestinal pathology of GvHR is paralleled by increased NK activity in the intestine (Borland *et al.*, 1983). Therefore, a major interest of this project was to examine the role of NK cells in the development of GvHR, as a means of assessing their potential contribution to the pathology which occurs in the intestine and other tissues.

The results in Chapter 4 demonstrated that in contrast to congenic B6 cells, beige spleen cells were unable to induce various forms of GvHR in different hosts. In the first experiments, it was shown that beige spleen cells could not to induce a GvHR in unirradiated (C3H X B6) $F_1$  or BDF $_1$  hosts when measured either as splenomegaly and NK activation in a proliferative GvHR, or as weightloss and mortality in a more acute GvHR. Furthermore, beige spleen cells were unable to induce a local GvHR in BDF $_1$  hosts, measured by the popliteal lymph node enlargement assay. During the course of these experiments, it was reported that beige mice had a decreased potential to induce GvHR (Halle-Pannenko and Bruley-Rosset, 1985).

Finally, the intestinal phase of GvHR in unirradiated BDF $_1$  mice given beige spleen cells was mild compared with the severe damage induced by B6 spleen cells. These defects were not due to an inherent inability of beige T lymphocytes to recognise and respond to alloantigens, as beige spleen cells were fully capable of inducing an acute GvHR in irradiated  $F_1$

recipients and, as shown in Chapter 5, proliferated in MLR.

Recent reports have confirmed that beige cells are defective in their ability to induce a GvHR (Lapp et al., 1985; Halle-Fannenko and Bruley-Rosset, 1985), although one of these groups found that beige cells also could not cause lethal GvHR in irradiated hosts. The reasons for this are not clear, but it could be due to the use of different numbers of donor cells or the dose of irradiation. The work of Lapp and his coworkers (1985) is of particular interest for my studies, as they examined the development of thymic lesions and immunosuppression during a GvHR in unirradiated (C3H X B6)F<sub>1</sub> mice after transfer of beige or bg/+ donor cells. Although these studies showed that beige donor cells could induce some splenomegaly and some pathology in the thymic cortex, only bg/+ cells induced significant, progressive lesions in the thymic medulla. It was concluded that initial T cell activation produced the early features of GvHR and this was normal in beige mice. However, it was suggested that donor NK cells were necessary for the development of persistent immune suppression and injury to epithelial tissues. This idea could therefore explain my findings that beige spleen cells only produced mild transitory intestinal changes, compared with the severe, persistent damage induced by B6 spleen cells. However, it is equally possible that the same mechanism is responsible for both the early and later phases of GvHR, with the more severe

pathology merely representing progression of the initial changes (Mowat et al., submitted). According to this hypothesis, the defect in beige mice would be quantitative, as indicated by results in showing that beige cells could induce a GvHR in irradiated mice. Therefore, it was of interest to examine whether an immunomodulator would allow beige cells to produce a GvHR in intact mice. However, although administration of rIL-2 to B6 mice enhanced their NK activity and increased their potential to induce GvHR, this had no effect on beige mice. There are no previous studies of the effect of IL-2 on beige mice, but my findings suggest that the beige defect affects the ability of NK cells or their precursors to respond to IL-2.

The findings that donor NK cells may play a role in GvHR are supported by the fact lethal GvHR can be prevented by treatment of mice with anti-asialo GM1 antibody (Charley et al., 1983). In addition, more recent studies from our laboratory have shown that the intestinal changes during a GvHR in unirradiated mice can be prevented by this antibody (Mowat and Felstein, 1987). Although it is not known how NK cells may contribute to the tissue damage, the possibility that NK cells are important components of cell mediated effector responses is supported by the presence of NK cell infiltrates in rejecting allografts (Nemlander et al., 1986) and by the fact that depletion of NK cells inhibits the induction of autoimmune diabetes in rats (Like et al., 1986). Nevertheless, it should be noted that most of these studies have implied a role for NK

cells on the basis of their elimination by anti-Asialo G<sub>M1</sub> and recent work has shown that T cells may also express this marker (Suttles *et al.*, 1986). Furthermore, the apparent importance of donor NK cells in GvHR, which has been suggested by experiments using beige, mice is difficult to explain when it is remembered that NK cells possess no known specific recognition ability.

Therefore, I decided it was important to examine the T cell functions in beige mice, as a defect in T cells would certainly explain the defective induction of GvHR.

The series of experiments described in Chapter 5 examined the T cell responses of beige mice, both *in vivo* and *in vitro*. Administration of allogeneic tumour cells induces augmentation of spleen NK activity as well as allospecific CTL activity (Herberman *et al.*, 1977; Cerottini and Brunner, 1974) and I used this regime to study the ability of beige mice to generate CTL *in vivo*. The results suggested that, compared with control B6 mice, beige mice had a defective ability to generate CTL responses *in vivo* after inoculation of P815 cells and this was paralleled by a similar defect in activation of NK cells in immunised beige mice. Beige spleen cells also had a defective ability to generate CTL responses *in vitro* during an MLR compared with the responses observed using B6 spleen cells. Although conflicting with initial findings (Roder and Duwe, 1979) these findings are supported by similar studies (Haller-

Pannenko and Bruley-Rosset, 1985; Saxena *et al.*, 1982).

Other T cell functions of beige mice have not been reported previously and, in Chapter 5, I examined the ability of beige mice to generate allospecific DTH *in vivo*. These studies showed that beige mice produced normal DTH both to allogeneic tumour cells and to semiallogeneic spleen cells. Other parameters, such as the proportion of T cell subsets and migration pathways were also normal in beige mice.

One finding of particular note was that beige cells had enhanced proliferative responses to Con A and in MLR *in vitro*. There are two possible explanations for this. First, NK cells are known to suppress antibody production (Nabel *et al.*, 1982; Tilden *et al.*, 1983), mitogen responses and MLR responses *in vitro* (Tilden *et al.*, 1983) and so lack of NK activity in beige mice could lead to abnormally high proliferative responses under these circumstances. Alternatively, these findings could be explained by the fact that beige mice generated low levels of CTL activity *in vitro*, compared with those generated by B6 cells. Thus, it could be suggested that the specific CTL responses generated by B6 responder cells in an MLR might lyse the allogeneic stimulator cells, with a resulting decrease in proliferation by responder cells, due to a lack of persistent stimulation. In contrast, the defective generation of specific CTL by beige responder cells would allow continued stimulation and therefore higher proliferative responses. This latter possibility is supported by the recent finding that compared with bg/+

mice, beige mice generate a greater number of  $\text{Lyt } 2^+$  cells in response to infection with lymphocytic choriomeningitis virus and this is accompanied by defective specific CTL activity by  $\text{Lyt } 2^+$  cells (Biron Pedersen and Welsh, 1987). In order to distinguish between the two mechanisms proposed above, it would be important to establish the numbers of stimulator cells at different times during an MLR in relation to the levels of CTL generated. Alternatively, the role of non-specific suppression by NK cells could be examined by assessing the ability of cultured beige and normal cells to suppress proliferative responses to unrelated antigens.

Together, these results indicate that the immune defect in beige mice is not restricted to NK cells, as was initially assumed but there is also a defect in some T cell functions which appears to affect CTL responses in particular. The overlapping defect is probably not surprising when one considers the nature of the abnormality in beige cells and the similarity between the functional mechanisms used by NK cells and CTL. All granule-containing cells of beige mice have abnormal lysosomal granules (Shultz and Sidman, 1987), including melanocytes, platelets, mast cells macrophages and LGL. Although the underlying cause of the lysosomal membrane abnormality is not well understood, there appears to be either a high rate of fusion among preexisting, normal lysosomal membranes or defective control of the size of lysosomes (Oliver and Essner, 1975). As a result,

lysosomal function is abnormal, with decreased lysosomal enzyme secretion from the kidney (Novak and Swank, 1979), and reduced synthesis of pigment granules in the melanocytes (Silvers, 1979). Recent evidence suggest that secretion of lysosomal granules is an important component of the lytic function of both NK and CTL and these cell types seem to use identical lytic mechanisms (Koren et al., 1987). Therefore, it might be anticipated that, as I have found, both NK and CTL activity would be similarly depressed in beige mice. These concepts remain to be proven by more detailed analysis of individual lymphocyte populations in beige mice and by further studies of the lytic mechanisms used by cytolytic lymphocytes. However, my results are consistent with the hypothesis that the induction of GvHR requires a subpopulation of donor lymphocytes which are capable of efficient lysosomal function and which are possibly cytolytic, at least in vitro. These findings are also supported by recent work showing that the induction of an acute GvHR in mice can be prevented by treating donor cells with leucyl-leucine O methyl ester (Thiele et al., 1987), an agent which disrupts lysosomes and kills cytotoxic cells. Alternatively, lymphokine secretion may also be dependent on normal lysosomal function, but this remains to be proved.

Finally, my findings indicate that studies using beige mice are complicated by the fact that these animals also have impaired CTL function. Therefore, it is important to stress the need for caution when using beige mice as a model for selective NK deficiency. In

view of other evidence that NK cells may indeed be involved in GvHR-induced pathology (see above), these studies should be repeated using models which allow more selective depletion of donor NK cells such as treatment with monoclonal antibodies specific for NK cells, like NK-1 (Reynolds and Ortaldo 1987).

#### Cell mediated effector functions of IEL.

The second part of this thesis was concerned with the possible role of IEL as effector cells in local CMI responses in the small intestine. Despite circumstantial evidence that IEL are closely involved in intestinal CMI reactions such as food hypersensitivity (Mowat and Ferguson, 1981), parasitic infections (MacDonald and Ferguson, 1978), GvHR (Mowat and Ferguson, 1982) and allograft rejection (Ferguson and Parrott, 1973; MacDonald and Ferguson, 1976), little is known of their biological function, or their role in host defence mechanisms. Furthermore, many studies of IEL function *in vitro* have yielded conflicting results. Therefore, I decided to use an *in vivo* approach to investigate the cell mediated effector responses of IEL. These experiments concentrated on the ability of IEL to mediate various forms of GvHR.

The results presented in Chapter 6 confirmed that IEL are able to induce a local GvHR as measured by popliteal lymph node hypertrophy (Dillon and MacDonald, 1984) and also gave preliminary indications that IEL could mediate splenomegaly after intraperitoneal

transfer in newborn mice. However, IEL were completely unable to induce a systemic GvHR in irradiated hosts. Therefore, the next experiments in this chapter were designed to investigate why IEL could not cause a lethal disease after iv transfer.

Proliferative responses by IEL *in vitro* are highly dependent on exogenous lymphokines, such as IL-2 (Dillon and MacDonald, 1984; Mowat *et al.*, 1986; McInnes *et al.*, in preparation) and IL-2 has been shown to enhance a systemic GvHR induced by other lymphoid cells (Jadus and Peck, 1983b). Nevertheless, treatment of irradiated host mice with rIL-2 did not allow IEL to induce a lethal GvHR despite evidence that the IL-2 had a biological effect *in vivo*. Responses by IEL *in vitro* may also require additional adherent, accessory cells (Mowat *et al.*, 1986) and it has been shown that bone marrow cells of donor or host origin can enhance the ability of lymphoid cells to induce a GvHR. This may be because bone marrow cells provide an additional source of donor T cells or because they have accessory cell functions such as increased presentation of host alloantigens (Korngold and Sprent, 1985). However, my experiments showed that neither type of bone marrow cell allowed IEL to induce a GvHR in irradiated hosts. Together, these findings indicate that intravenously injected IEL are incapable of alloreactivity *in vivo*. Nevertheless, this interpretation seemed unlikely in view of the ability of IEL to induce a local GvHR in the popliteal lymph node or in the spleen after intraperitoneal transfer. In addition, under appropriate

circumstances, IEL can recognise and proliferate in response to alloantigens in vitro (Dillon and MacDonald, 1984; Mowat et al., 1986).

Previous studies show that the induction of systemic GvHR requires donor cells not only to recognise host alloantigens but also that the recognition occurs after the donor lymphocytes have migrated into host lymphoid tissues (Ford 1975). Therefore, I considered the possibility that intravenously injected IEL could not induce a systemic GvHR because they were not capable of localising in host lymphoid tissues. Studies using <sup>51</sup>Cr labelled lymphocytes showed that the migration pattern of intravenously injected IEL was very different from that of other lymphoid populations. Thus, IEL failed to enter lymphoid tissues such as lymph nodes and showed less localisation in the spleen compared with MLN cells and spleen cells. Control experiments showed that the inability of IEL to migrate into lymphoid tissue was not due to lack of proliferative capacity, as mitomycin C-treated spleen cells migrated as efficiently as normal spleen cells. In addition, the isolation procedure to obtain IEL was not responsible for the failure of IEL to enter lymphoid tissue as this isolation procedure had no effect on the migration pattern of MLN cells.

A finding of particular interest was that in my studies, IEL did not migrate to the intestine or its lymphoid tissues, even after a period of 3-4 days. This is unusual compared with the known preference of other

gut-derived lymphocytes to return to the intestine (Parrott and Wilkinson, 1981) and contrasts with the earlier report of Guy Grand, *et al.*, (1978) who found that IEL appeared to migrate back to intestine 3-4 days after transfer. However, it should be noted that this earlier study used poorly defined populations of IEL and it is quite possible that the findings reflected contamination of the IEL preparations with LPL or FP cells.

IEL did localise reasonably efficiently in the spleen, but in view of their lack of migration to other peripheral lymphoid tissues, it is tempting to speculate that IEL were trapped non-specifically in the red pulp of the spleen, whereas most lymphocytes would be expected to localise in the white pulp (de Sousa, 1981). This idea would also explain the large accumulation of IEL in the liver and lungs, where cells could localise non-specifically in the vasculature. Nevertheless, IEL did not appear to remain in the bloodstream longer than other lymphoid cells and it would be important to establish the exact position of labelled IEL in the spleen and liver.

It is often assumed that lymphocytes only accumulate in the liver if they are dead and the liver does indeed remove effete lymphocytes from the circulation (Parrott and Wilkinson, 1981). Nevertheless, there is a substantial amount of evidence which suggests that large numbers of viable lymphocytes do leave the bloodstream in the liver via the lymphatics (Smith *et al.*, 1970; Parrott and Wilkinson, 1981). Of

particular interest is the fact that it seems to be mainly activated lymphocytes which localise in the liver (Ford, 1975), probably as a consequence of their greater locomotor and, or adhesive qualities (Parrott and Wilkinson, 1981).

Control experiments showed that the abnormal migration pattern of IEL was not due either to poor viability as heat killed MLN cells showed low up take of radiolabel and rapid clearance in contrast to IEL.

The best evidence that IEL were a viable population came from the experiments in Chapter 8, which showed that IEL exhibited a polarised morphology and had an excellent locomotor capacity in vitro. Indeed, IEL were much more active in vitro than spleen cells, despite the greater ability of spleen cells to recirculate in vivo. These results indicate that the defective migration of IEL in vivo is not because IEL do not possess a functional locomotor apparatus. Interestingly, LPL also had an excellent locomotor activity in collagen gels and thus, the majority of mucosal lymphocytes appear to be capable of moving readily through tissues. These findings are of obvious importance with respect to the possible role of mucosal lymphoid cells in local immune responses. It would also be of interest to define the phenotype of locomoting IEL, to establish whether this behaviour is a property of all IEL or only a subpopulation.

There are no previous detailed studies of the migration of IEL in vivo. However, one possible

explanation for their abnormal pattern of migration could be the fact that IEL lack the MEL-14 antigen (Jalkanen *et al.*, 1986). A similar correlation between failure to recirculate and absence of MEL-14 has been shown for activated T cells and thymocytes, and these findings suggested that the migration pattern of IEL might reflect their stage of differentiation (Jalkanen *et al.*, 1986). For these reasons, I compared the migration pattern of IEL with that of other "non-recirculating" MEL-14<sup>-</sup> cells. In these experiments, thymocytes were used as a representative population of immature lymphocytes. Although these cells also showed poor localisation in peripheral lymphoid tissues, thymocytes accumulated much better than IEL in the spleen, suggesting that IEL did not behave exactly as an immature cell. When the migration pathways of activated lymphocytes were examined, <sup>51</sup>Cr was used as a label since IEL do not take up DNA labels such as <sup>125</sup>IUdR and a direct comparison between IEL and activated cells was required. Using this protocol, the distribution of activated lymphocytes was not different from that of normal lymphocytes and was nothing like that of IEL. These findings suggest that IEL do not behave like activated lymphocytes in this respect.

Despite these differences, several features of IEL, such as trapping in the liver and polarised morphology with locomotor capacity, do suggest that their abnormal migration pattern may be because these cells are activated. For these reasons, I decided to test in a different way the possibility that IEL recirculated like

activated cells, by taking advantage of the fact that, although activated lymphoblasts exhibit abnormal pathways of redistribution through lymphoid tissues, they have the capacity to migrate non-specifically into sites of inflammation (Parrott and Wilkinson, 1981). Therefore, I investigated whether IEL would also behave in this manner, by studying their migration into inflamed skin or gut. The results in Chapter 9 demonstrated that IEL showed enhanced migration into inflamed ears compared with normal ears. In contrast,  $^{51}\text{Cr}$ -labelled MLN cells did not show this phenomenon, but localised more efficiently in the lymph nodes draining inflamed ears. As usual, IEL did not enter normal lymph nodes and only a slightly increased accumulation of IEL was found in draining lymph nodes. However, an unexpected finding in these studies was that IEL did not migrate to intestine which had been inflamed by either *Trichinella spiralis* or by an acute GvHR. Previous studies have shown that both these models induce intestinal inflammation which is maximal during the period I used in my experiments (Rose *et al.*, 1976a; Mowat, personal communication). Furthermore, I confirmed the presence of significant infection by *T. spiralis* by larval counts and mice with GvHR had marked splenomegaly. *Trichinella* has also been used extensively as means of demonstrating enhanced homing of lymphoblasts to gut (Rose *et al.*, 1976ab). Nevertheless, my experiments had important limitations. As others have shown (Rose *et al.*, 1976a), I found that

the  $^{51}\text{Cr}$  labelled MLN cells which I used as positive controls, did not reveal enhanced localisation in the inflamed intestine it and would have been more relevant to use  $^{125}\text{I}$ UdR labelled cells in this respect. However, as noted above, IEL did not take up this label in sufficient quantities to allow *in vivo* studies with this isotope and so I had to use  $^{51}\text{Cr}$  as label for both IEL and MLN cells. Unfortunately, time did not allow the use of  $^{125}\text{I}$ UdR labelled MLN cells in these or other migration experiments and it would be important to compare the migration pathways of IEL and other cells into normal and inflamed tissues using appropriate labels of this type.

My work on IEL leads me to propose the tentative conclusion that these cells may represent a population of cells which are activated *in situ*. DNA labelling studies show clearly that IEL are not true lymphoblasts and IEL do not express activation markers such as IL-2 receptors, transferrin receptors or Ia antigens (Selby *et al.*, 1983, 1984; Cerf-Bensussan *et al.*, 1984). Nevertheless many IEL have the morphological appearance of activated cells (Marsh, 1975). Finally, activated CTL have been shown to possess similar cytoplasmic granules and exhibit a polarised morphology (Yanneli *et al.*, 1986). Together with the evidence from the studies discussed above, these findings suggest that many IEL are activated. These findings would also explain why it is extremely difficult to stimulate freshly isolated, normal IEL with polyclonal mitogens or alloantigens, despite the fact that IEL from immunised animals will

exhibit antigen-specific CTL activity (Klein and Kagnoff 1984; Davies and Parrott 1980) and lymphokine secretion *in vitro* (Dillon *et al.*, 1986b) as well as DTH responses *in vivo* (Sheilds and Parrott, 1985). All these data are consistent with the idea that a large proportion of IEL are terminally differentiated, effector T cells which have been activated *in situ* by luminal antigens and hence are unresponsive to further, non-specific stimulation. It would also be anticipated that, as I have found, these cells would not recirculate like small, resting lymphocytes.

However, IEL are a heterogenous population and it is also important to consider the possibility that at least some IEL are not classical T lymphocytes (Mayrhofer, 1980; Mayrhofer and Wately, 1983). Therefore, my results could reflect the properties of other cell types. At least 60% of IEL possess cytoplasmic granules and are morphologically similar to the population of lymphocytes known as LGL (Guy Grand *et al.*, 1978; Ernst *et al.*, 1985). This is supported by the fact that although IEL do not have the phenotype of true NK cells and do not exhibit high levels of NK activity short term assays, reasonable NK activity can be revealed by a variety of immunological stimuli such as the induction of GvHR, or by systemic and enteric immunisation with allogeneic tumour cells (Klein and Kagnoff, 1984). The similarity between IEL and LGL is highlighted by the fact that LGL also fail to recirculate from blood to lymph *in vivo* and show a

similar pattern of migration to that reported here for IEL (Rostald et al., 1986). In addition, activation of NK cells by in vivo *C. parvum* or pyran copolymer (MVE-2) also causes a large accumulation of LGL in the liver (Wiltrout et al., 1984). LGL also exhibit a polarised morphology (Muse and Koren, 1982) and move rapidly through nitrocellulose filters in vitro (Bottazzi et al., 1985; Polentarutti et al., 1986). Interestingly, this activity can be enhanced by factors known to activate NK cells such as alpha, beta and gamma IFN and IL-2 (Polentarutti et al., 1986) and it would be of interest to examine the effects of these agents on locomotion by IEL. Although my findings suggest that IEL may be committed effector cells, their role in intestinal GvHR or other local CMI responses is still unclear. The possibility that these represent effector T cells supported by evidence that the majority of IEL in irradiated mice with GvHR are of donor origin. Furthermore, IEL from these mice have been shown to secrete lymphokines in response to specific antigens in vivo (Guy Grand and Vassalli, 1986) as do IEL from mice infected with *T. spiralis* (Dillon et al., 1986b). These findings are consistent with the hypothesis that IEL may be directly responsible for producing the soluble mediators which are believed to be important in the pathogenesis of these disorders (Mowat and Ferguson, 1981, 1982). However, an alternative possibility is that IEL are one component of the heterogenous population of lymphoid cells which are recruited into the mucosa by lymphokines released during local DTH responses. As

noted earlier, these cells may include NK cells and MMC and it has been shown that the NK cell activity of IEL is enhanced in mice with GvHR. Furthermore, the ability of anti-asialo  $G_{M1}$  antibody to prevent intestinal GvHR is associated with a decreased in IEL count (Mowat and Felstein 1987), while asialo- $G_{M1}^+$  IEL with non-specific cytotoxic activity appear to be important for protection against mouse enteric murine coronavirus virus (Carman et al., 1986). Together with the similarities between the morphology and recirculation of IEL and LGL, these features suggest that the IEL population contains a population of NK effector cells which are important during intestinal CMI responses.

As discussed above, NK cells are capable of wide variety of immunological functions in addition to non-specific cytotoxic activities and may play an important role against viral and parasitic infections, as well as in neoplasia (Lotzova and Herberman, 1986). All these characteristics make them ideally suited as non-specific effector cells for the protection of a site like the intestinal mucosa.

These considerations highlight the complex problem of understanding the functions of the heterogenous population of IEL. Clearly, these issues will only be resolved by examining the functional properties of highly purified, phenotypic subpopulations of IEL, both under normal and pathological conditions. In addition, the origin of these important cells will require analysis of T cell receptor expression.

In conclusion, the results of this thesis have indicated the complex types of effector cells which may be involved in the pathogenesis of tissue pathology during cell-mediated immune responses. In particular, I have provided some evidence that NK cells and IEL may be important, activated effector cells in intestinal CMI responses. Nevertheless, these preliminary findings require to be confirmed using models which allow specific determination of NK cell function and which can examine the exact nature of the different subpopulations of IEL.

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